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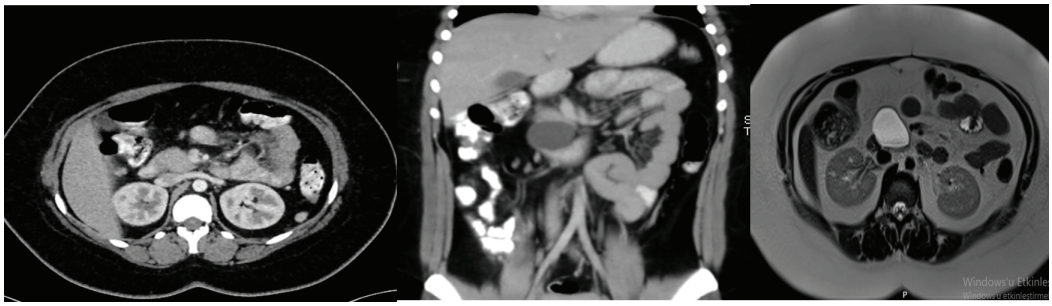
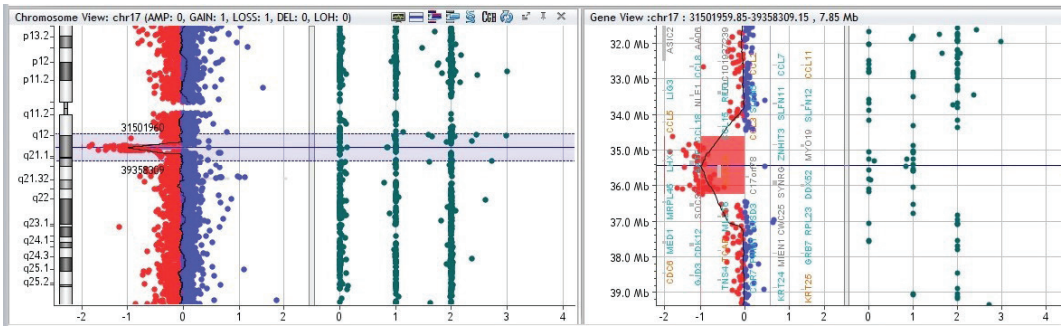


Abdominal computed tomography and magnetic resonance images demonstrating a cystic lesion in the head of the pancreas, and agenesis of the pancreatic neck, trunk and tail



Microarray analysis demonstrated a prominent, heterozygous deletion of a 1.63 Mb-spanning DNA sequence at chromosomal location 17q12, which included the HNF1B gene

A Case of Diabetes Mellitus Type MODY5 as a Feature of 17q12 Deletion Syndrome

Yaşar Köstek H et al.

Page: 205-210

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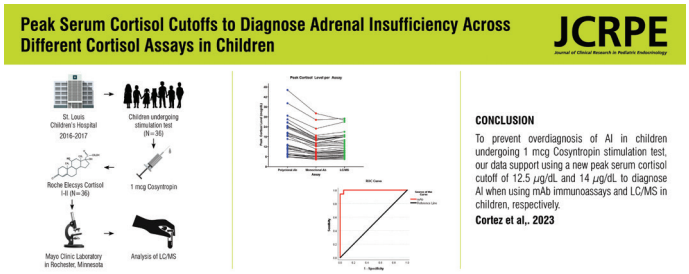

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Letters to the Editor

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REVIEW

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J Clin Res Pediatr Endocrinol 2024;16(2):123-136

Extensive Literature Review of 46,XX Newborns with Congenital Adrenal Hyperplasia and Severe Genital Masculinization: Should They Be Assigned and Reared Male?

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Abstract

46,XX individuals born with severely masculinized genitals due to congenital adrenal hyperplasia (CAH) who have been assigned male at birth and reared male can successfully establish a male gender identity/role, find employment, marry, function sexually with a female partner, and develop positive mental health status. While there were a few individuals who reportedly did not fare well or who changed gender to female, the majority of those identifying as males appear to have an overall good quality of life. Parental/family support, along with the support of others, appears essential to a positive outcome as a male, or as a female. This paper suggests that serious consideration should be given to male gender assignment and rearing and, in certain situations, is justified. Disorders of sex differentiation teams should inform parents about the option for male assignment and rearing in 46,XX CAH infants with severe genital masculinization, which is a rare condition. To provide this option is concordant with the principles of ethics, transparency and with the Endocrine Society Guidelines and the American Academy of Pediatrics' policy of fully informed consent.

Keywords: Masculinized genitalia, congenital adrenal hyperplasia, gender, sexuality, 46,XX males

Introduction

Originally described in 1865 (1), the congenital adrenal hyperplasias (CAH) are autosomal recessive disorders characterized by impaired cortisol synthesis. The worldwide incidence is estimated to be from 1:14,000 to 1:18,000 births (2). Most cases (>95%) are the result of 21-hydroxylase deficiency caused by a mutation in the *CYP21A2* gene (3). There are two classic forms of CAH, salt wasting (SW) and simple virilizing. CAH occurs in both sexes with external genital masculinization occurring in females. The degree of masculinization is indicated by the Prader Scale (4) with the most severe masculinization being graded four or five. Five indicates a fully formed penis with the meatus at the tip and a fully formed but empty scrotum. Internally, in 46,XX patients the reproductive organs are female.

The goal of the Pediatric Endocrine Society (PES) in formulating treatment guidelines has always been female assignment of all 46,XX CAH infants as females to "… preserve functional anatomy and fertility (2)". The 2005 consensus statement recommended more outcome data regarding male gender assignment for severely masculinized newborns diagnosed with 46,XX CAH (5). It shuld be noted that this statement was not a recommendation against male assignment (6). Historically some such individuals were assigned male before the diagnosis was made.

The purpose of this article is to summarize the published psychosexual (gender identity, gender role, sexual orientation) and behavioral outcome of adult 46,XX CAH individuals initially assigned and reared as males. The second purpose is to present the pros and cons of full

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disclosure regarding male gender assignment among severely masculinized infants.

