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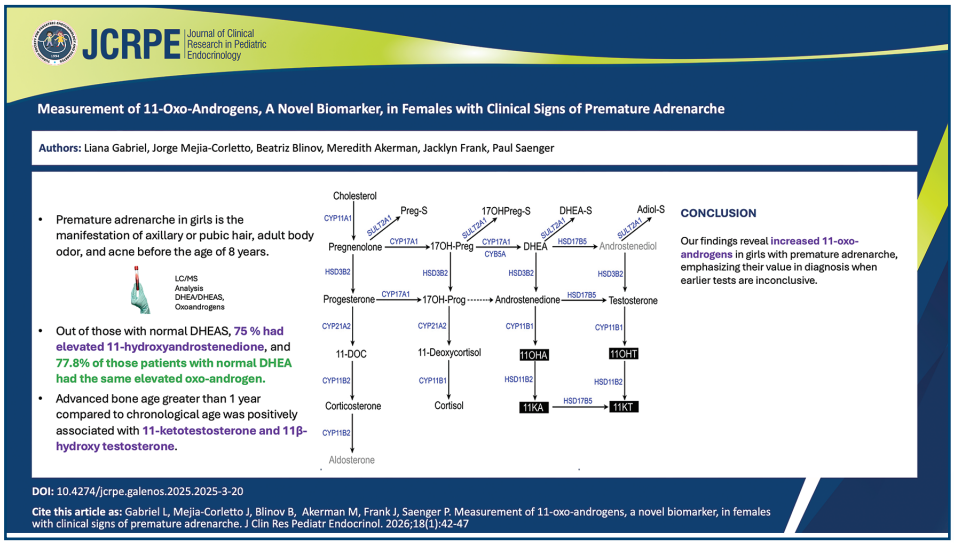

Measurement of 11-Oxo-Androgens, A Novel Biomarker, in Females with Clinical Signs of Premature Adrenarche

Gabriel L et al.

Page:42-47

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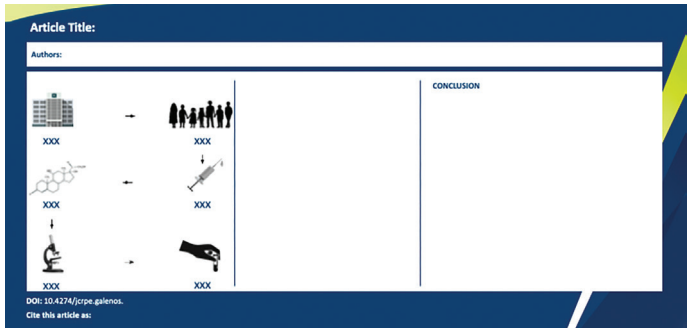

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REVIEW

JCRPE Journal of Clinical Research in Pediatric Endocrinology

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J Clin Res Pediatr Endocrinol 2026;18(1):1-10

Diabetic Peripheral Neuropathy in Children and Adolescents - Prevalence, Diagnostic Methods and Risk Factors

Marta Baszyńska-Wilk[1], Monika Nowacka-Gotowiec[2], Elżbieta Moszczyńska[1]

[1]The Children's Memorial Health Institute, Department of Endocrinology and Diabetology, Warsaw, Poland
[2]The Children's Memorial Health Institute, Electromyography and Evoked Potential Laboratory, Warsaw, Poland

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ABSTRACT

Diabetic peripheral neuropathy (DPN) is the most common form of acquired neuropathy. In children with type 1 diabetes, the reported prevalence of DPN varies widely, ranging from 3% to 62%, mainly due to differences in screening methodologies and patient population characteristics. While intraepidermal nerve fiber density assessment via skin biopsy remains the gold standard for detecting small fiber neuropathy, nerve conduction studies are the established diagnostic tool for large fiber involvement. However, several novel and non-invasive diagnostic tools have emerged recently, offering improved screening options for early-stage and subclinical DPN. The frequent presence of asymptomatic neuropathy in pediatric populations, combined with its limited treatment options, underscores the importance of early identification of modifiable risk factors thus reducing the risk of developing clinically significant DPN. This review provides a comprehensive overview of the current evidence on the prevalence, risk factors, and modern diagnostic approaches for DPN in children with diabetes.

Keywords: Diabetes type 1, diabetic neuropathy, children

Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes and the most common type of acquired neuropathy. Classically, DPN is defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in patients with diabetes after the exclusion of other causes (1). Typically, this is a chronic complication of diabetes but acute cases also occur (2). Sensory, motor, or autonomic nerves may be affected, often coexisting (1). There is no universally accepted classification for DPN. The American Diabetes Association classified diabetic neuropathies into three main categories: 1) diffuse neuropathy (distal symmetric polyneuropathy and autonomic); 2) mononeuropathy; and 3) radiculopathy or polyradiculopathy (3) (Figure 1). There are two stages of DPN, subclinical and clinical. Subclinical DPN implies neurophysiological changes in nerve function without clinical symptoms. The manifestation of nervous dysfunction characterizes clinical DPN (4).

The most common type of DPN is distal symmetric polyneuropathy (3). It may involve large-fiber nerves (related to touch, vibration, position perception, and muscle control) and small-fiber nerves (affected thermal perception, pain, and

Corresponding Author: Marta Baszyńska-Wilk, MD, The Children's Memorial Health Institute, Clinic of Endocrinology and Diabetology, Warsaw, Poland
E-mail: m.baszynska-wilk@ipczd.pl **ORCID:** orcid.org/0000-0001-8741-3518
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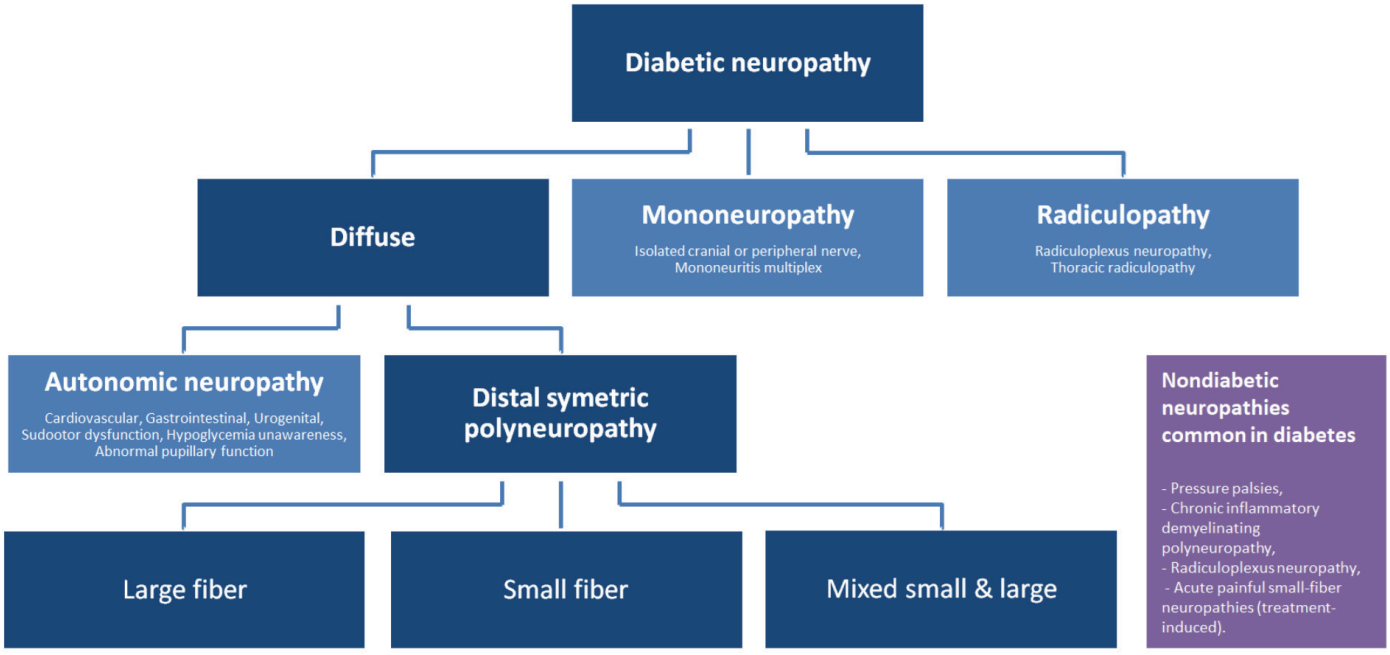

Figure 1. The classification of diabetic neuropathy modified from American Diabetes Association (3)

autonomic function) (Table 1). Most patients, however, have both large- and small-nerve fiber damage. Sensory symptoms include numbness, paresthesia, and neuropathic pain. Symptoms begin in the toes and fingers before progressing in a stocking and then a glove distribution as the disease progresses. In the pediatric population, most patients present with subclinical neuropathy. In the case of clinical manifestation, pain, and dysesthesias are the most common presentations (5).

The US Food and Drug Administration has not approved any casual treatments for DPN. Hence, prevention, reducing risk factors, and finding appropriate screening tests that enable diagnosis at an early stage of the disease are essential (3).

The review aims to summarize the data on the prevalence, diagnostic methods, and risk factors of DPN in children and adolescents, especially in type 1 diabetes (T1D).

Prevalence of Diabetic Neuropathy

The prevalence of DPN in T1D ranges widely from 3% to 62% (6,7,8). There is a variation between the results of the studies, which are limited and hard to compare due to differences in age ranges, clinical definitions and diagnostic tests for DPN. The data are summarized in Table 2. The Pittsburgh Epidemiology of Diabetes Complications Study revealed diabetic neuropathy in only 3% of patients under 18 years old, 18% for 18- to 29-year-old patients, and 58% for >30-year-old patients (6). The EURODIAB insulin-dependent diabetes mellitus complications study, one of the largest and most important epidemiological studies on T1D in children and adolescents in Europe, reported diabetic neuropathy in 19% of patients aged 15 to 29 years (9). The Danish Study Group reported subclinical neuropathy in 62% of patients aged 12-27 years, diagnosed by vibration detection threshold (VDT) (10). However, Nelson et al.'s (11) identified neuropathy in 57% of patients using one of the gold standard methods, a nerve conduction study (NCS). The SEARCH for diabetes in youth study (SEARCH) is a multicenter, population-based study designed to assess the incidence, prevalence, and complications of diabetes among children and adolescents in the United States. Data from SEARCH showed that 7% of adolescent patients had evidence of neuropathy, as diagnosed by the Michigan Neuropathy Screening Instrument (MNSI) (12).

Diagnostic Methods

International Society for Pediatric and Adolescent Diabetes recommends that screening for DPN in young people with T1D should start at puberty or from age 11 years if the child has had diabetes for 2-5 years and screening should be repeated annually (13).

Clinical findings suggestive of DPN include a loss of sensation to pinprick, temperature, vibration, and proprioception (5). Neurological examination should consist of reflex testing because loss of ankle reflexes can occur in the early stage of DPN. Weakness of small foot muscles and dorsiflexion is usually observed later (5).

Table 1. Diabetic peripheral neuropathy: small fiber vs. large fiber (4,5)

Nerve type	Fiber class	Nerve function	Fiber myelination	Function	Symptoms of neuropathy
Large-fiber	Aα	Motor	Myelinated	Muscle control	- Impaired vibration sense, - Loss of position sense, - Muscle weaknes, - Loss of deep tendon reflexes
	Aα/β	Sensory	Myelinated	Position perception, touch, vibration	
Small-fiber	Aδ	Sensory	Thinly myelinated	Cold, pain (fast and well-localized)	- Pain and paresthesia, - Loss of temperature perception - Normal tendon reflexes, - Autonomic signs and symptoms
	C	Sensory	Unmyelinated	Cold and warmth, pain (slow and poorly localized)	
	C	Autonomic	Unmyelinated	Blood pressure, heart rate, sweating, gastrointesinal and genitourinary system control	

Table 2. The summary of studies evaluating diabetic neuropathy in children and adolescents

Study	No. of patients	Diagnostic methods	Prevalence of DPN (age range)
Maser et al. (6) Pittsburgh Epidemiology of Diabetes complications study	400	- Clinical symptoms, - Neurological examination	3% (<18 years)
Tesfaye et al. (9) EURODIAB IDDM	3250	- Clinical symptoms, - Neurological examination, - Vibration perception threshold, - Autonomic dysfunction	19% (15-29 years)
Olsen et al. (10) Danish Study Group	339	- Vibration perception threshold	5% (10-15 years) 46.8% (15-20 years)
Nelson et al. (11)	73	- NCS	57% (mean age 13.7±2.6 years)
Jaiswal et al. (12) SEARCH	1734	- MNSI	7% (mean age 18±4 years)
Moser et al. (8)	151	- MNSI, - NCS (in case of a positive result of MNSI or in a high risk group).	10.6% (8-21 years)

IDDM: insulin-dependent diabetes melitus, NCS: nerve conduction studies, MNSI: Michigan Neuropathy Screening Instrument

Screening Tests

Touch Sensation Tests

Touch sensation tests assess the integrity of Merkel touch domes and Meissner corpuscles and their associated large-diameter fibers. The Semmes-Weinstein monofilament test, Neuropen, and the Ipswich touch test can be used to evaluate touch sensation. Frey's hair test may also allow thr examination of touch perception thresholds (14).

There are different types of monofilament, e.g., 0.5 g, 2 g, 10 g, 50 g, and 200 g. A 10 g monofilament test is the most common, in practice. The Neuropen combines an interchangeable 10 g monofilament and a calibrated sterile Neurotip for assessing pain sensation. The Ipswich touch test is a simple method where the tip of an index finger is used to elicit the sensation of light touch on the tips of the patient's first, third, and fifth toes for 1-2 seconds. Compared with the 10-g monofilament, the Ipswich Touch Test has a sensitivity and specificity of 76% and 90%, respectively, and can be done at home without equipment (7). Frey's hairs are based on a method similar to that of a monofilament; touch perception thresholds can be assessed by buckling the hair which corresponds to a predefined force determined by the filament's mechanical properties (nominal force of 0.026-110 g) with a force ranging from 0.026 g to 110 g (15).

Thermal Perception Testing

Thermal perception testing determines the function of free-nerve endings and their associated unmyelinated and thinly myelinated fibers. It is more valuable to test cold and warm perceptions separately. Only thermal threshold testing is capable of assessing small fiber dysfunction (5,14,16).

One of the simple screening methods for testing thermal perception is tip-therm. It is an instrument with two flat sides of a special polymer and a metal alloy. Due to the different thermal conductivity of both materials, the faces of the instruments are

perceived differently. Compared to the biothesiometer, tip-therm has high specificity (100%) and sensitivity (97.3%) in diagnosing diabetic neuropathy (16).

Vibratory Sensation Test

Vibration perception testing assesses function in the Meissner, and Pacinian corpuscles, and large-diameter fibers. Typical testing sites are the glabrous skin of the fingers and toes (5,14,17). For the vibration perception assessment, a 128-Hz standard tuning fork, vibra tip, Rydel-Seiffer fork, or biothesiometer are used (5).

A 128-Hz standard tuning fork determines the presence or absence of vibration perception. There are two methods of examination: on-off and timing method. In the first method, a tuning fork is applied to the bony prominence bilaterally situated at the dorsum of the first toe. The patient reports the start and the cessation of the vibration sensation. In the timing method, a tuning fork is applied to the same area, and the patient reports the time at which vibration diminished beyond perception. The results compare to the examiner's timing of vibration sensation (17).

There is also a pocket-size device, the VibraTip, for assessing vibration perception in DPN screening. It provides a stimulus of 128 Hz (5).

Quantitative methods, including the Rydel-Seiffer tuning fork (RSTF) and the biothesiometer (5), have been developed to determine VDT. The RSTF allows the testing of various vibration intensities by the patient to assess vibratory discrimination. A triangle and a scale from 0 to 8 imprinted on the weights allow assessment of the vibration threshold (18). After the tuning fork is struck to initiate vibration, its base is applied to a bony prominence. The patient is asked to indicate when the vibration sensation disappears, at which point the examiner records the corresponding value on the triangular scale displayed on the prongs of the tuning fork. Scores range from 8, indicating normal vibration perception, through 5-7 (mild reduction) and 3-4 (moderate impairment), to 0-2, reflecting severe sensory loss or complete absence of vibration perception (19).

The biothesiometer determines VDT on a scale from 0 to 50. It measures VDT by adjusting the amplitude of an electrical vibrator. In contrast to the RSTF, a biothesiometer measures the VDT as the point at which the first sensation of the vibration appears (20). Assessment of VDT using a biothesiometer is quick and reliable. Compared with a tuning fork, the biothesiometer has been reported to be more accurate (21).

New Quantitative Sensory Test

A quantitative sensory test (QST) determines the sensory threshold, which is defined as the minimal energy detected for a particular modality. Using direct patient feedback, QST measures sensory thresholds, such as vibration, cold, warmth, heat, or cold pain. There are two schemes: the method of limits and the method of levels. In the method of limits, a patient indicates as soon as an increasingly strong stimulus is detected or when a decreasing stimulus is no longer felt. In the method of levels, stimuli of defined intensity levels are tested, and the patient is asked to indicate if the stimulus is or is not detected. In the method of limits, the result is dependent on the rate of change of the stimuli and is more variable than the method of levels and will also be affected by a patient's reaction time (22).

Scoring System

Various clinical scoring systems are used to screen DPNs. They may involve symptom scoring, sign scoring, or both (Table 3) (23,24,25,26,27,28,29,30). The scoring system is a suitable and easy-to-perform method for the early detection of DPN. The MNSI is a widely used screening tool, including in the pediatric population. However, it has not been formally validated in children. Research in the adult population has shown a range of sensitivity (35-79%) and specificity (65-94%) compared to NCS, depending on the cut-off value used for abnormality in MNSI (23).

Gold Standard

Skin Biopsy

A skin biopsy is a relatively minimally invasive technique and the specimen is taken from the distal calf. Assessing intraepidermal nerve fibers (IENF) counts, lengths, and densities is key when identifying small fiber neuropathy (SFN). IENFs are the last endings of C and A-delta fibers, participating in pain perception and detection of thermal stimuli (31). There are two evaluation methods for IENFs: immunohistochemical (bright-field) or indirect immunofluorescence with and without confocal analysis. Nerve fiber staining is performed with protein gene product 9.5 (PGP9.5), a member of the ubiquitin hydroxylase system, and PGP9.5 is a non-specific pan-neuronal marker (31,32).

In clinical practice, IENF density (IENFD) is the gold standard for diagnosing conditions with small nerve fiber involvement. Diabetes is the most common cause of SFN, and a decrease in IENFD may be evident in the earliest stage of the disease. Moreover, IENFD can be used to monitor the progression of diabetic SFN, and a decrease in IENFD correlates with the severity of SFN (32).

Mechanoreceptors, innervated by A-beta fiber endings, can also be identified in skin biopsy samples. NCS is a gold standard for diagnosing large fiber neuropathy (LFN). However, skin biopsy allows assessment of early abnormalities of receptors and the distal part of myelinated fibers.

Table 3. Clinical scoring for DPN screening

Test	Description	Score
Signs and symptoms		
Michigan Neuropathy Screening Instrument (MNSI) (23,24)	Consist of 15 questions completed by patient, and physical assessment: appearance of feet, ulceration, ankle reflex, vibration perception at great toe, monofilament testing.	≥7/15 positive results of the questionnaire; ≥2/8 positive results of physical examination.
Toronto clinical neuropathy score (TCNS) (25)	Three items: symptomes (pain, numbness, tingling, weakness, ataxia, upper -limb symptoms), reflex (knee and ankle reflexes) and sensory test score (pinprick, temperature, light touch, vibration sense, position sense).	0-5 points without DPN; 6-8 points mild DPN; 9-11 points moderate DPN; 12 to 19 points severe DPN.
The Douleur Neuropathique en 4 (DN4) (26)	Includes questions for different types of neuropathic pain and a physical exam to test for touch and pin hypoesthesia and tactile dynamic allodynia.	≥4/10 positive result. Available pediatric version
Symptoms		
Neuropathy symptoms score (NSS) (27)	Seventeen items, symptoms of muscle weakness, sensory disturbances, autonomic symptoms .	≥1/17 positive result.
Diabetic neuropathy symptoms score (DNS) (28)	Four questions, simplified scoring system, assessing pain, numbness, tingling and ataxia.	≥1/4 positive result.
Signs		
Neuropathy disability score (NDS) (29)	Thirty-five items evaluating cranial nerve, sensation, reflexes, muscle strength. Revised NDS: ankle reflex, vibration, pinprick and temperature sensation at both sides of the largest toes.	≥2/10 positive result.
Diabetic neuropathy examination (DNE) (30)	Tree items: muscle strength (quadriceps femoris - extension of the knee; tibialis anterior - dorsiflexion of the foot), reflex (triceps surae), sensation index finger (pinprick) and big toe (pinprink, touch, vibration sensation, sensitivity to joint position).	>3/16 positive result.

DPN: diabetic peripheral neuropathy

Autonomic involvement can also be assessed through skin biopsy. In DPN, a significant loss of sudomotor (innervating the sweat glands) and pilomotor (controlling errector pili muscle contraction leading to hair follicle erection) nerve fibers has been demonstrated (32).

NCS

NCS is the gold standard for the diagnosis of LFN (5). The Toronto consensus for a firm diagnosis of LFN requires at least one symptom and/or at least one sign of neuropathy and abnormality on NCS (33). During the examination, small pads are inserted into the skin, deliver mild electric shocks, and detect electric signals. Routine NCS includes evaluation of the motor function of the median, ulnar, peroneal, and tibial nerves and the sensory function of the median, ulnar, radial, and sural nerves. Recommended attributes are amplitude, distal latency, distance, conduction velocity, and F-wave latency. Diagnostic criteria involve the abnormality of one or more attributes in two or more separate nerves to diagnose DPN (34). NCS may not be widely available for routine diagnostic evaluation of DPN. Furthermore, NCS is insensitive for the diagnosis of SFN (35).

Other Methods

Neuropad

A simple indicator test that can detect the early stage of peripheral neuropathy is Neuropad. The Neuropad is applied to the plantar aspect of the first metatarsal head and removed after 10 minutes. It assesses sweat production by the color change of a cobalt II compound from blue to pink. Color change indicates sudomotor dysfunction in DPN, which usually develops before sensory loss. The sensitivity of the test was reported to be 70-83% when validated against the Neuropathy Disability Score, NCS, and VDT (36).

Sudoscan

Sudoscan is a non-invasive, quantitative test to assess sudomotor function using reverse iontophoresis to measure electrochemical skin conductance (ESC) of sweat in the hands and feet. It evaluates the function of small C-fibers that innervate the sweat glands. Degeneration of these fibers results in reduced sweat gland function (37). Validation studies have confirmed good values for sensitivity (70-87.5%) when using foot ESC results to screen

for DPN (38). Global collaborative analysis established reference values in healthy subjects for different ethnic groups, ages, and by gender (39).

Corneal Confocal Microscopy

Corneal confocal microscopy (CCM) is a relatively new, non-invasive diagnostic tool for SFN. Nerve fibers from the trigeminal richly innervate the cornea. Corneal nerve fiber density, corneal nerve branch density, corneal nerve fiber length, and inferior whorl length are the main parameters used. A meta-analysis of over 3,000 participants revealed a reduction in the CCM parameter in patients with DPN compared with healthy controls and those without DPN. In addition, the authors also reported a lesser, but significant, decrease in CCM parameters in diabetic patients without DPN compared with controls, suggesting that CCM may identify subclinical DPN (40). Corneal nerve loss was also observed in patients with only impaired glucose tolerance (41) and in children with T1D (42).

Optical Coherence Tomography Angiography

Optical coherence tomography angiography (OCTA) is a non-invasive tool that enables quantitative assessment of retinal microcirculation. Research highlights the value of OCTA in monitoring neurodegeneration in patients with T1D. The potential of OCTA as an imaging biomarker for evaluating the progression of DPN has been suggested (43).

Risk Factors

Diabetic neuropathy develops as a result of various risk factors, which can be categorized into modifiable and non-modifiable. Identifying and assessing these factors is essential for preventing DPN.

Non-modifiable factors include disease duration, pubertal status, tall stature, ethnicity, and genetic predisposition. Modifiable factors include glycemic control, dyslipidemia, and hypertension.

Non-modifiable Factors

Duration of Diabetes

Long-standing diabetes is a confirmed risk factor for DPN. In SEARCH, in patients with DPN, diabetes duration was significantly longer, but HbA1C values were similar between groups, indicating that longer diabetes duration regardless of metabolic control is a crucial factor in DPN (12).

Tall Stature

In contrast to nephropathy and retinopathy (complications of diabetes, which more often occur in patients with short stature), tall stature was associated with a higher prevalence of neuropathy. Some hypotheses exist, such as patients with longer nerves (and thus a larger total axon surface area) who may be at greater risk for neurologic impairment and have a prolonged time requirement for complete regeneration. The association of height with peripheral neuropathy might be related to the increased hydrostatic pressure in tall patients during standing upright (44).

Puberty

Research has shown that puberty contributes to the development of late metabolic complications of diabetes, such as retinopathy and neuropathy (45). Peripheral nerve function is increasingly impaired during puberty. A cross-sectional study demonstrated an increasing subclinical motor nerve impairment detected during late puberty and after puberty. Reduced sensory nerve conduction velocity (NCV) and sensory nerve action potential amplitude were confirmed. The peripheral nerve fibers of pubertal and postpubertal patients may be the most vulnerable to nerve demyelination and axonal damage caused by poor metabolic control induced by pubertal hormonal changes and poor self-care during puberty (46).

Ethnicity

The prevalence of DPN varies according to ethnicity. Studies of multi-ethnic groups have found a greater prevalence of DPN occurring in Caucasian patients compared to other ethnic groups (47).

Genetic Markers

There are limited data on genetic predisposition to DPN among children with T1D. Single nucleotide polymorphism is a variation at a single position in the DNA sequence among individuals, which can be linked to a higher or lower risk of diseases. A Slovak study confirmed a strong association between the Cytochrome b-245 alpha chain (CYBA) polymorphism *rs4673* and DPN in children and adolescents with T1D. *CYBA* encodes the $p22^{phox}$ protein, a subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which plays a key role in the production of reactive oxygen species (48).

A study by Strokov et al. (49) reported that the polymorphic markers Ala(-9)Val in the *Superoxide Dismutase 2* (*SOD2*) gene and Arg213Gly in the *SOD3* gene were associated with a higher risk of DPN. *SOD2* and *SOD3* encode different isoforms of the enzyme SOD, which plays a key role in protecting cells from oxidative stress (49).

Vascular endothelial growth factor (VEGF) is a signaling protein that plays a critical role in angiogenesis. However, it has been reported that the distribution of a *VEGF* gene polymorphism in the promoter region (-7C/T) differed significantly between

patients with T1D with and without neuropathy, and may be implicated in the pathogenesis of DPN (50).

Modifiable Factors

Hyperglycemia

Hyperglycemia is a well-known risk factor for DPN. A study in children and youth with T1D confirmed an elevated DPN risk with poor glycemic control. EURODIAB determined the association of DPN with the duration of diabetes and highlights a strong relation with glycemic control independent of diabetes duration (9).

Glycemic Variability

In addition to hyperglycemia, glycemic variability (GV) may be another independent risk factor for diabetic complications, but results are inconsistent. Some studies propose potential pathophysiological mechanisms through which elevated GV contributes to the development of DPN. These include the activation of protein kinase C-dependent NADPH oxidase, which subsequently induces oxidative stress. Furthermore, an oxidative stress and inflammatory response is caused by activating the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (51,52).

The study by Oberhauser et al. (53), conducted among children and adolescents with T1D, confirmed that high GV was an unexpectedly strong predictor of slowed NCV. However, there are limited studies on the long-term outcomes of GV during the pediatric period, so further prospective research is needed (54).

Obesity

Obesity is an emerging risk factor for neuropathy, independent of hyperglycemia. Obesity may contribute to the development of neuropathy through several mechanisms, including oxidative stress, insulin resistance, metabolic inflammation, and ischemia, all of which can lead to nerve degeneration (55). Fat distribution is likely more important than general obesity in developing neuropathy. Among adolescents with obesity, the association of central adiposity with insulin resistance and inflammation has been confirmed. This effect is observed regardless of body mass index (56). Visceral adipose tissue is a source of pro-inflammatory mediators, including cytokines such as tumor necrosis factor-alpha and interleukin-6, and is associated with elevated levels of the acute-phase reactant C-reactive protein. These cytokines activate microglia and perivascular macrophages, ultimately promoting demyelination and axonal degeneration. Moreover, an excess of free fatty acids, particularly in central obesity, may cause direct damage to neural structures (57). Studies in childhood cohorts reveal a correlation between obesity and increased risk for neuropathy (58).

Dyslipidemia

Dyslipidemia increases the frequency and severity of micro- and macro-vascular complications in T1D. Large clinical studies like EURODIAB and SEARCH have confirmed the relationship between lipid disturbances and peripheral neuropathy. In EURODIAB, higher levels of total and low-density lipoprotein cholesterol (LDL-c) and triglycerides were significantly associated with the cumulative incidence of neuropathy (9). SEARCH found increased triglycerides, LDL-c, and lower levels of high-density lipoprotein cholesterol (HDL-c) as a risk factor for DPN in youth with T1D. The authors suggested that the lower HDL-c could be one of the crucial factors in the pathogenesis of DPN. HDL-c inhibits inflammation process, oxidation, and thrombosis, as well as vasodilatation via endothelial release of nitric oxide. Increasing HDL-c levels through lifestyle modification (dietary modification, aerobic exercise, weight loss) may be one of the therapeutic approaches (12).

Hypertension

Studies confirmed that hypertension was associated with the prevalence of neuropathy in young people with T1D (59). Hypertension is associated with impaired nerve conduction in T1D (60). Glycemic control has to be supported with strict blood pressure control to prevent and delay the onset of DPN. According to EURODIAB the risk factors for DPN in youth with T1D are increased diastolic blood pressure (9). The angiotensin-converting enzyme inhibitor (ACEI) may improve peripheral neuropathy even in normotensive patients with diabetes (61). Hyperglycemia increases tissue angiotensin II, causing oxidative stress, endothelial damage, and vascular changes. These contribute to DPN and can be diminished by blocking the renin-angiotensin system, which may explain the beneficial effect of therapy with ACEIs (62).

Conclusion

DPN is one of the most common complications of diabetes, which can result in foot ulcers and potentially necessitate amputation. The prevalence of neuropathy in patients with T1D differs, based on the screening methods used and the characteristics of the study groups. Nevertheless, the studies reviewed indicate a relatively high occurrence of subclinical neuropathy, highlighting the need for early detection of risk factors to prevent this complication. Of note, therapeutic options are currently very limited, so early screening and modification of risk factors is very important in patients with T1D.

Footnotes

Authorship Contributions

Concept: Marta Baszyńska-Wilk, Monika Nowacka-Gotowiec, Elżbieta Moszczyńska, Design: Marta Baszyńska-Wilk, Data Collection and Processing: Marta Baszyńska-Wilk, Monika Nowacka-Gotowiec, Analysis and Interpretation: Marta Baszyńska-Wilk, Monika Nowacka-Gotowiec, Elżbieta Moszczyńska, Literature Research: Marta Baszyńska-Wilk, Writing: Marta Baszyńska-Wilk, Elżbieta Moszczyńska.

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REVIEW

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Endocrine Implications of Congenital Disorders of Glycosylation

Yağmur Ünsal, Zeynep Alev Özön

Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye

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ABSTRACT

Glycosylation, attachment of monosaccharides or glycans to specific residues of proteins and lipids, is the most common post-translational modification. Defects among glycoprotein synthesis or modification pathways result in a genetically and clinically heterogenous group of metabolic disorders, congenital disorders of glycosylation (CDGs) with an estimated prevalence of 1/10,000. CDGs have multisystem involvement in which significant neurological dysfunction is frequent, with variable impairment of other organ functions. Most of the proteins responsible for endocrine homeostasis are essentially glycoproteins so disorders of glycosylation have an impact on hormone secretory pathways, changing hormone and carrier protein stability, circulatory half-live and abundance, alternating receptor configuration, activation, hormone-substrate affinity, and resetting endocrine control and feedback loops. Endocrine implications of CDGs are extensive and are described in up to 55% of all patients with CDGs during the natural course of the disease. This frequency is increased up to 85% in some CDG subgroups. Impacts on growth and growth factors, thyroid hormones, hypothalamo-pituitary-adrenal axis, hypothalamo-pituitary-gonadal axis, glucose metabolism, bone health and prolactin have been reported, yet clinical studies are scarce, with data mostly derived from case series. The aim of this review is to describe the current understanding of the endocrine implications of CDGs, focusing on both preclinical and clinical studies, highlighting the broad spectrum of findings. Clinical and laboratory findings of CDGs and the effect of current treatment strategies on endocrine function will be briefly discussed.

Keywords: Congenital disorders of glycosylation, endocrine, growth, bone, thyroid, adrenal, hypogonadism

Introduction

Glycosylation is the covalent attachment of monosaccharides or glycans (polysaccharides) to selected residues of target proteins and lipids, occurring in diverse subcellular locations but mostly within the endoplasmic reticulum and Golgi apparatus (1). Glycosylation is the most common post-translational modification of proteins. Its biological role is essential for normal protein maturation and function, ensuring maintenance of protein solubility, proper protein folding and conformation, which in turn maintains tissue structure, integrity and porosity (2,3). In addition, glycosylation of proteins controls leukocyte, extracellular and intracellular trafficking, protease resistance, cell-substrate and cell-cell interactions, and growth regulation (4). Therefore, glycosylation is central to normal development, growth, and functioning of the organism.

Defects among glycoprotein synthesis or modification pathways result in a genetically and clinically heterogenous group of metabolic disorders, known as congenital disorders of glycosylation (CDGs)

Corresponding Author: Yağmur Ünsal, MD, Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye
E-mail: yagmurunsal@yahoo.com **ORCID:** orcid.org/0000-0002-9113-8683
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(5). After the initial report in 1980, the clinical and biochemical features as well as the natural course of CDGs were identified in 1991. Initially, solely associated with phosphomannomutase deficiency, this was called "carbohydrate deficient syndrome" in 1997 (6). Advances in biochemical testing and exome sequencing enabled discovery of new forms and today, more than 130 separate CDGs have been described (7,8). Most of these monogenic diseases are autosomal recessive (AR) in inheritance, however autosomal dominant (AD) and X-linked forms have also been described (9).

CDGs exhibit multisystem involvement, usually with significant neurologic dysfunction in addition to variable impairment of other organ functions. There have been reports of cardiovascular, gastroenterological, renal, hematological, immunological, ophthalmological, and lipid metabolism impairments, as well as endocrinopathy. Since proteins involved in endocrine homeostasis are essentially glycoproteins, reports of endocrine symptoms are common. Alterations in glycosylation have an impact on hormone secretory pathways, modulating circulatory half-lives, changed stability, carrier protein availability and binding, alternating receptor configuration, binding, and activation, dysregulation of autocrine and paracrine actions, and resetting of endocrine control and feedback loops (10). Endocrine findings make up 5-7% of symptoms at presentation with hypoglycemia being the most common, reported in 7% of CDGs. Endocrine changes are diverse and are described in up to 55% of all patients with CDGs (n=280) during the natural course of the disease. This frequency is increased up to 85% in some CDG subgroups. In a recent natural history study, findings regarding virtually every endocrine axis have been described. Alterations in thyroid and adrenal function, growth, sexual development and bone and glucose metabolism have been reported (4).

In this review we will briefly address clinical and laboratory findings of CDGs, then focus on endocrine system involvement, highlighting the broad spectrum of findings. In addition, the effect of current treatment strategies on endocrine function will be discussed.

Epidemiology

The incidence and prevalence of all types of CDG is not well established, since some forms are quite rare. Globally, reports of CDGs from practically every ethnic group have been made and both sexes are equally affected (9). The estimated prevalence in European and African-American populations is 1/10,000 (11,12,13). The prevalence of the most common CDG, PMM2-CDG ranges from 1/20,000 in Dutch populations to 1/77,000 in Estonia based on isolated reports (9). However, to date, fewer than 100 cases have been reported for most CDG types (9).

Biochemical Classification, and Nomenclature

Historically, CDGs were classified by patterns of transferrin isoform analysis. Currently CDGs are classified into four groups: (I) *N*-linked glycosylation; (II) O-linked glycosylation; (III) combined N- and O-linked/multiple glycosylation; and (IV) lipid and glycosylphosphatidylinositol anchor biosynthesis defects (9).

CDG nomenclature was revised in 2008 to reflect molecular etiology considering the developments in molecular diagnostics. Currently, CDG nomenclature is denoted by the name of the affected gene, followed by-CDG (e.g. *PMM2-CDG*) (14).

Inheritance

The majority of CDGs are AR, while a small number of CDGs are AD, including GANAB-CDG, PRKCSH-CDG, EXT1/EXT2-CDG, POFUT1-CDG, and POGLUT1-CDG. A few are known to be X-linked and these are ALG13-CDG, SSR4-CDG, PIGA-CDG, SLC35A2-CDG, and ATP6AP1-CDG. Most dominant and some X-linked forms of CDG are due to *de novo* mutations (9). Genotype-phenotype correlations have been proposed, however, there is significant phenotypic variability, even within the same genotype (15).

Clinical Findings

Glycosylation is critical in many metabolic pathways, thus, there is considerable clinical heterogeneity in symptoms and findings (4). Age of onset and disease severity is diverse. Neonatal lethal forms, as well as nearly asymptomatic adults have been reported. The most frequent presentation is multi-systemic involvement within the first few years of life. Almost any organ system may be affected, the nervous system is the most frequent system involved (76%) (16,17). Mild to severe psychomotor retardation, hypotonia, cognitive difficulties, epileptic seizures, ataxia, polyneuropathy, and stroke-like events have been reported. Hypoglycemia, and various liver, eye, skin, gastrointestinal, immunological, skeletal, and coagulation disorders, are commonly noted. Nearly all patients experience feeding difficulties and failure to thrive (9,17,18,19).

The most common form, PMM2-CDG, causes neurologic symptoms with intellectual disability affecting 96% of patients, cerebellar ataxia and atrophy in 96%, hypotonia in 92% and peripheral neuropathy in 53%. PMM2-CDG has also been reported to involve cardiac (pericardial effusion in72% and cardiomyopathy in 25%) and gastrointestinal systems, with failure to thrive in 67%, hepatomegaly in between 18-100% of patients and hepatopathy again ranging widely from 12.5 to 100%. Thoracic deformities (84%) and kyphoscoliosis (53%), typical dysmorphia [inverted nipples (53%) and lipodystrophy (47%)], coagulopathy and recurrent infections (39%) have also been reported. Adults show stable neurologic findings, but kyphoscoliosis and osteoporosis are progressive (20,21,22,23).

Some CDGs affect only a single organ system. These include the retina in DHDDS-CDG; neuromuscular junction in ALG2-CDG, ALG14-CDG, CFPT1-CDG; brain in ST3GAL3-CDG, TUSC3-CDG; skin

or skeletal muscle in POGLUT1-CDG, POFUT1-CDG; cartilage in EXT1/EXT2-CDG; liver in TMEM199-CDG; and red blood cells in SEC23B-CDG (9). Clinical manifestations of CDGs are summarized in Table 1, in addition to endocrine manifestations.

Since many CDG subtypes are rare, the complete phenotype is still unclear. Therefore, in cases of multi-systemic disease, CDGs should be considered, particularly when there is developmental delay of unclear etiology. With a greater understanding of this subset of complex disorders, CDGs have become more frequently recognized in people with unexplained symptoms involving more than one system.

Diagnosis

Serum carbohydrate deficient transferrin analysis is the first-line screening test in patients suspected of CDG (9). This diagnostic test is performed by isoelectric focusing or capillary electrophoresis, gas chromatography/mass spectrometry, capillary electrophoresis/electrospray ionization/mass spectrometry or matrix-assisted laser desorption ionization/mass spectrometry to determine the presence and number of incomplete sialylated *N*-linked oligosaccharide residues linked to serum transferrin (24). Second-line tests include dolichol-linked glycan analysis and genetic testing, either by single gene, *CDG* gene panel or whole exome/whole genome sequencing.

Novel approaches studying whole plasma/serum N-glycome by various mass spectrophotometric techniques may identify defects on glycoproteins other than transferrin, which is especially useful in some CDGs characterized by normal transferrin profiles. Moreover, metabolomics and metallomics offer exciting prospects in both clinical practice (biomarkers for precision diagnostics, disease follow-up, treatment management) and in research (better understanding of the role of actors in glycosylation, application to the development of novel pharmacological agents) areas (25). Early and specific diagnosis of CDG subtype is imperative as there is a possibility of specific pharmacological treatment in some forms (8,9,26).

Endocrine Implications

In the initial report, most striking features involved the endocrine system. In 1980, Jaeken et al. (7) described identical twin-sisters with familial psychomotor retardation and endocrine findings proposing that these findings may be a part of a new syndrome. Born at 36 weeks with normal birthweight, the twins presented at two years of age with marked psychomotor retardation and a bone-age of one year. Physical examination and anthropometric measures were normal. Repeated investigations revealed markedly fluctuating serum prolactin (PRL), follicle stimulating hormone (FSH) and growth hormone (GH) levels, partial thyroxine binding globulin (TBG) deficiency, increased serum arylsulphatase A and increased cerebrospinal fluid protein, which were also present in the father (7). Since this initial report, findings in patients with CDGs have reported changes in virtually every endocrine axis. These CDG-associated alterations in endocrine systems are discussed in detail below.

Implications of CDG for Growth and Growth Factors

Growth rate of the initially described twin sisters were normal but later studies demonstrated growth failure in different CDG types due to a variety of reasons. Babovic-Vuksanovic et al. (27) reported a 30-month-old girl who presented with recurrent severe and persistent hypoglycemia, and who developed feeding difficulties, protein losing enteropathy and short stature (3rd percentile). She was diagnosed with MPI-CDG. Following specific treatment with mannose for six months, she exhibited catch-up growth (43rd percentile) (4,27). A similar patient with MPI-CDG whose anthropometric measures were at the 2nd percentile on admission, was reported by Hendriks et al. (28). Catch-up growth was attained after mannose treatment (25th percentile) (28). In a case reported by Miller et al. (29), a child with PMM2-CDG had severe growth failure, insulin like growth factor-1 (IGF-1) levels were low, and response to IGF-1 generation test was absent, suggestive of GH resistance. Catch-up growth was attained following treatment with recombinant human IGF-1 (29). Alsharhan et al. (5) reported two patients with ALG3-CDG diagnosed with panhypopituitarism [hypothyroidism, GH deficiency, and adrenal insufficiency (AI)]. Response to GH treatment has not been published yet.

Jaeken (30) summarized the natural course of growth in 29 patients with PMM2-CDG. In their study, birth weight, height, and growth rate in the first years of life were normal. Growth rate declined between the second and third years and a successive decline was noted in the following years. After adolescence, disproportionate short stature was observed in all cases, the majority of which were associated with marked skeletal deformities (kyphosis, kyphoscoliosis and vertebral fractures), leg length was normal when assessed (30). Similarly, the largest longitudinal study of children with PMM2-CDG concluded that anthropometric measures were within normal range at birth. Linear growth was restricted in the first six to nine months of life [-2.4 standard deviation score (SDS)]. Although a slight improvement was observed at the end of the second year (mean height -1.8 SDS), this improvement was not sustained and catch-up growth was not observed until 10 years of age. It was suggested that feeding difficulties could both contribute to and serve as the primary cause of severe growth failure (31).

The main determinant of growth early in life is quality and quantity of nutrition while this becomes endocrine regulation after the first two years (32). The GH/IGF cascade being the predominant factor, thyroid, adrenal hormones, and sex steroids regulate growth, differentiation, and metabolism. Circulating

IGFs (IGF-1 and IGF-2) which are GH-dependent, exert pleiotropic effects through activation of IGF receptors (IGF-1R and IGF-2R) and the insulin receptor (IR) signaling cascade (33). It is known that 10-15% of IGFs form binary complexes with IGF-binding proteins (IGFBPs: IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-6) while 80-90% form ternary complexes with IGFBPs (IGFBP-3, IGFBP-5) and acid-labile subunit (ALS) in the circulation. Formation of ternary complex is essential for stability and delivery of IGFs to the target tissues (34,35,36,37). Although IGFs, IGFBP-1 and IGFBP-2 are non-glycosylated, the components of the ternary complex are glycosylated with IGFBP-3 and ALS being N-glycosylated, and IGFBP-5 being O-glycosylated). IGFBP-4 and IGFBP-6 have also been shown to be glycosylated.

Previous *in vitro* studies presented the significance of optimum glycosylation on stability and function of the ternary complex. The number of N-glycosylation sites in ALS was proven to enable IGF1/IGFBP-3/ALS ternary complex formation (38,39,40). Enzymatic hypoglycosylation of ALS in mouse models reduced the affinity of ALS for IGFBP-3/IGF complexes by 50-100% depending on its extent (41). Despite *N*-linked glycans on IGFBP-3 being non-essential for ALS or IGF binding, it was postulated that glycosylation may modulate other biological activities of IGFBP-3, such as extracellular matrix binding (38). Indeed, non-glycosylated IGFBP-3 had a shorter half-life than glycosylated IGFBP-3 when administered to rats (41). In a study conducted on peripheral blood lymphocytes from 12 patients with CDG (patients were grouped according to previous nomenclature), compared to healthy controls, IGF-I levels were normal in CDG type-I and significantly reduced in CDG-II. IGF-1R was significantly reduced in lymphocytes and markedly reduced in carbohydrate content. Selective impairment in IGF-1-induced synthesis of DNA was reported. All patients displayed impaired mitogenic response. Although the study was limited to changes observed in lymphocytes, it was proposed by the authors that the results may be inferred to all tissues (42).

These *in vitro* findings were supported by *in vivo* studies. In a study of 26 patients (12 female and 14 male; 1-20 years of age) with CDG type 1 (7 longitudinally, 19 cross-sectionally) by de Zegher and Jaeken (10) it was reported that basal serum GH concentrations were normally low (GH: <10 mcg/L) in the majority of the boys (13/14) and 13.5 mcg/L in a 2 month-old boy, while basal serum GH concentrations were high in most of the girls (8/12) and extremely high in three girls (basal serum GH of one-month-old, two-month-old and 2-year-old girls were 192, 120 and 144 mcg/L, respectively). Longitudinal assessment of basal serum GH concentrations of three girl with high basal serum GH were documented to be <20 mcg/L within the following six months. Upon glucagon stimulation, biphasic GH hyper-responsiveness was observed in the initially described twin sisters. The same study reported that IGF-1 levels were low during infancy and low-normal during childhood and adolescence (10). Miller et al. (41) compared 12 patients with PMM2-CDG with age matched healthy controls in terms of the GH/IGF-1 axis. The study found that children with PMM2-CDG displayed significantly decreased (~50%) levels of both glycosylated (ALS and *IGFBP3*; 50%) and nonglycosylated (IGF-1 and IGF-2) components of ternary complex compared to controls (41). Reduced ALS glycosylation was shown to result in a neutral shift in the isoelectric point leading to a reduction in the affinity of ALS for IGFBP-3-IGF binary complex. Ternary complex formation was subsequently decreased. In this study, adequate nutrition, and oral mannose treatment in a child with MPI-CDG was shown to partially correct hypoglycosylation of ALS and IGFBP-3, enabling catch-up growth (41).

Growth failure is common in the majority of CDGs. It is likely that in addition to changes in GH/IGF-1 axis, feeding difficulties and nutritional factors as well as concomitant endocrine manifestations (hypothyroidism, AI, hypogonadism) and skeletal features lead to growth failure (Table 1). Management of these accompanying factors will help restore growth rate. Further studies are necessary to determine the extent to which protein hypoglycosylation negatively impacts function and stability of GH/IGF-1 axis, as well as to demonstrate natural growth and efficacy of GH treatment in children with CDG.

Implications of CDGs for Thyroid Hormones

Normal thyroid hormone biosynthesis starts with iodide uptake from circulation across the basolateral membrane of thyrocyte by the sodium-iodide symporter, which coordinates electrogenic symport of two sodium ions for one iodide ion down an electrochemical gradient generated by the Na+/K+ ATPase. Specific transporters (pendrin and anoctamin-1) then mediate iodide efflux into the follicular lumen. Synthesized by thyrocytes, thyroglobulin is the protein skeleton for thyroid hormone biosynthesis. Iodide is oxidized when hydrogen peroxide is present and incorporated into tyrosyl residues on the surface of thyroglobulin to form monoiodotyrosyl (MIT) and diiodottyrosyl (DIT) (organification). MIT and DIT couple to form thyroid hormones (T4 and triiodothyronine). Thyroid peroxidase (TPO) catalyzes hydrogen peroxide-dependent oxidation, organification and coupling of iodine. DUOX2 (a NADPH-oxidase) and its accessory protein, DUOXA2, are the predominant sources of hydrogen peroxide (43). Thyroglobulin is endocytosed back into thyroid follicular cell then cleaved and thyroid hormones are secreted into circulation. Thyroid hormone biosynthesis is regulated by thyrotropin [thyroid-stimulating hormone (TSH); secreted from the anterior pituitary] and thyroid hormones are carried by TBG albumin and transthyretin in the circulation.

Most of these proteins involved in thyroid hormone biosynthesis, regulation and secretion are essentially glycoproteins, and

glycosylation defects cause significant alterations to thyroid function and/or homeostasis. TBG hypoglycosylation has been shown to reduce the half-life by 15% (20). One of the initially described findings frequently reported in patients with CDG is low levels of TBG (75%) (44,45). Despite a decrease in total thyroid hormone levels, free hormone levels and TSH are reported to be normal (4,46). Although free hormone levels might be low via indirect measurement techniques from time to time, results with direct measurement techniques were normal (43). The most frequently described scenario is euthyroid with low TBG, however, as summarized above, proteins taking part in thyroid hormone biosynthesis, regulation and secretion are glycoproteins, and disorders of glycosylation may result in alterations in thyroid function as well (47). Furthermore, TBG, TPO, pendrin, DUOX2, and DUOXA2, which are essential for thyroid hormone biosynthesis, all achieve proper folding to three-dimensional functional conformation after post-translational modifications consisting of glycosylation (48,49,50,51,52,53,54). TSH was shown to have reduced bioactivity and receptor affinity due to lack of glycosylation (55,56,57). Given this high degree of glycosylation, CDGs may lead to several alterations in thyroid hormone levels, for example low total T4 and TBG with increased TSH which may raise suspicion of hypothyroidism, low free T4 (FT4) that is biochemical hypothyroidism but without clinical hypothyroidism (euthyroid). Clinical assessment of hypothyroidism may be difficult since developmental delay and neurologic findings are also common features of CDGs. However, for optimal development and metabolism, clinical hypothyroidism must be corrected (45).

Clinical hypothyroidism is rare in patients with CDG, however, abnormal thyroid function tests, like decreased TBG are frequently reported, especially in PMM2-CDG (4,46,58). Elevated TSH has been reported, especially during infancy (10,59). A study evaluating thyroid function in 18 patients with PMM2-CDG revealed positive neonatal screening test results in 10, elevated TSH levels in nine, and low FT4 in two. During follow-up, TSH elevation was transient in 3/9 and treatment was not started. Six patients were started on thyroid hormone replacement therapy (HRT) due to multi-organ failure in first few months of life (3/6), persistently elevated TSH during neonatal period (1/6) and elevated TSH accompanied by clinical signs of hypothyroidism such as low calorimetric measurements (decreased resting energy expenditure), low body temperature, constipation, and myxedema (2/6). Apart from the latter two patients with clinical findings of hypothyroidism, the reason for treatment was TSH elevation (60). A review of seven patients with PMM2-CDG, MPI-CDG, or ALG6-CDG reported low levels of serum TBG and T4 concentrations, but normal FT4 and TSH levels. Most of the reported ALG6-CDG patients were euthyroid (46,60). Two patients with MPI-CDG had TBG deficiency with normal TSH and FT4 (61). Alsharhan et al. (5) reported 10 patients with ALG3-CDG, four of which had central hypothyroidism and three required thyroid HRT. Other CDG subtypes with abnormal thyroid assessments include ALG1-CDG and ALG8-CDG (62).

In conclusion, hypoglycolysation of proteins essential for normal thyroid hormone biosynthesis may result in alterations of thyroid hormone and TSH levels. Thyroid function tests of individuals with CDG may be difficult to assess since clinical symptoms of hypothyroidism may be masked or mimicked by other, more severe CDG symptoms, and the diagnosis of hypothyroidism may be complicated (60). In critical circumstances (hypoalbuminemia, sepsis, protein-losing enteropathy, etc.), re-measurements of TSH and FT4 are recommended, and only patients with clinical signs of hypothyroidism should receive thyroid hormone replacement treatment after ruling out euthyroid sick syndrome (5). Long term follow-up for thyroid hormones is not established yet for CDG, but individuals with PMM2-CDG should have their thyroid hormone levels checked every six months, for the first two years of life, and once a year after that, to monitor for hypothyroidism (5). Considering the changes in thyroid hormone biosynthesis, it may be reasonable to generalize this suggestion to all CDGs (Table 2).

Implications of CDGs for Hypothalamo-Pituitary-Adrenal Axis

The hypothalamo-pituitary-adrenal axis (HPA) has a central role in regulating response to internal and external stressors. The hypothalamic parvocellular nucleus collects and integrates neuronal and humoral inputs to synthesize and secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the hypophyseal portal system (63). Activation of CRH-receptor 1 via CRH and AVP, initiates synthesis of adrenocorticotropic hormone (ACTH) from proopiomelanocortin. This step is catalyzed by prohormone convertase 1/3 enzyme and ACTH is then released from the anterior pituitary into the circulation (64,65). Upon reaching the adrenal cortex, ACTH binds to melanocortin 2 receptor (MC2R) which in turn stimulates synthesis and release of cortisol (66,67). Cortisol is mainly carried bound to proteins, such as albumin and corticosteroid binding globulin (CBG) in the circulation, and CBG regulates cortisol bioavailability (68). Proteins of the HPA such as CRHR1, MC2R, prohormone convertase 1/3, and CBG are N-glycosylated glycoproteins (69,70). The number and presence of *N*-linked glycosylation sites in the CRHR family were shown to have an important role in ligand binding and signal transduction. Disordered glycosylation could therefore lead to decreased ACTH and cortisol production (71). Furthermore, hypoglycosylation may decrease the steroid-binding capacity of CBG, diminishing total cortisol levels, and resulting in low to normal free cortisol.

The largest series evaluating the effect of CDGs on the HPA stemmed from an international natural course study with

contributions from many centers, observing 139 patients with PMM2-CDG longitudinally for four years with 6-monthly assessments (71). Čechová et al. (71) evaluated adrenal functions of 43 patients (20 girls) with available ACTH and cortisol data. Since cortisol was <5 mcg/dL without an increase in ACTH in 11 patients, central AI was suspected. Two patients were diagnosed with central AI and hydrocortisone was started. One of them was diagnosed during evaluation for hypoglycemia and accompanying GH deficiency as well as central hypothyroidism. Three of the remaining nine patients had normal peak cortisol response to low dose ACTH stimulation test. Despite slightly low response in one patient, hydrocortisone was not started. Low dose ACTH stimulation test was planned in five patients. Primary AI was not suspected in any of the patients (71). Another study described normal serum cortisol levels with diminished serum CBG in a patient with PMM2-CDG. These findings were attributed to loss of CBG due to protein-losing enteropathy (10). Other forms of CDG with reported AI are ALG6-CDG and ALG3-CDG.

In conclusion, glycosylation defects of proteins responsible for regulation of the HPA axis as well as hypoproteinemia due to protein losing enteropathy may lead to AI in patients with CDG. Annual evaluation of morning cortisol and ACTH were recommended in patients with PMM2-CDG. If abnormal, low dose ACTH stimulation test can be performed to evaluate the HPA axis (71) (Table 2).

Effects of CDG on the Hypothalamo-Pituitary-Gonadal Axis

Hypothalamic gonadotrophin-releasing hormone (GnRH) is the central regulator of synthesis and release of the pituitary gonadotrophins, luteinizing hormone (LH) and FSH which are essential for normal sexual development, maturation, and reproductive function (72). GnRH is synthesized in hypothalamic neurons and is secreted into the hypophyseal portal circulation to act primarily on the anterior pituitary by binding to the GnRH receptor (GnRHR) (73). Variations of GnRH pulsatility (pulse frequency) and amplitude have separate effects on FSH and LH (74). LH and FSH are secreted into peripheral circulation, acting on ovaries or testes to regulate folliculogenesis, ovulation, spermatogenesis, and steroidogenesis. FSH and LH are glycoprotein hormones with similarities in their chemical structures. Variation in glycosylation and glycan composition may lead to considerable heterogeneity in terms of half-life and bioactivity (75). Reduced FSH bioactivity and bioavailability was associated with delayed puberty (10,76). FSH and LH receptors (FSHR and LHR) are also glycosylated proteins. FSHR has more glycosylation sites than LHR and therefore it is presumed that FSHR function is more likely to be affected by impaired glycosylation and may result in hypergonadotropic hypogonadism (77). GnRHR is also a glycosylated protein, whether its glycosylation status has an influence on the pathophysiology of hypogonadism remains to be elucidated.

Impaired glycosylation of LH and FSH in patients with PMM2-CDG results in a variety of changes in the hypothalamo-pituitary-gonad axis. In the initial report of the twin sisters with CDG, fluctuating basal serum gonadotropin levels were described with respect to age. FSH was particularly elevated during infancy (normal, <5 IU/mL) and adolescence (normal, <20 IU/mL) while LH was consistently increased during adolescence (normal, <20 IU/mL). Following GnRH administration, at 2 and 13 years of age, a homogeneous hyper response of FSH and LH (serum levels at 13 years higher than that at 2 years) were noted. When secretory patterns were evaluated by deconvolution analysis of serum profiles during these tests, the serum half-life of LH was extremely long, and FSH bursts were delayed and prolonged. A woman with MPI-CDG and one with ALG6-CDG had normal levels of LH, FSH, and estradiol (61,77,78,79,80).

The effect of CDGs on puberty is variable. Delayed puberty, pubertal arrest, amenorrhea, hypergonadotropic and hypogonadotropic hypogonadism, as well as normal puberty and menstruation have been described (10,20,81). Patients with PMM2-CDG generally had hypergonadotropic hypogonadism (7/8) (30). Pérez-Dueñas et al. (82) followed two patients with PMM2-CDG until adulthood. One had hypergonadotropic hypogonadism while the other (milder phenotype) had a normal menstrual cycle (82). In other reports, 6/6 females with CDG syndrome type I had hypergonadotropic hypogonadism and the ovaries were not detected in 3/6 (76). Hypogonadotropic hypogonadism was described in four adolescent girls (10). Three woman with MPI-CDG developed normal puberty and delivered three healthy children after uncomplicated pregnancies (61,78,79,80). A patient with ALG6-CDG had regular menses with normal hormonal profile (77).

In contrast to females with CDG, males mostly expressed age appropriate secondary sex characteristics and underwent normal pubertal development (76). Some reports have described boys with normal puberty and virilization, but small testes, and low-normal testosterone values (10,76). A study described 2/3 male patients who passed 16 years of age and had normal puberty and secondary sex characteristics (30).

HRT in patients with hypogonadism is critical for initiation and progression of puberty, achieving appropriate body composition, optimum bone health, and increased health related quality of life. However, congenitally deficient or dysfunctional major natural anticoagulants (antithrombin III, protein S, and protein C) in CDGs are associated with life-threatening coagulopathies early in life. Though not always evident at birth, Eklund et al. (83) suggested that it would be prudent to consider CDGs in the category of thrombophilia wherein administrating HRT should be approached with caution. Guidelines for optimum HRT in CDG have not been established. Eklund et al. (83)

selected transdermal estrogen replacement in four patients with hypergonadotropic hypogonadism and PMM2-CDG to mimic physiology. All of them experienced deep vein thrombosis, even though one was on prophylactic anticoagulant therapy (20,83). It was suggested by the study that further research is necessary to determine the best combination of estrogen and anticoagulants, timing of treatment, and duration of primary prophylaxis (83).

Effects of CDG on Glucose Metabolism

Hypoglycemia was initially described in three patients with MPI-CDG. Hyperinsulinism that responded well to diazoxide was documented (9). Though infrequent, hypoglycemia has been described in other types of CDG. PMM2-CDG is the most common CDG to manifest with hypoglycemia. Vurallı et al. (84) reviewed these cases. Hypoglycemia was reported in 37 (3.4%) of the total of 1,060 patients published. While most of the cases presented with a wide spectrum of multisystem involvement, 24% was admitted solely due to symptoms of hypoglycemia, occurring in the first year or in the early months of life (84). In an MPI-CDG case series, hypoglycemia was present in majority of the patients and classified as a potential presenting sign, however it was rarely isolated. Hypoglycemia mainly manifested during infancy (first hypoglycemia observed from perinatal period to three years of age) with a mean age of 6.8 months (61).

The mechanism by which hypoglycosylation may cause hyperinsulinism in patients with CDG is yet to be explained. Since hyperinsulinemic hypoglycemia (HH) in patients with CDG usually responds well to diazoxide, hyperinsulinemia was suggested to have resulted because of disorders of glycosylation in the ATP-sensitive potassium (KATP) channel of the pancreatic beta cells (84). Physiologically, glucose metabolism alters intracellular the ADP/ATP ratio resulting in insulin secretion. ATP-channel binding induces channel closure, depolarization of the membrane, and activation of voltage-dependent calcium channels, leading to calcium influx, and exocytosis of insulin granules (85). So, KATP channels couple glucose metabolism to membrane electrical activity and insulin release. KATP channels containing four ion channels (Kir6.2) and four regulatory SUR1 receptors control glucose-stimulated release of insulin. Proper glycosylation of SUR1 is required for expression of KATP channels. Change in glycosylation of SUR1 in CDG is presumed to precipitate HH (4,86). Furthermore, insulin exerts its effects upon binding to the IR. The IR consists of two extracellular subunits and two transmembrane subunits which are both glycoproteins. Whether glycosylation (or hypoglycosylation) of IR has a role in HH pathogenesis still needs to be delineated (87).

Out of the 25 cases with hypoglycemia described by Vurallı et al. (84), (36%) were due to HH (9). Though patients responded well to diazoxide, one with permanent HH required pancreatectomy due to treatment side effects (20,84). While patients with transient HH were alive, 4/9 of the cases with permanent HH died (84). The most common cause of hypoglycemia was HH in MPI-CDG (61). Two cases with HH due to MPI-CDG were treated initially with a combination of frequent meals and diazoxide. However, after mannose was started, rapid tapering of diazoxide without recurrent hypoglycemia was possible (27,88). Rapid resolution of hypoglycemia following mannose administration in patients with MPI-CDG supports the notion that glycosylation is required for maintenance of normoglycemia. Other forms of CDG associated with HH are PGM1-CDG and ALG3-CDG (4,89,90). As HH was documented in several patients, one could also speculate that glycosylation is important in the regulation of insulin secretion, possibly via the SUR1 receptor. However, HH was not detected in three of the reported patients with hypoglycemia in another study (91). It should be kept in mind that poor feeding, feeding intolerance, vomiting, diarrhea, and hepatic dysfunction may also contribute to hypoglycemia. Optimization of feeding is advised for better glycemic control (84).

Skeletal Features and Effects of CDG on Bone Mineral Density

Skeletal abnormalities (kypho/scoliosis, severe spinal cord deformities and vertebral compression fractures), joint laxity or contractures and osteopenia are common in PMM2-CDG (15,92). Usually diagnosed during childhood, these findings effect health related quality of life (20). Fractures are common and appear to heal normally. Fibrillar collagen type I and II, which are initially synthesized as procollagens, are present in tendon, skin, epiphyseal growth plate and hyaline cartilage, making up the extracellular matrix of bone. Post-translational modification of procollagen I and procollagen II results in mature collagen I and II, characterized by a triple helical domain with fibrillar properties. This modification is achieved by N-glycosylation of C-terminal procollagen and subsequent cleavage of the N- and C-terminal propeptide domains. Since mutations of genes encoding type I and II collagen are known to cause skeletal dysplasia, it may be speculated that abnormal N-glycosylation at this step may result in a skeletal phenotype in some CDGs. However, skeletal features of CDGs are variable. This variability has been explained by the hypothesis that skeletal phenotype is a combined result of glycosylation defect and individual polymorphisms in genes responsible for bone and skeletal development (93).

In addition to that, dentin and extracellular matrix of bone contains non-collagenous proteins (osteopontin, bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein) which have a role in inhibition of bone mineralization and regulation of osteoblast activity. Defects in glycosylation will affect function of these glycoproteins, disrupting bone mineralization. Osteopenia as well as exostoses described in some forms of CDG may potentially be a result of the imbalance between bone formation and

resorption because of altered glycosylation of extracellular matrix proteins responsible for remodeling (92). Heparan sulphate, a glycosaminoglycan usually found on cell surface and extracellular matrix, and fibrillin, an extracellular matrix glycoprotein, play an integral role in the structural integrity of various tissues with roles in ligand binding, cell adhesion and cell singling (94,95). Whether hypoglycolysation of glycosaminoglycans and extracellular matrix glycoproteins play a role in the skeletal dysplasia phenotype is a subject worth investigating since the skeletal phenotype of CDGs and mucopolysaccaridoses are different from each other. It is known that bone phenotype in CDGs is multifactorial. Contributing factors are feeding difficulties, malabsorption, hepatic dysfunction, restrictions in movement as well as hypogonadism and short stature (15,93,96).

Skeletal dysplasia and associated short stature are well characterized in CDGs, including ALG12-CDG, ALG3-CDG, ALG9-CDG, ALG6-CDG, PGM3-CDG, COG7- CDG, COG1-CDG and COG8-CDG and TMEM165-CDG (Table 1) (97,98,99). Short stature, generalized osteopenia/osteoporosis and epi- and meta-physeal dysplasia were reported in patients with TMEM165-CDG (100,101,102). Osteopenia/osteoporosis in CDG was mostly reported in PMM2-CDG, TMEM165-CDG, B4GALT7-CDG and B3GAT3-CDG (15,99,103).

As portrayed, glycosylation is essential for proteins involved in the development of cartilage and bone, as well as in skeletal patterning pathways. CDGs should be considered in the differential diagnosis of skeletal dysplasias and defects in cartilage development, especially if systemic involvement is present (15). Furthermore, evaluation of bone health is generally overlooked in CDGs. Annual evaluation of conventional lateral lumbar radiography is advised in patients with normal levels of serum calcium, phosphate, and magnesium. If fractures are observed, measurement of bone mineral density via dual energy X-ray absorptiometry should be considered. Restoration of accompanying complications associated with CDG, like malnutrition, hypovitaminosis D, enteropathy, hypogonadism, coagulopathy, and hepatic dysfunction is crucial for bone health, and physical therapy will also improve bone mineralization. Patients with scoliosis should have regular orthopedic assessment and intervention, and assessment for atlantoaxial instability is also required. PGM1-CDG and TMEM165-CDG, characterized by under glycosylation of B-glycans were shown to improve glycosylation status following galactose supplementation (104,105). Mannose in MPI-CDG is known to restore other endocrine system functions as well as enteropathy (27,78). However, it is not yet clear whether these disease specific treatments have an impact on skeletal phenotype. Long-term prospective observational studies are needed to evaluate bone health in CDGs and to inquire whether disease specific treatment as well as supportive management has an influence on findings.

Effects of CDG on Prolactin Level and Function

Change in PRL level was one of the initially described endocrine manifestations of CDG (7). PRL is a protein hormone (106). Numerous variants of PRL have been identified, many of which result from post-translational modification (phosphorylation, glycosylation, sulfation and deamidation) of mature PRL protein (107). The PRL receptor is a class 1 receptor composed of three major domains (extracellular, transmembrane, and intracellular). The extracellular domain is known to be glycosylated. However, neither variants in the structure nor variation of the polysaccharides associated with the PRL receptor have been identified (108). Glycosylation defects are expected to alter serum PRL levels and PRL signaling because of the posttranslational modifications described in both PRL and its receptor. However, currently this area of endocrine manifestation remains largely unexplored.

Table 1. Summary of CDG with reported endocrine manifestations

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
Disorders of *N*-linked glycosylation						
PMM2-CDG (212065)		Phosphomannomutase 2	AR	-Failure to thrive, feeding difficulties -DD (normal in 10%, borderline in 2%; mild in 27%, moderate in 28%, severe in 30% and profound in 3%), microcephaly, seizures, hypotonia, ataxia, hyporeflexia, stroke like episodes -Strabismus, nystagmus, retinitis pigmentosa, optic hypoplasia, peripheral neuropathy, cerebellar atrophy/hypoplasia, olivopontocerebellar atrophy -Flat nasal bridge, large ears, thin upper lip, long philtrum, high arched palate, prominent jaw, retrognathia (in infancy), almond shaped eyes -Pericardial effusion, hypertrophic cardiomyopathy, cardiac failure, tamponade, conotruncal malformations -Inverted nipple, lipodystrophy, fat pads, p'eud orange, -Hepatomegaly, cirrhosis, liver steatosis, vomiting, protein losing enteropathy, diarrhea, GERD -Hydrops fetalis, non-immune, hydropic placenta, mirror syndrome -Edema and hypoalbuminemia, low cholesterol -Coagulopathy and thrombosis (factor II, V, VII, VIII, IX, X, XI, antithrombin III, protein C, protein S deficiency) -Recurrent infections, hypogammaglobulinemia, lack of response to vaccination -Hyperechoic kidneys, hydronephrosis, cysts, proteinuria, proximal tubulopathy -Disproportionate short stature, kyphoscoliosis, skeletal dysplasia (skeletal appearance were consistent with spondyloepiphyseal dysplasia congenita or Kniest dysplasia	-Panhypopituitarism -Hypothyroidism, decreased TBG, -Growth hormone resistance -Female: Delayed puberty, pubertal arrest, amenorrhea, hypergonadotropic and hypogonadotropic hypogonadism (normal puberty and menstruation have been described) -Male: Normal puberty and virilization but small testes, and low-normal testosterone, hypogonadism -Hyperprolactinemia -Hyperinsulinemic hypoglycemia -Adrenal insufficiency (in some patients) -Osteopenia, osteoporosis	(4,9,30,71,84,92,109)

Table 1. Continued

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
Disorders of N-linked glycosylation						
MPI-CDG (602579)	15q24.1-q24.2	Mannosephosphate Isomerase	AR	-Failure to thrive -Vomiting, diarrhea, villous atrophy, lymphangiectasia, protein-losing enteropathy -Hepatomegaly, hepatic fibrosis, cirrhosis, hepatic failure -Hypotonia -Anti-thrombin III, Protein C, Protein S deficiency, thrombosis, factor XI deficiency -Short stature	-Hyperinsulinemic hypoglycemia Treatment beyond symptomatic: Mannose 150 to 170 mg/kg/dose four to five times a day, po	(9,27,110,111)
ALG6-CDG (603147)	1p31.3	Alpha-1,3-glucosyltransferase	AR	-Failure to thrive -Large open fontanel, low-set ears, hypertelorism, macroglossia, brachydactyly distal phalangeal hypoplasia, scoliosis -Axial hypotonia, psychomotor retardation, areflexia, seizures, ataxia, strabismus -Decreased serum cholesterol, factor XI, antithrombin III and protein C	-Hypoglycemia -Hypothyroidism -Low corticosteroid binding globulin, normal cortisol	(9,15,112,113,114)
ALG3-CDG (608750)	3q27.1	Alpha-1,3-mannosyltransferase	??	-DD (mostly severe hence variable) -Failure to thrive -Strabismus and optic atrophy -Dilated aortic root -Craniofacial abnormalities (epicanthal folds, down slanting palpebral fissures, broad/flat nasal bridge, high palate, micrognathia, and dysplastic ears) -Skeletal dysplasia (arthrogryposis, scoliosis, club feet, hip dysplasia, camptodactyly, contractures, overlapping digits, and talipes, rhizomelic short stature, wide metaphysis, hypoplastic cervical vertebrae, narrow thorax, rounded iliac wings. chondrodysplasia punctata) -Feeding problems -Hypoalbuminemia, elevated transaminases, decreased factor XI, antithrombin III -Hypolipidemia	-Panhypopituitarism (central hypothyroidism, central adrenal insufficiency, growth hormone deficiency) -Hypoglycemia -Osteopenia and recurrent fractures	(5,15,114)

Table 1. Continued

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
Disorders of *N*-linked glycosylation						
ALG9-CDG (608776)	11q23.1	Alpha-1,2-Mannosyltransferase	AR	-Failure to thrive -Microcephaly, frontal bossing, long philtrum, low-set ears, hypertelorism, esotropia -Inverted nipples- DD, epileptic encephalopathy, seizures, intractable, hyperreflexia -Cortical and cerebellar atrophy, delayed myelination -Congenital heart defects, pericardial effusion	**Short stature** -Severe skeletal dysplasia (decreased ossification of the frontoparietal bones, thickening of the occipital bones, deficient ossification of cervical vertebral bodies and pubic bones, round pelvis, and short tubular bones with metaphyseal flaring). -Mild skeletal dysplasia have been reported (delayed bone age, mesomelic brachymelia with thickening of frontal and occipital bone, mild kyphosis of thoracolumbar spine, bilateral hip dislocation, round pelvis, brachycephaly, and shortening of greater sciatic notch.)	(15,98,115)
ALG12-CDG (607143)	22q13.33	Alpha-1,6-Mannosyltransferase	??	-Microcephaly, hypotonia, psychomotor retardation -Midface hypoplasia, broad nose, thin upper lip thick ears, sensorineural deafness -Retinal decollement -Patent foramen ovale, patent ductus arteriosus -Hypogammaglobulinemia -Severe skeletal dysplasia (interphalangeal dislocations, scoliosis, talipes equinovarus, rhizomelic limb shortening, short metacarpals, horizontal acetabular roof) -Short ribs with flared metaphysis, scoliosis -Short stature	-Hypoglycemia	(116)
PGM1-CDG (614921)	1p31.3	Phosphoglucomutase 1	AR	-Facial dysmorphism (hypertelorism, short neck, retrognathia, smooth philtrum and low set ears) bifid uvula/palate, -Hepatopathy, malignant hyperthermia -Imperforate anus -Rhabdomyolysis, exercise intolerance, axial hypotonia, dilated cardiomyopathy -Anti-thrombin III, Protein C, Protein S deficiency	-Hypoglycemia (ketotic or hyperinsulinemic) -GH deficiency, decreased IGF-1 and IGFBP-3 levels -High TSH, decreased TBG -Adrenal insufficiency -Hypogonadotropic hypogonadism Treatment beyond symptomatic: D-galactose 0.5 to 2.5 g/kg/day, five to six times a day, po (max: 50 g)	(117,118,119)

Table 1. Continued

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
Disorders of N-linked glycosylation						
TMEM165 (614727)	4q12	Transmembrane protein 165	AR	-DD, seizures, hypotonia -Microcephaly -Short stature -Skeletal dysplasia - Epi-metaphyseal dysplasia and joint destruction diagnosed as Desbuquois syndrome; pectus carinatum, kyphosis and scoliosis, short distal phalanges, genu varus, joint hyperlaxity, epi- and metaphyseal dysplasia with broad metaphysis	Generalized osteoporosis Treatment beyond symptomatic: Galactose 1 g/kg/day po	(15,99)
MAN2B2-CDG (NA)	4p16.1	Mannosidase alpha class 2B member 2	AR	-DD -Chronic diarrhea -Coagulopathy and multiple thrombotic strokes, pancytopenia -Immunodeficiency, small-vessel vasculitis	Short stature Treatment beyond symptomatic: HScT	(120)
O-linked glycosylation						
EXT1(133700) /EXT2-CDG (133701)	8q24.11/11p11.2	Exostosin Glycosyltransferase 1/ Exostosin Glycosyltransferase 2	AD	-DD, muscular dystrophy, hypotonia, polymicrogyria, lissencephaly -Loose skin, reticulate pattern of hyperpigmentation, hypermobility -Elevated CK, dysmorphic features	Skeletal dysplasia, multiple exostoses	(121)
B3GLCT-CDG (261540/ 610308)	13q12.3	Beta 3-glucosyltransferase	AR	Embryonic development of the eye is defective (corneal clouding and variable iridolenticulocorneal adhesions) -Cupid bow shape of the upper lip, cleft lip, cleft palate -Kyphoscoliosis, foot deformity, radioulnar synostosis	-Prenatal growth retardation, postnatal disproportionate short stature -Osteopenia	(15,122,123)
GALNT3-CDG (211900)	2q24.3	Polypeptide N-acetyl galactosaminyl transferase 3	AR	-Retinal angioid streaks, conjunctival irritation, eyelid calcifications	-Hyperphosphatemia associated with periosteal reaction and cortical hyperostosis (recurrent episodes of swelling, pain, and tenderness) -Elevated renotubular phosphate reabsorption -Increased serum FGF23 -Normal serum calcium -Normal serum parathyroid hormone	(124,125,126)

Table 1. Continued

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
O-linked glycosylation						
SLC35C1-CDG (605881)	11p11.2	Solute carrier family 35 member C1		-Severe mental retardation, cortical atrophy, seizures, hypotonia, microcephaly -Markedly reduced neutrophil motility, reduced neutrophil adherence	-Short stature Treatment beyond symptomatic: Fucose, po	(127)
CSGALNACT1-CDG (618870)	8p21.3	Chondroitin sulfate N-acetylgalactosaminyl transferase 1	AR		-Skeletal dysplasia -Micromelia, disproportionate short stature	(128,129)
EXT3-CDG (617425)	8p21.1	Exostosin like-glycosyltransferase 3	AR	-Neuro/immuno/skeletal (DD, seizures, SCID)	Various skeletal dysplasia (platyspondyly, severe, cervical spine malformation, cervical instability, progressive kyphoscoliosis, brachydactyly, delayed carpal ossification, epi-, metaphyseal dysplasia	(17)
Disorders of mixed glycosylation						
OGT-CDG (300997)	Xq13.1	O-GlcNAc transferase subunit p110	XLR	-Neuro/growth/ophthalmo (intellectual delay, hypotonia, eye abnormalities, hearing impairment, behavioural problems, dysmorphism)	Short stature	(130)
GPI anchor disorder						
PIGA-CDG (311770)	Xp22.2	Phosphatidylinositol Glycan Anchor Biosynthesis Class A	XLR	-Micrognathia, malar flattening, coarse facies, polyhydramnios and hydrops fetalis -Microcephaly, epileptic encephalopathy (hypsarrhythmia, burst-suppression pattern seen on EEG, irregular spike and slow waves, myoclonic seizures), severe DD, axial hypotonia, hyperreflexia, cerebellar hypoplasia, corpus callosum hypoplasia, cortical atrophy, spongy gliosis, delayed myelination	- Increased birth length (in some patients) and birth weight (in some patients) - Overgrowth	(131)
Disorders of multiple glycosylation pathways: disorders of Golgi pH and ion homeostasis						
SLC10A7-CDG (618363)	4q31.22	Solute carrier family 10 member 7	AR	-Round face, micrognathia, micro retrognathia, mandibular hypoplasia, cleft palate -Mild DD -Hearing and visual impairment -Amelogenesis imperfecta	-Disproportionate short stature, prenatal and postnatal (< -3 SD) -Obesity (in some patients) -Skeletal dysplasia (advanced bone age, proximal femur "Swedish key", short metacarpals and phalanges, irregular vertebra corpus, wide metaphysis, coxa valga -Osteoporosis	(132,133)

Table 1. Continued

CDG (OMIM)	Localization	Affected protein	Inheritance	Clinical manifestations	Endocrine manifestations and skeletal features	Reference
Disorders of multiple glycosylation pathways: disorders of Golgi pH and ion homeostasis						
PGM3-CDG (615816)	6q14.1	Phosphoglucomutase 3	AR	Hyper IgE syndrome (elevated serum IgE, recurrent skin and pulmonary infections, abscesses, eczema, and bronchiectasis)	-Severe skeletal dysplasia (radiographic pattern of Desbuquois dysplasia)	(97)
COG7-CDG (608779)	16p12.2	Component of oligomeric Golgi complex 7	AR	-Progressive microcephaly -Dysmorphic facial features (narrow, flat forehead, micrognathia, retrognathia, low-set ears, dysplastic ears) -Global DD, cerebral and cerebellar atrophy, hypoplasia of corpus callosum, delayed myelination	-Intrauterine growth retardation, failure to thrive, short stature -Variable skeletal anomalies (Adducted thumbs, overlapping, long fingers, Simian crease, contractures of the PIP and DIP joints with ulnar deviation of the hands)	(15,134)
COG1-CDG (611209)	17q25.1	Component of oligomeric Golgi complex 1	AR	-Progressive microcephaly -Dysmorphic facial features (midface hypoplasia, micrognathia, low-set ears, microtia, hypertelorism, thin upper lip, high arched palate, hearing loss) -Global DD -Cerebral and cerebellar atrophy -Anemia, thrombocytopenia -Recurrent infections	-Rhizomelic short stature -Rib fusions, rib abnormalities, vertebral abnormalities	(15,135)
COG8-CDG (611182)	16q22.1	Component of oligomeric Golgi complex 8	NA	-Microcephaly -Hypotonia, seizures, -Cortical atrophy	-Small hands and feet, hypoplasia of the first phalanx of some fingers and toes, sandal gap, clinodactyly	(136,137)

AR: autosomal recessive, AD: autosomal dominant, CDG: congenital disorders of glycosylation, CK: creatine kinase, DD: developmental delay, SCID: severe combined immunodeficiency, EEG: electroencephalogram, DIP: distal interphalangeal, GERD: gastro-esophageal reflux, HH: hyperinsulinemic hypoglycemia, HScT: hematopoietic stem cell transplantation, NA: not available, OMIM: Online Mendelian Inheritance in Man, PIP: proximal interphalangeal, TBG: thyroxine binding globulin, XLR: X-linked recessive, g: gram, po: per oral

Table 2. Recommended endocrine evaluation for patients with CDG

	On admission	First two years of life (six-month intervals)	After first two years (annually)
Height measurement	+	+	+
Glucose*	+	+	If needed
Calcium, phosphate, magnesium and vitamin D measurement	+	+	+
TSH, FT4	+	+	+
ACTH, cortisol**	+	Annually	+
FSH, LH, estradiol or testosterone	+	-	If needed
Lateral lumbar radiography	+	-	+

*If hypoglycemia is detected blood glucose should be measured biochemically with insulin, ACTH, cortisol, growth hormone, bicarbonate, lactate, acylcarnitine analysis, urine ketone analysis.
**If low, perform low dose ACTH stimulation test.
CDG: congenital disorders of glycosylation, TSH: thyroid-stimulating hormone, FT4: free T4, ACTH: adrenocorticotropic hormone, FSH: follicle stimulating hormone, LH: luteinizing hormone

In a case series of PMM2-CDG, basal serum PRL concentrations were reported to be normally elevated in three studied newborns and normally low (<I5 pg/L) in 22/26 patients examined after the neonatal period. Slightly elevated basal PRL levels were observed in four femaless (the initially described twin sisters (778-5652 U/mL, normal range <800) and two girls aged 2 years old (659 and 829 U/mL) (7,10). In the initially described twin sisters, prolactin decreased gradually over the course of 15 years and was sensitive to release-inhibitory action of L-dopa when they were 3 years old (10).

Conclusion and Future Prospects

Glycoproteins are essential for endocrine homeostasis where alterations in glycosylation change hormone and carrier protein stability, circulatory half-life and abundance, receptor configuration, activation, hormone-substrate affinity and reset endocrine control and feedback loops. Therefore, every endocrine axis may be affected in CDGs. It is known that CDG should be considered in patients with multisystemic disease, especially in cases with neurologic findings of nonspecific developmental delay with unclear etiology. CDGs should also be considered in the differential diagnosis of patients with endocrine manifestations of unclear etiology and multisystem involvement.

Clinical findings of CDG regarding endocrine system may be masked or mimicked by other, more severe CDG symptoms and endocrine problems may be overlooked. Evaluation of laboratory results in individuals with CDG may be difficult to interpret as structurally and functionally altered glycosylated proteins precede changed serum hormone levels. Given this, regular evaluation of endocrine manifestations is advised (Table 2). *In vitro* and clinical studies focusing on defects of glycosylation and its effects are emerging, hence there are some limitations in the existing literature that should be acknowledged. As seen, large-scale clinical studies focusing on implications of disorders of glycosylation on endocrine systems are scarce and data are mostly derived from case series. Studies focusing on alterations of glycosylation patterns and three dimensional structure of specific hormones may help clarify pathophysiology of hypogonadism, AI and defects of bone mineralization. Large scale proteomic and metabolomic profiling may help delineate these shortcomings in the literature and help illuminate specific endocrine effects as well as the natural course of disease. Long term studies with international participation focusing on growth, puberty, bone mineralization and thyroid and adrenal involvement are necessary. Results of disease specific treatments (ie. galactose, and mannose) focusing on endocrine systems should be sought.

Footnotes

Authorship Contributions

Concept: Zeynep Alev Özön, Design: Zeynep Alev Özön, Data Collection or Processing: Yağmur Ünsal, Zeynep Alev Özön, Analysis or Interpretation: Yağmur Ünsal, Zeynep Alev Özön, Literature Search: Yağmur Ünsal, Zeynep Alev Özön, Writing: Yağmur Ünsal, Zeynep Alev Özön.

Conflict of Interest: Zeynep Alev Özön is an Associate Editor of the Journal of Clinical Research in Pediatric Endocrinology. However, the reviewers evaluating this manuscript were blinded and were from different institutions. She was not involved in the editorial review of this manuscript to avoid prejudice that may disrupt impartiality. The other author declares no conflict of interest.

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Evaluation of Artificial Intelligence Answers for Short Stature in Paediatric Endocrinology by Paediatric Endocrinologists

Kamber Kaşali[1], Özgür Fırat Özpolat[2], Merve Ülkü[3], Ayşe Sena Dönmez[4], Serap Kılıç Kaya[4], Esra Dişçi[4], Serkan Bilge Koca[5], Ufuk Özkaya[4], Hüseyin Demirbilek[6], Atilla Çayır[7]

[1]Atatürk University Faculty of Medicine, Department of Biostatistics, Erzurum, Türkiye
[2]Atatürk University, Data Management Office, Erzurum, Türkiye
[3]Erzurum City Hospital, Clinic of Pediatric Endocrinology, Erzurum, Türkiye
[4]Erzurum City Hospital, Clinic of Pediatrics, Erzurum, Türkiye
[5]Kayseri City Training and Research Hospital, Clinic of Pediatrics, Division of Pediatric Endocrinology, Kayseri, Türkiye
[6]Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye
[7]Atatürk University Faculty of Medicine, Department of Pediatric Endocrinology, Erzurum, Türkiye

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What is already known about this?

Artificial intelligence (AI) is increasingly used in medical decision-making, including in pediatric endocrinology. AI models can help diagnose short stature by analyzing growth patterns and related factors, but not much is known about their accuracy and reliability.

What does this study adds?

This study evaluated AI-generated decisions about short stature by comparing them with expert opinions. It highlights the strengths and limitations of AI in clinical decision-making and identifies areas where AI is or is not in line with expert recommendations, particularly in the field of short stature.

Corresponding Author: Asst. Prof. Kamber Kaşali, Atatürk University Faculty of Medicine, Department of Biostatistics, Erzurum, Türkiye
E-mail: kmbrkasali76@gmail.com **ORCID:** orcid.org/0000-0002-2851-5263
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ABSTRACT

Objective: Artificial intelligence (AI) is increasingly used in medicine, including pediatric endocrinology. AI models have the potential to support clinical decision-making, patient education, and guidance. However, their accuracy, reliability, and effectiveness in providing medical information and recommendations remain unclear. The aim was to evaluate and compare the performance of four AI models, ChatGPT, Bard, Microsoft Copilot, and Pi, in answering frequently asked questions related to pediatric endocrinology.

Methods: Nine questions commonly asked by parents regarding short stature in pediatric endocrinology were selected, based on literature reviews and expert opinions. These questions were posed to four AI models in both Turkish and English. The AI-generated responses were evaluated by 10 pediatric endocrinologists using a 12-item Likert-scale questionnaire assessing medical accuracy, completeness, guidance, and informativeness. Statistical analyses, including Kruskal-Wallis and post-hoc tests, were conducted to determine significant differences between AI models.

Results: Bard outperformed other models in guidance and recommendation categories, excelling in directing users to medical consultation. Microsoft Copilot demonstrated strong medical accuracy but lacked guidance capacity. ChatGPT showed consistent performance in knowledge dissemination, making it effective for patient education. Pi scored the lowest in guidance and recommendations, indicating limited applicability in clinical settings. Significant differences were observed between AI models ($p<0.05$), particularly in completeness and guidance-related categories.

Conclusion: The present study highlights the varying strengths and weaknesses of AI models in an area of pediatric endocrinology. While Bard was effective in guidance, Microsoft Copilot excelled at accuracy, and ChatGPT was informative. Future AI improvements should focus on balancing accuracy and guidance to enhance clinical decision-support and patient education. Tailored AI applications may optimize the role of AI in specialized medical fields.

Keywords: Pediatric endocrinology, artificial intelligence (AI), clinical decision support, medical informatics

Introduction

Artificial intelligence (AI) has been rapidly expanding its applications in the field of medicine, including pediatric endocrinology. The complexity of clinical problems and the rapidly evolving need for information in pediatric endocrinology further enhance the potential of AI in this domain. This study evaluated the responses provided by AI systems to frequently asked questions about short stature. The published evidence demonstrates the applicability of AI in various areas of pediatric endocrinology, including growth disorders, obesity, diabetes management, and hormonal imbalances (1,2,3).

The integration of AI into pediatric endocrinology has become particularly prominent in diabetes management. Winkelman et al. (4) reported that AI had been successfully used for optimizing insulin dosing and predicting hypoglycemia risk. In addition, Zhang et al. (3) found that AI-assisted bone age analyses improve diagnostic accuracy in cases of growth hormone deficiency.

AI has also shown significant contributions to the early diagnosis of thyroid diseases. Otjen et al. (5) highlighted the high success rate of AI in the automated analysis of thyroid ultrasound images. Furthermore, AI models used in obesity and management of insulin resistance have facilitated personalized treatment approaches (1,2).

In terms of growth disorders, the accuracy of AI in bone age measurement and its impact on accelerated diagnostic processes are particularly noteworthy. Waikel et al. (6) showed that AI may serve as an effective educational tool for recognizing genetic syndromes.

The aim of this study was to analyze the accuracy of AI-generated responses to questions concerning short stature and the efficacy of growth hormone treatment by a panel of expert pediatric endocrinologists. The integration of AI into clinical practice has the potential to reduce the workload of healthcare professionals while playing an important complementary role in patient care and clinical decision-making. However, challenges such as data security, ethical concerns, and algorithmic accuracy remain key issues that need to be addressed.

Methods

First, a literature review and expert opinions were used to identify the nine most frequently asked questions by parents about short stature, which were then posed to AI models. Subsequently, the AI-generated responses were evaluated by 10 pediatric endocrinologists. A 12-item questionnaire was developed to assess these responses, and the endocrinologists were asked to complete it.

Participants

The study included 10 pediatric endocrinologists. The participants were selected randomly (using a simple random sampling method) from experts who had at least five years of experience in pediatric endocrinology and were actively engaged in clinical practice. No authors of the present study were eligible for inclusion on the expert panel.

Question Development

To determine the most commonly asked questions by the parents of pediatric endocrinology patients, a literature review was conducted, and expert opinions were sought. As a result, a total of nine questions were formulated. Each AI model was queried separately in both Turkish and English. The selected questions were:

1. What is short stature?
2. What are the causes of short stature?
3. How is growth velocity assessed in short stature?
4. How is bone age determined in cases of short stature?
5. What should be considered in the differential diagnosis of short stature?
6. Which laboratory parameters should be evaluated in cases of short stature?
7. What medications are used in the treatment of short stature?
8. How frequently should short stature be monitored?
9. What are the potential side effects of growth hormone therapy?

AI Models

The questions were posed to four different AI models: ChatGPT (developer: OpenAI; access: https://chat.openai.com), Bard/Gemini (developer: Google; access: https://gemini.google.com), Microsoft Copilot (developer: Microsoft; access: https://copilot.microsoft.com), and Pi (developer: Inflection AI; access: https://pi.ai). Each AI model was queried separately in both Turkish and English, and the responses were recorded for further analysis. Due to the rapid evolution of these models, the findings reported herein are strictly limited to the versions evaluated at the time of data collection.

Evaluation Process

The responses obtained from AI systems were evaluated by 10 pediatric endocrinologists. A 12-item Likert-type questionnaire was used for the assessment. For each AI-generated response, experts rated the following survey questions on a scale from 1 to 5:

1. Was a proper definition provided?
2. Was all necessary information included?
3. Was any essential information missing?
4. Was excessive information provided?
5. Was any irrelevant information included?
6. Was the medical information accurate?
7. Were recommendations given?
8. Was patient guidance provided?
9. Was a recommendation to consult a physician included?
10. Was the response sufficient for the patient?
11. Did the response aim to inform the reader?
12. Did the response aim to reassure the reader?

Statistical Analysis

The data are presented as mean, standard deviation, median, minimum, and maximum values. The obtained data were analyzed using SPSS, version 20.0 (IBM Inc., Armonk, NY, USA). The Kruskal-Wallis test was used to determine the significance of differences between the responses to the questions. In cases where significant differences were observed, post-hoc tests were conducted using the Kruskal-Wallis (k samples) test. A significance level of $p<0.05$ was considered statistically significant.

Results

A total of 10 pediatric endocrinologists specializing in pediatric endocrinology participated in the study. Table 1 presents the evaluation results of responses provided by four AI models [ChatGPT, Bard, Microsoft Copilot (MC), and Pi] to nine pediatric endocrinology related questions, as assessed by experts using a 12-item Likert type questionnaire. The expert evaluation results for each question posed to AI models are summarized as follows.

Evaluation of AI Models in Answering "What is Short Stature?"

Bard received the highest score for definition accuracy (5±1), though the difference among models was not significant ($p=0.139$). In the "Missing Information Provided" category, ChatGPT had a higher tendency for incomplete responses compared to Bard ($p=0.027$). Bard and MC performed best in "Recommendations Provided" and "Patient Guidance Provided" while Pi scored the lowest ($p<0.001$). Bard and MC also excelled in "Response Aims to Inform the Reader" with MC significantly outperforming ChatGPT ($p=0.007$). In "Response Aims to Reassure the Reader" Bard led, and Pi ranked lowest, with a significant difference between Bard and MC ($p<0.001$) (Table 1).

Evaluation of AI Models in Answering "What are the Causes of Short Stature?"

ChatGPT scored highest in the "All Necessary Information Provided" category (4±1), significantly outperforming MC and Pi ($p=0.001$). In "Essential Information Missing" Bard had the lowest score (2±1), with Pi and MC scoring higher ($p=0.011$). Bard and ChatGPT performed better in avoiding irrelevant information compared to MC ($p=0.011$). Bard excelled in "Recommendations Provided" (4±1) and "Patient Guidance Provided" (5±0), while

Table 1. Expert evaluation of AI-generated responses to pediatric endocrinology questions I

		Group						
		ChatGPT	Bard	MC	Pi			
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Kruskal-Wallis H	p	Post-hoc
What is short stature?	Was a proper definition provided?	4±1; 4±2	5±1; 5±1	4±1; 4±1	4±1; 4±0	5.498	0.139	
	Was all necessary information included?	3±1; 2.5±2	4±1; 4±1	3±1; 3±1	3±1; 3±1	4.707	0.195	
	Was any essential information missing?	4±1; 4±0	3±1; 3±2	3±1; 3±1	3±1; 4±2	9.150	0.027	Bard-ChatGPT
	Was excessive information provided?	2±1; 1.5±2	2±1; 2±3	2±1; 2±1	2±1; 2±1	1.558	0.669	
	Was any irrelevant information included?	2±1; 2±1	3±2; 3±3	2±1; 2±2	2±1; 2±3	0.650	0.885	
	Was the medical information accurate?	3±1; 2.5±3	4±1; 3.5±1	3±1; 3±2	3±1; 3±1	3.289	0.349	
	Were recommendations given?	2±1; 2±2	4±0; 4±0	4±1; 4±1	2±1; 2±1	29.005	**<0.001**	Pi-MC, Pi-Bard, ChatGPT-MC, ChatGPT-Bard
	Was patient guidance provided?	2±1; 2±1	4±1; 4±1	4±0; 4±0	2±1; 1.5±1	26.564	**<0.001**	Pi-MC, Pi-Bard, ChatGPT-MC, ChatGPT-Bard
	Was a recommendation to consult a physician included?	2±1; 2±1	4±1; 4±1	4±0; 4±0	1±0; 1±1	30.593	**<0.001**	Pi-MC, Pi-Bard, ChatGPT-MC, ChatGPT-Bard
	Was the response sufficient for the patient?	2±1; 2±1	3±1; 4±2	3±1; 3±2	3±1; 3±2	6.923	0.074	
	Did the response aim to inform the reader?	3±1; 3±2	4±1; 4±1	4±0; 4±0	3±1; 3±1	12.160	**0.007**	ChatGPT-MC
	Did the response aim to reassure the reader?	3±1; 3±1	4±1; 4±2	3±1; 2±2	2±1; 2±0	18.140	**<0.001**	Pi-Bard, MC-Bard
What are the causes of short stature?	Was a proper definition provided?	4±1; 4±3	3±1; 4±2	4±1; 3.5±1	3±1; 2±2	5.137	0.162	
	Was all necessary information included?	4±1; 4±1	3±1; 4±2	2±0; 2±0	2±1; 2±1	15.588	**0.001**	MC-ChatGPT, Pi-ChatGPT
	Was any essential information missing?	3±1; 2.5±1	2±1; 2±3	4±1; 4±1	4±1; 4±0	11.076	**0.011**	Bard-Pi
	Was excessive information provided?	2±1; 2±0	2±1; 2±2	3±1; 2±2	2±0; 2±1	5.013	0.171	
	Was any irrelevant information included?	2±0; 2±1	2±1; 2±1	3±1; 3±1	2±1; 2±2	11.176	**0.011**	Bard-MC, ChatGPT-MC
	Was the medical information accurate?	4±1; 4±2	4±1; 4±1	3±1; 3±1	3±1; 3.5±2	11.114	**0.011**	MC-ChatGPT
	Were recommendations given?	3±1; 3.5±2	4±1; 4±1	3±1; 3±1	2±1; 2±0	23.636	**<0.001**	Pi-MC, Pi-Bard
	Was patient guidance provided?	3±1; 3±2	5±0; 5±1	4±1; 4±1	2±1; 2±1	24.831	**<0.001**	Pi-MC, Pi-Bard, ChatGPT-Bard
	Was a recommendation to consult a physician included?	3±1; 3±2	5±1; 5±1	4±1; 4±1	2±1; 2±1	23.756	**<0.001**	Pi-MC, Pi-Bard
	Was the response sufficient for the patient?	4±1; 4±2	4±1; 4±1	3±1; 3±1	2±1; 2±2	20.325	**<0.001**	Pi-ChatGPT, Pi-Bard
	Did the response aim to inform the reader?	4±1; 4±1	4±1; 4±0	4±1; 4±1	3±1; 3±2	8.278	**0.041**	Pi-Bard
	Did the response aim to reassure the reader?	3±1; 3±1	4±2; 4.5±3	3±1; 2±1	3±1; 3±2	4.135	0.247	

Table 1. Continued

		Group						
		ChatGPT	Bard	MC	Pi			
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Kruskal-Wallis H	p	Post-hoc
How is growth rate evaluated in short stature?	Was a proper definition provided?	4±1; 4±1	3±2; 3±4	3±1; 2±2	3±1; 2±1	8.433	**0.038**	Pi-ChatGPT
	Was all necessary information included?	3±1; 4±2	4±1; 4±1	3±1; 3±1	4±1; 4±1	5.875	0.118	
	Was any essential information missing?	3±1; 2±3	2±1; 2±1	4±1; 4±1	2±1; 2±1	8.412	**0.038**	Bard-MC
	Was excessive information provided?	2±1; 2±0	3±1; 2±2	2±1; 2±1	2±0; 2±1	4.264	0.234	
	Was any irrelevant information included?	2±1; 2±1	2±1; 2±3	2±1; 2±0	3±1; 2±3	1.124	0.771	
	Was the medical information accurate?	4±1; 4±1	4±1; 3±1	4±0; 4±1	4±1; 4±0	3.487	0.322	
	Were recommendations given?	3±1; 2.5±2	4±0; 4±0	2±1; 1.5±1	3±1; 2.5±2	18.720	**<0.001**	ChatGPT-Bard, MC-Bard
	Was patient guidance provided?	3±1; 3.5±1	4±1; 4±2	2±1; 1.5±1	3±1; 2±2	12.110	**0.007**	MC-Bard
	Was a recommendation to consult a physician included?	4±1; 4±1	4±1; 5±2	2±1; 2±1	3±1; 3±2	13.789	**0.003**	MC-Bard
	Was the response sufficient for the patient?	4±1; 4±3	4±1; 4±2	2±1; 2±0	4±1; 4±2	8.428	**0.038**	MC-ChatGPT, MC-Bard, MC-Pi
	Did the response aim to inform the reader?	4±0; 4±1	4±1; 4±1	3±1; 3±1	4±1; 4±1	9.917	**0.019**	MC-Bard
	Did the response aim to reassure the reader?	3±1; 3.5±2	3±2; 4±3	2±1; 2±2	3±1; 2.5±2	4.339	0.227	
How is bone age determined in short stature?	Was a proper definition provided?	4±1; 4±1	3±1; 4±2	3±1; 4±2	3±1; 4±2	2.801	0.423	
	Was all necessary information included?	4±1; 3.5±2	4±1; 4±1	3±1; 3±1	3±1; 3±1	6.513	0.089	
	Was any essential information missing?	3±1; 3±3	3±1; 3±2	4±1; 4±1	3±1; 4±1	6.894	0.075	
	Was excessive information provided?	2±1; 2±0	3±1; 2±2	3±1; 3.5±2	2±0; 2±1	5.323	0.150	
	Was any irrelevant information included?	2±1; 2.5±1	3±1; 2±1	3±1; 3±2	2±1; 2±1	4.813	0.186	
	Was the medical information accurate?	4±1; 4±0	3±1; 3±1	3±1; 3±2	3±1; 3±1	6.781	0.079	
	Were recommendations given?	3±1; 3.5±1	4±1; 4±1	2±1; 2±1	2±1; 1.5±2	18.220	**<0.001**	Pi-Bard, MC-Bard
	Was patient guidance provided?	3±1; 3±1	4±0; 4±1	2±1; 2.5±2	2±1; 2±1	19.372	**<0.001**	Pi-Bard, ChatGPT-Bard, MC-Bard
	Was a recommendation to consult a physician included?	4±1; 3.5±2	5±1; 4.5±1	2±0; 2±1	2±1; 2±0	25.323	**<0.001**	Pi-Bard, MC-Bard, MC-ChatGPT
	Was the response sufficient for the patient?	3±1; 3.5±1	4±1; 4±1	3±1; 3±1	3±1; 3±1	8.178	**0.042**	MC-Bard
	Did the response aim to inform the reader?	4±0; 4±0	4±1; 4±1	3±1; 3±1	4±1; 4±0	7.096	0.069	
	Did the response aim to reassure the reader?	3±1; 2.5±2	2±1; 2±1	2±1; 2±1	2±1; 2±1	5.522	0.137	

Table 1. Continued

		Group						
		ChatGPT	Bard	MC	Pi			
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Kruskal-Wallis H	p	Post-hoc
What should be considered in differential diagnosis in short stature?	Was a proper definition provided?	4±1; 4±1	3±1; 2.5±2	4±1; 4±1	3±1; 3±2	8.321	**0.040**	Bard-ChatGPT
	Was all necessary information included?	4±1; 4±2	3±1; 4±1	3±1; 3.5±2	3±1; 3±1	1.606	0.658	
	Was any essential information missing?	3±2; 3±3	3±1; 3±2	2±1; 2±2	3±1; 4±2	4.436	0.218	
	Was excessive information provided?	2±1; 2±2	2±1; 2±1	2±1; 2±0	2±0; 2±1	2.172	0.537	
	Was any irrelevant information included?	2±1; 1.5±1	2±0; 2±0	2±0; 2±1	2±1; 2±2	1.461	0.691	
	Was the medical information accurate?	4±1; 4±2	4±0; 4±0	4±1; 4±1	3±1; 4±2	2.303	0.512	
	Were recommendations given?	4±1; 3±1	4±1; 4±2	4±0; 4±0	3±1; 3±2	6.707	0.082	
	Was patient guidance provided?	3±1; 2±2	4±0; 4±1	4±0; 4±0	3±1; 3±2	12.659	**0.005**	Pi-Bard, ChatGPT-Bard
	Was a recommendation to consult a physician included?	3±1; 3±2	5±1; 5±1	5±1; 4.5±1	3±1; 2.5±3	15.615	**0.001**	Pi-MC, Pi-Bard
	Was the response sufficient for the patient?	4±1; 4±2	4±1; 4±1	4±1; 4±1	3±1; 2.5±1	7.403	0.060	
	Did the response aim to inform the reader?	4±1; 4±1	4±1; 4±0	4±0; 4±0	3±1; 3±1	13.163	**0.004**	Pi-ChatGPT
	Did the response aim to reassure the reader?	3±1; 2±2	2±1; 2±3	3±1; 2±1	2±1; 2±0	0.598	0.897	

AI: artificial intelligence, MC: Microsoft Copilot, SD: standard deviation, IQR: interquartile range

Pi scored the lowest (p<0.001). In "Recommendation to Consult a Physician" Bard and MC led, whereas Pi performed significantly worse (p<0.001). ChatGPT, Bard, and MC scored highest in "Response Aims to Inform the Reader", with Pi performing worse (p=0.041) (Table 1).

Evaluation of AI Models in Answering "How is Growth Rate Evaluated in Short Stature?"

In the "Definition Provided" category, ChatGPT scored higher (4±1) than Pi (3±1), demonstrating superior definition clarity (p=0.038). In "Essential Information Missing" Bard (2±1) performed better than MC (4±1), highlighting Bard's ability to provide more complete responses (p=0.038). Bard excelled in "Recommendations Provided" (4±0), while MC scored the lowest (2±1) (p<0.001). In "Patient Guidance Provided" Bard (4±1) outperformed MC (2±1) (p=0.007). Similarly, in "Recommendation to Consult a Physician" Bard (4±1) led, while MC (2±1) performed poorly (p=0.003). Lastly, in "Response Was Sufficient for the Patient" MC had a significantly lower score than other models (p=0.038), indicating its weaker performance in providing satisfactory responses (Table 1).

Evaluation of AI Models in Answering "How is Bone Age Determined in Short Stature?"

In the "Definition Provided" category, no significant difference was found between the models (p=0.423), with ChatGPT scoring highest (4±1). In "Recommendations Provided" Bard (4±1) significantly outperformed Pi and MC (2±1) (p<0.001), confirming its superiority in offering guidance. In "Patient Guidance Provided" Bard (4±0) excelled, significantly outperforming all other models (p<0.001). Similarly, in "Recommendation to Consult a Physician" Bard (5±1) led, while Pi (2±0) and MC (2±1) performed the worst (p<0.001). Finally, in "Response Was Sufficient for the Patient" Bard (4±1) was the most effective, while MC (3±1) scored significantly lower (p=0.042), indicating Bard's stronger ability to meet users' informational needs (Table 1).

Evaluation of AI Models in Answering "What Should be Considered in Differential Diagnosis in Short Stature?"

In the "Definition Provided" category, ChatGPT scored highest (4±1), significantly outperforming Bard (3±1) (p=0.040). In "Patient Guidance Provided" Bard and MC (4±0) excelled, while Pi and ChatGPT (3±1) performed lower, with significant differences between Pi-Bard and ChatGPT-Bard (p=0.005). For "Recommendation to Consult a Physician" Bard and MC (5±1) were the most effective, while Pi (3±1) performed the weakest, with significant differences between Pi-MC and Pi-Bard (p=0.001). In

"Response Aims to Inform the Reader" ChatGPT, Bard, and MC (4±1) performed well, whereas Pi (3±1) lagged, showing a significant difference from ChatGPT (p=0.004) (Table 1).

Table 2 presents the evaluation results of responses provided by the four AI programs to nine pediatric endocrinology-related questions, as assessed by experts using a 12-item Likert-type questionnaire. The expert evaluation results for each question posed to AI models are summarized as follows.

Evaluation of AI Models in Answering "What Should be Considered in Laboratory Parameters in Short Stature?"

In the "Definition Provided" and "All Necessary Information Provided" categories, all models received similar scores, with no significant differences (p=0.595 and p=0.446, respectively). Although ChatGPT scored highest, the variations were not significant. In "Patient Guidance Provided" Bard (4±1) outperformed MC (2±1) and Pi (3±1), with a significant difference between MC and Bard (p=0.030), again indicating Bard's stronger guidance ability. Similarly, in "Recommendation to Consult a Physician" Bard (3±1) and ChatGPT (3±1) scored higher than MC (2±1), with Bard significantly outperforming MC (p=0.014). For "Response Was Sufficient for the Patient" ChatGPT (4±1) led, while MC (2±1) and Bard (3±1) scored lower, with a significant difference between MC and ChatGPT (p=0.018). Lastly, in "Response Aims to Inform the Reader" ChatGPT (4±1) significantly outperformed MC (3±1) (p=0.033), confirming ChatGPT's superior capacity for providing informative responses (Table 2).

Evaluation of AI Models in Answering "Which Drugs are used in the Treatment of Short Stature?"

In the "All Necessary Information Provided" category, MC scored the lowest (2±1), significantly underperforming compared to Bard and ChatGPT (4±1) (p<0.001). This suggests MC was less effective in providing comprehensive information. In "Essential Information Missing" MC (4±1) had the highest score, indicating a greater tendency to provide incomplete information. ChatGPT (2±1) and Pi (2±1) scored lower, with MC significantly differing from these programs (p=0.002). In "Medically Accurate Information Provided" MC (3±1) slightly but significantly outperformed ChatGPT (p=0.036), highlighting MC's relative strength in medical accuracy. For "Recommendations Provided" Bard (4±1) scored highest, with a significant difference from MC (3±1) and Pi (3±1) (p=0.027), confirming Bard's superiority in offering guidance. In "Response Was Sufficient for the Patient" ChatGPT (4±1) led, while MC and Pi (2±1) scored

Table 2. Expert evaluation of AI-generated responses to pediatric endocrinology questions II

		Group						
		ChatGPT	Bard	MC	Pi	Kruskal-Wallis H	p	Post-hoc
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR			
What should be considered in laboratory parameters in short stature?	Was a proper definition provided?	4±2; 4±3	3±1; 3±2	3±1; 2±2	3±1; 3±2	1.894	0.595	
	Was all necessary information included?	4±1; 3.5±3	3±1; 3±1	3±1; 3±2	3±1; 4±1	2.669	0.446	
	Was any essential information missing?	3±2; 3±3	3±1; 3±1	3±1; 3.5±3	3±1; 3±2	1.189	0.756	
	Was excessive information provided?	2±1; 2±3	2±1; 2±1	3±1; 2±2	3±1; 2±3	1.543	0.672	
	Was any irrelevant information included?	3±1; 3.5±2	2±1; 2±1	3±1; 4±2	3±1; 2±3	3.593	0.309	
	Was the medical information accurate?	3±1; 4±2	3±1; 3.5±2	3±0; 3±0	3±1; 3±2	0.598	0.897	
	Were recommendations given?	3±1; 3±1	3±1; 3±1	2±1; 2±2	3±1; 3±1	5.295	0.151	
	Was patient guidance provided?	3±1; 3±2	4±1; 4±1	2±1; 2±1	3±1; 3±2	8.962	**0.030**	MC-Bard
	Was a recommendation to consult a physician included?	3±1; 2±2	3±1; 4±2	2±1; 1±1	3±1; 3±2	10.588	**0.014**	MC-Bard
	Was the response sufficient for the patient?	4±1; 4±2	3±1; 4±2	2±1; 2±1	3±1; 3.5±1	10.111	**0.018**	MC-ChatGPT
	Did the response aim to inform the reader?	4±1; 4±1	4±1; 4±1	3±1; 3±1	4±0; 4±0	8.726	**0.033**	MC-ChatGPT
	Did the response aim to reassure the reader?	3±1; 2±2	2±1; 2±1	3±1; 2.5±2	3±1; 2±1	0.944	0.815	

Table 2. Continued

		Group						
		ChatGPT	Bard	MC	Pi			
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Kruskal-Wallis H	p	Post-hoc
Which drugs are used in the treatment of short stature?	Was a proper definition provided?	4±2; 4.5±3	3±2; 4±3	2±1; 2±1	3±1; 3±2	6.971	0.073	
	Was all necessary information included?	4±1; 4±2	4±1; 4±1	2±1; 2±0	3±1; 2±2	19.178	**<0.001**	MC-Bard, MC-ChatGPT
	Was any essential information missing?	2±1; 2±2	3±1; 3±2	4±1; 4±2	2±1; 2±1	15.036	**0.002**	ChatGPT-MC, Pi-MC
	Was excessive information provided?	2±1; 1.5±3	3±2; 2±3	2±0; 2±1	2±1; 2±2	2.103	0.551	
	Was any irrelevant information included?	2±1; 2±1	2±1; 2±1	2±1; 2±2	2±1; 2±2	0.600	0.896	
	Was the medical information accurate?	4±1; 4±1	3±1; 4±2	3±1; 2.5±1	3±0; 3±1	8.565	**0.036**	MC-ChatGPT
	Were recommendations given?	3±1; 3.5±1	4±1; 4±1	3±1; 3±1	3±1; 2.5±2	9.182	**0.027**	MC-Bard
	Was patient guidance provided?	3±1; 3.5±1	4±1; 4±0	3±1; 3±1	3±1; 2.5±2	11.977	**0.007**	Pi-Bard, MC-Bard
	Was a recommendation to consult a physician included?	3±1; 4±2	3±2; 4±3	3±1; 4±2	3±1; 3±0	3.347	0.341	
	Was the response sufficient for the patient?	4±1; 4±1	3±1; 3±2	2±1; 2±1	2±1; 2±2	14.788	**0.002**	MC-ChatGPT
	Did the response aim to inform the reader?	4±0; 4±0	3±1; 4±1	3±1; 3±2	4±1; 3.5±1	11.880	**0.008**	MC-ChatGPT
	Did the response aim to reassure the reader?	3±1; 3±2	2±1; 2±2	2±1; 2±1	2±1; 2±1	10.577	**0.014**	Pi-ChatGPT, MC-ChatGPT
How often should short stature be monitored?	Was a proper definition provided?	3±1; 4±2	3±1; 2±2	2±1; 2±0	3±1; 3±2	6.086	0.108	
	Was all necessary information included?	3±1; 3.5±1	3±1; 4±2	2±1; 2±0	3±1; 3±1	10.044	**0.018**	MC-ChatGPT
	Was any essential information missing?	3±1; 2.5±3	3±1; 3±0	4±1; 4±0	3±1; 4±2	10.087	**0.018**	ChatGPT-MC
	Was excessive information provided?	2±1; 2±1	2±1; 2±2	2±0; 2±1	2±1; 2±1	1.419	0.701	
	Was any irrelevant information included?	2±1; 2±2	2±0; 2±1	3±2; 2.5±4	3±1; 2±3	3.018	0.389	
	Was the medical information accurate?	4±1; 4±0	3±1; 3±1	3±0; 3±0	4±1; 3.5±1	10.702	**0.013**	MC-ChatGPT, Bard-ChatGPT
	Were recommendations given?	4±1; 4±0	4±0; 4±0	3±1; 3±1	3±1; 2±1	18.475	**<0.001**	Pi-ChatGPT, Pi-Bard, MC-Bard, MC-ChatGPT
	Was patient guidance provided?	4±1; 4±0	4±0; 4±0	4±1; 4±1	2±1; 2±1	14.169	**0.003**	Pi-ChatGPT, Pi-Bard
	Was a recommendation to consult a physician included?	4±0; 4±0	4±1; 4.5±1	4±0; 4±1	2±1; 2±0	20.644	**<0.001**	Pi-ChatGPT, Pi-Bard
	Was the response sufficient for the patient?	4±1; 4±1	4±1; 4±1	2±1; 2±0	3±1; 2.5±1	13.867	**0.003**	MC-ChatGPT, MC-Bard
	Did the response aim to inform the reader?	4±1; 4±1	4±0; 4±0	4±1; 3.5±1	3±1; 3±2	6.194	0.103	
	Did the response aim to reassure the reader?	3±1; 2±2	3±1; 3±3	2±1; 2±0	2±1; 2±1	4.128	0.248	

Table 2. Continued

		Group						
		ChatGPT	Bard	MC	Pi	Kruskal-Wallis H	p	Post-hoc
		Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR	Mean±SD; Med±IQR			
What are the side effects that can be seen after growth hormone treatment?	Was a proper definition provided?	3±2; 4±3	3±1; 2.5±3	2±1; 2±2	3±1; 2±2	2.667	0.446	
	Was all necessary information included?	3±1; 3±2	3±1; 2.5±2	3±1; 3±2	2±1; 2±1	2.281	0.516	
	Was any essential information missing?	3±1; 2.5±3	3±1; 3±2	3±1; 2±2	3±1; 4±2	1.942	0.585	
	Was excessive information provided?	2±1; 2±1	3±1; 4±2	4±1; 4±0	2±1; 1.5±3	10.448	**0.015**	Pi-MC, ChatGPT-MC
	Was any irrelevant information included?	2±1; 2±2	3±1; 2±2	3±1; 3±1	2±1; 2±2	5.300	0.151	
	Was the medical information accurate?	4±1; 4±1	3±1; 3±2	3±1; 3±2	3±1; 2±2	5.665	0.129	
	Were recommendations given?	4±0; 4±0	4±1; 4±2	3±1; 3.5±2	2±1; 2±1	17.006	**0.001**	Pi-ChatGPT, Pi-Bard
	Was patient guidance provided?	4±0; 4±0	4±1; 4±2	3±1; 3.5±2	2±1; 2±1	17.790	**<0.001**	Pi-ChatGPT, Pi-Bard
	Was a recommendation to consult a physician included?	4±1; 4±1	4±1; 4±1	3±1; 3.5±2	2±0; 2±1	22.334	**<0.001**	Pi-ChatGPT, Pi-Bard
	Was the response sufficient for the patient?	3±1; 4±2	3±1; 3±2	3±1; 3±2	2±1; 2±2	7.628	0.054	
	Did the response aim to inform the reader?	4±0; 4±0	4±1; 4±1	4±1; 4±0	3±1; 3±1	8.965	**0.030**	Pi-ChatGPT
	Did the response aim to reassure the reader?	3±1; 2.5±2	2±1; 2±1	2±1; 1.5±1	2±1; 2±1	6.925	0.074	

AI: artificial intelligence, MC: Microsoft Copilot, SD: standard deviation, IQR: interquartile range

lower. MC performed significantly worse than ChatGPT (p=0.002), demonstrating ChatGPT's stronger ability to meet users' informational needs. Lastly, in "Response Aims to Inform the Reader" ChatGPT (4±0) significantly outperformed MC (3±1) (p=0.008), reinforcing ChatGPT's superiority in delivering informative responses (Table 2).

Evaluation of AI Models in Answering "How Often Should Short Stature be Monitored?"

In the "All Necessary Information Provided" category, MC scored the lowest (2±1), while ChatGPT and Bard performed better (3±1). A significant difference was observed between MC and ChatGPT (p=0.018), indicating MC's weaker performance in delivering comprehensive information. In "Essential Information Missing" MC (4±1) had the highest score, showing a greater tendency to omit details, with a significant difference from ChatGPT (p=0.018). In "Medically Accurate Information Provided" ChatGPT (4±1) significantly outperformed MC (3±0) (p=0.013), highlighting ChatGPT's superior accuracy. For "Recommendations Provided" Bard (4±1) and ChatGPT (4±0) led, while Pi (3±1) performed significantly worse (p<0.001). In "Response Was Sufficient for the Patient" ChatGPT and Bard (4±1) excelled, while MC (2±1) and Pi (3±1) scored lower. A significant difference was found between ChatGPT and MC (p=0.003), emphasizing ChatGPT's stronger ability to meet users' informational needs. Lastly, in "Recommendation to Consult a Physician" Bard (4±0) and ChatGPT (4±1) performed best, while Pi (2±1) had the lowest score, with significant differences between Pi and the other models (p<0.001) (Table 2).

Evaluation of AI Models in Answering "What are the Side Effects that can be Seen after Growth Hormone Treatment?"

In the "Excessive Information Provided" category, MC (4±1) had the highest score, significantly differing from ChatGPT (2±1) and Pi (2±1) (p=0.015), indicating MC's greater tendency to provide excessive details. In "Recommendations Provided" Bard (4±0) and ChatGPT (4±1) scored the highest, while Pi (2±1) performed the worst. A significant difference was observed between Pi and the other programs (p=0.001), suggesting Pi's limitations in providing recommendations. For "Patient Guidance Provided" Bard (4±0) and ChatGPT (4±1) again excelled, while Pi (2±1) lagged significantly (p<0.001), demonstrating Bard and ChatGPT's superior ability to offer guidance. In "Recommendation to Consult a Physician" Bard (4±1) and ChatGPT (4±1) performed best, while Pi (2±0) had the lowest score. Post-hoc analysis confirmed Pi's significantly weaker performance compared to

the other models ($p<0.001$), reinforcing Bard and ChatGPT's reliability for clinical guidance in this area. Lastly, in "Response Aims to Inform the Reader" Bard (4±0) and ChatGPT (4±1) scored the highest, while Pi (3±1) performed worse, with a significant difference between Pi and ChatGPT ($p=0.030$), highlighting ChatGPT's strength in delivering informative responses (Table 2).

Table 3 presents the expert evaluation of responses provided by the four AI programs to questions related to pediatric endocrinology.

A statistically significant difference was found the four programs for the "Was a definition provided?" question ($p=0.028$). The significance was for the differences between MC-ChatGPT and Pi-ChatGPT. For the "Was all necessary information provided?" question, Bard (3.5±0.4) had the highest score, while MC (2.7±0.5) had the lowest. A significant difference was detected between the applications ($p=0.002$), with differences specifically identified between MC-ChatGPT and MC-Bard. For the "Essential Information Missing?", "Was excessive information provided?", "Was irrelevant information provided?", and "Was the information medically accurate?" questions, the respective p values were 0.074, 0.178, 0.486, and 0.12, indicating no differences between the AI programs. In the "Were recommendations provided?" question, Bard (4±0.3) had the highest score, while Pi (2.4±0.6) had the lowest. A significant difference was observed between the AI programs ($p=0.001$), with significant differences identified between MC-Bard and Pi-Bard. Similarly, in the "Was patient guidance provided?" criterion, Bard (4.1±0.3) had the highest average score, while Pi (2.3±0.6) had the lowest. A significant difference was found between the AI programs ($p<0.001$), with the difference primarily between Pi and Bard. For the "Was a recommendation to consult a physician provided?" question, Bard (4.2±0.5) received the highest score, and a significant difference was identified between Pi and Bard ($p<0.001$). For the "Was the response sufficient for the patient?" question, Bard (3.5±0.3) and ChatGPT (3.4±0.5) had similar values, receiving the highest scores. A significant difference was found between the AI programs ($p=0.004$), with differences being identified between MC-ChatGPT and MC-Bard. A significant difference was also detected for the "Does the response aim to inform the reader?" question ($p=0.045$), with the difference identified between Pi and ChatGPT. Finally, for the "Does the response aim to reassure the reader?" question, ChatGPT (2.8±0.2) had the highest value, and a significant difference was found between the AI programs ($p=0.007$). The observed differences were between MC-ChatGPT and Pi-ChatGPT. The highest reliability, with an ICC value of 0.774 (0.682-0.844), was observed to the question "Was an appropriate definition provided?", while the lowest reliability, with an ICC value of -0.047 (-0.306-0.197), was observed for the question "Was any recommendation given?" (Table 3).

Discussion

In this study, responses provided by four different AI programs (ChatGPT, Bard, MC, and Pi) to questions related to pediatric endocrinology concerning short stature were evaluated by experts based on specific criteria. The findings indicated significant differences between the programs included in terms of medical information accuracy, guidance capacity, and user informativeness. The Bard model distinguished itself in categories requiring guidance and direction receiving the highest scores. Moreover, Bard was the model that omitted the least essential information. This suggests that Bard possessed a strong ability to deliver supportive and guiding responses. The literature highlights that AI models focusing on guidance enhance user confidence and support medical decision-making processes (7). MC excelled in providing accurate and medically reliable information. In categories such as "Was medically accurate information provided?" and "Was all necessary information provided?", it performed similarly to or even outperformed Bard and ChatGPT. This suggested that the version of MC tested was a reliable model for areas requiring medical accuracy. However, its lower guidance capacity suggested that it may not be sufficient for clinical applications in the version tested. Published evidence also supports the suggestion that AI programs with strong medical accuracy capabilities may be effectively utilized in clinical decision support systems (8). ChatGPT demonstrated consistent performance in providing information and educating users. It received high scores in the "Does the response aim to inform the reader?" category, highlighting its reliability as an informational source. However, it lagged behind Bard and MC in categories relating to guidance. This suggested that while ChatGPT was effective in knowledge dissemination, it required further development in terms of user guidance. AI applications with strong user education capabilities are known to play an important role in patient education and information dissemination (9). The Pi model exhibited acceptable performance in basic informational categories but received the lowest scores in terms of user guidance and recommendation. This suggests that Pi was inadequate for guidance-focused clinical decision-making processes. AI programs with limited guidance capacities are generally considered more suitable for handling basic queries rather than facilitating detailed information provision (10). Overall, Bard emerged as the most effective model in terms of guidance and recommendations at the time of testing, making it a more suitable AI for specialized fields, such as pediatric endocrinology, where expert guidance is essential. MC was a medically accurate application, but it requires improvement in its guidance capabilities. ChatGPT demonstrated strong informational capabilities, and if its guidance capacity is enhanced, which may have now happened, it could have broader applications. Meanwhile, Pi showed significant limitations in guidance and recommendations,

Table 3. Comparison of expert evaluation averages for AI-generated responses to questions

Questions	ChatGPT	Bard	MC	Pi				
	Mean±SD; Median (Min-Max)	Mean±SD; Median (Min-Max)	Mean±SD; Median (Min-Max)	Mean±SD; Median (Min-Max)	Kruskal-Wallis H	p	Post-hoc	ICC; 95% CI (L-U)
Was a proper definition provided?	3.7±0.3; 3.6 (3.3-4.1)	3.2±0.6; 3.1 (2.7-4.5)	3±0.8; 2.7 (2.1-4)	3.1±0.5; 3.1 (2.5-4.1)	9.066	**0.028**	MC-ChatGPT, Pi-ChatGPT	0.774 (0.682-0.844)
Was all necessary information included?	3.4±0.3; 3.5 (2.8-3.8)	3.5±0.4; 3.4 (2.9-3.9)	2.7±0.5; 2.8 (1.9-3.3)	2.9±0.5; 3 (2.2-3.5)	14.596	**0.002**	MC-ChatGPT, MC-Bard	0.523 (0.343-0.664)
Was any essential information missing?	2.7±0.5; 2.6 (2.2-3.9)	2.8±0.3; 2.9 (2.3-3.1)	3.3±0.7; 3.6 (2.2-4)	3±0.5; 3.2 (2-3.8)	6.922	0.074		0.611 (0.463-0.726)
Was excessive information provided?	2.1±0.2; 2.1 (1.8-2.3)	2.4±0.4; 2.4 (1.9-3)	2.4±0.8; 2.2 (1.7-4)	2±0.3; 1.9 (1.7-2.5)	4.912	0.178		0.525 (0.345-0.666)
Was any irrelevant information included?	2.3±0.4; 2.4 (1.7-3)	2.2±0.4; 2.2 (1.6-2.8)	2.6±0.6; 2.7 (1.7-3.3)	2.3±0.2; 2.4 (1.8-2.5)	2.443	0.486		0.536 (0.360-0.674)
Was the medical information accurate?	3.6±0.4; 3.8 (2.8-4)	3.4±0.4; 3.4 (3-4)	3.1±0.4; 3.1 (2.5-3.8)	3.7±1.4; 3.3 (2.6-7.4)	5.842	0.12		0.444 (0.237-0.607)
Were recommendations given?	3.3±0.6; 3.3 (2.1-4.1)	4±0.3; 4 (3.2-4.4)	2.9±0.8; 2.8 (1.8-4.1)	2.4±0.6; 2.5 (1.6-3.2)	17.659	**0.001**	MC-Bard, Pi-Bard	-0.047 (-0.306-0.197)
Was patient guidance provided?	3.2±0.6; 3.1 (2.3-4.1)	4.1±0.3; 4 (3.6-4.7)	3.1±0.9; 3.4 (1.8-4.2)	2.3±0.6; 2.3 (1.5-3.1)	18.498	**<0.001**	Pi- Bard	-0.030 (-0.272-0.202)
Was a recommendation to consult a physician included?	3.2±0.6; 3.4 (1.9-4.1)	4.2±0.5; 4.4 (3.4-4.6)	3.1±1.1; 3.2 (1.5-4.5)	2.2±0.6; 2.1 (1.3-3.1)	17.992	**<0.001**	Pi-Bard	0.049 (-0.181-0.268)
Was the response sufficient for the patient?	3.4±0.5; 3.5 (2.1-3.9)	3.5±0.3; 3.6 (3-4)	2.6±0.6; 2.5 (1.8-3.7)	2.8±0.6; 2.7 (2-3.6)	13.305	**0.004**	MC-ChatGPT, MC-Bard	0.341 (0.112-0.527)
Did the response aim to inform the reader?	3.9±0.4; 4.1 (2.9-4.4)	3.9±0.3; 4 (3.4-4.4)	3.6±0.4; 3.5 (2.8-4.2)	3.5±0.4; 3.4 (3-4.1)	8.048	**0.045**	Pi-ChatGPT	0.364 (0.135-0.548)
Did the response aim to reassure the reader?	2.8±0.2; 2.8 (2.5-3.1)	2.8±0.7; 2.4 (2.3-4.1)	2.2±0.4; 2.2 (1.6-2.7)	2.3±0.4; 2 (1.8-2.9)	12.059	**0.007**	MC-ChatGPT, Pi-ChatGPT	0.523 (0.345-0.663)

AI: artificial intelligence, MC: Microsoft Copilot, SD: standard deviation, Min-Max: minimum-maximum, ICC: intraclass correlation coefficient, CI: confidence interval, L: lower bound, U: upper bound

making the version tested insufficient for clinical applications requiring decision support.

The findings of this study highlight the strengths and weaknesses of different AI programs and shed light on their potential applications in medical decision-making processes. For instance, Bard, with its strong guidance capacity, could be beneficial in patient management, while MC may be more effective in areas that require medical accuracy. ChatGPT stood out as a suitable model for patient education and general information sharing.

Study Limitations

The answers produced may have changed due to the updating of the AI programs used in our study. The answers of the experts making the evaluations may be subjective. The lack of real patient data in our study can be considered as a limitation. More studies are needed for the integration of AI in clinical applications. The fact that AI programs are subject to rapid change and are constantly evolving may lead to differences in the results of the study if the same analysis was performed now.

Conclusion

This study demonstrated that different AI programs exhibited varying performances in the field of pediatric endocrinology at the time of the study. The Bard model excelled in guidance and recommendation categories, while MC proved to be strong in medical accuracy. ChatGPT emerged as a reliable option for information dissemination and user education, whereas Pi showed limited applicability in this domain, due to its insufficient guidance capacity. Future research should focus on improving AI models to achieve a more balanced performance in both guidance and medical accuracy. In addition, optimizing these programs to align with user needs is recommended to enhance patient trust and integrate AI effectively into clinical decision-support processes. Evidence has shown that while AI holds great potential in supporting patient care processes, this potential can only be fully realized through a careful balance in model design (11,12). These findings underscore the need for development of customized AI solutions, involving both software developers and experts in the field to produce programs tailored to the needs of specialized subjects, such as pediatric endocrinology.

Ethics

Ethics Committee Approval: This study does not require ethical committee approval.

Informed Consent: All experts agreed to participate.

Footnotes

Authorship Contributions

Concept: Kamber Kaşali, Özgür Fırat Özpolat, Design: Kamber Kaşali, Özgür Fırat Özpolat, Merve Ülkü, Data Collection or Processing: Kamber Kaşali, Ayşe Sena Dönmez, Serap Kılıç Kaya, Esra Dişçi, Serkan Bilge Koca, Analysis or Interpretation: Kamber Kaşali, Özgür Fırat Özpolat, Atilla Çayır, Literature Search: Kamber Kaşali, Ufuk Özkaya, Writing: Kamber Kaşali, Hüseyin Demirbilek.

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Measurement of 11-Oxo-androgens, A Novel Biomarker, in Females with Clinical Signs of Premature Adrenarche

Liana Gabriel[1], Jorge Mejia-Corletto[1], Beatriz Blinov[2], Meredith Akerman[3], Jacklyn Frank[4], Paul Saenger[1]

[1]NYU Langone Hospital-Long Island, Division of Pediatric Endocrinology, New York, United States
[2]Driscoll Children's Hospital, Endocrine and Diabetes Center, Texas, United States
[3]Northwell Health, Department of Biostatistics, New York, United States
[4]Kidcrew Medical, Toronto, Canada

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What is already known on this topic?

Adrenarche is characterized by the activation of androgens precursors dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) which are released from the zona reticularis of the adrenal gland. Recent studies have demonstrated that adrenal production of 11-oxygenated steroids are also potent adrenal androgens that can bind to androgen receptors and therefore likely play a role in the clinical picture of adrenarche.

What this study adds?

Our study has indicated that these traditional markers like DHEA and DHEA-S are not sensitive enough. 11-oxo androgens could be used as novel biomarkers to evaluate premature adrenarche in female patients in whom previous biochemical evaluation yielded inconclusive results. Perhaps early identification of these patients will permit early therapy, in hopes to prevent metabolic syndrome, type II diabetes mellitus and polycystic ovarian syndrome associated with premature adrenarche.

ABSTRACT

Objective: Endocrine findings in premature adrenarche have been characterized by elevated dehydroepiandrosterone (DHEA) DHEA-sulfate (DHEA-S) levels in the past.

Methods: We reviewed female patients, aged 4 to 8 years, with premature adrenarche who were seen at a single center between 2019 and 2023. Data were collected on the traditional androgens (DHEA and DHEA-S) and novel 11-oxo-androgens, which were measured using liquid chromatography/tandem mass spectrometry assays in commercial laboratories (Lab Corp).

Corresponding Author: Liana Gabriel, DO, NYU Langone Hospital-Long Island, Division of Pediatric Endocrinology, New York, United States
E-mail: liana.gabriel@nyulangone.org **ORCID:** orcid.org/0009-0009-6424-6281
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Results: The study included 44 girls. The majority, 89% of patients from the youngest group (4-5 years olds), presented with apocrine odor as the only symptom of premature adrenarche. We have demonstrated that DHEA and DHEA-S levels were within the normal range in many girls with premature adrenarche, whereas 11-oxo-androgens, particularly 11-hydroxyandrostenedione and 11-beta-hydroxytestosterone, were elevated. Out of those with normal DHEA-S, 75% had elevated 11-hydroxyandrostenedione, and 77.8% of those patients with normal DHEA had the same elevated oxo-androgen. Moreover, advanced bone age greater than 1 year compared to chronological age was positively associated with 11-ketotestosterone [Spearman rho=0.32, 95% confidence interval (CI): 0.01-0.57, p=0.0429] and 11β-hydroxy testosterone (Spearman rho=0.32, 95% CI: 0.01-0.58, p=0.0395).

Conclusion: We propose that 11-oxo-androgens are a more sensitive steroid to be measured when premature adrenarche is suspected.

Keywords: Oxo-androgens, adrenarche, child, premature

Introduction

Adrenarche is characterized by the activation of androgen precursors, which are released from the zona reticularis of the adrenal gland (1). Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEA-S) are both thought to be responsible for the clinical signs of adrenarche (2).

Pubarche is the appearance of pubic hair, which may also occur with the appearance of axillary hair and the presence of apocrine odor. This process is considered premature if it occurs before the age of 8 years in girls and before 9 years in boys (3,4,5,6,7).

The event of adrenarche occurs only in humans and higher primate species, such as chimpanzees, gorillas, and old world monkeys (3,4,5,6,7). It is important to differentiate premature adrenarche from precocious puberty by a lack of progressive breast development in girls or testicular enlargement in boys.

The absence of a relationship of testosterone levels to adrenarche, as in hirsutism, has raised the possibility of other bioactive androgens circulating in humans. Recent studies have demonstrated that adrenal production of 11-oxygenated steroids, such as 11-ketotestosterone (11KT) and 11-beta-hydroxytestosterone (11β-OHT) (11, are also potent adrenal androgens that can bind to androgen receptors and, therefore, likely play a role in the clinical picture of adrenarche (see Figure 1) (8).

Both DHEA and DHEA-S are typically elevated in premature adrenarche, though not in all patients. It has long been thought that these steroids may act as precursors for the increased production of testosterone in hair follicles and genital skin that exhibit the phenotypic effects associated with adrenarche (8,9,10,11,12,13).

One of the 11-oxo-androgens is 11-keto-testosterone, which has indeed been identified as the dominant bioactive androgen in children during adrenarche (11,12,13). Its androgenic capacity exceeds that of both DHEA and DHEA-S, which may well be at normal levels in premature adrenarche. Herein, we present data on 11-oxo-androgens, novel biomarker exclusively secreted by the adrenal gland with no admixture by the ovaries or testes.

Methods

We conducted a study and looked at laboratory and anthropometric data of female patients who presented to the Pediatric Endocrinology Outpatient Center at NYU Langone-Long Island from September 1, 2019, to April 15, 2023, with a history of clinical signs of premature adrenarche before 8 years of age. The study was approved by the NYU Winthrop/Long Island School of Medicine Institutional Review Board (approval no.: i20-01685, date: 05.11.2020), which granted us permission to look at the data retrospectively and also for current patients as they presented for evaluation to our center. Laboratory results and bone age results, as well as demographics, were obtained from the Epic Electronic Medical Record software program, made by Epic Systems Corporation, in Verona, Wisconsin, USA. NYU Langone-Long Island manages all of the aforementioned electronic medical records. These records were accessible to the study team members as part of their clinical responsibilities. The data collected was de-identified and stored in REDCap electronic data capture, according to NYU Langone Health's policy on data storage.

Inclusion Criteria

Female patients who are 10 years old or younger with a history and clinical signs of premature adrenarche and not showing signs of puberty (breast development).

Exclusion Criteria

1) Male patients, as there is no reference range available for male patients.

2) Patients with concomitant precocious puberty.

3) Patients with congenital adrenal hyperplasia.

4) Patients with an adrenal tumor.

The levels of 11-oxo-androgens were measured using liquid chromatography/tandem mass spectrometry (LC/MS-MS) assays in commercial laboratories (Lab Corp). DHEA-S and DHEA levels were also measured by LC/MS-MS assays. The reference values to evaluate normal levels of DHEA and DHEA-S were determined using reference ranges provided by the Esoterix manual of pediatric

endocrinology (Lab Corp). Reference ranges for DHEA levels were <68 ng/dL for 1-5-year-olds, <111 ng/dL for 6-7-year-olds, and <186 ng/dL for 8-10-year-olds. Reference ranges for DHEA-S levels in prepubertal children were <57 ng/dL for 1-5 years old, <72 ng/dL for 6-7 years old, and <193 ng/dL for 8-10 years old (Lab Corp).

Currently, commercial laboratories do not provide a reference range for 11-oxo-androgens in the pediatric population by age. Rege et al. (8) measured levels of 11β-OHT, 11KT, and 11-hydroxyandrostenedione (11OHA) in pediatric female patients with normal and premature adrenarche. We have used the mean range described in their study for 11-oxo-androgens as the reference range for our patients, as described below:

11β-OHT (ng/dL): Ages 4-5 years, mean 3.0 (2.6-6.5); ages 6-8 years, mean 4.6 (3.2-6.7); and ages 9-10 years, mean 5.5 (4.1-6.5) (8).

11OHA (ng/dL): Ages 4-5 years, mean 17.6 (11.2-34.0); ages 6-8 years, mean 27.0 (20-39.7); and ages 9-10 years, mean 26.1 (17.4-44.8) (8).

11KT (ng/dL): Ages 4-5 years, mean 8.6 (7.3-10.9); ages 6-8 years, mean 13.4 (10.3-18.1); and ages 9-10 years, mean 17.6 (14.2-22.5) (8).

The recovery rate of the oxoandrogen assay is as follows: 11KT: 101.1-107.7%, 11OHA: 97.8-113.5%, and 11β-OHT: 101.1-110.9%. The laboratory does not routinely determine lower limits of detection values for these assays. The lower limit of quantification is 3 ng/dL for each analyte. This is the lowest value where precision is <20% and accuracy is within 20% of the target. The laboratory validated linearity with x2, x5 and x10 dilutions. Matrix effects are mitigated by the use of heavy isotope internal standards for each analyte. Specimen stability was determined to be at least 14 days at ambient and refrigerated conditions, and at least one year at frozen temperatures. Intra-assay coefficient of variability for 11β-OHT was from 2.5% to 10.9%, for 11OHA (2.9-7.8%), and 11KT (2.4-4.2%) (Esoterix/LabCorp, Calabasas Hills, CA, USA).

Statistical Analysis

Descriptive statistics (mean, standard deviation, median, 25th and 75th percentiles, minimum and maximum values for continuous variables; frequencies and percentages for categorical variables) were calculated separately by group (normal vs. elevated levels of DHEA and DHEA-S). The two groups were compared using the chi-square test or Fisher's exact test, as deemed appropriate, for categorical variables and the two-sample t-test or Mann-Whitney test for continuous data, as appropriate. Spearman correlation coefficients were used to assess the association between each of the biomarkers and advanced bone age. A result was considered statistically significant at the $p<0.05$ level of significance. All analyses were performed using SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

The cohort included 44 patients in total. The characteristics of the patients in our study are shown in Tables 1 and 2. Patients were divided into three different age groups. Interestingly, the majority, 89% (8 out of 9), of patients from the youngest group (4-5-year-olds) presented with apocrine odor as the symptom of premature adrenarche. Out of 44 patients, 25 had elevated body mass index (BMI) percentile (>85th), which is 57% of our patient cohort (Table 2).

The groups were further divided into patients with normal DHEA and DHEA-S values. We then determined the number of patients with normal DHEA and DHEA-S values and elevated 11-oxo-androgens. The median values, 25th, and 75th quartiles for 11-oxo-androgens (in ng/dL) for each age group are presented in Table 3.

Out of 44 patients, 36 (81.8%) had normal DHEAS levels. Two of those had elevated 11β-hydroxy testosterone ($p=0.0349$, Fisher's exact test). There was no relationship between the 11OHA values and increased BMI percentile. However, advanced bone age greater than 1 year compared to chronological age was positively associated with 11KT (Spearman correlation coefficient=0.32, 95% CI: 0.01-0.57, $p=0.0429$) and 11OHT (Spearman correlation coefficient=0.32 (95% CI: 0.01-0.58, $p=0.0395$). Out of 44 patients, 22 (50%) had bone age advancement of more than 1 year (Table 2). In addition, our data demonstrated that 77.8% (14/18) of those patients with normal DHEA had elevated 11-OHA. Out of those with normal DHEAS, 75% have elevated 11OHA. Figure 2 depicts the DHEAS values of the whole cohort plotted against 11OHA levels on the secondary y-axis on the left

Table 1. Patient demographics

Age groups		**4-5 y.o.**	**6-7 y.o.**	**8 y.o.**
n value (% of the total group)		9 (21%)	23 (52%)	12 (27%)
Race	Caucasian/Non-hispanic	3 (33%)	12 (52%)	6 (50%)
	Hispanic	3 (33%)	5 (22%)	2 (17%)
	African American	2 (22%)	4 (18%)	0 (0%)
	Unknown	1 (11%)	2 (8%)	4 (33%)

Table 2. Patient characteristics

Characteristics	Age groups		
	4-5 y.o.	6-7 y.o.	8 y.o.
BMI in the overweight range n value (% of the total group in that category)	1 (11%)	8 (35%)	1 (8%)
BMI in the these obese range	4 (44%)	7 (31%)	4 (33%)
Bone age advancement >1 year	4 (44%)	12 (52%)	6 (50%)
Presence of pubic hair	3 (33%)	13 (30)	8 (67%)
Presence of axillary hair	1 (11%)	10 (44%)	5 (42%)
Presence of body odor	8 (89%)	15 (65%)	5 (42%)
Presence of acne	3 (33%)	1 (4%)	2 (17%)

BMI: body mass index, y.o.: years old

Table 3. Median oxo-androgen levels by age group in girls with premature adrenarche

	Age groups		
	4-5 years	6-7 years	8 years
11KT (ng/dL)	18.7 (16.0, 34.3)	20.1 (13.1, 27.3)	18.9 (16.9, 28.2)
11OH (ng/dL)	2.7 (1.5, 3.9)	3.0 (2.1, 3.6)	4.4 (2.6, 5.5)
11OHA (ng/dL)	75.0 (50.8, 84.6)	67.7 (35.4, 79.3)	63.8 (52.9, 82.4)

*Data presented as median (25^{th}-75^{th} percentiles)
11KT: 11-ketotestosterone, 11OH: 11-hydroxytestosterone, 11OHA: 11-hydroxyandrostenedione

side of the figure. The upper normal of 11OHA was indicated on the left, and the y-axis was arranged in multiples of that upper normal value (8). Our data shows that 69.6% (16/23) of those with normal levels of both DHEA and DHEAS had elevated 11OHA.

Discussion

We have demonstrated that DHEA and DHEA-S were in the normal range in many girls with premature adrenarche, while 11-oxo-androgens, particularly 11OHA and 11β-OHT, were elevated. That said, 48% of the patient population in our study were Caucasian girls from Long Island, NY, which could explain the finding and may not necessarily apply to the more diverse patient population of different ethnicities and races. This finding indicates that clinical symptoms of androgen excess were caused by 11-oxo-androgens as opposed to elevations of DHEA and DHEA-S, as previously thought. In addition, we did not see any correlation between BMI percentiles and 11-oxo-androgens. We would likely need a larger population size to confirm any correlation between BMI and 11-oxo-androgens, as it is often seen that children with premature adrenarche have increased BMI percentiles.

Rege et al. (8) identified 11KT as the dominant bioactive androgen during normal and premature adrenarche. Further prospective studies need to be conducted to determine if females with premature adrenarche with elevated oxo-androgens have a higher risk of developing polycystic ovarian syndrome (PCOS) in the future, as it has been previously shown that adolescent girls with PCOS have higher 11-oxo-androgen values when compared to non-PCOS controls (14,15).

Conventional measurements of weaker androgens (DHEA, DHEA-S), which were previously used in patients with premature adrenarche, may not give clinicians relevant biological information about the extent of androgen excess (2,16,17).

In recent studies in patients with congenital adrenal hyperplasia, androstenedione was used as the key marker for adrenal-derived androgen excess, while it is well known that androstenedione has a mixed gonadal and adrenal origin (15,18). Therefore, when studying adrenally derived androgen excess, 11-oxo-androgens are the preferred method for clinical assessment (18), and as suggested in personal communication with R. Auchus, 2025.

Study Limitations

One of our main limitations was a small sample size for the study population. Further studies with a larger number of patients of diverse racial and ethnic backgrounds are needed to investigate if a correlation of other oxo-androgens can be found in females with premature adrenarche who have otherwise normal DHEA and DHEA-S.

Conclusion

Our data provides clinical data about 11-oxo-androgen levels in girls with premature adrenarche. Our results suggest the

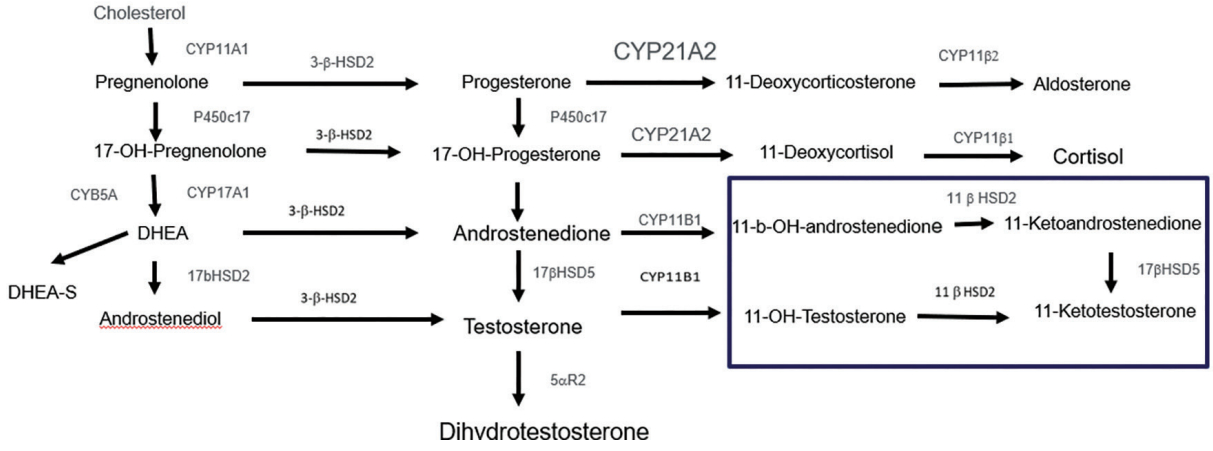

Figure 1. Steroidogenic pathway of adrenal gland [modified from Figure 3 Rosenfeld et al. (19)]
11-oxygenated androgens are enclosed in a box

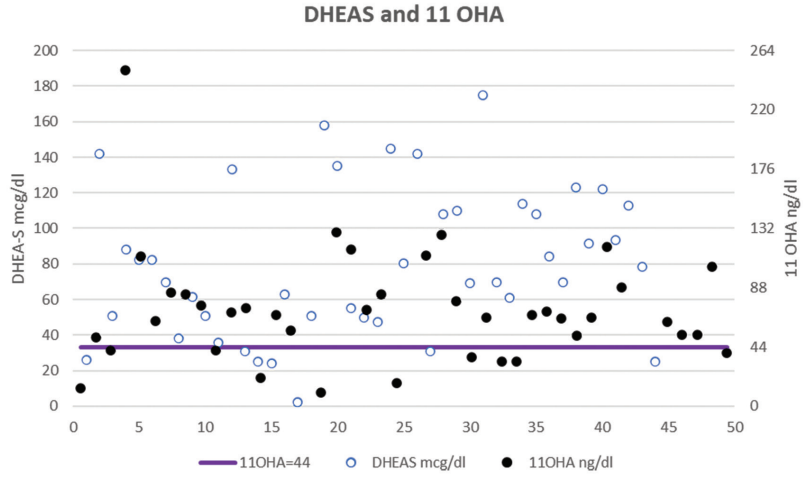

Figure 2. Relationship between 11-oxoandrogens and DHEAS levels
11OHA 44 ng/dL-upper normal value [Rege et al. (8)]

usefulness of measuring 11-oxo-androgens in a frequent clinical condition of androgen excess, in which conventional biochemical evaluation yielded inconclusive results. Perhaps early identification of these patients with premature adrenarche will permit early therapy, such as healthy lifestyle modifications, in hopes of preventing long-term complications such as metabolic syndrome, type 2 diabetes mellitus, and PCOS that are associated with premature adrenarche.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the NYU Winthrop Hospital/NYU Long Island School of Medicine Institutional Review Board (approval no.: i20-01685, date: 05.11.2020).

Informed Consent: We reviewed data retrospectively of our patients who were seen in our clinic and had this lab work done as part of their routine endocrine care.

Footnotes

Authorship Contributions

Concept: Liana Gabriel, Paul Saenger, Design: Liana Gabriel, Paul Saenger, Data Collection or Processing: Liana Gabriel, Jorge Mejia-Corletto, Beatriz Blinov, Jacklyn Frank, Analysis or Interpretation: Liana Gabriel, Beatriz Blinov, Jacklyn Frank, Meredith Akerman, Literature Search: Liana Gabriel, Paul Saenger, Beatriz Blinov, Writing: Liana Gabriel, Paul Saenger, Jorge Mejia-Corletto, Beatriz Blinov, Meredith Akerman.

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Improving Diabetes Care Through Teamwork, Comprehensive Education, Tighter Goals, and Technology: Single-Center Data from Türkiye

Elif Eviz[1,2], Kağan Ege Karakuş[2], Tuğba Gökçe[3], Ecem Can[3], Gül Yeşiltepe Mutlu[1,2], Şükrü Hatun[1,2]

[1]Koç University Faculty of Medicine, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye
[2]Koç University Faculty of Medicine, Medicine, İstanbul, Türkiye
[3]Koç University Hospital, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye

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What is already known on this topic?

Achieving better glycaemic control while maintaining a quality of life similar to that of peers is a challenging issue in the management of type 1 diabetes. Use of diabetes technologies helps to achieve better metabolic control in type 1 diabetes.

What this study adds?

Holistic approaches that focus on patient behaviors, comprehensive education, teamwork, written individualized treatment plans, and tighter metabolic targets are effective in achieving better glycemic outcomes. Most of the glycemic metrics of automated insulin delivery (AID) users were significantly better compared to multiple dose insulin and continuous glucose monitoring users and non-AID pump users.

ABSTRACT

Objective: The management of type 1 diabetes (T1D) in children aims to achieve an hemoglobin A1c (HbA1c) of <7%, a good quality of life and a life similar to that of their peers. While the HbA1c <7% target may be difficult to achieve, it is possible that national programs, quality control programs and setting team targets can achieve significant reductions in HbA1c.

Methods: The records of children with T1D followed up in our department between 2020 and 2022 were analyzed. Children and their families received a comprehensive education including an "Individual Treatment Plan", nutrition and carbohydrate counting. All HbA1c measured during follow-up were averaged for each child separately. Continuous glucose monitoring (CGM) data from the last visit was evaluated in terms

Corresponding Author: Elif Eviz, MD, Koç University Faculty of Medicine, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye
E-mail: evzelf@gmail.com **ORCID:** orcid.org/0000-0002-8889-6811
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of achieving CGM consensus targets. To assess the effect of CGM use and automated insulin delivery (AID) system use, subjects were divided into 3 groups as multiple dose insulin and CGM users, non-AID pump users and AID users and evaluated.

Results: The 480 children included in the study had a mean HbA1c of 7.8±1.5% at the first visit. The median HbA1c value during the two-year follow-up was 7.1%. Of the participants, 43% had an HbA1c <7%. Evaluating cases by treatment modalities and glucose measurement methods revealed that AID users having the lowest mean HbA1c (7±0.7%).

Conclusion: While diabetes technologies have significantly improved T1D treatment, we believe that holistic approaches focusing on patient behaviors, comprehensive education, teamwork, written individualized treatment plans, and tighter metabolic goals are effective in achieving better glycemic outcomes.

Keywords: Carbohydrate counting, diabetes technologies, individual treatment plan, type 1 diabetes

Introduction

The management of type 1 diabetes (T1D) in childhood requires a holistic approach that encompasses both glycemic outcomes and quality of life, enabling children and their families to lead daily lives similar to those of their peers (1). Current targets for glycemic outcomes reflect the need to minimize hyperglycemia as safely as possible and include a hemoglobin A1c (HbA1c) target of <7% (HbA1c target <6.5% in stage 3 T1D and remission periods, in those with access to advanced technology, and in those followed up in clinics providing advanced education/services), coefficient of variation (CV) of blood glucose of <36%, a glucose value in the range of 70-180 mg/dL, time in range (TIR) >70%, and a fasting glucose target of 70-144 mg/dL (2).

Despite targets being increasingly tightened over the years, the management of T1D in children remains a challenging issue, with mean/median HbA1c levels of 7.5% and above in almost all countries across the globe (3). In a recent study including 8004 children younger than 6 years old with T1D from the United States of America (USA), Europe and Australia, it was highlighted that more than half of the children were not able to achieve the target HbA1c value of <7.0% despite the high rate (57% to 85%) of continuous glucose monitoring (CGM) use (4). In contrast, centers in countries such as Slovenia, Norway, Sweden and other centers in Australia have achieved significant reductions in HbA1c levels within a period of 10-12 years (ranging from 9.26% to 7.75% in Slovenia, 8.2% to 7.2% in Norway, and an average of 6.7-6.8% in Sweden and Australia) due to nationwide practices, quality control programs, team goal setting and benchmarking (5,6,7,8). Promisingly, the 4T project in the USA has clearly demonstrated the multifaceted positive effects of structured programs involving teamwork, goals, technology and tight control in diabetes management, especially in regard of HbA1c (9,10).

Since there is no national registration system in Türkiye, metabolic control data is limited. In a study published in 2013 involving 1032 cases from various centers at the national level, the mean HbA1c was found to be 8.5%, and in another study involving 498 cases at the national level and published in 2016, this figure was 8.6% (11,12). In a recent cohort study of the data of 2730 children from 42 centers between 2018 and 2023, the median HbA1c was reported as 8.4% (13). This data shows that the average HbA1c in Türkiye is higher than the intended target and, perhaps more worryingly, that there has been no improvement in the last 10 years.

The aim of this study was to present the results of our program, the main components of which are teamwork, comprehensive training, tightening of targets and use of technology, as a basis for a putative national diabetes program.

Methods

The records of children with T1D who were followed up in the Department of Pediatric Endocrinology and Diabetes at Koç University Hospital between June 2020 and June 2022 were collected retrospectively. These children and their families had received comprehensive training, including education on nutrition, and practice in carbohydrate counting. During the comprehensive training, children and their families are first informed about what T1D is, general lifestyle recommendations (doing sports, not consuming junk food, daily life order), diabetes management during fasting, diabetes management during the postprandial period, additional dose application strategies, international targets (such as for HbA1c) in T1D and what value the TIR should be; the "10 Basic Recommendations" are explained (14,15). Then, an individualized written treatment plan according to the weight of the child is given to the family. Afterwards, during the interview with the diabetes education nurse, which lasts for 1-2 hours, how to measure blood glucose, insulin injection technique, injection sites and the importance of site rotation, hyperglycemia management, hypoglycemia management, glucagon application, ketone monitoring, management of sick days, CGM and pump types available in Türkiye are explained. During the dietitian meeting, which lasts three sessions, each lasting one hour, carbohydrate counting is first explained. In the second meeting, sample menus are prepared by giving individualized insulin-carbohydrate ratios to the child and family who come with a food consumption form. In the final meeting, the effects of protein and fat on blood glucose, and exercise management are explained. In the psychologist

interview, acceptance of T1D, how diabetes can be explained to young children, and a depression scale is completed for children older than 8 years old. The Children's Depression Inventory (CDI) scale was used. Motivational interviewing sessions are provided to support families and children coping with diabetes-related burnout. The frequency of psychologist meetings is determined according to individual needs. The doctor's interview is repeated every three months and the family's education is reviewed by the diabetes education nurse during each visit. The dietitian visit is repeated every six months.

The inclusion criteria for the study were having T1D for at least one year, attending at least two outpatient clinic visits and having a follow-up period of at least six months. Insulin dose adjusted HbA1c value was calculated and if ≤9%, the cases were considered to be in the honeymoon period and excluded from the study. The formula HbA1c (percent) + [4 x insulin dose (units per kilogram per 24 h)] was used to calculate this value (Figure 1) (16). Children's age, gender, duration of diabetes, blood glucose measurement methods [self-monitoring of blood glucose (SMBG), flash-CGM (f-CGM), real-time CGM (rt-CGM)], treatment modalities [multiple dose insulin (MDI), automated insulin delivery (AID), non-AID insulin pump], and total daily insulin doses (TDI) were collected from electronic health records. The AID pump used in this study was Minimed™ 780G advanced hybrid closed loop (AHCL) system, and non-AID pumps were sensor augmented Minimed™ 640G and Minimed Paradigm® Veo™ 754, Medtronic, Northridge, CA, USA. The patch pump was Omnipod DASH®, Insulet, Corporation, Acton, MA, USA. All HbA1c measurements were collected over a 2-year study period where the mean HbA1c was calculated for individuals and grouped as follows: <6.5%; 6.6-7%; 7.1-8%; 8.1-9%; and >9%. The last 14 days of CGM data for the last visit were evaluated in terms of achieving the international CGM consensus targets [TIR (70-180 mg/dL), time above range (TAR) 1 (180-250 mg/dL), TAR2 (>250 mg/dL), time below range (TBR) 1 (54-70 mg/dL), TBR2 (<54 mg/dL), mean sensor glucose (mean SG), CV, glucose management indicator (GMI) parameters] and TIR >70% and CV <36% (14).

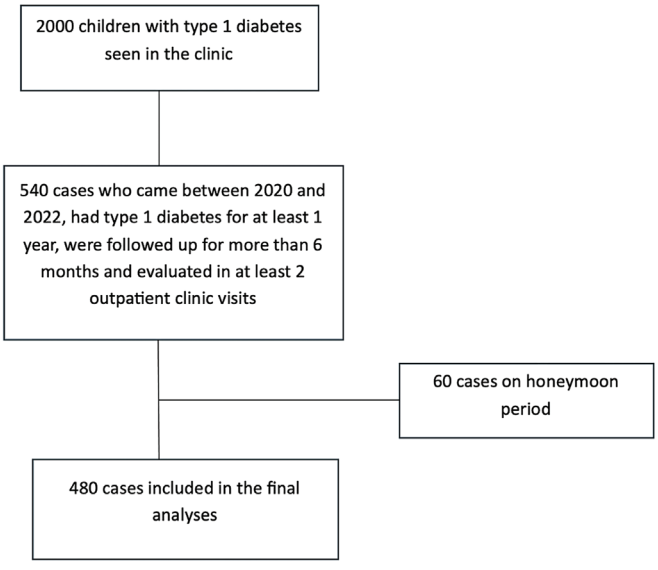

Figure 1. The flow chart of the cases

HbA1c and CGM metrics were compared between pump users and MDI users. In order to evaluate the effect of CGM use and AID use on metabolic control separately, the subjects were divided into three groups: those who used MDI and CGM; those who used non-AID pump; and those who used AID. These three groups were then compared in terms of the parameters listed above. In a separate analysis, the metabolic parameters of 203 children using CGM were compared according to the type of sensor they used, f-CGM (Abbott FreeStyle Libre) and rt-CGM (Dexcom G6, Medtronic Guardian Connect), and evaluated in terms of achieving international CGM use consensus targets (14).