Methods

A systematic search of PubMed was performed using these key words: 46,XX CAH; adrenogenital syndrome; assigned male; reared male; Prader stage 4; Prader stage 5; gender identity; gender role/expression; sexual orientation; sexual function; and mental health. Inclusion criteria were: cases in English of 46,XX CAH adults; 17 years and older; and assigned male at birth because of extremely masculinized external genitalia. These cases provided outcome data on gender identity, gender role, sexual orientation, work, marriage, and mental health. Excluded case reports were those that described: [1] children who died in infancy; [2] males who were too young to provide outcome data; and [3] those who were initially assigned as male then reassigned female at an age too young to provide outcome data (6,7). Also excluded were reports with only aggregate data (8,9).

Patients identified from publications from PubMed search (n = 131)	→	Patients removed based on exclusion criteria n = 78	→	Patients retained by inclusion criteria n = 53 from 18 publications

The Results and Discussion sections are identified by the reference numbers and, when pertinent, case numbers.

Results

Accurate Diagnosis Delayed

Delay of diagnosis of 46,XX CAH is a hallmark of individuals born with 46,XX CAH with severely masculinized genitalia (Table 1). Delay ranged from 0.1 to 35 years. The median was 6.8 years, although given the range of years and dates of the included reports, this figure is less meaningful and we refer the reader to Table 1. Surprisingly, delay has been documented even with accurate diagnosis (10,11).

Penile Length

Seventeen measurements of stretched or erect penile length were found in seven reports, including two separate measurements on three patients (12, Case 1,2,3) and are shown in Figure 1, plotted on a normal distribution population graph (13) based on data from Schonfeld and Beebe (14). They are also found in Table 1. The median length was 6.5 cm. although this includes those who had endogenous or exogenous androgen exposure.

Gender Identity

Table 1 shows all 53 adults who were initially assigned males: 46 living/identifying as males; 4 living/identifying as females; and 3 reassigned to female but self-gender changed back to male. Hence, of the 53, 92% identify as male. Being male was firmly established in the only report using questionnaires assessing outcome in adult life (7). This is consistent with a report of three adult patients reared male, reporting happiness and satisfaction without regrets (12, Case 1, 2, 3). Similar responses are published for 18- (15, Case 1) and 26-year-old men (16, Case 1). Another case (17, Case 2) reported that parents and surgeon did not accept the sex reversal. The surgeon removed Mullerian structures but refused to remove the "large clitoris similar to adult phallus". At age 36, the case was short (147 cm/4 feet 9 inches), depressed, expressed regret and cannot find an "appropriate job" (17, Case 2). A case identified as "bi-gender" has had "hard social adjustment" (18, Case 4).

Four individuals were reassigned and continued to live as females. One person lived as a male until age four when a CAH diagnosis was made and 46,XX karyotype was found. Change was recommended because the "tiny" penis precluded a normal male sex life "whereas fertile life in the female sex was clearly possible". This individual was initially reported to have behaved "more or less like a normal boy" except sitting to urinate (19). At age 22, she married, being fully aware of her medical history and considered the error had been corrected. At age 26 she conceived and gave birth via Caesarean section (20). The second female was reared as a male until age 12 years when she was evaluated for breast development and vaginal bleeding. With the help of her parents, she accepted the recommended gender change "easily". She later married and had two biological children and is "satisfied with her gender" (17, Case 1). The third individual, reported at age 26 years, lived as a male until age 16 years when adrenal hyperplasia was diagnosed.

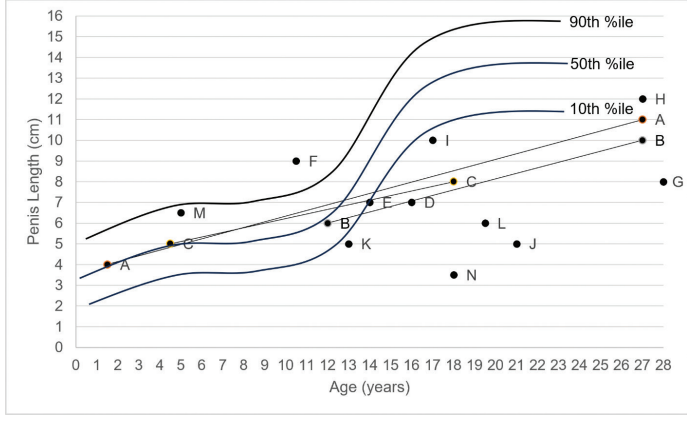

Figure 1. Penile growth graph

Table 1. CAH 46,XX adults (≥17 years) with severe virilization assigned male

Reference & country	Apóstolos et al. (12) (2018) Brazil			Bin-Abbas et al. (16) (2014) Saudia Arabia			Dewhurst and Gordon (19) (1984) USA	Gillenwater et al. (10) (1970) USA
	Case 1	Case 2	Case 3	Case 1	Case 2	Case 3		
Prader score	IV	V	IV	IV	V	V	V*	V
CAH diagnosis	SV 21-OH	SV 21-OH	SV 21-OH	CYP11B1 (Novel)	CYP11B1 (Novel)	CYP11B1 (Novel)	CAH	
Delay (years) 46,XX diagnosis	1.67	12	5	16	14	10	4	6
Age informed of diagnosis (years)				16	14	10		
Current age reported (years)	27	27	18	26	24	20	26	21
Gender change? (age in years)	No	No	No	Yes (17)	No	No	Yes (4)	No
Surgery (feminizing or masculinizing)	M	M	M	F	M	M	F	M
Gender identity	Male	Male	Male	Female	Male	Male	Female	Male
Gender role	Male	Male	Male	Female	Male	Male	Female	Male
Sexual orientation	Homo-sexual	Homo-sexual	Homo-sexual	Hetero-sexual			Hetero-sexual	
Sexual intercourse	Yes	Yes	No		No	No	Yes	
Masturbation	Yes	Yes	Yes		Yes	Yes	Yes	
Work	Computer technician	General Service–Pharmacy						
Marriage	No	No		No	No	No	Yes	
Children reported	No	No	No	No	No	No	Yes (1)	No
Mental health				Adjusting well				
Parental support of gender assignment				Yes	Yes	Yes	Yes	
Reported height (cm)	163	133	140	138	140	154		
Penile length (cm); (age at time of measurement in years)	4 (1.67)	6 (12)	5 (5)	7 (16)	7 14)	9 (10)		