In addition, cases were grouped according to the duration of diabetes technology (CGM/pump) use; those who had used it for ≤2 years and those who had used it for >2 years. Then the effect of increasing duration of diabetes technology use on glycemic control was evaluated.

The protocols were conducted according to the declaration of Helsinki principles and were approved by the Koç University Social Sciences Research Ethics Committee (approval no.: 2025.139.IRB3.060, date: 24.03.2025).

Statistical Analysis

All analyses were conducted using SPSS, version 26 (IBM Corp, Armonk, NY, USA). The Kolmogorov-Smirnov test was performed to determine whether the variables were normally distributed. Mean±standard deviation values were used to describe normally distributed continuous variables, and median and interquartile ranges were used to describe non-normally distributed continuous variables. Frequency and percentage were used to describe categorical variables. In paired group comparisons, Student's t-test was used for independent continuous variables with normal distribution and p value was determined according to Levene's analysis of variance (ANOVA) and the Mann-Whitney U test was used for non-normally distributed independent continuous variables. In comparisons of more than two normally distributed independent groups, if the sample difference between the groups was large, variance analysis was performed with the Levene's test. One-way ANOVA test was performed if there was equality of variance, otherwise the Welch-ANOVA test was performed. The groups between which the difference occurred were evaluated with Games Howell post-hoc analysis. The Kruskal-Wallis test was used for comparisons of more than

two non-normally distributed groups, and the groups between which the difference occurred were evaluated using the Mann-Whitney U test with Bonferroni correction. The chi-square test was used for comparing categorical variables. A value of $p<0.05$ was considered statistically significant.

Results

Of the 480 children included in the study, 50% were male, the mean age at the time of data collection was 11.4±4.2 years, the mean age at diagnosis of diabetes was 6.9±3.9 years, and the cases presenting to our clinic for the first time had a median of 0.4 (0.06-2.4) years after the diagnosis of diabetes. The mean number of visits was 4.2±1.7 and they were followed up for a mean of 2.7±1.4 years.

Demographic and metabolic parameters at baseline are given in Table 1, 72% (n=344) were using MDI and 28% (n=136) were using AID or non-AID pump. Of the MDI users, 40% had SMBG, 41% with f-CGM, 19% rt-CGM (17% with Dexcom G6, and 2% with Guardian Connect). Of the pump users, 43% were using AID (AHCL), 57% were using a non-AID pump (32% Minimed™ 640G, 15% Minimed Paradigm® Veo™ 754, and 9% Omnipod DASH®).

The mean TDI of the whole group was 0.8±0.2 U/kg/day. The mean HbA1c level was 7.8±1.5% at baseline, the mean number of HbA1c measurements during follow-up was 3.1±1.5, and the mean and median HbA1c values were 7.3±1.1% and 7.1%, respectively. Of the measured HbA1c values, 21% were <6.5%, 22% between 6.6-7%, 37% between 7.1-8%, 13% between 8.1-9%, and 7% >9%. In the CGM users, the mean TIR was 66.2±13.8%, TAR1 20.2±9.3%, mean SG 149.5±23 mg/dL, CV 39±7%, GMI 6.8±0.5%, median TAR2 6%, TBR1 4%, and TBR2 1%.

When the cases were divided into groups according to treatment modalities and glucose measurement methods, those who were on MDI and CGM (n=203), those who used a non-AID pump (n=77) and those who used AID (n=59), the lowest mean HbA1c value was found in AID users (7±0.7%), although there was no difference between groups (p=0.060). The ratio of there being an HbA1c <7% was highest in AID users with 58%. Of those using AID, 88% achieved the TIR >70% target. All of the glycemic metrics of AID users were significantly better compared to other treatment modalities and glucose monitoring methods, the TIR values of MDI users with CGM and non-AID pump users were 62.4±12.6% and 66.3±13.5%, and the TIR of AID users was 79.6±8.5% (p<0.001). The mean TAR1 values of MDI users with CGM was 21.1±8.4%, in non-AID pump users this was 24.2±11.2%, in AID users this was 13.7±6.5%, and was significantly lower in AID users compared to the other two groups (p<0.001). The median TAR2 was 2% in AID users, 6% in non-AID pump users, and 8% in those using MDI with CGM, and was again significantly lower in AID users compared to the other two groups (p<0.001). The respective median TBR1 and TBR2 values were 2% and 0% in AID users, 2% and 1% in non-AID pump users and 5% and 1% in MDI users with CGM glucose monitoring; the results for the MDI users with CGM were significantly worse than for the other two groups (both p<0.001) (Figure 2). The mean SG was 135.2±14.1 mg/dL in AID users, 155.3±22.1 mg/dL in non-AID pump users and 152.2±23.9 mg/dL in MDI users with AID, and was significantly lower in AID users compared to the other two groups (p<0.001). The mean CV was 33.7±5.1% in AID users, 37.4±5.4% in non-AID pump users, 41.5±6.9% in MDI users (p<0.001). The ratio of individuals with a CV<36% was significantly higher among AID users compared to non-AID pump users and MDI users with CGM (66%, 34%, and 21%, respectively; p<0.001). Mean GMI was also significantly lower in AID users compared with non-AID pump

Table 1. Demographic features and metabolic parameters of the cases in all groups

Number of the participants	480
Gender (Male) (%)	50
Age at diagnosis of diabetes (years), mean±SD	6.9±3.9
Diabetes duration in the first visit (years), median (IQR)	0.4 (0.06-2.4)
Follow-up time (years), mean±SD	2.7±1.5
Number of visits, mean±SD	4.2±1.7
TDI (U/kg/day), mean±SD	0.8±0.2
HbA1c in the first visit (%), mean±SD	7.8±1.5
HbA1c (%)†, mean±SD median (IQR)	7.3±1.1‡ 7.1 (6.6-7.8)
Number of HbA1c measurements, mean±SD	3.1±1.5
HbA1c<6.5% (%)	21
HbA1c 6.6-7% (%)	22
HbA1c 7.1-8% (%)	37
HbA1c 8.1-9% (%)	13
HbA1c>9% (%)	7
TIR (70-180 mg/dL) (%), mean±SD	66.2±13.8
TAR1 (180-250 mg/dL) (%), mean±SD	20.2±9.3
TAR2 (>250 mg/dL) (%), median (IQR)	6 (2-11.7)
TBR1 (54-70 mg/dL) (%), median (IQR)	4 (2-7)
TBR2 (<54 mg/dL) (%), median (IQR)	1 (0-2)
Mean SG (mg/dL), mean±SD	149.5±23
CV (%), mean±SD	39±7
GMI (%), mean±SD	6.8±0.5

†The HbA1c value given here is the average of HbA1c values during follow-up.
‡There was a statistically significant difference between Hba1c at baseline and mean Hba1c (p<0.001).
CV: coefficient of variation, GMI: glucose management indicator, Mean SG: mean sensor glucose, TAR: time above range, TBR: time below range, TDI: total daily insulin, TIR: time in range, HbA1c: hemoglobine A1c, IQR: interquartile range, SD: standard deviation

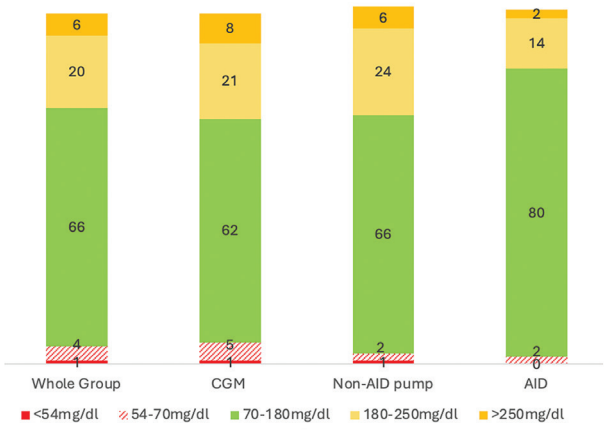

Figure 2. CGM metric values in cases with sensor data
CGM: continuous glucose monitoring, AID: automated insulin delivery

users and MDI users with CGM glucose monitoring (6.5±0.3% vs 7±0.5% vs 6.9±0.6%, p<0.001) (Table 2).

When the glycemic outcome was evaluated regarding insulin treatment modality, the mean HbA1c was 7.2±0.9% in pump users and 7.4±1.2% in MDI users (p=0.048); according to the glucose monitoring method for MDI users, the mean HbA1c was 7.8±1.4% in those who performed SMBG and 7.1±0.9% in those who used CGM (p<0.001).

When the glycemic parameters of individuals using MDI and CGM were compared in terms of the type of CGM used, i.e. f-CGM (FreeStyle Libre, n=140) and rt-CGM [Dexcom G6, (n=58) and Guardian Connect (n=5), n=63], it was observed that the use of rt-CGM provided better glycemic outcomes. The mean HbA1c of rt-CGM users was 6.7±0.7%, while the mean HbA1c of f-CGM users was 7.2±0.8% (p<0.001). The mean TIR was 68.1±12.4% in rt-CGM users and 59.2±11.7% in f-CGM users (p<0.001). Mean TAR1 was significantly lower in rt-CGM users compared to f-CGM

Table 2. Metabolic parameters of the patients according to treatment modalities and glucose monitoring methods

	MDI+CGM (n=203)	Non-AID pump users (n=77)	AID users (n=59)	p value
HbA1c (%), mean±SD†,‡	7.1±0.9	7.3±1	7±0.7	0.060
HbA1c<6.5% (%)§	25	16	24	<0.001
HbA1c<6.6-7% (%)§	24	26	34	<0.001
HbA1c 7.1-8% (%)§	37	39	36	<0.001
HbA1c 8.1-9% (%)§	10	13	5	<0.001
HbA1c>9% (%)§	4	5	0	<0.001
TIR (70-180 mg/dL) (%), mean±SD‡	62±12.6	66.3±13.5	79.6±8.5	<0.001
TIR>70% (%)§	29	45	88	<0.001
TAR1 (180-250 mg/dL) (%), mean±SD‡	21.1±8.4	24.2±11.2	13.7±6.5	<0.001
TAR2 (>250 mg/dL) (%), median, (IQR)¶	8 (3-13)	6 (2-10.5)	2 (1-4)	<0.001
TBR1 (54-70 mg/dL) (%), median, (IQR)¶	5 (3-8)	2 (1-4)	2 (1-4)	<0.001
TBR2 (<54 mg/dL) (%), median, (IQR)¶	1 (1-3)	1 (0-1)	0 (0-1)	<0.001
Mean SG (mg/dL), mean±SD‡	152.2±23.9	155.3±22.1	135.2±14.1	<0.001
CV (%), mean±SD‡	41.5±6.9	37.4±5.4	33.7±5.1	<0.001
CV being<36%, (%)§	21	34	66	<0.001
GMI (%), mean±SD‡	6.9±0.6	7±0.5	6.5±0.3	<0.001

†The HbA1c value given here is the average of HbA1c values during follow-up.
‡Levene analysis of variance was performed due to differences in sample size between groups. Welch ANOVA test was performed for all parameters except CV since Levene's variance was not equal between the groups. One-Way ANOVA was performed due to the equality of variance between the groups in CV. The Games-Howell test was used as post-hoc analysis to determine which groups the difference occurred between. There was a significant difference in CV between all three groups. For other parameters, there was a significant difference between AID users and the other two groups, but no significant difference between CGM with MDI users and non-AID pump users.
§The difference between the groups was analyzed using the chi-square test.
¶The significance of the difference between groups was assessed using Kruskall-Wallis analysis. Mann-Whitney U analysis with Bonferroni correction was performed to determine which groups the difference occurred between. The difference in TAR2 was between AID users and the other two groups. The difference in TBR1 and TBR2 was between CGM with MDI users and the other two groups.
AID: automated insulin delivery, CGM: continuous glucose monitoring, CV: coefficient of variation, MDI: multiple dose insulin, GMI: glucose management indicator, IQR: interquartile range, Mean SG: mean sensor glucose, TAR: time above range, TBR: time below range, TIR: time in range, HbA1c: hemoglobin A1c, SD: standard deviation. ANOVA: analysis of variance

users (19.4±7.8 vs 21.8±8.6, p=0.038). Median TBR1 and TBR2 values were significantly lower in rt-CGM users than in f-CGM users (4% vs 5%, p1<0.001; 1% vs 2% p2=0.004, respectively). The CV value was 38.6±5.5% in rt-CGM users and 42.6±7% in f-CGM users (p=0.001). The rate of individuals with a TIR >70% was significantly higher in rt-CGM users compared to f-CGM users (46% vs 21%, p=0.001) (Table 3).

When evaluated according to the duration of diabetes technology use, the mean HbA1c level was 6.9±0.8% in those with ≤2 years of technology use and 7.3±0.9% in those with >2 years (p<0.001). The mean TIR value was significantly higher in those with ≤2 years of technology use (68.2±13.8%) compared to those with >2 years (64.6±13.6%) (p=0.026). There was no difference between them in terms of TBR1 (p=0.671) and TBR2 values (p=0.312) (Table 4).

Table 3. Metabolic parameters according to the type of CGM used

	f-CGM (n=140)	rt-CGM (n=63)	p value
HbA1c (%), mean±SD	7.2±0.8	6.7±0.7	<0.001
TIR (70-180 mg/dL) (%), mean±SD	59.2±11.7	68.1±12.4	<0.001
TAR1 (180-250 mg/dL) (%), mean±SD	21.8±8.6	19.4±7.8	0.038
TAR2 (>250 mg/dL) (%), median, (IQR)	9 (3-14)	7 (3-12)	0.091
TBR1 (54-70 mg/dL) (%), median, (IQR)	5 (4-8)	4 (2-6)	<0.001
TBR2 (<54 mg/dL) (%), median, (IQR)	2 (1-4)	1 (1-1)	0.00
Mean SG (mg/dL), mean±SD	153.8±24.8	148.7±21.4	0.175
CV (%), mean±SD	42.6±7	38.6±5.5	0.001
GMI (%), mean±SD	6.9±0.6	6.8±0.5	0.222
TIR (70-180 mg/dL) being>70% (%)	21	46	0.001
TAR (180-250 mg/dL) being<25% (%)	36	48	0.121
TBR (<70 mg/dL) being<5% (%)	38	57	0.018
CV being<36% (%)	18	30	0.082

The difference between the groups was analyzed with Independent Samples t-test for HbA1c, TIR, TAR1, Mean SG, CV and GMI; with Mann-Whitney U test for TAR2, TBR1 and TBR2, and with chi-square test for TIR>70%, TAR>25%, TBR<5% and CV<36%.
CV: coefficient of variation, f-CGM: flash-continuous glucose monitoring, GMI: glucose management indicator, IQR: interquartile range, mean SG: mean sensor glucose, rt-CGM: real-time continuous glucose monitoring, TAR: time above range, TBR: time below range, TIR: time in range, HbA1c: hemoglobin A1c, SD: standard deviation

Table 4. Metabolic parameters according to the duration of diabetes technology use

	≤2 years (n=132)	>2 years (n=207)	p value
HbA1c (%), mean±SD	6.9±0.8	7.3±0.9	**<0.001**
TIR (70-180 mg/dL) (%), mean±SD	68.2±13.8	64.6±13.6	**0.026**
TAR1 (180-250 mg/dL) (%), mean±SD	19.1±9.9	21.1±8.8	0.076
TAR2 (>250 mg/dL) (%), median, (IQR)	4 (2-10)	7 (2-13)	**0.032**
TBR1 (54-70 mg/dL) (%), median, (IQR)	4 (2-6)	4 (2-7)	0.671
TBR2 (<54 mg/dL) (%), median, (IQR)	1 (1-2)	1 (0-2)	0.312
Mean SG (mg/dL), mean±SD	145.4±22.7	152.6±22.9	**0.007**
CV (%), mean±SD	38.5±6.9	39.4±7.1	0.310
GMI (%), mean±SD	6.7±0.6	6.9±0.5	**0.019**
TIR (70-180 mg/dL) being>70% (%)	49	39	0.067
TAR (180-250 mg/dL) being<25% (%)	58	44	**0.018**
TBR (<70 mg/dL) being<5% (%)	53	57	0.428
CV being<36% (%)	35	33	0.728

The difference between the groups was analyzed with Independent Samples t-test for HbA1c, TIR, TAR1, Mean SG, CV and GMI; with Mann-Whitney U test for TAR2, TBR1 and TBR2, and with chi-square test for TIR >70%, TAR >25%, TBR <5% and CV <36%.
CV: coefficient of variation, f-CGM: flash-continuous glucose monitoring, GMI: glucose management indicator, IQR: interquartile range, mean SG: mean sensor glucose, rt-CGM: real-time continuous glucose monitoring, TAR: time above range, TBR: time below range, TIR: time in range, HbA1c: hemoglobin A1c, SD: standard deviation

Discussion

In this single center study examining the glycemic outcomes of children with T1D, 480 children with regular follow-up between 2020 and 2022 had a median HbA1c of 7.1%, where 43% of cases had a HbA1c <7%, and only 7% had a HbA1c above 9%. These values are lower than the previously reported mean HbA1c levels from Türkiye (8.5%, 8.6% and 8.4%) and it is noteworthy that the rate of HbA1c >9%, which was 7% in our cohort was much lower than in the earlier studies, 36.9% and 35.7%, respectively (11,12,13).

Our data shows that the best metabolic results, especially TIR and HbA1c, were obtained in the group using an AID. The T1D cases followed in our department use AHCL as AID and in this group, the mean HbA1c was 7% and the mean TIR was 79.6%, providing better glycemic results than all groups using sensors. The most important contribution of AID to diabetes management is that it provides adaptive basal insulin according to the basal insulin requirement that varies according to many factors during the day, as well as making small adjustments every five minutes instead of making large adjustments at infrequent intervals (17). Recently published studies have shown that these systems, when set optimally, can achieve targets not only for TIR but also for TITR, regardless of country (18,19). Our data also support these findings and that, in the long term, all children with T1D should use AID, which is currently the most physiological method of insulin delivery available.

The use of CGM leads to better glycemic parameters compared to SMBG (20). In our cohort, the mean HbA1c of those with SMBG was significantly worse than the mean HbA1c of those using CGM was 7.1±0.9. When an evaluation was made between CGMs, HbA1c was 6.7±0.7% and TIR were significantly better in rt-CGM than in those using f-CGM. In the CORRIDA study evaluating the effect of f-CGM and rt-CGM on metabolic parameters, similar to our data, rt-CGM improved metabolic parameters (21). This suggests that the difference in glycemic parameters was due to sensor use in AID users.

However, as the duration of diabetes increased, glycemic parameters may worsen in individuals with T1D due to loss of motivation and burnout, and the solution to this also requires a multidisciplinary team approach (22). In our study, metabolic control of the cases worsened as the duration of diabetes increased, but follow-up of these cases is ongoing and long-term results may become better after identification of the problem and additional multidisciplinary team care.

The pediatric diabetes program in our department was started in 2016 with the establishment of a new center and so far around 2000 children with T1D have been seen. Our department has a pediatric diabetes team consisting of two physicians, one fellow, two nurses, one dietician and one psychologist. Each case is allocated an hour of time by the physicians in the first interview and topics such as individual treatment recommendations, glucose targets, insulin dose calculations (insulin/carbohydrate ratios and correction factor according to meals), rules to be followed before going to bed at night, reverse dawn phenomenon and management, hypoglycemia management, timing and calculating correction doses, optimal carbohydrate amount, “diabetes team at home” and the role of fathers are emphasized. All recommendations are made for each child according to the age and characteristics of the child and given to the family in writing as an “Individual Treatment Plan”. In addition, a basic diabetes education update is provided at the first visit and nutrition/carbohydrate counting training is provided at a separate appointment for each case.

In addition to the relatively better conditions of the cases admitted to our department, we believe that the comprehensive education provided, teamwork, and “10 Basic Recommendations” that set the basic goals and the use of technology are effective in achieving these glycemic results (15). In Türkiye, sensors were not reimbursed at the time this study was conducted and there is limited support for insulin pump therapy. However, the rate of self-provided sensor use in the cases followed up in our department is higher than the national average, and our data show that sensor use leads to better HbA1c control in cases on MDI therapy. Previously published studies from Sweden and the Czech Republic, and more recently from the USA and Norway, show that equal access to CGM immediately after diagnosis of T1D can be a first step towards improving HbA1c for all young people with T1D (3,8,23,24). Our data and these studies show that the most important step to be taken in changing the lives of around 30,000 children with T1D in Türkiye and to ensure that they live normal and healthy lives, like their peers, is to provide unconditional CGM support to all children with T1D, regardless of income, through the social security system and global reimbursement.

Currently, glycemic parameters are not meeting the recommended targets and this appears to be largely due to glucose fluctuations during daylight hours and the attitudes of people with T1D and/or their families. In most cases, attitudes such as the impracticality of treatment recommendations, unclear communication on glycemic targets, and incompatibility between the goals of diabetes teams and families are common (25). Additional issues include the habit of eating three main meals and three snacks, which was recommended when regular insulin was used, variations in education on nutrition (26), not administering or delaying the correction dose, going to bed with high glucose levels due to fear of hypoglycemia (27), and

neglecting carbohydrate counting and meal composition. Failure to achieve recommended targets leads to a loss of motivation and inertia, characterized by a gradual move away from long-term goals (22). In our department, carbohydrate counting is taught starting from diagnosis, children with T1D and their families are encouraged to be an active part of insulin dose adjustments and food management from the very beginning, correcting glucose elevation >145 mg/dL if possible, going to bed with normal glucose, and avoiding snacks unless necessary, are emphasized as routine practices. We observe that the previously mentioned "10 Basic Recommendations" (15), which are easy to keep in mind, and its written form in the "Individual Treatment Plan" enable families of children with T1D to follow a roadmap and start by knowing what to do and why, which, together with the information provided by the sensors, helps families and patients master the condition and facilitates better metabolic control. We suggest that this "mastering" process had a significant impact on the relatively better metabolic results they obtained and we reported and that our patients and families adhered to their T1D treatment routines with the motivation they gained from seeing success; a positive feedback cycle. At this point, we would like to state that we believe it is also important to focus on helping families overcome the fear of hypoglycemia and glucagon injection (28,29) and that we have an educational approach that enables them to manage diabetes with knowledge, not fear.

Study Limitations

One limitation of this study is that not all HbA1c measurements were performed at the same intervals, due to its retrospective design. Since the study was conducted in a private hospital, not all cases were able to attend follow-up visits every three months, and HbA1c measurements could not be obtained at every visit. One possible reason for these less frequent visits may be the financial burden associated with receiving care in a private setting; however, we do not have direct evidence to confirm this. In addition, factors such as family education, sociocultural background, and acceptance of the diabetes diagnosis may also influence glycemic control. Due to the retrospective design of the study, data on the educational, socio-cultural, and socio-economic characteristics of the families were not available in the outpatient clinic records, and thus their potential impact could not be evaluated. Furthermore, no validated questionnaires or assessment tools were used to evaluate the level of diabetes acceptance by the children or their families. These are acknowledged as important limitations of our study. Furthermore, since the families attending this center generally have middle and upper socio-economic level, the data may not reflect the entire population. When the cases were evaluated according to the duration of use of diabetes technologies, it was observed that glycemic control was worse in those who used diabetes technologies for a longer period of time. Thus, the lack of longer follow-up data can be considered as another limitation of this study. However, as mentioned in the introduction, we firmly believe that these results are encouraging and may be used to help us all build our own national program.

Conclusion

In conclusion, although the use of technology, especially CGM, has made a major difference in the treatment of T1D, there remains a need for holistic approaches that encourage the use of diabetes technology as widely as possible, focus on the behavior of people with T1D, especially nutrition, and that a full complement of specialists are included in diabetes teams to ensure this.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Koç University Social Sciences Research Ethics Committee (approval no.: 2025.139.IRB3.060, date: 24.03.2025).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Elif Eviz, Kağan Ege Karakuş, Gül Yeşiltepe Mutlu, Şükrü Hatun, Concept: Elif Eviz, Kağan Ege Karakuş, Tuğba Gökçe, Ecem Can, Gül Yeşiltepe Mutlu, Şükrü Hatun, Design: Elif Eviz, Tuğba Gökçe, Ecem Can, Gül Yeşiltepe Mutlu, Şükrü Hatun, Data Collection or Processing: Elif Eviz, Kağan Ege Karakuş, Gül Yeşiltepe Mutlu, Şükrü Hatun, Analysis or Interpretation: Elif Eviz, Kağan Ege Karakuş, Gül Yeşiltepe Mutlu, Şükrü Hatun, Literature Search: Elif Eviz, Tuğba Gökçe, Ecem Can, Gül Yeşiltepe Mutlu, Şükrü Hatun, Writing: Elif Eviz, Kağan Ege Karakuş, Gül Yeşiltepe Mutlu, Şükrü Hatun.

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Association of Obesity and Overweight with Early Puberty in Boys: A Meta Analysis

Xiou Wang†, Yi Song†, Ziqin Liu

Capital Center for Children's Health, Capital Medical University, Capital Institute of Pediatrics, Department of Endocrinology, Beijing, China
†Authors contributed equally to this work and should be considered co-first authors.

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What is already known on this topic?

While obesity is a well-documented risk factor for early puberty in girls, its association with male pubertal timing remains controversial. Existing studies show conflicting results, ranging from earlier onset to no effect or even delayed puberty in severe obesity. The post-coronavirus disease-2019 era has seen a global rise in idiopathic cases coinciding with increasing childhood obesity rates, though underlying mechanisms remain unclear. Emerging evidence suggests potential adverse effects on male genital development.

What this study adds?

While obesity is an established risk factor for precocious puberty in females, its role in male pubertal development remains controversial. Our meta-analysis confirms that childhood obesity significantly increases the risk of early puberty in males. Notably, JCEM studies indicate obesity may reduce pubertal penile growth by approximately 10% while lowering testosterone levels. These findings collectively suggest a dual-effect paradigm of adiposity in male development: obesity appears to both accelerate sexual maturation while potentially compromising optimal genital development.

ABSTRACT

Objective: To evaluate the published associations between obesity, overweight, and central obesity and the risk of early puberty in boys.

Methods: A comprehensive systematic search was conducted in accordance with PRISMA guidelines using the Web of Science and PubMed databases up to December 31, 2024. Study quality was assessed using the Newcastle-Ottawa Scale. Statistical analyses were performed using R software (version 4.4.2), with odds ratios (ORs) and 95% confidence intervals (CIs) calculated.

Corresponding Author: Ph.D. Ziqin Liu, Capital Center for Children's Health, Capital Medical University, Capital Institute of Pediatrics, Department of Endocrinology, Beijing, China
E-mail: lendocrinology@126.com **ORCID:** orcid.org/0000-0003-3310-4861
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Results: A total of 15,452 studies were initially identified, of which six high-quality studies (n=64,485) met the inclusion criteria after screening. The analysis found that obesity [defined by body mass index (BMI)] was significantly associated with an increased risk of testicular enlargement (OR=1.27, 95% CI: 1.19-1.36). Overweight also increased the risk of testicular enlargement (OR=1.20, 95% CI: 1.11-1.29). Obesity was significantly associated with an increased risk of pubarche (OR=1.37, 95% CI: 1.23-1.53). Funnel plots and sensitivity analyses indicated no significant publication bias, and the results remained robust.

Conclusion: This study demonstrated that obesity and overweight are reported to be associated with an increased risk of early puberty in boys. Childhood obesity appears to be an important determinant of earlier pubertal onset, though the relationship may follow a non-linear pattern at extreme BMI levels. The potential implications for adult reproductive health warrant further investigation.

Keywords: Obesity, overweight, central obesity, early puberty, testicular enlargement, pubarche, meta-analysis

Introduction

Central precocious puberty (CPP) is defined as the onset of puberty before the age of 8 years in girls and 9 years in boys, resulting from premature activation of the hypothalamic-pituitary-gonadal axis (1,2). In recent decades, the incidence of CPP has shown a significant upward trend globally, with a notable surge observed following the coronavirus disease-2019 (COVID-19) pandemic (3,4,5). The etiology of CPP is multifactorial, encompassing genetic predispositions, environmental influences, and metabolic factors, among which obesity has emerged as an important contributor (6,7,8). An earlier onset of puberty in children has been documented in many countries, with precocious puberty representing a prevalent endocrine disorder in childhood (1). The concurrent trends of declining age at puberty onset and increasing prevalence of obesity have generated interest in the association between obesity and pubertal timing (5).

Epidemiological cross-sectional and longitudinal studies have consistently demonstrated that overweight and obesity are strongly associated with earlier puberty onset and menarche in girls (4,6,7,9). A meta-analysis has further identified obesity as a significant risk factor for the early onset of puberty in girls (10). However, the relationship between obesity and CPP in boys remains poorly understood, with limited and inconsistent evidence available (8,11,12). The incidence of precocious puberty differs markedly between boys and girls, with girls exhibiting a significantly higher prevalence than boys (8). While previous studies have consistently highlighted a strong association between early puberty in girls and elevated body mass index (BMI) or obesity, the evidence in boys remains inconclusive and subject to debate (8,11,12).

To address this gap, we conducted a comprehensive analysis of the existing literature to determine whether obesity or overweight status is similarly associated with early puberty in boys. The aim of this systematic review was to evaluate the potential association between obesity and CPP in boys, providing clearer evidence about an important and understudied aspect of pubertal development.

Methods

All methods used in this systematic review and meta-analysis were conducted in accordance with the PRISMA guidelines (13).

Search Strategy

A comprehensive literature search was conducted on Web of Science and PubMed. The search strategy included key terms related to obesity, such as “Obesity”, “obese”, “adiposity”, “overweight”, “bodyweight”, “BMI”, “body mass index”, “body fat”, or “body fat mass”, combined with terms related to early puberty, including “pubertal timing”, “puberty timing”, “sexual precocity”, “sexual prematurity”, “premature pubarche”, “first spermatorrhea”, “gonadarche”, or “precocious puberty”. The search was executed on both Web of Science and PubMed databases to ensure a thorough retrieval of relevant studies.

Selection Criteria

This systematic review and meta-analysis included studies that met the following criteria: (i) cohort or case-control studies focusing on children; (ii) an exposure group comprising children classified as obese by the study authors, compared with a control group of children with normal weight; (iii) the primary outcome measured was the onset of secondary sexual characteristics, specifically including testicular enlargement, first ejaculation, and the initial appearance of pubic hair.

Quality Assessment of Literature

The methodological rigor of the included cohort studies was evaluated using the Newcastle-Ottawa Scale (NOS), which assigns a maximum score of 9 points. Studies were categorized as low (0-3 points), medium (4-6 points), or high quality (7-9 points) (14). Two independent researchers (AA, BB) performed the quality assessments. Any disagreements were resolved through discussion or adjudication by a senior author (AA).

Statistical Analysis

All statistical analyses were conducted using R software, version 4.4.2. The odds ratio (OR) was used as the primary measure of effect size for count data, accompanied by a 95% confidence interval (CI). Heterogeneity among studies was assessed using the chi-square test, with the p value and I^2 statistic providing measures of heterogeneity. A fixed-effects model was employed when the studies exhibited low heterogeneity ($p \geq 0.05$, $I^2 \leq 50\%$), while a random-effects model was used in the presence of significant heterogeneity ($p < 0.05$, $I^2 > 50\%$). Publication bias was assessed through funnel plots and Egger's test. Sensitivity analysis was performed to evaluate the robustness of the meta-analysis results by systematically excluding each study and analyzing its influence on the overall effect, with statistical significance set at $p < 0.05$.

Results

The systematic search of databases identified 15,452 potential studies (Figure 1). Following the removal of duplicates and an initial screening of titles and abstracts, 132 full-text articles were selected for detailed eligibility assessment. Studies were excluded based on predefined criteria: 40 were excluded for not focusing on precocious puberty, 65 were excluded due to the absence of both obesity exposure and control groups, and 21 were excluded for not adhering to case-control or cohort study designs. Ultimately, six studies meeting all inclusion criteria were included in the quantitative analysis, as summarized in Table 1. These studies were conducted in Chile, the United States, and China, and included participants of Asian, Black, White, and Caucasian race/ethnicity. The methodological quality of the six cohort studies was evaluated using the NOS, with scores ranging from 7 to 9, indicating high-quality studies (Table 2). The total sample size across all studies combined was 64,485 participants.

BMI-defined obesity was significantly associated with an increased risk of testicular enlargement (≥4 mL, OR=1.27, 95% CI: 1.19-1.36; Figure 2). However, substantial heterogeneity was observed across studies (I^2=54.8%, p=0.0237). Overweight, also defined by BMI, similarly increased the risk of testicular enlargement (≥4 mL, OR=1.20, 95% CI: 1.11-1.29; Figure 3), with high heterogeneity (I^2=58%, p=0.0266). Two studies examined the association between central obesity, defined by waist circumference, and testicular enlargement, but the

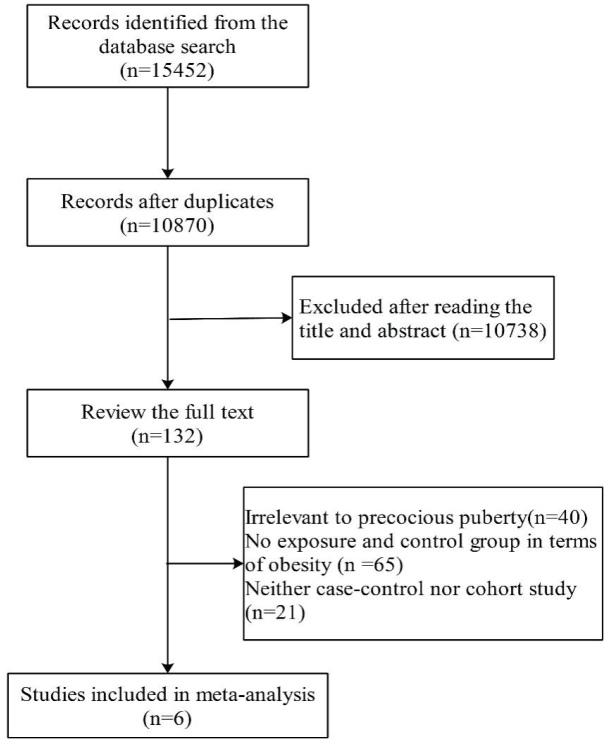

Figure 1. PRISMA flow diagram

Table 1. Characteristics of studies included in the meta-analysis

Study, year	Country	Ethnicity	Design	Sample size	Obesity	Outcome
Pereira et al. (15), 2021	Chile	White	Cohort	345	BMI>+2SD WC>90th	Gonadarche
Li et al. (19), 2018	China	Asian	Cohort	644	BMI>27.9	Gonadarche
Li et al. (16), 2022	China	Asian	Cohort	645	BMI>27.9 WC≥90th	Gonadarche
Liu et al. (17), 2021	China	Asian	Case-control	525	BMI>P95th	Central precocious puberty
Aghaee et al. (20), 2022	California	White, Black, Hispanic, Asian	Cohort	62190	BMI≥P95th	Gonadarche Pubarchec
Deardorff et al. (21), 2021	California	White	Cohort	136	BMI≥P95	Gonadarche Pubarche

Table 2. Newcastle-Ottawa scale for assessing the methodological quality of cohort studies

Study	Selection				Comparability	Outcome		
	Representativeness of the expose cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts	Score
Pereira et al. (15), 2021	1	1	1	1	1	1	1	0	7
Li et al. (19), 2018	1	1	1	1	1	1	1	1	8
Li et al. (16), 2022	1	1	1	1	2	1	1	1	9
Aghaee et al. (20), 2022	1	1	1	1	1	1	1	1	8
Deardorff et al. (21), 2021	1	1	1	1	2	1	1	1	9

results were not significant (OR=2.38, 95% CI: 0.92-6.19; Figure 4). Obesity was significantly associated with an increased risk of pubarche (OR=1.37, 95% CI: 1.23-1.53; Figure 5), with high heterogeneity (I^2=75.9%, p=0.0023). Overweight also increased the risk of pubarche (OR=1.26, 95% CI: 1.18-1.36; Figure 6), with moderate heterogeneity (I^2=55.8%, p=0.06). Funnel plots indicated no significant publication bias, and the adjusted effect size remained statistically significant (OR=1.24, 95% CI: 1.17-1.32; Figure 7). Sensitivity analyses confirmed the robustness of the results, as the exclusion of any single study did not alter the significance of the findings (Figure 8).

Discussion

Our systematic review ultimately included six high-quality studies that met the predefined eligibility criteria. The findings of this analysis demonstrated a significant reported association between overweight/obesity and precocious puberty in boys, consistent with trends previously observed in girls. These results are also consistent with multiple domestic and international studies (1,3,6,9), further supporting the important role of obesity in the onset of precocious puberty.

The relationship between obesity/overweight and earlier pubertal initiation in girls has been well-documented in prior research (4,7,9). In contrast, studies focusing on boys remain limited, likely due to the lower incidence of precocious puberty in males compared to females, as well as the historical emphasis on pathological etiologies in male cases. However, emerging evidence suggests a rising trend in idiopathic male precocious puberty globally, particularly following the COVID-19 pandemic. This trend parallels observations reported in China and other regions (15,16,17,18,19).

The relationship between childhood obesity and precocious puberty in boys has attracted increasing attention amid global trends of declining pubertal age and increasing pediatric adiposity. This systematic analysis of six contemporary studies revealed both converging and conflicting evidence regarding this association, highlighting the need for nuanced interpretation of biological and environmental interactions.

Supportive Evidence for Obesity-Puberty Link

Multiple longitudinal studies demonstrate measurable associations between adiposity and earlier gonadarche in boys. Pereira et al. (15) found that total body fat [β=-0.32 years/standard deviation (SD), p<0.01] and central adiposity (waist-height ratio β=-0.41 years/SD, p<0.001) independently predicted earlier testicular enlargement in a multiethnic cohort, with obese boys experiencing gonadarche 1.1 years earlier than lean peers. These findings align with the Chinese longitudinal study of Li et al. (16), showing boys in the highest adiposity trajectory had 2.3-fold increased risk of precocious pubarche (95% CI: 1.4-3.8) compared to normal-weight counterparts.

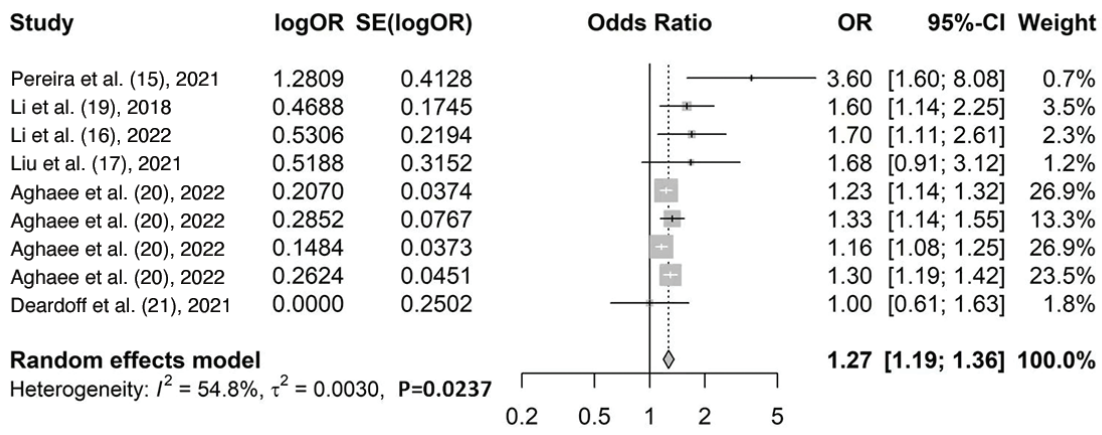

Figure 2. Forest plot of obesity (BMI-defined) and testicular enlargement
OR: odds ratio, CI: confidence interval, SE: standard error, BMI: body mass index

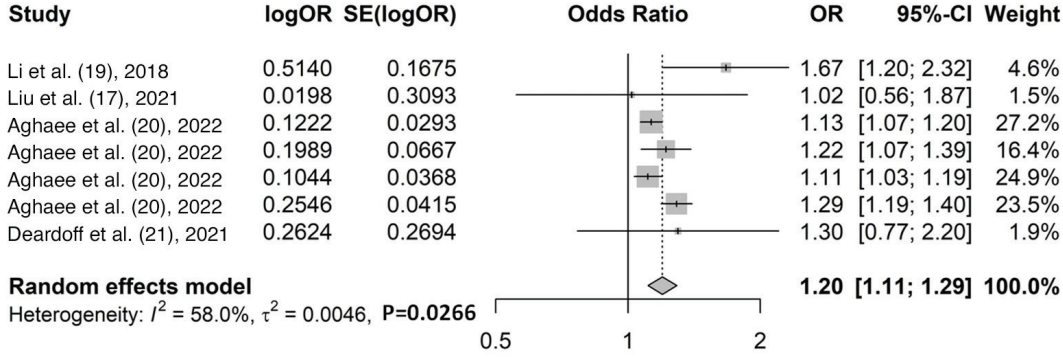

Figure 3. Forest plot of overweight (BMI-defined) and testicular enlargement
OR: odds ratio, CI: confidence interval, SE: standard error, BMI: body mass index

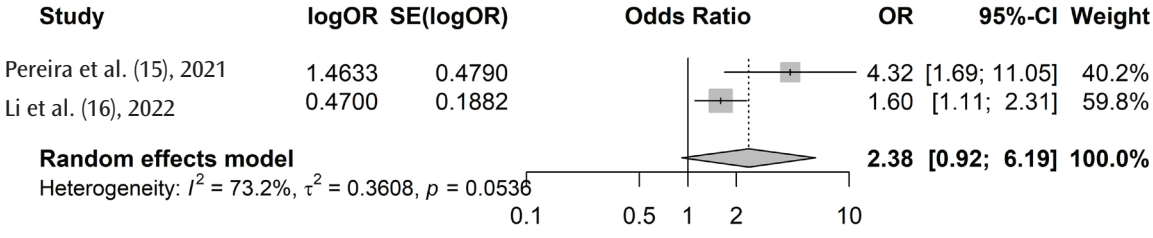

Figure 4. Forest plot of central obesity (waist circumference-defined) and testicular enlargement
OR: odds ratio, CI: confidence interval, SE: standard error

Mechanistically, Liu et al. (17) identified elevated leptin levels (OR=1.8, p=0.02) and leptin-to-adiponectin ratios (OR=2.1, p=0.01) as potential mediators in their case-control analysis of CPP. These mechanistic insights suggest a dual-effect model of adiposity in male puberty. Obesity may promote hypothalamic-pituitary-gonadal axis activation leading to earlier pubertal onset (testicular enlargement OR=1.27). Paradoxically, obesity may simultaneously impair genital development as shown by

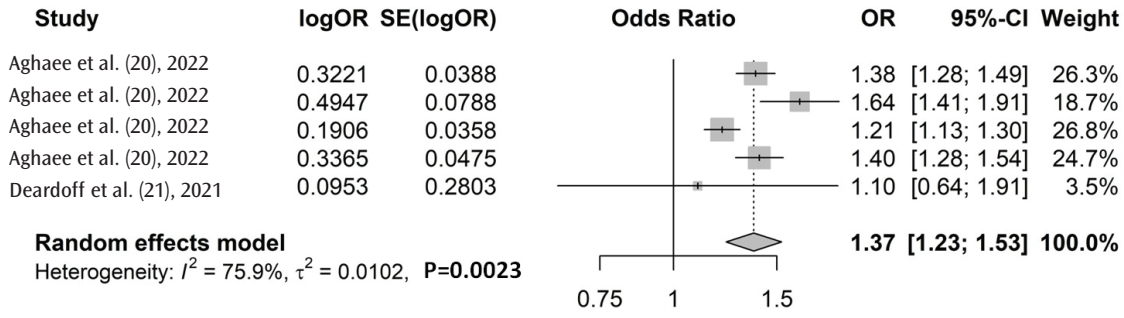

Figure 5. Forest plot of obesity (BMI-defined) and pubarche
OR: odds ratio, CI: confidence interval, SE: standard error, BMI: body mass index

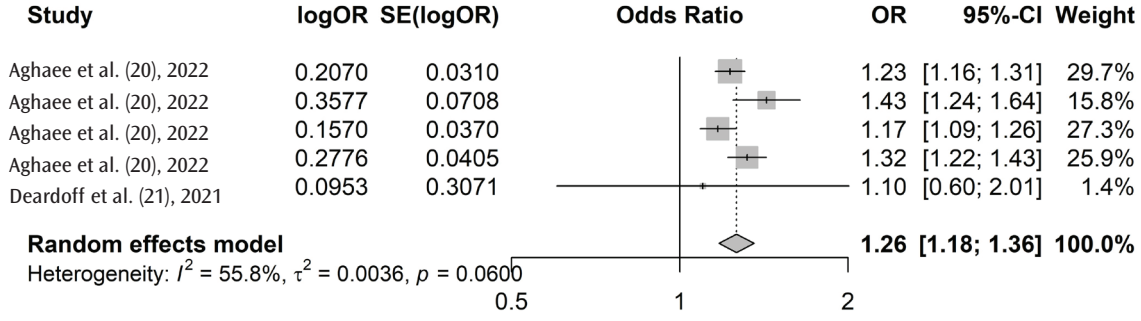

Figure 6. Forest plot of overweight (BMI-defined) and pubarche
OR: odds ratio, CI: confidence interval, SE: standard error, BMI: body mass index

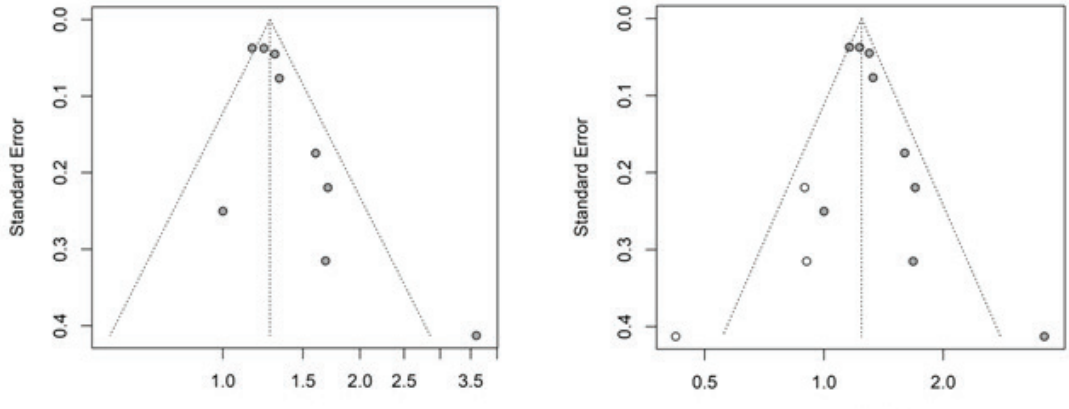

Figure 7. Funnel plot for publication bias assessment

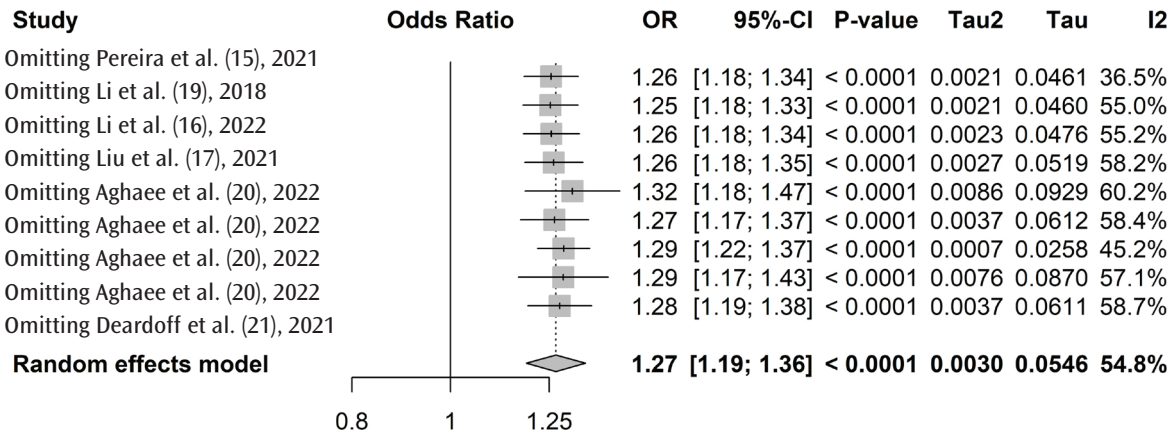

Figure 8. Sensitivity analysis (sequential exclusion of each study followed by re-analysis)
OR: odds ratio, CI: confidence interval

10% reduced penile growth in obese boys (18). This effect likely contributes to the observed heterogeneity across studies.

Contradictory Findings and Methodological Considerations

Despite some studies (16,17,18) suggesting a link between obesity/overweight and earlier puberty in boys, the remaining studies challenged this consensus. Li et al. (19) reported no significant association between prepubertal obesity and earlier voice breaking in Chinese boys (β=0.08 years, p=0.42), despite strong correlation in girls. Similarly, Aghaee et al. (20) found race/ethnicity modified this relationship, with obesity accelerating pubertal timing in Hispanic boys [hazard ratio (HR)=1.4, p=0.03] but not in non-Hispanic white peers (HR=0.9, p=0.61). These discrepancies may stem from varying outcome measures (clinical vs self-reported puberty markers) and population-specific genetic/environmental factors. Notably, Deardorff et al. (21) observed paradoxical associations in Mexican-American boys, where severe obesity (BMI≥99th percentile) correlated with delayed pubic hair development (HR=0.7, p=0.04), suggesting potential threshold effects of adiposity.

Emerging Post-Pandemic Patterns

Recent epidemiological shifts post-COVID-19 warrant special consideration. Wang et al. (12) documented a 23% surge in idiopathic precocious puberty cases across Asian and European centers, paralleling accelerated weight gain during lockdowns. This trend is consistent with biological mechanisms where adipose tissue aromatase activity may convert androgens to estrogens, thus lowering the hypothalamic-pituitary-gonadal axis activation threshold (22). Nevertheless, Rosenfield (23) caution against overattributing idiopathic cases to obesity alone, given the historical predominance of pathological etiologies (e.g., CNS lesions) in male precocious puberty diagnoses.

Study Limitations

Current research on the relationship between adiposity and puberty has notable limitations, including variability in measurement methods, for example inconsistent use of markers of male puberty, such as testicular volume vs. pubic hair, and adiposity indices, including BMI vs. central adiposity, which hinders direct comparisons across studies (15,16,21). Moreover, many studies focus primarily on concurrent associations rather than exploring the likely important prepubertal period that may have a stronger influence on pubertal timing (18,20). Another key limitation is the insufficient adjustment for potential confounders, such as endocrine-disrupting chemicals and socioeconomic factors, both of which are known to independently affect pubertal development (24). These methodological gaps highlight the need for more standardized assessments, longitudinal designs, and rigorous control of confounding variables in future investigations. Finally, while BMI remains the standard adiposity metric, its inability to distinguish fat from muscle mass may obscure true associations. Although we analyzed waist circumference data, the limited available studies precluded definitive conclusions regarding the effect of central obesity.

Conclusion

Although accumulating evidence suggests adiposity promotes earlier pubertal onset in boys, with a notable association in high-obesity populations, this association demonstrates greater context-dependency than has been reported in girls. Clinicians should be aware of the non-linear nature of this relationship, where extreme obesity may paradoxically delay specific pubertal markers while accentuating others, and the need for individualized assessment considering population-specific adiposity patterns. Future research must incorporate

standardized genital morphometry and body composition profiling to elucidate these complex interactions. Furthermore, evolving global childhood adiposity trends in the post-pandemic era (21) necessitate dynamic monitoring to inform precision prevention strategies.

Implications

These findings suggest that it will be important to recognize that obesity is a probable significant risk factor for precocious puberty in boys, mirroring patterns seen in girls. The increasing prevalence of idiopathic male precocious puberty, particularly in the post-pandemic era, highlights the need for further research to confirm the findings and elucidate underlying mechanisms, thus informing preventive strategies.

Ethics

Ethics Committee Approval: Not applicable (since this is a meta-analysis based on published literature).

Informed Consent: Not applicable.

Footnotes

Authorship Contributions

Concept: Ziqin Liu, Design: Ziqin Liu, Data Collection and Processing: Xiou Wang, Analysis or Interpretation: Xiou Wang, Yi Song, Literature Research: Xiou Wang, Yi Song, Ziqin Liu, Writing: Yi Song, Ziqin Liu.

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Comparison of Methods used for Final Height Prediction in Patients with Central Precocious Puberty

Nisa Nur Turan[1], Aşan Önder Çamaş[2], Burçin Çiçek[2], Merve Nur Hepokur[2], Hamdi Cihan Emeksiz[2]

[1]İstanbul Medeniyet University, Göztepe Prof. Dr. Süleyman Yalçın City Hospital, Department of Pediatrics, İstanbul, Türkiye
[2]İstanbul Medeniyet University, Göztepe Prof. Dr. Süleyman Yalçın City Hospital, Department of Pediatric Endocrinology, İstanbul, Türkiye

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What is already known on this topic?

The Bayley-Pinneau and Roche-Wainer-Thissen methods are commonly used for predicting final/target height.

What this study adds?

The Bone Age Percentile Curve Projected Height Estimation (BAPCPHE) method is a method of estimating final height by extrapolating the bone age percentile to the end of the percentile curve at 18 years of age. The BAPCPHE is more practical for use in outpatient settings, and was found to be effective in predicting target height.

ABSTRACT

Objective: Various methods may be used to estimate target height in patients diagnosed with precocious puberty. These methods include the Bayley-Pinneau (BP) and Roche-Wainer-Thissen (RWT) methods. In addition to these methods, in our clinic, we routinely use a practical approach based on the percentiles in growth charts. In this method, the bone age percentile is projected to the end of the percentile curve (at 18 years of age) to estimate the final adult height. We have named this method Bone Age Percentile Curve Projected Height Estimation (BAPCPHE). The aim of this study was to retrospectively compare the effectiveness of these three methods in predicting target height in patients treated for central precocious puberty and who have reached their final height in our pediatric endocrinology clinic.

Methods: Fifty female patients were included. The predicted adult heights (PAH) were calculated at treatment initiation, at the end of the first, second, and third years of treatment, and at the time of final height attainment using the BP, RWT, and BAPCPHE methods, based on the patients' heights and bone ages.

Corresponding Author: Assoc. Prof. Aşan Önder Çamaş, İstanbul Medeniyet University, Göztepe Prof. Dr. Süleyman Yalçın City Hospital, Department of Pediatric Endocrinology, İstanbul, Türkiye
E-mail: asanonder@yahoo.com **ORCID:** orcid.org/0000-0002-5730-3198
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Results: When the agreement between the PAH calculated by three methods and the final height was analyzed using intraclass correlation coefficient, significant agreement was found for PAH using the BAPCPHE method in the third year. Among the methods, the strongest agreement with final height and PAH was observed with the BP method at the end of treatment, followed by the BAPCPHE method.

Conclusion: The BAPCPHE method allows estimation of PAH quickly, making it a valuable tool in the outpatient setting. Given its simplicity and accuracy, we find the BAPCPHE method preferable.

Keywords: Precocious puberty, predicted adult height, bone age, Bayley-Pinneau, Roche-Wainer-Thissen

Introduction

Puberty is a transitional phase in children characterized by accelerated growth, the development of secondary sexual characteristics, and physical and psychosocial maturation (1). Precocious puberty (PP) refers to the onset of secondary sexual characteristics before the age of 8 years in girls and 9 years in boys (2). Early initiation of treatment in central PP (CPP) management is effective in preserving adult height and hence, the assessment of predicted target height is important in the follow-up of patients with CPP (3).

Various methods are used to estimate predicted adult height (PAH) in patients diagnosed with PP. The Bayley-Pinneau (BP) method estimates final height using the child's current height and bone age, determined according to the Greulich and Pyle bone atlas (4). The Roche-Wainer-Thissen (RWT) method predicts adult height based on height, weight, mid-parental height (MPH) (calculated from measured parental heights), and bone age recorded during a single pediatric visit (5). Given that the BP and RWT methods are time consuming in practice, we sought a faster, more practical method. It should also be kept in mind that the bone atlas data used were based on data from the 1930-1950 period in the United States of America (USA), when puberty started later. The final height estimates made with the data from this atlas may not be suitable for the children from different populations and the present time. In addition to these methods, a practical approach employed in our clinic involves a method based on growth percentile curves, numerically defined by Neyzi and Saka (6). Using the percentile curves, bone age is plotted, and the projection of the percentile line at age 18 years is considered the predicted final height. We have termed this method the Bone Age Percentile Curve Projected Height Estimation (BAPCPHE).

In this study, we aimed to retrospectively compare the effectiveness of BAPCPHE and two other commonly used methods (BP, RWT) for estimating PAH in patients diagnosed with CPP who underwent treatment and had achieved their final height.

Methods

Patients diagnosed with CPP, followed up and treated, without any additional chronic diseases, and who had reached their final height (defined as bone age ≥14 years in girls and growth velocity <2 cm/year) who gave consent fort his study were included. Leuprolide acetate 3.75 mg/month or 11.25 mg/3 months was used as the treatment in all cases. Exclusion criteria were defined as the presence of additional chronic diseases, history of mass/trauma/radiotherapy in the hypothalamic-pituitary region, syndromic disorders, or treatment for other conditions. Patients who discontinued treatment were also excluded from the study.

A total of 2,000 patients who applied for suspected early puberty, to the pediatric endocrinology outpatient clinic of our hospital between 2015 and 2023 were screened using patient files and the hospital information system. From these, female patients meeting the above study criteria were selected.

Ethical approval for the study was obtained from İstanbul Medeniyet University, Göztepe Prof. Dr. Süleyman Yalçın City Hospital Clinical Research Ethics Committee (approval number: 2023/0966, date: 20.12.2023). Informed consent was also obtained from the participating patients.

Demographic characteristics, medical history, anthropometric measurements, pubertal findings (Tanner stages), laboratory results, imaging studies, parental heights, and MPH values of the patients were retrospectively collected from patient files. The heights of the parents of the patients who came to our outpatient clinic were measured in our clinic. In rare cases, the heights of parents who could not come to our outpatient clinic were measured in a health institution close to them and recorded.

The treatment initiation date was considered as month 0. Heights, height standard deviation scores (SDS), body weights, body weight SDS values, and bone ages were recorded at months 12, 24, and 36 following the start of treatment, as well as at the end of treatment.

MPH was calculated using the following formula:

- For girls: [mother's height (cm)+father's height (cm)-13]/2
- For boys: [mother's height (cm)+father's height (cm)+13]/2

The age at final height attainment, final height, and final height SDS values were recorded for all patients.

Height was measured using a Harpenden stadiometer with a precision of 0.1 cm (SECA, Hamburg, Germany). Height SDS were calculated using reference data prepared for Turkish children through the Anthropometry Calculation Program (Child Metrics), an online tool developed by the Pediatric Endocrinology and Diabetes Association (*Çocuk Endokrinolojisi ve Diyabet Derneği*-ÇEDD) based on the standards published by Neyzi et al. (7).

Body mass index (BMI) was classified as follows: underweight (<5th percentile), normal weight (5th-85th percentile), overweight (85th-95th percentile), and obese (>95th percentile). A BMI SDS>2 SDS was defined as obesity (8,9).

Breast and pubic development were classified using the Tanner staging system during physical examinations performed by a pediatric endocrinologist (10,11). The presence or absence of axillary hair was also recorded.

Basal levels of follicle stimulating hormone (FSH), luteinising hormone (LH) and estradiol (E2), as well as stimulated LH and FSH levels, were assessed. Basal LH, FSH, and E2 tests were conducted between 8:00 and 10:00 AM. A basal LH level of ≥0.3 IU/L was considered significant for diagnosis. In cases with non-diagnostic basal LH levels and/or ambiguous clinical findings, a luteinizing hormone releasing hormone stimulation test was administered. A peak LH level of ≥5 IU/L or a peak LH/FSH ratio >0.66 was considered consistent with PP (12,13,14).

A single-view radiograph of the left hand and wrist was obtained for all patients. All bone age measurements were determined by two pediatric endocrinologists (Reader 1 and Reader 2) using the Greulich-Pyle bone age atlas (15). A bone age-to-chronological age ratio of >1.2 was considered indicative of CPP, and a reduction in this ratio during follow-up was interpreted as a positive response to treatment (16).

Final height predictions based on patients' heights and bone ages at the start of treatment, at the first, second, and third years of treatment, and at the time of final height attainment were calculated using the BP, RWT, and BAPCPHE methods.

BP and RWT predictions were performed using the PAH calculation tool integrated into the Child Metrics application (4,5).

For the BAPCPHE method, PAH was calculated, as illustrated in Figure 1. The patient's current bone age and height were plotted on Neyzi's growth percentile chart for Turkish children. The corresponding percentile was then tracked along the growth curve until the age of 18 years. The final projected value was recorded as the patient's PAH.

Statistical Analysis

Descriptive data in the study are presented as frequency and percentage, and continuous data are expressed as mean±standard deviation or median (minimum-maximum)

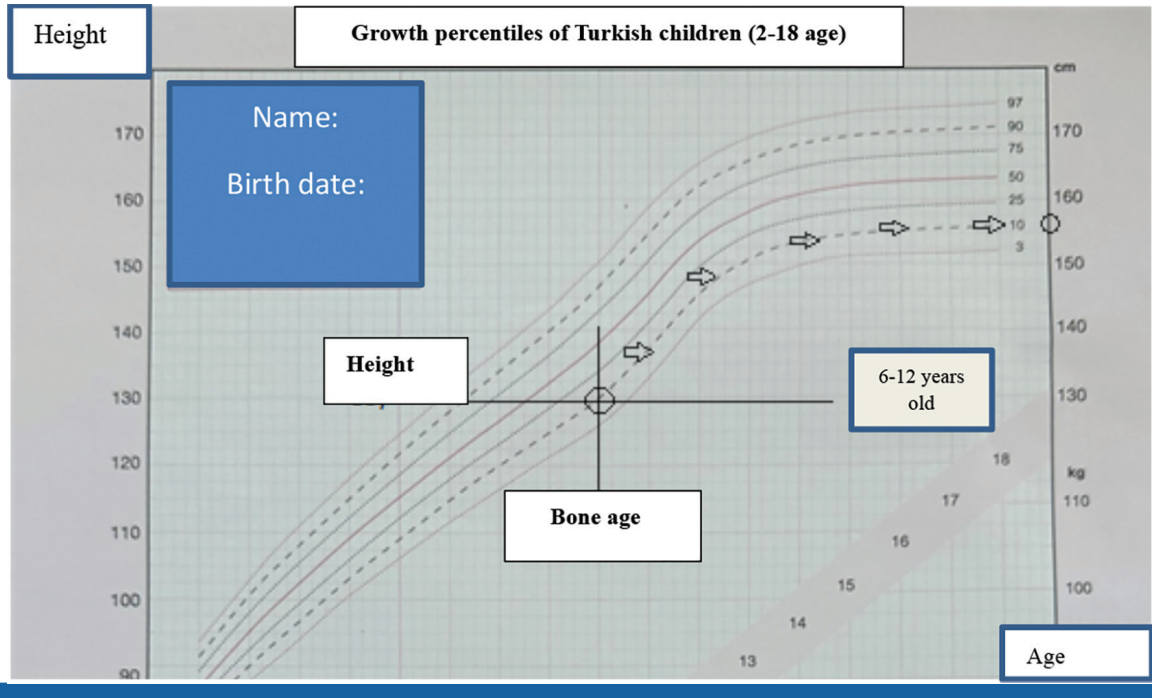

Figure 1. BAPCPHE method for calculating predicted target height
BAPCPHE: Bone Age Percentile Curve Projected Height Estimation

values, as appropriate. For categorical variables, the McNemar-Bowker test was used for comparisons of dependent groups, while the chi-square and Fisher's exact tests were applied for independent groups where appropriate. The normality of the distribution of measurements was assessed using the Kolmogorov-Smirnov test and histogram plots. The difference between measurements with a normal distribution was compared using a one-sample t-test. For measurements that did not show normal distribution, the Mann-Whitney U test was used for comparisons between groups. The level of agreement between two pediatric endocrinologists to determine bone age was assessed using the intraclass correlation coefficient (ICC). ICC was also used to assess the agreement between the PAH, calculated using the three different prediction methods (BP, RWT, and BAPCPHE), and the actual final height achieved by the same individuals. Bonferroni correction was applied for post-hoc analyses. Analyses were performed using IBM SPSS Statistics, version 20 (IBM Inc., Armonk, NY, USA).

Results

The study was conducted with a total of 50 female patients. The mean age at which the first symptoms appeared was 7.16±0.84 years. The mean age at onset of thelarche was 7.3±0.7 years, while the mean age at onset of pubarche was 8.7±1.6 years. The mean age at onset of axillary hair was 9.3±1.6 years. The mean height SDS at the start of treatment was 1.43±1.24, the mean weight SDS was 1.13±0.92, and the mean BMI SDS was 0.69±0.74.

The mean bone age at the start of treatment was calculated to be 9.7±1.7 years.

When evaluating Tanner stages, 72% (n=36) of patients were in stage T2 and 28% (n=14) were in stage T3. In terms of pubarche stages, 56% (n=28) were in stage P1, 36% (n=18) in stage P2, 6% (n=3) in stage P3, and 2% (n=1) in stage P4. Regarding axillary hair presence, 60% (n=30) of patients had no axillary hair, while 40% (n=20) had axillary hair.

When examining BMI distributions at presentation, no patients were classified as underweight. Most (n=34, 68%) of patients were within the normal weight range, 22% (n=11) were overweight, and 10% (n=5) were obese.

The mean age at the start of treatment was 8.3±1.0 years, and the mean treatment duration was 28.4±11.9 months.

The mean age at the end of the treatment was 10.65±0.27 years. At this time, the median height SDS was 0.84 (-1.06-3.2) SDS, the mean weight SDS was 1.17±0.82, and the mean BMI SDS was 0.93±0.

The mean bone age at the end of the treatment was 11.4±0.9 years. The average age of final growth was achieved at 14.1±0.7 years, and the mean final height was 163.0±6.4 cm. The final height SDS was 0.46±1.10.

The mean difference between final height and MPH was 4.3 (-5.6-19) cm, and the mean difference between final height SDS and MPH SDS was 1.29±0.94 SDS. The mean difference between MPH and initial height was 24.4±20.3 cm, and the mean difference between MPH SDS and initial height SDS was -2.11±1.49 SDS. The mean difference between final height and initial height was 26.5±7.6 cm, and the mean difference between final height SDS and initial height SDS was -0.96±1.08 SDS.

The ICC between Reader 1 and Reader 2 for the bone age measurements at the first, second and third year of treatment were 0.986 [95% confidence interval (CI)=0.939-0.981, p<0.001], 0.976 (95% CI=0.945-0.989, p<0.001) and 0.975 (95% CI=0.857-0.995, p<0.001), respectively.

The relation between final height prediction techniques and final height was evaluated using the ICC. At the end of the third year of treatment, all parameters, except for the PAH using the BAPCPHE method, had a significant correlation with final height. The level of agreement was ranked from highest to lowest, and the highest correlation with final height was observed with the BP model at treatment completion. There was a high agreement between final height and PAH calculated according to BP model at treatment completion, the RWT model at the end of the second year, and the BP model at the end of the third year, respectively. A poor agreement was found between final height and PAH calculated according to BAPCPHE model at the end of the first year and at the start of treatment, and a moderate level of agreement was observed with other parameters, subsequently (Table 1).

When statistical significance of differences between final height and PAH using three different methods was examined, it was found that final height was significantly shorter than the BP predicted adult height at the end of the second year, third year, and at treatment completion (p=0.007, p=0.036, and p=0.004, respectively). No significant difference was found between final height and the other two model predictions of adult height (Table 2).

When comparing the treatment initiation age according to the achievement of target height, patients who reached the BP predicted adult height at the end of the first year had a significantly lower median treatment initiation age compared to those who did not (p=0.032). Patients who reached the RWT predicted adult height at the end of the third year had a significantly higher median treatment initiation age compared to those who did not (p=0.038). No significant relationship was found between treatment initiation age and other target height achievements.

When comparing the treatment duration according to the achievement of target height, patients who reached the RWT predicted adult height at the end of the third year had a significantly shorter median treatment duration compared to those who did not (p=0.038). No significant relationship was found between treatment duration and other target height achievements.

Table 1. Analysis of the agreement between predicted adult height calculation methods and final height

	ICC	p value
BP predicted adult height at treatment initiation	0.504	**<0.001**
RWT predicted adult height at treatment initiation	0.639	**<0.001**
BAPCPHE predicted adult height at treatment initiation	0.262	**0.032**
BP predicted adult height at the end of the 1st year of treatment	0.582	**<0.001**
RWT predicted adult height at the end of the 1st year of treatment	0.656	**<0.001**
BAPCPHE predicted adult height at the end of 1st year of treatment	0.268	**0.030**
BP predicted adult height at the end of the 2nd year of treatment	0.686	**<0.001**
RWT predicted adult height at the end of the 2nd year of treatment	0.734	**<0.001**
BAPCPHE predicted adult height at the end of 2nd year of treatment	0.449	**0.003**
BP predicted adult height at the end of the 3rd year of treatment	0.727	**0.006**
RWT predicted adult height at the end of the 3rd year of treatment	0.608	**0.024**
BAPCPHE predicted adult height at the end of 3rd year of treatment	0.488	0.076
BP predicted adult height at the end of treatment	0.749	**<0.001**
RWT predicted adult height at the end of treatment	0.676	**<0.001**
BAPCPHE predicted adult height at the end of treatment	0.566	**<0.001**

p<0.005 is considered statistically significant.
ICC: intraclass correlation coefficient, BP: Bayley-Pinneau method, RWT: Roche-Wainer-Thissen method, BAPCPHE: Bone Age Percentile Curve Projected Height Estimation

Table 2. Analysis of the difference between final height and predicted target heights assessed by three different methods

	Target height	Final height	p value*
	Target height-Final height	Target height-Final height	
BP predicted adult height at treatment initiation	163.2±6.4	163.0±6.4	0.861
RWT predicted adult height at treatment initiation	164.1±4.9	163.0±6.4	0.250
BAPCPHE predicted adult height at treatment initiation	163.1±5.6	163.0±6.4	0.948
BP predicted adult height at the end of the 1st year of treatment	164.0±6.2	163.0±6.4	0.297
RWT predicted adult height at the end of the 1st year of treatment	163.3±4.4	163.0±6.4	0.776
BAPCPHE predicted adult height at the end of 1st year of treatment	163.4±6.0	163.0±6.4	0.694
BP predicted adult height at the end of the 2nd year of treatment	**165.6±7.4**	**163.0±6.4**	**0.007**
RWT predicted adult height at the end of the 2nd year of treatment	163.0±4.9	163.0±6.4	0.965
BAPCPHE predicted adult height at the end of 2nd year of treatment	164.2±6.4	163.0±6.4	0.208
BP predicted adult height at the end of the 3rd year of treatment	**165.0±5.1**	**163.0±6.4**	**0.036**
RWT predicted adult height at the end of the 3rd year of treatment	163.0±2.8	163.0±6.4	0.965
BAPCPHE predicted adult height at the end of 3rd year of treatment	164.3±4.2	163.0±6.4	0.172
BP predicted adult height at the end of treatment	**165.8±6.1**	**163.0±6.4**	**0.004**
RWT predicted adult height at the end of treatment	162.9±4.4	163.0±6.4	0.878
BAPCPHE predicted adult height at the end of treatment	164.4±4.9	163.0±6.4	0.141

*p<0.005 is considered statistically significant.
BP: Bayley-Pinneau method, RWT: Roche-Wainer-Thissen method, BAPCPHE: Bone Age Percentile Curve Projected Height Estimation

Discussion

In the present study, we retrospectively evaluated 50 female patients diagnosed with idiopathic CPP, treated with gonadotropin releasing hormone (GnRH) analogs, and followed until they reached their final height. Our aim was to assess the accuracy of three different methods for estimating final height.

The study by Baek et al. (17) in South Korea, which included 71 female CPP patients, reported an average treatment duration of 27.9±9.0 months, a mean treatment initiation age of 8.5±0.7 years, and a mean MPH of 161.6±3.6 cm. Their findings indicated a significant increase in PAH from 158.7±4.1 cm before treatment to 163.8±4.7 cm afterward, by using the BP method (17). Similarly, in our study, the PAH at treatment initiation was 163.2±6.4 cm using the BP method, increasing to 165.8±6.1 cm in the post-treatment period. These findings suggest that treatment effectively halts bone age advancement, contributing to increased PAH, in line with the literature.

Wu et al. (18) developed a predictive model in 2023 to estimate target height in 258 Chinese girls with idiopathic CPP. This model incorporated variables, such as height SDS at diagnosis, bone age-adjusted height SDS, and MPH. Unlike traditional models, it used bone age-adjusted height SDS instead of the peak LH/FSH ratio as a diagnostic factor. Bone age was assessed using the Greulich-Pyle atlas and Tanner-Whitehouse (TW) methods. The model's predicted target heights closely matched the final heights observed in the cohort.

Studies comparing different methods for predicting final height have shown variability in accuracy. For instance, a study including short-statured girls who did not receive GnRH therapy found that the BP method was the most accurate among three methods (BP, TW, and RWT) (19). Joss et al. (20) reported that the BP method provided reliable predictions, while the TW method overestimated final height by 3.9 cm and the RWT method by 6.3 cm. In contrast, Brämswig et al. (21) argued that BP, TW, and RWT methods were equally inadequate in predicting adult height in patients with PP.

Quiroga et al. (22) compared the BP and RWT methods in a cohort of 93 girls with CPP who reached their final height without GnRH treatment. They found that the BP method underestimated the predicted target height by 1.01 cm, while the RWT method overestimated it by 0.96 cm. Despite these differences, they recommended the BP method for its simplicity and practical application in predicting height in cases of early puberty (22).

Akın Kağızmanlı et al. (23) found that while the RWT method provided predictions close to the final height, the BP method produced the smallest difference between PAH and final height, making it the preferred method.

Jang et al. (24) studied 206 patients with CPP and reported an MPH of 160.26±3.62 cm. Using the BP method, PAH at diagnosis was 155±5.71 cm, while the final height was 159.3±4.26 cm. The mean initial height was 133.9±5.15 cm, with a mean final height increase of 25.4 cm (24).

In a study by Matias et al. (25) involving 138 patients, the BP and TW methods were compared. The mean final height was 173.6±5.31 cm. The TW method predicted a mean target height of 168.6±6.17 cm and the BP method predicted 172.5±5.12 cm. The BP method's predictions were significantly closer to the final height (25).

In the present study, the mean difference between final height and MPH was 4.3 (-5.6-19) cm, while the final height SDS-MPH SDS difference was 1.29±0.94 SDS. The mean difference between final height and initial height was 26.5±7.6 cm, and the SDS difference was -0.96±1.08 SDS. PAH at treatment initiation was 163.2±6.4 cm using the BP method, 164.1±4.9 cm using the RWT method, and 163.1±5.6 cm using the BAPCPHE method, respectively. ICC analysis revealed that the BP method showed the highest correlation with final height, followed by the RWT and BAPCPHE methods. All three methods demonstrated satisfactory accuracy in predicting final height.

When assessing the ICC between target height prediction methods and final height at the third year of treatment, all parameters except the BAPCPHE-predicted target height showed significant correlation. The BP method based on post-treatment bone age exhibited the highest agreement with final height. Strong agreement was observed between final height and post-treatment BP predictions, second-year RWT predictions, and third-year BP predictions. Moderate correlation was noted with other parameters, while a weak correlation was found with first-year and baseline BAPCPHE predictions.

The differences between all these studies can be attributed to the content of the methods and the patient profile. For example, the inclusion of weight in the RWT method causes obesity to affect the assessment of PAH. We did not evaluate our obese patients with subgroup analyses. The age at presentation of obese patients may have influenced the relationship between RWT/BP methods and age at treatment initiation.

There are a few published studies evaluating the efficacy of PAH methods within the treatment period. In the present study, we found that all three methods were effective and gave similar PAH results during treatment. The method we use is not affected by either obesity or MPH. In addition, the fact that bone ages were evaluated by two different specialists and consistency was found between the evaluations strengthened the results of our study. We believe that the BAPCPHE method has benefits because it is easy to use in practice and the final height estimates are in

agreement with the final height during the whole treatment process.

Study Limitations

This study has several limitations. It is a retrospective study involving a relatively small patient cohort receiving varying doses of GnRH analogs at different pubertal stages. The RWT method recommends horizontal height measurement, whereas our study used standing height measurements due to its retrospective design. Factors such as obesity-related bone age advancement were not analyzed, limiting insights into its potential contribution to final height. CPP is more prevalent in girls. Our study cohort had no male patients achieving final height. The heights of the parents who could not come to our outpatient clinic were not measured in our outpatient clinic but in another health institution close to them. This may have created an error in the calculation of mid parenteral height.

Conclusion

In clinical practice, the BAPCPHE method's practical application allows for quick and easy target height estimation, making it a valuable tool in outpatient settings. Given its simplicity and accuracy, we found the BAPCPHE method preferable.

Ethics

Ethics Committee Approval: Ethical approval for the study was obtained from İstanbul Medeniyet University, Göztepe Prof. Dr. Süleyman Yalçın City Hospital Clinical Research Ethics Committee (approval number: 2023/0966, date: 20.12.2023).

Informed Consent: Informed consent was obtained from the participating patients.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Nisa Nur Turan, Aşan Önder Çamaş, Burçin Çiçek, Merve Nur Hepokur, Hamdi Cihan Emeksiz, Concept: Nisa Nur Turan, Aşan Önder Çamaş, Design: Nisa Nur Turan, Aşan Önder Çamaş, Data Collection or Processing: Nisa Nur Turan, Aşan Önder Çamaş, Burçin Çiçek, Merve Nur Hepokur, Hamdi Cihan Emeksiz, Analysis or Interpretation: Nisa Nur Turan, Aşan Önder Çamaş, Literature Search: Nisa Nur Turan, Aşan Önder Çamaş, Merve Nur Hepokur, Writing: Nisa Nur Turan, Aşan Önder Çamaş, Hamdi Cihan Emeksiz.

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Real-World Experience from Türkiye: Genetic and Therapeutic Insights in Pediatric Heterozygous Familial Hypercholesterolemia

Havva Yazıcı[1], Esra Er[2], Fehime Erdem[1], Ayşe Yüksel Yanbolu[1], Sakina Mammadova[1], Sedef Alpdoğan[1], Merve Yoldaş Çelik[1], Yasemin Atik Altınok[3], Ebru Canda[1], Ayça Aykut[4], Haluk Akın[4], Sema Kalkan Uçar[1], Mahmut Çoker[1]

[1]Ege University Faculty of Medicine, Department of Pediatrics, Division of Metabolism and Nutrition, İzmir, Türkiye
[2]University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, Clinic of Pediatrics, Division of Metabolism and Nutrition, İzmir, Türkiye
[3]İzmir Tınaztepe University Faculty of Health Sciences, Department of Nutrition and Dietetics, İzmir, Türkiye
[4]Ege University Faculty of Medicine, Department of Medical Genetics, İzmir, Türkiye

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What is already known on this topic?

Familial hypercholesterolemia (FH) is a common inherited lipid disorder that increases atherosclerotic risk from childhood.

What this study adds?

This is the first comprehensive Turkish cohort analysing both genetic and therapeutic aspects of pediatric heterozygous FH. The study found major shortcomings in early screening, treatment acceptance, and follow-up. The study also identified three novel *LDLR* variants.

ABSTRACT

Objective: Familial hypercholesterolemia (FH) is an inherited metabolic disorder that increases cardiovascular risk from childhood. Despite its frequency, pediatric diagnosis and treatment remain limited, particularly in developing countries.

Corresponding Author: Assoc. Prof. Ebru Canda, Ege University Faculty of Medicine, Department of Pediatrics, Division of Metabolism and Nutrition, İzmir, Türkiye
E-mail: ebru.canda@ege.edu.tr **ORCID:** orcid.org/0000-0002-9175-1998
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Methods: Retrospective analysis of pediatric patients with genetically confirmed heterozygous FH (HeFH). Genetic testing included sequencing of the genes *LDLR*, *APOB*, and *PCSK9*. Clinical features, treatment responses, statin use, and adverse events were assessed and a comparative analysis was conducted between different statin types.