Table 1. Continued

Reference & country	Jones (42) (2004) USA	Khattab et al. (24) (2017)		Kiviat and Leonard (47) (1978) USA	Lee et al. (5) (2010) USA *(Continued)*			
		Case 1 Pakistan	Case 2 Brazil		Case 1	Case 2	Case 3	Case 4
Prader score	IV*	IV	IV	IV*	IV*	V*	IV*	V*
CAH diagnosis		SV CYP21A 2; Homozygous In2/In2	SV CYP321A 2; Compound heterozygote Ex1 In2 Ex3/ In2	21-OH	21-OH	21-OH	21-OH	21-OH
Delay (years) 46,XX diagnosis	11	3	1.5-2***		3	3-12***	4	4
Age informed of diagnosis (years)	31							
Current age reported years)	31	28	27	17	35	36	45	45
Gender change? (age in years)	No	No	No	No	No	No	No	No
Surgery (feminizing or masculinizing)	M	M	M	M	M	M	M	M
Gender identity	Male	Male	Male	Male	Male	Male	Male	Male
Gender role	Male	Male	Male		Male	Male	Male	Male
Sexual orientation	Hetero-sexual	Hetero-sexual	Hetero-sexual		Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual
Sexual intercourse	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Masturbation	Yes	Yes	Yes		Yes	Yes	Yes	Yes
Work	Proprietor gas station	Soccer player			Business	Executive	Laborer	Welder
Marriage	No (attempted)	Yes	No		No	Yes	Yes	Yes
Children reported	No	No	No		No	No	No	No
Mental health	Suicide	Satisfied			Good		Good	Good
Parental support of gender assignment	Yes	Yes	Yes		Yes			Yes
Reported height (cm)		151	160	142	162	167.5	150.5	150
Penile length (cm); (age at time of measurement in years)		8 (28)	12 (27)	10 (17)				

Table 1. Continued

Reference & country	Lee et al. (5) (2010) USA *(Continued)*							
	Case 5	**Case 6**	**Case 7**	**Case 8**	**Case 9**	**Case 10**	**Case 11**	**Case 12**
Prader score	IV*	IV*	IV*	IV*	V*	V*	IV*	IV*
CAH diagnosis	21-OH	21-OH	21-OH	21-OH	21-OH	21-OH	21-OH	21-OH
Delay (years) 46,XX diagnosis	3-12***	12	3	3-12***	3	3-12***		
Age informed of diagnosis (years)								
Current age reported (years)	47	49	49	53	57	69	35	49
Gender change? (age in years)****	No	No	M-F-M	No	No	No	M-F-M	
Surgery (feminizing or masculinizing)	M	M	M	M	M	M		
Gender identity	Male	Male	Male	Male	Male	Male	Male	Male
Gender role	Male	Male	Male	Male	Male	Male	Male	Male
Sexual orientation	Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual	Hetero-sexual
Sexual intercourse	Yes	Yes	Yes	Yes	Yes			
Masturbation	Yes	Yes	Yes	Yes	Yes		Yes	
Work	Computer programmer	Welder	Disabled	No	Insurance Salesman	Priest	Artist	Laborer
Marriage	Yes	Yes	Yes	Yes	Yes			
Children reported	No	No	No	No	No	No		
Mental health		Good	Poor	Poor	Poor adjustment			
Parental support of gender assignment		Yes	No		Yes			
Reported height (cm)	155	150	163	160	152	160	155	155
Penile length (cm); (age at time of measurement in years)								

Table 1. Continued

Reference & country	Madsen (21) (1963) Germany		Maxted et al. (25) (1965) USA	Money and Daléry (15) (1976) USA			Money (22) (1991) USA	Peris (23) (1960) USA
	Case 1	Case 2		Case 1	Case 2	Case 3		
Prader score	V*	V*	V*	V*	V*	V*	IV*	V*
CAH diagnosis								
Delay (years) 46,XX diagnosis	35		21	12.17	1 month	7.42	< 1 month	18
Age informed of diagnosis (years)	35			Partially informed	Partially informed	Partially informed	18	Never
Current age reported (years)	35		21	18.17	24.25	26.5	24	18
Gender change? (age in years)****	Yes (35)	No	No	No	No	No	M-F-M	No
Surgery (feminizing or masculinizing)	No	No		M	M	M	M	M
Gender identity	Female	Male	Male	Male	Male	Male	Male	Male
Gender role	Male	Male		Male	Male	Male	Male	Male
Sexual orientation	Hetero-sexual	Hetero-sexual		Hetero-sexual	Hetero-sexual	Hetero-sexual		Hetero-sexual
Sexual intercourse		Yes		Yes	Yes	Yes	No	Yes
Masturbation		Yes		Yes, slightly dissatisfied	Yes	Yes		Yes
Work	Army, Monastery			Farm supply business	Construction	Factory, manual work		
Marriage		Yes	Yes		Yes	Yes	No	
Children reported	No	No			No		No	No
Mental health		Fair					Better after surgery	Satisfactory
Parental support of gender assignment				Yes			Yes	
Reported height (cm)	152	152		152	160	152		160
Penile length (cm); (age at time of measurement in years)			5 (21)	5x2 (13)	6x2.5 (19.5)	6.5x2.5 (5.5)		3.5x1.5 (18)

Table 1. Continued

Reference & country	Razzaghy-Azar et al. (17) (2017) Iran		Rosler and Leiberman (26) (1984) Israel *(Continued)*					
	Case 1	Case 2	Case 1 Morocco	Case 2 Morocco	Case 3 Morocco	Case 4 Morocco	Case 5 Morocco	Case 6 Tunisia
Prader score	V	IV	V*	V*	V*	V*	V*	V*
CAH diagnosis	11-beta OHD	11-beta OHD	11-beta OHD	11-beta OHD	11-beta OHD	11-beta OHD	11-beta OHD	11-beta OHD
Delay (years) 46,XX diagnosis	Birth	6	1.8	1.9	1	1.9	5.6	1
Age informed of diagnosis (years)		6						
Current age reported (years)	34	36	20	26	24	21	17	25
Gender change? (age in years)	Yes (12)	No	No	No	No	No	No	No
Surgery (feminizing or masculinizing)	F	M	M	M	M	M	M	M
Gender identity	Female	Male	Male	Male	Male	Male	Male	Male
Gender role	Female	Male						
Sexual orientation	Hetero-sexual							
Sexual intercourse	Yes							
Masturbation	Yes							
Work		Unemployed						
Marriage	Yes							
Children reported	Yes (2)	No						
Mental health	Satisfied	Depressed, expresses regret						
Parental support of gender assignment	Yes	Yes						
Reported height (cm)		147	143-157	143-157	143- 157	143-157	143-157	143-157
Penile length (cm); (age at time of measurement in years)								