Results: Among the cohort of 124 patients only 28.2% of patients were diagnosed via routine lipid screening, though 90.3% had a positive family history. After diagnosis, 16.1% declined treatment and 41.1% were lost to follow-up. Most genetic diagnoses involved pathogenic *LDLR* variants; only a few cases involved *APOB* and *PCSK9*. Three novel *LDLR* variants were identified. Among treated patients, atorvastatin led to a greater median low density lipoprotein-cholesterol (LDL-C) reduction. A higher (though not statistically significant) proportion of pitavastatin users achieved LDL-C targets. LDL-C reduction was positively correlated with baseline LDL-C levels. For the majority of patients, statins were well tolerated; five patients had transient creatine kinase elevations that resolved with treatment interruption.

Conclusion: This is the first large pediatric HeFH cohort study from Türkiye and provides data on both genetic background and treatment outcome. Despite genetic confirmation, significant gaps remain in early diagnosis, treatment acceptance, and long-term follow-up. Both atorvastatin and pitavastatin proved to be safe and effective. These results suggest a need for national screening programmes, family education, dietary counselling, and consistent follow-up.

Keywords: DNA sequencing, heterozygous familial hypercholesterolemia, *APOB, LDLR, PCSK9,* paediatrics

Introduction

Familial hypercholesterolemia (FH) is attributed to mutations in genes that are critical for the receptor-mediated endocytosis of low-density lipoprotein cholesterol (LDL-C). This impairment compromises the body's ability to effectively clear LDL-C from the circulation, resulting in hyperlipidemia that significantly elevates the risk of premature cardiovascular disease (CVD). As such, early identification and prompt initiation of therapeutic interventions are of paramount importance (1).

FH occurs in two distinct clinical forms: heterozygous FH (HeFH) and homozygous FH. HeFH is associated with monoallelic mutations in the autosomal semi-dominant genes *LDLR*, *APOB*, and *PCSK9*. *LDLR* gene variants are most commonly found in patients with FH, while variants in *APOB* and *PCSK9* genes are less frequently observed (2,3). Scientific organisations, including the European Atherosclerosis Society (EAS) Consensus Panel, Simon Broome Register Group, and Dutch Lipid Clinic Network, have established well known diagnostic criteria based on scores assigned to family history and laboratory parameters (4,5,6,7).

Current guidelines recommend universal lipid screening for pediatric patients aged 9 to 11 years and 17 to 21 years. For those outside these age ranges, a selective screening approach is preferred, which involves screening individuals who have risk factors or a family history of early CVD (8,9,10). The EAS Familial Hypercholesterolemia Studies Collaboration has indicated that approximately 450,000 children are born annually worldwide with FH. Nevertheless, only 2.1% of adults affected by this condition receive a diagnosis before the age of 18 years (11). Despite these international insights, data on the national burden of FH have been scarce. A recent large-scale study from Türkiye, using electronic health records of over 83 million citizens, revealed a notably high FH prevalence of 0.63% among adults (~1/159) and 0.37% (~1/270) among children and adolescents. Despite its inherited nature, the lower prevalence of FH observed in childhood compared to adulthood suggests a significant gap in early diagnosis during the pediatric period in Türkiye (12).

In the absence of sufficient lipid-lowering therapy (LLT), individuals with HeFH, which affects an estimated 1 in 100 to 1 in 500 people, face a 20-fold increased risk of developing CVD when their LDL-C levels exceed 5.5 mmol/L, compared to unaffected individuals with LDL-C levels below 3.5 mmol/L (13). In terms of treatment for FH in children, conflicting opinions remain among healthcare professionals about when to initiate LLT, particularly at what age, at what lipid thresholds and what the target lipid levels should be. Statins and ezetimibe are conventional LLTS. A widely acceptedpublished approach recommends initiating statin therapy in pediatric patients with LDL-C levels of 160 mg/dL or higher, particularly when additional risk factors or comorbidities are present. Moreover, treatment is also advised for children with LDL-C levels exceeding 190 mg/dL, even without other risk factors (14). Current treatment recommendations suggest achieving LDL-C reduction of at least 50% and targeting an LDL-C level below 130 mg/dL in children and adolescents with FH, although aiming for levels below 100 mg/dL may offer greater protection against CVD over the lifespan (14,15). Moreover, the earlier the initiation of treatment, the more favourable the long-term prognosis tends to be (1).

All commercially available statins are FDA-approved (16) and are generally well-tolerated in children, with adverse events being rare, mild, and typically reversible without requiring discontinuation (17). Multiple randomised controlled trials, Cochrane reviews, and long-term studies, including a 20-year follow-up in children with FH (18), have confirmed statins' short- and long-term safety in the pediatric population (16,19,20,21). Though uncommon, potential adverse effects include liver enzyme elevations and muscle symptoms (22).

Therefore, this study aimed to present comprehensive data on the identification of FH, underlying molecular defects, and treatment approaches in a large pediatric patient cohort from Türkiye, where such data are limited when compared to developed countries in Europe and North America.

Methods

Study Population

Our research adopted a retrospective cohort design, focusing on patients under 18 years of age. A total of 450 patients who were referred to a tertiary centre for evaluation of FH were initially assessed. After excluding secondary causes of hypercholesterolemia, patients with confirmed heterozygous mutations in the *LDLR*, *APOB*, or *PCSK9* genes were included in the study. The baseline for the study was established at the point when a clinical diagnosis of HeFH was made.

Lifestyle change recommendations were made, focusing on reducing eating frequency outside the home, snacking habits, and unhealthy food choices while encouraging physical activity. In all cases, lifestyle modifications were implemented, starting with the CHILD-1 diet, followed by a gradual transition to the CHILD-2 diet (8). The study was approved by the Medical Research Ethics Committee of Ege University Faculty of Medicine (approval number: E.2371714, date: 28.03.2025).

When statins were prescribed, adherence was assessed based on information obtained from patients and their families regarding the regular intake of statin therapy. The target LDL-C level was defined as 130 mg/dL (14,15).

Sequencing of FH-related Genes

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of the extracted DNA was quantified using the Qubit™ double-stranded DNA (dsDNA) HS Assay Kit on the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). This approach provides high sensitivity and accuracy in quantifying dsDNA, ensuring reliable results for downstream applications.

First, *LDLR* was analysed using Sanger sequencing. Only after confirming negative results for *LDLR*, a targeted next-generation sequencing (NGS) panel was used to analyse the following genes: *ABCA1*, *ABCG5*, *ABCG8*, *ACTA2*, *ACVRL1*, *AGL*, *ALMS1*, *ANGPTL3*, *APOA1*, *APOA5*, *APOB*, *APOC2*, *APOE*, *BMPR1B*, *BMPR2*, *CAV1*, *CBS*, *CETP*, *COL3A1*, *CREB3L3*, *CYP27A1*, *ENG*, *FBN1*, *FBN2*, *GHR*, *KCNK3*, *LCAT*, *LDLR*, *LDLRAP1*, *LIPA*, *LIPC*, *LMF1*, *LPA*, *LPL*, *MYH11*, *PCSK9*, *SCARB1*, *SLC2A10*, *SMAD2*, *SMAD3*, *SMAD9*, *TGFB2*, *TGFB3*, *TGFBR1*, *TGFBR2*, *USF1*, and *GPIHBP1*. The study was conducted based on the analysis of these genes. The use of NGS allowed simultaneous analysis of multiple genes with high precision, enabling the identification of a broad spectrum of genetic variants.

Identification of Disease-Causing Variants

Detected variants were classified for their pathogenicity following the guidelines of the American College of Medical Genetics and Genomics (ACMG), ensuring that the interpretation was clinically relevant and accurate. The minor allele frequencies of the variants were assessed using publicly available databases, such as National Center for Biotechnology Information dbSNP and the Genome Aggregation Database (gnomAD). Disease-associated variant information was retrieved from databases, including ClinVar, which provides insights into genetic variants linked to diseases, and Online Mendelian Inheritance in Man, a detailed resource for genetic disorders and traits.

Novel variants identified during NGS were systematically analysed for their pathogenicity, mode of inheritance, and association with clinical phenotypes. Variants were examined for their potential impact on protein function, focusing on missense variants affecting evolutionarily conserved amino acid residues within critical protein domains.

To confirm the accuracy of candidate pathogenic variants identified through NGS, Sanger sequencing was employed using the Applied Biosystems, Inc. PRISM 3500 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). This gold-standard method provided reliable verification of the identified variants. Furthermore, segregation analyses were performed, where applicable, to determine the inheritance patterns of the variants within affected families, strengthening the link between the variants and observed clinical phenotypes.

Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 28.0 (IBM Corp., Armonk, NY, USA). Continuous variables, including baseline LDL-C, LDL-C reduction (mg/dL), statin initiation age, treatment duration of statin, and age at the last visit, were assessed for normality using visual inspection and tested for distribution. Since most variables were not normally distributed, results are presented as medians with interquartile ranges (IQR, 25^{th}-75^{th} percentiles). Comparisons between atorvastatin and pitavastatin groups were performed using the Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables, such as LDL target achievement, statin dose adjustment, and statin adherence. Correlations between baseline LDL-C and absolute LDL-C reduction were evaluated using Spearman's rank correlation coefficient for the total cohort and within each treatment subgroup. A two-sided p value <0.05 was considered statistically significant.

Results

Patient Demographics and Clinical Characteristics

A total of 124/450 (27.5%) patients were included in the study, with 45.2% (n=56) female and 54.8% (n=68) male. The median age at diagnosis was 7.9 (4.8-11.0) years. The most common reason for referral was family screening (46.0%, n=57), followed by routine screening (28.2%, n=35) and other causes (25.0%, n=31). Xanthoma was noted in only one patient (0.8%). A positive family history of hypercholesterolemia was present in 90.3% (n=112) of cases, and 32.3% (n=40) had a family history of premature CVD. The median body mass index standard deviation score at diagnosis was 0.22 (-0.79 to 1.0) kg/m^2, and the median LDL-C level at diagnosis was 234.5 (197.5-270.8) mg/dL. At the time of analysis, 29.0% (n=36) were on pitavastatin, 26.6% (n=33) on atorvastatin, 28.2% (n=35) had not yet started statin treatment, and 16.1% (n=20) had declined treatment. The median age at the last follow-up visit was 13.0 (8.6-15.7) years) (Table 1). In terms of follow-up, 42.7% (n=53) of patients remained under regular follow-up, 7.3% (n=9) were poorly compliant, 8.1% (n=10) had transitioned to adult care, 41.1% (n=51) were lost to follow-up, and 0.8% (n=1) were followed at another centre.

Clinical Characteristics of Patients under Statin Treatment

Lifestyle modifications and dietary interventions were implemented in all cases, beginning with the CHILD-1 diet and transitioning to the CHILD-2 diet as needed. Statin therapy was initiated in patients who did not achieve adequate lipid control through these measures. Among the 69 patients receiving statin therapy, 53.3% (n=36) were treated with pitavastatin and 46.7% (n=33) with atorvastatin. Although other statin preparations are available in Türkiye, these two remained consistently accessible and were continuously provided to the cohort throughout the study period. The median age at statin initiation was 11.3 (8.3-12.4) years, with no difference between the pitavastatin group [11.0 (7.9-12.0) years] and the atorvastatin group [11.3 (9.5-13.3) years; p=0.216]. Median baseline LDL-C level at statin initiation was significantly higher in the atorvastatin group [274.0 (247.0-298.0) mg/dL] compared to the pitavastatin group [225.5 (202.8-262.0) mg/dL; p<0.001]. The overall duration of statin treatment was 2.6 (1.4-3.4) years, with no difference between groups (p=0.263). The median age at the last follow-up was significantly older in the atorvastatin group [15.2 (13.3-16.4) years] than in the pitavastatin group [13.0 (10.8-15.9) years; p=0.037]. Overall adherence to statin therapy was 46.4% (n=32), with higher rates observed in the pitavastatin group (55.6%, n=20) compared to the atorvastatin group (36.4%, n=12) (Table 2). There was no significant difference between the atorvastatin and pitavastatin groups regarding the requirement for dose adjustment or statin adherencece (chi-square test, p=1.0; Mann-Whitney U test, p=0.148, respectively).

Firstly, when evaluating the treatment response to atorvastatin in terms of dosage, the median drop in LDL-C levels were: for 5 mg/day (n=17), 0.345 (0.294-0.476); for 10 mg/day (n=23), 0.494 (0.332-0.538); and for 20 mg/day (n=4), 0.369 (0.224-0.476). The number of cases in the 20 mg/day group was limited, and one of these patients had poor statin adherence. Similarly, when evaluating the treatment response to pitavastatin in terms of dosage, the median values were: for 1 mg/day (n=25), 0.450 (0.307-0.517); for 2 mg/day (n=19), 0.508 (0.315-0.578); and for 4 mg/day (n=2), 0.314 (0.307-0.321). Both patients in the 4 mg/day group had poor statin adherence.

The median absolute reduction in LDL-C was significantly greater in the atorvastatin group compared to the pitavastatin group (133.0 mg vs. 101.0 mg; p=0.048) (Figure 1A), while no difference was found in percentage LDL-C reduction between the groups (53.8% vs. 43.4%; p=0.778). Since the atorvastatin group had higher baseline LDL-C levels, the observed difference in absolute LDL-C reduction was affected by these initial values.

Table 1. Overview of patient demographics and baseline characteristics

Gender, % (n)	
Female	45.2% (56)
Male	54.8% (68)
Diagnosis age (y), median (IQR)	7.9 (4.8-11)
Reason for examination, % (n)	
Family screening	46.0% (57)
Screening	28.2% (35)
Others	25.0% (31)
Xanthoma	0.8% (1)
Hypercholesterolemia in family	
Yes	90.3% (112)
No	9.7% (12)
Premature CVD in family, % (n)	
Yes	32.3% (40)
No	67.7% (84)
BMI SDS at diagnosis, median (IQR)	0.22 (-0.79-1.0)
LDL-C at diagnosis, median (IQR), mg/dL	234.5 (197.5-270.8)
Statin, % (n)	
Pitavastatin	29.0% (36)
Atorvastatin	26.6% (33)
Not started yet	28.2% (35)
Declined	16.1% (20)
Age at last visit (y), median (IQR)	13 (8.6-15.7)

y: years, IQR: interquartile range, CVD: cardiovascular disease, BMI: body mass index, SDS: standard deviation score, LDL-C: low density lipoprotein-cholesterol

Table 2. Overview of clinical characteristics of patients undergoing statin treatment

	Overall 100% (69)	Pitavastatin 53.3% (36)	Atorvastatin 46.7% (33)	p*
Starting age of statin (y), median (IQR)	11.3 (8.3-12.4)	11.0 (7.9-12.0)	11.3 (9.5 -13.3)	0.216[m]
LDL-C at initiating statin, median (IQR)	247.0 mg/dL (217.0-285.0)	225.5 mg/dL (202.8-262.0)	274 mg/dL (247.0-298)	0.001[m]
Treatment duration under statin, median (IQR)	31.0 m (17.2-41)	31.0 m (13.5-38.2)	31.5 m (18.8-67.2)	0.263[m]
Age at last visit (y), median (IQR)	14.3 (12.2-16.4)	13.0 (10.8-15.9)	15.2 (13.3-16.4)	0.037[m]
Adherence with statin, % (n)	46.4% (32/69)	55.6% (20/36)	36.4% (12/33)	0.148[m]

*p values refer to comparison between statin sub-groups; [m]Mann-Whitney U test, y: years, IQR: interquartile range, LDL-C: low density lipoprotein-cholesterol

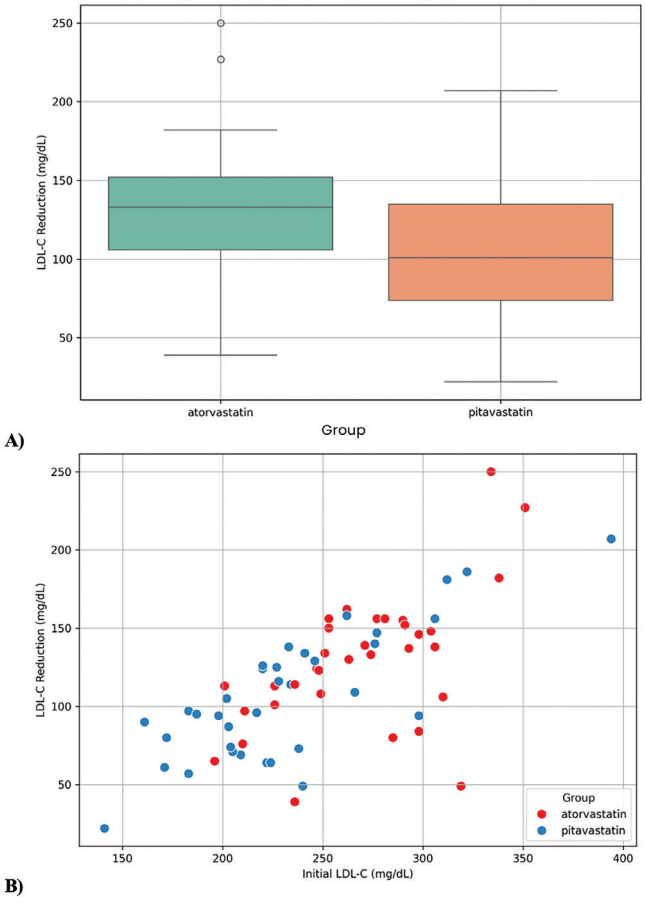

Figure 1. A) Comparison of absolute LDL-C reduction (mg/dL) between atorvastatin and pitavastatin groups. Each box represents the median and interquartile range (IQR) of LDL-C reduction. The atorvastatin group showed a greater median reduction compared to the pitavastatin group (p=0.048). **B)** Correlation between baseline LDL-C and absolute LDL-C reduction in patients receiving atorvastatin or pitavastatin. Each dot represents an individual patient. A significant positive correlation was observed in the total cohort (p=0.675, p<0.0001), as well as in both treatment subgroups (atorvastatin: p=0.502, p=0.003; pitavastatin: p=0.709, p<0.0001)
LDL-C: low density lipoprotein-cholesterol

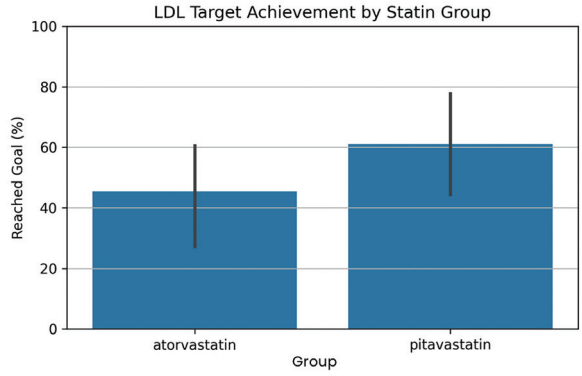

Figure 2. Comparison of LDL-C target achievement rates between atorvastatin and pitavastatin groups. The percentage of patients who reached their LDL-C goal was higher in the pitavastatin group (61.1%) compared to the atorvastatin group (45.5%), though the difference was not significant (p=0.231). Error bars represent 95% confidence intervals
LDL-C: low density lipoprotein-cholesterol

A correlation analysis was performed between initial LDL-C and the absolute LDL-C reduction following statin therapy to investigate whether baseline LDL-C levels influenced treatment response. A significant positive correlation was observed in the total cohort (p=0.675, p<0.0001), indicating that patients with higher baseline LDL-C tended to experience greater absolute reductions. This association remained significant within both treatment subgroups; for atorvastatin (p=0.502, p=0.003) and for pitavastatin (p=0.709, p<0.0001) (Figure 1B).

While a higher proportion of patients in the pitavastatin group achieved LDL-C targets compared to the atorvastatin group (61.1% vs. 45.5%), the difference was not significant (p=0.2318) (Figure 2). Regarding atorvastatin treatment, among the patients receiving 5 mg/day (n=17), 5 (29.4%) patients achieved the target. In the 10 mg/day group (n=23), 9 (39.1%) patients reached the target, while in the 20 mg/day group (n=4), only 1 (25%) patient met the target. As for pitavastatin, 11 (44%) out of 25 patients receiving 1 mg/day achieved the target. In the 2 mg/day group (n=19), 11 (57.9%) patients reached the target. In contrast, neither (0%) of the 2 patients on 4 mg/day achieved the target.

Adverse Events during Statin Therapy

Elevated creatine kinase (CK) levels were observed in five statin therapy patients (Table 3). One male patient (P1) on atorvastatin 10 mg/day developed two separate CK elevations at ages 17.5 and 18 years, with CK levels reaching 1631 U/L and 1262 U/L, respectively. Before statin initiation, his CK level was 73 U/L, which rose to 154 and 227 U/L after discontinuation. The remaining four cases occurred in patients receiving pitavastatin 2 mg/day. CK elevations ranged from 504 U/L to 5105 U/L, and all patients showed increases relative to baseline values. In these patients, pre-statin CK levels ranged from 65 to 184 U/L, while levels measured after statin discontinuation ranged from 118 to 149 U/L. Notably, the highest CK elevation (5105 U/L) occurred on pitavastatin in a male patient (P3). No adverse effects on growth or pubertal development were observed in any of the patients within the statin-treated cohort.

Molecular Results

Genetic analysis identified a wide spectrum of variants, predominantly in the *LDLR* gene, with additional variants detected in *APOB* and *PCSK9*. A total of 59 distinct *LDLR* variants were found, most of which were classified as pathogenic based on ACMG criteria. Three novel *LDLR* variants were identified, including c.1551delC (p.Lys518Serfs30)*, c.1528A>C (p.Thr510Pro), and c.1749del (p.Ser584ProfsTer81), all of which were considered likely pathogenic. A notable finding was that none of these novel detected variants were present in the gnomAD database, highlighting their rarity or novelty within the general population. In addition, novel variants were also found in *APOB* (c.9217A>G, p.Asn3073Asp and c.10238C>A, p.Thr3413Asn) and were interpreted as variants of uncertain significance (VUS).

Among the pathogenic variants in *LDLR*, c.1729T>C (p.Trp577Arg) was the most frequently observed (n=13), followed by c.1646G>A (p.Gly549Asp) (n=6), and c.1432G>A (p.Gly478Arg) and c.81C>G (p.Cys27Trp) (each n=5). Large rearrangements were also detected, consistent with structural mutations (exon 1-2 and exon 1-18 deletions). One pathogenic variant was identified in *PCSK9* (c.286C>T, p.Arg96Cys), a known mutation associated with autosomal dominant hypercholesterolemia.

The molecular diagnosis confirmed a high proportion of pathogenic or likely pathogenic variants, supporting the clinical diagnosis and justifying the initiation or continuation of LLT in this cohort (Table 4).

Discussion

This study presents an overview of the diagnostic approach and molecular characteristics of a large pediatric Turkish population diagnosed with HeFH. The findings support that initiating statin therapy at an early age is safe and effective, with no severe adverse effects observed. Despite growing awareness, published data on statin use in children and adolescents remain limited. To the best of our knowledge, this study represents the largest pediatric HeFH cohort from Türkiye to systematically investigate the etiology, clinical follow-up, and treatment course.

Screening Gaps

Only 28.2% of patients in our cohort were diagnosed through lipid screening, indicating that routine or opportunistic screening for FH in children is still underutilised. Although a large proportion of patients had a family history of hypercholesterolemia, less than half were diagnosed through family screening, and only a minority were identified through routine lipid screening. This suggests that both cascade and opportunistic lipid screening remain underutilised in the Turkish pediatric population, despite clear familial risk aligned with the previous reports (23,24). Global registry data from the EAS Familial Hypercholesterolemia Studies Collaboration showed that only 2% of participants were diagnosed before the age of 18 years (25). Furthermore, only 3.6% of individuals under 18 registered in the same cohort were from non-high-income countries (11). The observed gap highlights missed opportunities for early diagnosis and timely intervention, particularly in non-high-income countries, such as Türkiye.

Table 3. Clinical characteristics of patients with elevated CK levels under statin therapy

Number	Gender	Statin	Age at initiation of statin (y)	Event			CK before statin	CK off statin
				Statin dose	Age (y)	CK		
P1	Male	Atorvastatin	9.6	10 mg/day	17.5	1631 U/L	73 U/L	154 U/L
				10 mg/day	18	1262 U/L	73 U/L	227 U/L
P2	Female	Pitavastatin	8.6	2 mg/day	9.1	569 U/L	184 U/L	126 U/L
P3	Male	Pitavastatin	12.0	2 mg/day	14.9	5105 U/L	120 U/L	137 U/L
P4	Male	Pitavastatin	11.5	2 mg/day	15.8	504 U/L	65 U/L	149 U/L
P5	Female	Pitavastatin	5.8	2 mg/day	7.3	562 U/L	98 U/L	118 U/L

y: years, CK: creatine kinase

Table 4. Characterization of detected genetic variants by gene, DNA, and protein changes

Gene	DNA	Protein	Novelty	ACMG	n
LDLR	c.1729T>C	p.Trp577Arg	Known	Pathogenic	13
LDLR	c.1646G>A	p.Gly549Asp	Known	Pathogenic	6
LDLR	c.1432G>A	p.Gly478Arg	Known	Pathogenic	5
LDLR	c.81C>G	p.Cys27Trp	Known	Pathogenic	5
LDLR	c.1730G>C	p.Trp577Ser	Known	Pathogenic	4
LDLR	c.858C>A	p.Ser286Arg	Known	Pathogenic	4
LDLR	c.1678A>T	p.Ile560Phe	Known	Pathogenic	4
LDLR	c.1048C>T	p.Arg350*	Known	Pathogenic	3
LDLR	c.1551delC	p.Lys518Serfs*30	Novel	Likely pathogenic	3
LDLR	c.157C>T	p.Gln53*	Known	Pathogenic	3
LDLR	c.415G>A	p.Asp139Asn	Known	Pathogenic	3
LDLR	c.1061C>A	p.Asp354Gly	Known	Pathogenic	2
LDLR	c.1246C>T	p.Arg416Trp	Known	Pathogenic	2
LDLR	c.1151A>C	p.Gln384Pro	Known	Pathogenic	2
LDLR	c.1324T>C	p.Tyr442His	Known	Pathogenic	2
LDLR	c.1463T>C	p.Ile488Thr	Known	Pathogenic	2
LDLR	c.1807A>T	p.Lys603*	Known	Pathogenic	2
LDLR	c.2311+1G>A		Known	Pathogenic	2
LDLR	c.2389+5G>T		Known	VUS (PM2 PP3 BP6)	2
LDLR	c.2389G>A	p.Val797Met	Known	Pathogenic	2
LDLR	c.339_343delGTTTC	p.Phe114Leufs*14	Known	Pathogenic	2
LDLR	c.378delC	p.Phe126fs	Known	Pathogenic	2
LDLR	c.530C>G	p.Ser177Trp	Known	Likely pathogenic	2
LDLR	c.664T>C	p.Cys222Arg	Known	Pathogenic	2
LDLR	c.682G>C	p.Glu228Gln	Known	Pathogenic	2
LDLR	c.761A>C	p.Gln254Pro	Known	Pathogenic	2
LDLR	Exon 7-12 del		Known	Pathogenic	2
APOB	c.9217A>G	p.Asn3073Asp	Novel	VUS (PM2 BP4)	2
APOB	c.10238C>A	p.Thr3413Asn	Novel	VUS (PM2)	1
LDLR	c.1135T>C	p.Cys379Arg	Known	Pathogenic	1
LDLR	c.1195G>A	p.Ala399Thr	Known	Pathogenic	1
LDLR	c.1216C>T	p.Arg406Trp	Known	Pathogenic	1
LDLR	c.1285G>A	p.Val429Met	Known	Pathogenic	1
LDLR	c.1322T>A	p.Ile441Asn	Known	Pathogenic	1
LDLR	c.1478_1479delCT	p.Ser493Cysfs*42	Known	Pathogenic	1
LDLR	c.1528A>C	p.Thr510Pro	Novel	Likely pathogenic	1
LDLR	c.1567G>A	p.Val523Ile	Known	Pathogenic	1
LDLR	c.1601C>A	p.Thr534Asn	Known	Pathogenic	1
LDLR	c.1664_1674delTGGTGACTGAAinsCC	p.Leu555_Glu558delinsPro	Novel	Likely pathogenic	1
LDLR	c.1720C>T	p.Arg574Cys	Known	Likely pathogenic	1
LDLR	c.1747C>T	p.His583Tyr	Known	Pathogenic	1
LDLR	c.1749del	p.Ser584ProfsTer81	Novel	Likely pathogenic	1

Table 4. Continued

Gene	DNA	Protein	Novelty	ACMG	n
LDLR	c.1775G>A	p.Gly592Glu	Known	Pathogenic	1
LDLR	c.1823C>T	p.Pro608Leu	Known	Pathogenic	1
LDLR	c.1898G>A	p.Arg633His	Known	Pathogenic	1
LDLR	c.1946C>T	p.Pro649Leu	Known	Likely pathogenic	1
LDLR	c.2093G>T	p.Cys698Phe	Known	Pathogenic	1
LDLR	c.268G>A	p.Asp90Asn	Known	Pathogenic	1
LDLR	c.40dupT	p.Leu14Phefs*38	Known	Pathogenic	1
LDLR	c.41dup	p.Leu14fs	Known	Pathogenic	1
LDLR	c.460C>T	p.Gln154Ter	Known	Pathogenic	1
LDLR	c.502delG	p.Asp168Thrfs*38	Novel	Likely pathogenic	1
LDLR	c.506delA	p.Asn169Thrfs*37	Novel	Likely pathogenic	1
LDLR	c.694+2T>C		Known	Pathogenic	1
LDLR	c.763T>G	p.Cys255Gly	Known	Pathogenic	1
LDLR	c.796G>A	p.Asp139Asn	Known	Pathogenic	1
LDLR	c.846C>A	p.Phe282Leu	Known	Pathogenic	1
LDLR	c.859G>A	p.Gly287Ser	Known	Likely pathogenic	1
LDLR	c.888C>A	p.Cys296Ter	Known	Pathogenic	1
APOB	c.9068C>T	p.Ala3023Val	Novel	VUS (PM2)	1
LDLR	c.977C>G	p.Ser326Cys	Known	Pathogenic	1
LDLR	c.97C>T	p.Gln33*	Known	Pathogenic	1
LDLR	Exon 1-2 del		Known	Pathogenic	1
LDLR	Exon 1-18 del		Known	Pathogenic	1
PCSK9	c.286C>T	p.Arg96Cys	Known	Pathogenic	1

ACMG: American Journal of Medical Genetics, VUS: variant unknown significance

Parental Treatment Refusal

In addition to the gaps in early diagnosis, our study also identified barriers to treatment initiation. In 16.1% of cases within the study cohort, statin therapy was recommended, but the parents refused to initiate statin treatment in their children. Unfortunately, the retrospective study design did not permit investigation of the reasons behind parental refusal of statin therapy. However, numerous previous studies have explored and highlighted parental concerns regarding the use of statins in children. These studies consistently report that parents' concerns primarily revolve around the potential side effects and long-term safety of statin therapy, the perceived medicalization of childhood, and the uncertainty regarding the necessity of early treatment initiation (26,27,28). Despite the availability of long-term data demonstrating the safety of statin therapy in pediatric populations, parental hesitation and concerns remain a persistent barrier to treatment initiation. A nationwide study based on electronic health records reported LLT coverage as low as 1.5% among Turkish pediatric patients (12).

One of the strengths of this study was the relatively long and carefully monitored follow-up period. The median age at diagnosis was 7.9 years, with statin therapy initiated at a median age of 11.3 years and the last follow-up recorded at 13.0 years. These findings highlight the continuity of care and the structured long-term monitoring of the cohort, which allowed for a more comprehensive assessment of treatment response and disease progression. Statin therapy was introduced in cases where dietary and lifestyle interventions did not lead to sufficient lipid control. However, due to limitations in the consistency and completeness of lifestyle-related data collected from families and patients, these findings were not included in the analysis.

A comparative analysis of atorvastatin and pitavastatin was performed to evaluate differences in lipid-lowering efficacy and target attainment. The atorvastatin group showed a significantly greater median absolute reduction in LDL-C levels. In contrast, a higher proportion of patients in the pitavastatin group achieved their LDL-C targets, although this difference was not significant. This trend may suggest a difference in pharmacologic response rather than adherence, as no significant differences

were observed in adherence or the need for dose adjustments. Although not significant, the higher rate of target attainment in the pitavastatin group may still be clinically relevant, as even modest improvements in LDL-C goal achievement during childhood could contribute to reduced lifetime cardiovascular risk. This observation warrants confirmation in larger, prospective pediatric studies.

Differences in LDL-C reduction between the two statins may reflect their pharmacodynamic profiles, baseline LDL-C levels, or differential metabolism in pediatric patients. Further prospective studies are warranted to confirm these trends and inform statin selection.

A correlation analysis was performed to further investigate factors influencing treatment response between baseline LDL-C levels and the absolute LDL-C reduction. A significant positive correlation was observed across the total cohort indicating that higher initial LDL-C levels were associated with greater absolute reductions in both treatment groups. Our findings suggest that baseline LDL-C may be a key determinant of statin response, regardless of the statin type used. Both atorvastatin and pitavastatin have efficacy in the treatment of FH in children (14,15,18,29). In addition, it has been consistently reported that higher baseline LDL-C levels are associated with greater absolute reductions, indicating that starting lipid levels may significantly influence the therapeutic response (15).

A total of six adverse events were observed in five patients during statin therapy, with one patient experiencing two separate episodes. All events were muscle-related and asymptomatic, consistent with previous reports (30,31). In each case, statin treatment was temporarily interrupted and re-initiated after CK levels normalized. Although the literature suggests that muscular symptoms, when present, typically resolve spontaneously without requiring discontinuation of therapy (30,31), treatment was paused in our cases due to parental concerns. During follow-up visits, adverse events should be actively assessed, and even in asymptomatic cases, organ-specific markers should be monitored to detect subclinical effects and guide clinical decision-making.

In this cohort, the majority of molecular diagnoses were associated with pathogenic or likely pathogenic variants in the *LDLR* gene, consistent with previous studies identifying *LDLR* as the most commonly affected gene in FH 2 and in Turkish patients (32). The high frequency of c.1729T>C (p.Trp577Arg), c.1646G>A (p.Gly549Asp), and c.1432G>A (p.Gly478Arg) variants aligns with earlier findings from Turkish (32,33,34,35) and Mediterranean populations (36), supporting the notion of population-specific founder mutations.

Importantly, three novel *LDLR* variants were identified, all predicted to be pathogenic, expanding the mutational spectrum of FH and contributing to the understanding of genetic heterogeneity in this condition. Detecting structural rearrangements, such as exon deletions, further highlights the need for comprehensive molecular testing that includes sequencing and Multiplex Ligation-dependent Probe Amplification. Although *APOB* and *PCSK9* variants were less frequent, their identification highlights the importance of including these genes in genetic testing panels, especially for cases with a negative or inconclusive *LDLR* result. The two novel *APOB* variants, currently classified as VUS, warrant functional validation studies to clarify their role in LDL-C metabolism.

Overall, the high diagnostic yield of molecular testing in this study reinforces the utility of genetic analysis in guiding clinical decision-making, cascade screening, and risk stratification in pediatric FH populations.

Study Limitations

This retrospective study design may have limited the completeness and consistency of clinical and lifestyle data. Treatment adherence was based on self-reports and could not be objectively verified. Moreover, functional validation of the novel genetic variants identified in this study was not performed, and therefore their pathogenicity could not be conclusively established. This gap should be addressed in future studies using *in vitro* or *in vivo* assays to confirm variant effects. The high rate of loss to follow-up may have introduced bias in treatment outcome estimates and limits the generalizability of our findings. In addition, long-term follow-up data into adulthood were lacking, underscoring the need for future studies to evaluate treatment continuity and cardiovascular outcomes beyond childhood.

Conclusion

This study presented the diagnostic, genetic, and therapeutic characteristics of the first and largest pediatric HeFH cohort reported from Türkiye. Despite confirmed diagnoses, substantial gaps persist in early detection, treatment acceptance, and long-term follow-up. Both atorvastatin and pitavastatin were safe and effective, underscoring the importance of national screening, family education, and sustained care to reduce lifelong cardiovascular risk.

Ethics

Ethics Committee Approval: The study was approved by the Medical Research Ethics Committee of Ege University Faculty of Medicine (approval number: E.2371714, date: 28.03.2025).

Informed Consent: Our research adopted a retrospective cohort design, focusing on patients under 18 years of age.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Havva Yazıcı, Esra Er, Fehime Erdem, Ayşe Yüksel Yanbolu, Sakina Mammadova, Sedef Alpdoğan, Merve Yoldaş Çelik, Concept: Havva Yazıcı, Design: Havva Yazıcı, Ebru Canda, Data Collection or Processing: Yasemin Atik Altınok, Mahmut Çoker, Analysis or Interpretation: Ayça Aykut, Haluk Akın, Literature Search: Ebru Canda, Sema Kalkan Uçar, Writing: Havva Yazıcı, Ebru Canda.

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Associations of Urinary Metabolites of Parabens and Bisphenol a with Premature Thelarche among a Sample of Iranian Girls

Nafiseh Mozafarian[1], Mahin Hashemipour[1,2*], Mohammad Reza Maracy[3,4*], Hamid Galehdari[5], Roya Kelishadi[1]

[1]Isfahan University of Medical Sciences, Research Institute for Primordial Prevention of Non-Communicable Disease, Child Growth and Development Research Center, Isfahan, Iran
[2]Isfahan University of Medical Sciences, Metabolic Liver Disease Research Center, Isfahan, Iran
[3]Isfahan University of Medical Sciences, School of Public Health, Department of Biostatistics and Epidemiology, Isfahan, Iran
[4]Isfahan University of Medical Sciences, Research Institute for Primordial Prevention of Non-Communicable Disease, Environment Research Center, Isfahan, Iran
[5]Isfahan University of Medical Sciences, Skin Diseases and Leishmaniasis Research Center, Department of Dermatology, Isfahan, Iran
*Co-corresponding authors

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What is already known on this topic?

Endocrine-disrupting chemicals might influence the process of puberty including the development of premature thelarche.

What this study adds?

Exposure to bisphenol A and methyl paraben and ethyl paraben is related to increased odds of early breast development in girls.

ABSTRACT

Objective: Endocrine-disrupting chemicals may influence the process of puberty including the development of premature thelarche (PT). Our aim was to investigate the relationship between exposure to bisphenol A (BPA) and parabens with PT among a sample of Iranian girls.

Methods: This case-control study was conducted in 2022-2023 on girls with a mean (standard deviation) age of 7.5 (0.6) years in Isfahan, Iran. Participants were 90 newly diagnosed PT cases and 114 healthy controls. Spot urine samples were collected from both groups to measure the

Corresponding Authors: Prof. Mahin Hashemipour, Isfahan University of Medical Sciences, Research Institute for Primordial Prevention of Non-Communicable Disease, Child Growth and Development Research Center; Isfahan University of Medical Sciences, Metabolic Liver Disease Research Center, Isfahan, Iran - Prof. Mohammad Reza Maracy, Isfahan University of Medical Sciences, School of Public Health, Department of Biostatistics and Epidemiology; and Isfahan University of Medical Sciences, Research Institute for Primordial Prevention of Non-Communicable Disease, Environment Research Center, Isfahan, Iran

E-mail: mahin.hashemipour@gmail.com - maracy@med.mui.ac.ir **ORCID:** orcid.org/0000-0002-6032-5523 - orcid.org/0000-0002-3695-0863

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levels of BPA and paraben metabolites. Analyses of BPA and paraben metabolites included methyl paraben (MeP), ethyl paraben (EtP), propyl paraben, and butyl paraben and benzyl paraben and were performed by gas chromatography-mass spectrometry. The association between concentrations of creatinine-standardized urinary BPA and parabens and PT was analyzed with multiple logistic regression models, after adjusting for potential confounders.

Results: The results showed that individuals in the highest quartile of MeP [odds ratio (OR)=4.3, 95% confidence interval (CI): 1.2-14.9, p=0.023], EtP (OR=4.7, 95% CI: 1.3-17.2, p=0.018) and BPA (OR=5.03, 95% CI: 1.4-17.9, p=0.013) had a significantly higher odds for PT compared to those in the lowest quartile.

Conclusion: The findings of this study suggest that exposure to BPA, MeP and EtP is related to increased odds of early breast development in girls. Limiting the exposure to these chemicals may help to reduce the risk of PT.

Keywords: Bisphenol A, parabens, early puberty, thelarche, girls

Introduction

Puberty is a stage of development marked by significant physical and physiological changes. The early onset of secondary sexual characteristics in girls, particularly breast development, before the age of 8 is termed precocious puberty (1). Recent global data showed a downward trend in the age of thelarche in girls over recent decades (2).

As genetic factors remain relatively constant in this short period of time, this declining trend may be related to other factors including improved health and nutrition status, as well as various biological and lifestyle-related factors such as birth weight, sleep duration, physical activity levels, vitamin D status, socioeconomic status (SES), and maternal age at menarche and environmental exposures (3,4,5,6,7,8,9).

A particular concern is that exposure to endocrine disruptor chemicals (EDCs) might change hormonal balance (10), and may thus be related to this widely observed decrease in the age of onset of puberty (11). However, the consequences of exposure to EDCs on child reproductive development have not been comprehensively described.

Several materials with endocrine disrupting activity have been recognized, like bisphenol A (BPA) and parabens. According to the available literature, BPA and parabens have estrogenic and anti-androgenic properties (12,13,14,15). Exposure to these chemicals is widespread in the world. Humans are exposed to BPA and parabens through oral intake, as the major route, as well as inhalation and dermal absorption (16,17,18,19). Children may be exposed to BPA and parabens through various common sources encountered in daily life.

BPA, an organic monomer, is widely used in the production of epoxy resin and polycarbonate plastics. Epoxy resin is used in the inner lining of cans and jar caps. Polycarbonate plastics are used in a wide range of consumer goods, such as food packaging and plastic bottles, medical equipment, thermal paper and toys (20). Parabens are widely used as antibacterial preservatives in a diverse range of cosmetic and personal care products (21). Parabens are found in more than half of personal care products and nearly 90% of processed foods and beverages (22,23).

Several biomonitoring studies conducted in Iran have reported detectable levels of BPA and parabens in urine samples from both children and adults, indicating widespread exposure across the population. For example, a cross-sectional study by Malakootian et al. (24), involving 96 women in Kermanshah, detected methyl paraben (MeP), ethyl paraben (EtP), propyl paraben (PrP), and butyl paraben (BuP) in 100% of urine samples. Among these, PrP had the highest mean concentration, while BuP had the lowest (24). Similarly, a 2020 cross-sectional study among 117 pregnant women in Isfahan found detection rates of MeP, EtP, PrP, and BuP in 92%, 36%, 65%, and 89% of urine samples, respectively (25). Furthermore, Kiani Feizabadi et al. (26) reported widespread exposure of Iranian adolescents to paraben compounds.

Exposure to EDCs such as BPA and parabens is concerning because these compounds can mimic or interfere with endogenous hormone activity, potentially disrupting the finely tuned hypothalamic-pituitary-gonadal axis signaling. Furthermore, evidence suggests that EDCs may influence gene expression through epigenetic mechanisms, such as DNA methylation and histone acetylation, without altering the underlying DNA sequence (27,28,29). These disruptions may ultimately lead to alterations in the timing of pubertal onset.

Due to well-established evidence regarding the harmful health effects of BPA, several countries have implemented restrictions on its use in consumer products. For instance, the European Union has banned BPA in baby bottles and children's toys (30,31). Similarly, the U.S. Food and Drug Administration has prohibited the use of BPA in the manufacture of baby bottles, training cups, and packaging for infant foods, citing concerns about its potential biological effects (32,33). In addition, the European Union regulates the use of parabens in cosmetic and personal care products, setting a maximum allowable concentration of 0.8% for mixtures of parabens and 0.4% for any individual paraben (34). Furthermore, in Denmark, the use of PrP and

BuP in products intended for children has been completely prohibited (35).

During the last few decades, several human and animal studies have investigated the potential impact of chemicals on the odds of precocious puberty in girls. Some studies have indicated a significant relationship between BPA (10,36,37,38) and concentrations of parabens (39) in urine and precocious puberty in girls. However, the results of other studies showed that BPA exposure may be weakly related to pubertal timing in girls (40,41,42). In addition, very few studies have examined the association between exposure to parabens and timing of pubertal development in girls (39,43). As far as we know, the association between BPA and parabens with PT have not been previously evaluated among Iranian girls. Therefore, our goal was to evaluate the associations between exposure to BPA and parabens with PT among a sample of Iranian girls.

Methods

This case-control study was performed from 2022 to 2023 on girls with a mean [standard deviation (SD)] age of 7.5 (0.6) years in Isfahan. This research received ethical approval from Isfahan University of Medical Sciences (code: IR.MUI.MED.REC.1399.176, project number: 398986). Informed consent was obtained from the parents and their daughters involved in the study, after they were fully informed about the research objectives. The parents were assured that their personal information will be kept confidential. The present study was carried out with the cooperation of the Department of Education and the Health Center of Isfahan province.

Girls with newly diagnosed PT as cases were selected by consecutive sampling method from pediatric endocrinology clinics.

Control subjects, girls without premature thelarche, were selected from seven elementary schools in five educational districts of Isfahan city. The sampling method has been described previously (44). Briefly, the schools were selected randomly. Then, girls aged 6-8 years were invited to participate in the study as control group, Students who were willing to give a urine sample were included in the study.

Participants with a history of chronic diseases and genetic syndromes or any long-term medication use (such as use of gonadotropin releasing hormone agonist) were excluded. Those participants who refused the clinical examination were also excluded. All participants were of Iranian nationality.

Data were collected through clinical examinations, laboratory measurements and questionnaires. The questionnaires were completed during an interview with the mothers of selected students.

Anthropometric Measurements

Anthropometric variables including height and weight of participants were measured according to the standard protocols using validated instruments. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). According to the World Health Organization guidelines, we classified the adolescents' weight status using the BMI-for-age and gender. The following cut off points were used: underweight: BMI <5th percentile; normal weight: 5-84.9th percentile, overweight: 85-94.9th percentile, and obesity: ≥95th percentile (45).

Clinical Examination

Clinical breast tanner staging was assessed by pediatric endocrinologists for both case and control groups using Tanner's rating scale. Breast development was examined through both visual inspection and palpation (46). The first appearance of breast buds (B2) was considered as the onset of puberty (47). Achieving B2 before age 8 years was considered as precocious puberty (1).

Measurement of Urinary BPA and Parabens

Spot urine samples were collected from case and control groups to measure the levels of BPA, MeP, EtP, PrP and BuP and also benzyl paraben (BzP), as well as urinary creatinine concentrations. Samples were collected in polypropylene containers and were stored at -20 °C until analysis of the metabolites.

To extract parabens and BPA from urine samples, dispersive liquid-liquid microextraction (DLLME) approach was used (48).

The gas chromatography-mass spectrometry (GC-MS) device used was manufactured by Agilent (USA), model 7890, equipped with an Agilent mass spectrometer model 5975 and a Split/Splitless inlet (49). The mass spectrometer is of the quadrupole type. Separation was carried out using a capillary column made of silica, coated with poly (dimethylsiloxane) [HP-5 MS (5% phenyl)-95%] with dimensions of 30 m × 0.25 mm I.D. and a film thickness of 0.25 µm. For tuning of the mass spectrometer, perfluorotributylamine (PFTBA) was used. Selected Ion Monitoring (SIM) mode was applied for each target compound. In this mode, instead of scanning a wide range of m/z values, only a limited number of user-defined m/z values with the highest abundance are detected, thus enhancing sensitivity and making it more suitable for quantitative measurement. The device software was MSD ChemStation, version E.02.01.1177. The figure below shows an image of the GC-MS system.

The injection was performed in splitless mode with an injection volume of 1 µL, and the inlet temperature was set at 290 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature program started at 60 °C (held for 2 minutes), followed by an increase at a rate of 6 °C

per minute up to 280 °C, where it was held for an additional 2 minutes. The interface temperature was set at 290 °C, while the ion source and quadrupole temperatures were maintained at 230 °C and 150 °C, respectively.

Isotopically labeled internal standards were used in the analysis. Specifically, we used ^{13}C12-BPA for BPA and D4-MeP, D4-EtP, D4-PrP, and D4-BuP for the respective parabens.

Quality Assurance and Quality Control (QA/QC)

The GC/MS method was validated following the ICH guidelines (50). To assess precision, samples were analyzed in triplicate, and the standard deviations were calculated and reported as relative SD. Accuracy was evaluated by performing triplicate analyses using high-performance liquid chromatography-grade water as a blank substitute for human urine. The limits of detection (LOD) and quantification (LOQ) were determined by injecting diluted standard solutions with known concentrations, where LOD and LOQ corresponded to signal-to-noise ratios of 3 and 10, respectively.

The detection rates of BPA, MeP, EtP and PrP ranged between 93.2 and 98%. Urine concentrations of the metabolites lower than LOD were replaced by LOD/2 (51).

The detection rates of BuP and BzP were only 70.2 and 60%. Consequently, concentrations below the LOD were replaced with random values from a uniform distribution between zero and the respective LOD (52).

To minimize bias from variations in urine dilution, creatinine concentrations were measured using a calorimetric method (Jaffe) on a Mindray BS-800 Chemistry Analyzer. The concentrations of BPA and parabens were expressed as micrograms per gram of creatinine (µg/g Cr).

Then, the urine concentrations of BPA and parabens were categorized into quartiles to estimate the relationship between the biomarkers and odds of PT in girls. The first quartile (the lowest concentration) was considered as a reference group in the analysis.

Assessment of Physical Activity and Screen Time

We assessed physical activity levels in participants using the Physical Activity (PA) Questionnaire.

The questionnaire's validity and reliability were previously confirmed in an Iranian population (53). PA scores were obtained from various items about the activities of the students during the preceding week, including various sports (16 items), and as well as subjects' activities during physical education classes, school breaks, lunch hours, after school, in the evenings, on weekends and in general. Then, we classified the score to a dichotomous variables: PA score: 1-1.9 as low PA level; and PA score: 2-5 as high PA level, as previously described (54).

To measure screen time (ST), the hours of watching TV and using a personal computer (PC) or playing electronic games were asked separately for weekdays and weekends. Then, the weighted average of these hours was calculated as ST activity. Then ST was again categorized into two groups: <2 and ≥2 hours/day (55).

Moreover, the parents reported that their daughters usually spend outdoors per day between 10 AM and 4 PM on weekdays and on weekends. The weighted average hours of sun exposure were calculated for each participant.

In addition, mothers were asked how many hours their daughter usually sleeps at night. Sleep duration was categorized as a dichotomous variable. Long sleep was defined as sleep duration >8 hours/day (56).

SES

Family SES was estimated using a validated questionnaire; the method and variables were previously reported (57). Mothers were asked about parents' education, parents' occupation, owning a private car, type of school (public/private), type of home (private/rented) and having a PC at home. The variables were combined as one main component of SES by principle component analysis (PCA). Then, this main component was classified into quartiles, with the first quartile being considered as the "lowest SES" and the fourth quartile as the "highest SES" group (58).

Statistical Analysis

Data analysis was done using STATA 10 software (Stata Corp, College Station, Texas, USA). A $p<0.05$ was considered significant. Continuous variables were reported as mean and median (25^{th}-75^{th} percentile) and geometric mean. Categorical variables are presented as frequency (%). Independent t-test and chi-square and/or Fisher's exact test were used to compare continuous and categorical variables between two the groups. Urinary BPA and paraben levels were compared between cases and controls groups using the Mann-Whitney U test.

We performed multiple logistic regressions to examine associations between urinary paraben metabolites or BPA concentrations and PT in girls. Based on this regression, parabens and BPA were considered as independent.

Potential confounders were selected after literature review (3,4,5,6,7). These included birth order, birth weight, season of birth, sun exposure, type of delivery, maternal weight before pregnancy, height of mother, maternal age at delivery, breast feeding duration, feeding with soymilk, feeding method in the first year of life, is the child a single or one of multiple twins?,

variables of SES and health behaviors of girls (watching TV, computer time, physical activity, sun exposure time and sleep duration).

All the variables with differences between the case and control groups at the level of p<0.2 were included in the multiple logistic regression analyses as confounding variables.

Since the normal range of urinary creatinine is 0.3-3 g/L (59,60), in a further analysis we excluded 11 participants (case=4 and control=7) with urinary creatinine less than 0.3 g/L.

Results

In this case-control study, 90 newly diagnosed PT cases and 114 healthy controls were included. The mean (SD) ages of participants were 7.7 (0.6) and 7.3 (0.6) years for the case and control group, respectively. Table 1 presents the characteristics of the participants in both groups.

The parameters of Quality Assurance/Quality Control (QA/QC) for BPA and parabens determination is presented in Table 2.

Table 1. Characteristics of the children with and without premature thelarche

Characteristics	Category	Total n=195	Control n=110	Case n=85	p*
Child characteristics					
Age (year)[a]		7.5 (0.6)	7.3 (0.6)	7.7 (0.6)	<0.001
Child BMI (kg/m^2)	Underweight	11 (5.4)	8 (7)	3 (3.3)	0.003**
	Normal weight	123 (60.3)	79 (69.3)	44 (48.9)	
	Overweight	28 (13.7)	12 (10.5)	16 (17.8)	
	Obese	42 (20.6)	15 (13.2)	27 (30)	
Child BMI (kg/m^2)	Underweight and normal	134 (65.7)	87 (76.3)	47 (52.2)	<0.001
	Overweight and obese	70 (34.3)	27 (23.7)	43 (47.8)	
Breastfeeding duration	Not breastfed	7 (3.6)	2 (1.8)	5 (5.8)	0.357**
	<6 months	13 (6.6)	7 (6.3)	6 (7)	
	≥6 months	177 (89.8)	102 (91.9)	75 (87.2)	
Feeding with soymilk	No	189 (95.9)	106 (95.5)	83 (96.5)	1.000**
	Yes	8 (4.1)	5 (4.5)	3 (3.5)	
Feeding method in the first year of life	Breastfeeding	147 (74.6)	87 (78.4)	60 (69.8)	0.350**
	Formula	8 (4.1)	4 (3.6)	4 (4.7)	
	Mixed feeding	42 (21.3)	20 (18)	22 (25.6)	
Is the child under investigation a twin?	Singleton	191 (96)	110 (99.1)	81 (92)	0.023**
	Twins and more	8 (4)	1 (0.9)	7 (8)	
Birth weight, g	<2500	21 (10.6)	5 (4.5)	16 (18.2)	0.002
	≥2500	178 (89.4)	106 (95.5)	72 (81.8)	
Season of birth	Spring	57 (27.9)	34 (29.8)	23 (25.6)	0.152
	Summer	64 (31.4)	41 (36)	23 (25.6)	
	Fall	44 (21.6)	19 (16.7)	25 (37.8)	
	Winter	39 (19.1)	20 (17.5)	19 (21.1)	
Birth order	First born	110 (55.8)	49 (44.1)	61 (70.9)	<0.001
	Second born or later	87 (44.2)	62 (55.9)	25 (29.1)	
ST duration (hour)[a]		3.3 (1.5)	3.02 (1.4)	3.8 (1.6)	0.001

Table 1. Continued

Characteristics	Category	Total n=195	Control n=110	Case n=85	p*
Sleep duration (hour)	≤8	50 (25.4)	33 (29.7)	17 (19.8)	0.111
	>8	147 (74.6)	78 (70.3)	69 (80.2)	
Physical activity[a]		1.9 (0.6)	2.0 (0.7)	1.8 (0.5)	0.021
Physical activity	Low PA	122 (61.9)	64 (57.7)	58 (67.4)	0.161
	High PA	75 (38.1)	47 (42.3)	28 (32.6)	
Sun exposure time (hour)	<1	22 (11.2)	16 (14.4)	6 (7)	0.157
	1-2	134 (68)	69 (62.2)	65 (75.6)	
	2-3	20 (10.2)	14 (12.6)	6 (7)	
	>3	21 (10.7)	12 (10.8)	9 (10.5)	
Sociodemographic characteristics of parent					
SES	Q1 (lowest SES)	49 (24.9)	35 (31.5)	14 (16.3)	0.057
	Q2	49 (24.9)	22 (19.8)	27 (31.4)	
	Q3	51 (25.9)	29 (26.1)	22 (25.6)	
	Q4 (highest SES)	48 (24.4)	25 (22.5)	23 (26.7)	
Maternal age at menarche (years)	<12	19 (9.6)	9 (8.1)	10 (11.5)	0.016
	12-13	95 (48)	45 (40.5)	50 (57.5)	
	>13	84 (42.4)	57 (51.4)	27 (31)	
Type of delivery	Natural birth	57 (28.9)	30 (27)	27 (31.4)	0.502
	Cesarean section	140 (71.1)	81 (73)	59 (68.6)	
Mother's height		161.7 (7.17)	162.4 (8.1)	160.9 (5.8)	0.142
Maternal weight before pregnancy (kg)[a]		61.6 (10.5)	61.2 (11.0)	62.2 (9.9)	0.524
Maternal prepregnancy BMI (kg/m^2)	Underweight	11 (5.6)	9 (8.2)	2 (2.4)	0.311**
	Normal weight	127 (65.1)	72 (65.5)	55 (64.7)	
	Overweight	44 (22.6)	22 (20)	22 (25.9)	
	Obese	13 (6.7)	7 (6.4)	6 (7.1)	
Maternal age at delivery (years)	<25	42 (21.4)	18 (16.2)	24 (28.2)	0.085
	25-29	84 (42.9)	48 (43.2)	36 (42.4)	
	>30	70 (35.7)	45 (40.5)	25 (29.4)	

[a]Data are presented as mean (SD) other data are presented as number (%)
*p values using the chi-square ($\chi 2$) and t-test between the case and control group (where appropriate). **p values using Fisher's exact test.
BMI: body mass index, ST: screen time, PC: personal computer, TV: television, PA: physical activity, SES: socioeconomic status

Distribution of urinary concentrations of BPA and parabens among case and control groups is presented in Table 3.

Table 4 presents results of the multiple logistic regression models to estimate the association between urinary BPA and parabens levels with PT.

After adjusting for age, BMI, birth order, birth weight, season of birth, maternal age at menarche, maternal age at delivery, mother's height, SES, ST, sleep duration, physical activity and time of sun exposure, significant positive association was found between the highest quartile for BPA and PT (OR=3.1; 95% CI: 1.0-9.5, p=0.046).

In addition, after adjustment for confounding variables, the highest concentrations of EtP were associated with 3.2-fold increased odds of PT (OR=3.2, 95% CI: 1.02-9.97, p=0.045) compared to those in the lowest quartile for this analyte.

Table 2. The parameters of Quality Assurance/Quality Control (QA/QC) for bisphenol A and parabens determination

Compound name	RT (min)	Units	LOD*	LOQ**	R2***	RSD****
Methyl paraben	13.82	ppb	0.10	0.33	0.992	8.6
Ethyl paraben	15.49	ppb	0.10	0.34	0.997	5.9
Propyl paraben	17.69	ppb	0.09	0.28	0.996	10.1
Butyl paraben	19.89	ppb	0.10	0.33	0.991	6.7
Benzyl paraben	23.01	ppb	0.06	0.19	0.998	7.7
Bishphenol A	28.82	ppb	0.10	0.33	0.996	6.7

*Limit of detection, **Limit of quantitation, ***R-squared correlation, ****RSDs% (relative standard deviation)
RT: retention time, LOD: limit of detection, LOQ: limit of quantitation, RSD: R-squared correlation

Table 3. Concentrations of parabens and bisphenol A in urine of case and control groups

Compound (µg/g creatinine)	%> LOD	Mean (SD)	GM (95% CI)	Min.	Max.	Total* (n=204)	Control* (n=114)	Case* (n=90)	p
MeP	94.6	5.4 (10.96)	3.06 (2.60-3.60)	0.035	107.85	3.26 (2.34-5.14)	3.17 (2.19-4.57)	3.28 (2.42-6.25)	0.462
EtP	94.1	5.4 (10.0)	3.17 (2.71-3.73)	0.035	88.75	3.37 (2.38-5.43)	3.31 (2.30-4.90)	3.47 (2.59-6.39)	0.382
PrP	93.2	4.41 (9.77)	2.32 (1.95-2.76)	0.032	101.10	2.60 (1.86-4.08)	2.84 (1.95-3.83)	2.33 (1.51-4.45)	0.177
BuP	70.2	2.62 (5.49)	0.76 (0.58-0.99)	0.005	55.10	1.79 (0.14-2.92)	1.91 (0.48-2.96)	1.67 (0.07-2.69)	0.149
BzP	60	2.57 (7.17)	0.46 (0.33-0.63)	0.002	92.30	1.47 (0.04-2.95)	2.13 (0.99-3.30)	0.05 (0.02-1.95)	<0.001
BPA	98	6.32 (24.4)	3.09 (2.72-2.80)	0.045	323.57	2.86 (2.08-4.61)	2.93 (2.01-4.22)	2.86 (2.17-5.39)	0.337

p values using Mann-Whitney U test, *Data are represented as median (IQR, interquartile range)
Min: minimum, Max: maximum, GM: geometric mean, LOD: limit of detection, MeP: methyl paraben, EtP: ethyl paraben, PrP: propyl paraben, BuP: butyl paraben, BzP: benzyl paraben, BPA: bisphenol A

The results showed a lower odds ratio of PT in participants who were in the third and fourth quartile for BzP, compared to those in the lowest quartile ($p<0.05$).

The results indicated a higher odds ratio of PT in participants who were in the fourth quartile for MeP (OR=4.3, 95% CI: 1.2-14.9, p=0.023) and EtP (OR=4.7, 95% CI: 1.3-17.2, p=0.018) and BPA (OR=5.03, 95% CI: 1.4-17.9, p=0.013), compare to those in the first quartile.

Discussion

The purpose of the present study was to compare the urinary concentrations of five parabens and BPA in girls with or without PT. We found that exposure to these EDCs was common among Iranian girls from Isfahan.

In the present study, the geometric mean (GM) of BPA was 3.09 (2.72-3.52) µg/g creatinine, and BPA was detectable in 98% of the samples. Various studies have also been conducted in other countries (Table 5). For example, a 2021 study in Spain found a GM BPA level of 0.90 ng/mL, detectable in 63% of samples (59). In China (2020), the GM BPA levels in 3- and 7-year-old girls were 2.88 and 4.66 µg/g creatinine, respectively (61). In the U.S. (2019), BPA was detected in 97.5% of samples, with a GM (SD) of 1.23 (0.06) µg/g creatinine (62).

Furthermore, the GM urinary concentrations of MeP, EtP, and PrP were relatively high and detectable in most samples, while BuP and BzP were found in about 60% of the samples (Table 6). Although the concentrations of MeP and EtP were higher in the case group than in the control group, the difference was not significant. Previous studies conducted in countries including Spain (59), California (39), and Iran (26) have also shown that MeP and PrP are detected in a high percentage of children and adolescents. However, exposure levels in Iran, particularly for MeP, were reported to be significantly higher than in European and Asian countries (26).

Our findings suggest that exposure to BPA and MeP and EtP might be linked to early breast development ($p<0.05$). A small number of human studies have assessed the link between prenatal exposure to BPA and the stages of puberty (63,64,65). For instance, a cohort study in Mexico City in 120 girls aged 8-13 years in 2017 reported that BPA levels in the second trimester were related to an increased risk of early breast development (63).

In line with the present study, studies also evaluated urinary levels of BPA in children. Some studies found significant associations between urinary BPA levels and precocious puberty.

Table 4. Association of bisphenol A and paraben concentrations (µg/g creatinine) with premature thelarche

Compound		Range	Crude models (n=204) case=90, control=114		Adjusted model1 (n=195) case=85, control=110		Adjusted model[2]* (n=184) case=81, control=103	
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
MeP	Q1	<2.34	1		1		1	
	Q2	2.35-3.26	1.4 (0.6-3.0)	0.424	1.6 (0.5-4.9)	0.421	1.6 (0.5-5.1)	0.431
	Q3	3.27-5.14	0.8 (0.4-1.9)	0.682	1.1 (0.3-3.5)	0.881	1.2 (0.4-4.1)	0.754
	Q4	>5.14	1.9 (0.9-4.1)	0.114	2.7 (0.9-8.1)	0.077	4.3 (1.2-14.9)	0.023
EtP	Q1	<2.38	1		1		1	
	Q2	2.39-3.37	1.2 (0.5-2.6)	0.687	1.6 (0.5-4.8)	0.433	1.5 (0.5-4.8)	0.486
	Q3	3.38-5.43	1.2 (0.5-2.6)	0.687	1.7 (0.5-5.3)	0.390	1.7 (0.5-5.8)	0.371
	Q4	>5.43	1.6 (0.7-3.5)	0.324	3.2 (1.02-9.97)	0.045	4.7 (1.3-17.2)	0.018
PrP	Q1	<1.86	1		1		1	
	Q2	1.87-2.60	0.7 (0.3-1.5)	0.323	0.7 (0.2-2.1)	0.524	0.7 (0.2-2.2)	0.565
	Q3	2.61-4.08	0.3 (0.1-0.6)	0.003	0.4 (0.1-1.2)	0.099	0.4 (0.1-1.5)	0.187
	Q4	>4.08	0.9 (0.4-1.9)	0.692	1.1 (0.4-3.3)	0.807	1.7 (0.5-5.5)	0.398
BuP	Q1	<0.14	1		1		1	
	Q2	0.14-1.79	0.4 (0.2-0.8)	0.011	0.2 (0.05-0.6)	0.006	0.2 (0.05-0.6)	0.009
	Q3	1.80-2.92	0.6 (0.3-1.3)	0.167	0.3 (0.09-1.1)	0.072	0.3 (0.08-1.1)	0.076
	Q4	>2.92	0.5 (0.2-0.997)	0.049	0.31 (0.09-1.0)	0.053	0.4 (0.1-1.4)	0.137
BzP	Q1	<0.04	1		1		1	
	Q2	0.05-1.47	0.4 (0.2-0.9)	0.033	0.3 (0.09-1.1)	0.080	0.2 (0.06-0.9)	0.029
	Q3	1.48-2.95	0.08 (0.03-0.21)	<0.001	0.08 (0.02-0.3)	<0.001	0.05 (0.01-0.2)	<0.001
	Q4	>2.95	0.14 (0.06-0.3)	<0.001	0.14 (0.04-0.5)	0.003	0.15 (0.04-0.6)	0.007
BPA	Q1	<2.08	1		1		1	
	Q2	2.09-2.86	1.3 (0.6-2.8)	0.550	1.6 (0.5-5.1)	0.397	1.7 (0.5-5.4)	0.407
	Q3	2.87-4.61	0.7 (0.3-1.6)	0.413	1.1 (0.4-3.6)	0.821	1.3 (0.4-4.2)	0.687
	Q4	>4.61	1.7 (0.8-3.8)	0.167	3.1 (1.0-9.5)	0.046	5.03 (1.4-17.9)	0.013

Model 1 and 2 are adjusted for age, child BMI, birth order, birth weight, season of birth, maternal age at menarche, maternal age at delivery, mother's height, socioeconomic status, screen time, sleep duration, physical activity and time of sun exposure.
*The subjects with urinary creatinine less than 0.3 g/L were excluded from the model[2].
MeP: methyl paraben, EtP: ethyl paraben, PrP: propyl paraben, BuP: butyl paraben, BzP: benzyl paraben, BPA: bisphenol A, Q: quartile, OR: odds ratio, CI: confidence interval

For example, a study that was conducted in 2022 in China on 76 girls showed that urinary BPA levels in the case group were significantly higher than those in the control group (66). In addition, a case-control study in 2018 in China on 272 girls reported that BPA exposure was also related to a higher odds of precocious puberty (38). However, other studies reported no significant association between BPA and precocious puberty (40,41). For example, a Chinese case control study in 2023 that was conducted among 120 girls with precocious puberty (cases) and 145 healthy girls (controls) did not find significant association between exposure to BPA and odds of early puberty (41). Similarly, a cohort study in 2017 among 1051 American girls aged 6-8 years did not document a significant association between exposure to BPA and age of menarche (67). However, some other studies reported significant relationship between BPA exposure with delayed menarche. A cross-sectional study on 655 girls aged 9-18 years from Shanghai in 2017 showed BPA exposure was related to delayed menarche (68). Similarly, a cross-sectional study conducted in the USA involving 987 adolescent girls aged 12-19 years demonstrated an association between urinary BPA levels and delayed menarche (36).

To date, few studies have been conducted to assess the link between paraben exposure and timing of puberty, and have reported inconsistent results. A longitudinal cohort study in 2019 in USA showed that peripubertal exposure to MeP was related to earlier thelarche, pubarche and menarche. The results also suggested an association of peripubertal PrP with earlier

Table 5. Bisphenol A concentration in a range of studies for comparison with results of the present study

First author	Publication year	Location	Study design	Sample size	Age	Median	GM (g/g crμ)
Dualde et al. (59)	2021	Spain	Cross-sectional	562	5-12		0.90*
Guo et al. (61)	2020	China	Longitudinal	229	3	2.59	2.88
				412	7	2.41	4.66
Jacobson et al. (62)	2019	U.S.	Cross-sectional	894	6-19		1.23
Çok et al. (72)	2020	Türkiye	Cross-sectional	125	3-6	0.60	1.05
Zhou et al. (66)	2022	China	Case-control	Case=30	7.1 (0.7)	5.87	
				Control=46	7.3 (0.7)	0.24	
Supornsilchai et al. (73)	2016	Thailand	Case-control	Case=41	7.44 (1.03)	1.44	
				Control=47	7.44 (1.03)	0.59	
Buttke et al. (40)	2012	U.S.	Cross-sectional	440	12-16		2.25
Current study	2024	Isfahan	Case-control	Case=90	6-8	2.86	3.04
				Control=114	6-8	2.93	3.17

*ng/mL
GM: geometric mean

Table 6. Paraben concentrations in some studies

First author	Publication year	Location	Study design	Sample size	Age (year)	Statistic	Unit	MeP	EtP	PrP	BuP	BzP
Present study	2024	Isfahan	Case-control	204	6-8	Median	μg/g cr	3.26	3.37	2.60	1.79	1.47
						GM	μg/g cr	3.06	3.17	2.32	0.76	0.46
Dualde et al. (59)	2021	Spain	Cross-sectional	562	5-12	GM	ng/mL	1.4	<0.2	0.39	<0.2	-
Lu et al. (74)	2019	China	Cross-sectional	255	3-11	Median	μg/L	2.3	0.33	0.50	0.02	0.03
Kiani Feizabadi et al. (26)	2020	Isfahan	Cross-sectional	100	12-20	Median	μg/g cr	92.2	8.46	12.26	8.42	-
						GM	μg/g cr	93.6	4.37	6.13	5.59	-
Harley et al. (39)	2019	California	Cohort	179	9-13	GM	ng/g cr	44.9	-	4.9	-	-
Guth et al. (75)	2021	Canada	Cross-sectional	382	6-17	GM	μg/g cr	10.7	0.84	1.8	0.23	-

MeP: methylparaben, EtP: ethylparaben, PrP: propylparaben, BuP: butylparaben, BzP: benzylparaben, BPA: bisphenol A, GM: geometric mean

pubarche in girls (39). A recent systematic review of seven studies reported higher peripubertal paraben exposure was related to precocious puberty but the effect sizes were very small (43). However, a cross-sectional study in 2012, on American girls aged 12-16 years reported total parabens were not related to age of menarche (40). Similarly, another cohort study of 200 Chilean girls in 2018 found no link between concentrations of MeP and PrP and earlier menarche (69). In 2015, a prospective study was conducted among 1239 girls aged 6-8 years in the USA. They were followed annually for 7 years. After adjustment for confounding factors, including race/ethnicity and caregiver education, paraben levels were not linked with earlier thelarche and pubarche (70). A cohort study of 1151 American girls aged 6-8 years at enrollment reported that after adjusting for some confounding variables, urinary paraben concentrations were not linked with breast and pubic hair development (71).

Several factors may be behind these differences in the results of various studies. First, methods to assess sexual maturity were different across studies. Some studies used maternal and self-assessment and some used clinical examinations for assessing pubertal status. Second, the controlled confounding factors varied between studies. Moreover, the differences in study methodology, study design, sample size, statistical analysis methods and adjustment for urine creatinine may also contribute to the inconsistent results of different studies.

To the best of our knowledge, no previous study has examined the association between urinary paraben and BPA levels with premature thelarche among Iranian girls. However, the present study had some potential limitations. The cross-sectional study design limits assessment of causal relationships between the chemicals and onset of puberty. One spot urine was collected for the measurement of concentration of BPA and parabens so exposure misclassification may have affected our findings.

Conclusion

Our findings provide evidence of an association between higher exposure to BPA, MeP and EtP and precocious puberty among a cohort of Iranian girls. Considering that the evidence related to this topic is scarce and controversial, further cohort studies with large sample sizes and more repeated measures of the chemicals during the prepubertal years are suggested. This will enable a more reliable assessment of the clinical importance of the current findings. In addition, future research should explore potential mechanisms of action and interactions with nutritional, and lifestyle factors.

Ethics

Ethics Committee Approval: The study protocol was approved by the ethics committee of Isfahan University of Medical Sciences with code of IR.MUI.MED.REC.1399.176 and project number 398986.

Informed Consent: Informed consent was obtained from the parents and their daughters.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: Mahin Hashemipour, Roya Kelishadi, Concept: Nafiseh Mozafarian, Mahin Hashemipour, Mohammad Reza Maracy, Hamid Galehdari, Roya Kelishadi, Design: Nafiseh Mozafarian, Mahin Hashemipour, Mohammad Reza Maracy, Hamid Galehdari, Roya Kelishadi, Data Collection or Processing: Nafiseh Mozafarian, Mahin Hashemipour, Mohammad Reza Maracy, Hamid Galehdari, Roya Kelishadi, Analysis or Interpretation: Nafiseh Mozafarian, Mohammad Reza Maracy, Literature Search: Nafiseh Mozafarian, Mahin Hashemipour, Mohammad Reza Maracy, Writing: Nafiseh Mozafarian, Mahin Hashemipour, Mohammad Reza Maracy, Roya Kelishadi.

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Growth Hormone Strongly Induces h*SMN2* Promoter Driving Construct Gene Expression in Mammalian Cells

Dilara Yücedal[1], Ahmet Arman[2]

[1]Marmara University School of Medicine, Institute of Health Sciences, Department of Medical Genetics, İstanbul, Türkiye
[2]Marmara University School of Medicine, Department of Medical Genetics, İstanbul, Türkiye

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What is already known on this topic?

Prolactin increases survival motor neuron (SMN) expression and survival in a mouse model of severe spinal muscular atrophy (SMA) via the STAT5 pathway. STAT5 constitutive activation rescues defects in SMA. Human growth hormone (hGH) increases SMN expression and survival in severe SMA mouse model. hGH treatment upregulates SMN protein in NT2 cells (shown total SMN protein in the manuscript, it is not known how much SMN is coming from *SMN1* gene or how much SMN protein is coming from *SMN2*). GH majorly activates STAT5 activation.

What this study adds?

Nobody shows effect of GH on human *SMN2* promoter using luciferase specific gene expression in mammalian cells. We did the first GH-*SMN2* Promoter study in the world and our study shows GH specifically-strongly affect *SMN2* promoter. Results showed that luciferase activity of the GH-treated pGL3-human *SMN2* (h*SMN2*) promoter 1 region increased 191.6-fold, GH-treated pGL3-h*SMN2* promoter 2 region increased 348-fold and GH-treated pGL3-h*SMN2* promoter 3 region increased 133-fold compared to GH-treated plasmid alone. These fold increases are too huge amount. GH may be used to increase *SMN2* gene expression to treat SMA.

ABSTRACT

Objective: Spinal muscular atrophy (SMA) is the most common neurodegenerative disease caused by the absence or insufficiency of the survival motor neuron (SMN) protein. Human *SMN1* (h*SMN1*) produces fully functional SMN protein but h*SMN2* produces only about 10% functional protein. Deletion or mutation in h*SMN1* gene leads to SMA, while the h*SMN2* copy number modifies disease severity. Increasing h*SMN2* expression has emerged as a potential therapeutic approach. In this study, we investigated the effect of growth hormone (GH) on h*SMN2* promoter activity using a reporter in Chinese hamster ovary (CHO) cells.

Corresponding Author: Ahmet Arman, PhD, Marmara University School of Medicine, Department of Medical Genetics, İstanbul, Türkiye
E-mail: ahmetarman@marmara.edu.tr **ORCID:** orcid.org/0000-0001-5547-0024
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Methods: Three different h*SMN2* promoter regions (588 bp, 1036 bp and 1705 bp) were used to show the effect on gene expression of reporter response to GH in this study. Promoters were amplified by polymerase chain reaction (PCR) and cloned into the pGL3 luciferase reporter vector. The ligation reactions were transformed into DH5α cells and positive colonies containing specific h*SMN2* promoter inserts were confirmed by PCR with h*SMN2*-primers. The plasmids carrying h*SMN2* promoters were transfected into CHO cells. After transfection, the cells were treated with GH for 24 hours and luciferase activity was measured to assess promoter activity.

Results: All h*SMN2* promoter constructs responded to GH. The 1036 bp promoter construct showed the highest luciferase expression upon GH treatment. However, the 1705 bp promoter construct exhibited reduced gene expression compared to the control vector treated with GH.

Conclusion: These findings suggest that GH can modulate h*SMN2* expression in h*SMN2* promoter dependent manner. GH may be a candidate hormone for SMA treatment by enhancing h*SMN2* expression.

Keywords: Spinal muscular atrophy, growth hormone, survival motor neuron protein, survival motor neuron 2 promoter, genetic disease

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease characterized by degeneration of spinal cord motor neurons associated with proximal muscle weakness and muscular atrophy (1). SMA affects 1 in 6,000 to 1 in 10,000 individuals worldwide (2). Based on the age of onset and severity of the clinical course, childhood-onset SMA can be classified into four types (types I-IV). The most severe and most common (45%) type is type 1, which presents in infancy, and the mildest form is type 4, which is present in adults (3). Muscle weakness and impaired mobility are characteristic features of SMA (4). SMA is caused by a deficiency of the survival motor neuron (SMN) protein (1). The gene responsible for encoding the SMN protein maps to the reverse duplication site on 5q11.2-q13.3 and is called the *SMN* gene (5). In humans, there are two copies of the *SMN* gene, identified as human SMN1 (h*SMN1*) (telomeric) and h*SMN2* (centromeric) and this is unique to Homo sapiens (6). All forms of SMA result from homozygous loss of the hSMN1 gene due to gene deletion, conversion or mutation (7). Consistent with this gene duplication being a very recent evolutionary event, the h*SMN1* and h*SMN2* genes share more than 99.8% sequence homology over a 30 kb segment containing the entire coding region (8). h*SMN1* is composed of nine exons, 1, 2a, 2b, 3, 4, 5, 6, 7 and 8 (untranslated exon 8) encoding a 294 amino acid (aa) protein with a molecular weight of 38 kDa (9). h*SMN1* gene is transcribed into a full-length (FL) messenger RNA (mRNA). However, h*SMN2* is primarily transcribed into alternatively and naturally spliced mRNA lacking exon 7. This alternative splicing is caused by a silent mutation (C to T) in exon 7 of the h*SMN2* gene, which results in the loss of an exon splicing enhancer or the creation of an exon splicing repressor (1). The h*SMN2* gene mainly produces 90% transcripts lacking exon 7 and 5-10% wild type transcripts (10). When the h*SMN1* gene is deleted or mutated, the h*SMN2* gene cannot produce sufficient levels of functional SMN protein, resulting in SMA. All SMA patients have one or more copies of h*SMN2* gene and there is an inverse correlation between SMA severity and h*SMN2* copy number. The relationship between disease severity and copy number for h*SMN2* correlates with an increase in the FL SMN produced by each additional h*SMN2* gene (11). Increasing expression level of the h*SMN2* gene is considered an important strategy in the treatment of SMA.

Growth hormone (GH) has long been known to be a regulator of growth and sugar-fat metabolisms, but mechanisms of the transcription regulation by GH for some specific genes, such as h*SMN2*, are not described. GH binds to two GH receptors (GHR) and this ternary complex activates GHR-associated Janus kinase 2 (JAK2), which in turn phosphorylates tyrosines residues in itself, on the GHR and on intracellular proteins. Phosphorylated tyrosines on the receptor form docking sites for a number of signaling proteins, including members of the signal transducers and activators of transcription (STAT) family. Phosphorylated STAT proteins are released from the receptor and then they are dimerized, migrate to the nucleus and play an important role in the regulation of gene transcription (12). The role of the JAK/STAT signaling pathway in the regulation of h*SMN2* expression has also been demonstrated (5). STAT5 transcription factor in the STAT family plays an important role in the JAK2/STAT5 pathway. The PRL JAK2/STAT5 pathway is known to be involved in the regulation of h*SMN2* gene expression (5). GH is plays a major role in activation of STAT5 but there is no information about increased h*SMN2* gene expression by GH through the JAK-STAT5 signaling pathway in humans.

There is currently no cure for SMA. There are only treatments that slow the progression of disease severity and reduce symptoms. Recent studies have indicated that up-regulating h*SMN2* gene expression may be a possible treatment for SMA.

Previous *in vivo* studies have suggested that GH may influence SMN expression through STAT5 pathway activation. In particular, MacKenzie et al. (13) demonstrated that systemic administration of human GH (hGH) in severe SMA mouse models increased SMN protein levels in the brain and spinal cord, improved disease phenotype, and significantly prolonged survival. These results identified hGH as a potential therapeutic compound acting via STAT5 signaling (13). Building upon these findings, our study focused on the direct transcriptional regulation of the h*SMN2*

promoter by GH in a cell culture system, aiming to provide mechanistic evidence for the promoter-level responsiveness of *SMN2* to GH.

In this study, our purpose was to determine whether or not GH specifically increased the expression level of luciferase gene for the reporter vectors containing three different promoter regions of h*SMN2* gene in Chinese hamster ovary (CHO) cells.

Methods

Bioinformatic Analysis

The promoter regions of h*SMN1* and h*SMN2* genes were analyzed using data from the National Center of Biotechnology Information (NCBI) and The Eukaryotic Promoter Database (EPD). The promoter sequence of the two genes was compared and differences were determined using the bioinformatic tool, VectorBuilder (VectorBuilder Inc. 1010 W 35th Street, Suite 515 Chicago, IL 60609, USA Tel: +1 800-517-2189; https://en.vectorbuilder.com/). STAT5 transcription sites in promoter region of h*SMN2* gene were analyzed using EPD. A restriction enzyme map in the h*SMN2* promoter region was analyzed using NEBcutter 3.0 (a tool provided by New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723, USA; Tel: +1 978-927-5054; Email: info@neb.com; Website: https://www.neb.com/). Restriction enzyme cut sites were determined for cloning, based on restriction enzyme analysis of h*SMN2* promoter and the pGL3 vector cloning site. Restriction enzymes used in the study were NheI and XhoI (New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723, USA). Primers for h*SMN2* promoter regions were designed specifically and NheI and XhoI restriction sequences were added to 5' site of the primers. The primers for h*SMN2* promoter regions were analyzed for Tm and GC values using OligoAnalyzer™ Tool (Integrated DNA Technologies, Inc., Coralville, IA 52241, USA). Specificity and size of amplicons were also checked with NCBI Primer Blast Tool.

Molecular Biology Studies

DNA Isolation and PCR

Genomic DNA was isolated from blood using a salting out technique (14). DNA concentration and purity were measured with a NanoDrop (Thermo Fisher, USA) and DNA with an A260/A280 ratio between 1.8-2.0 was used for polymerase chain reaction (PCR). The PCR reaction for promoter regions 1 and 2 of h*SMN2* was prepared in a total volume of 25 µL and composed of 1x PCR Buffer, 1.5 mM MgCl2, 200 µM dNTP mixture, 0.5 µM h*SMN2* Forward1 or 2 primer, 0.5 µM h*SMN2* reverse primer, 500 ng genomic DNA, 1U Taq polymerase (Promega, USA). PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94 °C 2 min (1X); first cycle: 94 °C 30s, 61 °C 30s, 72 °C 1 min (5X); following second cycle: 94 °C 30s, 65 °C 30s, 72°C 1 min. (25x); final extension: 72 °C 10 min. The long-range PCR for h*SMN2* promoter region 3 was performed in a total volume of 50 µL and composed of 1x PCR Buffer with MgCl2, 200 µM dNTP mixture, 0.5 µM h*SMN2* Forward3 primer, 0.5 µM h*SMN2* Reverse primer, 500 ng Genomic DNA, 1U Taq polymerase (Takara, Japan). PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94°C 2 min (1X), 94 °C 30s, 65 °C 30s, 72 °C 2 min (5x); following cycle: 94 °C 30s, 68 °C 30s, 72 °C 2 min, Final extension: 72 °C 10 min. PCR products were analyzed by 1% agarose gel electrophoresis and the ethylene bromide-stained gel was visualized using the Gel Imaging System (Biolab, UK).

PCR Purification

PCR purification was performed using the High Pure PCR Product Purification Kit (Roche, Switzerland). Concentration of the purified PCR products was measured by NanoDrop (Thermo Fisher, USA).

Double Digestion

Double cut was performed for the h*SMN2* promoter PCR products and pGL3 vector using 25 µL PCR product (2 µg) or pGL3 vector (2 µg), 5 µL 10X Buffer (rCutSmart), 1 µL XhoI (20U), 1 µL NheI (20U) with a total volume of 50 µL. The digestion reactions were incubated at 37 °C for 4 hours (Bacterial Incubator, Binder, Germany). Digested pGL3 vectors and PCR products were purified as previously described. Concentrations of double cut PCR products and pGL3 vector were measured and analyzed by agarose gel electrophoresis.

Ligation

The double cut h*SMN2* PCR promoter products were ligated into double cut pGL3 vector using T4 DNA ligase (3U, Promega, USA). The ligation reactions were performed in 1/1, 1/3 and 1/5 ratios and incubated at +4 °C overnight. Restriction enzymes were inactivated 80 °C and 65 °C for 20 min respectively. Then, transformation was performed using heat shock method with 5 µL ligation product and 100 µL DH5α competent cells (Takara, Japan). Transformed products were plated on bacterial plates with ampicillin and incubated at 37 °C overnight (Bacterial Incubator, Binder, Germany).

Colony PCR

Direct colony PCR was performed to determine insertion of promoter regions of h*SMN2* gene in the pGL3 vectors on colonies. The transformed colonies on the plates were transferred into tubes containing 10 µL distilled, DNA-free water (dwater), from which 4 µL bacterial aliquot were taken into sterile tubes. The remaining 6 µL were incubated at 95 °C for 10 minutes. For direct colony PCR content: 2 µL template, 1.5 µL 10X PCR Buffer, 1.5 mM

MgCl2, 200 µM dNTP, 0.4 µM forward primer for vector, 0.4 µM reverse primer for vector, 0.5U Taq DNA Polymerase (Promega), 7.5 µL dwater. PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94 °C 2 min (1X), 94 °C 30s, 55 °C 30s, 72 °C 90 sec (25X), final extension: 72 °C 5 min. PCR products were analyzed by 1% agarose gel electrophoresis and visualized using the Gel Imaging System (Biolab, UK).

Plasmid Isolation

Positive colonies were cultured overnight at 37 °C and the plasmid isolation was performed using a Genopure Plasmid Isolation Kit (Roche, Switzerland) following manufacturer protocol and DNA concentrations were measured.

Specific PCR for plasmids obtained from positive colonies. PCR was performed using 100 ng/µL plasmids, 1.5 µL 10X PCR Buffer, 1.5 mM MgCl2, 200 µM dNTP, 0.5 µM forward primer (h*SMN2* forward 1 or h*SMN2* forward 2 or h*SMN2* forward 3), 0.5 µM reverse primer (h*SMN2* reverse), 1U Taq DNA Polymerase and 8.5 µL dwater. PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: Initial denaturation: 94 °C 2 min, 94 °C 30s, 67 °C 30s, 72 °C 90 sec (30x), Final extension: 72 °C 5 min.

Cell Culture Studies

CHO cells were cultured in T25 flask containing High Glucose with L-Glutamine (500 mL, PAN-Biotech, Germany), 10% Fetal Bovine Serum, heat inactivated (500 mL, Wisent Inc, Canada) and 500 µL penicillin + streptomycin and incubated at 37 °C with 5% CO_2 (Mammalian cell culture incubator, Binder CB150. Germany).

Transfection

CHO cells were plated into 24 well plates containing $0.05x10^6$ cells. After 24 hours incubation, transfection was done using TransIT®-2020 Transfection Reagent (Mirus, USA) based on company protocol. Transfection was performed by 400 ng GHR, 400 ng STAT5 and 300 ng different reporter constructs containing different promoter regions of the h*SMN2* gene. Transfected CHO cells were incubated at 37 °C with 5% CO_2 for 24 hours.

GH Treatment

After 24 hours of transfection, the medium of the transfected CHO cells was removed and the cells were washed 3 times with DMEM. The transfected cells were starved with 0.5 mL DMEM for 1 hour at 37 °C in a 5% CO_2 incubator. The transfected cells were treated with GH (Genotropin Goquick, 5.3 mg/mL); (1000 ng/mL for each well) for 24 hours at 37 °C with 5% CO_2.

Cell Culture Lysis and Luciferase Assay

Cell Culture Lysis Buffer 5X Reagent (Promega, USA) was used for the lysis of cells. Medium in 24-well plate was removed and the cells were washed 3 times with cold 1X PBS. 100 µL Luciferase Cell Culture Lysis Reagent (1X) was added to each well and shaken for 15 min. The cell lysates were transferred into sterile centrifuge tubes and luciferase activity was performed using Promega Luciferase Assay System protocol. 100 µL of luciferase substrate and 20 µL of cell lysate were added into each well of a 96-well plate and luciferase activities were measured on EnSpire Multimode Plate Reader (PerkinElmer Inc., USA).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 30.0.0.0 (IBM Inc., Armonk, NY, USA). Normality of the data was assessed using the Shapiro-Wilk tests. A one-way ANOVA was performed to compare the luciferase activity among the four experimental groups. A post-hoc test (Tukey) was conducted to identify specific group differences. A p value<0.05 was considered statistically significant.

Results

STAT5 binding sites in the promoter region of h*SMN2* gene were analyzed using the EPD tool and binding sites were located at -26, -334, -523, -750, -1631, -1686 from transcription start site. NheI and XhoI restriction sites were chosen for cloning based on pGL3 vector cloning sites and no presence in the h*SMN2* promoter. Three h*SMN2* promoter regions from transcription start site were amplified successfully by specific h*SMN2* promoter primers shown in Table 1. Amplified PCR products were analyzed by agarose gel electrophoresis and size of PCR products was 588 bp, 1036 bp and 1705 bp fragments, respectively (Figure 1).

Table 1. PCR primers used for h*SMN2* promoter amplification and for determination of insert in the pGL3 vector in this study

Name	Sequence (5'-3')
h*SMN2* reverse	TTAACTCGAGCGTCCCTTCTTAAGAGTGACGACTTC
h*SMN2* forward 1	ATTGCTAGCTAAGGATCTGCCTTCCTTCCTGC
h*SMN2* forward 2	ATTGCTAGCGGGCTGAGGCAGAATTGCTTG
h*SMN2* forward 3	ATTGCTAGCCCCGAGTTCAAGTGATTCTCCTGG
RV3 forward	CTAGCAAAATAGGCTGTCCC
GL2 reverse	CTTTATGTTTTTGGCGTCTTCCA

Oligomer Biotechnology Inc.
h*SMN2*: human survival motor neuron 2, PCR: polymerase chain reaction

The amplified h*SMN2* PCR promoter products and pGL3 vector were digested by NheI and XhoI restriction enzymes. The digested PCR products were ligated into NheI and XhoI sites located in front of luciferase gene in pGL3 vector producing three pGL3-h*SMN2* promoter1, pGL3-h*SMN2* promoter2 and pGL3-h*SMN2* promoter3 constructs. In order to determine h*SMN2* promoter insert in the transformed colonies on amp plates, colony PCR was performed successfully using pGL3 vector primers shown in Table 1 and Figure 2 shows the agarose gel electrophoresis results of colony PCR. The size of PCR products including vector sequence were 788 bp, 1236 bp and 1905 bp respectively and these were the expected sizes for h*SMN2* promoters plus part of vector.

In order to confirm specific h*SMN2* promoter inserts in positive colonies, plasmids were isolated from cultured bacteria and they were amplified by specific h*SMN2* primers and PCR-agarose gel electrophoresis analysis were performed (data not shown). Results showed that inserts in plasmids were specific h*SMN2* promoter sequences and the sizes were correct.

In order to determine the effect of hGH on h*SMN2* promoters driving luciferase gene expression, transfected CHO cells expressing pGL3 alone, pGL3-h*SMN2* promoter1, pGL3-h*SMN2* promoter2 and pGL3-h*SMN2* promoter3 were treated with hGH and then their luciferase activities were measured. Luciferase results showed that GH strongly induced luciferase reporter gene expression for all reporter constructs driven by h*SMN2* promoters compared to luciferase expression of pGL3 vector alone, with or without GH treatment. Although the pGL3-h*SMN2*

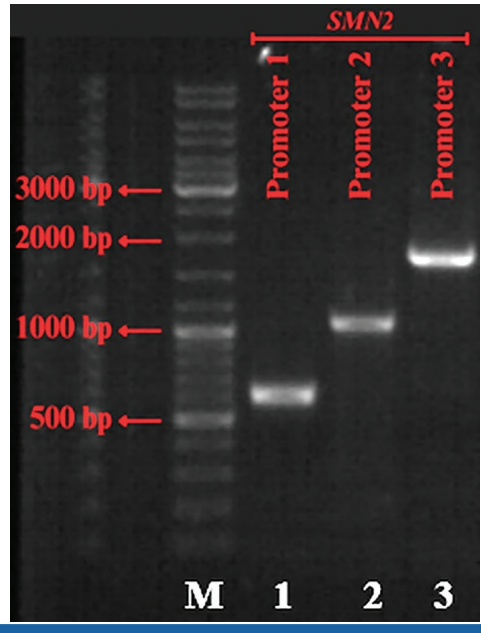

Figure 1. Agarose gel electrophoresis of purified h*SMN2* promoter PCR products. h*SMN2* promoter regions 1 and 2 were amplified by normal PCR and h*SMN2* promoter region 3 was amplified with long-range PCR. Purified h*SMN2* promoter PCR products were run on a 1% agarose gel. M shows GeneRuler DNA Ladder Mixture, line 1 shows h*SMN2* promoter 1 region corresponding to 588 bp, line 2 shows h*SMN2* promoter 2 region corresponding to 1036 bp and line 3 shows h*SMN2* promoter region 3 corresponding to 1705 bp DNA fragments, respectively
hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction

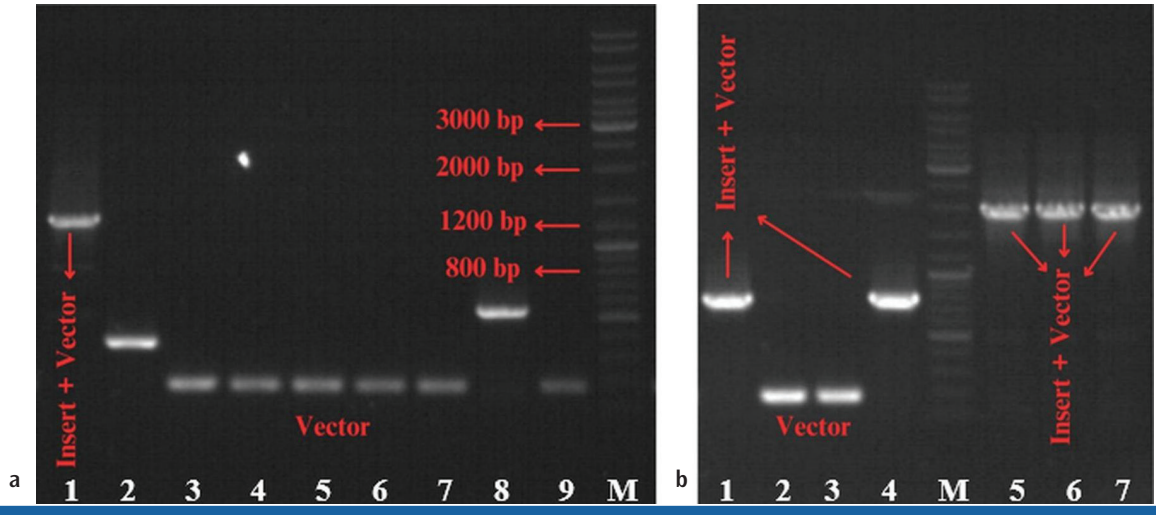

Figure 2. Agarose gel electrophoresis of colony PCR for h*SMN2* promoter regions. Transformed colonies on the bacterial plate were screened by colony PCR using pGL3 vector primers. The colony PCR products were analyzed on 1% agarose gel. **a)** Line 1 shows h*SMN2* promoter 2 region corresponding to 1236 bp (vector + insert), lines 3-7 and 9 show vector alone and line 2 and 8 show different h*SMN2* promoter insert sizes into the vector. **b)** line 1 and 4 show h*SMN2* promoter 1 region corresponding 788 bp (vector + insert), line 2-3 show 200 bp vector alone, line 5-7 show h*SMN2* promoter region 3 corresponding to 1905 bp DNA fragment (vector + insert). M shows GeneRuler DNA ladder mixture
hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction

promoter2 construct induced by hGH produced the highest level of luciferase gene expression, the pGL3-h*SMN2* promoter3 construct suppressed luciferase gene transcription, as shown in Figure 3.

Statistical analysis was performed by assessing the normality of the data using the Shapiro-Wilk test. To assess the normality of each group's data, the Shapiro-Wilk test was conducted. All groups showed p-values greater than 0.05 [pGL3 vector (+): p=0.824, pGL3-h*SMN2* Promoter 1 (+): p=0.567, pGL3-h*SMN2* Promoter 2 (+): p=0.943, pGL3-h*SMN2* Promoter 3 (+): p=0.846], indicating that the data were normally distributed. However, due to the small sample size (n=3 per group), the results of the normality tests should be interpreted with caution. A one-way ANOVA was performed to compare the luciferase activity among the four experimental groups [(1) pGL3 Vector (+), (2) pGL3-h*SMN2* Promoter 1 (+), (3) pGL3-h*SMN2* Promoter 2 (+), (4) pGL3-h*SMN2* Promoter 3 (+)]. The analysis revealed a statistically significant difference between the groups (p=0.002). Post-hoc analyses using Tukey's HSD test revealed significant differences between Group 1 and Group 2 (p=0.007), Group 1 and Group 3 (p=0.001), and Group 3 and Group 4 (p=0.030). No significant differences were found between the other group pairs. These results are presented in Table 2.

Discussion

SMA is an inherited autosomal recessive neurodegenerative disease presenting with variable phenotype and is characterized by the loss of motor neurons from the anterior horn cells of the spinal cord, resulting in progressive muscle loss and respiratory failure (7). Most cases of SMA (95%) have a homozygous deletion in the h*SMN1* gene on chromosome 5q13.

SMA is one of the most common autosomal recessive neuromuscular disorders. However, clinical heterogeneity in disease phenotype depends on h*SMN1* gene (Telomeric) and h*SMN2* (Centromeric) genes, specifically the varying copy

Table 2. Tukey post-hoc test results

Group 1	Group 2	p value
pGL3 vector (+)	pGL3-h*SMN2* promoter 1 (+)	**0.007**
pGL3 vector (+)	pGL3-h*SMN2* promoter 2 (+)	**0.001**
pGL3 vector (+)	pGL3-h*SMN2* promoter 3 (+)	0.141
pGL3-h*SMN2* promoter 1 (+)	pGL3-h*SMN2* promoter 2 (+)	0.531
pGL3-h*SMN2* promoter 1 (+)	pGL3-h*SMN2* promoter 3 (+)	0.210
pGL3-h*SMN2* promoter 2 (+)	pGL3-h*SMN2* promoter 3 (+)	**0.030**

h*SMN2*: human survival motor neuron 2

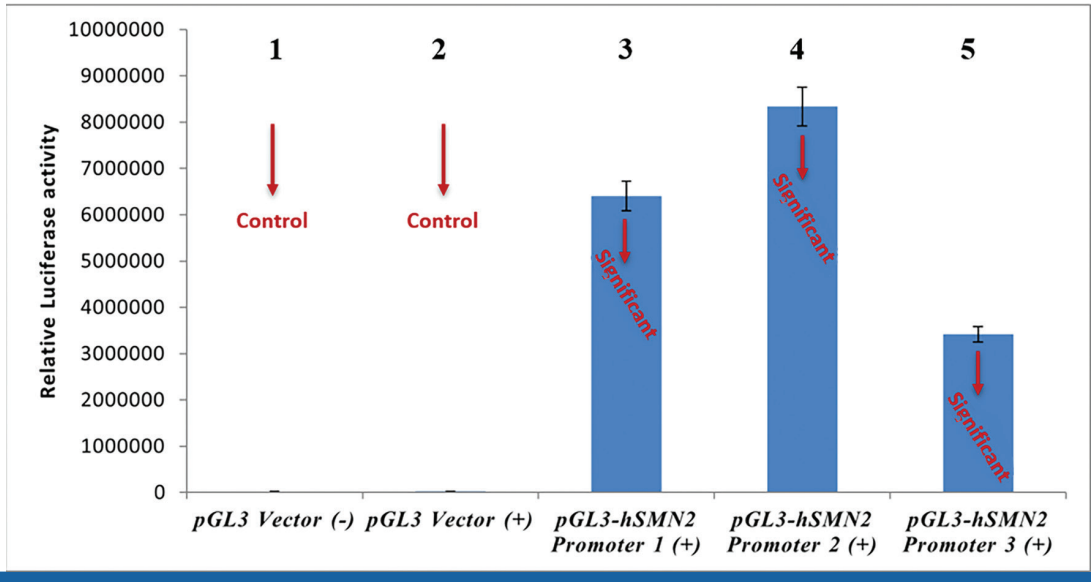

Figure 3. Relative luciferase activity of different pGL3-h*SMN2* promoter constructs in response to growth hormone stimulation (1000 ng/mL). Line 1 shows pGL3 vector alone without GH treatment, line 2 shows pGL3 vector alone with GH treatment, line 3 shows pGL3-h*SMN2* promoter 1 construct with GH treatment, line 4 shows pGL3-h*SMN2* promoter 2 construct with GH treatment, and line 5 shows pGL3- h*SMN2* promoter construct 3 with GH treatment luciferase activities respectively. Data are presented as mean±standard deviation
hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction, GH: growth hormone

Table 3. Luciferase activity results for pGL3 vector with or without GH treatment and pGL3-h*SMN2* promoter constructs with GH treatment

Promoter	Mean relative light units (RLU)	
pGL3 vector (-)	19,169	
pGL3 vector (+)	27,923	
h*SMN2* promoter 1 (+)	5,350.000	191.6 fold
h*SMN2* promoter 2 (+)	9,715.000	348 fold
h*SMN2* promoter 3 (+)	3,715.000	133 fold

Compared to the pGL3 vector (+), luciferase activity increased 191.6-fold in the pGL3-h*SMN2* promoter 1 (+), 348-fold in the pGL3-h*SMN2* promoter 2 (+) and 133-fold in the pGL3-h*SMN2* promoter 3 (+).
h*SMN2*: human survival motor neuron 2, GH: growth hormone

number of h*SMN2*. There is only one base difference between h*SMN1* and h*SMN2* genes and C residue at exon 7 in h*SMN1* is converted to T residue in *SMN2* gene (c.840C>T) disrupting the exogenic splicing enhancer (1). C->T substitution causes abnormal splicing resulting in removal of exon 7 (1). Exon 7 deleted mRNA gives truncated non-functional protein. SMA patients lacking h*SMN1* are dependent on the amount of residual h*SMN2* functional SMN protein for alpha motor neuron function. Several reports have shown that there is a strong positive correlation between SMA phenotype severity and the number of copies of h*SMN2* gene. Patients carrying high copy number of h*SMN2* show milder SMA (11).

There are several approaches to treatment of SMA, one of which increases h*SMN2* gene expression. There are numbers of studies that have been shown to increase h*SMN2* expression levels (1,5,7,13,15,16). Andreassi et al. (7) investigated the effect of 4-phenylbutyrate (PBA) treatment on h*SMN2* gene expression in fibroblast cell cultures obtained from SMA patients and PBA increased FL h*SMN2* transcript levels and SMN protein in cells from patients with all SMA types (Type I, II and III). Grzeschik et al. (1) showed the effect of hydroxyurea (HU) treatment on h*SMN2* gene expression in lymphoblastoid cell lines derived from SMA patients and HU was shown to increase the FL h*SMN2* transcript ratio in a dose and time-dependent manner. In addition to this, a significant increase in SMN protein levels and significantly increased nuclear gem (Gemini of Cajal bodies), which are SMN-containing nuclear structures were shown with treatment using HU (1). Biondi et al. (15) reported that NMDA receptor activation accelerated motor neuron maturation, reduced apoptosis and increased h*SMN2* gene expression in SMA model mice. It was reported that GH induced SMN expression in an SMA animal model (16). Previous studies have shown that GH activates the JAK/STAT pathway (15). However, the direct effect of GH on h*SMN2* promoter activity has not been previously reported in mammalian cells. Our study is first study to show that transfected cells incubated in the presence of hGH strongly increased h*SMN2* promoter driving gene expression of luciferase gene of construct in mammalian cells.

Although it has been reported that GH can regulate motor neuron function through the JAK/STAT pathway (5), the present study fills an important evidence gap by demonstrating the potential for GH to be used as a therapeutic target in the treatment of SMA.

Our results, showing a strong and specific activation of the h*SMN2* promoter by GH *in vitro*, are consistent with the findings of MacKenzie et al. (13) who reported that GH treatment increased SMN protein levels and extended survival in severe SMA mouse models. While their work demonstrated the therapeutic relevance of GH *in vivo*, our data provides mechanistic support at the transcriptional level by confirming that GH-linked signaling pathways can directly activate the h*SMN2* promoter. Together, these complementary studies strengthen the rationale for further investigation of GH or GH-related STAT5 activators as candidate therapeutic agents in SMA. However, translation from promoter-reporter assays to clinical application requires additional validation in motor neuron-derived cells, *in vivo* studies and eventually patient-based models.

Our h*SMN2* promoter studies showed that h*SMN2* promoter regions exhibited different levels of transcriptional activity in response to hGH treatment. pGL3-h*SMN2* Promoter 2 driving construct exhibited the highest luciferase activity among the h*SMN2* promoters, as shown in Figure 3. However, pGL3-h*SMN2* Promoter 3 driving reporter constructed lowered promoter activity, indicating a potentially suppressor regulatory role in promoter region. Luciferase activity of the hGH-treated pGL3-h*SMN2* promoter 1 region increased 191.6-fold, GH-treated pGL3-h*SMN2* promoter 2 region increased 348-fold and GH-treated pGL3-h*SMN2* promoter 3 region increased 133-fold compared to GH-treated plasmid alone, as shown in Table 3. The different transcriptional activities of the three h*SMN2* promoters may indicate the existence of enhancers and suppressor sequences located at the promoter regions for binding sites for transcription factors activated by GH. The 669 bp hSMN2 promoter region between residues 1036 and 1705 contains a suppressor sequence. Addition to *in vitro* studies on CHO cells, GH-induced hSMN2 expression studies should be performed *in vitro* in human motor neurons cell or human fibroblast cell

cultures obtained from patients with different type of SMA or in *in vivo* models.

Study Limitations

One of the limitations of our study was that we did not perform an electrophoretic mobility shift assay to demonstrate the binding of GH-induced STAT5 or other transcription factors to the *SMN2* promoter. Due to limited funding, we were unable to utilize radioactive labeling of the *SMN2* promoter, and we also lacked the necessary equipment to carry out this analysis. Therefore, we could not directly assess the transcription factors involved in the regulation of SMN2 expression.

Conclusion

These findings suggest that GH may be a potential therapeutic target in the treatment of SMA, but this needs to be confirmed *in vivo* in large animals. It is hoped that this study will stimulate investigation of a new therapeutic approach for SMA by demonstrating the effects of GH on h*SMN2* expressions. Our findings are among the first to identify the effects of GH on h*SMN2* promoter regions and this may provide a basis for further studies.

Ethics

Ethics Committee Approval: This research was approved by the Marmara University Faculty of Medicine Non-Drug and Non-Medical Device Research Ethics Committee (approval number: 09.2024.640, date: 10.07.2024). All human subjects' rights in this research were protected and any necessary approval was secured from the ethics committee.

Informed Consent: Informed consent was obtained from the volunteer included in the study.

Footnotes

Authorship Contributions

Concept: Ahmet Arman, Design: Ahmet Arman, Data Collection and Processing: Dilara Yücedal, Analysis or Interpretation: Ahmet Arman, Literature Research: Dilara Yücedal, Writing: Dilara Yücedal, Ahmet Arman.

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Young Turkish Adults Show a Continuing Positive Secular Change of Height but an Alarming Increase of Overweight in Males: Pilot Study for the Initiation of Updated Growth Charts

Ozge Bayrak Demirel[1], Cansu Koc[1], Nur Mine Sukur[2], Asli Derya Kardelen[1], Melek Yildiz[1], Sukran Poyrazoglu[1], Firdevs Bas[1], Jan M. Wit[3]*, Feyza Darendeliler[1]*

[1]İstanbul University, İstanbul Faculty of Medicine, Department of Pediatrics, Pediatric Endocrinology Unit, İstanbul, Türkiye
[2]İstanbul University, Child Health Institute, İstanbul, Türkiye
[3]Willem-Alexander Children's Hospital; Leiden University Medical Center, Department of Pediatrics, Division of Pediatric Endocrinology, Leiden, Netherlands
*These authors share last authorship.

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What is already known on this topic?

Current Turkish growth charts are based on data from 1989 to 2002. Positive secular trends in height are observed globally, and updating growth references every 20 years is recommended. Obesity is a global issue with rising prevalence, and monitoring body mass index (BMI) is crucial due to its long-term health implications.

What this study adds?

The present study shows that Turkish young adults' height increased by 1.8 cm over two decades, consistent with a continuing positive secular trend. The study demonstrated that females' BMI remained stable, while males showed a significant and alarming increase in BMI, with 58% classified as overweight or obese.

ABSTRACT

Objective: Turkish growth reference charts are based on 1989-2002 data. Globally, positive secular trends in height have been observed, and updating growth charts every 20 years is recommended. Additionally, obesity is a rising health issue worldwide. This study investigates if there

Corresponding Author: Ozge Bayrak Demirel, MD, İstanbul University, İstanbul Faculty of Medicine, Department of Pediatrics, Pediatric Endocrinology Unit, İstanbul, Türkiye
E-mail: ozge.bayrakdemirel@istanbul.edu.tr **ORCID:** orcid.org/0000-0001-7780-0231
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has been a further increase in young Turkish adults' mean height and body mass index (BMI) compared to previous national data (TK2002) and Turkish-origin young adults in the Netherlands. It also explores the association between adult height and BMI with socioeconomic status (SES) and geographical region.

Methods: This cross-sectional study (2023-2024) included 217 females and 248 males, aged 18-26, voluntarily recruited randomly selected from İstanbul University, representing all regions of Türkiye. Height, weight, and SES were recorded. The top two SES groups were combined for analysis.

Results: Sample distribution aligned with Türkiye's regional population distribution. Mean height was 1.8 cm taller in females ($p=0.003$) and males ($p<0.001$) compared to TK2002, and also taller (2.3 and 0.5 cm, respectively, $p<0.001$ and $p=0.03$) than in Netherlands measured in 2009 (NL2009). BMI was significantly higher in males than in TK2002 and NL2009 ($p<0.001$).

Conclusion: Final height of Turkish students increased by 1.8 cm in both sexes over two decades. Males' BMI was alarmingly high (58% overweight or obese). A population growth study to generate updated growth charts from birth to young adulthood and prevention programs to reduce obesity are needed.

Keywords: BMI, final height, growth, obesity, secular trend

Introduction

Anthropometric measurements play a central role in tracking children's growth and development. Growth references are benchmarks for assessing children's health and comparing their growth with peers. A population's height is influenced by environmental factors, particularly nutrition and socioeconomic status (SES) (1), which can result in a so-called "secular trend". Therefore, up-to-date national references for height assessment are needed (2). Body mass index (BMI) is even more strongly affected by environmental influences, as illustrated by the obesity epidemic in the last five decades (3).

The first growth references in Türkiye (1968-1970) were collected in 0-18 year-old youngsters from families with high SES living in İstanbul (4). The subsequent study (TK2002) included 6-18 year-old students attending primary and secondary schools located in six districts of İstanbul city in 1989-2002 and showed a mean height increase of 1.4 cm and 2.7 cm in 17-year-old males and females, respectively. Notably, height differences were compared using data from 17-year-olds, as the first study lacked sufficient height data for the 18-year-old group (5). Mean weight increased by 4.5 and 1.5 kg in 18-year-old males and females, respectively.

It has been recommended that growth references should be reassessed every 5-10 or 15-20 years for populations where large (≥1 cm/decade) or little secular change is expected, respectively (6). Before embarking on an expensive nation-wide growth study, we performed a pilot study in young adults to determine whether the positive secular trend of height has continued and to assess current BMI. While a positive secular change of height is generally considered a positive indicator of a population's health status, a BMI increase is associated with negative consequences of overweight and obesity for individuals and society (3). Young adulthood can be considered a suitable life stage to assess final height and BMI, as it reflects the cumulative outcomes of childhood growth and nutrition.

The primary aim of this study was to test our hypothesis that mean height and BMI of young Turkish adults is higher compared with previous national data (TK2002). The secondary aim was to compare current height and BMI of young Turkish adults with those of young adults of Turkish origin living in the Netherlands measured in 2009 (NL2009) (7,8,9). Thirdly, we aimed to explore the association between final height and BMI with SES and geographical region.

Methods

Study Setting

A prior power analysis (95% power) indicated that at least 122 subjects of each sex were needed to detect a 1.8 cm height increase ($p=0.05$) vs TK2002. Sample size calculation was performed using G-Power 3.1.3 (Faul, University of Kiel, Kiel, Germany). From 65,000 students at İstanbul University, originating from every region of Türkiye in 2023-2024, we recruited a sample of 529 volunteer students. Inclusion criteria for the study were: (a) age between 18 and 26 years; (b) being a student at İstanbul University; and (c) volunteering to participate in the study. Exclusion criteria were: (a) diagnosis of a chronic disease; (b) history of surgery that can affect linear growth; (c) diagnosis of growth retardation; (d) history of growth hormone administration; (e) having a parent born outside of Türkiye; (f) birth length or weight less than -2 standard deviation score (SDS); and (g) current height or BMI less than -3 SDS or greater than +3 SDS (Figure 1). The students' medical history, birthplace, high school graduation province, parental education level and occupation and household income were recorded through a questionnaire.

Measurements

Height was measured with bare feet using a portable stadiometer (SECA™ 213, Hamburg, Germany) and documented to the nearest 0.1 cm. Height measurements were repeated twice, and

the mean value was calculated. If the difference exceeded 0.3 cm, a third measurement was taken, and the average of the two closest values was used. Individuals were weighed wearing light clothing using a digital scale (SECA™ 813, Hamburg, Germany), recorded to the closest 0.1 kg. BMI was calculated as kg/m^2. Two trained technicians performed all measurements.

Classification of SES

After collecting all data, the participants were classified into four groups according to SES, based on educational level of both parents and father's occupation. The SES classification ranged from SES 1 (highest scoioeconomic group) to SES 4 (lowest). For example, SES 1 included individuals with university-educated parents and professional occupations, while SES 4 included those with limited education and unskilled jobs (Supplementary Material 1) (7,8,9). Compared with the TK2002 questionnaire, the educational level of parents was increased by one level because of the 26% average increase in the duration of education in Türkiye between 2008 and 2022 (10). Since height and BMI were not different between the top two SES groups in previous (4,5) nor in the current study (all p>0.2), students from both classes (151 females and 157 males) were combined for this analysis.

Geographical Subgroup Analysis

Geographical subgroups were defined in two different ways: based on the participant's birthplace and based on the region where they completed high school. Each participant was categorized into one of the seven official geographical regions of Türkiye (1: Marmara, 2: Aegean, 3: Mediterranean, 4: Black Sea, 5: Central Anatolia, 6: Eastern Anatolia, 7: Southeastern Anatolia). Analyses were conducted separately for both definitions, and corresponding results were reported in tables and Supplementary Materials 2 and 3.

Comparative Analyses

Height and BMI of the present study population were compared with those of the TK2002 study, in which data for 18-year-olds were collected from measurements of senior high school students in six affluent districts of İstanbul. The technical specifications and measurement methods of the instruments used in this study are identical to those used in TK2002, ensuring comparability and consistency across both studies. Final height was compared with 21-year-olds of Turkish origin living in the NL2009, while BMI was compared with 18-year-olds from the same cohort (7,8,9).

Statistical Analysis

Statistical analyses were performed using SPSS, version 29.0 (IBM Corp., Armonk, NY, USA). Normality was assessed using the Shapiro-Wilk test. Descriptive data are presented as frequencies, percentages, means ± SDs, or ranges. Normally distributed data were compared using Student's t-tests, while non-normally distributed data were analysed with the Mann-Whitney U test. To investigate the association between the geographical regions and height and BMI, a Multivariate Analysis of Variance (MANOVA) was performed. Multiple group comparisons were conducted using one-way ANOVA. For significant ANOVA results, Levene's test for homogeneity of variances and Tukey's honestly significant difference test for post-hoc comparisons were used. Age, SES, and geographical region were used to conduct subgroup analyses. Age groups were divided into 18-22 and 23-26 years.

Results

The study included 465 young adults (217 females, 248 males) (1). Table 1 shows the results of anthropometric measurements for SES groups. In females, mean height tended to decrease with lower SES (p=0.4) but no such pattern was noted for weight and BMI. In males, mean height of the SES 4 group was lower (by 4 cm) than that of the other three SES groups (p=0.002), weight and BMI tended to decrease with lower SES. Data obtained from the SES 1 and 2 groups were used for further analyses.

Supplementary Materials 3 and 4 show the percentages of the study sample for region of birth and of high school completion, respectively, in comparison with Türkiye's overall population distribution (11). In general, the regional distribution of our sample is consistent with that of the Turkish population, although percentages of individuals born in the Marmara and Mediterranean regions are slightly over-represented and Aegean and Central Anatolia regions slightly under-represented.

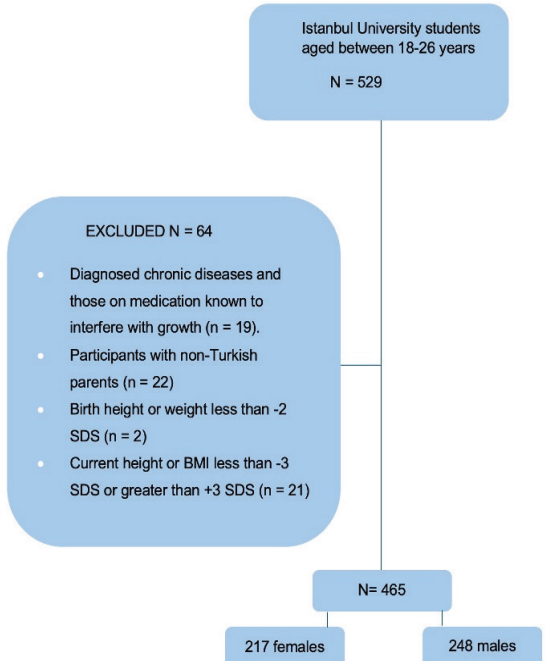

Figure 1. Flow chart of the study participants
SDS: standard deviation scores, BMI: body mass index

Table 2 presents mean height, weight and BMI in the high SES group by region. In females, birthplace was not associated with height and BMI [MANOVA, Wilks' Lambda, F(12, 298)=1.572, p=0.099], in contrast to the region of high school completion [Wilks' Lambda, F(12, 288)=2.120, p=0.016]. Further analysis revealed an effect of the region of high school completion on height [F(6, 150)=2.91, p=0.005] but not on BMI [F(6, 150)=0.97, p=0.412]. Post-hoc analysis showed differences in height between Marmara and Central Anatolia regions (p=0.011) and between Central Anatolia and Eastern Anatolia (p=0.04). In males, results indicated no association between birthplace or of high school completion and height and BMI [Wilks' lambda, F(12, 298)=1.572, p=0.099 and Wilks' Lambda, F(12, 298)=0.933, p=0.514, respectively].

Supplementary Material 4 shows mean ± SD of anthropometric measurements in the high SES group according to two age groups (18-22 years versus 19-26 years). No notable differences were observed, but BMI in males showed a trend of increasing with age (p=0.12).

Table 3 and Figures 2a and 2b show 3-10-25-50-75-90-97th percentiles of TK2024, TK2002 and NL2009 for height in both sexes. Mean height was 1.8 cm taller in females (p=0.003) and males (p<0.001) compared to TK2002.

Table 1. Anthropometric measurement results according to SES groups

	SES1	SES2	SES3	SES4	SES1&2	All
Females (n, %)	49 (23%)	102 (47%)	42 (19%)	24 (11%)	151 (69%)	217
Age (years)	22±1.5	21.8±1.6	22.1±1.4	21.2±1.5	21.8±1.6	21.8±1.5
Height (cm)	164.9±5.9	164.3±5.5	164.1±6.5	163.3 ± 6.7	164.5±5.6	164.3±5.9
Weight (kg)	59±9.7	60.1±8.4	60±9.4	60±7.4	59.7±8.8	59.8±8.7
BMI (kg/m^2)	21.6±2.8	22.2±2.8	22.4±3.2	22.5±2.4	22.1±2.8	22.1±2.8
Males (n, %)	54 (22%)	103 (41%)	62 (25%)	29 (12%)	157 (63%)	**248**
Age (years)	21.8±1.9	21.5±1.9	21.6±1.7	21.1±1.7	21.6±1.9	21.5±1.8
Height (cm)	178.3±6.2	178.8±6.3	178.2±7	174.6±6	178.6±6.3	178.1±6.5
Weight (kg)	83.5±12.3	81.5±12.4	79.8±11.8	77.8±15.5	82.2±12.3	81.1±12.6
BMI (kg/m^2)	26.2±3.3	25.5±3.5	25.1±3.3	25.5±4.8	25.7±3.4	25.6±3.6

BMI: body mass index, SES: socioeconomic status

Table 2. Anthropometric measurements of participants by regions (high SES)*

Females					Males			
n	**Height (cm)**	**Weight (kg)**	**BMI (kg/m^2)**	**Region**	**n**	**Height (cm)**	**Weight (kg)**	**BMI (kg/m^2)**
52 (66)	165.9±6 (165.9±5.7)	60.6±9.7 (61.2±10.2)	22±2.9 (22.2±3)	1	66 (72)	178.9±6.5 (178.7±6.3)	83.7±11.8 (84.1±11.9)	26.2±3.3 (26.3±3.2)
14 (12)	164.2±6.1 (164.6±5.4)	58.6±9.2 (57.2±7.3)	21.6±2.6 (21.1±2.4)	2	5 (5)	179.4±5.3 (181±3)	70.7±9 (74.3±13.4)	22±3.1 (22.7±22.7)
22 (24)	164.2±4.4 (163.9±5.2)	59.2±6.7 (61.3±6.9)	21.9±2.2 (22.8±2.7)	3	31 (33)	179.9±5.5 (179±6)	81.5±12 (81.6±14.4)	25.2±3.6 (25.4±4.3)
17 (16)	164.9±6.4 (163.7±6.2)	62.9±11.4 (60.3±9)	23±3.4 (22.4±2.9)	4	16 (13)	175.7±6.5 (176.3±6.9)	78.6±14 (78.8±11.8)	25.3±3.4 (25.3±3.1)
16 (11)	161.1±4.3 (160±4.3)	56.2±6.8 (55.9±7)	21.7±2.5 (21.9±2.4)	5	12 (8)	179.6±5 (178.9±5.1)	87.1±10.6 (84±7.9)	26.9±2.6 (26.2±2.5)
14 (8)	166.8±4.2 (167.4±2.9)	61.5±8.1 (58.7±9)	22.2±3.5 (21±3.6)	6	10 (11)	177.2±6 (178.8±6.9)	76.4±12.1 (77.7±10.3)	24.3±3.3 (24.3±2.9)
16 (14)	161.5±4.6 (161.7±4.8)	59.7±6.3. (55.4±4.1)	22±2.6 (22.1±1.7)	7	17 (15)	178±7.2 (178.4±7.5)	84.5±13.2 (82.7±12.1)	26.6±3.6 (25.7±2.6)

*Data are presented as mean ± standard deviation. The first values are the results of the groups categorized by birthplace, and the values in brackets are the results of the groups formed according to the region of high school completion. Regions: 1: Marmara, 2: Aegean, 3: Mediterranean, 4: Black Sea, 5: Central Anatolia, 6: Eastern Anatolia, 7: Southeastern Anatolia.
SES: socioeconomic status, BMI: body mass index

Table 3. Height data of young adults in TK2024 compared with TK2002 and NL2009 (5,8,9)								
Percentiles		**3**	**10**	**25**	**50**	**75**	**90**	**97**
Females (cm)	**TK2024**	152.6	157.5	160.0	164.9	168.0	171.9	174.2
	TK2002	152.0	155.6	159.1	163.1	167.1	170.7	174.2
	NL2009	151.3	154.9	158.6	162.6	166.6	170.3	173.9
Males (cm)	**TK2024**	166.7	170.9	174.4	178.0	182.3	187.5	192.7
	TK2002	164.5	168.2	172.0	176.2	180.4	184.2	187.9
	NL2009	164.7	168.8	172.9	177.5	182.1	186.2	190.3

Table 4. BMI data of young adults in TK2024 compared with TK2002 and NL2009 (7,13)								
Percentiles		**5**	**15**	**25**	**50**	**75**	**85**	**95**
Females	**TK2024**	18.4	19.2	19.7	21.6	23.8	25.1	27.6
	TK2002	19.0	19.9	20.5	21.8	23.3	24.3	26.1
	NL2009	17.9	19.6	20.7	23.1	26.0	27.9	31.5
Males	**TK2024**	20.3	22.7	23.7	25.5	27.7	29.3	31.4
	TK2002	19.2	20.5	21.3	23.1	25.2	26.6	29.4
	NL2009	18.2	19.7	20.7	22.9	25.5	28.9	30.6
BMI: body mass index								

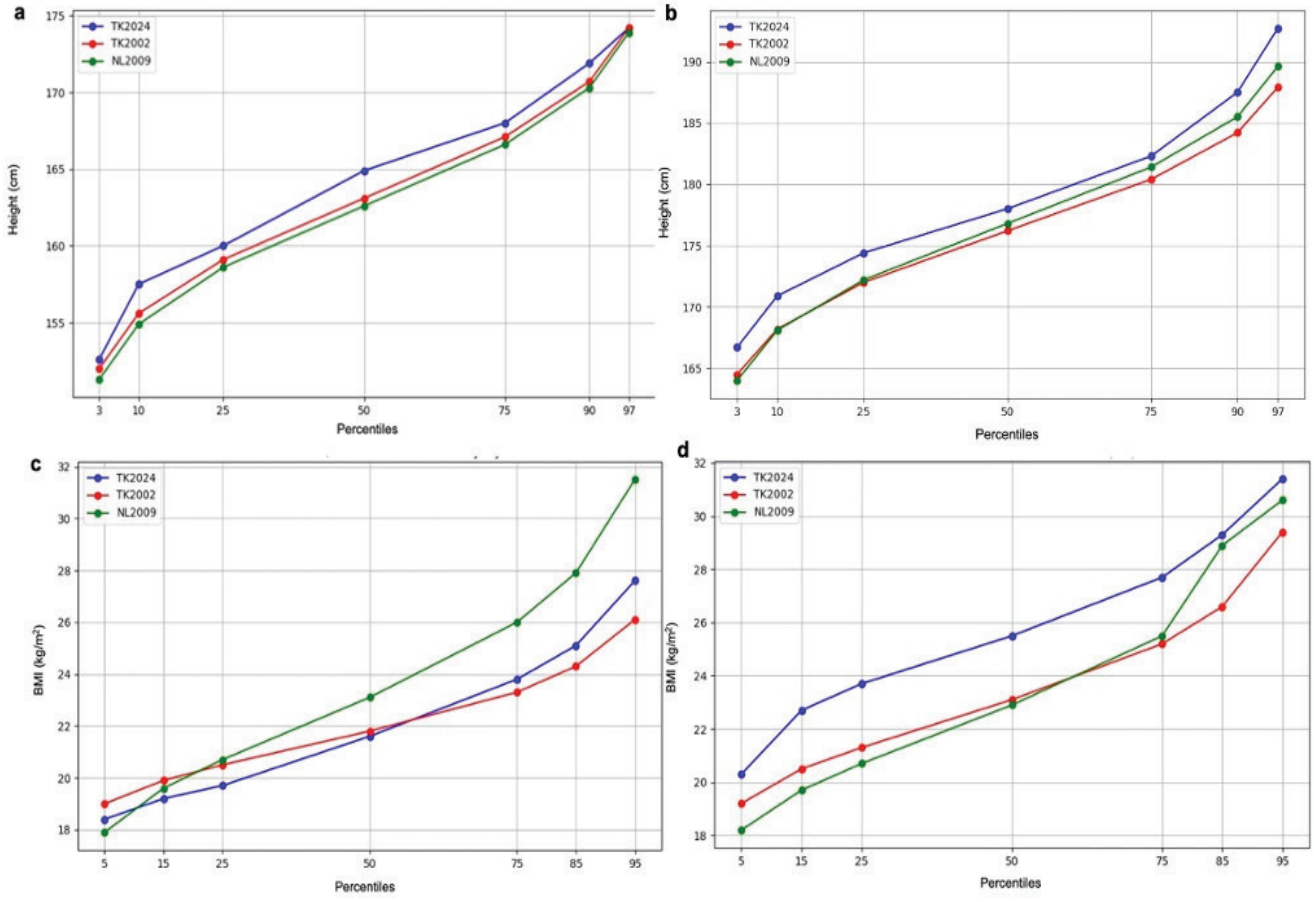

Figure 2. Height data of females (a) and males (b) and BMI data of females (c) and males (d) in TK2024 compared with TK2002 and NL2009
BMI: body mass index

Table 4 and Figures 2c and 2d show these percentiles for BMI versus earlier growth studies. In females, BMI was similar to TK2002 and lower than NL2009 ($p<0.001$). BMI was higher in males than in TK2002 and NL2009 ($p<0.001$).

Discussion

Our study shows a notable increase in mean height of young adult males and females by 1.8 cm over two decades, showing a continuing positive secular trend in both sexes. Compared to previous national growth studies, secular growth change continues at a similar rate (4,5). Notably, males show a concerning increase in BMI, with 58% classified as overweight or obese, while females' BMI has remained stable. Compared to the 2003 Turkey Demographic Health Survey (TDHS), the weight-for-height ratio of 0-5 year-olds increased in the 2018 TDHS, suggestive of an increasing BMI in childhood (18,19). Regional variations in height were present among females but not males. We speculate that the ongoing secular trend of height is associated with improved living and nutritional conditions, even in the best-off segments of the population, and improved child health and immunization programs.

İstanbul (population>15 millions) has the highest number of migrants from all regions of Türkiye, predominantly young individuals aged 20 to 24 moving for educational purposes (12). Previous national growth reference studies used data from children in affluent neighbourhoods of İstanbul (4,5,13). İstanbul University, with a student body of 63,790 from across Türkiye, accurately reflects the country's demographic composition (14). The alignment between our sample distribution and Türkiye's regional demographics ensures the generalizability of our findings. The only regional height variation was observed among females when classified by the region where they completed high school (shorter height in Central Anatolia compared with Marmara and Eastern Anatolia), consistent with a 2007-2008 study in Kayseri, Central Anatolia (15).

The main factors that influence the assessment of secular change of final height are the SES, geography and age of the male study sample. While SES and geography of TK2002 and TK2024 were comparable, the age of measurement of males was 18 and 18-26 years, respectively. Since in the NL2009 study mean male height increased by 0.5 cm between 18 and 21 years (9), we cannot exclude that the unbiased secular trend in males is 1.3 cm. Current mean height of Turkish young adult males is also taller than that of offspring of Turkish immigrants living in the Netherlands 15 years earlier (NL2009) (8), probably mainly associated with the time interval.

In developing countries, it is advised to exclude malnourished children of low socioeconomic backgrounds from population growth studies, as they may not accurately represent the overall population's growth potential. We therefore included students with higher SES in the study, in line with the previous national growth references (TK2002) and WHO growth standards (5,21). This "prescriptive" or "normative" approach aims to create references that can be used to identify inappropriate growth rather than to define the current population averages (6). In the present study, the mean height of males in the lowest SES group was lower than that of the other SES groups. In addition, a trend of a height decrease from high to low SES was observed in females. We realize that this approach has disadvantages, for example that it obscures disparities within the country, overestimates the "real" mean final height and secular trend (if mean height in the highest SES cohort increases more than in lower SES groups) or underestimates it if the opposite phenomenon would occur. Furthermore, it causes a bias if the height of the Turkish population is compared with that of other countries.

A very concerning result of our study is the increase in male BMI (58% overweight or obese). Male BMI had increased compared to TK2002 and the NL2009 data (7,13), indicating that the Turkish population is at risk of a severe obesity epidemic that starts at least in young adulthood, but likely even before this. Factors such as sedentary lifestyles and unhealthy dietary patterns may contribute to this increase. The two TDHSs (2013 and 2018) in 0-5 year-olds (19,22) reported overweight prevalences of 11 and 8%, respectively, while the prevalence peaked at 6-36 months and 12-17 months of age, respectively. For future BMI reference charts, it is important to present normative data derived from growth studies performed before the overweight epidemic, as done in the current USA, WHO and Dutch charts (16,23,24,25,26).

Interestingly, while males showed a marked increase in BMI, the BMI of females remained relatively stable over the same period. This sex-specific difference may be influenced by societal pressure regarding body image and stronger motivation for weight control among females, as reported in previous studies (27). Moreover, females often show healthier dietary preferences than males (28). These behavioral differences might partly explain the stable BMI trend observed in females in our study.

When the sample was divided into age groups (18-22 years and 23-26 years), no differences were found in height. This suggests that the chosen age range of young adults for assessing final height was appropriate and did not introduce bias related to age within the specified range. BMI in males showed a non-significant age-related increase ($p=0.12$); however, this finding should be interpreted cautiously and does not indicate a strong trend.

Our study has notable strengths, such as using a clearly defined sample from İstanbul University, which includes participants from all regions of Türkiye. Thus, we believe that our findings can be generalized to the Turkish population. Moreover, using standardized anthropometric measurements and detailed statistical analyses ensures the reliability and accuracy of the data. Furthermore, the SES questionnaire used in this study was based on scales from previous national studies, ensuring comparability and relevance for the analysis. This methodological consistency allows for reliable comparisons to previous research, strengthening the validity of our findings. The study's design meets the criteria recommended by Waterlow and recognized by the World Health Organization. These criteria necessitate that the reference population be adequately nourished, the sampling procedure be clearly defined and reproducible, the sample size be sufficient, the measurements be relevant and high quality, and the data be appropriately processed (21,29).

Study Limitations

A potential limitation of our study is that the sample consists only of university students. Although we acknowledge that this does not fully represent all young adults in Türkiye, the large and diverse student body of İstanbul University from all regions of Türkiye significantly reduces this concern. Furthermore, the current national growth curves are based on measurements taken from students in schools in well-off İstanbul districts with high SES (13). Since the sample that best reflects the growth potential of the society should be selected when constructing growth references, we believe that the study sample was appropriate for our purpose and that the results can be generalized to the Turkish population.

Another potential limitation is the age range of 18-26 years in our study. The previous national study (TK2002) with which we compared our results includes data only up to 18 years old. Despite these limitations, the study provides valuable insights into the anthropometric measurements of Turkish young adults.

Conclusion

Our results have demonstrated that the average height of Turkish young adults has increased by 1.8 cm over the past two decades. Therefore, we suggest that this increase be considered when interpreting target height, to reflect current trends in during growth evaluation. Future research is needed to assess the age period in which this height increase has mainly occurred. Furthermore, while the BMI of young female adults did not change, males have reached alarmingly high BMI levels, with 58% being classified as overweight or obese, highlighting a rising public health issue. This indicates the importance of enhancing prevention programs to promote healthier habits and lower obesity rates, especially among boys.

Ethics

Ethics Committee Approval: This research was approved by İstanbul University, İstanbul Faculty of Medicine Clinical Research Ethics Committee (approval no.: 2018362, date: 10.08.2023).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Footnotes

Authorship Contributions

Concept: Ozge Bayrak Demirel, Asli Derya Kardelen, Melek Yildiz, Firdevs Bas, Jan M. Wit, Feyza Darendeliler, Design: Ozge Bayrak Demirel, Asli Derya Kardelen, Sukran Poyrazoglu, Firdevs Bas, Jan M. Wit, Feyza Darendeliler, Data Collection or Processing: Ozge Bayrak Demirel, Cansu Koc, Nur Mine Sukur, Melek Yildiz, Sukran Poyrazoglu, Firdevs Bas, Jan M. Wit, Analysis or Interpretation: Ozge Bayrak Demirel, Cansu Koc, Firdevs Bas, Jan M. Wit, Feyza Darendeliler, Literature Search: Ozge Bayrak Demirel, Jan M. Wit, Feyza Darendeliler, Writing: Ozge Bayrak Demirel, Sukran Poyrazoglu, Firdevs Bas, Jan M. Wit.

Conflict of Interest: One author of this article, Feyza Darendeliler, is a member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, she did not involved in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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Associations Between Dietary Diversity Score and Adiposity Indexes in Obese Adolescents

Rukiye Bozbulut, Mehmet Ali Oktay, Ulaş Akçay, Esra Döğer, Aylin Kılınç Uğurlu, Mahmut Orhun Çamurdan, Aysun Bideci

Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye

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What is already known on this topic?

Visceral adipose tissue is considered an independent risk factor for cardiometabolic risk. Diet and lifestyle changes will affect visceral adipose tissue. However, the relationship between dietary diversity and adiposity-related biomarkers used to determine visceral adiposity and predict cardiometabolic risk is unknown.

What this study adds?

High dietary diversity was associated with lower insulin resistance and lower visceral adiposity, triglyceride/glucose, and lipid accumulation product indices, all of which predict cardiometabolic risk.

ABSTRACT

Objective: Nutrition may affect visceral adipose tissue, but the effect of dietary diversity on visceral adiposity is unknown. Our aim was to investigate the relationship between dietary diversity and visceral adiposity indices and biochemical parameters in obese adolescent.

Methods: Subjects were obese adolescents. Participants' biochemical parameters, anthropometric measurements, and blood pressures were measured. Two days of retrospective food intake records were collected, and dietary diversity scores (DDS) were calculated and divided into tertiles. A DDS score of <4.09 was classified as tertile 1 (low); 4.09-4.96 as tertile 2 (medium); and >4.96 as tertile 3 (high). Visceral adiposity, triglyceride/glucose, lipid accumulation product, and body shape indexes were calculated according to previously published formulas.

Results: The study included 141 obese adolescents (70 males, 49.6%) aged between 12 and 18 years. Insulin and Homeostasis Model assessment for Insulin Resistance (HOMA-IR) values were higher in individuals in Tertile 1 compared to those in other tertiles (p<0.001). The triglyceride/glucose index was lower in individuals in Tertile 3 compared to those in Tertile 1 (p=0.028). In individuals in Tertile 3, fibre (p=0.002), vegetable

Corresponding Author: Rukiye Bozbulut, PhD, Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye
E-mail: dyt_rukiye@hotmail.com **ORCID:** orcid.org/0000-0003-4317-9226
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(p<0.001), and whole grain (p<0.001) intake were higher than in other tertiles, while refined grain (p<0.001) and meat consumption (p=0.013) were lower than in other tertiles. A negative correlation was found between the DDS and fasting blood glucose (rho=-0.177; p=0.036), insulin (rho=-0.633; p<0.001), triglycerides (rho=-0.223; p=0.008), HOMA-IR (rho=-0.656; p<0.001), visceral adiposity index (rho=-0.228; p=0.007), triglyceride/glucose index (rho=-0.251; p=0.003), and lipid accumulation product index (rho=-0.200; p=0.018). When confounding factors were controlled for, fasting blood glucose emerged as a significant factor affecting DDS.

Conclusion: High DDS in obese adolescents are associated with lower visceral adiposity, and lower triglyceride/glucose and lipid accumulation product indexes, indices associated with visceral obesity. As DDSs increased, fasting blood sugar, insulin, triglyceride, and HOMA-IR levels decreased.

Keywords: Dietary diversity, visceral adiposity, triglyceride/glucose index, lipid accumulation product, body shape indices

Introduction

Obesity has emerged as a global health concern because chronic obesity will affect cell metabolism and is associated with increased adipose tissue deposition, thereby increasing the risk of metabolic syndrome, which in turn is known to increase the risk of cardiovascular disease, hypertension, and type 2 diabetes (1). The increase in the prevalence of obesity among children and adolescents is a risk factor for chronic diseases in adulthood and affects future morbidity/mortality. Therefore, identifying risk factors for obesity in adolescents is important as it will facilitate intervention to prevent the development of persistent obesity and overweight which will reduce the risk of cardiovascular disease and other metabolic conditions (2).

In recent years, the location and distribution of fat in the body has been shown to be potentially more important than the total amount of fat in terms of metabolic risk. In general, visceral obesity is reported to play a central role in the development of chronic disease compared to regional or general obesity (3). Visceral adipose tissue is a hormonally active component of the body's fat mass, stored in the abdominal cavity, near the digestive organs (4). It is considered an independent risk factor for metabolic syndrome due to its role in regulating glucose, lipid metabolism, and blood pressure (5). It has been found to be associated with cardio-metabolic pathologies, and the amount and activity of visceral fat is a clinically useful biomarker when determining risk for these diseases (6). To date, body composition variables used as predictors of metabolic syndrome include body mass index (BMI), waist circumference (WC), or waist-to-hip ratio. In addition, visceral fat tissue can be measured using costly and less practical methods, such as bioelectrical impedance analysis, dual-energy X-ray absorptiometry, computed tomography, and magnetic resonance imaging (7,8). For example, BMI is a widely used auxological parameter but it cannot distinguish between muscle mass and body fat mass, an increase in muscle mass may be diagnosed as excess weight and mis-classified as obesity. Therefore, additional anthropometric indicators are needed to assess abdominal visceral obesity (9). In the past few years, some lipid and visceral obesity-related indices, such as visceral adiposity index, triglyceride/glucose index, lipid accumulation product (LAP) index, atherogenic index of plasma, cardiometabolic index, and body roundness index, have also been proposed as supplementary indices to to estimate the presence of obesity and the distribution pattern of adipose tissue, especially visceral adiposity (10). Visceral adiposity index, triglyceride/glucose index, LAP index, and body shape index have been shown to be better predictors of insulin resistance and metabolic syndrome risk than traditional indices in pediatric population (11). These indices are mathematical models calculated using anthropometric and biochemical data. They are used to indicate visceral adiposity, adiposity dysfunction, the homeostasis model assessment for insulin resistance (HOMA-IR), metabolic dysfunction, and cardiometabolic risk. Thus, they can help predict significant health risks with a simple formulation and facilitate early intervention (11).

Current evidence shows that lifestyle changes and diet will affect deposition of visceral adipose tissue (4). When examining the effects of diet on health, the importance of dietary diversity has been highlighted. Dietary diversity ensures a more balanced intake of nutrients and other non-nutrient components into the body (12). Studies have found a negative correlation between greater dietary diversity and the incidence of cardiovascular disease, cancer, metabolic syndrome, and osteoporosis (13,14,15). A study found that increased consumption of plant-based diets was associated with better anthropometric measurements, increased high-density lipoprotein (HDL) cholesterol levels, and reduced LAPs (3). Another study suggested that increased dietary protein intake and animal-derived monounsaturated fatty acids may be positively associated with changes in visceral fat dysfunction and visceral adiposity index (16). In contrast, another study found no effect of Western-style, healthy, and combined diets on triglyceride/glucose index and visceral fat levels (4). However, the relationship between dietary diversity and adiposity-related biomarkers is unknown. Therefore, the aim of the present study was to investigate the effects of dietary diversity on visceral adiposity, triglyceride/glucose, LAPs, and body shape indexes, which are used to determine visceral adiposity and predict cardiometabolic risk, in obese adolescents.

Methods

Study Design, Setting, and Participants

This study included obese adolescents aged 12-18 years who attended the Clinic of Pediatric Endocrinology Outpatient at Gazi University Faculty of Medicine between February 2025 and May 2025. The inclusion criteria were obese adolescents, defined as a BMI ≥95th percentile, who had no previously diagnosed chronic condition, did not take hormone therapy, and did not use medication. Exclusion criteria included having any concomitant chronic medical condition (syndromic, metabolic, or neurological), except for metabolic syndrome secondary to obesity, or not having clinically normal mental faculty.

Clear explanations were provided about the purpose of the study, after which written informed consent was obtained from the adolescents in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Gazi University Ethics Committee (approval number: 2025-164, date: 05.02.2025).

Data Collection and Evaluation

Data was collected in face-to-face interviews through a questionnaire that included adolescent socio-demographics, dietary habits, anthropometric measurements, body composition analyses, biochemical findings, dietary diversity score (DDS) table, and two-day food consumption records (4,6).

Anthropometric Measurements and Body Composition Analysis

Body weight measurement and body composition analysis [fat mass, percentage of fat, fat-free mass (FFM)] were conducted with the InBody 720 (1-1000 kHz) a combination scale and body composition analyzer (InBody Co., Korea). Height was measured (cm) with feet close together and the head in Frankfort plane with a portable stadiometer with a 0.1 cm accuracy. BMI was calculated as weight (kg)/height (m^2). BMI-standard deviation score (SDS) and body weight-SDS were calculated according to the standards established for Turkish children (17). WC (cm) was measured from the midpoint between the lowest rib and the iliac crest.

Biochemical Parameters and Blood Pressure

The levels of fasting blood glucose, fasting insulin, total cholesterol, low density lipoprotein-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglyceride, and liver transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] of the participants, which are routinely analyzed at Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, were recorded. Venous blood samples were obtained from all patients from the antecubital region between 8.00 and 8.30 am after an 8-12 hour overnight fast. Fasting glucose was measured with the enzymatic ultraviolet (UV) (hexokinase) method using an AU5800 autoanalyzer (Beckman Coulter Inc., Brea, CA, USA). HDL-C, LDL-C, total cholesterol, and triglyceride levels were also measured on the AU5800 using enzymatic colorimetric methods. Insulin levels were measured with a one-step principle enzymatic immunoassay method using a Beckman UniCel DxI 800 (Beckman Coulter Inc., Brea, CA, USA). Serum AST and ALT levels were measured by kinetic UV method on the AU5800 (Beckman Coulter Inc., Brea, CA,USA). Blood pressure measurements of the adolescent were taken by the researchers in accordance with the standard measurement protocol (18). HOMA-IR value was calculated using the standard fasting blood glucose (mg/dL) x fasting insulin (µU/mL)/405 formula (19). Unit changes have been made in the parameters in accordance with the formulas.

Cardio-Metabolic Risk Markers

Cardio-metabolic risk markers were calculated as:

Visceral adiposity index=in girls [WC/((36.58)+(1.89 x BMI))] x (TG/0.81) x (1.52/HDL-C),

in boys=[WC/((39.68+(1.88 x BMI))] x (TG/1.03) x (1.31/HDL-C) (20)

Triglyceride/glucose index=ln [fasting triglyceride (mg/dL) x fasting blood glucose (mg/dL)/2] (21)

LAP index=(WC-58) x TG in girls, (WC-65) x TG in boys (22)

Body shape index=WC/($BMI^{⅔}$ x $height^{½}$) (23).

Dietary Intake and Calculation of DDS

DDS is most often determined by counting the number of selected food groups consumed by individuals over a reference period, which usually ranges between 1-7 days (24). Two-day food consumption records were obtained from the participants. Adolescents were taught by the dietitian on how to keep food consumption records. The Food and Nutrient Photo Catalogue was used to ensure that patients correctly specified the amount of food they consumed. The food diversity score table was filled in by the researcher according to retrospective food consumption records, using data from one weekday and one day from a weekend. The DDS score was calculated according to the completed food diversity table.

The food diversity score table consists of five main food groups: grains, vegetables, fruits, meat, and dairy products. Under these five groups, 23 food sub-groups were evaluated in terms of score. These subgroups were:

1. Grains group with seven subgroups: white bread, biscuits, pasta, whole grains, cereals, rice, refined grains,

2. Fruits were divided into 2 subgroups: berries and citrus, and other fruits and juices.

3. Vegetables group with seven subgroups: potatoes, tomatoes, other starchy vegetables (sweetcorn, pea, eggplant, squash),

legumes (pease, beans, - lentils), yellow vegetables (carrots and pumpkin), and green vegetables (bell peppers, all kinds of cabbage, broccoli, celery, cucumbers, garlic, onion, green beans, zucchini, leeks, parsley, lettuce, radish, spinach, turnips).

4. The meat group was divided into four subgroups: red meat, poultry, fish, and eggs.

5. The dairy group was divided into three subgroups: milk (low-fat and full-fat), yogurt (low-fat and full-fat), and cheese.

Fats and sugars were excluded from the DDS calculation.

To be considered as a consumer of a food group, it is necessary that at least a half-serving of that food group should be consumed per day by individuals. Each of the five main groups is evaluated at two scores. If all food groups-are consumed, the DDS is 10 points. This two scores was divided among the subgroups. For example; There are 7 subgroups in the grain group. Someone who consumes 5 of the grain groups; (2/7)×5=1.43 points. The points obtained from five groups are summed, and the total DDS score is obtained (2).

Statistical Analysis

Data were analyzed using SPSS, version29.0 (IBM Corp, Armonk, NY, USA). Compliance with normal distribution was examined using Shapiro-Wilk and Kolmogorov-Smirnov tests. Mean ± standard deviation and median [interquartile range (IQR) Q1-Q3] values were used in descriptive statistics for continuous variables. Independent two-sample t-test was used to compare normally distributed data according to paired groups, and Mann-Whitney U test was used to compare non-normally distributed data. To create balanced groups according to DDS, dietary diversity status was divided into tertiles based on 33.3% and 66.6% percentiles of the series. Individuals in the top 33.3 percent were classified as having low dietary diversity, while those in the 33.3 percent to 66.6 percent decile were considered to have moderate dietary diversity. Those with values above 66.6 percent were classified as having high dietary diversity. A DDS <4.09 was classified as tertile 1 (46 individuals), 4.09-4.96 as tertile 2 (48 individuals), and >4.96 as tertile 3 (47 individuals). One-way analysis of variance (ANOVA) was used to compare normally distributed data for groups of three and more than three, and multiple comparisons were analyzed with the Tukey HSD test. Kruskal-Wallis test was used to evaluate non-normally distributed data for groups of three or more, and multiple comparisons were subsequently analyzed with Dunn's test, if indicated. Pearson correlation analysis was used for normally distributed data, and Spearman correlation analysis was used for non-normally distributed data. Multiple Linear Regression Analysis was performed to determine the effect of independent variables on the dependent variable (DDS). Variables that showed a significant correlation with DDS were included in the multiple regression model, taking into account confounding factors (age, gender, BMI-SDS, total energy intake, Statistical significance was accepted if $p<0.05$.

Results

The mean age of the adolescents was 14.81±1.94 years. Of the individuals, 70 (49.6%) were boys. Table 1 shows the distribution of general characteristics of the individuals according to gender. A statistically significant difference was found between genders for BMI-SDS, body weight-SDS, fat percentage, systolic and diastolic blood pressure, visceral adiposity index, and body shape index ($p<0.05$). The BMI-SDS and body weight-SDS scores of boys were lower than those of girls ($p<0.05$). It was found that girls had higher fat percentage ($p=0.012$) and lower systolic and diastolic blood pressure than boys ($p<0.05$). The visceral adiposity index was higher in girls ($p=0.048$), while the body shape index was higher in boys ($p=0.020$). Other demographic data, anthropometric measurements, biochemical findings, and index scores were similar between genders ($p>0.05$).

DDS by gender is shown in Table 2. Boys have higher fruit group DDS ($p=0.005$) and milk group DDS ($p=0.018$) than girls. No significant difference was found between the meat group DDS, the vegetable group DDS, the grain group DDS, and total DDS by gender ($p>0.05$).

Comparison of anthropometric measurements, biochemical findings, and index scores by DDS are presented in Table 3. Significant differences were observed between tertiles in terms of insulin ($p<0.001$), HOMA-IR ($p<0.001$), and triglyceride/glucose index ($p=0.034$) values. Insulin and HOMA-IR values differed between the three tertiles, with individuals in Tertile 1 having higher insulin and HOMA-IR values than those in the other tertiles ($p<0.001$). The triglyceride/glucose index value was found to be lower in individuals in Tertile 3 compared to those in Tertile 1 ($p=0.028$). In individuals in Tertile 3, fibre ($p=0.002$), vegetable ($p<0.001$), and whole grain ($p<0.001$) intake was higher than in other tertiles, while refined grain ($p<0.001$) and meat consumption ($p=0.013$) were lower than in other tertiles. There were no significant differences between individuals for the other parameters according to tertiles ($p>0.05$).

Table 4 shows the relationship between DDS and anthropometric measurements, biochemical findings, and index scores. DDS was negatively correlated with fasting blood sugar (rho=-0.177; $p=0.036$), insulin (rho=-0.633; $p<0.001$), triglycerides (rho=-0.223; $p=0.008$), HOMA-IR (rho=-0.656; $p<0.001$), visceral adiposity index (rho=-0.228; $p=0.007$), triglyceride/glucose index (rho=-0.251; $p=0.003$), and LAP index (rho=-0.200; $p=0.018$).

The results of the multiple regression analysis are shown in Table 5. In the regression analysis, at least one of the independent variables was found to be a significant factor (DDS: F=6.917 and

p<0.001). Fasting blood glucose (B=-0.017; p=0.039) was found to have significant effects on DDS and explained approximately 35% of the variance (R^2adj=0.355).

Discussion

Identifying risk factors for obesity in adolescents will be important for identifying the most appropriate way to intervene, ideally before obesity, and reduce cardiovascular risk (2). Diet, insulin resistance, and adiposity, particularly visceral fat, contribute significantly to the development of obesity (4). Previous studies have examined the potential contribution of diet to serum insulin levels and body (4,25,26). However, there are few studies on the effect of diet composition on visceral adiposity, and triglyceride/glucose, LAP, and body shape indexes, which are strong predictors of cardiometabolic risk. This study found that as the DDS increased, the scores for the visceral adiposity index, triglyceride/obesity index, and LAP index, used to predict metabolic obesity, decreased. DDS is an important parameter

Table 1. Distribution of general characteristics of individuals by gender

	Boys (n=70)	Girls (n=71)	Total (n=141)	p
Age (years)	14.50±1.82	15.11±2.02	14.81±1.94	0.061*
Mother's age (years)	40.47±5.64	41.25±5.98	40.87±5.81	0.426*
Father's age (years)	43.96±5.32	44.90±5.64	44.43±5.48	0.308*
BMI-SDS	2.01 (1.61-2.55)	2.39 (1.76-2.99)	2.16 (1.72-2.75)	0.012**
Body weight-SDS	1.73±0.91	2.35±1.42	2.04±1.23	0.003*
Fat mass (kg)	24.30 (19.90-34.80)	26.20 (21.20-32.70)	25.50 (20.70-33.10)	0.561**
Fat percentage (%)	33.65±8.28	36.65±5.44	35.16±7.13	0.012*
Fasting blood sugar (mg/dL)	90 (84-96)	88 (82-93)	89 (84-94.60)	0.178**
Insulin (µU/L)	20.61 (13.04-31.11)	23.15 (13.70-29.38)	21.70 (13.70-30.75)	0.928**
Total cholesterol (mg/dL)	161.81±33.68	164.01±32.06	162.92±32.78	0.692*
LDL (mg/dL)	94 (78-108.92)	93 (77-102)	93 (78-105)	0.585**
HDL (mg/dL)	43 (38.30-49.70)	46.50 (40.21-51.50)	44 (39.50-50.70)	0.136**
Triglycerides (mg/dL)	110.30 (84.90-159.90)	101 (76-131.60)	102.60 (80.80-150.80)	0.158**
Systolic blood pressure (mmHg)	130 (120-140)	130 (120-130)	130 (120-140)	0.022**
Diastolic blood pressure (mmHg)	75 (70-80)	70 (70-75)	70 (70-80)	0.006**
HOMA-IR	4.48 (2.93-6.96)	5.03 (3.15-6.92)	4.87 (3.13-6.93)	0.916**
Visceral adiposity index	1.55 (1.01-2.38)	1.82 (1.37-2.68)	1.68 (1.13-2.50)	0.048**
Triglyceride/glucose index	8.58 (8.26-8.84)	8.35 (8.13-8.68)	8.43 (8.17-8.82)	0.104**
Lipid accumulation product index	44.60 (33.91-70.23)	38.43 (28.39-64.67)	41.72 (30.17-65.33)	0.362**
Body shape index	0.0834±0.0045	0.0814±0.0054	0.0824±0.0051	0.020*

*Independent two-sample t-test value is given as mean ± standard deviation, **Mann-Whitney U test value is given as median (Q1-Q3: IQR-interquartile range).
BMI-SDS: body mass index standard deviation score, VA-SDS: body weight standard deviation score, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

Table 2. Distribution of dietary diversity scores by gender

	Boys (n=70)	Girls (n=71)	Total (n=141)	p
Meat group DDS	1.13 (0.72-1.43)	1.23 (0.87-1.51)	1.19 (0.81-1.46)	0.126**
Fruit group DDS	1.00 (0.66-1.49)	0.76 (0.39-1.12)	0.88 (0.51-1.23)	0.005**
Vegetable group DDS	0.92 (0.51-1.15)	0.90 (0.55-1.29)	0.90 (0.51-1.18)	0.479**
Dairy group DDS	0.71 (0.41-1.00)	0.54 (0.37-0.77)	0.65 (0.39-0.90)	0.018**
Cereal group DDS	0.90 (0.29-1.33)	0.91 (0.48-1.46)	0.90 (0.43-1.37)	0.349**
Total DDS	4.56±1.12	4.43±0.79	4.50±0.97	0.425*

*Independent two-sample t-test value given as mean ± standard deviation, **Mann-Whitney U test value given as median (Q1-Q3: IQR-interquartile range), p<0.05.
DDS: dietary diversity score

Table 3. Comparison of anthropometric measurements, biochemical findings and index scores according to tertiles of dietary diversity score

	1st tertile [<4.09] low (n=46)	2nd tertile [4.09-4.96] medium (n=48)	3rd tertile [>4.96] high (n=47)	p
BMI-SDS	2.17 (1.76-2.75)	2.11 (1.72-2.77)	2.20 (1.71-2.79)	0.946**
Body weight-SDS	2.12±1.24	2.17±1.08	1.84±1.36	0.371*
Fat mass (kg)	25.75 (21.20-34.00)	25.75 (21.40-32.85)	24.40 (19.30-33.90)	0.716**
Fat percentage (%)	35.03±7.45	35.04±7.15	35.40±6.93	0.961*
Fasting blood sugar (mg/dL)	91 (87-96)	86 (80.15-94.30)	88 (83-93)	0.072**
Insulin (µU/L)	31.72 (23.17-42.83)[a]	21.70 (14.84-27.37)[b]	13.04 (10.38-20.68)[c]	<0.001**
Total cholesterol (mg/dL)	161.92±34.53	167.31±26.82	159.41±36.54	0.489*
LDL (mg/dL)	93.50 (79-108.28)	90 (77.50-103)	94 (77.80-107.20)	0.782**
HDL (mg/dL)	42.65 (39.90-48)	46.15 (40-53)	43.70 (37.20-50.70)	0.266**
Triglycerides (mg/dL)	111.75 (89.40-166.50)	102.80 (81.85-155.90)	98.30 (66.10-131.60)	0.074**
Systolic blood pressure (mmHg)	130 (120-140)	130 (120-140)	125 (120-140)	0.369**
Diastolic blood pressure (mmHg)	70 (70-80)	70 (70-80)	70 (70-80)	0.910**
HOMA-IR	7.07 (5.03-10.05)[a]	4.79 (3.46-6.49)[b]	2.80 (2.28-4.40)[c]	<0.001**
Visceral adiposity index	2.03 (1.26-2.77)	1.60 (1.39-2.53)	1.52 (0.95-2.30)	0.081**
Triglyceride/glucose index	8.57 (8.27-8.90)[a]	8.46 (8.17-8.87)[a,b]	8.31 (8.05-8.63)[b]	0.034**
Lipid accumulation product index	48.31 (34.44-71.50)	45.04 (32.32-72.03)	37.93 (25.70-61.16)	0.106**
Body shape index	0.0816±0.004	0.0829±0.004	0.0828±0.005	0.435*
Energy (kcal)	2118.26±103.44	2133.61±188.13	2097.81±216.22	0.319*
Carbohydrates (%)	49.08±3.5	48.62±2.9	48.79±3.5	0.459*
Proteins (%)	14.04±2.66	13.87±3.04	14.21±2.15	0.501*
Fats (%)	35.92±2.74	36.23±3.12	35.61±2.18	0.662*
Fiber (g)	20.61.±2.19[a]	19.74±3.15[a]	24.69±2.57[b]	0.002*
Whole grains (g)	103.17.±25.23[a]	147.47±15.19[a]	239.52±13.08[b]	<0.001*
Refined grains (g)	352.14±18.35[a]	271.22±20.15[a]	205.35±31.28[b]	<0.001*
Fruits (g)	296.55±126.17	319.63±149.37	336.13±105.40	0.103*
Vegetable (g)	288.24±100.15[a]	269.38±93.63[a]	361.79±65.48[b]	<0.001*
Dairies (g)	455.86±76.84	478.5±101.62	446.32±92.17	0.516*
Meat (g)	171.77±62.11[a]	163.80±54.22[a]	127.52±55.13[b]	0.013*
Legumes (g)	19.88±10.14[a]	17.65±11.29[a]	23.93±14.86[b]	0.004*

*One-way analysis of variance value is given as mean ± standard deviation, **Kruskal-Wallis test value is given as median (Q1-Q3: IQR-interquartile range). [a-c]: There is no difference between groups with the same letter.
BMI-SDS: body mass index-standard deviation score, BW-SDS: body weight standard deviation score, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

used to assess nutrient adequacy, overall diet quality, and the diet-disease relationship. It has been reported that a higher DDS is closely associated with a healthy diet with better nutrient adequacy and diet quality (2). It has been shown that DDS has an inverse relationship with metabolic syndrome and also with high blood pressure, high triglyceride levels, and abnormal glucose homeostasis (27). In the present study, a negative relationship was identified between DDS and fasting blood sugar, insulin, triglycerides, and HOMA-IR. When we excluded confounding factors, the single factor affecting DDS was fasting blood glucose. Visceral adipose tissue is considered an independent risk factor for cardiovascular diseases due to its role in regulating glucose, lipid metabolism, and blood pressure (9). The visceral adiposity index has been identified as a new cardiometabolic risk marker in recent decades because it reflects abdominal fat distribution and dyslipidaemia. The triglyceride/glucose index and LAP index are good markers of insulin sensitivity and are associated with insulin resistance (4). It has been reported that visceral adipose tissue is affected by changes in diet and lifestyle (4,9). In the present study, as the DDS scores of obese adolescents increased,

Table 4. Relationship between dietary diversity score and anthropometric measurements, biochemical findings, and index scores

	Dietary Diversity Score	
	r	**p**
Body weight-SDS	-0.080	0.347*
Fat percentage (%)	-0.024	0.780*
Total cholesterol (mg/dL)	-0.062	0.468*
Body shape index	0.074	0.384*
Energy (kcal) (day)	-0.077	0.369*
	rho	**p**
BMI-SDS	-0.009	0.920**
Fat mass (kg)	-0.075	0.379**
Fasting blood sugar (mg/dL)	-0.177	0.036**
Insulin (µU/L)	-0.633	<0.001**
LDL (mg/dL)	-0.018	0.834**
HDL (mg/dL)	0.047	0.580**
Triglycerides (mg/dL)	-0.223	0.008**
Systolic blood pressure (mmHg)	-0.144	0.088**
Diastolic blood pressure (mmHg)	-0.038	0.657**
HOMA-IR	-0.656	<0.001**
Visceral adiposity index	-0.228	0.007**
Triglyceride/glucose index	-0.251	0.003**
Lipid accumulation product index	-0.200	0.018**

*Pearson correlation analysis, **Spearman correlation analysis.
BMI-SDS: body mass index-standard deviation score, BW-SDS: body weight standard deviation score, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

Table 5. Multiple regression results for different factors affecting the dietary diversity score

		Unstandardized coefficients		Standardized coefficients		
Dependent	**Independent**	**B±SE**	**95% CI**	**Beta**	**p**	**Significance of the model**
Dietary diversity score	Constant	9.505±2.862	3.841/15.169		0.001	F=6.917 p<0.001 R^2=0.355
	Fasting blood sugar (mg/dL)	-0.017±0.008	-0.032/-0.001	-0.204	0.039	
	Insulin (µU/L)	-0.035±0.018	-0.071/0.001	-0.592	0.057	
	Triglycerides (mg/dL)	-0.008±0.005	-0.018/0.003	-0.594	0.155	
	HOMA-IR	0.022±0.078	-0.133/0.177	0.087	0.782	
	Visceral adiposity index	0.094±0.106	-0.116/0.305	0.212	0.378	
	Triglyceride/glucose index	-0.017±0.394	-0.796/0.762	-0.009	0.966	
	Lipid accumulation product index	0.006±0.006	-0.005/0.018	0.299	0.279	

B: unstandardized coefficient, SE: standard error, CI: confidence interval, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

the visceral adiposity index, triglyceride/glucose index, and LAP index used to predict metabolic obesity decreased. In an earleir study examining the effects of different dietary patterns on body composition, it was found that the Western-style dietary pattern positively affected the fat mass index/FFM index ratio, while the "vegetable and fruit"-based dietary pattern negatively affected the fat mass index/FFM index ratio (28). It has been reported that adolescents with low DDS have a higher body fat percentage than adolescents with high DDS (29). However, in our study, no relationship was found between DDS and anthropometric measurements, such as BMI, fat percentage, fat mass. This may be explained because our study sample consisted of obese individuals and that energy, carbohydrate, fat, and protein intake were similar across DDS groups which may have contributed to this finding.