Table 1. Continued

Reference & country	Rosler and Leiberman (26) (1984) Israel		Sharma and Gupta (18) (2012) India					
	Case 7 Morocco	Case 8 Turkey	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Prader score	V*	V*	IV*	IV*	IV*	IV*	IV*	V*
CAH diagnosis	11-beta OHD	11-beta OHD	21-OH	21-OH	21-OH	21-OH	21-OH	21-OH
Delay (years) 46,XX diagnosis	2	28	13	15	13	14	14.5	21
Age informed of diagnosis (years)								
Current age reported (years)	23	33	29.1	26.3	23.5	20	18.8	30
Gender change? (age in years)	No	No	No	No	No	Yes (N/A)	No	No
Surgery (feminizing or masculinizing)	M	M	M	M	M	M	M	M
Gender identity	Male	Male	Male	Male	Male	Bigender	Male	Male
Gender role								
Sexual orientation								
Sexual intercourse								
Masturbation								
Work								
Marriage								
Children reported								
Mental health			Good	Good	Good	Poor social adjustment	Good	Good
Parental support of gender assignment			Yes	Yes	Yes		Yes	Yes
Reported height (cm)	143-157	143-157						
Penile length (cm); (age at time of measurement in years)								

Table 1. Continued

Reference & country	Wesselius (2017) Netherlands	Wolfe-Christensen et al. (57) (2002) Germany			
		Case 1	Case 2	Case 3	Case 4
Prader score	V**	V*	V*	V*	V*
CAH diagnosis	21-OH	2-OH, SW	21-OH, SW	11-beta OHD	21-OH
Delay (years) 46,XX diagnosis	12	2.17	0.08		
Age informed of diagnosis (years)					
Current age reported (years)	64	36	31	49	33
Gender change? (age in years)	No	No	No	No	No
Surgery (feminizing or masculinizing)	M	M	M	M	M
Gender identity	Male	Male	Male	Male	Male
Gender role		Male	Male	Male	Male
Sexual orientation					
Sexual intercourse					
Masturbation	Yes				
Work					
Marriage					
Children reported					
Mental health					
Parental support of gender assignment					
Reported height (cm)					
Penile length (cm); (age at time of measurement in years)					

Notes: grey space indicate data not reported.
Surgery marked M indicates masculinizing surgery which may include testicular implants, hysterectomy, and hypospadias repair.
Surgery marked F indicates feminizing surgery such as vaginoplasty.
*Prader score based on description of external genitals.
**Prader score based on no mention of hypospadias.
***Only range given.
****Male-to-female-to-male.
SV: simple virilizing, CAH: Congenital adrenal hyperplasia, N/A: not applicable

The parents reported that their child "…behaved as a female since early childhood… playing with girls, although attending a male school". A pediatric psychiatrist "confirmed a female gender identity and a strong wish to be converted to female. At last report, she was 26 years old, having a "strong desire to marry a man and be a mother" (16, Case 1). The fourth person, at the age of 35 years and being convinced he was a woman, was admitted to hospital, requesting to be changed. He had served in the army in the Second World War but started menstruating at age 26 (21, Case 1). This case died during surgery. Mullerian structures and a hypertrophied right adrenal were found at autopsy, a postmortem diagnosis.

Three individuals initially assigned male had two changes of gender, first to female and then back to male again. One infant was initially assigned male, then two weeks later reassigned to female when CAH was diagnosed, had no surgery and was lost to follow up until age 12 (22). He chose masculinizing surgery. By age 17, he had more friends and appeared happier according to a public health nurse. The remaining two individuals were reported in 2010 (7, Cases 2, 7). The first, age 35, was reassigned in infancy and later self-reassigned as a male after he fought to be reassigned "for 18 years". The second person, age 49, was previously married as a female but eventually self-reassigned to male.

Gender Role/Expression

All male-identified individuals in Table 1 dressed in stereotypical male clothes, had no interest in female toys such as dolls, but in "traditional" male activities. One individual had interests and perspectives that were so "palpably" masculine "that any attempt at reassignment would be disastrous" (23). A subject who was named and reared as a boy, based on the external genitalia, indicated he was content as a minor (16). He proudly behaved and dressed as a male, played with boys and participated in "boys" sports.

Work

These males had typical "male" jobs for the times, including playing soccer (a sole family breadwinner), construction, laborer, pharmacist, businessman, welder, insurance salesman, computer programmer, priest, executive, and computer technician. Only one of four individuals living as women had held a paying job (as a male in the army). Two women were married and mothers. Of the three individuals who changed gender twice, one was an artist, one was a manual laborer, and the third worked as a teacher's aide.

Mental Health

Most cases lacked reports of mental health status. Among the 21 cases that mentioned this, 10 indicated good mental health, 5 satisfactory, and 6 poor. One study (7) included questionnaires with psychometric characteristics. The measures employed were the Rosenberg Self-Esteem Scale, Body Esteem Scale, Masculine Gender Identity Survey, Social Adjustment Self-Report, and Symptom Checklist (SCL-90). Only six of 12 completed these questionnaires. Three who completed them reported that their parents supported them, scored within the average range for self-esteem, body esteem, work, extended family on the SCL-90 global severity index (7, Cases 1, 5, 8). The other three who scored below average on these questionnaires did not have supportive parents (7, Cases 2, 3, 6). A 36-year-old male reported depression and regret (17, Case 2).

Sexual Orientation/Sexual Function

The majority of 46,XX CAH males reported being persistently attracted to and aroused by females and had had vaginal intercourse, with orgasm with women who perceived them as males. Masturbation and non-masturbation sexual fantasies were of females only. Satisfactory sexual function was achieved in all but one case, who reported "slight dissatisfaction" (16). One male had several different girlfriends involving "kissing and petting" beginning at 13 years old (15, Case 3). All six individuals who completed questionnaires reported female sexual partners (7, Cases 1, 3, 4, 6, 8). Sexual function, activity, and satisfaction persisted among those with a male gender rearing. Two males having had sexual intercourse with women completed the International Index of Erectile Function, reporting no problems and one case reported sexual satisfaction including orgasms, which was confirmed by his wife (12, Cases 1, 2, 3).