The inverse relationship between DDS and metabolic risks may be attributed to the increased consumption of healthier food groups associated with high DDS (2).The present study found that although adolescents'energy intakes were similar regardless of their DDS scores, adolescents with high DDS consumed more fibre, vegetables, whole grains, legumes, and less refined grains and meat. Vizzuso et al. (11) found that energy intake was positively associated with BMI z-score, but no association was found with visceral adiposity index. In this study, while total energy intake was not associated with cardiometabolic risk markers, it was observed that meal pattern affected DDS and DDS was associated with cardiometabolic risk markers. In another study, healthy plant-based diet index scores were found to be associated with better anthropometric measurements and HDL-C levels compared to unhealthy plant-based diet index scores, and were also found to reduce LAP levels (3). It has been reported that healthy diet models had no effect on triglyceride/glucose indices compared to a Western-style diet or a mix of healthy and Western-style diets, but they did cause a decrease in LAP levels. Individuals with the highest healthy diet model scores were 71% less likely to have high LAP levels compared to those in the lowest category (4). Mazidi et al. (26) also reported positive correlations between the visceral adiposity index and glucose/insulin homeostasis markers and the consumption of carbohydrates and sugar, total fat and saturated fatty acids, as well as negative correlations between fibre, vitamin, and mineral intake and the visceral adiposity index and LAP indices, findings similar to those of the present study. In addition, it has been noted that there is an inverse relationship between a diet rich in monounsaturated and polyunsaturated fatty acids and fasting blood glucose and LAP indices (26). Studies have reported a negative relationship between the Dietary Approaches to Stop Hypertension diet index, which is based on increasing the consumption of vegetables, fruits, whole grains, legumes, and white meat, while reducing the consumption of red meat, refined carbohydrates, and sugary beverages, and the visceral adiposity index (30). and a negative relationship between the anti-inflammatory diet and triglyceride/glucose indices (4). A study examining the relationship between whole grain consumption and insulin resistance, glucose homeostasis, and inflammation found that high whole grain consumption was associated with lower C-reactive protein, apolipoprotein B, fasting blood glucose, insulin, homeostatic model assessment of insulin resistance (HOMA-β), hemoglobin A1c, and glucose levels when comparing the group with high whole grain product consumptionto the group with low whole grain product consumption (26).

Adolescents are generally influenced by their peers, have greater freedom of choice in food, and tend to choose unhealthy foods. Good growth and development require a variety of foods from various food groups (vegetables, fruits, whole grains, and animal-based foods) and a balanced intake of vitamins. Dietary diversity consists of all food groups (grains, vegetables, fruits, meat, and dairy products) necessary for growth and development, and high dietary diversity is associated with healthy food groups, such as vegetables, fruits, and fibre (2). In the present study, individuals with high dietary diversity were found to have a reduced risk of metabolic syndrome. The proposed mechanism explaining the results obtained from implementing a dietary model with high dietary diversity is that the higher fibre content of vegetables, whole grain products, and legumes may lead to lower nutrient absorption or energy intake, affecting total fat mass and visceral fat accumulation (1,26). Higher fibre intake has been shown to improve insulin resistance and reduce visceral adiposity (4,31). High fibre intake, which is broken down into short-chain fatty acids by the gut microbiota, is known to improve insulin sensitivity or insulin resistance (3). Low-glycemic index carbohydrates found in vegetables, whole grains, and legumes may also reduce insulin resistance (32). The chronic low grade inflammatory state is a common condition in obesity and associated with multiple metabolic complications (1,32). Moreover, the anti-inflammatory and antioxidant properties of vegetables, fibre, and legumes may be associated with lower systemic inflammation. Soluble fibre, in particular, binds to bile acids in the small intestine, increasing the excretion of bile salts in the feces, lowering cholesterol, and regulating postprandial insulinemic and glycemic responses (33).

High intake of antioxidants and micronutrients from plant-based foods also represents another potential cardioprotective mechanism. This antioxidant capacity, combined with the potential to modulate nitric oxide production, enhances the ability of polyphenolic compounds to maintain vascular homeostasis (34). In the present study, refined grain and meat consumption were significantly reduced in the tertile with the highest dietary diversity. Low intakes of animal protein and saturated fatty acids have been reported to effectively prevent obesity. In addition, animal proteins are rich in other nutrients,

such as iron, sodium, and nitrites obtained from processed meats, increasing the risk of cardiometabolic diseases (35). Refined grains have high carbohydrate content, which leads to a high dietary glycemic load. Compared to whole grain products, refined grains are rapidly absorbed due to their high glycemic load, leading to increased fasting blood sugar and insulin resistance. Unlike refined grains, whole grain products are high in dietary fibre, trace elements, and phytochemicals, and their nutrients and nutritional components have beneficial effects on metabolic syndrome (36).

Study Limitations

This study has some limitations. The study included only obese adolescents from a single tertiary center. Without a healthy control group, the ability to generalize the findings or assess relative risk is limited. The diet history method has some limits in accuracy. All self-reported dietary assessment methods are subject to both random and systematic measurement errors. However, retrospective food consumption records do not have the potential recall bias caused by food consumption frequency questionnaires. The cross-sectional nature of the study, preventing any causal inferences, the division of DDS into tertiles, potentially leading to misclassification in the data, and the small sample size are the other limitations of the study. This study also has strengths. It is the first study to examine the effect of dietary diversity on cardiometabolic risk markers, such as visceral adiposity, triglyceride/glucose, LAP, and body shape indices. Other strengths include the control of a wide range of potential confounding factors to obtain an independent relationship, the calculation of DDS from food consumption records by a trained dietitian, and the homogeneity of the sample due to the adolescents participating in the study being from the same geographical region and sharing similar cultures, lifestyles, and eating habits. Finally, using food combinations rather than single foods to examine the specified relationships provided more accurate information and should be considered an additional strength.

Conclusion

DDS were inversely associated with visceral adiposity, triglyceride/glucose, and LAP indexes in this cohort of obese adolescents. Furthermore, as dietary diversity scores increased, fasting blood sugar, insulin, triglyceride, and HOMA-IR levels decreased. Increased dietary diversity was found to be positively associated with biomarkers of metabolic syndrome. Strategies aimed at increasing dietary diversity through nutritional interventions may have a positive effect, particularly on insulin resistance and cardio-metabolic risk. Extensive prospective studies focusing on different populations are needed to confirm these findings.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Gazi University Ethics Committee (approval number: 2025-164, date: 05.02.2025).

Informed Consent: Clear explanations were provided with regard to the purpose of the study, after which written informed consent was obtained from the adolescents in accordance with the Declaration of Helsinki.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Aysun Bideci, Mahmut Orhun Çamurdan, Aylin Kılınç Uğurlu, Esra Döğer, Mehmet Ali Oktay, Ulaş Akçay, Concept: Rukiye Bozbulut, Mehmet Ali Oktay, Ulaş Akçay, Esra Döğer, Aylin Kılınç Uğurlu, Mahmut Orhun Çamurdan, Aysun Bideci, Design: Rukiye Bozbulut, Mehmet Ali Oktay, Ulaş Akçay, Esra Döğer, Aylin Kılınç Uğurlu, Mahmut Orhun Çamurdan, Aysun Bideci, Data Collection or Processing: Rukiye Bozbulut, Mehmet Ali Oktay, Ulaş Akçay, Analysis or Interpretation: Esra Döğer, Aylin Kılınç Uğurlu, Rukiye Bozbulut, Mehmet Ali Oktay, Ulaş Akçay, Literature Search: Rukiye Bozbulut, Writing: Rukiye Bozbulut.

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ORIGINAL ARTICLE

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The Course of Progranulin Levels at Admission and During Early Period of Insulin Treatment in Children with Newly Diagnosed Type 1 Diabetes Mellitus

Ayşe Sena Dönmez[1], Atilla Çayır[2], Esra Laloğlu[3], Alev Lazoğlu Özkaya[4], Esra Dişci[1], Serap Kılıç Kaya[1], Kamber Kaşali[5], Serkan Bilge Koca[6], Hüseyin Demirbilek[7]

[1]University of Health Sciences Türkiye, Erzurum City Hospital, Clinic of Pediatrics, Erzurum, Türkiye
[2]Atatürk University Faculty of Medicine, Department of Pediatric Endocrinology, Erzurum, Türkiye
[3]Atatürk University Faculty of Medicine, Department Medical Biochemistry, Erzurum, Türkiye
[4]University of Health Sciences Türkiye, Erzurum City Hospital, Clinic of Medical Biochemistry, Erzurum, Türkiye
[5]Atatürk University Faculty of Medicine, Department of Biostatistics, Erzurum, Türkiye
[6]University of Health Sciences Türkiye, Kayseri City Hospital, Clinic of Pediatrics, Division of Pediatric Endocrinology, Kayseri, Türkiye
[7]Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye

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What is already known on this topic?

Progranulin (PGRN) is a growth factor involved in inflammation, insulin resistance, and glucose metabolism. Increased serum PGRN levels have been reported in adults with both type 2 and type 1 diabetes. However, the relationship between PGRN levels and metabolic status in children with type 1 diabetes is not clearly understood. There are very few studies in the pediatric age group evaluating the dynamic changes in PGRN levels during the early treatment period of type 1 diabetes.

What this study adds?

Our findings indicate that serum PGRN levels are markedly elevated in children with newly diagnosed type 1 diabetes during diabetic ketoacidosis and remain higher than in healthy controls even after early glycemic stabilization, suggesting a potential role for PGRN in the metabolic response to acute disease presentation.

Corresponding Author: Res. Asst. Ayşe Sena Dönmez, University of Health Sciences Türkiye, Erzurum City Hospital, Clinic of Pediatrics, Erzurum, Türkiye
E-mail: sertugay@hotmail.com **ORCID:** orcid.org/0000-0001-7024-8157
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ABSTRACT

Objective: Progranulin (PGRN), a growth factor, modulates cell proliferation, wound repair, and inflammation. It is also involved in glucose metabolism and is associated with insulin resistance and diabetes mellitus (DM). In the present study, PGRN levels were measured at admission and during follow-up in children with newly diagnosed type 1 DM (T1DM) and compared to healthy controls.

Methods: Children with T1DM and healthy controls were included. The age, weight, height, body mass index (BMI), severity of acidosis, glucose, insulin, C-peptide, and diabetes-specific autoantibodies of children with newly diagnosed T1DM were collected. PGRN was measured in children with T1DM at admission, at first week of follow-up, and in healthy controls.

Results: A total of 49 children were included; 25 with T1DM [12 Female/13 Male (12F/13M)] and 24 healthy controls (10F/14M). There was no differences in age (11±3.9 years vs 12.1±3.1 years, p=0.269) and BMI standard deviation (SD) score (-0.11±1.49 SD vs. 0.10±0.82 SD, p=0.540) of children with T1DM and healthy controls. The mean basal PGRN level of children with newly diagnosed T1DM was higher than in controls (90.8±17.3 ng/mL vs. 30±11.5 ng/mL, p<0.001). In children with T1DM, mean basal PGRN at admission had declined significantly (58.4±16.9 ng/mL; p<0.001) in the first week after glycemic regulation was achieved but remained significantly higher than in controls (p<0.001).

Conclusion: These findings suggest that elevated PGRN levels in children with newly diagnosed T1DM may reflect either an acute inflammatory response to diabetic ketoacidosis or a persistent alteration in metabolic regulation, or both of these, highlighting the potential role of PGRN as a biomarker in the early course of T1DM.

Keywords: Diabetic ketoacidosis, pediatric, progranulin, PGRN, type 1 diabetes mellitus

Introduction

Progranulin (PGRN), also called granulin-epithelin precursor, acrogranin, proepithelin, GP88, and prostate cell-derived growth factor, is a growth factor that is comprised of 593 amino acids with a molecular weight of 75-80 kDa (1,2,3). It modulates cell proliferation, tissue regeneration, and wound repair, thus being involved in mechanisms of tumorigenesis, inflammation, and fibrosis (4,5,6). PGRN acts as an endogenous antagonist for TNF-α by competitively binding to its receptor (7). It is an adipokine involved in glucose metabolism and associated with insulin resistance, diabetes mellitus (DM), and metabolic complications (8,9). PGRN is encoded by the *GRN* gene, mapped on the chromosomal region 17q21.32, and has 12 exons (10).

Although PGRN exhibits anti-inflammatory activity, some granulin peptides derived from the proteolytic cleavage of PGRN stimulate inflammation (11,12). Besides, increased PGRN expression in adipocytes disrupts insulin signalling and induces inflammation (11). Elevation in circulating PGRN levels has been shown in patients with type 2 DM (T2DM) and reported mainly associated with impaired glucose tolerance rather than impaired fasting glucose (11).

Studies conducted in adults have shown that PGRN levels are increased in individuals with type 1 DM (T1DM) (13). In a study conducted in children, PGRN levels were shown not to differ between children with newly diagnosed T1DM, those with good and poor metabolic control, and healthy controls (14).

In the present study, the aim was to evaluate PGRN levels measured at admission and during follow-up in children with newly diagnosed T1DM who presented with diabetic ketoacidosis (DKA) and compare these with healthy controls.

Methods

Subjects

This cross-sectional case-control study was conducted on patients admitted to Pediatric Endocrinology Outpatient Clinics, Inpatient Clinics, and Pediatric Emergency Services of University of Health Sciences Türkiye, Erzurum City Hospital between September 2023 and September 2024.

Anthropometric measurements [weight, height, and body mass index (BMI)] were recorded. Age- and sex-specific reference ranges and standard deviation scores (SDS) were calculated (15). Patients with concomitant endocrinological problems, such as hypothyroidism, Cushing syndrome, or familial hyperlipidemia, those diagnosed with hypertension or chronic liver disease, celiac disease and those taking medication, such as corticosteroids, were excluded. The control group consisted of healthy children who were attended pediatric outpatient clinics for routine health evaluations and found to have no significant illness.

Laboratory Measurements

Blood samples collected from the patient and control groups were left in tubes in a vertical position for 30 min for coagulation. They were then centrifuged at +4 °C for 7 min at 4500 rpm. The serum specimens obtained were aliquoted and placed into a deep freeze at -80 °C until the day of analysis.

Biochemical measurements were performed using the Beckman Coulter AU 5800 (Beckman Coulter, CA, USA) analyzer. Insulin levels were measured with the Beckman Coulter DXI 800 (Beckman Coulter, CA, USA) device. The glycated haemoglobin (HbA1c) levels were measured by a high-performance liquid chromatography method (Lifotronic H9, Lifotrophic Technology,

Shenzhen, China). The ABL 800 Flex (Radiometer, Copenhagen, Denmark) device, which is available as a blood gas analyzer in our laboratory, provides quantitative measurement of parameters, such as pH, pCO_2, pO_2, Na^+, K^+, Cl^-, iCa^{++}, glucose, L-lactate, total hemoglobin, hematocrit, and hemoglobin saturation. Children with T1DM were classified as "mild", "moderate", "severe" and "without acidosis" according to the pH and HCO_3 levels in blood gas at admission (16). The specific crieria are shown below:

- Mild acidosis: venous pH <7.3 or serum bicarbonate <18 mmol/L,

- Moderate acidosis: pH <7.2 or serum bicarbonate <10 mmol/L,

- Severe acidosis: pH <7.1 or serum bicarbonate <5 mmol/L.

Blood samples for biochemical examinations of PGRN measurement were collected after 6-8 hours of overnight fasting where applicable. Serum, obtained from whole blood samples collected, was analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) using the Human PGRN ELISA Kit (BT LAB, Cat. No. E1755Hu, China) according to the manufacturer's instructions. The kit measurement range for PGRN was 10-700 ng/mL, and the sensitivity of this assay was 5.12 ng/mL. Intra- and interassay coefficients of variation for PGRN were <5% and <10%, respectively. Briefly, the samples and standards were added to wells pre-coated with human PGRN antibodies. PGRN present in the samples was bound by the antibodies coating the wells. A biotinylated human PGRN antibody was then added to bind to the bound PGRN, followed by streptavidin-horseradish peroxidase (HRP) to bind to the biotinylated PGRN antibody. After incubation, the unbound streptavidin-HRP was washed away. Substrate solution was added and color developed proportionately to the amount of human PGRN in the well. The reaction was terminated by adding an acidic stop solution and absorbance was measured at 450 nm. PGRN concentrations were determined by comparing the optical density in the sample wells with a standard curve constructed with included kit reagents.

The study was approved by the Scientific Research Ethics Committee of Health Sciences University Erzurum Faculty of Medicine (approval no.: 05/105, date: 05/08/2024) and carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from the participants or their legal guardians.

Statistical Analysis

Data were analyzed by using SPSS, version 24.0 (IBM Corporation, Armonk, NY, USA). The mean, SD, minimum and maximum values of the numeric variables were calculated. Categorical variables are presented as frequency and percentage (%). Shapiro-Wilk test was used to evaluate the normality assumption. However, variables with kurtosis and skewness values within the range of -2 to +2 were considered to have a normal distribution. Histogram and Q-Q plot graphs were examined. The chi-square test was used to compare categorical variables, and the Student's t-test was used to compare independent variables. One-way analysis of variance was used to compare numerical variables in more than two independent groups. An examination of repeated measurements was performed using the paired t-test. The relationship between normally distributed variables was evaluated with Pearson correlation analysis, and those without normal distribution were evaluated with Spearman's rank test. A p value less than 0.05 was considered statistically significant.

A post-hoc G-power analysis was conducted, using a study comparing children with newly diagnosed, well-controlled, or poorly controlled type 1 diabetes with a healthy control group as reference (14). The effect size was 0.85, the critical t-value was 2.01, the degrees of freedom were 44, and the power was 81% if the study was conducted with a total of 46 children, 23 in each group.

Results

A total of 49 children, 25 with T1DM [12 Female/13 Male (12F/13M)] and 24 healthy controls (10F/14M) were recruited. There was no difference in age (11±3.9 years vs. 12.1±3.1 years, p=0.269) or BMI SDS (-0.11±1.49 SD vs. 0.10±0.82 SD, p=0.540) between children with T1DM and healthy controls. In terms of severity of acidosis at admission, 10 (40%) patients had mild acidosis, nine (36%) patients had moderate acidosis, and four (16%) patients had severe acidosis. Two patients (8%) did not have acidosis at the time of admission. Laboratory characteristics of patients with T1DM are displayed in Table 1. The number of patients with at least one serologically positive diabetes autoantibody was 17 (68%). The mean basal PGRN level of children with newly diagnosed T1DM at admission was higher than in controls (90.8±17.3 ng/mL vs. 30±11.5 ng/mL, p<0.001). There was no significant difference between male and female newly diagnosed diabetics according to age (11.3±3 years vs. 10.7±4.8 years, p=0.737), BMI SDS (-0.18±1.2 SD vs -0.04±1.8 SD, p=0.819), or basal PGRN levels (92.5±15.8 ng/mL vs. 88.8±19.4 ng/mL, p=0.609), respectively.

Furthermore, no significant difference was found in PGRN levels when comparing the patients with mild, moderate, and severe acidosis at admission (p=0.940).

The white blood cell (WBC) counts of children with T1DM were higher than healthy controls at admission, presumably due to dehydration, stress response, and systemic inflammatory activation associated with DKA (12.622 WBCs/µL vs. 8.545 WBCs/µ, p=0.015). A weak positive correlation was observed between PGRN level and WBC count (r=0.292, p=0.042).

Comparison of the PGRN levels measured at admission and after stabilization of blood glucose levels under insulin therapy showed that PGRN levels measured at admission were significantly higher than both their 1st-week measurements and than those of healthy controls (both p<0.001) (Figure 1). Of note, despite a significant decrease in PGRN levels over the first week of treatment, these one-week levels in the children with newly diagnosed T1DM remained signifiacntly higher than in the control group (p<0.001). None of the variables that may have affected the change in PGRN levels over the first week of insulin treatment in children with T1DM was found to be significant (Table 2).

Discussion

In the present study evaluating PGRN levels in patients presenting with newly diagnosed T1DM, a significantly higher PGRN level in children at first admission with DKA compared to healthy controls was found. The level of PGRN declined during an average follow-up period of one week when blood glucose stabilization was achieved using insulin therapy.

Although a decline was observed in the PGRN levels in the first week of admission, it remained significantly higher than in healthy controls. Nevertheless, we did not detect a relationship between clinical characteristics (age and anthropometry) and laboratory parameters (glucose, insulin, c-peptide, HbA1c,

Table 1. Laboratory characteristics of patients with type 1 diabetes mellitus

	Mean±SD	Median (Q1-Q3)	Min.	Max.
VBG pH	7.17±0.11	7.19 (7.14-7.24)	6.93	7.37
VBG HCO3 (mmol/L)	11.08±3.54	10.4 (8.65-12.75)	6.6	21.9
Base deficit (mmol/L)	-18.1±5.64	-19.4 (-22.9– -14.1)	-26.4	-1.9
Glucose (mg/dL)	487.6±196.2	412 (342-593.5)	219	930
Urine pH	6.06±0.30	6 (6-6)	5	6.5
Urine density	1031±7.9	1031 (1026-1036)	1022	1053
HbA1c (%)	13.1±2.1	13.1 (11.2-14.4)	10	18
Insulin (mU/L)	2.41±1.93	1.9 (1.2-3.3)	0.6	9
C-peptide (µg/L)	0.37±0.26	0.39 (0.14-0.50)	0.06	0.93
PGRN (1st day) ng/mL	90.8±17.3	97.1 (75.4-105)	50.9	108.7
PGRN (1st week) ng/mL	58.4±16.9	60.2 (48.8-69.9)	14.9	88.6

SD: standard deviation, VBG: venous blood gas, PGRN: progranulin, HbA1c: glycated haemoglobin

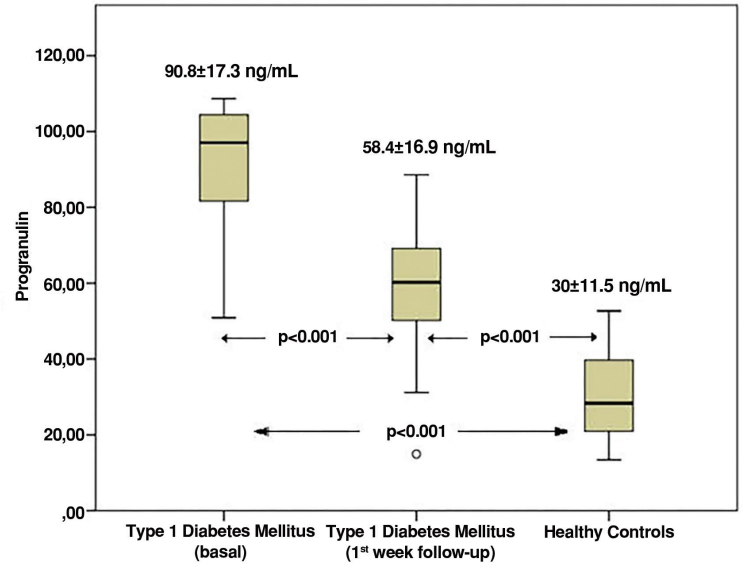

Figure 1. The comparison of progranulin levels in type 1 diabetes mellitus and healthy controls according to measurements at admission and at the first week

Table 2. Factors that may affect the alteration of PGRN levels over time (decline between admission and first week PGRN value) in children with type 1 diabetes mellitus

	Correlation coefficient	p
Age	-0.169	0.418$^{\phi}$
Height SDS	0.211	0.311$^{\phi}$
Weight SDS	0.032	0.881$^{\phi}$
BMI SDS	-0.101	0.633$^{\phi}$
VBG pH	0.087	0.680$^{\phi}$
VBG HCO3	0.075	0.723$^{\Psi}$
Glucose	-0.248	0.232$^{\phi}$
Insulin	0.381	0.060$^{\Psi}$
HbA1c	-0.137	0.515$^{\phi}$
C-peptide	-0.226	0.278$^{\phi}$
ICA	-0.165	0.429$^{\Psi}$
Anti-GAD	0.193	0.355$^{\phi}$
Anti-insulin	0.323	0.115$^{\Psi}$

$^{\phi}$Pearson correlation analysis, $^{\Psi}$Spearman correlation analysis.
PGRN: progranulin, SDS: standard deviation score, VBG: venous blood gas, ICA: islet cell antibody, GAD: glutamic acid decarboxylase antibody

degree of acidosis, and diabetes autoantibodies) and the decline in PGRN level over time. Although the elevation in PGRN levels can be attributed to the inflammatory or immune response-related increase in PGRN in children with T1DM due to acute DKA, since PGRN levels remained higher than those of healthy controls, we could not exclude the role of diabetes in elevated PGRN levels. When we divided the subgroups according to the degree of acidosis, we observed no difference in terms of age, sex, BMI SDS, or WBC count. However, our small sample size and the even smaller numbers in subgroups may also explain why there was no significant difference in between DKA severity subgroups in terms of PGRN levels. Further studies with larger case series and longer-term follow up are required to elucidate the role of the overlapping factors.

There was a positive correlation between the PGRN level and the WBC count at admission. Since sensitive C-reactive protein was not measured in most of the patients, this relationship could not be further evaluated to determine whether it is due to the high WBC count, dehydration or inflammation.

In a study conducted in China comparing PGRN levels in obese and healthy controls, PGRN levels were found to be higher in obese children, but these authors did not find a significant relationship between PGRN levels and HOMA-IR, HOMA-B, and dynamic parameters derived from the oral glucose tolerance test (insulinogenic index, ΔI30/ΔG30 and C-peptide index, ΔC30/ΔG30) (17).

In another study comparing the PGRN levels of a group of healthy controls and children with T1DM (newly diagnosed, those with good metabolic control, and those with poor metabolic control), no difference was observed in PGRN levels (14). Nevertheless, in that study, there was a difference in age and BMI of the patient groups. The authors also reported a negative correlation between PGRN levels and age, as well as between PGRN levels and BMI, in newly diagnosed T1DM patients (14). In our study, although age, sex, and BMI SDS were similar between patients and controls, PGRN levels were higher in the T1DM patients compared to the healthy controls.

In a study in patients with T2DM, serum PRGN level were reported to be associated with the severity of diabetic nephropathy (DN) and diabetic retinopathy (9). The authors suggested that serum PGRN level could be used as an early biomarker of DN in patients with decreased estimated glomerular filtration rate but without albuminuria (9). Another explanation for the increased serum PGRN level in patients with DN, similar to what we observed in patients with DKA, could be a compensatory mechanism that reduces renal impairment, as PGRN can alleviate inflammation in an acute situation (18). Schlatzer et al. (19) investigated PGRN in the urine of 74 patients with T1DM and concluded that it can be used in a panel together with three protein levels (urinary Tamm-Horsfall glycoprotein, clusterin, and human α-1 acid glycoprotein) to predict early signs of diabetic kidney disease. In another study conducted on young adults between the ages of 20 and 30, PGRN levels in type 1 diabetics were significantly higher than in healthy controls, while no relationship was found between diabetic microvascular complications (retinopathy, nephropathy, neuropathy) and PGRN levels (13).

The half-life of PGRN is approximately 40 hours. In our study, early elevation of PGRN levels in newly diagnosed T1DM patients, followed by a decline in the first week of glycemic control, may suggest that the PGRN molecule acts as an acute-phase reactant. However, higher PGRN levels in T1DM patients compared to healthy controls during follow-up, suggests that the relationship of PGRN levels and T1DM remains unknown and merits further investigation (20).

Study Limitations

The limitations of our study include the small number of participants and the cross-sectional assessment of PGRN levels. Although we compared initial and short-term follow-up PGRN levels, longitudinal studies with larger number of cases and long-term courses of PGRN levels are needed to explore how PGRN levels alter over time with disease duration and various treatment regimens. Data on the relationship between PGRN and BMI has been published. Although we had anthropometric measurements at the first and third months in our study, PGRN levels were not measured at these time points. The strength of

our study was that PGRN levels were assessed during DKA and the early period when glucose regulation was achieved.

Conclusion

In this cross-sectional small-scale study, we showed an elevated PGRN level in children with T1DM who presented with DKA, and which declined shortly after achieving normoglycemia and stabilization of the acute phase of presentation with T1DM. None of the clinical or laboratory parameters investigated was associated with the change between PGRN measured at the time of admission and at follow-up. However, the one-week PGRN level remained higher than in healthy controls, suggesting a need to clarify whether elevated PGRN was due to diabetes-specific metabolic changes or an increased inflammatory response to the acute phase of DKA. Larger-scale longitudinal studies performed in T1DM children are required to elucidate this relationship.

Ethics

Ethics Committee Approvel: The study was approved by the Scientific Research Ethics Committee of Health Sciences University Erzurum Faculty of Medicine (approval no.: 05/105, date: 05/08/2024) and carried out in accordance with the principles of the Declaration of Helsinki.

Informed Consent: Informed consent was obtained from the participants or their legal guardians.

Footnotes

Authorship Contributions

Concept: Ayşe Sena Dönmez, Atilla Çayır, Esra Laloğlu, Esra Dişci, Serap Kılıç Kaya, Serkan Bilge Koca, Hüseyin Demirbilek, Design: Alev Lazoğlu Özkaya, Esra Dişci, Serap Kılıç Kaya, Kamber Kaşali, Serkan Bilge Koca, Hüseyin Demirbilek, Data Collection or Processing: Ayşe Sena Dönmez, Esra Laloğlu, Alev Lazoğlu Özkaya, Kamber Kaşali, Serkan Bilge Koca, Analysis or Interpretation: Atilla Çayır, Esra Dişci, Serap Kılıç Kaya, Kamber Kaşali, Writing: Ayşe Sena Dönmez, Atilla Çayır, Esra Laloğlu, Alev Lazoğlu Özkaya, Hüseyin Demirbilek.

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Founder Pathogenic Variant in *LMNA* with Diverse Phenotypic Manifestations in Mandibuloacral Dysplasia: Insights from a Turkish Cohort

Zehra Manav Yiğit[1], Mustafa Altan[2], Göksel Tuzcu[3], Gökay Bozkurt[1], Ahmet Anık[4]

[1]Aydın Adnan Menderes University Faculty of Medicine, Department of Medical Genetics, Aydın, Türkiye
[2]Ankara Bilkent City Hospital, Clinic of Medical Genetics, Ankara, Türkiye
[3]Aydın Adnan Menderes University Faculty of Medicine, Department of Radiology, Aydın, Türkiye
[4]Aydın Adnan Menderes University Faculty of Medicine, Department of Pediatric Endocrinology, Aydın, Türkiye

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What is already known on this topic?

Mandibuloacral dysplasia (MAD) is associated with mutations in the *LMNA* gene, which plays an important role in maintaining nuclear structure. Previous research has documented various phenotypic manifestations of MAD, but the relationship between specific mutations and clinical variability remains poorly understood, particularly in diverse populations.

What this study adds?

This study identifies a common founder variant in the *LMNA* gene within a Turkish cohort and highlights the significant phenotypic variability observed among affected individuals. By correlating genetic findings with clinical presentation, helping to improve the understanding of the genotype-phenotype relationship in MAD, it is hoped that more personalized diagnostic and therapeutic strategies will emerge.

ABSTRACT

Objective: Mandibuloacral dysplasia (MAD) is a rare genetic disorder characterized by distinctive skeletal abnormalities, metabolic issues, and skin changes, often linked to pathogenic variants in the *LMNA* gene, which encodes lamin A/C. This study investigates a specific founder mutation within a Turkish cohort and explores its impact on phenotypic expressivity.

Methods: We conducted a comprehensive analysis involving genetic testing for *LMNA* variants in patients diagnosed with MAD. Clinical evaluations documented a wide range of phenotypic features, including facial dysmorphism, skeletal anomalies, and metabolic abnormalities. We also collected family histories to assess inheritance patterns and potential environmental influences.

Corresponding Author: Prof. Ahmet Anık, Aydın Adnan Menderes University Faculty of Medicine, Department of Pediatric Endocrinology, Aydın, Türkiye
E-mail: ahmet.anik@yahoo.com **ORCID:** orcid.org/0000-0002-7729-7872
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Results: Our findings identified a common founder mutation in the *LMNA* gene among the cohort, which was present in a significant percentage of participants. Notably, phenotypic expressivity varied significantly, with some individuals exhibiting classic MAD features, while others showed atypical manifestations, such as additional endocrine disorders and variable severity of skeletal anomalies. This variability underscores the complexity of the genotype-phenotype relationship.

Conclusion: This study highlights the significance of the founder mutation in *LMNA* and its diverse phenotypic outcomes in MAD. Our results contribute to the understanding of how genetic mutations can lead to a spectrum of clinical presentations, emphasizing the necessity for personalized clinical approaches in managing this condition. Further research is warranted to elucidate the underlying mechanisms of phenotypic variability and to improve diagnostic and therapeutic strategies.

Keywords: Founder effect, *LMNA* gene, mandibuloacral dysplasia type A, partial lipodystrophy, progeroid syndrome

Introduction

Mandibuloacral dysplasia (MAD) is a rare autosomal recessive progeroid disorder characterized by postnatal growth retardation, mandibular and clavicular hypoplasia, acro-osteolysis of the terminal phalanges, delayed cranial suture closure, joint contractures, lipodystrophy, skin atrophy, alopecia, and mottled skin pigmentation (1,2,3). Patients with MAD present either with partial lipodystrophy [type A; MADA; Online Mendelian Inheritance in Man (OMIM)#248370] or generalized lipodystrophy (type B; MADB; OMIM#608612), resulting from biallelic variants in either the *LMNA* gene, encoding lamin A/C (4,5), or the *ZMPSTE24* gene, encoding zinc metalloprotease (6), respectively. A third type of MAD progeroid syndrome [mandibular dysplasia progeroid syndrome (MDPB) OMIM #619127] due to biallelic variants in the *MTX2* gene encoding metaxin-2 (MTX2) has recently been described (7).

In addition to the cutaneous and skeletal manifestations, MAD patients are predisposed to metabolic complications, including insulin resistance, diabetes mellitus, and hypertriglyceridemia (4,6). While MADA is rarely associated with hepatomegaly or hepatic steatosis, a few cases have been reported (1,4,8). To date, there are approximately 40 patients reported for MADA and 20 patients reported for MADB (9). Furthermore, there are eight patients reported for the recently described MDPS (10). Information regarding genotype-phenotype correlations and the natural progression of MAD subtypes remains limited. Additionally, most existing literature focuses on children and young adults, resulting in a paucity of data on clinical manifestations and metabolic complications in older adults.

Therefore, we present a comprehensive characterization of four newly identified MADA patients from Türkiye, including a 61-year-old female, all carrying the same homozygous pathogenic variant in the *LMNA* gene.

Patients and Methods

Patient 1 (P1)

A 9-year-old female of Turkish descent presented with dysmorphic facial features, skeletal dysplasia of the hands, and a preliminary diagnosis of scleroderma. She was the second child born to healthy consanguineous parents. Her developmental milestones were reported as normal, and her older sister was healthy.

Anthropometric measurements revealed a weight of 36 kg (78th percentile), a height of 140 cm (77th percentile), and a head circumference of 52 cm (72nd percentile). She exhibited increased fat deposition in the periumbilical region and around the neck, with reduced subcutaneous fat in both upper and lower extremities. Additional features included microretrognathia, a bird-like nose, a bifid jaw, acral osteolysis, and increased radiolucency of the distal clavicle on chest X-ray (Figures 1, 2). Skinfold thickness measurements using a Holtain Skinfold Caliper were: biceps, 3.2 mm; triceps, 4.4 mm; subscapularis, 7 mm; and suprailiac, 3.2 mm.

Liver enzymes were elevated, and there was evidence of mild hypertriglyceridemia alongside low levels of high-density lipoprotein (HDL) cholesterol (Table 1). Fasting blood glucose levels were elevated, although glycated hemoglobin (HbA1c) remained within the normal range. Fasting serum insulin levels were notably high. A bone density assessment by dual-energy X-ray absorptiometry (DXA) revealed decreased bone density (Figure 1).

An abdominal ultrasound showed grade 1 hepatic steatosis.

At the most recent follow-up of the patient who has been under surveillance for eight years, and taking metformin 2000 mg/day, omega-3, vitamin E 400 IU/day, and vitamin D 600 IU/day, she had a chronological age of 17 years, a height of 158 cm [-0.8 standard deviation score (SDS)], a weight of 51 kg (-1.2 SDS), a body mass index (BMI) of 20.2 kg/m^2 (-0.7 SDS), and hirsutism was detected with a Ferriman-Gallwey score of 16. Laboratory tests revealed mild transaminase elevation [alanine

aminotransferase, 67 U/L (normal range: 0-55); aspartate aminotransferase, 37 U/L (normal range: 0-34)], high insulin [77.50 µU/mL (normal range: 2-25)], high triglyceride [267 mg/dL (normal range: <150)], low HDL [22 mg/dL (normal range: >40)], and hyperandrogenism [total testosterone, 200 ng/dL (normal range: 0-50), free androgen index 25 (normal range: <5)]. Pelvic ultrasonography findings were consistent with polycystic ovary syndrome, with right and left ovarian volumes measuring 14 and 15 mL, respectively.

Patient 2 (P2)

This 13-year-old male of Turkish origin, the second child of healthy consanguineous parents (first cousins), was a dizygotic twin. He presented with intellectual disability, skeletal dysplasia, and dysmorphic facial features. His birth weight was 2300 g, and his physical appearance was unremarkable at birth. On the second day of life, he developed physiological jaundice, necessitating phototherapy for one week. Postnatally, he required umbilical cord surgery due to an infection and underwent inguinal hernia repair at three months of age.

His twin sister was healthy, however his 25-year-old older brother exhibited similar dysmorphic features (P3). Developmental milestones of P2 were delayed, particularly in gross motor skills. He had achieved independent sitting at 9 months, had ambulated independently at 18 months, and had spoken his first word at 18 months; however, he was unable to form complete sentences. Bladder and bowel control were attained at three years of age. Due to intellectual disability, he required special education. His pubertal development corresponded to Tanner stage 1.

At 13 years of age, his physical examination revealed a height of 136 cm (<3rd percentile) and a head circumference of 53 cm (3-10th percentile). His hand length was 15 cm, and the length of his third finger was 5.5 cm, ruling out brachydactyly or

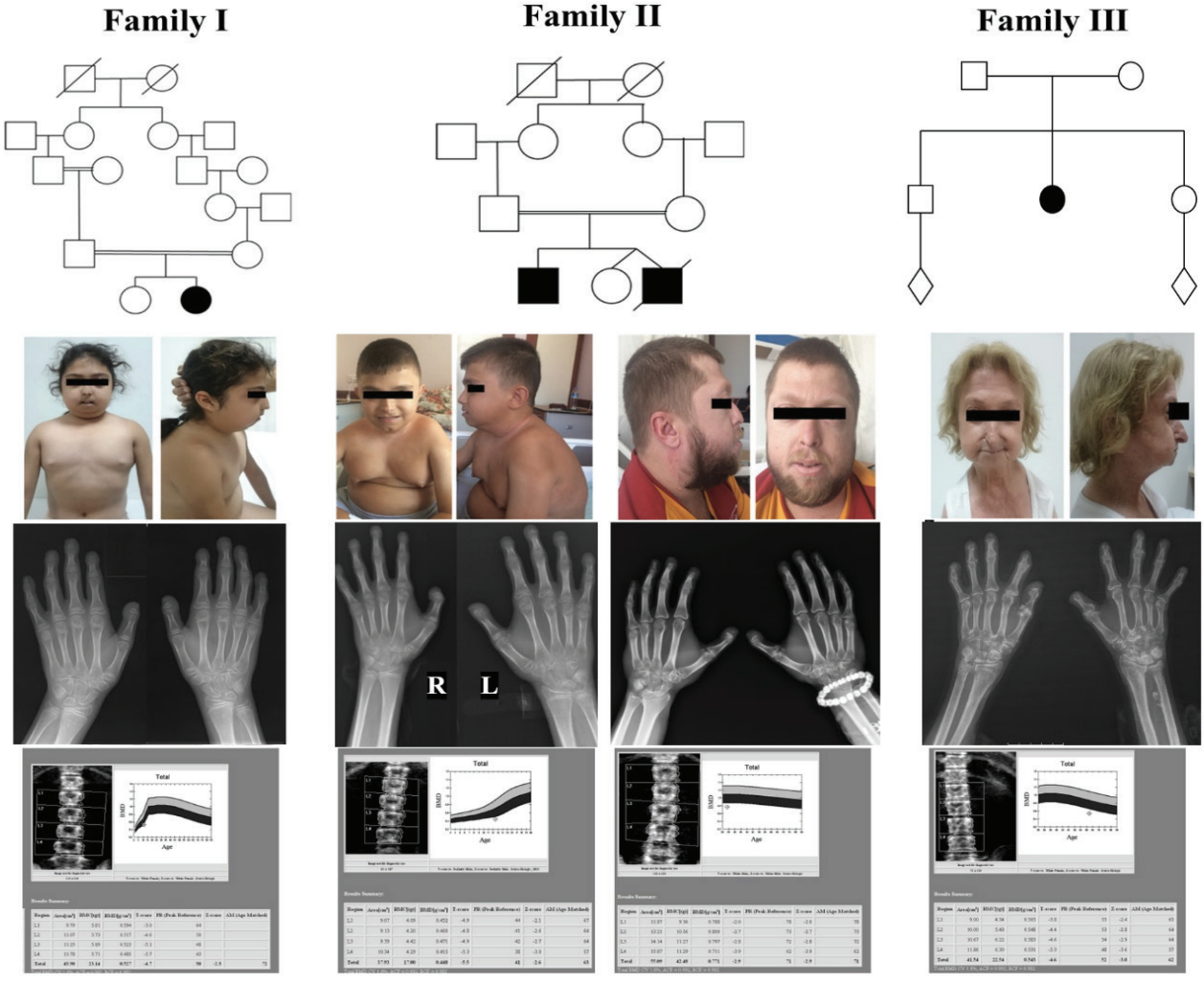

Figure 1. From top to bottom: pedigrees of three families, photographs of patients at the time of diagnosis, hand radiographs demonstrating acral osteolysis, and DXA scans indicating osteoporosis/osteopenia
DXA: dual-energy X-ray absorptiometry

arachnodactyly. His father measured 185 cm tall and weighed 90 kg (BMI SDS: 0.94), while his mother was 165 cm tall and weighed 110 kg (BMI SDS: 4.07). His affected brother was 163 cm tall and weighed 75 kg (BMI SDS: 1.39), whereas his unaffected sister measured 170 cm in height and weighed 80 kg (BMI SDS: 2.07).

The patient exhibited dysmorphic features similar to those of P1, but additionally presented with dental anomalies, gynecomastia, diffuse mottled pigmentation on the trunk, and kyphosis. Cardiological assessments revealed grade 1 mitral insufficiency and dilated cardiomyopathy, with asymmetric left ventricular prominence on cardiac magnetic resonance imaging. Laboratory investigations demonstrated elevated plasma pro-brain natriuretic peptide (418 pg/mL; normal range: 0-100 pg/mL), troponin I (53.6 ng/mL; normal range: 0-36.6 ng/mL), creatine kinase (1118 U/L; normal range: 30-200 U/L), and lactate dehydrogenase (325 U/L; normal range: 125-243 U/L). Serum transaminase and glucose levels were also elevated, though lipid and lipoprotein profiles were within normal limits (Table 1).

Abdominal ultrasonography demonstrated a thickened (5 mm) and trabeculated bladder wall, as well as hepatic steatosis. Bone density assessment by DXA showed decreased bone density (Figure 1). The patient was treated with enalapril (5 mg daily), digoxin (0.25 mg daily), and furosemide (40 mg every other day). Electromyography findings were normal, and there was no evidence of muscle weakness. His karyotype was 46,XY, and an array comparative genomic hybridization study yielded normal results.

At 13 years of age, the patient developed cardiac symptoms and subsequently experienced sudden death.

Patient 3 (P3)

This 25-year-old male, the older brother of P2, exhibited postnatal growth retardation. His dysmorphic features included bird-like facial features, full cheeks, prominent eyes, a pinched nose, joint stiffness, acroosteolysis of the distal phalanges, rounded fingertips, mottled pigmentation, skin atrophy, and partial lipodystrophy characterized by loss of subcutaneous fat from the extremities with increased fat deposition around the trunk and neck.

Radiographic studies confirmed acral osteolysis, although the clavicles appeared normal (Figures 1, 2). Despite these phenotypic abnormalities, his metabolic parameters remained within normal limits. Abdominal ultrasonography revealed grade 2 hepatic steatosis (Table 1). Bone density assessment by DXA identified osteoporosis in the lumbar spine and osteopenia

Table 1. Clinical and laboratory characteristics of the patients at the age of diagnosis

	Patient 1	Patient 2	Patient 3	Patient 4
Age (year)	9	13	25	61
Weight (kg)	36	42	75	33
Weight-SDS	0.71	-0.08	-	-
Height (cm)	140	136	163	134
Height-SDS	0.67	-1.84	-	-
BMI (kg/m^2)	18.37	22.71	27.8	18.4
BMI-SDS	0.58	1.02	-	-
Glucose (mg/dL)	81	94	81	88
Insulin (µU/mL)	42.2	25.1	22.9	8.5
HbA1c (%)	5	5.6	5.6	5.2
ALT (U/L)	190	70	40	12
AST (U/L)	134	47	31	24
Total cholesterol (mg/dL)	207	176	200	214
Triglyceride (mg/dL)	206	108	63	133
HDL cholesterol (mg/dL)	27.8	41.1	46.2	53.8
LDL cholesterol (mg/dL)	138	113	141	134
Creatine kinase (U/L)	72	1118	-	87
Abdominal USG	Grade 1 hepatic steatosis	Grade 1 hepatic steatosis	Grade 2 hepatic steatosis	Normal

SDS: standard deviation score, BMI: body mass index, ALT: alanine aminotransferase, AST: aspartate aminotransferase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, USG: ultrasonography

in the left hip (Figure 1). He did not exhibit cardiomyopathy or intellectual impairment.

At 20 years of age, he was diagnosed with polycythemia vera; however, Janus kinase 2 analyses was performed, and the result was negative. At 21 years old, he developed nephrolithiasis. At 22 years, he underwent surgical excision of a pleomorphic adenoma of the parotid gland.

Patient 4 (P4)

This 61-year-old female of Turkish origin presented with short stature, skeletal dysplasia, and dysmorphic facial features. She weighed 33 kg, measured 134 cm in height, and had a BMI of 18.4 kg/m².

She exhibited dysmorphic features similar to those observed in affected individuals (Figure 1), including joint stiffness in the hands, knees, and elbows, along with prominent eyes, and she had previously been misdiagnosed with rheumatoid arthritis. She had marked irregularity and increased radiolucency of the distal clavicle on chest X-ray (Figure 2). Bone density assessment by DXA revealed osteoporosis in the lumbar spine and osteopenia in the left hip (Figure 1). Complete blood count and biochemical tests, including vitamin D, parathyroid hormone, liver enzymes, and renal function tests, were within normal limits (Table 1).

She had no history of diabetes mellitus or coronary heart disease. Her parents were from the same small village, and she has one healthy brother and a sister. She has no children.

Methods

Written informed consent for the use of genetic information for research purposes was obtained from patients or their legal guardians who underwent genetic testing at Aydın Adnan Menderes University. Since this is a retrospective study, ethical approval was obtained from the Aydın Adnan Menderes University Faculty of Medicine Non-Interventional Clinical Research Evaluation Committee (protocol no.: 2025/96; date: 20.03.2025).

Genomic DNA was extracted from the peripheral blood leukocytes of the probands. Sanger sequencing of the *LMNA* gene was performed on all patients who had been clinically diagnosed with MADA. The entire coding region, along with the highly conserved exon-intron boundaries, was amplified by polymerase chain reaction and sequenced in both directions. The amplicons were analyzed via direct sequencing using the ABI Genetic Analyse 3500 (Life Technologies, Waltham, MA, USA). Results were interpreted using Mutation Surveyor software (Softgenetics, USA).

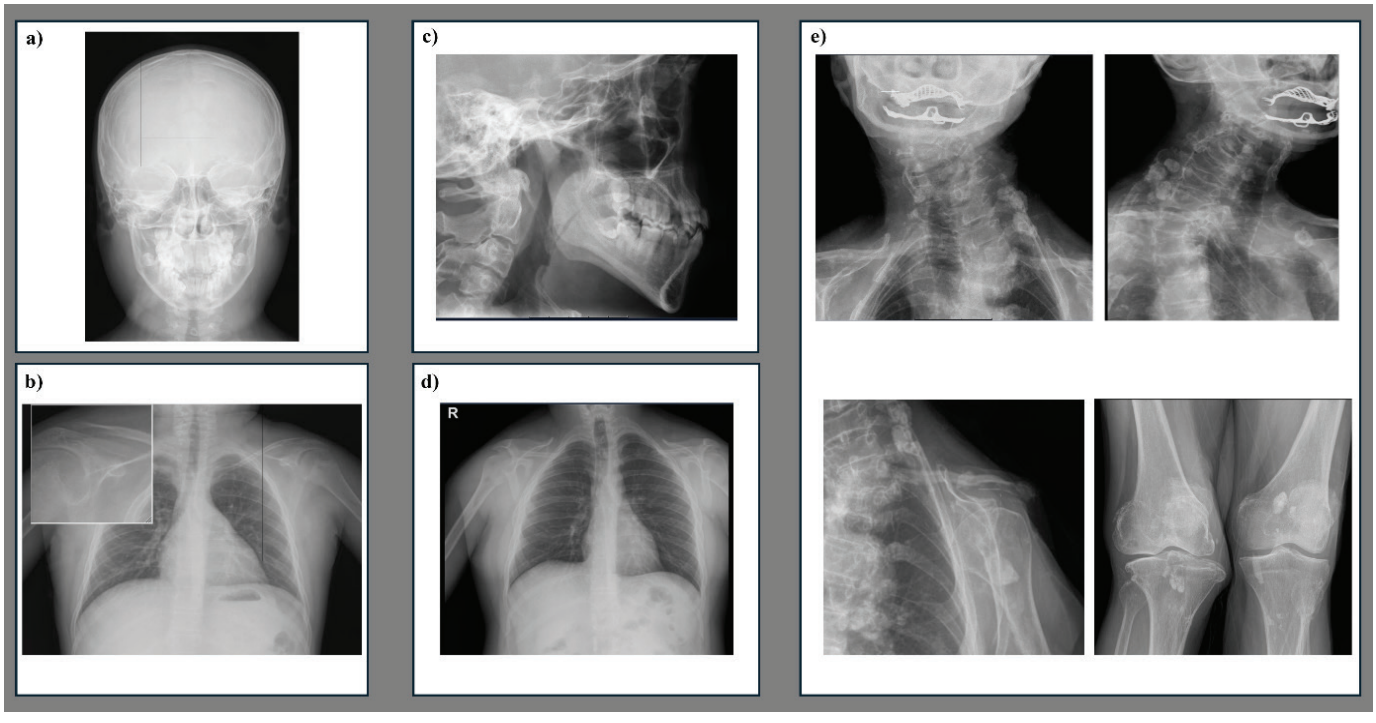

Figure 2. a) Patient 1; skull X-ray demonstrating mandibular hypoplasia. b) Patient 1; increased radiolucency of the distal clavicle. c) Patient 3; lateral skull X-ray demonstrating the absence of mandibular hypoplasia. d) Patient 3; normal clavicle. e) Patient 4; mandibular hypoplasia, soft tissue calcifications, marked irregularity and increased radiolucency of the distal clavicle, advanced degeneration of joint-facing bone surfaces, osteoporosis in bone structures, and vertebral height loss

Whole body magnetic resonance imaging scans (MRIs) of 3 patients were performed with T1- and T2-weighted sequences using PHILIPS Achieva 1.5T, (Philips Inc., Milwaukee, WI, USA).

Statistical Analysis

Descriptive statistics were used to summarize clinical and laboratory findings across the four patients. For continuous variables such as age, height, BMI, insulin, triglyceride, HDL cholesterol, and liver enzyme levels, minimum, maximum, and median values were calculated. Raw data were obtained from Table 1, and mean values were computed by summing individual patient data and dividing by the total number of patients (n=4). These calculations were performed manually to demonstrate phenotypic variability and to illustrate the range and average severity of metabolic and clinical findings within the cohort.

Results

All affected patients were found to harbor the same homozygous variant on *LMNA* gene; chr1:156137210, LMNA (NM_170707.4) c.1586 C>T; which is predicted to result in the substitution of alanine at position 529 with valine (p.Ala529Val). (Clinvar variation ID: 14513, dbSNP: rs60580541).

Among the four patients from three families with MADA harboring the homozygous *LMNA* p.Ala529Val variant, clinical and metabolic parameters demonstrated notable variability. At diagnosis, ages ranged from 9 to 61 years (median: 19 years), while heights varied between 134 cm and 163 cm (median: 138 cm), and BMIs ranged from 18.37 to 27.8 kg/m² (median: 20.56 kg/m²).

Whole-body MRI imaging revealed mandibular hypoplasia, acroosteolysis in the carpal bones, and regional fat accumulation in the gluteal and abdominal side walls in P1. In P3, acroosteolysis was evident in the carpal bones, and focal fat accumulation was observed in the submandibular region, chest, and abdominal side walls. P4 had mandibular hypoplasia, acroosteolysis of the carpal bones, focal fat accumulation in the bilateral submandibular region and gluteal regions, soft tissue calcifications, significant osteoporosis, and decreased corpus heights of the vertebrae (Figure 2, Figure 3).

Discussion

Several biallelic missense variants in *LMNA* have been reported in patients with MAD type A. Among these, the most common variant is p.R527H, frequently identified in Italian patients in a homozygous state (11). This prevalence is attributed to a founder effect and the high rate of consanguinity among the parents. Similarly, four patients from Türkiye have previously been reported to harbor the homozygous p.A529V variant, suggesting a similar founder effect (3,12).

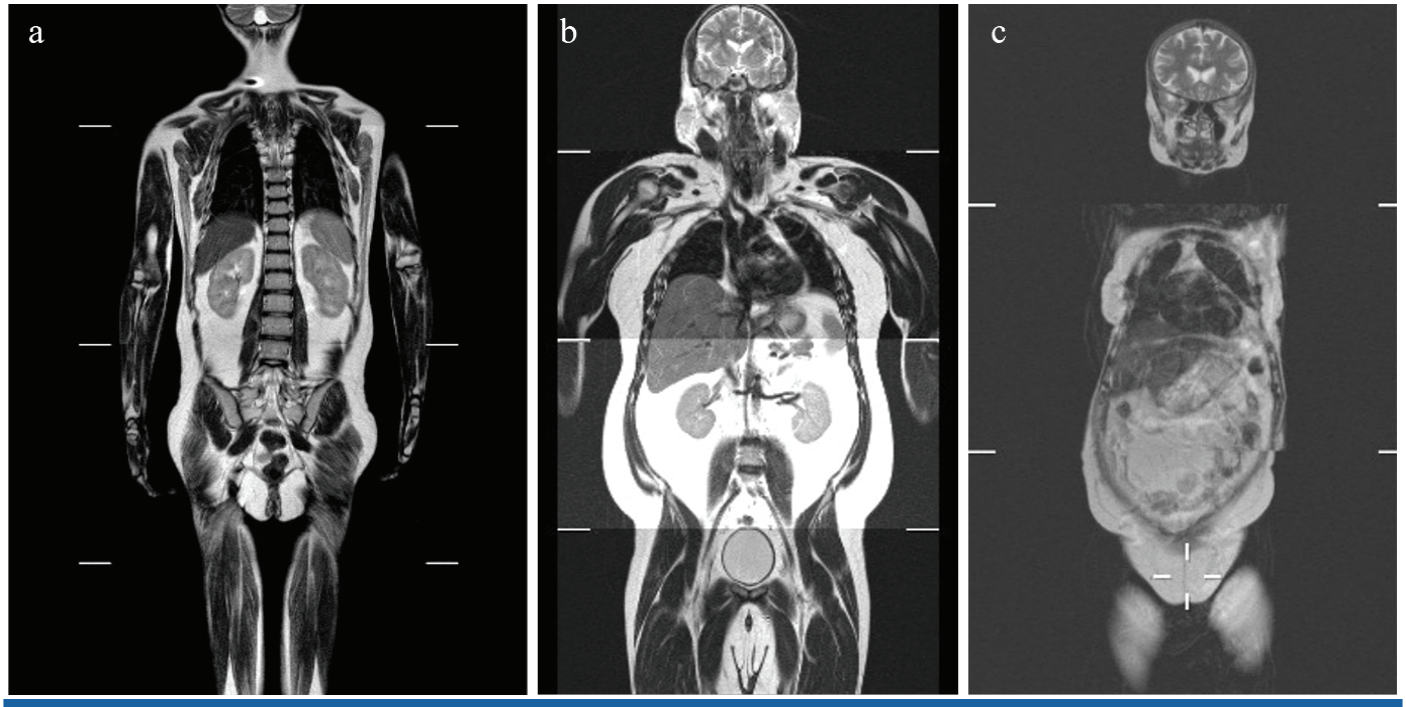

Figure 3. Whole body MRI of the patients. a) Patient 1; regional fat accumulation in the gluteal and abdominal side walls. b) Patient 3; focal fat accumulation in the submandibular region, chest, and abdominal side walls. c) Patient 4; focal fat accumulation in the bilateral submandibular region and gluteal region
MRI: magnetic resonance imaging

In this context, it is necessary to discuss the social circumstances of the four patients presented in this report and their families. These individuals belong to a Turkmen community who historically associated with woodworking. They are an insular society that has preserved its unique cultural and spiritual practices (13). According to the patients and their families, these cultural practices support the hypothesis of a founder effect for the *LMNA* mutation, as marriages have traditionally occurred exclusively within the community. This observation further reinforces the suggestion that a shared ancestor within this closed community likely explains why these three unrelated families, residing in different locations, carry the same pathogenic variant.

This study also provides an opportunity to compare the physical and metabolic profiles of patients from Türkiye with those of Italian patients carrying the homozygous p.R527H variant. Turkish patients exhibited similar skeletal manifestations but had milder metabolic complications (14). Notably, three out of four patients in this cohort showed hepatic steatosis and abnormal liver function tests; however, none developed diabetes mellitus, and hypertriglyceridemia was observed in only one patient.

The occurrence of cardiomyopathy in the proband of pedigree P2 is noteworthy and appears to be unrelated to the *LMNA* variant, as his affected brother does not exhibit this phenotype. However, the absence of cardiomyopathy in the brother does not definitively rule out a connection, as cases of MADA with cardiomyopathy have been reported previously (14). Further insights into this question may emerge as additional MADA cases are reported and our understanding of the *LMNA* gene continues to advance.

Intellectual impairment has not been previously reported in MADA cases associated with *LMNA* variants. This observation suggests that the intellectual disability in this patient may result from the co-inheritance of another undetermined rare genetic variant. Unfortunately, further investigations could not be conducted due to the patient's demise.

When reviewing reports of patients diagnosed with MADA and found to have biallelic variations in the *LMNA* gene to date, only one case has been reported in which the patient did not exhibit micrognathia or mandibular hypoplasia (15). It is therefore noteworthy that P3 did not present with signs of micrognathia and mandibular hypoplasia was not observed on radiographic examination. This phenomenon may be attributed to phenotypic expressivity. The etiology of polycythemia vera in P3 also remains unclear. Additional genetic studies are required to further clarify the findings observed in these patients.

Finally, P4, the oldest MADA patient to date, offers valuable insights into the long-term complications of MADA due to the p.A529V variant. Despite her advanced age, she has not developed diabetes, dyslipidemia, abnormal liver function tests, coronary heart disease, or cardiomyopathy. The absence of metabolic complications despite the patient's advanced age may be attributed to her nutritional habits and environmental factors. According to her medical history, she has maintained a traditional diet characterized by a low intake of processed foods and refined sugars, regular consumption of home-cooked meals, and limited excess calories. Additionally, she has led a physically active life, maintaining sustained daily mobility without prolonged sedentary periods. She has also not been exposed to known cardiometabolic risk factors, such as smoking or chronic occupational stress. These observations, though anecdotal, suggest that favorable dietary habits and a low-risk living and working environment may have mitigated the development of metabolic complications in this patient. This highlights the potential role of environmental modifiers in the phenotypic expression of MADA.

To date, two studies from Türkiye have reported cases of MADA patients who are homozygous for the *LMNA* p.Ala529Val variant (3,12). These earlier cases presented with the classic skeletal and cutaneous features consistent with those observed in our cohort. However, certain findings observed in our patients, such as cardiomyopathy, polycythaemia vera, and intellectual disability, have not previously been described in association with this specific variant. Furthermore, mandibular hypoplasia is considered a hallmark of MADA but it was absent in one of our patients, further illustrating phenotypic variability. Previous studies have consistently noted the absence of breast development in female patients, suggesting that this may be a frequent manifestation of the A529V genotype. However, this finding was not observed in our female patients. These inter-individual differences, despite an identical *LMNA* variant, highlight the possible influence of genetic modifiers or environmental factors on disease expression.

The clinical phenotype associated with the p.Ala529Val variant appears to represent as a distinct, potentially milder form of MADA compared to other *LMNA* variants, such as Val440Met, Arg471Cys, Arg527Leu, Thr528Met, Ala529Thr, Met540Ile, and Met540Thr (11). While core findings, such as acroosteolysis and lipodystrophy, were consistently observed in all patients with the Ala529Val variant, dermatological and metabolic features were notably less prominent. Remarkably, none of the patients exhibited alopecia, and only one showed insulin resistance without progression to diabetes, contrasting with variants such as Lys542Asn and Ala529Thr, which have been associated with more frequent metabolic disturbances. Furthermore, clavicular hypoplasia varied, and the overall severity of lipodystrophy was moderate. Most strikingly, one individual was diagnosed at the age of 61 years, which appears to be the latest age at diagnosis reported for MADA in the available literature. The presence of intellectual disability and polycythemia vera, which have not

previously been described in association with *LMNA*-related MADA, may suggest novel, variant-specific manifestations. However, it remains unclear whether these features are directly attributable to the p.Ala529Val variant or whether they arise from unrelated etiologies. Further research is needed to establish whether these findings broaden the phenotypic spectrum of MADA or represent coincidental comorbidities. Overall, these findings suggest that the p.Ala529Val variant may contribute to a less penetrant, and clinically atypical subgroup within the broader MADA spectrum.

Study Limitations

This study has several limitations. Firstly, the sample size is small, as MADA is an extremely rare disorder, limiting the generalizability of the findings. Secondly, functional studies were not conducted to further investigate the molecular mechanisms of the *LMNA* p.A529V variant. Thirdly, due to the retrospective nature of the study, some clinical and metabolic parameters were not consistently available across all patients.

Conclusion

This study expands the clinical and molecular understanding of MADA caused by the homozygous p.A529V variant in *LMNA*. The identification of a probable founder effect within the patients' community underscores the significance of cultural and genetic factors in the epidemiology of rare diseases. The reported cohort provides unique insights into the metabolic, skeletal, and other atypical complications of MADA, including the first description of intellectual disability associated with *LMNA* mutations. These findings highlight the necessity for comprehensive and multidisciplinary approaches to improve diagnosis, management, and genetic counseling for affected patients.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Aydın Adnan Menderes University Faculty of Medicine Non-Interventional Clinical Research Evaluation Committee (protocol no.: 2025/96; date: 20.03.2025).

Informed Consent: Written informed consent for the use of genetic information for research purposes was obtained from patients or their legal guardians who underwent genetic testing at Aydın Adnan Menderes University.

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Footnotes

Authorship Contributions

Concept: Zehra Manav Yiğit, Ahmet Anık, Design: Zehra Manav Yiğit, Gökay Bozkurt, Ahmet Anık, Data Collection or Processing: Zehra Manav Yiğit, Mustafa Altan, Göksel Tuzcu, Analysis or Interpretation: Zehra Manav Yiğit, Mustafa Altan, Göksel Tuzcu, Literature Search: Zehra Manav Yiğit, Mustafa Altan, Göksel Tuzcu, Gökay Bozkurt, Ahmet Anık, Writing: Zehra Manav Yiğit, Göksel Tuzcu, Gökay Bozkurt, Ahmet Anık.

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Diagnostic Value of Peak-to-Basal Difference or Ratio of Growth Hormone in Children with Growth Hormone Deficiency

Özge Köprülü[1], Elif Gökçe Basa[1], İbrahim Mert Erbaş[1,2], Fatma Yavuzyılmaz Şimşek[1], Özlem Nalbantoğlu[1,2], Hüseyin Anıl Korkmaz[1,2], Behzat Özkan[1,2]

[1]University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, Clinic of Pediatric Endocrinology, İzmir, Türkiye
[2]University of Health Sciences Türkiye İzmir Faculty of Medicine, Department of Pediatrics, İzmir, Türkiye

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What is already known on this topic?

Growth hormone (GH) deficiency is a relatively rare but important cause of short stature in children, and its diagnosis remains challenging due to the limitations of GH stimulation tests.

What this study adds?

In this study, we evaluated the diagnostic performance of basal-to-peak ratio and basal-to-peak difference derived from L-Dopa and clonidine stimulation tests. Our findings indicate that ΔGH (peak-to-basal difference), particularly in the clonidine test, demonstrated excellent diagnostic performance and may serve as a reliable adjunct to conventional peak GH cut-offs in clinical practice.

ABSTRACT

Objective: Growth hormone (GH) deficiency (GHD) is a rare but important cause of short stature in children. Although GH stimulation tests remain the gold standard for diagnosis, establishing a definitive diagnosis continues to be challenging. Our aim was to evaluate the diagnostic performance of the peak-to-basal ratio and difference for identifying GHD in children.

Methods: Patients with short stature who were evaluated for GHD with GH stimulation tests were retrospectively assessed. ΔGH was defined as the difference between peak and basal GH levels. The GH ratio was calculated as the ratio of peak to basal GH levels.

Results: Data were collected from 265 patients (182 prepubertal) with a median age at presentation of 10.6 years (interquartile range: 6.13-12.42), of whom 46.7% were female. In total, 146 patients met the diagnostic criteria for GHD. ΔGH and GH ratio during the L-Dopa and Clonidine stimulation tests were significantly lower in the GHD group ($p<0.001$). A ΔGH cut-off of ≤7.08 in the clonidine test demonstrated

Corresponding Author: Özge Köprülü, MD, University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, Clinic of Pediatric Endocrinology, İzmir, Türkiye
E-mail: ozgeguclu@hotmail.com **ORCID:** orcid.org/0000-0002-0598-3494
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excellent discriminative ability, with both sensitivity and specificity above 80%, and an area under the curve close to 0.9, suggesting that this parameter may may provide supportive diagnostic information for GHD.

Conclusion: To the best of our knowledge, ΔGH has been explored only in a limited number of studies. This study investigated diagnostic accuracy of difference (ΔGH) or ratio of peak-to-basal GH on a large cohort of children with short stature. The supportive diagnostic performance observed in our cohort suggests that ΔGH is clinically useful in routine practice.

Keywords: GH ratio, growth hormone deficiency, growth hormone stimulation tests, short stature, ΔGH

Introduction

Short stature is one of the most common reasons for referral to pediatric endocrinology clinics (1). Short stature is defined as a height below -2 standard deviation (SD) scores (SDS) for age and sex (2).

Growth hormone (GH) deficiency (GHD) is one of the most important causes of short stature in children, and accounts for approximately 10% of cases presenting with short stature. Its prevalence ranges from 1/4,000 and 1/10,000 according to reports from around the world (3,4). Although relatively rare, an accurate and early diagnosis of GHD is important, as recombinant human GH (rhGH) replacement therapy is highly effective. Conversely, a misdiagnosis may lead to unnecessary economic costs and expose patients to avoidable adverse effects (5).

The diagnosis of GHD typically relies on evidence from clinical, auxological, radiological, and biochemical assessments and endocrine dynamic tests (1). Although the assessment of spontaneous GH release is considered the best approach, difficulties associated with technicalities and standardization of results make it challenging (6). A diagnosis of GHD requires a failure to respond to two separate stimulation tests (1).

GH stimulation tests are still the gold standard for the diagnosis of GHD, but controversies remain regarding diagnostic criteria (7). One of the major challenges in the diagnostic process is the uncertainty regarding the cut-off values used to define GHD. The limited availability of reference data on GH secretion in normally growing children and the variability in assay methodologies over time both contribute to this uncertainty (6).

With the advent of monoclonal antibody testing and the implementation of newer standards, GH assay results are approximately 40% lower than those obtained with older immunoassay-based methods. Consequently, the diagnostic cut-offs for GHD should be reduced accordingly. However, no universally accepted threshold has yet been established (1).

In this study, we aimed to evaluate the diagnostic performance of the peak-to-basal ratio and peak-to-basal difference for identifying GHD in children.

Methods

Study Design and Patients

We retrospectively analyzed patients with short stature who were evaluated for GHD with GH stimulation tests at the University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital between December 2022 and August 2025. All patients were followed up in our pediatric endocrinology clinic at a tertiary referral hospital in western Türkiye. A structured questionnaire was used to systematically evaluate all clinical, hormonal and radiological data. The SDS for weight, height, body mass index (BMI), and midparental height were measured according to Turkish children's reference values (8).

Patients with chronic systemic illnesses, chronic conditions affecting growth, untreated or inadequately treated hypothyroidism or other endocrine disorders were excluded from the study. Mild, well-controlled hypothyroidism was allowed if thyroid function had been normalized before testing. In addition, patients with incomplete data regarding GH stimulation tests or biochemical parameters were also excluded from the study.

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Non-Interventional Research Ethics Committee of University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital (approval number: 2025/15-07, date: 02.10.2025). Informed consent was obtained from all subjects and parents involved in the study. Written informed consent has been obtained from the patients and parents to publish this paper.

Hormonal and Biochemical Measurements

Serum GH concentrations were measured by chemiluminescent immunoassay, using Siemens Healthineers IMMULITE 2000 xpi Immunoassay System (Siemens Healthineers USA, 40 Liberty Blvd, Malvern, PA 19355, US). Results were expressed in ng/mL. After an overnight fast, L-Dopa was administered orally at a dose of 10 mg/kg (maximum 500 mg), and blood samples were obtained at 0, 30, 60, 90, and 120 minutes for GH measurement. Following overnight fasting, clonidine was administered orally at a dose of 0.15 mg/m² (maximum 300 mg) body surface area between 08:00 and 09:00 and blood samples were collected at baseline and at

30, 60, 90, and 120 minutes. The highest GH value obtained during the test was defined as the peak GH concentration. Children with peak GH value <10 ng/mL in the first stimulation test underwent a second stimulation test on a separate day. GHD was diagnosed when the peak GH concentration was <10 ng/mL in at least two different stimulation tests. ΔGH was calculated as the difference between peak and basal GH levels in the L-Dopa and Clonidine stimulation tests. The GH ratio was calculated as the ratio of peak to basal GH levels during the L-Dopa and Clonidine stimulation tests.

Statistical Analysis

Statistical analyses of the data were performed using SPSS for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Distribution of data was evaluated using the Kolmogorov-Smirnov test. For numerical comparisons, the Student's t-test or Mann-Whitney U tests were used to assess differences between the two groups according to the normal distribution of the measured parameters. Categorical variables were analyzed with the chi-square test. Receiver operating characteristic (ROC) curves were used to define the cut-off values for the ratios and delta of GH levels in clonidine and L-Dopa tests that yielded the highest sensitivity and specificity. Data are presented as mean±SD or median and interquartile range (IQR, 25th-75th percentile). In all statistical tests, p values <0.05 were considered as statistically significant.

Results

A total of 317 patients with short stature who underwent GH stimulation testing were included in the analysis. Fifty-two patients with a peak GH >10 ng/mL in the first stimulation test were excluded from the study. Data were collected from the remaining 265 patients, of whom 182 were prepubertal and 46.7% were girls, with a median age at presentation of 10.6 years (IQR: 6.13-12.42). In total, 146 (55.1%) patients met the diagnostic criteria for GHD.

The cohort with GHD consisted of 146 children, including 79 (54.1%) male and 67 female, with a median age of 10.3 years (IQR: 6.3-12.6). These children had a median height SDS of -2.64 (IQR: -3.02– -2.37) and a mean BMI SDS of -0.55±1.07. The GH peak responses to L-Dopa and Clonidine stimulation were 3.09 and 5.14 ng/mL, respectively.

Table 1 summarizes the demographic, clinical, and laboratory findings of the patients, comparing those diagnosed with GHD to those without GHD. Chronological age, age by height, bone

Table 1. The demographic, clinical, and laboratory findings of the patients

	GH deficiency (n=146)	Normal (n=119)	p value
Gender (male/female)	79/67	63/56	0.846
Prepubertal/Pubertal	102/45	80/38	0.781
Chronological age (years)	10.3 (6.3-12.6)	9.0 (5.9-12.3)	0.398
Age by height (years)	7.59 (4.45-9.98)	6.84 (3.83-9.45)	0.321
Bone age (years)	8.0 (4.0-11.0)	7.0 (3.5-11.0)	0.468
Weight, SDS*	-1.89±1.04	-2.19±0.88	0.008
Height, SDS	-2.64 (-3.02–-2.37)	-2.69 (-3.16–-2.29)	0.863
BMI, SDS*	-0.55±1.07	-0.83±0.88	**0.020**
MPH, SDS*	-1.26±0.98	-1.39±0.91	0.342
IGF-1, SDS	-1.61 (-2.27–-1.15)	-1.46 (-2.21–-0.79)	0.121
IGFBP-3, SDS	-0.41 (-1.05-0.27)	-0.22 (-0.63-0.34)	0.051
L-Dopa			
Peak GH, L-Dopa	3.09 (1.79-4.60)	6.49 (4.11-11.25)	**<0.001**
ΔGH, L-Dopa	2.37 (0.53-3.77)	5.15 (2.21-10.70)	**<0.001**
GH ratio, L-Dopa	7.78 (1.69-28.19)	16.6 (5.2-91.6)	**0.001**
Clonidine			
Peak GH, Clonidine	5.14 (3.20-7.11)	13.05 (11.4-15.70)	**<0.001**
ΔGH, Clonidine	4.59 (2.34-6.44)	11.79 (9.59-14.40)	**<0.001**
GH ratio, Clonidine	12.77 (4.10-38.8)	26.15 (8.80-68.5)	**0.006**

*Normal distribution (Student's t-test). Data are given as mean ± SD or median (IQR 25-75 percentile).
ΔGH: peak GH-basal GH in the L-Dopa and Clonidine provocation tests.
GH ratio: ratio of peak-to-basal GH in the L-Dopa and Clonidine provocation tests.
SDS: standard deviation score, GH: growth hormone, BMI: body mass index, MPH: midparental height, IGF-1: insulin-like growth factor 1, IGFBP-3: insulin-like growth factor binding protein 3

age, weight SDS, height SDS, mid-parental height SDS, insulin-like growth factor-1, SDS, and IGF-binding protein-3 SDS were similar between the groups.

As expected, peak GH responses during both the L-dopa and clonidine stimulation tests were significantly lower in patients with GHD compared with those without GHD. ΔGH (the difference between peak and basal GH levels) in the stimulation tests were significantly lower in the GHD group ($p<0.001$). GH ratio (the ratio of peak-to-basal GH levels) during the L-Dopa and Clonidine stimulation tests were significantly lower in the GHD group ($p<0.001$).

ROC analysis revealed that the cut-off value of GH ratio in the L-Dopa stimulation test ≤9.98 supported good diagnostic prediction with 57.2% sensitivity and 63.3% specificity [area under the curve (AUC)± standard error (SE), 0.627±0.038; $p=0.001$] (Figure 1). ROC curve analysis also identified a cut-off value of ≤4.04 for the ΔGH in the L-Dopa stimulation test which yielded 82.2% sensitivity and 60.9% specificity (AUC±SE, 0.735±0.036; $p=0.001$) (Figure 2).