Marriage/Children

Fifteen males who always lived as males were married to women. Eight (7, Cases 2, 3, 4, 5, 6, 7, 8, 9) had been married between 7 to 34 years with an average duration of 20 years; two (15, Cases 2, 3) without reported duration; one (24, Case 1) for two years; one (21, Case 2) married at age 30; one (11, Case 3) reported in 2002, one (25) reported in 1965 and one (26, Case 7) in 1984.

There were also two individuals who had been gender reassigned twice who either had a serious romantic relationship or had married. One person was initially assigned male then female then reassigned himself to male (7, Case 2). He had a 14-year relationship with a "partner". The second person (7, Case 7) previously married as a female before self-changing to male. There were two individuals initially assigned male but were reassigned to female and had children. One person who changed gender to female at age 12 years eventually married and had two children (17, Case 1). The other person had one child after gender reassignment to female at age four years (19). One wife became pregnant by artificial insemination (26, Case 7) and one wife was beginning artificial insemination procedures (15, Case not identified).

Discussion

Cases of 46,XX CAH having less severe masculinization than those with Prader 4/5 developed a male gender identity and male role expression (8). This shift in behavior has been called Gendered Behavior (27). Research has shown that this "shift" toward male behavior is influenced by androgen exposure, severity of CAH as SW CAH patients generally have significantly more masculinized genitalia and more male gender role/expressions than those without (28,29,30,31), and those with the *CYP21A2* genotype, especially those with the null genotype (3,30). Sexual orientation toward females (27) occurs in 46,XX CAH, with lower sexual attraction to men than controls (32).

Most who established a male gender identity appeared to live successfully in spite of a delayed diagnosis. The diagnosis occurred because of a range of reasons including parents seeking orchidopexy because of the presumption of bilateral cryptorchidism, signs of puberty at a very young age or "hematuria" (menstrual blood) (26). This delay occurred in poor or rural areas and before newborn screening programs were available. Reported cases have been from many countries (Table 1).

A second defining aspect is the male dominant (9) culture in which individuals were born (33,34,35). Examples include a low socioeconomic Pakistani family who urged their financially successful soccer player child to accept male gender assignment (24, Case 1). Some parents preferred a male child, even after an accurate diagnosis and female

gender reassignment was suggested because of possible fertility (17, Case 2, 24, Case 2, 25). Retainment of male gender in India meets socioeconomic needs (18).

Historically, gender assignment for an infant born with DSD was considered a medical emergency, requiring prompt gender assignment, commonly without full disclosure with a warning that birth status should be kept secret from the child. One rural family agreed to female genital normalizing surgery thinking it was emergency therapy (34).

In part, positive psychosexual outcome may have been because parents and professionals were certain regarding maleness and being unaware of the diagnosis. Parental rearing practice studies on gendered behavior are lacking. In one report (36), parents encouraged less girl-typical and more boy-typical toy play in CAH girls. Investigators suggested that the girls' toy preference was influencing their parents' perceptions.

The American Academy of Pediatrics (37) recommends all 46,XX individuals be assigned female with full disclosure and full participation of parents in decision-making for the newborn, but does not address those with delayed diagnosis. The PES Clinical Practice Guidelines (2) state pros and cons of gender assignment and emphasizes that fertility implications must be completely discussed. Surgical decisions must be made with parents and the child, if old enough to provide assent. Experienced consultants are recommended who consider family values, religion and culture. Professionals need to be aware that how information is "framed" impacts on parents' decisions (38,39,40,41) and how and when such knowledge is discovered by patients. One person discovered her diagnosis at the age of 35 years and immediately self-reassigned (21, Case 1). A second, sadly, committed suicide after learning at age 31 years that he was not allowed to marry another genetic female (42).

The best predictor of adult gender identity is initial gender assignment (43) but this is not absolute, since those reported herein changed gender after initially being assigned male at birth. Gender identity may be more fluid than originally thought. We consider it ethically mandatory to inform parents of their option to assign as males if they so choose. Such a position can create controversy within multidisciplinary teams (44) and challenges the PES's Clinical Practice Guidelines (2). These guidelines still take the position that with excellent suppression therapy with glucocorticoids or other medications, such patients can be fertile. However, this has been the perception since the first patients were treated with glucocorticords and this has seldom been demonstrated. Delaying genital surgery is a choice that allows for time to assess gender development with the growing child, providing potential autonomy for the child. Gonadotropin Releasing Hormone analog therapy at or just before pubertal onset can delay puberty allowing more time for monitoring gender development and other domains.

Advantages to temporary assignment as male in infancy without surgery include retaining external genitalia, allowing for later decisions regarding surgery and providing functional anatomy for the child and for sexual function later. While living as a male, he needs to be informed of his diagnosis and its consequences, in an age-appropriate manner, in order to make a fully informed decision at some point in the future. If a male gender develops, feminizing surgery has been avoided while a functional penis is maintained. Negative aspects are increased risk of short adult stature, later surgery to remove female reproductive organs, infertility, cryopreservation, and later hypospadias surgery if necessary and also prostate cancer which was reported in two men (45,46) with one further report (47) of prostatic tissue in a 46,XX male. Testosterone treatment will be needed to induce puberty.

Advantages of female assignment is the potential for normal puberty if adrenal androgens have been well suppressed overtime, which often does not occur sufficiently (2). Feminizing surgery, while challenging and associated with a number of potential problems (48,49), will allow for menstrual flow and intercourse if desired. Feminizing surgery, especially in infants with severe virilization, does not appear to improve nor hamper psychosexual outcome (50). Fertility is possible among the minority with regular menstrual cycles, but studies (51,52,53,54) have documented low frequency of pregnancy, especially those with severely masculinized genitalia at birth and those with SW associated with the null mutation complete 21-hydroxylase deficiency. Anovulation and psychosexual development issues both contribute to infertility (50). Conversely, male genitalia cannot be restored after feminizing surgery should a male gender identity develop or the individual decide to reassign male.