ROC curve analysis showed that a GH ratio cut-off value of ≤27.4 in the Clonidine stimulation test provided good diagnostic performance, with 66% sensitivity and 49.4% specificity (AUC±SE, 0.610±0.039; $p=0.001$) (Figure 3). ROC curve analysis demonstrated that a cut-off value of ΔGH ≤7.08 in the clonidine stimulation test also provided good diagnostic accuracy, with 81.3% sensitivity and 86.2% specificity (AUC±SE, 0.892±0.029; $p<0.001$) (Figure 4).

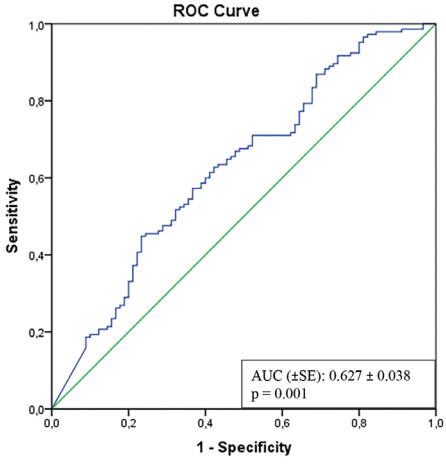

Figure 1. ROC curve analysis of the GH ratio in the L-Dopa stimulation test
ROC: receiver operating characteristic, AUC: area under the curve, SE: standard error, GH: growth hormone

Discussion

In this study, we investigated the diagnostic performance of the GH ratio and ΔGH on L-Dopa and clonidine stimulation tests for identifying GHD in children. Our findings demonstrated that ΔGH and GH ratio may provide additional supportive evidence, particularly ΔGH in the clonidine test.

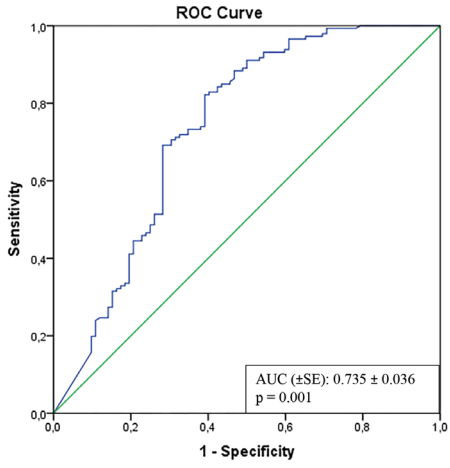

Figure 2. ROC curve analysis of the ΔGH in the L-Dopa stimulation test
ROC: receiver operating characteristic, AUC: area under the curve, SE: standard error, GH: growth hormone

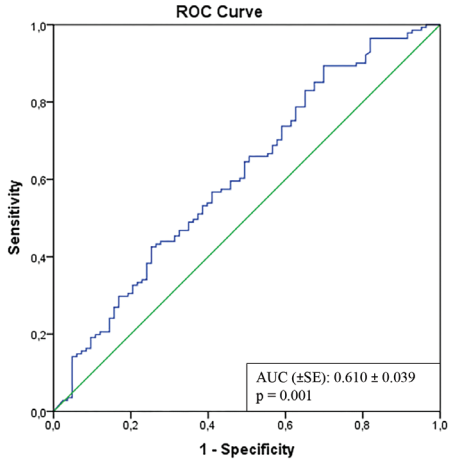

Figure 3. ROC curve analysis of the GH ratio in the Clonidine test
ROC: receiver operating characteristic, AUC: area under the curve, SE: standard error, GH: growth hormone

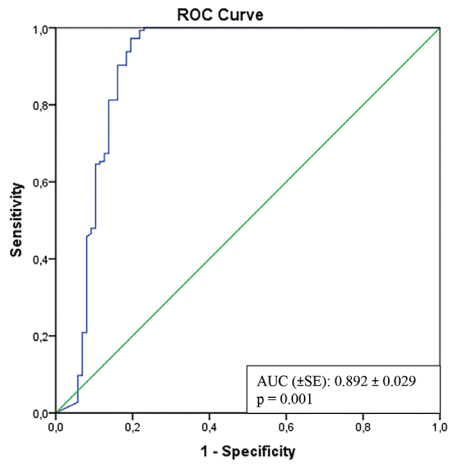

Figure 4. ROC curve analysis of the ΔGH in the Clonidine stimulation test
ROC: receiver operating characteristic, AUC: area under the curve, SE: standard error, GH: growth hormone

A diagnosis of GHD is established when peak GH responses are subnormal in at least two independent stimulation tests (4,9). As expected, in our cohort, the peak GH levels were lower in children with GHD than in non-GHD.

GHD accounts for only a small proportion of children with short stature, but misdiagnosis is common and may expose children to unnecessary treatment (6). Despite the dramatic changes in GHD treatment since the 1960s, diagnosing GHD remains challenging (6,10). Previous studies have also reported the challenges of GH stimulation tests. Furthermore, GH secretion may be influenced by factors such as obesity, undernutrition, sex, age and puberty (1,6,11). In fact, due to the inherent limitations of GH stimulation tests, the Pediatric Endocrine Society guidelines recommend against using GH stimulation test results as the sole diagnostic criterion for GHD in children and emphasize the importance of integrating auxological, biochemical, and imaging findings in the diagnostic process (12).

GH stimulation tests remain controversial, due to their low sensitivity and specificity, which further reduce their diagnostic reliability (13). Traditionally, a peak GH cut-off of <10 µg/L has been used in children. However, some experts have proposed lowering this threshold to <7 µg/L. Although diagnostic guidelines have been revised over the past decades and peak GH cut-off values have been modified accordingly, these thresholds remain largely arbitrary, particularly in pediatric populations (14).

The difficulties in performing GH stimulation tests, the potential adverse effects, reference data for GH secretion in normally growing children, the high false-positive rates and the uncertainty of consensus on cut-off values have led researchers to explore new diagnostic strategies (4,7,11,15,16).

The rationale for using ΔGH or GH ratio lies in their ability to capture dynamic responsiveness rather than relying on a single stimulated peak, which may be influenced by pre-test conditions, body composition, or assay variability. In our cohort, ΔGH improved the discrimination of the tests, supporting the concept that such dynamic indices may offer supplementary information rather than serving as independent diagnostic tools.

One possible explanation for false-negative results in GH stimulation testing is the occurrence of a spontaneous physiological GH peak shortly before the test (9,17), which may blunt the stimulated response. In such cases, considering the increase relative to the basal value may provide a better reflection of the pituitary reserve and secretory capacity than absolute peak concentrations alone.

In children with peak GH responses <5 ng/mL, the diagnosis of GHD is clearer (2,7,9,12). However, when peak GH values is in the range of 5-10 ng/mL, the diagnosis becomes more challenging and requires additional values and supportive criteria. Our study extends previous findings by identifying a ΔGH cut-off of ≤7.08, although its clinical utility remains dependent on the reference peak GH threshold used to define GHD. In our cohort, a ΔGH value of ≤7.08 in the clonidine stimulation test demonstrated reasonable discriminative performance, with sensitivity (81.3%) and specificity (86.2%) both exceeding 80% and an AUC approaching 0.9. These results suggest that ΔGH may provide supportive diagnostic information, particularly when evaluating children within the borderline peak GH range of 5-10 µg/L. However, although ΔGH showed discriminative ability in this subgroup, it did not confer a clear diagnostic advantage over conventional peak GH criteria. Therefore, ΔGH should be interpreted as an adjunctive rather than a primary diagnostic parameter.

To the best of our knowledge, ΔGH has rarely been investigated in the diagnostic work-up of pediatric GHD. Borges et al. (18) addressed this parameter and reported that both GH peak concentrations and ΔGH were significantly lower in children with GHD compared to non-GHD groups. Our study extends these findings by identifying a ΔGH cut-off (≤7.08) with supportive performance, thereby providing novel evidence that this parameter may serve as a reliable criterion.

Study Limitations

This study has certain limitations. First, the retrospective, single-center design inherently restricts the generalizability of our findings, as patient characteristics, clinical approaches, and stimulation protocols may differ across institutions. Second, only L-Dopa and clonidine stimulation tests were used; the proposed ΔGH and GH ratio thresholds were not validated against more reliable reference tests, such as the insulin tolerance test (ITT).

Third, subgroup analyses by pubertal stage, sex, BMI and magnetic resonance imaging (MRI) characteristics were limited. Another limitation of our study is that GHD was defined based on stimulation test results rather than structural or genetic confirmation. However, this definition is consistent with most clinical studies in the field, as the ITT or MRI-based criteria are not routinely available for all patients. Finally, longitudinal outcomes, particularly growth response to rhGH treatment, were not available. Prospective, multicenter studies are needed to examine whether baseline ΔGH and GH ratio predict rhGH treatment response.

Conclusion

The strong supportive diagnostic performance observed in our cohort suggests that ΔGH is clinically useful in routine practice. However, validation in larger, multicenter studies with other supporting diagnostic data, such as cranial MRI and/or ITT, is needed before it can be widely adopted.

Ethics

Ethics Committee Approval: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Non-Interventional Research Ethics Committee of University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital (approval number: 2025/15-07, date: 02.10.2025).

Informed Consent: Informed consent was obtained from all subjects and parents involved in the study. Written informed consent has been obtained from the patients and parents to publish this paper.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Özge Köprülü, Concept: Özge Köprülü, İbrahim Mert Erbaş, Behzat Özkan, Design: Özge Köprülü, İbrahim Mert Erbaş, Behzat Özkan, Data Collection or Processing: Özge Köprülü, Elif Gökçe Basa, Fatma Yavuzyılmaz Şimşek, Özlem Nalbantoğlu, Hüseyin Anıl Korkmaz, Analysis or Interpretation: Özge Köprülü, Elif Gökçe Basa, Literature Search: Özge Köprülü, Writing: Özge Köprülü, İbrahim Mert Erbaş, Behzat Özkan.

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ORIGINAL ARTICLE

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Nailfold Capillaroscopy: A Non-Invasive Tool for Early Detection of Microvascular Alterations in Children with Type 1 Diabetes Mellitus

Gözde Akın Kağızmanlı[1], Tuncay Aydın[2], Kübra Yüksek Acinikli[1], Rana İşgüder[2], Zehra Kızıldağ Karabacak[2], Korcan Demir[1], Ece Böber[1], Şevket Erbil Ünsal[2], Ayhan Abacı[1]

[1]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye
[2]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Rheumatology, İzmir, Türkiye

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What is already known on this topic?

Nailfold capillaroscopy (NC) is a non-invasive and practical method for assessing microvascular structures and has been widely used to detect vascular alterations in various systemic diseases. In type 1 diabetes mellitus (T1DM), microvascular complications such as retinopathy, nephropathy, and neuropathy are well-established consequences of chronic hyperglycemia. However, microvascular structural and functional abnormalities can develop during childhood and adolescence, even in the absence of clinically evident vascular disease. Early detection of these changes is essential for timely intervention and the prevention of long-term complications. Although NC has the potential to identify early microvascular alterations in pediatric T1DM patients, data in this population remain limited. Most existing studies have focused on adults, underscoring the need for further research to clarify the role of NC in early diagnosis, monitoring, and the characterization of diabetes-related microvascular changes in children and adolescents.

What this study adds?

This study demonstrates that microvascular structural and functional abnormalities can develop in children and adolescents with T1DM even before the appearance of clinically evident vascular complications. It highlights the significant association between poor glycemic control, longer disease duration, and capillaroscopic alterations, particularly reduced capillary density. The findings emphasize the potential of NC as a valuable, non-invasive tool for the early detection and monitoring of microvascular changes in pediatric T1DM populations. Early identification of these alterations may facilitate timely interventions aimed at preventing the progression of vascular complications.

Corresponding Author: Prof. Ayhan Abacı, MD., Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye
E-mail: ayhan.abaci@deu.edu.tr **ORCID:** orcid.org/0000-0002-1812-0321
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ABSTRACT

Objective: Nailfold capillaroscopy (NC) is a non-invasive tool that can detect microvascular changes in the early stages of vascular disease. To assess capillary microarchitecture in children with type 1 diabetes mellitus (T1DM) and its relationship with clinical characteristics, laboratory findings, and glycemic control.

Methods: We included children and adolescents with T1DM, aged 6-18 years, and diagnosed for at least one year and an equal number of age- and sex-matched healthy controls. For all patients with T1DM, data on diabetes duration were collected, and the average annual HbA1c value was calculated for the four measurements made at routine follow-up in the preceeding year. In patients using 24-hour continuous glucose monitoring (CGM) devices, glycemic data from the previous three months were analyzed. The capillaroscopic findings were evaluated by two different researchers with experience in the field of pediatric rheumatology. Capillaroscopic parameters were compared based on glycemic control (HbA1c ≥7.5% vs. <7.5%), disease duration (<5 vs. ≥5 years), time in range (TIR≥70% vs. <70%), and glucose variability (CV≤36% vs. >36%).

Results: The median age of the 55 patients with T1DM was 14.5 (11.3-17.2) years, with a median disease duration of 3.8 (2.3-6.7) years. Compared to controls, patients with T1DM had significantly lower capillary density and more frequent dilated, tortuous, cross-linked, and abnormal capillaries ($p<0.001$, $p<0.001$, $p<0.001$, $p=0.01$, and $p=0.03$, respectively). Capillary density was significantly lower in patients with poor glycemic control ($p<0.001$) and those with longer disease duration ($p=0.02$). A negative correlation was observed between capillary density and disease duration ($r=-0.3$, $p=0.02$). After adjusting for age, gender, body mass index, and diabetes duration, capillary density remained negatively correlated with average HbA1c ($r=-0.4$, $p=0.004$). Among CGM users (n=22), capillary density showed a positive correlation with TIR ($r=0.5$, $p=0.04$), even after adjustment for confounders.

Conclusion: Children with T1DM exhibited significantly higher microvascular changes, mostly associated with poor glycemic control, compared to healthy controls. NC may be a useful technique for detecting early alterations in the capillary structures of children with T1DM, even in the absence of overt clinical microvascular complications.

Keywords: Diabetic vasculopathy, morbidity, hyperglycemia, insulin, screening

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease that affects the microvasculature as well as other systems. Chronic hyperglycemia causes various molecular and biochemical changes, leading to a chronic proinflammatory process that damages capillaries. These changes become evident in the initial stages of vascular disease, before the development of diabetic macro- and microvascular complications (1,2). Although these complications are uncommon in childhood and adolescence, early vascular functional and structural deterioration may occur during this period (3). Given that the majority of diabetes-related complications typically manifest later in life, the early detection of subclinical vascular changes in childhood offers an important opportunity for earlier intervention. Early identification and appropriate management may delay or even prevent the progression of microvascular and cardiovascular complications, thereby reducing morbidity and mortality (3,4).

Various factors, such as genetics, gender, pubertal stage, duration of diabetes, glycemic control, and lifestyle, play a role in the development of vascular complications. It is important to identify vascular disease in patients with diabetes before the onset of clinically evident complications (3). Different techniques are used to detect early microvascular damage, including Doppler flowmetry, ophthalmoscopy, optical coherence tomography and 24-hour ambulatory blood pressure monitoring (5,6,7). However, these methods have several limitations. For instance, while Doppler flowmetry offers real-time measurement of blood flow, it is limited in its ability to provide structural information about microvessels and may be affected by movement artifacts, making it less reliable in clinical practice (8,9). Ophthalmoscopy, although effective for detecting retinopathy, is restricted to assessing retinal vessels and does not provide a comprehensive view of systemic microvascular health (10). Similarly, 24-hour ambulatory blood pressure monitoring focuses on blood pressure fluctuations but does not directly assess microvascular structure or function. This method can also be costly and impractical for repeated use due to patient compliance issues, and physical activity throughout the day can affect the accuracy of readings (11). In contrast to these techniques, nailfold capillaroscopy (NC) is a non-invasive, simple, cost-effective, and reproducible imaging technique that assesses both quantitative and qualitative characteristics of nailfold microvasculature. It allows for the direct visualization of capillary structure, making it a valuable tool for detecting subtle microvascular changes. In recent years, NC has gained importance for diseases that affect vascular structure and function, such as diabetes, in addition to connective tissue diseases (12).

Few published studies have assessed the effectiveness of the NC method in detecting early vascular disease in children and adolescents with T1DM (5,6,13,14). Moreover, to the best of our knowledge, there is no data available on the use of this technique in clinical practice for patients using a 24-hour continuous glucose monitoring (CGM) system. The objective of this study was to examine the presence of nailfold capillary abnormalities in children with T1DM by comparing them to age- and sex-matched healthy controls.

Methods

Patients

This cross-sectional, single-center study involved patients aged 6-18 years diagnosed with T1DM and followed up in our outpatient clinic for at least a year, along with an equal number of healthy individuals of similar age and sex who were referred to the pediatric endocrinology clinic for various reasons. Diagnosis of T1DM was made according to the criteria of the International Society of Pediatric and Adolescent Diabetes (15). Children with other types of diabetes, such as type 2 diabetes, maturity-onset diabetes of youth, and secondary diabetes, as well as patients with connective tissue disease or autoimmune diseases, those with traumatic lesions in the periungual fold of the finger, recent infections, or those using medications known to affect microcirculation (e.g., vasodilators, antihypertensive drugs), were excluded from the study. Informed consent was obtained from each patient or their legal guardians.

Clinical Evaluation

A thorough analysis of medical records was conducted retrospectively to collect information such as the anthropometric measurements, physical examinations, duration of diabetes, insulin therapy, use of insulin pumps and CGM devices, as well as the history of diabetes-related complications such as retinopathy, neuropathy, or nephropathy.

Height was measured with a Harpenden stadiometer capable of measuring with an accuracy of 0.1 cm. Body weight was measured in underwear without shoes using an electronic scale (SECA, Hamburg, Germany) to the nearest 0.1 kg. The standard deviation (SD) scores for height, weight, and body mass index (BMI) were calculated using an online tool (Child Metrics), according to the Turkish standards established by Neyzi et al. (16).

Laboratory tests consisted of the most recent measurements of glycated hemoglobin (HbA1c), and fasting lipid profile, which included triglycerides (TG), total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol, as well as urinary albumin creatinine ratio measured from an early morning, fasting urine sample. In addition, the annual mean HbA1c was calculated, and glycemic control was defined based on the mean HbA1c levels. Mean annual HbA1c was calculated by averaging the four most recent measurements made at three-monthly routine follow-up clinic visits. Patients with HbA1c <7.5% were considered to have good glycemic control, while those with HbA1c ≥7.5% were classified as having poor glycemic control. Furthermore, patients with diabetes were divided into two subgroups according to disease duration (≥5 years vs. <5 years) when assessing microvascular alterations.

In diabetic patients using CGM devices, the glucose data for the last three months were obtained. Time in range (TIR) was defined as the percentage of readings and time per day within the target glucose range of 70 to 180 mg/dL. Achieving a percentage of over 70% for the time in the target glucose range was accepted as indicating good glycemic control. Glycemic variability [coefficient of variation (CV)] was defined as the percentage fluctuation in blood glucose. The glucose profile was considered stable when CV was ≤36%. Patient subgroups were created using CGM data based on TIR (≥70% vs. <70%) and CV (≤36% vs. >36%) (17).

Capillaroscopic Evaluation

All patients underwent a comprehensive assessment by two different researchers experienced in the field of pediatric rheumatology. The researchers were blinded to the participants' group status (patients with T1DM or healthy controls) to minimize potential bias and ensure objectivity in the evaluations. Each image was independently evaluated by both observers using the predefined criteria of the European League Against Rheumatism developed in 2020 for the standardization of NC in the evaluation of patients with Raynaud's phenomenon and systemic sclerosis were used for qualitative assessment (18). In cases of disagreement, the images were re-examined and discussed collaboratively until a consensus was reached. However, there were no significant discrepancies between the two assessors. The mean of the two observers' measurements was used for data analysis. This approach was designed to eliminate inter-rater reliability and ensure consistency and accuracy in the final evaluation of the capillaroscopic findings.

To standardize evaluations, capillaroscopy was performed under controlled conditions, following a 15-20-minute rest period at room temperature. Fingers affected by recent local trauma were excluded from the analysis. Images were captured at 200× magnification using the Dino-Lite CapillaryScope 200 Pro/MEDL4N Pro, a validated digital capillaroscopy device, and analyzed with DinoCapture 2.0, version 1.5.49.B software (Dino-Lite Europe, IDCP B.V., The Netherlands). For optimal visualization, a drop of immersion oil was applied to the nail bed to improve image resolution. Each examination involved the assessment of eight fingers, excluding the thumbs, with two distinct images obtained from the midline of each finger.

The capillaroscopic examination consisted of both quantitative and qualitative analyses. A quantitative assessment was performed on images obtained from a 1 mm length area in the distal row of capillaries. This analysis evaluated several parameters, including capillary density, width, and length, intercapillary distance, capillary morphology, and the presence of microhemorrhages and avascular areas. The qualitative analysis included general pattern recognition, where the images were classified as either showing a scleroderma pattern or a non-scleroderma pattern, which could be normal or exhibiting non-specific abnormalities.

"Capillary density" refers to the number of capillaries per millimeter, and ≤7 capillaries per mm indicates a decrease in capillary number. A capillary diameter (arterial, venous, or apical) <20 µm was considered normal; an increase from 20 µm to 50 µm was classified as an enlarged capillary; and a diameter ≥50 µm was classified as a giant capillary. Giant capillaries have been described as potentially indicative of an underlying scleroderma spectrum disorder. An intercapillary distance of more than 500 µm in the distal row of capillaries was considered an "avascular area." Microbleeding around the capillary appears in dark masses adjacent to the distal row, known as "micro-hemorrhages". Capillaries exhibiting a hairpin shape or a "tortuous" shape in which the afferent and efferent limbs bend but do not intersect, as well as a shape that is "cross-linked" once or twice, were defined as normal capillary morphology. All other shapes were defined as having "abnormal" morphology (Figure 1).

For each participant, 16 images obtained from eight fingers were analyzed for the presence or absence of capillaroscopic parameters, including decreased capillary density, the presence of enlarged or giant capillaries, cross-linked capillaries, tortuosity, abnormal morphology, avascular area, and microhemorrhages. In both groups, capillary abnormalities were defined as the presence of signs in at least two fingers. In addition, capillary density, length, width, and distance between capillaries in each image were measured and calculated as the mean.

Ethics

This study was approved by the Dokuz Eylül University Non-Interventional Research Ethics Committee (approval no: 2023/41-02, date: 20.12.2023) and performed in line with the principles of the Declaration of Helsinki.

Statistical Analysis

Statistical analyses were performed using the SPSS program for Windows, version 24.0 (IBM Co., Armonk, NY, USA). The data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Descriptive statistics for categorical variables are presented as a number (%), while continuous variables are reported as a mean±SD for normally distributed data and as medians with the respective 25-75th percentile (interquartile range) values for non-normally distributed data. Comparisons between categorical variables were conducted using the Pearson chi-square test or Fisher's exact test, as appropriate. The Student's t-test was used to compare normally distributed continuous variables between the T1DM and control groups. The Pearson correlation test was used to assess correlations between capillary density and continuous variables. Then, any correlation was investigated among the identified significant variables after adjusting for age, gender, and BMI. Multivariate linear regression analysis was performed to examine the association between capillary density and potential confounding factors, including age, gender, BMI, duration of T1DM, insulin dose, average HbA1c, and LDL-C and TG levels as independent variables, with capillary density as the dependent variable. A two-sided p-value of <0.05 was regarded as statistically significant.

Effect Size and Power Analysis

The effect size in this study was calculated based on capillary density, a key parameter in assessing microcirculation. It was determined by calculating the standardized difference between the two means, divided by the SD for the two independent groups (19). The analysis revealed a large effect size (Cohen's d=1.6), indicating a significant difference in capillary density between the T1DM group and the control group. Moreover, a post-hoc power analysis was performed using G*Power (version 3.1.9.4).

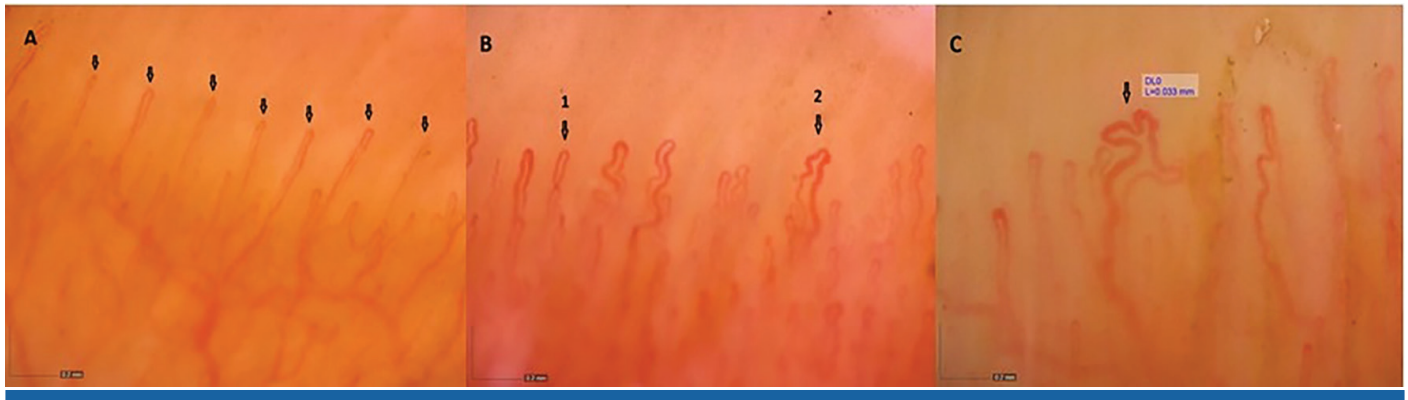

Figure 1. Examples of capillaroscopy findings in the analyzed groups of healthy controls (A and B) and a patient with T1DM (C). **A)** Capillaries with a normal "hairpin" shape; **B)** capillaries that display crossing (1) and tortuosity (2) patterns are considered not indicative of any pathological conditions; **C)** abnormal morphology and enlarged capillary with an increase in capillary diameter (20-50 µm)
T1DM: type 1 diabetes mellitus

With 55 subjects in both the T1DM and control groups, and an effect size of 1.6, the analysis demonstrated that the study achieved a statistical power of greater than 90% at a significance level of α=0.05.

Results

The Baseline Characteristics of Study Subjects

The study included 55 patients with T1DM, with a median age of 14.5 (11.3-17.2) years. We compared their data with those of 55 age-matched healthy controls [13.0 (10.2-16.0) years, p=0.2]. The female/male ratio in the control group was 28/27, while it was 24/31 in the diabetes group, demonstrating similar ratios (p=0.5). In addition, the pubertal stage, height, weight, and BMI SD scores were similar between the two groups (p=0.5, p=0.8, p=0.4, and p=0.3, respectively). The clinical and laboratory characteristics of the subjects with T1DM are listed in Table 1.

NC

Table 2 presents the comparison of capillaroscopic parameters between the two groups. Patients with T1DM exhibited a significantly lower capillary density than healthy individuals (p<0.001). In addition, T1DM patients had significantly increased arterial, venous, and apical diameters compared to healthy individuals (p<0.001). Furthermore, in the T1DM group, there were more dilated, tortuous, cross-linked, and abnormally morphological capillaries (p<0.001, p<0.001, p=0.01, and p=0.03, respectively). Moreover, non-specific abnormalities were also more common in the T1DM group (p<0.001).

Capillaroscopic parameters were found to be similar in both male and female patients with diabetes. When the patients were categorized by age (under and 12 years and over), no significant differences were observed in qualitative and quantitative capillaroscopic features, except for larger arterial and apical diameters in the older group (p=0.02 and p=0.03, respectively). Similarly, no significant differences were detected between the prepubertal and pubertal T1DM groups, except for greater apical, arterial, and venous diameters in the pubertal group (p=0.03, p=0.01, and p=0.04, respectively).

Table 3 shows the capillaroscopy findings in patients with diabetes, categorized based on HbA1c levels and disease duration. Patients with poor glycemic control, defined as average HbA1c≥7.5% in the previous year, exhibited significantly lower capillary density compared to those with good glycemic control (p<0.001). Capillary density was found to be negatively correlated with the average HbA1c (r=-0.5, p<0.001) (Figure 2). After adjusting for age, gender, BMI, and diabetes duration, the negative correlation between capillary density and average HbA1c persisted (r=-0.4, p=0.004). In addition, the group with poor glycemic control demonstrated significantly greater venous diameters (p=0.02). However, all other quantitative parameters, including arterial and intercapillary diameters, as well as abnormal capillary morphology, were similar between the two groups (p=0.1, p=0.2, and p=0.7, respectively). Individuals with poor control showed increased frequencies of dilated, tortuous, and cross-linked capillaries (p=0.03, p=0.002, and p=0.002, respectively). Moreover, non-specific abnormalities were more frequently found in this group (p=0.01).

Table 1. The clinical and laboratory characteristics of the patients (n=55)

Age, years	14.5 (11.3-17.2)
Female/male, n (%)	24 (43.6%)/31 (56.4%)
Pubertal, n (%)	43 (78.2%)
Family history of T1DM, n (%)	11 (20%)
The presence of DKA	22 (40%)
Duration of T1DM, years	3.8 (2.3-6.7)
The presence of autoantibodies, n (%)	40 (72.7%)
Height, SD score	0.3±1.1
Weight, SD score	0.4±1.3
BMI, SD score	0.2±1.2
Insulin dose, unit/kg/day	0.8±0.2
Treatment with pump, n (%)	6 (10.9%)
The use of CGM, n (%)	22 (40%)
TIR, %	54.3±22.3
CV, %	37.1±6.5
Recent HbA1c, %	8.0±1.4
Average HbA1c, %	8.2±1.4
TC, mg/dL	163.0 (147.0-183.0)
HDL-C, mg/dL	54.0 (47.0-63.0)
LDL-C, mg/dL	90.4 (80.6-107.2)
TG, mg/dL	76.0 (64.0-104.0)
Dyslipidemia, n (%)	5 (9.1%)
Autoimmune thyroiditis, n (%)	2 (3.6%)
Celiac disease, n (%)	4 (7.3%)
Urinary albumin creatinine ratio, mg/g	10.2 (7.5-15.4)
Microvascular complication	
Retinopathy, n (%)	0 (0%)
Nephropathy, n (%)	2 (3.6%)
Neuropathy, n (%)	1 (1.8%)

Data were presented as mean±standard deviation for normal distribution and median (25-75p) for those not distributed normally.
Reference values: Total cholesterol, 111-202 mg/dL; HDL, 31-68 mg/dL; LDL, 45.6-131 mg/dL; TG, 38-143 mg/dL.
T1DM: type 1 diabetes mellitus, DKA: diabetic ketoacidosis, SD score: standard deviation score, BMI: body mass index, CGM: continuous glucose monitoring, TIR: time in range, CV: glycemic variability, HbA1c: glycated hemoglobin, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, TG: triglyceride

Table 2. Comparative analysis of nailfold capillaroscopy findings between patients with T1DM and the control group

NC parameters	Patients with T1DM (n=55)	Healthy controls (n=55)	p
Quantitative			
Capillary density, capillary/mm	6.5±0.5	7.3±0.5	**<0.001**[a]
Reduced capillary density (<7/mm), n (%)	45 (81.8%)	15 (27.3%)	**<0.001**[b]
Capillary length, µm	507.5±75.1	425.5±51.8	**<0.001**[a]
Arterial diameters, µm	12.3±1.8	10.6±1.3	**<0.001**[a]
Venous diameters, µm	16.0±2.5	13.4±2.0	**<0.001**[a]
Apical diameters, µm	16.7±2.9	14.5±2.0	**<0.001**[a]
Intercapillary diameters, µm	194.6±21.5	178.2±16.1	**<0.001**[a]
Presence of dilated capillaries, n (%)	26 (47.3%)	0 (0%)	**<0.001**[b]
Giant capillaries, n (%)	0 (0%)	0 (0%)	-
Tortuosity, n (%)	42 (76.4%)	24 (43.6%)	**<0.001**[b]
Cross-linked capillaries, n (%)	22 (40%)	10 (18.2%)	**0.01**[b]
Abnormal capillary morphology, n (%)	7 (12.7%)	0 (0%)	**0.03**[b]
Presence of avascular areas, n (%)	0 (0%)	0 (0%)	-
Presence of microhemorrhages, n (%)	0 (0%)	0 (0%)	-
Qualitative			
Pattern, n (%)			
Normal	7 (12.7%)	42 (76.4%)	**<0.001**[b]
Non-specific abnormalities	48 (87.3%)	13 (23.6%)	

Data were presented as mean±standard deviation for normal distribution. [a]Student's t-test, [b]Pearson's chi-square test, p<0.05.
T1DM: type 1 diabetes mellitus, NC: nailfold capillaroscopy

Table 3. Comparative analysis of nailfold capillaroscopy findings based on HbA1c levels in patients with T1DM

NC parameters	Patients with HbA1c <7.5 (n=21)	Patients with HbA1c >7.5 (n=34)	p	Duration of T1DM <5 years (n=33)	Duration of T1DM >5 years (n=22)	p
Quantitative						
Capillary density, capillary/mm	6.7±0.4	6.4±0.5	**0.01**[a]	6.7±0.4	6.4±0.5	**0.02**[a]
Capillary length, µm	489.4±85.0	518.6±67.2	0.2[a]	497.1±73.9	523.1±75.9	0.2[a]
Arterial diameters, µm	11.9±1.7	12.6±1.9	0.1[a]	12.3±1.7	12.4±2.1	0.8[a]
Venous diameters, µm	15.0±2.4	16.6±2.4	**0.02**[a]	15.6±2.2	16.6±2.8	0.1[a]
Apical diameters, µm	15.6±2.6	17.4±2.9	**0.02**[a]	16.8±2.9	16.5±2.9	0.7[a]
Intercapillary diameters, µm	189.9±23.2	197.6±20.1	0.2[a]	194.4±21.6	195.1±21.7	0.9[a]
Presence of dilated capillaries, n (%)	6 (28.6%)	20 (58.8%)	**0.03**[b]	13 (39.4%)	13 (59.1%)	0.2[b]
Giant capillaries, n (%)	0 (0%)	0 (0%)	-	0 (0%)	0 (0%)	-
Tortuosity, n (%)	11 (52.4%)	31 (91.2%)	**0.002**[b]	22 (66.7%)	20 (90.9%)	**0.04**[b]
Cross-linked capillaries, n (%)	3 (14.3%)	19 (55.9%)	**0.002**[b]	12 (36.4%)	10 (45.5%)	0.5[b]
Abnormal capillary morphology, n (%)	2 (9.5%)	5 (14.7%)	0.7[b]	2 (6.1%)	5 (22.7%)	0.1[b]
Presence of avascular areas, n (%)	0 (0%)	0 (0%)	-	0 (0%)	0 (0%)	-
Presence of microhemorrhages, n (%)	0 (0%)	0 (0%)	-	0 (0%)	0 (0%)	-
Qualitative						
Pattern, n (%)						
Normal	7 (33.3%)	0 (0%)	**0.01**[b]	7 (21.2%)	0 (0%)	**0.03**[b]
Non-specific abnormalities	14 (66.7%)	34 (100%)		26 (78.8%)	22 (100%)	

Data were presented as mean±standard deviation for normal distribution. [a]Student's t-test, [b]Pearson's chi-square test, p<0.05.
T1DM: type 1 diabetes mellitus, HbA1c: glycated hemoglobin

Among patients with diabetes, those with a T1DM duration ≥5 years exhibited a significantly lower capillary density than those with a T1DM duration <5 years (p=0.02). Capillary density showed a negative correlation with the duration of T1DM (r=-0.3, p=0.02) (Figure 2). However, after adjustment for age, gender, and BMI, the relationship between capillary density and disease duration became non-significant (p=0.08). Nevertheless, patients with a longer duration of T1DM displayed significantly higher rates of non-specific abnormalities (p=0.03).

In the multivariate linear regression analysis (Table 4), capillary density was evaluated as the dependent variable, while adjusting for potential confounders, including age, gender, BMI, duration of T1DM, insulin dose, average HbA1c, LDL-C, and TG levels. The analysis revealed that only average HbA1c levels were significantly associated with capillary density (β-coefficient=-0.358, p=0.03), indicating that higher HbA1c levels correlated with reduced capillary density. Other variables, including age, gender, BMI, duration of T1DM, insulin dose, LDL-C, and TG levels, did not show significant associations with capillary density (p>0.05). The overall model explained 29.1% of the variance in capillary density (r^2=0.291, p=0.032), suggesting that glycemic control plays a key role in influencing capillary density.

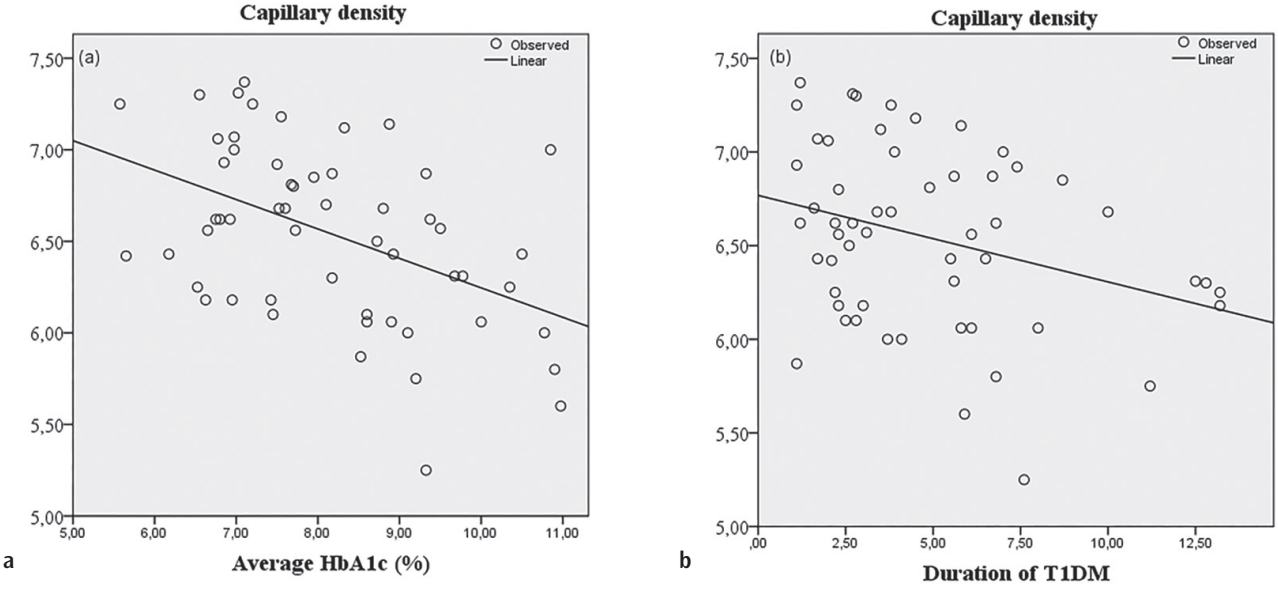

Figure 2. The correlation between capillary density and (**a**) average HbA1c (r=-0.5, p<0.001), (**b**) duration of T1DM (r=-0.3, p=0.02)
T1DM: type 1 diabetes mellitus, HbA1c: glycated hemoglobin

Table 4. Multivariate linear regression analysis (dependent variable: capillary density)

Variable	B (95% CI)	SRC (ß)	T	p
Age, years	-0.014 (-0.057/0.030)	-0.111	-0.620	0.5
Gender (female/male)	-0.068 (-0.329/0.193)	-0.071	-0.526	0.6
BMI, kg/m^2	0.011 (-0.031/0.052)	0.100	0.521	0.6
Duration of T1DM, years	-0.015 (-0.063/0.032)	-0.106	-0.657	0.5
Insulin dose, unit/kg/day	-0.418 (-1.014/0.179)	-0.204	-1.409	0.2
Average HbA1c, %	-0.123 (-0.229/-0.016)	-0.358	-2.316	0.03
LDL-C, mg/dL	0.001 (-0.005/0.006)	0.030	0.214	0.8
TG, mg/dL	-0.001 (-0.003/0.002)	-0.083	-0.559	0.6

T1DM: type 1 diabetes mellitus, BMI: body mass index, HbA1c: glycated hemoglobin, LDL-C: low-density lipoprotein cholesterol, TG: triglyceride, B: coefficient of regression, SRC: standardized regression coefficient (r^2=0.291, p=0.032, Durbin Watson=1,887), CI: confidence interval

Among the subjects with T1DM, 22 (40%) used CGM devices. The median age of these patients was 13.3 (9.7-15.6) years, with a median disease duration of 3.1 (1.7-5.7) years. Within this group, 16 patients (72.7%) exhibited a TIR of <70%, while 12 individuals (54.5%) showed a CV of >36%. The capillaroscopic characteristics did not show a significant difference between patients with a TIR of ≥70% and those with <70%. Likewise, these findings were similar in both patients with a CV of ≤36% and those with >36% (data not shown). However, there was a positive correlation between capillary density and TIR (r=0.5, p=0.01). This correlation persisted after adjusting for age, gender, BMI, and disease duration (r=0.5, p=0.04), indicating that diabetic children with better TIR had higher capillary density. However, there was no significant correlation between TIR and apical, arterial, or venous diameters (r=-0.4, p=0.07; r=-0.3, p=0.2; and r=-0.4, p=0.09, respectively). Similarly, no significant correlation was present between CV and capillary density, apical, arterial, or venous diameters (r=0.02, p=0.9; r=-0.2, p=0.3; r=-0.08, p=0.7; and r=-0.07, p=0.7, respectively).

Discussion

In the current study, it was found that children and adolescents with T1DM exhibited significant microvascular changes, even in the absence of diabetes-related microvascular complications. In particular, these abnormal microvascular alterations were associated with poor glycemic control and longer diabetes duration. The results of our study are in line with previously reported effects of diabetes on capillary structure and function in both adult and pediatric populations (5,6,14,20). Of note, our study was the first to assess the utility of NC in CGM users and found a positive correlation between capillary density and TIR, suggesting that patients with better TIR had a better microvascular structure.

In the review of the literature, few studies have focused on capillaroscopy changes in children with T1DM. Although a typical diabetic pattern has not yet been identified, these studies have reported characteristic morphological capillaroscopic features in diabetic patients, including reduced capillary density, increased capillary diameter, tortuous and cross-linked capillaries, microhemorrhagic areas, and avascular zones (5,6,12,13,14,21). For instance, in a study involving children and adolescents with T1DM, Hosking et al. (5) identified microhemorrhages and avascular zones as the most frequent microvascular alterations, with patients who had microvascular complications exhibiting more avascular areas on NC. Similarly, Bogusz-Górna et al. (6) reported that the most common lesions among juveniles with T1DM were enlarged, tortuous, bushy, elongated vessels, and hemorrhages. Another study found that diabetic adolescents had a higher prevalence of tortuous, cross-linked, and giant capillaries, as well as avascular areas, compared to healthy controls (14). Furthermore, in a study investigating the relationship between microangiopathic lesions in retinal vessels and capillary alterations in adults with T1DM and T2DM, Barchetta et al. (20) observed that patients with T1DM exhibited more capillaroscopic abnormalities, including reduced capillary density, than those with T2DM. They also noted these changes in nearly 50% of patients with diabetes without retinopathy, indicating early capillary abnormalities (20). Our study confirmed previously published findings that children with T1DM had significantly lower capillary density, increased capillary diameters, and a higher prevalence of dilated, tortuous, cross-linked, and abnormal morphological capillaries compared to healthy individuals. However, microhemorrhagic and avascular areas were not observed. These findings might suggest that alterations in the peripheral microvasculature are associated with end-organ damage in diabetes, even without any microvascular complications. However, it is worth noting that while capillary density is an important parameter of microcirculation, indicating disease severity, capillaroscopic variations, such as tortuous or cross-linked capillaries, can occur in healthy individuals without accompanying damage or symptoms (18,22,23,24,25).

The development of diabetes-related complications is associated with various risk factors, including puberty, elevated HbA1c levels, high glycemic variability, and longer disease duration (3,26,27). Some studies have shown that adolescent girls have a higher incidence of microvascular complications compared to boys (28,29,30). It has been reported that the duration of diabetes before puberty has less influence on complications (31). However, several studies have suggested that individuals who develop diabetes during puberty are at a higher risk of vascular complications compared to those who develop diabetes after puberty (29,32,33). Consistent with previous studies, female patients had a higher frequency of abnormal morphological parameters than male patients in a study evaluating microvascular alterations in diabetic patients (34). Similarly, Kaminska-Winciorek et al. (13) found that an increased number of vessels, indicating possible neoangiogenesis, occurred more frequently in female juveniles with diabetes. In contrast to these earlier studies, the current study found similar structural changes in both male and female patients. Moreover, there were no significant differences observed, except for larger capillary diameters in the pubertal T1DM group compared to the prepubertal group.

Chronic hyperglycemia, along with the accumulation of advanced glycation end products, oxidative stress, and inflammatory cytokines, leads to dysregulation in vascular tone, hemostasis, and intercellular communication, resulting in vascular endothelial damage (35). This endothelial dysfunction, characterized by a pro-inflammatory and pro-thrombotic state,

plays a major role in the development of microangiopathy (2,36,37). These mechanisms can explain the capillaroscopic alterations seen in diabetic patients, even before the manifestation of overt vascular complications. Several studies evaluating the relationship between capillaroscopy findings, diabetes duration, and metabolic control have demonstrated increased morphological changes in patients with higher HbA1c levels or longer diabetes duration, further supporting the role of hyperglycemia in driving microvascular damage (5,13,14). However, there have also been conflicting results regarding the association between capillaroscopic abnormalities and disease duration, or glycemic control (6). Abdelmaksoud et al. (14) reported a significant positive correlation between microvasculature changes and longer diabetes duration, as well as poor glycemic control. In another study, Kaminska-Winciorek et al. (13) observed that elevated HbA1c levels were associated with increased dilated capillaries and reduced capillary density. Furthermore, these authors noted that the presence of abnormal capillaries correlated with the duration of diabetes (13). Kuryliszyn-Moskal et al. (35) showed that diabetic adults with poor metabolic control also had severe capillary changes. Once again, they found that the disease duration was longer in patients with severe capillaroscopic changes compared to those with mild or moderate microvascular abnormalities (35). In contrast, Bogusz-Górna et al. (6) found no significant relationship between the presence of capillaroscopic changes and the duration of diabetes or metabolic control.

The current study demonstrated that patients with poor glycemic control had significantly lower capillary density and more dilated capillaries. In addition, patients with a longer disease duration exhibited significantly reduced capillary density. We also found a negative correlation between capillary density and both average HbA1c levels and disease duration. While there was a positive correlation between capillary density and TIR, the capillaroscopic characteristics were found to be similar between CGM users with a TIR of ≥70% and those with <70%. Similarly, there was no significant difference in capillaroscopic findings between patients with a CV of ≤36% and those with >36%. This observation may be attributed to the small sample size of patients evaluated.

Several published studies have demonstrated a close association between capillaroscopic alterations and diabetes-related complications, such as retinopathy, nephropathy, and neuropathy, in both T1DM and T2DM patients (5,36,38,39,40). In one of these studies, Hosking et al. (5) reported a higher frequency of avascular areas in T1DM patients with microvascular complications. Similarly, Chang et al. (41) identified a correlation between diabetic retinopathy and the presence of tortuous, ramified, and dilated capillaries, with these alterations increasing as retinopathy progressed. Abdelmaksoud et al. (14) found that diabetic nephropathy and neuropathy were independently associated with NC changes, with more significant capillary abnormalities observed in patients with vascular complications compared to those without. In addition, Kuryliszyn-Moskal et al. (35) showed abnormal capillaroscopic findings in 81% of adults with diabetes, noting that more severe changes were present in over half of those diagnosed with microvascular complications.

In the present study, only three patients exhibited microvascular complications: two with albuminuria and one with neuropathy. These patients displayed reduced capillary density and abnormal capillary morphology. However, due to the limited number of patients with complications, a detailed comparison could not be performed. Given that micro- and macrovascular complications are rare in the pediatric population, our findings emphasize the need for long-term follow-up studies. Although most patients did not present with overt complications, the observed capillaroscopic changes, particularly decreased capillary density and abnormal morphology, are suggestive of early subclinical endothelial dysfunction. Considering the progression of diabetic microangiopathy from silent structural alterations to clinically manifest disease, these findings may hold prognostic significance. Identifying microvascular alterations at an early stage may provide a valuable opportunity for timely interventions that could delay or prevent the development of long-term complications, such as retinopathy, nephropathy, and neuropathy.

NC has the potential to be implemented as a routine screening tool in pediatric diabetes care due to its simplicity, non-invasive nature, ease of use, repeatability, low cost, minimal equipment requirements, and ability to provide high-quality images for both qualitative and quantitative assessments of peripheral microvascular abnormalities (12,22). Current clinical guidelines recommend initiating annual screening for microvascular complications, such as retinopathy, nephropathy, and peripheral neuropathy in children with T1DM starting at puberty, or age 11 years, after 2 to 5 years of diabetes duration (3). Within this framework, NC could be integrated into annual routine follow-up visits as a complementary tool, especially in patients with poor glycemic control or longer disease duration. Longitudinal studies are necessary to further explore the predictive value of early capillaroscopic changes in T1DM patients. However, for NC to be widely adopted as a screening tool, standardized protocols, appropriate clinician training, and further research are required to validate its diagnostic accuracy and prognostic utility in larger patient cohorts.

Study Limitations

The current study has several limitations. First, it was conducted on a relatively small group of diabetic patients and CGM users, which may limit the generalizability of the findings. Future

studies with larger sample sizes for both groups could provide more definitive results. Second, as a cross-sectional study, it did not allow for the assessment of the progression of microvascular changes over time. Prospective longitudinal studies are necessary to explore how early capillaroscopic changes relate to long-term clinical outcomes in T1DM patients. Third, inter-rater reliability metrics, such as the intraclass correlation coefficient could not be calculated because individual scoring data from each observer were unavailable; only the averaged values were used in the final dataset. Although factors such as age and pubertal status were considered in subgroup and adjusted analyses, larger datasets are needed to more accurately assess their impact on microvascular structure. In addition, the type of insulin therapy was not evaluated, which may represent another potential confounding factor. Future studies should explore treatment modalities more comprehensively to determine their independent effects on microvascular health.

Conclusion

These capillaroscopic findings in patients with T1DM suggest that microvascular structural and functional abnormalities, primarily associated with poor glycemic control, can develop during childhood and adolescence, even in the absence of clinically evident vascular disease. This highlights the importance of early diagnosis and monitoring of microvascular health in pediatric populations with diabetes. NC may provide valuable data for detecting vascular damage before diabetes progresses and complications arise. Future prospective longitudinal studies with larger sample sizes are needed to determine the role of this method and to better characterize diabetes-related microvascular features in patients with T1DM.

Ethics

Ethics Committee Approval: This study was approved by the Dokuz Eylül University Non-Interventional Research Ethics Committee (approval no: 2023/41-02, date: 20.12.2023).

Informed Consent: Both patients and parents were required to sign the informed consent form to participate in the study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Gözde Akın Kağızmanlı, Concept: Gözde Akın Kağızmanlı, Tuncay Aydın, Kübra Yüksek Acinikli, Rana İşgüder, Zehra Kızıldağ Karabacak, Korcan Demir, Ece Böber, Şevket Erbil Ünsal, Ayhan Abacı, Design: Gözde Akın Kağızmanlı, Kübra Yüksek Acinikli, Rana İşgüder, Zehra Kızıldağ Karabacak, Ayhan Abacı, Data Collection or Processing: Gözde Akın Kağızmanlı, Tuncay Aydın, Analysis or Interpretation: Gözde Akın Kağızmanlı, Tuncay Aydın, Rana İşgüder, Zehra Kızıldağ Karabacak, Korcan Demir, Ece Böber, Şevket Erbil Ünsal, Ayhan Abacı, Literature Search: Gözde Akın Kağızmanlı, Kübra Yüksek Acinikli, Korcan Demir, Ece Böber, Şevket Erbil Ünsal, Ayhan Abacı, Writing: Gözde Akın Kağızmanlı, Ayhan Abacı.

Conflict of Interest: One of the author of this article, Korcan Demir is member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, he was not involved in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions.

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BRIEF REPORT

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Glucocorticoid Dose and Type are Associated with Depression Scores in Youth with Classical Congenital Adrenal Hyperplasia

Mark Chih-Wei Liang[1,2], Nicole Fraga[2], Nare Minaeian[2,3], Megan M. Herting[4], Mitchell E. Geffner[2,3,5], Tania A. S. S. Bachega[6], Mimi S. Kim[2,3,5]

[1]University of California Irvine School of Medicine, California, United States of America
[2]Center for Endocrinology, Diabetes and Metabolism; Children's Hospital Los Angeles (CHLA), Los Angeles, CA, United States of America
[3]Keck School of Medicine of University of Southern California, Los Angeles, CA, United States of America
[4]University of Southern California, Department of Population and Public Health Sciences, California, United States of America
[5]The Saban Research Institute at CHLA, Los Angeles, CA, United States of America
[6]Universidade de São Paulo Faculdade de Medicina, Hospital das Clínicas, Division of Endocrinology, Laboratory of Hormones and Molecular Genetics (LIM-42), São Paulo, Brazil

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What is already known on this topic?

Patients with classical congenital adrenal hyperplasia (CAH) exhibit a higher lifetime prevalence of depression, increased prevalence of anxiety in youth, adult-onset mood disorders, personality disorders, alcohol misuse and rates of adult suicidality. Additionally, structural brain alterations have been observed in patients with CAH, with relevance in emotional dysregulation and mood disorders.

What this study adds?

Both glucocorticoid dose and type, specifically dexamethasone, were found to be associated with higher depression scores on the Children's Depression Inventory (CDI) in youth with CAH. In addition, glucocorticoid dose predicts CDI scores (total score and multiple subscales).

ABSTRACT

Objective: Adults with classical congenital adrenal hyperplasia (CAH) exhibit a higher lifetime prevalence of depression, but little is known about onset or etiology of mood disorders in this population. We therefore aimed to assess depression in youth with CAH, compared to controls, using the Children's Depression Inventory (CDI).

Corresponding Author: Mimi S. Kim, M.D., M.Sc., Center for Endocrinology, Diabetes and Metabolism; Children's Hospital Los Angeles (CHLA), Los Angeles, CA, United States of America
E-mail: mskim@chla.usc.edu **ORCID:** orcid.org/0000-0002-6266-3569
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Methods: Youth with classical CAH due to 21-hydroxylase deficiency and age- and sex-matched controls completed the CDI and had analyte and genetic testing.

Results: A total of 31 patients with CAH and 36 controls were included. Youth with CAH exhibited CDI measures that differed significantly by glucocorticoid dose and type. For glucocorticoid dose, significant correlations were found between CDI total T-score ($r=0.42$, $p<0.05$), as well as multiple subscores. Dose also predicted total T-score ($\beta=1.75$), Emotional-Problems ($\beta=1.41$), Negative-Self-Esteem ($\beta=1.91$), Functional-Problems ($\beta=1.90$), Ineffectiveness ($\beta=1.56$), and Interpersonal-Problems ($\beta=2.11$) (all $p<0.01$). For glucocorticoid type [dexamethasone n=7, hydrocortisone (HC) n=24], scores were higher in patients treated with dexamethasone for total T-score [dexamethasone: 59 (53.5-72), HC: 50 (43.75-55.75)], Emotional-Problems [dexamethasone: 63 (51.0-67.0), HC: 45 (42.0-56.5)], and Negative-Self-Esteem [dexamethasone: 53 (50.0-73.5), HC: 44 (44.0-51.0)] (all $p<0.05$).

Conclusion: Higher HC doses and use of dexamethasone were both found to be associated with higher CDI scores in children and adolescents with classical CAH.

Keywords: Depression, 21-hydroxylase deficiency, congenital adrenal hyperplasia

Introduction

Classical congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is characterized by cortisol and aldosterone deficiencies necessitating lifetime glucocorticoid replacement (1). Patients with classical CAH exhibit a higher lifetime prevalence of depression, as well as an increased prevalence of anxiety in youth and adult-onset psychiatric disorders (2,3). In addition, patients with CAH exhibit structural brain alterations, including prefrontal cortex, amygdala, and hippocampal volumes, as well as altered white matter microstructure (4,5,6). Differences in these regions have shown relevance to emotional dysregulation and mood disorders, especially in adolescent populations (7).

Despite these observations, there are few studies on mood disorders in youth with CAH. The timeline for the emergence of mood symptoms in patients with CAH is unclear, with conflicting studies on depression in pediatric patients with CAH (8,9). In addition, cortisol replacement may transition from immediate-release hydrocortisone (HC) to longer-acting glucocorticoids when patients near completion of growth, with little known about the relationship between glucocorticoid type, dose, and mood disorders. Thus, we sought to compare depression survey scores in youth with and without CAH, and to examine relationships with biochemical, genetic, and clinical markers in youth with CAH.

Methods

Participants filled out the Children's Depression Inventory (CDI, 2nd Edition) with higher scores quantifying increased depressive symptomology (10). We report CDI T-scores which are standardized for age; T-scores above 60 are considered above average. In patients with CAH, medical history was collected from the medical record and genotyping performed as described previously (11). All patients with CAH had 21-OHD as confirmed by biochemical testing and/or genotyping of *CYP21A2*. Age- and sex-matched controls were recruited via flyers posted at the Children's Hospital Los Angeles and University of Southern California. Written informed consent was obtained from parents/legal guardians of participants <18 years old and participants >14 years old. All minors up to 14 years of age gave assent. Glucocorticoid daily dosing was recorded as HC equivalents (HCe=dexamethasone dose x 60) (1).

Statistical Analysis

Data were analyzed using R (v4.0.3, R Foundation for Statistical Computing, Wirtschaftsuniversität Wien, Welthandelsplatz 1 1020, Vienna, Austria). Group comparisons were assessed using chi-square, with Mann-Whitney U tests for CDI scores. Fisher's exact test was used for group comparisons of genetics due to small sample size. Pearson correlations were used to assess associations between continuous variables. CDI scores are reported as median with interquartile ranges unless otherwise noted. Multiple linear regression analysis was used to examine the relationship between glucocorticoid usage and CDI scores, independent of disease severity.

Results

Study Population

The study included 31 youth with CAH and 36 healthy controls (8-18 years; age- and sex-matched) (Table 1). All patients on dexamethasone had been switched from HC due to poor disease control secondary to medication non-compliance, and average duration of dexamethasone therapy prior to the study visit was 29.3±22 months.

Glucocorticoid Dose and Depression in CAH

Group comparisons between CAH and control youth showed no overall differences in CDI (total T-scores or subscores). However, within the CAH group, glucocorticoid dose as HCe was positively correlated with CDI total T-score ($r=0.42$, $p<0.05$) (Figure 1), Negative-Self-Esteem ($r=0.57$, $p<0.001$), Functional-

Problems (r=0.43, p<0.05), Ineffectiveness (r=0.36, p<0.05), and Interpersonal-Problems (r=0.37, p<0.05). Average glucocorticoid dose for patients with CDI total T-score ≥60 was 18.1±6.33 mg/m²/day.

When controlling for markers of disease severity [bone age standard deviation (SD), highest 17-hydroxyprogesterone (17-OHP) at diagnosis, waist-to-height ratio (WHtR)] by including them in the regression model, glucocorticoid dose at the study visit still predicted total T-score (β=1.75, p<0.001), Emotional-Problems (β=1.41, p<0.001), Negative-Self-Esteem (β=1.91, p<0.001), Functional-Problems (β=1.90, p<0.001), Ineffectiveness (β=1.56, p<0.01), and Interpersonal-Problems (β=2.11, p<0.001).

Table 1. Study participant characteristics

	CAH (n=31)	Controls (n=36)	p
Sex, female	18 (58.1)	21 (58.3)	1.0
Age, years Range	12.6±3.2 8.4-18.9	12.9±2.8 8.7-18.9	0.5
CAH phenotype			
Salt-wasting	29 (93.5)	--	
Simple-virilizing	2 (6.5)	--	
CAH genotype			
Null (0% enzymatic activity) Non-null: A (<2% activity) B (3-7% activity)	14 (45.2) -- 11 (35.5) 5 (16.1)	-- -- -- --	
Treatment			
Dexamethasone Hydrocortisone Glucocorticoid dose, mg/m²/day Concurrent fludrocortisone	7 (22.6) 24 (77.4) 16.3±4.38 --	-- -- -- --	
Highest 17-OHP at birth, nmol/L Highest 17-OHP at birth, ng/dL 17-OHP at visit, nmol/L 17-OHP at visit, ng/dL Total testosterone, nmol/L Total testosterone, ng/dL Androstenedione, nmol/L Androstenedione, ng/dL Plasma renin activity, ng/mL/h Bone age, SD Waist-to-height ratio	480.3 (622.1) 15200 (19689) 67.14 (139.7) 2125 (4420.5) 2.51±2.45 72.3±70.8 2.83 (3.94) 81 (113) 3.52±2.89 0.87±1.30 1.59±0.83	-- -- -- -- -- 0.16±0.62 0.82±0.95	 **0.01** **0.001**

Mean±SD, Median (IQR), or n (%).
CAH: congenital adrenal hyperplasia, 17-OHP: 17-hydroxyprogesterone, SD: standard deviation, IQR: interquartile range

A sensitivity analysis of the subset of patients on HC, without any history of dexamethasone usage, showed that glucocorticoid dose still predicted the CDI total T-score (β=1.89, p<0.05). There was no group differences between CDI total T-score or its subscores with patients with ambiguous genitalia (n=17) at birth.

Glucocorticoid Type and Depression in CAH

When patients with CAH were stratified by glucocorticoid type [dexamethasone (n=7), HC (n=24)], those on dexamethasone exhibited higher scores on the CDI (Figure 2) compared to those on HC. There was no relationship between duration of

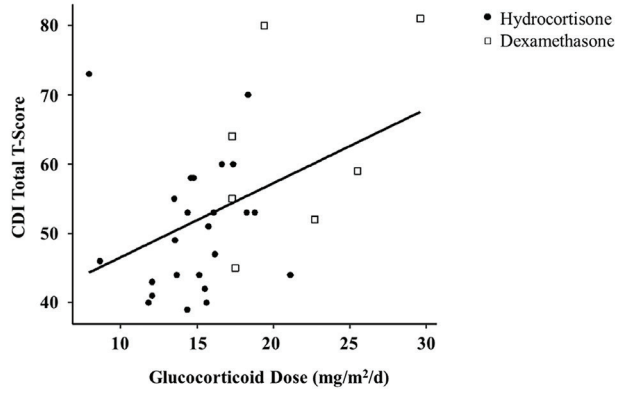

Figure 1. Depression associated with GC dose and type. Glucocorticoid dose (mg/m²/day; dexamethasone converted to hydrocortisone equivalents) was positively associated with CDI Total t-scores for youth with classical CAH (r=0.42, p<0.05)
CDI: Children's Depression Inventory, GC: glucocorticoid, CAH: congenital adrenal hyperplasia

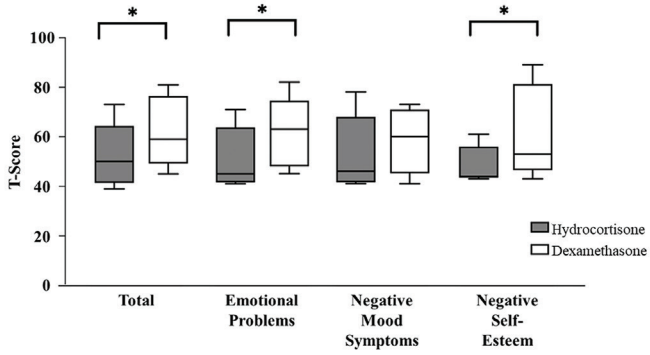

Figure 2. CDI T-scores by GC type. Youth with classical CAH on dexamethasone treatment (white) exhibit higher CDI total T-scores, as well as subset scores for Emotional Problems and Negative Self-Esteem, compared to youth with CAH on hydrocortisone (gray). *p<0.05
CDI: Children's Depression Inventory, GC: glucocorticoid, CAH: congenital adrenal hyperplasia

dexamethasone treatment and CDI scores. Statistical differences between dexamethasone- and HC-treated groups were reported for total T-score [dexamethasone: 59 (53.5-72.0), HC: 50 (43.8-55.8), $p<0.05$], Emotional-Problems [dexamethasone: 63 (51.0-67.0), HC: 45 (42.0-56.5), $p<0.02$], and Negative-Self-Esteem [dexamethasone: 53 (50.0-73.5), HC: 44 (44.0-51.0), $p<0.05$].

When controlling for markers of disease severity [bone age SD, highest 17-OHP at diagnosis, WHtR], patients on dexamethasone had higher total T-scores ($\beta=18.5$, $p<0.001$) compared to those taking HC.

Genetics and Depression in CAH

Null and non-null patients did not exhibit differences in CDI total score ($p=0.9$), subscores, GC dosage ($p=0.34$) or the GC treatment type that they were receiving ($p=0.4$).

Discussion

The main findings of our study show that both glucocorticoid dose and type were associated with higher depression scores on the CDI in youth with classical CAH. Our findings support prior observations that increased glucocorticoid doses are associated with higher CDI scores in CAH adolescents (12). Notably, we found the use of dexamethasone for glucocorticoid replacement to be associated with higher depression scores in youth with classical CAH.

Dexamethasone use could select for patients with a high degree of disease severity, poor control of disease, and/or non-adherence to medication use. A major question is whether high GC dose and/or type directly leads to depression symptoms in CAH, or reflects disease severity that can increase the propensity for psychiatric morbidity. As was seen in our cohort of patients, dexamethasone is often given to older adolescents and adults with CAH who struggle with hormonal control as an effective, potent, long-acting glucocorticoid for suppressing the excess production of adrenal androgens. However, dexamethasone has major physical side effects, with long-term use potentially leading to modification of the hypothalamic–pituitary–adrenal axis in psychiatric etiology, a direct effect on emotion regulatory networks, and/or the potential to lead to higher psychiatric morbidity and decreased quality of life (1,13). Confounders of disease severity in youth with CAH on dexamethasone may also include altered brain structural volumes with white matter changes that could put them at risk of increased psychiatric disorders (5,7).

To further study disease severity, we examined genotype and showed no significant relationship with depression or glucocorticoid dose/type. We and others have also found that 17-OHP values, another indicator of disease severity, are a poor marker for anxiety or depression symptoms in CAH. Impaired mental health could inherently impact hormonal control and thereby the intensity of glucocorticoid treatment needed in the patient. In our cross-sectional study, however, our findings suggest that disease severity is less likely to be a main contributor to the differences seen in depression scores in CAH youth (8).

There may be inherent differences in CAH youth that make them susceptible to depression, and it is also possible that depression and stress lead to increased ACTH levels, thereby leading to increase in androgens and sub-optimal disease control, necessitating higher HC doses or switching to dexamethasone, as seen in our patient population. These changes likely occur over the lifetime, and the adolescent population studied may be too young to significantly see these effects compared to controls. Brain structural changes in CAH patients start as early as *in utero* due to displaced hormonal pathways. Such structural changes, nevertheless, likely do not translate to differences in psychiatric disorders until late adolescence, which would be an older cohort than our patients studied (7). To assess inherent susceptibility to depression versus effects of medication on mood in this patient population, future longitudinal research is merited to examine patients with CAH as their own controls, thereby monitoring CDI values throughout adolescence and young adulthood with a focus on changes in glucocorticoid dosing.

Study Limitations

Our study had some other limitations, including a relatively small sample size, and two patients slightly older than 17 years, the upper limit of validity for the CDI survey. It would be useful to study a larger number of patients with CAH treated with dexamethasone, including a broader age range and longitudinal measures of glucocorticoid dosing and neuroimaging. The majority of studies examining glucocorticoid and CDI-based evaluations of depression are related to anti-inflammatory treatments in other conditions, with dexamethasone attributed to depression scores in conditions such as pediatric inflammatory bowel disease (14). As studies continue to emerge in patients with primary adrenal insufficiency, where glucocorticoid treatment is used for replacement of cortisol deficiency, our understanding of the relationship between lifelong cortisol replacement and depression will improve.

Conclusion

In conclusion, our findings suggest that both the dose and type of glucocorticoid the patient is taking are associated with higher scores for depression in youth with classical CAH. Further studies are merited to assess the frequency and natural history of clinical depression in adolescents and young adults with CAH, especially in patients utilizing dexamethasone for glucocorticoid replacement therapy. Regardless of etiology, the utilization of dexamethasone should be reconsidered as novel therapeutics

emerge, for example modified-release HC, and corticotropin-releasing hormone receptor antagonists, for the optimal replacement of cortisol and control of excess adrenal androgens.

Ethics

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

Acknowledgments

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Footnotes

Authorship Contributions

Concept: Mark Chih-Wei Liang, Megan M. Herting, Mitchell E. Geffner, Mimi S. Kim, Design: Mark Chih-Wei Liang, Megan M. Herting, Mitchell E. Geffner, Mimi S. Kim, Data Collection or Processing: Mark Chih-Wei Liang, Nicole Fraga, Nare Minaeian, Megan M. Herting, Tania A. S. S. Bachega, Analysis or Interpretation: Mark Chih-Wei Liang, Nicole Fraga, Nare Minaeian, Megan M. Herting, Tania A. S. S. Bachega, Literature Search: Mark Chih-Wei Liang, Writing: Mark Chih-Wei Liang, Nicole Fraga, Nare Minaeian, Megan M. Herting, Mitchell E. Geffner, Mimi S. Kim.

Conflict of Interest: MEG receives research support from Ascendis, Novo Nordisk, and Pfizer; serves on advisory boards or as a consultant for Adrenas Therapeutics, Ascendis, Eton Pharmaceuticals, Neurocrine Biosciences, Novo Nordisk, Pfizer, Spruce Biosciences, Theratechnologies Inc., and Tolmar; serves as an adjudication committee member for ICON Clinical Research, LLC/Aeterna Zentaris; and receives royalties from McGraw-Hill and UpToDate. MSK receives research support from Neurocrine Biosciences, Spruce Biosciences, and Diurnal; served on an advisory board for Eton Pharmaceuticals; and receives royalties from UpToDate.

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CASE REPORT

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Hyperinsulinemia in Sotos Syndrome with a *de novo NSD1* Deletion

Elena Lundberg[1], Magnus Burstedt[2], Irina Golovleva[2]

[1]Umeå University, Institute of Clinical Science, Department of Pediatrics, Umeå, Sweden
[2]Umeå University, Institute of Medical Biosciences, Department of Medical and Clinical Genetics, Umeå, Sweden

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What is already known on this topic?

Sotos syndrome belongs to a group of congenital overgrowth disorders. Most of the cases with Sotos syndrome are due to intragenic mutations and deletions of the NSD1 which is located at chromosome 5q35. To date, over 600 disease-associated variants in NSD1 have been reported to the Human Gene Mutation Database. Most of the variants are missense mutations, followed by small deletions and gross deletions. Congenital hyperinsulinemic hypoglycaemia (CHI) has been described as an uncommon feature of Sotos syndrome, initially reported as transient CHI in 1990. A few cases of Sotos patients with transient CHI and point mutations in the *NSD1* gene were described. NSD1 is not known to be directly involved in regulating insulin secretion but patients with Sotos syndrome have alterations in the IGF-1 axis which could play a role in β-cell hyperplasia.

What this study adds?

Our case reports a patient with Sotos syndrome and prolong CHI due to de novo, novel large genomic deletion encompassing 24 OMIM genes including the entire *NSD1* gene that has never been presented before. In this case CHI that persisted for almost two years. After treatment with diazoxide was started, the patient responded with a serious side effect, leading to heart failure. A treatment changed to Octreotide with no response. Diazoxide was then resumed at a low dose, less than 5 mg/kg/day, because of the risk of cardiac complications. Doses were required for nearly 2 years and were sufficient to avoid hyperinsulinemia and to ensure normoglycemia. Our proposal is that, in neonatal diagnostics, the phenotypic spectrum of Sotos syndrome should include HI as a significant feature.

ABSTRACT

Sotos syndrome belongs to the group of diseases characterised by features such as facial dysmorphism, intellectual disability, hypotonia and overgrowth. Usually, Sotos syndrome is caused by heterozygous mutations in the *NSD1* gene at chromosome 5q35 or by large genomic deletions of the same region. Genotype-phenotype correlations have mainly been reported as an association of significant or major abnormalities and presence of 5q35 deletions rather than intragenic deletions or point mutations in *NSD1*. Congenital hyperinsulinemic hypoglycaemia (CHI) has been described as an uncommon feature in the presentation of Sotos syndrome. Most of the patients with Sotos syndrome and transient CHI were carriers of 5q35 deletions, while persistent CHI has been recently reported in individuals with point mutations or small *NSD1* deletions. We report the clinical features and medical treatment in a new-born child with Sotos syndrome and CHI that was present for almost two years. Genetic cause of Sotos syndrome in this case was a novel, large genomic deletion encompassing 24 Online Mendelian Inheritance in Man genes including the entire *NSD1* gene and six other potentially morbid genes. Our report describes challenges in diagnosis and management of this rare genetic condition. We propose, that in neonatal diagnostics, the phenotypic spectrum of Sotos syndrome should include CHI as a characteristic feature and molecular genetic testing should be done by whole genome analysis.

Keywords: Hyperinsulinemia, hypoglycaemia, *NSD1*, overgrowth, Sotos syndrome

Corresponding Author: Assoc. Prof. Elena Lundberg, Umeå University, Institute of Clinical Science, Department of Pediatrics, Umeå, Sweden
E-mail: elena.lundberg@umu.se **ORCID:** orcid.org/0000-0001-6010-351X

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Introduction

Sotos syndrome (SOS) [Online Mendelian Inheritance in Man (OMIM) #117550] belongs to a group of congenital overgrowth disorders characterised by facial dysmorphism, brain involvement, prenatal and postnatal overgrowth, cardiac defects, kidney problems, scoliosis and loss of vision and hearing (1,2).

SOS is caused by haploinsufficiency of the *NSD1* gene at 5q35.2-q35.3 coding for nuclear receptor binding SET domain 1 protein. The NSD1 protein functions as a transcriptional regulator of chromatin through the histone methyltransferase activity (1,3). To date, 623 disease-associated variants in *NSD1* have been reported to the Human Gene Mutation Database (HGMD Professional) (https://my.qiagendigitalinsights.com/bbp/view/hgmd/pro/gene.php?gene=NSD1). Most of the variants are missense mutations (n=263), followed by small deletions (n=142) and gross deletions (n=63). Gross deletions may result in removal of a single or several exons or the entire *NSD1* gene with adjoining genes. Deletions have been reported to vary from 3.8 kb to 5 Mb, according to HGMD Professional. The majority of the large genomic deletions including *NSD1* appear *de novo* while familial cases with missense mutations in the *NSD1* gene have also been reported (1,4).

Molecular techniques, such as genome-wide genotyping or chromosomal microarray (CMA) and multiplex ligation-dependent probe amplification are usually used for detection of large genomic deletions encompassing *NSD1* or the *NSD1* intragenic deletions, that can also be confirmed by fluorescence *in situ* hybridization (FISH).

Congenital hyperinsulinemic hypoglycaemia (CHI) due to inappropriate insulin secretion leading to severe hypoglycemia may be an isolated finding or a feature of the syndrome. CHI has been described as an uncommon feature of SOS, and was initially reported as transient CHI in 1990 (1,5). Over the last decade, the number of reported cases of SOS with 5q35 deletions and transient CHI became more numerous (6,7,8). Most of the cases of SOS with CHI were caused by microdeletions but Sotos patients with transient CHI and point mutations in the *NSD1* gene have also been described (6,9). Thus, Grand et al. (6) presented seven patients, all carriers of *NSD1* point mutations, three of whom demonstrated persistent CHI while five of them had atypical features of SOS. These authors concluded that the CHI present in Sotos patients with *NSD1* point mutations could not be explained by the deletion of additional genes in the deleted 5q35 region.

A large difference in the frequency of 5q35 microdeletions causing SOS was observed in Japanese (49%) and non-Japanese (6%) patients (10,11). A partial or whole *NSD1* gene deletions were present in ~10% of 30 Brazilian Sotos patients of non-Japanese ancestry (12). In a cohort of Sotos patients from France and the UK, 5q35 the frequency of microdeletions was 18% and 5%, respectively, while intragenic *NSD1* mutations responsible for Sotos phenotype were detected in 49% of French and in more than 70% of British patients (13,14).

In this report, we present clinical features, molecular diagnostics and medical treatment of persistent CHI in a patient with SOS caused by a *de novo* large genomic deletion encompassing 24 OMIM genes including the entire *NSD1* gene.

In this report, we describe the clinical features and medical management of a newborn with SOS complicated by congenital hyperinsulinism, a condition that persisted for nearly two years. We also highlight the diagnostic challenges and therapeutic considerations associated with this rare genetic presentation.

According to national regulations, presentation of this case report did not require approval from an ethics committee. Informed consent for publication was obtained from the patient's parents.

Case Presentation

A full-term male baby [gestational age of 39 weeks, birth weight 3855 g (+1 standard deviation score, SDS), birth length 53 cm (+1SDS), head circumference 37 cm (+1SDS)], the second child of non-consanguineous Caucasian parents, was born by emergency Cesarean section because of a pathological cardiotocography trace. Antenatal scans showed polyhydramnios, abnormal flow in the umbilical cord and in the arteria cerebri media, as well an abnormal brain morphology. Apgar score was 1-5-10 min: 3-7-8p. Directly after delivery, the patient was found to be hypotonic and hypoglycaemic (P-glucose 0.6 mmol/L) and was admitted to the neonatal intensive care unit. He required intravenous (IV) high concentration glucose infusions with a utilization rate of 13-14 mg/kg/min and, due to tachypnea, he was treated with positive pressure therapy, using continuous positive pressure therapy.

Physical Characteristics

Clinical examination revealed syndromic features, including macrocephaly with prominent forehead, hypertelorism, posteriorly rotated low set ears, short philtrum, flat nasal bridge, and general hypotonia. SOS was suspected.

Systemic Event

At six days of age, he developed repeated seizures, not linked to hypoglycaemia, confirmed with video electroencephalography. Treatment with antiepileptics, phenobarbitone and phenytoin was started. Neuroimaging of brain showed hypo-myelinization, ischemia, a periventricular white matter lesion and reduction of

the corpus callosum. A cardiac ultrasound showed a muscular ventricular-septal defect.

Glycemic Event and Treatment

Recurrent hypoglycaemia required continuous glucose infusion and nutritional intake by breastfeeding and nasogastric tube feeding. Repeated diagnostic fast tolerance test was done at the age of 15 days. A critical sample was obtained that revealed plasma-glucose 2.6 mmol/L, C-peptide 0.36 nmol/L, and p-insulin 2.1 mIU/mL. Metabolic investigation for carnitine, methylmalonate, methionine and free amino acids was normal. Plasma beta- hydroxybutyrate was not analyzed and an ammonium level was 67 umol/L, however this was taken at another occasion. The clinical presentation did not resemble hypopituitarism and this diagnosis was excluded because of clinical and laboratory findings.

Diazoxide, as a first-line treatment for CHI, was initiated at a dose 10 mg/kg/day on day 15 with normalisation of p-glucose at day 18. However, due to fluid retention and development of severe pulmonary hypertension with lung oedema and heart failure, diazoxide was discontinued on day 19. The condition of the patient was critical, requiring intubation and respiratory treatment and was regarded as diazoxide "toxicity" affecting the heart. At the intensive care unit, normoglycemia 4-7 mmol/L was observed until patient's age of 29 days, when he was discharged to the pediatrics care unit.

At day 30, a persistent hypoglycaemia re-occurred and so IV glucose with a utilization rate to 6 mg/kg/min was started. Due to the suspicion of possible diazoxide heart toxicity, octreotide treatment was initiated on day 35, at a dose of 3.5 µg/kg/day and increased by 2 µg/kg/day up to 20 µg/kg/day over 10 days. However, episodes of hypoglycemia persisted. Octreotide treatment was considered ineffective and was discontinued at day 45. On some occasions, diazoxide was carefully re-initiated at low doses of 1 and later 2 mg/kg/day. These doses were well tolerated and therefore were increased to 5 mg/kg/day for maintained normoglycemia. The baby was fed every 2.5 hours and was able to fast for five hours. He maintained normoglycemia at day 46 when glucose infusion was discontinued. Patient's treatments during the first two months of life are presented in Figure 1.