In all of the scenarios, signs of gender dysphoria can appear and should be addressed. Whether assignment is male, female or not assigned, periodic psychological assessments for gender dysphoria are indicated by at least periodic screening. Parents also remain at risk for emotional and other difficulties as the child develops (55,56,57,58). Adolescents and adults can demonstrate problems (59,60). Continued professional support through young adulthood can be helpful (61). In the case of those assigned and being reared male, periodic assessment of gender development is required to determine whether or not genital surgery should

be delayed or recommended in the case of female gender development. The emphasis is upon continuing affirmation of gender identity, realizing that there are multiple variations in gendered behavior. For example, gender identity may be male while sexual orientation may not be typically heterosexual (3,32,43).

The limitations of this report include assessing a small number of retrospective clinical case publications without information from medical records and questionnaires regarding psychometric information, some of which date from 60 years ago. Systematic evaluations are lacking regarding mental health, social life, knowledge of CAH and gender assignment options, general health, drug and alcohol use. Difficulties in achieving compliance among those 46,XX or 46,XY is beyond the scope of this paper.

Conclusion

This study summarizes all outcome data from the global literature on 46,XX CAH whether raised male or female. We believe that the accumulated evidence, though limited, indicates that a 46,XX CAH infant with severely masculinized genitals can successfully establish an adult male gender identity with a reasonable quality of life. Therefore, careful assessment of the person, parents and their cultural beliefs is necessary before reassignment to female is considered. It is highly unlikely that a systematic study, which is needed, comparing overall quality of life outcome of those with severe genital masculinization at birth reared male or female can be accomplished in the foreseeable future. Until then, parents should be fully informed of all options and possible consequences.

Since 1976, the question of whether or not to assign and rear "chromosomal females born with a penis has been an issue of competing values, preserving fertility as a female versus a smoother path through adolescence to adult psychosexual maturity and function as a male" (15). The medical profession still remains divided. We also note that a significant number of CAH patients were not found using neonatal screening testing (62) so it is possible that the Prader 4 and 5 46,XX patients may be missed in countries with screening testing as well as those from rural areas and third world countries.

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Ethics

Authorship Contributions

Concept: Tom Mazur, Peter A. Lee, Design: Tom Mazur, Peter A. Lee, Data Collection or Processing: Jennifer O'Donnell, Peter A. Lee, Analysis or Interpretation: Tom Mazur, Peter A. Lee, Literature Search: Jennifer O'Donnell, Peter A. Lee, Writing: Tom Mazur, Jennifer O'Donnell, Peter A. Lee.

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Screening of Mutations in Maturity-onset Diabetes of the Young-related Genes and *RFX6* in Children with Autoantibody-negative Type 1 Diabetes Mellitus

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What is already known on this topic?

Maturity-onset diabetes of the young (MODY) subtypes differ in terms of the age of diabetes onset, the pattern of hyperglycemic presentation, the response to treatment, and the association with extra pancreatic manifestations. Although genetically identifying the MODY subtype has benefits, a large number of patients have not been tested. Mutations in *GCK, HNF1A,* and *HNF4A* are the most common causes of MODY.

What this study adds?

This study established three additional novel mutations in different *MODY* genes. The etiology has not yet been elucidated in 71% patients diagnosed with autoantibodies-negative type 1 diabetes mellitus.

Abstract

Objective: Maturity-onset diabetes of the young (MODY) is the most common type of monogenic diabetes. To date, mutations have been identified in 14 different genes of patients with a clinical diagnosis of MODY. This study screened mutations in 14 MODY-related genes and the regulator factor X6 (*RFX6*) gene in children

Methods: The presence of clinical features of MODY and negative results for three autoantibody markers of type 1 diabetes mellitus (T1DM) in children and adolescents were used as inclusion criteria for genetic testing. The screening panel for next-generation sequencing included 14 MODY-related genes (*GCK*, *HNF4A*, *HNF1A*, *HNF1B*, *PDX1*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, and *APPL1*) and the *RFX6* gene.

Results: Twenty-four different variants in MODY-related genes were identified in 49 children diagnosed with autoantibody-negative T1DM. Twelve variants were classified as pathogenic/likely pathogenic (P/LP) while 12 were interpreted as variant of unknown significance. Nine of the P/LP variants were found in *GCK*, two in *HNF1B*, and one in *ABCC8*. Three variants were novel, and one was a *de novo* variant. All but one of the variants exhibited heterozygotic inheritance.

Conclusion: The frequencies of the MODY subtypes differed from previous reports. Although GCK-MODY was the most frequent mutation in Turkish children, similar to previous studies, the second most prevalent MODY subtype was HNF1B-MODY. This study also established three additional novel mutations in different *MODY* genes.

Keywords: Diabetes mellitus, mutation, MODY, *RFX6*, Turkish children

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Introduction

Maturity-onset diabetes of the young (MODY) is characterized by an autosomal dominant genetic defect in beta-cell function with at least two consecutively affected generations of diabetes, typically before the age of 25-years, and the absence of beta-cell autoimmunity (1). Heterozygous mutations in various transcription factors that play a role in development and maturation of pancreatic beta-cells and mutations in enzymes acting on glucose sensing of the beta-cell result in MODY. MODY is currently categorized into 14 subtypes, each caused by mutations in different genes (2). Regulatory factor X6 (RFX6) is a transcription factor encoded by the *RFX6* gene located on chromosome 6 that is expressed in pancreatic beta-cells and small-intestinal cells. It plays a key role in the differentiation of pancreatic beta cells in mice and human, and regulates insulin secretion by modulating Ca^{2+} homeostasis in human beta-cells. Homozygous mutations in this gene are associated with Mitchell-Riley syndrome, which is characterized by neonatal diabetes with pancreatic hypoplasia, duodenal or jejunal atresia, and gall bladder agenesis. However, heterozygous *RFX6* mutations, which are associated with MODY with reduced penetrance and without intestinal atresia, have been reported (3,4).

The MODY subtypes differ in terms of the age of diabetes onset, the pattern of hyperglycemic presentation, the response to treatment, and the association with extra pancreatic manifestations. Management of MODY is subtype-specific and includes diet, oral antidiabetic drugs (OADs), or insulin. Therefore, the correct genetic diagnosis is important for lifelong treatment and patient prognosis. Although genetically identifying the MODY subtype has benefits, a large number of patients have not been tested (5).

This study screened mutations in the 14 MODY-related genes and the regulatory factor X6 (*RFX6*) gene in Turkish children diagnosed with autoantibody-negative type 1 diabetes mellitus (T1DM).

Methods

This was a prospective cross-sectional study performed between January 2005 and January 2022. The following criteria were used to diagnose diabetes mellitus (6): fasting plasma glucose or random plasma glucose levels ≥126 mg/dL or ≥200 mg/dL, respectively, glycated hemoglobin (HbA1c) ≥6.5%, and C-peptide <0.6 ng/mL. The clinical diagnosis of MODY was made using the classical criteria of impaired fasting glucose or the development of diabetes before the age of 25-years, negative results for T1DM markers [islet cell antibodies (ICAs), glutamic acid decarboxylase antibodies (GADAs), and insulin autoantibodies (IAAs)] and a family history of diabetes mellitus for at least two consecutive generations. The 14 MODY-related genes and *RFX6* were screened in all patients with a clinical diagnosis of MODY. Blood samples were collected to obtain fasting or random glucose measurements with concomitant C-peptide, HbA1c%, and three antibody tests (ICA, GADA, and IAA) at the time of the T1DM diagnosis.

Peripheral blood samples were collected into EDTA tubes for genetic testing. Genomic DNA was isolated from peripheral blood samples using the MagPurix robotic system (Zinexts, New Taipei city, Taiwan). Primary quality control of isolated DNA samples was performed using a NanoDrop spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and samples with an A260/280 value between 1.8 and 2 were included in the study.

Twenty-seven patients with clinically diagnosed MODY were included in next-generation sequencing (NGS) analyses. A panel consisting of the 14 MODY-related genes (*GCK*, *HNF1A*, *HNF4A*, *HNF1B*, *ABCC8*, *KCNJ11*, *INS*, *NEURD1*, *CEL*, *APPL1*, *PDX1*, *KLF11*, *PAX4*, and *BLK*), and the *RFX6* gene was designed using Ion Ampliseq Designer software. The MODY panel contained 384 amplicons and was optimized as two pools by Thermo Fisher Scientific (Waltham, MA, USA). Amplicons prepared using the Ion Ampliseq Library v2.0 commercial kit were loaded into the Ion Chef (Ion torrent, Thermo Fisher Scientific) instrument. Template creation, enrichment, and chip loading stages were performed automatically by the Ion Chef device. The sequencing reaction step was performed on the Ion S5 (Ion Torrent, Thermo Fisher Scientific) NGS device. As a result of sequencing, "single-end array" raw data (*fast or UBAM) were used in the NGS platform within the scope of a bioinformatics analysis.

Variant Analyses

Sequenced reads were aligned to the reference genome (GRCh37/hg19) using the Ion Torrent platform-specific pipeline software Torrent Suite 4.2. The Ion Reporter 4.0 (Thermo Fisher Scientific), Integrative Genomics Viewer, and Varsome (http://www.varsome.com) (7) software were used to analyze the data. Variants with a minor allele frequency >0.1% in the Genome Aggregation Database (http://gnomad.broadinstitute.org/) were filtered out. Mutation Taster (http://www.mutationtaster.org/), prediction of effects of human nsSNPs (http://genetics.bwh.harvard.edu/pph2/), and scale-invariant feature transform (http://sift.jcvi.org/) were used to evaluate the effect of nonsynonymous variants on protein function and structure. Human Splicing Finder

(http://www.umd.be/HSF/) was used to predict the effect of the mutations on splicing. We interpreted the variants using The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and a literature search. The variants were named according to Human Genome Variants Society (http://www.hgvs.org) nomenclature. The variants were classified as "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," or "benign" following the American College of Medical Genetics and Genomics Standards and Guidelines (ACMG) (8).

Informed consent was obtained from all subjects or their parents. This study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the Eskişehir Osmangazi University (approval number: E-2022-263, date: 20.12.2022).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS), version 18.0, (SPSS Inc., Chicago, IL, USA) was used for descriptive data analysis. Descriptive statistics of the clinical and laboratory findings are expressed as mean ± standard deviation, numbers, or percentages. Comparative statistics were not performed because the number of patients in the MODY subgroups was limited and not homogeneously distributed.

Results

The three antibodies (GADA, ICA, and IAA) were negative in 49 (12%) of 408 patients diagnosed with T1DM between January 2005 and January 2022. These 49 patients were screened for the 14 MODY-related genes and *RFX6*. The MODY diagnosis was confirmed by NGS in 14 of 49 (29%) patients, as pathogenic (P) or likely pathogenic (LP). The clinical and genetic characteristics of the MODY patients are given in Table 1 and Table 2, respectively. The mean age of the MODY patients was 9.5 ± 4.3 years (range, 3.5-17.8 years). The mean HbA1c level of MODY patients was 6.9 ± 1.1% (range, 6.2-9.8%). The lowest mean HbA1c level was seen in patients with GCK-MODY [mean, 7.01 ± 0.34%, (range, 6.6-7.8%)]. The clinical and genetic characteristics of 13 patients diagnosed with autoantibody-negative T1DM and genetic identification with variants of uncertain significance (VUS) are given in Table 3 and Table 4, respectively. The mean age of the children with VUS variants in MODY related genes and in *RFX-6* was 6.4 ± 4.0 years (range, 2.5-14 years). The mean HbA1c level in these patients was 8.1 ± 1.1% (range, 6.9-10.2%). A heterogeneous clinical presentation was observed according to the *MODY* genes. Patients with GCK- and ABCC8-MODY presented with hyperglycemia, whereas, HNF1B-MODY presented with diabetic ketoacidosis and multicystic kidneys. A heterogeneous clinical presentation was also seen in diabetic patients with VUS variants in MODY related genes and in *RFX-6*. Patients with VUS variations in *HNF1A-*, *HNF4A-*, *BLK-*, and *ABCC8* presented

Table 1. Clinical characteristics of 14 patients diagnosed with confirmed maturity-onset diabetes of the young (MODY)