Follow-up

The patient was discharged from hospital at 12 weeks of age with diazoxide treatment at dose of 5 mg/kg/day. He was mainly fed by nasogastric tube, which was discontinued at six months of age. He was growing at 0SDS for both height and weight, based on Swedish reference and genetic potential was a target height of 0SDS. His head circumference was +3SDS according to the expectations for those with SOS. He was diagnosed with mild mental retardation. At 15 months of age, the patient showed positive progress in motor development. He still required a small dose of diazoxide of 1.5 mg/kg/day and at 18 months diazoxide at the same dose was needed only during infections. At two years of age hypoglycemic episodes completely resolved. Currently he is on a normal diet and can tolerate overnight fast without hypoglycaemia. His heart function was stable at follow-

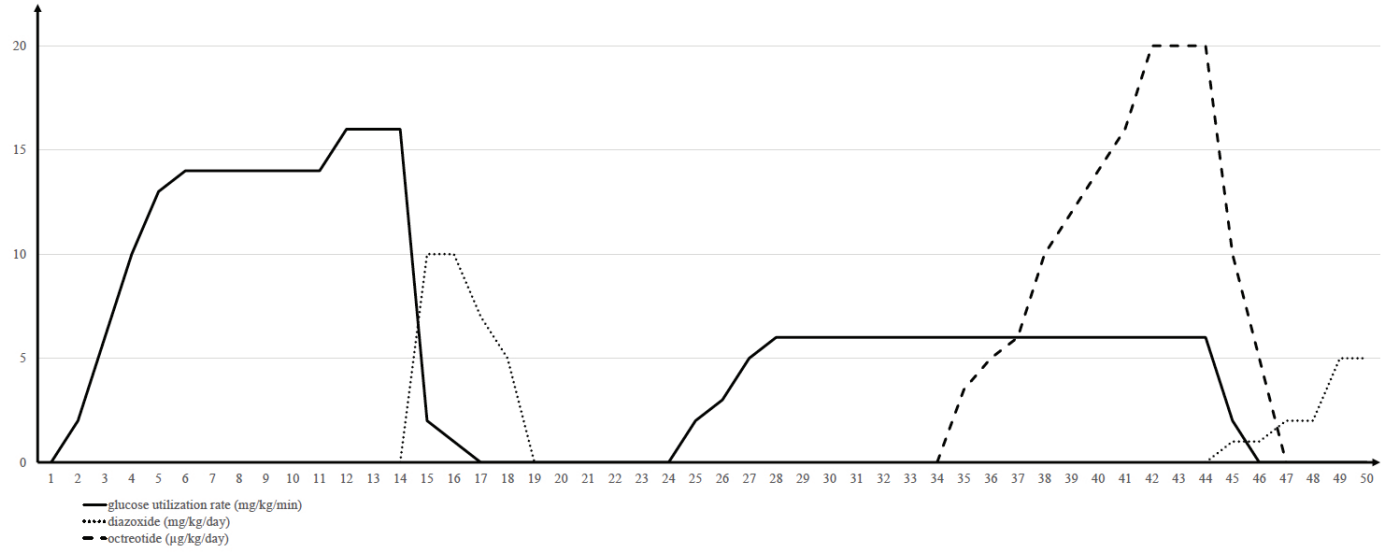

Figure 1. Glucose utilization, use of medication during treatment NICU. Blackline-glucose utilization rate (mg/kg/min), dotted line-diazoxide doses (mg/kg/day), dashed line-octreotide doses (µg/kg/day)
NICU: neonatal intensive care unit

up appointments. The patient was diagnosed with bronchial asthma and treated with conventional inhalation steroid. He has frequently been affected by viral infections complicated by mucus plugs in airways due to hypotonus and has often required short-term hospitalizations. The patient needs a team of specialists for his associated anomalies and development delay.

Materials and Methods

Clinical features, biochemical data, and medical treatments were collected from the patient's medical records and from personal observations of clinical follow up. Height, weight and head circumference were measured at each visit, and SDS were calculated using current Swedish National references (15,16).

Genetic Findings

The patient's DNA analyzed by CMA and revealed a 1349-1354 kb deletion on chromosome 5: arr[GRCh38] 5q35.2q35.3(176,597,879-177,949,621)x1 (Figure 2). The deleted region overlapped 36 HGNC and 24 OMIM genes: *GPRIN1, SNCB, UNC5A, HK3, UIMC1, ZNF346,* fibroblast growth factor receptor 4 *(FGFR4) gene, NSD1, RAB24, MXD3, PRELID1, LMAN2, RGS14, SLC34A1, PFN3, F12, GRK6, DBN1, PDLIM7, DOK3, DDX41, FAM193B, PRR7* and *B4GALT7* (Table 1).

The deletion of the *NSD1* gene that would result in haploinsufficiency represents the major cause of SOS. Therefore, this loss was interpreted as a pathogenic copy number variant (CNV), causing the syndrome in our patient. The deletion was confirmed by FISH with a locus specific *NSD1* probe (Figure 3). The specific signal for *NSD1* signal was seen on only one homologous chromosome 5. Subsequent FISH analysis of parental samples did show normal signal pattern with presence of the *NSD1* signals on both chromosomes. Thus, we concluded that the deletion in this case appeared to be *de novo*.

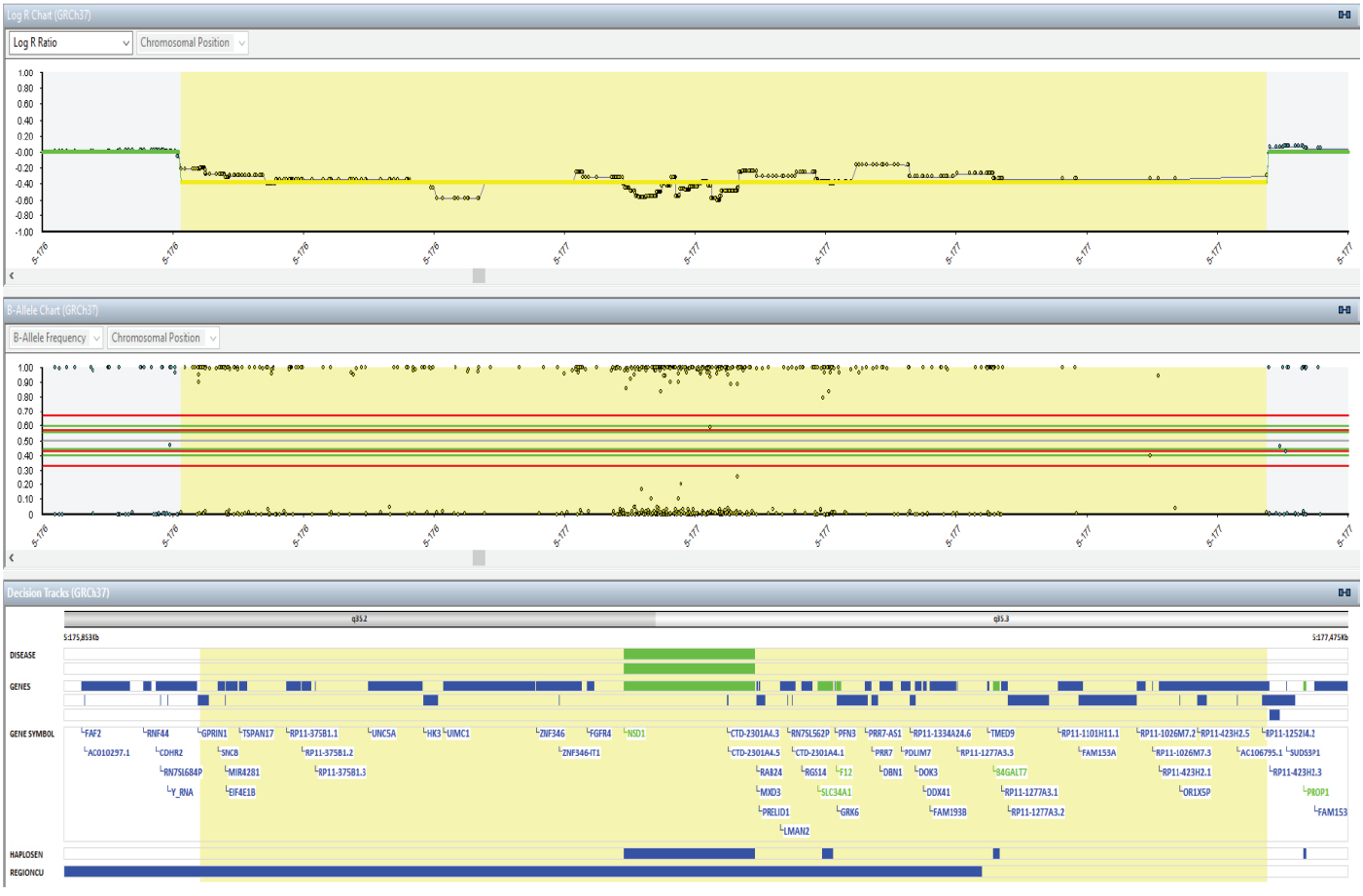

Figure 2. Deletion on chromosome 5q35.2q35.3 [arr(GRCh38) 5q35.2q35.3(176,597,879-177,949,621)x1] detected by genome wide genotyping and CNV analysis. Details of SNP array analysis are presented in Materials and Methods. The data are visualized in BlueFuse Multi software as Log2Ratio (upper panel), B-allele frequency (middle panel) and Decision Tracks. The deletion covers 24 genes, *NSD1* is shown in green. Boundaries of the deletion are shown as a yellow box. The list of all genes is available in Table 1

CNV: copy number variant, SNP: single nucleotide polymorphism

Table 1. The genes located at 5q35.2-q35.2 deleted in the patient

Gene		Start	End	OMIM	Morbid
B4GALT7	Beta-1,4-galactosyltransferase 7	177600132	177610330	604327	Ehlers-Danlos syndrome, spondylodysplastic type, 1 (AR)
DBN1	Drebrin 1	177456608	177474401	126660	-
DDX41	DEAD-box helicase 41	177511577	177516961	608170	-
DOK3	Docking protein 3	177501904	177511274	611435	-
EIF4E1B	Eukaryotic translation initiation factor 4E family member 1B	176630618	176646644	-	-
F12	Coagulation factor XII	177402133	177416583	610619	Angioedema, hereditary, 3 (AD) Factor XII deficiency (AR)
FAM153A	Family with sequence similarity 153 member A	177707981	177784435	-	-
FAM193B	Family with sequence similarity 193 member B	177519789	177554586	615813	-
FAM193B-DT	FAM193B divergent transcript	177554824	177555364	-	-
FGFR4	Fibroblast growth factor receptor 4	177086905	177098144	134935	Cancer progression/ metastasis (Unknown inheritance)
GPRIN1	G protein regulated inducer of neurite outgrowth 1	176595802	176610156	611239	-
GRK6	G protein-coupled receptor kinase 6	177403204	177442901	600869	-
HK3	Hexokinase 3	176880869	176899346	142570	-
LINC01574	Long intergenic non-protein coding RNA 1574	176743205	176743871	-	-
LMAN2	Lectin, mannose binding 2	177315805	177351840	609551	-
MIR4281	microRNA 4281	176629439	176629500	-	-
MXD3	MAX dimerization protein 3	177301461	177312757	609450	-
NSD1	Nuclear receptor binding SET domain protein 1	177131830	177300213	606681	Sotos syndrome (AD)
OR1X5P	Olfactory receptor family 1 subfamily X member 5 pseudogene	177836434	177837646	-	-
PDLIM7	PDZ and LIM domain 7	177483394	177497606	605903	-
PDLIM7-AS1	PDLIM7 antisense RNA 1	177494995	177503647	-	-
PFN3	Profilin 3	177400109-	177400661	612812	-
PRELID1	PRELI domain containing 1	177303799	177306949	605733	-
PRMT1P1	Protein arginine methyltransferase 1 pseudogene 1	177265580	177266588	-	-
PRR7	Proline rich 7, synaptic	177446445	177456286	618306	-
PRR7-AS1	PRR7 antisense RNA 1	177438503	177447982	-	-
RAB24	RAB24, member RAS oncogene family	177301198	177303744	612415	-
RGS14	Regulator of G protein signaling 14	177357924	177372596	602513	-
SLC34A1	Solute carrier family 34 member 1	177379235	177398848	182309	Fanconi renotubular syndrome 2 (AR) Hypercalcemia, infantile, 2 (AR) Nephrolithiasis/osteoporosis, hypophosphatemic, 1 (AD)
SNCB	Synuclein beta	176620082	176630556	602569	Dementia, Lewy body; DLB (AD)
TMED9	Transmembrane p24 trafficking protein 9	177592203	177597242	-	-
TSPAN17	Tetraspanin 17	176647387	176659054	-	-
UIMC1	Ubiquitin interaction motif containing 1	176905005	177022633	609433	-
UNC5A	Unc-5 netrin receptor A	176810519	176880898	607869	-
ZNF346	Zinc finger protein 346	177022696	177081189	605308	-
ZNF346-IT1	ZNF346 intronic transcript 1	177051714	177052963	-	-

Genomic positions according GRChr38/hg38.
AR: autosomal recessive, AD: autosomal dominant, OMIM: Online Mendelian Inheritance in Man

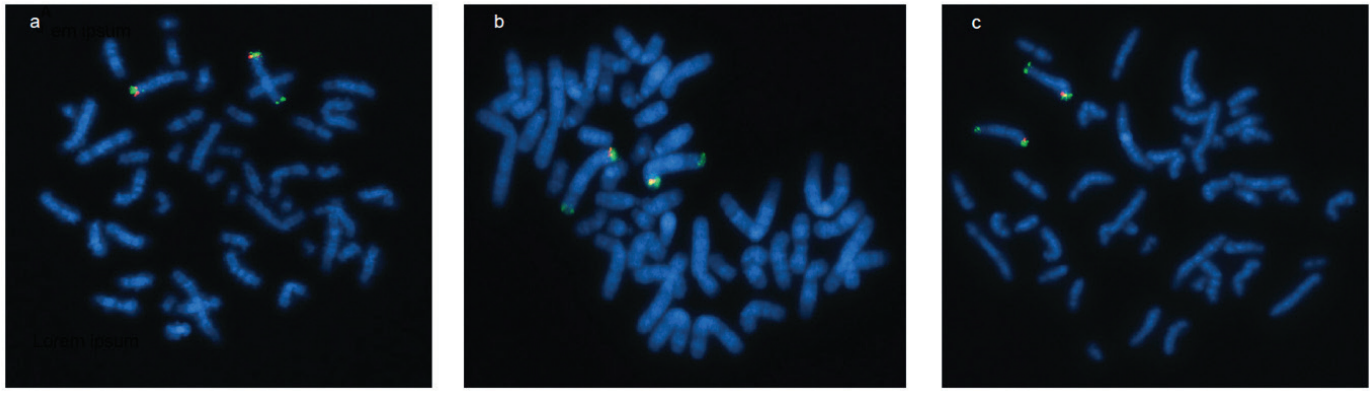

Figure 3. Fluorescence *in situ* hybridization results with *NSD1* specific probe (5q35) (Cytovision Technologies). a) *NSD1* deletion is seen in metaphase derived from the patient's peripheral blood. FISH on parental blood samples show presence of two signals on both chromosomes [b) paternal sample, c) maternal sample]

Molecular genetic analyses targeted next generation seuencing with a congenital hyperinsulinism sequencing panel with CNV detection did not identify any pathogenic variants. Minimum NGS coverage ≥20X for all exons and ±10bp of flanking DNA, and ≥10x from 11-20bp of flanking DNA. Average NGS coverage was 165x and fraction of bases covered with NGS was 99.5%. The following genes, *ABCC8, GCK, GLUD1, HADH, HNF1A, HNF4A, SLC16A1,* and *UCP2* were analyzed.

High resolution CMA was performed on peripheral blood collected in EDTA tubes using standard procedure, and DNA was isolated from 200 µL of whole blood using the QiaSymphony (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA was quantified using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

CMA or genome-wide genotyping used for detection of CNVs was performed with the Infinium CytoSNP-850K v1.2 Beadchip (Illumina, San Diego, CA, USA) containing approximately 850,000 single nucleotide polymorphisms markers over the entire genome with an average probe spacing of 1.8kb. Two hundred nanogrammes of ng DNA was hybridized on a beadchip after whole-genome amplification, followed by scanning on the HiScan machine (Illumina). Genotyping results were visualized, normalized and clustered using the Genotyping module of the GenomeStudio software (Illumina) and by BlueFuse Multi software (v.4.4). The cnvPartition 3.2.0 (Illumina) was applied for CNV detection by retrieving Log R Ratio (LRR, the ratio between the observed and the expected probe intensity) and the B allele frequency (BAF). When a CNV is absent, the LRR is around zero, and the BAF is 0, 0.5, or 1 depending on genotypes AA, AB, and BB. Deviations from the expected values indicate copy number alterations. Human genome GRCh38 (NCBI)/hg38 (UCSC) was used for assigning all chromosomal positions. CNVs overlapping with a region of known microdeletion or microduplication syndromes and/or disease-causing genes were classified as pathogenic. The Database of Genomic Variants, the OMIM, and the dbVar Genome Browser and Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources were used to access known microdeletion and microduplication syndromes.

FISH analysis specific for chromosome 5 was performed on metaphase slides according to the manufacturer's standard protocol (Cytocell Technologies, Cambridge, UK). Probes detecting Cri-du-Chat syndrome on 5p15.2 (*CTNND2* in red) and 5p15.31 (*UBE2QL1* in green) were used as control probes. The third probe in this mix was a *NSD1* specific probe on 5q35 labelled in green. The slides were dehydrated and co-denatured with the probes at 73 °C for 5 min. Hybridization was done overnight at 37 °C using Hybrite™ (Vysis, Downers Grove, IL, USA). The slides were counterstained with 4',6-diamidino-2-phenylindole (Vysis). The images were captured by Leica Microscope and analyzed using Cytovision Image Analysis and Capture System (version 7.5) (Leica Biosystems, Maarn, NL).

Discussion and Conclusion

We present a patient with characteristic features of SOS, persistent CHI and a *de novo* genomic deletion encompassing 24 OMIM genes including the entire *NSD1* gene. Among the 24 OMIM genes, seven were classified as morbidity-associated (Table 1). Notably, one of the deleted genes, *HK3*-hexokinase, a member of the hexokinase family, is involved in the first step of glucose metabolism (17). However, neither sequence variants in *HK3* gene or haploinsufficiency have been linked with hyperinsulinism. *HK3* sequence variants have been suggested to be associated with ovarian failure and affect the glycolysis important in the development and progression of different neoplasms (18,19,20). Another interesting gene is the *FGFR4*. Sequence variants in *FGFR4* gene or haploinsufficiency have

been suggested to be associated with diverse phenotypes but not with CHI (12,21).

A heterozygous mutation in the *NSD1* gene (MIM 606681) identified in more than 75% of cases is a common genetic cause of SOS (22). Previously, CHI was reported as an unusual presentation in Sotos patients (5,23,24), but in recent years the number of studies reporting CHI in this condition has increased (7,8). The concurrent presence of *NSD1* defects and CHI in SOS has also been reported. Transient neonatal CHI was described in Japanese patients with SOS where 7 of 8 patients harboured a 5q35 microdeletion but only 3 of 8 required diazoxide treatment (7,8). In a national Japanese survey, CHI was present in about 10% of children with SOS, indicating strong association between these two features (25). Furthermore, CHI was reported in seven patients with SOS caused by point mutations in *NSD1* (26). In 3 of 7 patients, CHI persisted for more than one year. These results challenge the previous hypothesis that CHI in SOS is due to the deletion of additional genes in the 5q35 region. Moreover, *NSD1* is proposed to play a role in glucose homeostasis. *NSD1* was known as a histone methyltransferase and is implicated in the regulation of chromatin and gene expression (6). However, *NSD1* is expressed in human pancreatic beta cells, as demonstrated by bulk islet cell analyses and single-cell RNA-sequencing (6,27,28). Association between SOS, response to diazoxide treatment and CHI disappearance over time was also described by Kapoor et al. (29), although the exact mechanisms are still not completely understood.

The present case exhibited resolution of his CHI, but it had persisted for almost two years; this is in line with previous publications that reported a similar association between SOS and CHI. The definition of transient hyperinsulinemic hypoglycaemia was poorly defined in earlier studies, and is characterized by spontaneous resolution within a few days but as late as six months of life (29). According to this definition, the transient CHI was prolonged in our case. It is unclear if this is due to the relatively large deletion. The patient required extra feeding and for almost two years was on medication with diazoxide, a ATP-sensitive potassium channel opener, the first-line therapy for CHI (30). It is important to note that our patient responded poorly to diazoxide, which led to heart failure. This meant that when diazoxide was reinitiated, it was at a low dose of less than 5 mg/kg/d because of the risk of heart complications. Nevertheless, the doses were sufficient to avoid hyperinsulinemia and to ensure normoglycaemia. Compared to our patient who required treatment with diazoxide for almost 2 years, previous reports have found diazoxide treatment was required for shorter periods, although this was up to 8 months of age in one report (31) and three children with point mutations in *NSD1* were treated over one year (6).

As practice shows, neonatal CHI needs the correct diagnosis and an adequate treatment to avoid neurological consequences. The presented patient with SOS also exhibited a broad spectrum of clinical features, especially in terms of CHI.

The identification of *NSD1* abnormalities in most patients with SOS makes a molecular diagnosis possible and helps to confirm a clinical diagnosis of SOS. Despite hypoglycemia being described as a minor feature in SOS, several reports on a genotype-phenotype correlation were published that warrant further research. We propose that, in neonatal diagnostics, the phenotypic spectrum of SOS should include HI as a significant feature.

This case demonstrates that early clinical diagnosis of this rare condition may be challenging and depends on subjective clinical experience and judgement. Experiences and lessons from our management may merit inclusion within medical discourse, and it is hoped this case report will serve as a reference for the diagnosis and treatment of similar patients in the future.

Ethics

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

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Data Availability Statement

The dataset generated by genome wide genotyping using SNP-array (Illumina) is available upon request.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Elena Lundberg, Concept: Elena Lundberg, Magnus Burstedt, Irina Golovleva, Design: Elena Lundberg, Irina Golovleva, Data Collection or Processing: Elena Lundberg, Genetic analysis Magnus Burstedt, Irina Golovleva, Analysis or Interpretation: Elena Lundberg, Magnus Burstedt, Irina Golovleva, Writing: Elena Lundberg, Magnus Burstedt, Irina Golovleva.

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CASE REPORT

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Atypical Presentation and Course of ACTH-Independent Cushing's Syndrome in Two Families

Kübra Yüksek Acınıklı[1], Sezer Acar[2], Ahu Paketçi[3], Özgür Kırbıyık[4], Mert Erbaş[5], Özge Besci[1], Gözde Akın Kağızmanlı[1], Deniz Kızmazoğlu[6], Oktay Ulusoy[7], Erdener Özer[8], Kutsal Yörükoğlu[8], Ayhan Abacı[1], Handan Güleryüz[9], Ece Böber[1], Korcan Demir[1]

[1]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye
[2]Manisa City Hospital, Clinic of Pediatric Endocrinology, Manisa, Türkiye
[3]Medipol Mega University Hospital, Department of Pediatric Endocrinology, İstanbul, Türkiye
[4]University of Health Sciences Türkiye, İzmir Tepecik Training and Research Hospital, Genetic Diagnosis Center, İzmir, Türkiye
[5]University of Health Sciences Türkiye, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, Clinic of Pediatric Endocrinology, İzmir, Türkiye
[6]Dokuz Eylül University Institute of Oncology, Department of Pediatric Oncology, İzmir, Türkiye
[7]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Surgery, İzmir, Türkiye
[8]Dokuz Eylül University Faculty of Medicine, Department of Pathology, İzmir, Türkiye
[9]Dokuz Eylül University Faculty of Medicine, Department of Radiology, Division of Pediatric Radiology, İzmir, Türkiye

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What is already known on this topic?

Primary pigmented nodular adrenocortical disorder (PPNAD), which is rarely seen children while it is the most prevalent cause of adrenocorticotropic hormone (ACTH) independent Cushing's syndrome, is typically a micronodular disease and mainly associated with Carney complex (CNC). CNC is a rare autosomal dominant syndrome, characterized by pigmented lesions of the skin and mucosa, cardiac, cutaneous and other myxomas and multiple endocrine tumors.

What this study adds?

The findings of reported families provide information for a better understanding of the genetic pathogenesis, diagnosis and clinical management of CNC. Analytic variability in ACTH assays should be kept in mind during interpretation of ACTH levels. One case developed Hodgkin lymphoma five year after adrenalectomy, this association was not previously reported with CNC. Two cases had macronodules contrary to what is generally seen in cases with PPNAD.

Corresponding Author: Prof. Korcan Demir, Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye
E-mail: korcandemir@gmail.com **ORCID:** orcid.org/0000-0002-8334-2422
Received: 19.09.2023 **Accepted:** 10.12.2023 **Epub:** 12.12.2023 **Publication Date:** 13.03.2026

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ABSTRACT

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare genetic disease mainly associated with Carney complex (CNC), which is caused by germline mutations of the regulatory subunit type I-alpha of the cAMP-dependent protein kinase (*PRKAR1A*) gene. We report three cases suffering from CNC with unique features in diagnosis and follow-up. All cases had obesity and a cushingoid appearance and exhibited laboratory characteristics of hypercortisolism. However biochemical and radiological examinations initially suggested Cushing's disease in one case. All of the cases were treated surgically; two of them underwent bilateral adrenalectomy at once, one of them had unilateral adrenalectomy at first but required contralateral adrenalectomy after nine months. Contrary to what is usually known regarding PPNAD, the adrenal glands of two cases (Case 2 and 3) had a macronodular morphology. Genetic analyses revealed pathogenic variants in *PRKAR1A* (Case 1: c.440+5 G>A, not reported in the literature; cases 2 and 3: c.349G>T, p.V117F). One case developed Hodgkin lymphoma five year after adrenalectomy, this association was not previously reported with CNC. The findings of these families provides important information for a better understanding of the genetic pathogenesis, diagnosis, and clinical management of CNC. Hodgkin lymphoma may be a component of CNC.

Keywords: Cushing's disease, cancer, myxoma, lentigo, PEG precipitation, macronodule

Introduction

Endogenous Cushing's syndrome (CS) in children is rare, with an incidence of 1-1.5 per million population per year. Adrenocorticotropic hormone (ACTH)-independent CS accounts for 15-20% of endogenous CS (1). Bilateral nodular adrenocortical diseases have been detected in 1-2% of these patients, resulting in ACTH-independent CS (2). In primary pigmented nodular adrenocortical disease (PPNAD), both adrenal glands are involved, and there are small, brown-black nodules separated by the atrophic adrenal cortex. Nodules are typically smaller than 1 cm and demonstrate micronodular hyperplasia (3). Carney complex (CNC) is a rare autosomal dominant syndrome characterized by pigmented lesions of the skin and mucosa, cardiac, cutaneous, and other myxomas, and multiple endocrine tumors, the most common of which is PPNAD (4). CNC is caused by mutations in the *PRKAR1A* (OMIM 188830) coding for the regulatory subunit type I-alpha (RIα) of protein kinase A (PKA). To establish a diagnosis of CNC, a patient must exhibit two of the disease's manifestations or exhibit one of these manifestations and have an affected first-degree relative or an inactivating *PRKAR1A* mutation. No direct correlation has been identified between all *PRKAR1A* mutations and the various phenotypes to date (5).

The present report describes three cases of PPNAD. Two of the cases were distantly related, and we present two pedigrees involving cases and currently healthy *PRKAR1A* mutation carriers. The cases had unique diagnostic and follow-up features. To the best of our knowledge, this report presents the first CNC case who developed Hodgkin lymphoma during follow-up.

Cases

Case characteristics are shown in Table 1 and pedigrees in Figure 1.

Case 1

The proband was an 11-year-old girl. She presented to our institution with weight gain over the preceding two years. She was pubertal. The family history revealed no consanguinity. On physical examination, her height, weight, and body mass index (BMI) were 132.5 cm [-1.5 standard deviation score (SDS), 44.8 kg (0.92 SDS), and 25.5 (+1.8 SDS)], respectively. Moon face, abdominal adiposity, buffalo hump, acne, and stria were evident. The hormonal assessment showed a suppressed plasma ACTH in the presence of high morning serum cortisol and an altered circadian cortisol level. The hormonal and clinical signs indicated ACTH-independent CS. A computed tomography (CT) scan of the adrenals revealed bilateral micronodular hyperplasia (shown in Figure 2A). PPNAD was suspected, and bilateral adrenalectomy was performed. Hydrocortisone and fludrocortisone were initiated. The histopathological findings showed numerous cortical hyperplastic nodules (less than 4 mm), compatible with PPNAD (Figure 3A). She progressively lost weight, and signs of CS regressed. The molecular genetic analysis of *PRKAR1A* identified a heterozygous splice site mutation within exon 4a (c.440+5 G>A), which was pathogenic according to the American College of Medical Genetics and Genomics criteria and which was not reported in the medical literature. This variation has not been associated with any protein alterations. Nevertheless, the occurrence of a different variation within the same identical exon (c.440+5 G>C) was reported to result in a premature stop codon (TGA) and modification of the secondary structure of the RIα domain (6). Regarding CNC, cardiac examination and thyroid ultrasound (US) were normal, and there were no signs of skin lesions. Other pituitary hormone levels were normal. Genetic analysis of her mother (44 years old) and maternal aunt (35 years old) was performed, and they had the same variant in *PRKAR1A* without any symptoms or signs of CNC. Her father and other family members could not undergo genetic analysis

Table 1. Case characteristics

	Case 1	Case 2*	Case 3*
Gender	Female	Female	Female
Age (years)	11	16	12
Serum cortisol (µg/dL)			
Morning, basal	17.8	30	25
Midnight (at 23:00)	15	NA	19
Morning, after 1 mg dexamethasone	17.8	13.8	13
24 h UFC (µg/m²/day, NR<70)	NA	207	233
ACTH (pg/mL, NR 0-63)	<5	<1	<5
Adrenal CT scan	Symmetrical, bilateral nodular hyperplasia (bilateral micronodular)	Asymmetrical (L>R) bilateral nodular hyperplasia (left side macronodular)	Asymmetrical (L>R), bilateral nodular hyperplasia (left side macronodular)
***PRKAR1A* variant**	c.440+5G>A	c.349G>T	c.349G>T
IGF-1 (ng/mL, NR 143-506)	NA	215	203
DHEA-S (µg/dL, NR 25-460)	NA	169	345
Echocardiography	Normal	Normal	Minimal hypertrophic IVS
Thyroid ultrasound	Normal	Normal	Normal

*Cases 2 and 3 were distant relatives.
UFC: urinary free cortisol, NR: normal range, CT: computed tomography, IGF-1: insulin like growth factor-1, DHEA-S: dehydroepiandrosterone-sulfate, L: left, NA: not available, R: right, IVS: interventricular septum

because of social reasons. Reportedly, subjects III.11, IV.4, and IV.5 were diagnosed with CS and had an adrenalectomy. At the age of 16 years, the patient developed persistent cervical and supraclavicular lymphadenopathy associated with weight loss and was diagnosed with nodular sclerosing type Hodgkin lymphoma five years after adrenalectomy. At the most recent follow-up of Case 1, she was 17 years and 8 months old; her physical examination was normal with weight of 45 kg (-1.75 SDS), height of 150 cm (-2.21 SDS), BMI of 20 kg/m² (-0.25 SDS), and other manifestations of CNC were not present.

Case 2

This case was a 16-year-old girl who presented with a 4-year history of significant weight gain, hirsutism, and irregular menstrual periods. Pubertal development was already complete, with spontaneous menarche starting at 12 years. The family history revealed no consanguinity. On physical examination, her height, weight, and BMI were 147.5 cm (-2.58 SDS), 85.6 kg (3.29 SDS), and 39.5 (3.83 SDS), respectively. She displayed striae, moon face, abdominal adiposity, buffalo hump, acne, and hirsutism. There were no lentigines or blue nevi on skin examination. Serum and 24-hour urinary cortisol levels were high. At first, plasma ACTH level (Siemens, a solid phase, two-site enzyme chemiluminescent system, IMMULITE® 2000 XPI) was found to be 14 pg/mL (normal, 7-63 pg/mL). Pituitary magnetic resonance imaging revealed an adenoma (4 mm). High dose dexamethasone suppression test revealed unsuppressed ACTH level of 10.6 pg/mL. Clinical and biochemical incompatibility suggested ACTH interference and ACTH level was undetectable after polyethylene glycol precipitation (PEG). Furthermore, ACTH level was undetectable (<1 pg/mL) when measured with a different analytical platform (Roche Cobas E411 Diagnostics, a solid-phase, two site electrochemiluminescence immunoassay platform). Adrenal CT scanning showed bilateral nodular lesions characteristic of hyperplasia, which were more prominent and macronodular in the left adrenal gland (shown in Figure 2B). Bilateral adrenalectomy was performed. Hydrocortisone and fludrocortisone were initiated. The histopathological findings showed PPNAD with bilateral micro- and macro-nodules (greater than 10 mm), while revealing no discernible signs of necrotic or hemorrhagic regions (shown in Figures 3B and 4). The molecular genetic analysis of the *PRKAR1A* gene identified a heterozygous, previously reported c.349G>T (p.V117F) which was pathogenic according to the ACGM criteria. Additional investigations for CNC features were negative. Her mother, father, and brother underwent genetic analysis and only her father had the same variant but was asymptomatic. At the most recent follow-up of Case 2, she was 21 years and 6 months old, her blood pressure was normal without any medication but she was obese (weight: 72.5 kg, height: 149 cm, BMI: 32.4 kg/m²). Other manifestations of CNC were not present. The size of pituitary adenoma had not changed during follow-up.

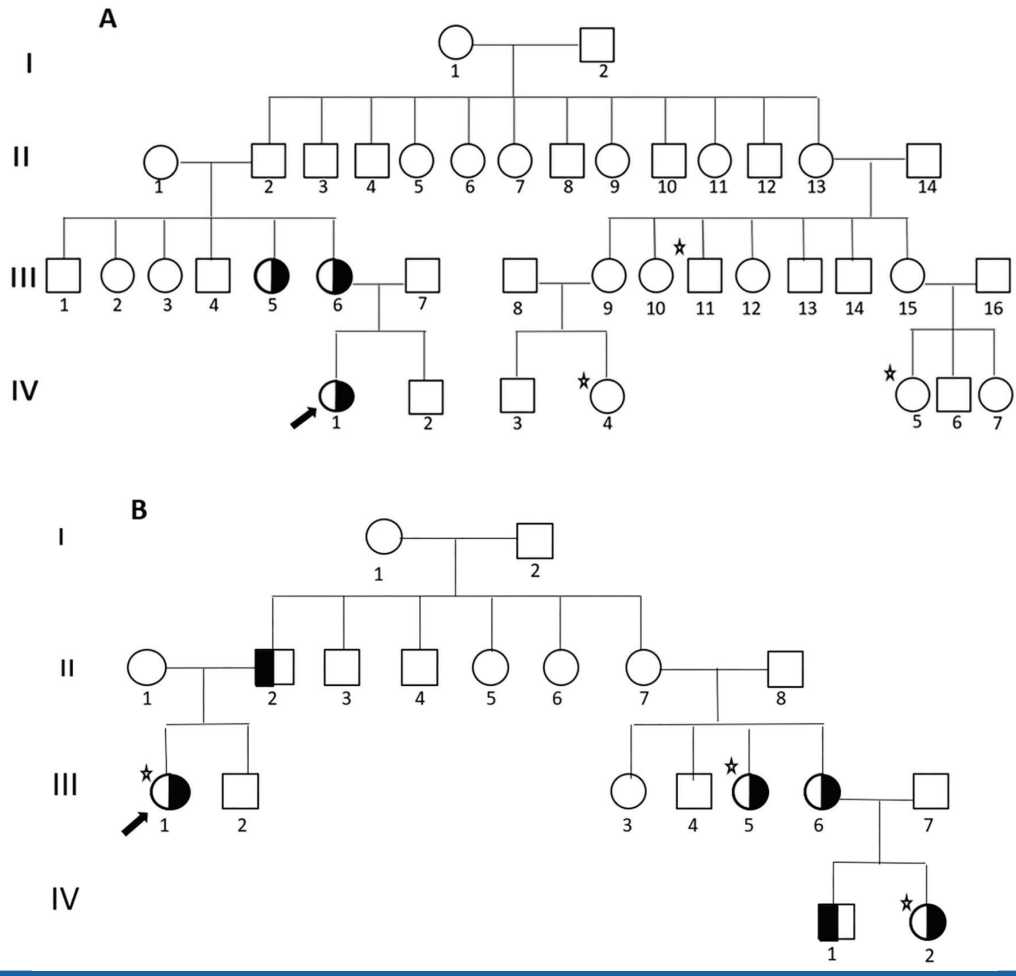

Figure 1. Pedigrees of the two families with PPNAD. Half-filled squares and circles represent heterozygous variant carriers. Individuals who were reported to have Cushing syndrome and underwent an adrenalectomy are indicated with a star. **(A)** Case 1 is subject IV.1 (c.440+5 G>A). Genetic analyses were made in subjects II.1, III.5, III.6, and IV.1. **(B)** Case 2 is subject III.1 (c.349G>T) and Case 3 is subject IV.2 (c.349G>T). Genetic analyses were made in subjects II.1, II.2, III.1, III.2, III.5, III.6, III.7, IV.1, and IV.2
PPNAD: primary pigmented nodular adrenocortical disease

Case 3

Case 3 was a 12-year-old girl who presented to another institution complaining of hirsutism, significant acne, and weight gain over the preceding two years. Her parents were second cousins. On physical examination, her height, weight, and BMI were 146 cm (-1.16 SDS), 102.4 kg (4.68 SDS), and 48 (+4.5 SDS), respectively. Pubertal development was Tanner stage 3, without menarche. She displayed striae, a moon face, abdominal adiposity, and a buffalo hump, and there were no lentigines or blue nevi on skin examination. She had persistent hypertension and was receiving treatment with enalapril, spironolactone, and valsartan. Hormonal assessment showed a suppressed plasma ACTH in the presence of high serum cortisol with increased levels of 24-hour urinary free cortisol (UFC). Adrenal CT evaluation revealed bilateral nodular lesions, which were more prominent on the left (shown in Figure 2C). She underwent left-side adrenalectomy at the same institution. However, her clinical picture and hypercortisolism did not improve, and she was referred to our center. Hormonal assessment showed a suppressed plasma ACTH level in the presence of high serum cortisol. A right-side adrenalectomy was performed at our institution nine months after the first surgery. Hydrocortisone and fludrocortisone were initiated. Histopathological assessment revealed bilateral micro- and macro-nodular hyperplasia. *PRKAR1A* analysis detected the same mutation as in Case 2 (c.349G>T, p.V117F). Her mother and brother had the same variant without any symptoms or signs of CNC. Her maternal aunt, who had the same *PRKAR1A* mutation, was diagnosed with ACTH-independent CS. Her adrenal CT revealed bilateral adenoma (right 25x27 mm, left 10x10 mm). She underwent a right adrenalectomy in another center, and histopathological assessment revealed adrenocortical oncocytoma. At the most recent follow-up of Case 3, she was 14

years and 4 months old, her blood pressure was normal without any medication, but she was obese [weight: 93.7 kg (+4.2 SDS), height: 152.5 cm (-1.59 SDS), BMI: 40.2 kg/m^2 (+4.2 SDS)] and had hirsutism. Other manifestations of CNC were not present.

Follow-up

We recommended annual laboratory and imaging evaluations for both affected subjects and the asymptomatic carriers, with an echocardiogram for cardiac myxoma, a thyroid US for thyroid nodules, a testicular US for boys, and the measurement of insulin-like growth factor-1 and prolactin beginning in adolescence to screen for pituitary overactivity. For asymptomatic carriers, an annual measurement of 24-hour UFC excretion was planned. At the time of writing, no additional characteristics of CNC have been identified in either affected subjects or asymptomatic carriers (7).

Discussion

The diagnosis of CS is not straightforward and so approximately 2.5 to 3 years of delay are reported in the literature (8). The diagnosis of CS was further complicated in Case 2 due to a falsely unsuppressed ACTH level. Available ACTH assays exhibit considerable differences in terms of sensitivity and lead to a wide variability in ACTH measurements, especially when low concentrations of ACTH are present. In the presented case, measurement of the ACTH level was initially conducted with a kit that was reported to fail in detecting low ACTH levels in 19% of cases in a multicenter study (9,10). Treatment of plasma and serum samples with PEG has been shown to precipitate immunoglobulins, including heterophile antibodies. Falsely high values are prevented, and the true level may be measured (10,11). Thus, a PEG procedure was performed using our patient's serum, and an undetectable ACTH level was demonstrated. This finding was confirmed by measurement on a different analytical platform. Regardless of their sensitivity and specificity, immunoassays are susceptible to occasional analytical errors. An astute clinician should keep in mind the potential for interference in cases where there is a discrepancy between clinical and laboratory findings.

A contrast-enhanced CT scan should be the next diagnostic step after hormonal evaluation in patients with ACTH-independent CS (1). The appearance of the adrenal glands on imaging in

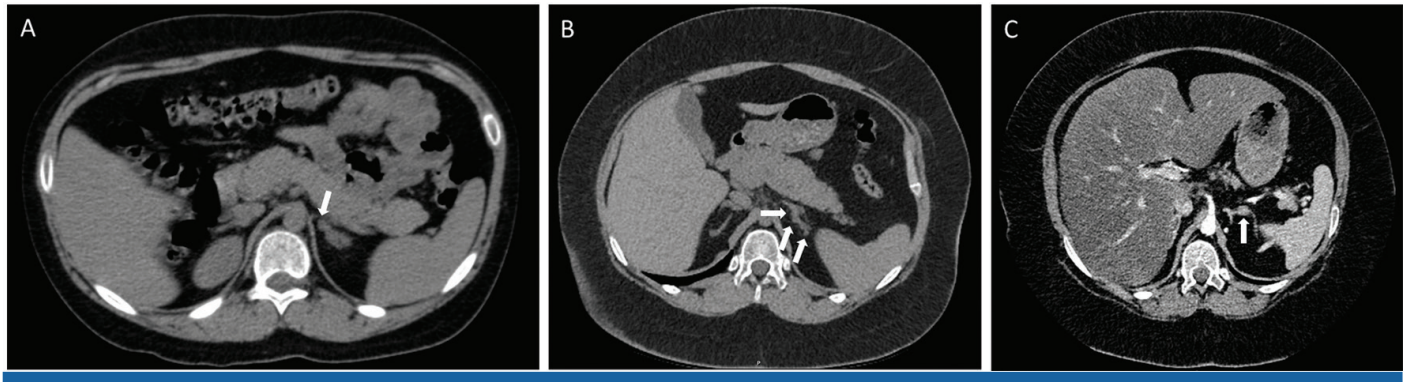

Figure 2. Adrenal CT images showing numerous adrenal nodules of varying sizes: **A)** Case 1, **B)** Case 2, and **C)** Case 3
CT: computed tomography

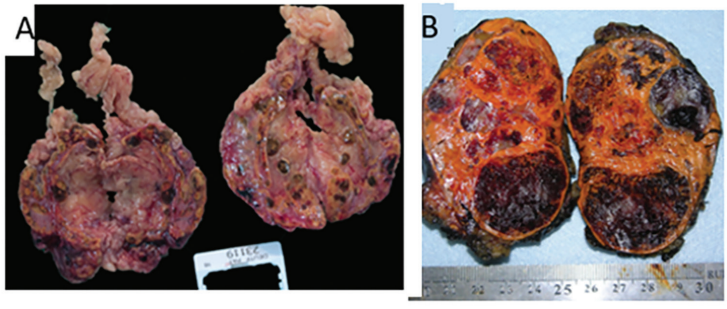

Figure 3. Gross view of a resected adrenal glands showing nodular changes. **A)** Case 1, **B)** Case 2. Multiple tan-brown nodules [**A)** micronodules, **B)** macro and micronodules] were seen in the cortex

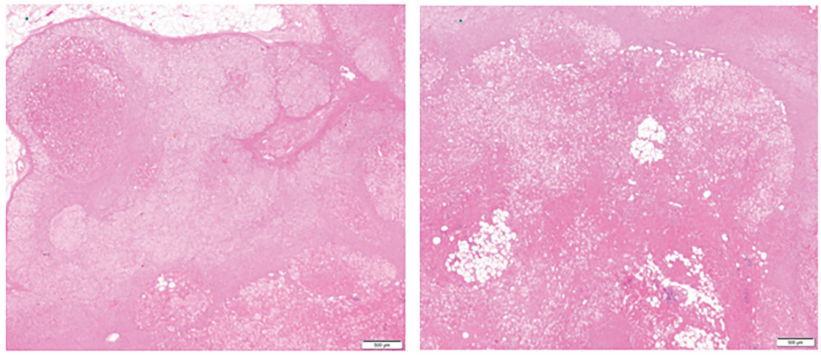

Figure 4. Histopathological images of the adrenal of Case 2. Nodules are composed of clear and compact cells with variable lipid. No mitotic figures or atypical cells were present

patients with PPNAD is often initially interpreted as normal, which differs from other ACTH-independent disorders where relatively large tumors are easily seen. It is important that the clinician and radiologist have expertise in evaluating radiological findings in these cases. Nodules are typically less than 1 cm in size (micronodules) (12). However, histopathological evaluation of the adrenal glands in cases 2-3 demonstrated macronodules. Contrary to what is generally known, macronodular appearance may rarely be detected in cases of PPNAD. A report of 11 patients with PPNAD due to *PRKAR1A* variants found one patient (26 years of age) with macronodules (2.5 cm), while three (between 25 and 55 years of age) of four patients with PPNAD without a *PRKAR1A* mutation had macronodules (3). An 18-year-old female who had a monoallelic (c.102G>A) germline *PRKAR1A* mutation was reported to have macronodular PPNAD. In addition, a somatic mutation in *PRKAR1A* (16-bp deletion of the acceptor splice site of exon 4B, IVSdel-17→-2) was found in the macronodule. This somatic mutation was not present in the tissue adjacent to this nodule, in the left adrenal, or in leukocyte DNA (13). The exact etiology of macronodules in PPNAD is unclear, but in general, somatic *CTNNB1* mutations are suggested to play a role in the formation of macronodules by accumulation of β-catenin (5).

The variant in Case 1 (c.440+5 G>A) was also present in her asymptomatic mother and maternal aunt, while individuals III.11, IV.4, and IV.5 within this family were reported to have CS and underwent adrenalectomy. However, as these patients reside in another country, we were unable to gather additional clinical and genetic information about them. Typically, the overall penetrance of CNC in individuals with a pathogenic *PRKAR1A* variant exceeds 95% by the age of 50 years. However, certain splice site variants may lead to a milder phenotype or reduced penetrance of CNC (5). To date, there is only one report in the literature of the *PRKAR1A* variant detected in cases 2 and 3, and in these cases, the adrenal phenotype was highly variable (14). According to this report, the c.349G>T splice site mutation is predicted to lead to exon 4 skipping, and the resulting frameshift would lead to a premature stop codon. The *PRKAR1A* mutation was found in three asymptomatic individuals within this familial cohort, who did not exhibit any clinical signs associated with CNC. The authors concluded that the c.349G>T variation has low penetrance, resulting in incomplete clinical expression. The absence of CNC symptoms in asymptomatic carriers might be related to both the young age of the carriers and the low penetrance of some variants in *PRKAR1A*.

Of note, Case 1 developed Hodgkin lymphoma during follow-up. *PRKAR1A* is the gene encoding the type 1A regulatory subunit of PKA, which modulates various events during cell proliferation in combination with cAMP, and deregulation of these effector molecules is associated with the development of different cancers via multiple pathways (15). Functionally, loss of *PRKAR1A* is associated with excess PKA signaling in tumors from patients, although the exact mechanism by which this aberrant signaling causes tissue-specific tumorigenesis is unknown (16). Patients with a *PRKAR1A* mutation were more likely to develop other cancers, including growth hormone-secreting pituitary tumors, gonadal tumors, and thyroid neoplasms, at an earlier age (8). However, Hodgkin lymphoma has never been reported in the context of CNC, despite the fact that PKA is obviously involved in the regulation of the immune system (17). To the best of our knowledge, there is only one animal study suggesting this association. In a mouse model of CNC, mice with antisense-*PRKAR1A* expression were found to have B-cell lymphoma, but the *PRKAR1A* knock-out mice did not develop such a proliferative disease. In addition, *PRKAR2A*-knockout mice (absence of another PKA subunit) developed lymphoma (16). In view of this experimental evidence, we suggest that the *PRKAR1A* mutation in Case 1 may have played a role in the development of her Hodgkin lymphoma.

In summary, our study broadens the genotypic and phenotypic spectrum of *PRKAR1A* mutations associated with CNC. For the first time, the coexistence of PPNAD and lymphoma in humans has been reported. We believe that the findings from these families provide important information for a better understanding of the genetic pathogenesis, diagnosis, and clinical management of CNC.

Ethics

Informed Consent: Informed consent was obtained.

Footnotes

Authorship Contributions

Concept: Kübra Yüksek Acınıklı, Sezer Acar, Ahu Paketçi, Özgür Kırbıyık, Mert Erbaş, Özge Besci, Gözde Akın Kağızmanlı, Deniz Kızmazoğlu, Oktay Ulusoy, Erdener Özer, Kutsal Yörükoğlu, Ayhan Abacı, Handan Güleryüz, Ece Böber, Korcan Demir, Design: Kübra Yüksek Acınıklı, Sezer Acar, Ahu Paketçi, Özgür Kırbıyık, Mert Erbaş, Özge Besci, Gözde Akın Kağızmanlı, Deniz Kızmazoğlu, Oktay Ulusoy, Erdener Özer, Kutsal Yörükoğlu, Ayhan Abacı, Handan Güleryüz, Ece Böber, Korcan Demir, Data Collection or Processing: Kübra Yüksek Acınıklı, Korcan Demir, Analysis or Interpretation: Kübra Yüksek Acınıklı, Korcan Demir, Literature Search: Kübra Yüksek Acınıklı, Korcan Demir, Writing: Kübra Yüksek Acınıklı, Korcan Demir.

Conflict of Interest: One author of this article, Korcan Demir, is a member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, he did not involved in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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CASE REPORT

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Floating-Harbor Syndrome in a Korean Patient with Short Stature and Early Puberty: A Case Report

Jooyoung Jeon, Eu-seon Noh, Il Tae Hwang

Hallym University College of Medicine, Kangdong Sacred Heart Hospital, Department of Pediatrics, Seoul, South Korea

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What is already known on this topic?

Floating-Harbor syndrome (FHS) is a rare genetic disorder characterized by short stature, language deficits, and distinctive facial appearance, caused by mutations in the *SRCAP* gene. Due to its non-specific clinical symptoms, there is poor awareness of FHS, leading to difficult and delayed diagnoses. Only two FHS cases were previously reported in Korea, with facial dysmorphism and intellectual disabilities, but not all showing short stature or early puberty.

What this study adds?

This study adds new insights by reporting the first Korean case of FHS with both early puberty and short stature, highlighting the effectiveness of combined human recombinant growth hormone and gonadotropin-releasing hormone agonist therapy for such cases. It emphasizes the significance of genetic testing, particularly *SRCAP* gene mutation analysis, for accurate FHS diagnosis and contributes to a better understanding of FHS's clinical spectrum and management.

ABSTRACT

Floating-Harbor syndrome (FHS) is a rare autosomal dominant genetic disorder characterized by proportionately short stature, lack of expressive language, and distinctive facial features, including a large nose, long eyelashes, deeply set eyes, and a triangular face. We present a case of an 11-year-old Korean girl who was initially suspected of having Noonan-like syndrome but was later diagnosed with FHS. The patient exhibited short stature, developmental language delay, dysmorphic facial features, and early puberty. Targeted exome sequencing revealed a heterozygous mutation, c.7303C>T (p.Arg2435Ter), in the *SRCAP* gene, confirming a diagnosis of FHS. She responded well to human recombinant growth hormone and gonadotropin-releasing hormone agonist, effectively suppressing bone maturation and improving her height standard deviation score from -4.6 to -2.4.

Keywords: Floating-Harbor syndrome, growth hormone therapy, short stature

Corresponding Author: Prof. Il Tae Hwang, Hallym University College of Medicine, Kangdong Sacred Heart Hospital, Department of Pediatrics, Seoul, South Korea
E-mail: ithwang83@kdh.or.kr **ORCID:** orcid.org/0000-0002-3885-4322

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Introduction

Floating-Harbor syndrome [FHS, (MIM 136140)] is a rare condition characterised by short stature, delayed osseous maturation, language deficits, and a distinctive facial appearance. Mutations in the *SRCAP* gene which codes for SNF2-related CREBBP activator protein cause truncation of the SRCAP protein, leading to FHS.

To date, approximately 100 cases have been reported in medical literature. Poor awareness of the condition and the non-specific clinical symptoms of FHS make its diagnosis difficult and delayed. Only two cases of FHS have been reported in Korea (1,2). Both patients had facial dysmorphism and intellectual disabilities, but one patient did not exhibit short stature (1) and the other did not experience early puberty (2). In this report, we describe the clinical features and molecular analysis of an 11 years and 5-month-old girl with an *SRCAP* mutation who presented with short stature, developmental language delay, dysmorphic facial features, and early puberty.

Case Report

The female patient was born at 38 weeks of gestation through normal vaginal delivery with a birth weight of 2840 g [-0.89, Korean standard deviation score (SDS)]. No perinatal complications were observed. Her mother had a height of 162.5 cm (0.29 SDS), and the father had a height of 174 cm (-0.08 SDS), resulting in a predicted adult height, calculated using mid-parental height, of 161.7 cm (0.13 SDS) (see Figure 1A). Moreover, there was a family history of short stature, as evidenced by the maternal grandmother's recorded height of 155 cm (-1.21 SDS) (see Figure 1B). The patient had no other significant family history. She exhibited persistent short stature from birth and so chromosomal analysis was performed at the age of 10 months. However, only an inversion p11-q13 in chromosome 9, which could be interpreted as a normal mutation, was identified. At the age of 16 months, Noonan syndrome was suspected at another hospital because of her short stature and large ears. Subsequently, genetic testing for Noonan syndrome was performed, and no abnormalities were found. Due to the initial suspicion of a Noonan-like syndrome, recombinant growth hormone (rhGH) therapy was started at 4 years of age. At that time, her height was 83.4 cm (-4.63 SDS), her weight was 9.4 kg (-5.19 SDS), and her insulin-like growth factor 1 (IGF-1) level was 55.3 ng/mL (reference range: 43.8-239.7). The patient's language development progressed slowly, and she commenced speech therapy at the age of five and was able to speak sentences at approximately six years of age. As the patient's growth was tracked, there was a consistent pattern of lagging bone age progression. However, at the age of 8 years and 7 months, the bone age assessment exhibited a significant acceleration, revealing a bone age of 7 years and 9 months, a markedly rapid increase compared to prior assessments. Furthermore, during a follow-up physical examination conducted six months later, at the age of 9 years and 3 months, bilateral breast development was observed, indicating significant progression. Remarkably, within just one year, the bone age had advanced by 2 years and 3 months. In response to these developments, starting at the age of 9 years and 10 months, a gonadotropin-releasing hormone (GnRH) agonist was administered in combination with the rhGH therapy to suppress bone maturation.

At the age of 11 years and 5 months, she was transferred to our hospital to continue treatment for her short stature and early puberty. Her height was 131.5 cm (-2.59 SDS), and her weight was 28.4 kg (-2.0 SDS) (Figure 1A). During physical examination, several distinctive features were noted that differed from those of her parents. These included large ears, a short neck, a long nose with a narrow nasal bridge and wide nostrils, mild cubitus valgus, and clinodactyly. Breast development was well suppressed, and bone age was determined to be 11 years, which was six months less than her chronological age. In addition, there were no anomalies detected in her blood biochemistry or thyroid function tests.

We conducted targeted exome sequencing (TES) to identify the genetic causes of her persistent short stature and facial dysmorphism. DNA samples were obtained from peripheral blood leukocytes using the Chemagic™ Magnetic Separation Module I method (PerkinElmer Chemagen, Baesweiler, Germany) with a DNA blood 200 mL kit. The G-Mendeliome panel (Celemics, Inc., Seoul, South Korea) was used for library preparation, and sequencing was performed using the DNBSEQ-G400 (MGI Tech Co., Ltd., Shenzhen, China), generating 2×100 bp paired-end reads. The sequence reads obtained were aligned to the reference sequence based on the public human genome build GRCh37/UCSC hg19 using BWA-mem (version 0.7.17). Duplicate reads were marked with biobambam2, and base quality recalibration and variant calling was performed using the Genome Analysis Toolkit (GATK, version 4.1.8). Annotation was performed using variant effect predictor (VEP101) and dbNSFP v4.1.

TES revealed a heterozygous variant, NM_006662.3: c.7303C>T, p (Arg2435Ter), in *SRCAP* (Figure 2). The pathogenicity of this mutation was assessed following the guidelines established by the American College of Genetics and Genomics. Based on the criteria PVS1, PM2, and PP5, this variant was classified as pathogenic. Furthermore, it was not detected in the Genome Aggregation Database (gnomAD). Consequently, we have determined that this is a pathogenic variant causing a non-sense mutation, leading to the conversion of the arginine residue into a stop codon.

These results confirmed the presence of the *SRCAP* mutation, which ultimately led to a diagnosis of FHS. Notably, no *SRCAP*

variants were identified in the patient's father, mother, or sister. The patient was maintained on a regimen of 45 mg/kg/day of rhGH, which had been consistently administered at another hospital. GnRH agonists were discontinued at the age of 12 years. The patient consulted a doctor for the assessment of hyperopia, strabismus, and conductive hearing loss, which may be present in FHS. However, the findings of this assessment were unremarkable. The echocardiogram showed favourable results, and renal ultrasound showed a difference in size between the two kidneys but no other abnormalities.

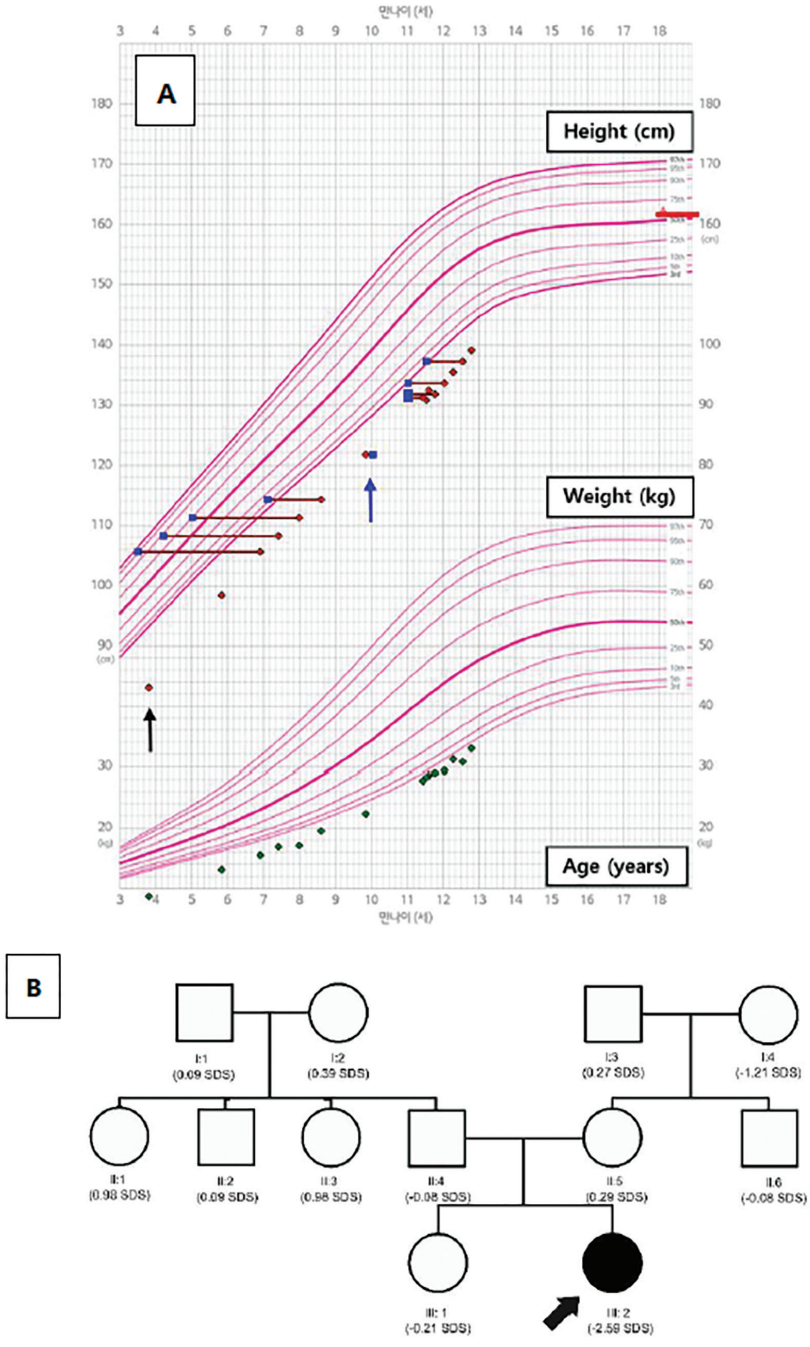

Figure 1. A) Reference growth chart for Korean females (3-18 years). Heights are marked with red dots, while weights are denoted by green dots. The blue dots represent the bone age. The mid-parental height trend is illustrated by a continuous red line. Significant milestones in the patient's medical treatment are highlighted: the commencement of recombinant human growth hormone therapy is marked by a black arrow, and the initiation of gonadotropin-releasing hormone agonist treatment is indicated by a blue arrow. **B)** Patient's family pedigree. The patient highlighted by a black arrow, and the height SDS corresponding to the age of each family member is also displayed
SDS: standard deviation score

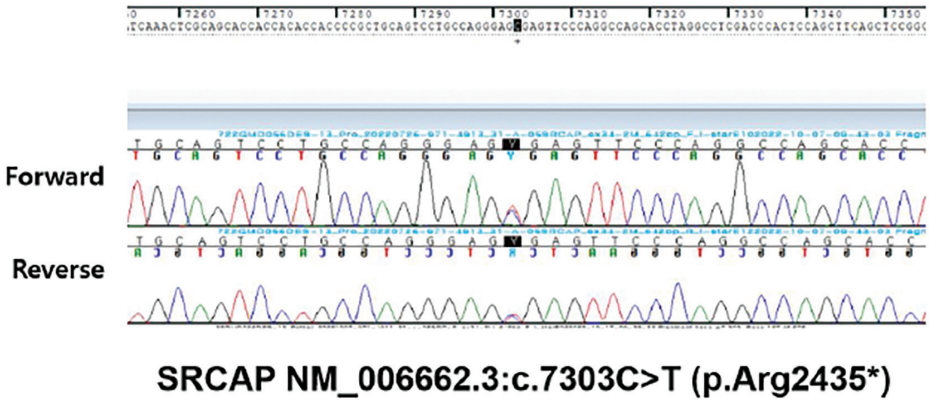

Figure 2. Results of Sanger sequencing of the *SRCAP* gene in the patient. NM_006662.3: c.7303C>T, p.(Arg2435Ter), heterozygote, non-sense

The patient is currently 13 years and 4 months old, with a height of 141.9 cm (-2.5 SDS) and a weight of 36.2 kg (-1.8 SDS). She is undergoing rhGH (60 mg/kg/day) therapy and receiving continuous speech therapy for delayed language development.

Discussion

In this report, we confirmed the presence of a heterozygous *SRCAP* variant using TES. The patient exhibited symptoms of short stature, developmental language delay, dysmorphic facial features, and early puberty.

FHS can present with symptoms resembling those seen in other genetic conditions. Noonan syndrome, 3M syndrome, Rubinstein-Taybi syndrome, and Silver-Russell syndrome must be differentiated from FHS. Noonan syndrome, which shares facial features and short stature with FHS, can be differentiated from FHS by a variety of physical abnormalities, including heart defects (3,4). Further, unlike in FHS, patients with 3M syndrome typically exhibit a large head and normal intelligence and speech development, with the possibility of hypogonadism in affected men (5). For Rubinstein-Taybi syndrome, patients often display a round face, severe intellectual decline, and normal bone age (6). In Silver-Russell syndrome, there are different features, such as an asymmetric body, café-au-lait spots, and blue sclera (7). As these conditions can present with symptoms resembling those of FHS, accurate diagnosis and genetic testing by a specialist are necessary.

Only two cases of FHS have been reported in Korea. Both patients had facial dysmorphism and intellectual disabilities, but one patient did not have short stature (1), and the other did not have early puberty (2). Therefore, our patient was the first in Korea to receive concurrent rhGH therapy and GnRH agonist therapy because of a short stature and early puberty.

The pathogenesis of short stature in FHS is not completely understood. It has been argued that GH deficiency, GH neurosecretory dysfunction, and IGF-1 signalling defects may be related to FHS, yet the evidence is limited due to the rarity of cases and lack of extensive scientific research (8). Contrasting with these uncertainties, another study has shown that the effects of rhGH therapy on FHS are modest at best, suggesting that the major molecular pathology of FHS is not caused by reduced GH secretion or activity (9). This highlights the necessity for additional research into the pathological mechanisms. In recent literature summarizing the experiences of 22 patients with FHS who received rhGH therapy, no side effects were reported. Notably, except for four individuals, there was a meaningful increase in height SDS compared to before treatment (8). In our case, the patient received rhGH therapy (45-60 mg/kg/day) for more than eight years. The final recorded height of the patient was 141.9 cm (SDS=-2.5), and no adverse effect of rhGH therapy was reported. In our patient, the growth rate improved. Given the rarity of FHS, there is limited information on the outcome of long-term treatment with rhGH. Further studies are necessary to clarify the longitudinal growth pattern and the real effectiveness and safety of rhGH therapy.

Recent studies have suggested a potential association between FHS and early puberty (9). However, the mechanisms underlying early puberty in FHS are again poorly understood. Several cases of precocious puberty in FHS patients have been reported, and some patients have undergone treatment with GnRH agonists (10,11,12). Treatment for early puberty in FHS resembles that for other forms of precocious puberty; however, its effectiveness requires further investigation. Our patient exhibited signs of puberty at the age of 9 years and 10 months. The rapid onset of puberty prompted the initiation of GnRH agonist therapy. This treatment successfully suppressed bone maturation. In FHS patients having symptoms of short stature and early puberty,

we speculate that GnRH agonist therapy could potentially delay bone maturation, thereby extending the duration of rhGH therapy.

At the initial diagnosis of FHS, the growth rate should be evaluated, and renal ultrasonography, blood pressure measurement, ophthalmic examination, hearing testing, dental examination, and genitourinary examination should be performed (8). For men, it is necessary to check for undescended testes. Orthopedic examination and evaluation of motor and language development are required for detecting hip dysplasia or other anomalies, as well as for genetic counselling.

Poor awareness of the condition and the non-specific clinical symptoms of FHS make its diagnosis difficult and often delayed. Patients with short stature, dysmorphic facial features, and developmental delays should undergo genetic investigation, including consideration of conditions such as FHS. In cases like ours, where FHS is accompanied by early puberty and short stature, rhGH therapy and GnRH agonist therapy may be beneficial.

Ethics

Informed Consent: Written informed consent was obtained from the patient's parents.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Jooyoung Jeon, Il Tae Hwang, Concept: Jooyoung Jeon, Eu-seon Noh, Il Tae Hwang, Design: Jooyoung Jeon, Il Tae Hwang, Data Collection or Processing: Jooyoung Jeon, Il Tae Hwang, Analysis or Interpretation: Jooyoung Jeon, Literature Search: Jooyoung Jeon, Writing: Jooyoung Jeon, Eu-seon Noh, Il Tae Hwang.

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CASE REPORT

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Schwartz-Jampel Syndrome Type 1: Compound Heterozygosity of Two Novel Variants

Fatma Güliz Atmaca[1], Özlem Akgün Doğan[2], Büşra Kutlubay[3], Heves Kırmızıbekmez[1]

[1]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye
[2]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Genetics, İstanbul, Türkiye
[3]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Neurology, İstanbul, Türkiye

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What is already known on this topic?

Schwartz-Jampel syndrome (SJS) type 1 is characterized by myotonic myopathy, chondrodystrophy, short stature, facial and eye abnormalities. SJS type 1 develops due to variations in the *HSPG2* gene, which encodes the perlecan protein, one of the main proteoglycans of the basement membrane.

What this study adds?

Our patient had "two novel" heterozygot variants in *HSPG2* together with the clinical symptoms of the syndrome, demonstrating that the "compound heterozygosity" may cause the disease. In cases of myotonia with muscle stiffness, limitation of joint movement, especially squinting in the eyes and difficulty in opening the mouth with an accompanying short stature, SJS should definitely be considered. However, it may take years for them to become recognizable, as the clinical findings of our patient were subtle until the age of 3.5.

ABSTRACT

Schwartz-Jampel syndrome (SJS) type 1 (OMIM; #255800), a rare cause of skeletal dysplasia, is characterized by myotonic myopathy, chondrodystrophy, short stature, facial and eye abnormalities. SJS type 1 develops due to variations in the *HSPG2* gene which produces the "perlecan" molecule, one of the main proteoglycans of the basement membrane. A 6-year-old girl presented with short stature, a mask face, shrunken lips, narrow palpebral opening due to blepharospasm, stiffness of facial muscles, micrognathia, overlapping teeth, a short neck, and a bell-shaped thorax due to myotonic myopathy. She was diagnosed with SJS type 1 due to compound heterozygosity of two novel variations in the *HSPG2* gene. In patients with short stature and an accompanying myotonic myopathy SJS should be considered. Compound heterozygosity may cause typical clinical findings of SJS. In case of suspicion creatinine kinase levels can be measured, and the determination of myotonia may

Corresponding Author: Prof. Heves Kırmızıbekmez, University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye

E-mail: heveskirmizibekmez@yahoo.com **ORCID:** orcid.org/0000-0003-2678-3886

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require evaluation with electromyography. Once the diagnosis is made, patients should be carefully monitored in terms of growth, neuromuscular disorders, joints problems and bone health.

Keywords: *HSPG2* gene, myotonia, short stature, skeletal dysplasia

Introduction

Schwartz-Jampel syndrome (SJS) type 1 (OMIM; #255800) is characterized by myotonic myopathy, chondrodystrophy, short stature, and facial and eye abnormalities (1,2). Due to clinical heterogeneity, SJS has been classified into three types. SJS type 1A is associated with moderate bone dysplasia, which is usually recognized in childhood. Type 1B may be present at birth and the clinical picture is more severe. SJS type 1 develops due to variations in the *HSPG2* gene, which encodes the perlecan protein, one of the main proteoglycans of the basement membrane. Perlecan regulates cellular processes including bone and cartilage formation, inflammation, and angiogenesis. It binds growth factors and cell membrane receptors, regulates intracellular signals, and plays a critical role in endochondral bone formation by promoting angiogenesis for cartilage matrix remodeling and formation of endochondral bone (2). Type 2, due to variations in the *LIFR* gene, is the most severe, rarest type with a very high mortality (3,4).

Almost all patients with SJS type 1 have short stature and dysmorphic features, including mask-like face, epicanthal folds, blepharospasm, ptosis and blepharophimosis, retrognathia, upturned nose, long philtrum, short neck, low ears, and high arched palate. The mask-like face and limited ability to open the mouth widely, which is more prominent when the patient is crying, are the major clues for clinical diagnosis. Other features may include toe walking, mild kyphosis, contractures in the elbow, spine, pelvis, metaphyseal deformities, lumbar lordosis, limitation of movement in large joints, hydrocephalus, and carpal tunnel syndrome. Complications such as myelopathy, recurrent infections, stridor, and mental retardation can also be present (5,6,7).

This presented case report describes two new variations in *HSPG2*, and will serve to remind colleagues of the importance of evaluating myotonia when investigating dysmorphic findings in children with short stature.

Case Report

A 6-year-old girl was referred to the pediatric endocrinology clinic for short stature. She also had a complaint of progressive squinting in her eyelids. She was born in the 39th week of gestation with a birth weight of 3350 g to healthy, non-consanguineous parents. Her medical history showed no record of chronic disease. Her growth rate was reported to have declined over the years. Her developmental milestones were compatible with peers until 3.5 years, she walked at one year of age, she could run at two, and jump at three years of age. However, from the age of 3.5, parents recognized progressive blepharospasms, wide based gait, joint stiffness, and progressive restriction of range of motion. She frequently had a duck-like gait following prolonged immobilization, which lasted for a few minutes and resolved spontaneously. No family history regarding the same medical problems was reported. She had no history of bone fracture or severe bone pain.

On physical examination, a mask-like appearance with long philtrum, pursed lips, narrow palpebral fissures, blepharospasm, long eyelashes, thick eyebrows, short forehead, short neck, micrognathia, crowded teeth and narrow thorax were evident. Hypertrophy of deltoid, biceps and brachioradialis muscles, joint stiffness, and restriction of range of motion were also present. She had long and thin fingers with no significant deformity in hands and feet (Figure 1). The height and body mass index were 104.8 cm [-2.25 standard deviation score (SDS)] and 14.1 kg/m^2 (-0.9 SDS), respectively. The maternal height was 155 cm, the paternal height was 170 cm (mid-parental height: -1.21 SDS) (8). The sitting height/height ratio was 0.56 (0.0 SDS) (9). The bone age was 4.5 years, and growth velocity was 2.3 cm in the previous 5 months. The skeletal survey was normal except for slightly increased lumbar lordosis. The results of routine laboratory tests for growth retardation, including whole blood count, biochemical tests, thyroid hormones, tissue transglutaminase antibodies, insulin-like growth factor-1 (IGF-1) and IGF-binding protein-3, were all in normal ranges. However elevated levels of creatinine kinase (514 U/L) were detected, supporting myotonia. Electromyography (EMG) revealed electrophysiological changes in the conduction and response of peripheral nerves in the lower and upper extremities, also consistent with myotonia. A clinical diagnosis of SJS was considered and clinical exome sequencing was performed.

Methods and Results

Automatic DNA isolation was performed by the standard protocols of the QIAamp DNA Mini (Qiagen GmbH, 40724 Hilden, Germany) kit from peripheral blood samples. The sequencing was done on an Illumina NextSeq 500 platform using SOPHIA Clinical Exome Solution (SOPHiA GENETICS SA, Rue du Centre 136, Switzerland) and Illumina V2 chemicals (5200 Illumina Way, California 92122, USA). The Sophia-DDM-V5.2 bioinformatics analysis software was used to perform variant calling and data analysis. The interpretation of the variants was performed

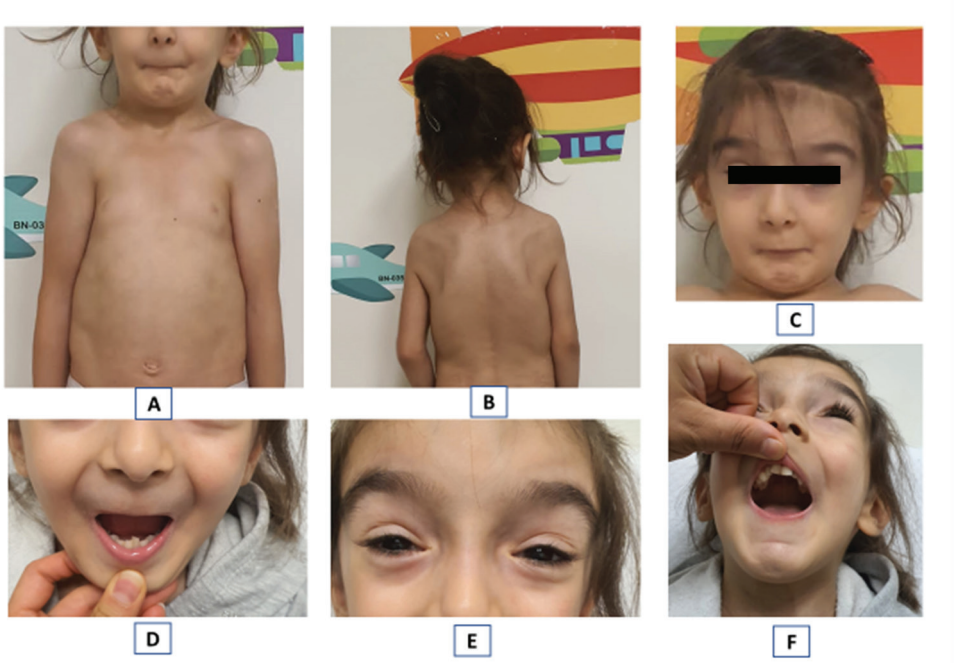

Figure 1. A) Short neck and a bell-shaped thorax, **B)** hypertrophy of muscles such as deltoid, biceps and brachioradialis leaded to a Herculean appearance, **C)** mask face with long philtrum and shrunken lips, **D)** limited mouth opening, stiffness of facial muscles, micrognathia, and overlapping teeth, **E)** narrow palpebral opening due to blepharospasm, long eyelashes, thick eyebrows, a straight and short forehead, **F)** low ears, high arched palate and overlapping teeth (consent was obtained from the parents for the use of the patient's photographs for medical and scientific purposes)

according to the 2015 American College of Medical Genetics and Genomics (ACMG) standards and guidelines. GnomAD, 1000 genome projects, dbSNP data were used as the control population. In silico prediction programs such as SIFT, Polyphen, EIGEN, FATHMM-MKL, MutationTaster, and GERP were used for variant pathogenicity predictions.

In CES (solo) analysis, *HSPG2* (NM_005529.7), c.4651C>T, p.(Arg1551Cys), heterozygous, missense variant and c.16_22dup, p.(Ala8Glyfs*31) heterozygous, frameshift variant were detected. Both variants were novel and classified as likely pathogenic according to the 2015 ACMG standards and guidelines. Segregation analysis by Sanger sequencing in parents confirmed the compound heterozygosity of the variants and thus the diagnosis of SJS.

Discussion

SJS, which is a very rare condition, was first described by Aberfeld et al. (10) in 1965, in a brother and sister with short stature, myotonic myopathy, dystrophy of epiphyseal cartilages, joint contractures, blepharophimosis, unusual pinnae, myopia, and "pigeon breast". These patients had previously been reported by Schwartz and Jampel (11) in 1962, who focused especially on the blepharophimosis. Huttenlocher et al. (12) described low muscle potassium suggesting an improper gradient of sodium and potassium due to a membrane defect. Myotonic EMG abnormalities have been described in patients. These EMG findings were also described in asymptomatic parents and siblings, while some of the patients with the syndrome had normal EMG findings (13,14,15). Minor abnormalities of toes and joints, severe microcephaly, and disproportion between skull and facial structures were described in female monozygotic twins with SJS (16). Spranger et al. (17) described four patients with SJS after further analyses who had previously been described as Kniest dysplasia, kyphomelic dysplasia, or Burton syndrome.

SJS may present with growth retardation and dysmorphic findings caused by increased muscle tone in some parts of the body. Even if these findings begin in early childhood, it may take years for them to become recognizable. Here we present a patient with novel variants in *HSPG2*, whose clinical findings were subtle until the age of 3.5 years.

Our patient had two novel heterozygote variants in *HSPG2*, together with the clinical symptoms of SJS, demonstrating that compound heterozygosity had caused the syndrome. The parents and sister of the patient had no clinical finding of skeletal dysplasia or myotonia. Similarly, Yan et al. (18) reported a 10-year-old female with SJS-1 from a Chinese family, with short stature, joint contractures, pigeon breast, and myotonia that led

to progressive stiffness of the face and limbs. They performed whole exome sequencing and Sanger sequencing for the proband and family members, finding two novel mutations (c.8788G>A; p.Glu2930Lys and c.11671+5G>A) in the *HSPG2* gene, suggesting that compound heterozygousity may be responsible for SJS-1 (18).

The *HSPG2* gene is located on chromosome 1 p34-36.1, and encodes perlecan, an important component of basement membranes. Decreased production of perlecan due to loss of function variants in *HSPG2* results in increased acetylcholine concentration at the neuromuscular junction, stimulating neuroexcitatory activity and myotonic discharges (19). Perlecan is also found in cartilage and bone marrow stromal cells and plays an important role in cartilage development and bone repair. It acts as a mechanical sensor for bone to detect external loading, and deficiency of perlecan increases the risk of osteoporosis. The skeletal abnormalities and pseudo fractures in SJS may be associated with defects in perlecan production (20,21,22,23,24).

In cases of myotonia with muscle stiffness, limitation of joint movement, but especially squinting in the eyes and difficulty in opening the mouth widely with an accompanying short stature, should suggest that SJS should be considered. Once the diagnosis is made, patients should be carefully monitored in terms of growth, neuromuscular disorders, joints problems and bone health.

Ethics

Informed Consent: Informed consent was obtained from the parents for the use of the patient's photographs for medical and scientific purposes.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Fatma Güliz Atmaca, Özlem Akgün Doğan, Büşra Kutlubay, Heves Kırmızıbekmez, Concept: Fatma Güliz Atmaca, Özlem Akgün Doğan, Heves Kırmızıbekmez, Design: Fatma Güliz Atmaca, Heves Kırmızıbekmez, Data Collection or Processing: Fatma Güliz Atmaca, Özlem Akgün Doğan, Analysis or Interpretation: Özlem Akgün Doğan, Literature Search: Fatma Güliz Atmaca, Özlem Akgün Doğan, Büşra Kutlubay, Heves Kırmızıbekmez, Writing: Fatma Güliz Atmaca, Özlem Akgün Doğan, Heves Kırmızıbekmez.

Conflict of Interest: One author of this article, Özlem Akgün Doğan, is a member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, she did not involved in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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