No	*MODY* gene	Age at diagnosis (year)	Gender	Family history for MODY/generations	Clinical presentation	HbA1c % level at diagnosis	Follow-up treatment
1	*GCK*	14.2	Male	+/3	Hyperglycaemia	6.3	Diet
2	*GCK*	6.9	Male	+/3	Hyperglycaemia	6.6	Diet
3	*GCK*	7.5	Male	+/2	Hyperglycaemia	6.4	Diet
4	*GCK*	14.3	Male	+/3	Hyperglycaemia	6.3	Diet
5	*GCK*	7.1	Male	+/3	Hyperglycaemia	6.5	Diet
6	*GCK*	8.4	Female	+/3	Hyperglycaemia	6.3	Diet
7	*GCK*	6.1	Male	+/3	Hyperglycaemia	6.2	Diet
8	*GCK*	10.4	Male	NA	Hyperglycaemia	6.8	Diet
9	*GCK*	17.8	Female	+/3	Hyperglycaemia	6.2	Diet
10	*GCK*	3.8	Female	-/1	Hyperglycaemia	6.3	Diet
11	*GCK*	8.5	Female	+/2	Hyperglycaemia	6.8	Diet
12	*HNF1B*	9.5	Male	+/2	Diabetic ketoacidosis Multicystic kidneys	8.8	Insulin
13	*HNF1B*	13,5	Female	+/3	Diabetic ketoacidosis Multicystic kidneys	9.8	Insulin
14	*ABCC8*	3.5	Female	+/2	Hyperglycaemia	7.6	Sulfonylurea

OADs: oral antidiabetic drugs, NA: not available

Table 2. Mutation analysis screening of the *MODY* genes in Turkish children diagnosed with autoantibody negative type 1 diabetes mellitus

Gene OMIM ID MODY type	cDNA rs ID Types of mutation	Protein	Zygosity	*In silico* prediction	MAF %	ACMG class	N
GCK 125851/MODY2	c.109C > T rs762263694 missense	p.(Arg37Trp)	Hetero	PbD/Del/DC	0.00119	P	2
GCK	c.679G > A rs148311934 missense	p.(Val227Met)	Hetero	PbD/Del/DC	0.000796	P	2
GCK	c.116A > C rs1064794268 missense	p.(Gln39Pro)	Hetero	PbD/Del/DC	NA	LP	1
GCK	c.575G > A rs886042610 missense	p.(Arg192Gln)	Hetero	PbD/Del/DC	NA	LP	1
GCK	c.686C > T rs80356655 missense	p.(Thr229Met)	Hetero	PbD/Del/DC	0.000399	P	1
GCK	c.1181T > C missense	p.(Met394Thr)	Hetero	PD/Del/DC	NA	P	1
GCK	c.45 + 1G > C novel splice site	p.?	Hetero	NA/NA/DC	NA	LP	1
GCK	c.1090_1091insGCTGCGACCCTCGACCACCG novel insertion	p.(Asp364Glyfs Ter46)	de-novo	NA/NA/DC	NA	LP	1
GCK	c.536G > A rs886039380 missense	p.(Gly179Glu)	Hetero	PbD/Del/DC	NA	P	1
HNF1B 137920/MODY5	c.1024T > C rs1282596664 missense	p.(Ser342Pro)	Hetero	PD/Tol/DC	0.000402	LP	1
HNF1B	c.1045 + 1G > A novel splice site	p.?	Hetero	NA/NA/DC	NA	LP	1
ABCC8	c.4369G > A rs72559717 missense	p.(Ala1457Thr)	Hetero	PbD/Del/DC	NA	LP	1

In silico prediction: PolyPhen-2, SIFT: mutation tester, respectively; Polyphen predictions: B: bening, PD: possibly damaging, PbD: probably damaging, SIFT predictions: Del: deleterious, Tol: tolerated, mutation tester predictions: DC: disease-causing, Poly: polymorphism, NA: not available, ACMG Class, P: pathogenic, LP: likely pathogenic
Transcripts: GCK(NM_033507.3), HNF1B(NM_000458.4), ABCC8(NM_000352.6)

with hyperglycemia, with *KLF11* and *RFX6* variants with diabetic ketosis, and in *HNF1B-*, *PAX4-*, and *INS* variants with diabetic ketoacidosis.

An investigation of family history revealed that 23 of 27 (85%) patients (including MODY patients and patients with VUS) had one or two consecutive affected generations. Overall, 11 of the 27 diabetic patients whose family history revealed only mild hyperglycemia in two generations and who had not required insulin therapy, were presumptively diagnosed with GCK-MODY and the diagnosis was confirmed by genetic testing. GCK-MODY patients were managed with diet treatment only, ABCC8-MODY patients with oral sulfonylurea treatment only, whereas HNF1B-MODY were managed with insulin treatment. In patients with VUS in MODY related genes; HNF1A- and ABCC8-MODY were managed by sulfonylurea therapy only, whereas all remaining patients with VUS in *MODY* genes were managed by insulin treatment.

All MODY patients, except one, were heterozygotes, and one was a *de novo* mutation. The distribution of mutations according to the MODY related genes was as follows: nine different P/LP variants in the *GCK* gene, and one LP in *ABCC8* (Table 2). Seven of the nine mutations in the *GCK* gene were missense and two were novel splice site and insertion mutations. One of two LP mutations in *HNF1B* was a missense mutation, and the other was a novel splice-site mutation. The distribution of VUS variants according to genes is shown in Table 4. Two of the 12 different VUS variants in MODY related genes and *RFX6* were novel variants, and the other 10 variants were reported previously (Table 4).

Table 3. Clinical characteristics of 13 patients diagnosed with autoantibody-negative type 1 diabetes mellitus and genetic identification with variants of uncertain significance detected according to According to the American College of Medical Genetics and Genomics

No	*MODY* gene	Age at diagnosis (year)	Gender	Family history for MODY/ generations	Clinical presentation	HbA1c % level at diagnosis	Follow-up treatment
1	*HNF1A*	10.1	Male	+ /3	Hyperglycaemia	8.4	Diet, sulfonylurea
2	*HNF1A*	14	Female	+ /3	Hyperglycaemia	7.1	Diet, sulfonylurea, insulin
3	*HNF1B*	4.0	Male	-/1	Diabetic ketoacidosis	8.9	Insulin
4	*HNF1B*	8.3	Female	NA	Diabetic ketoacidozis	9.4	Insulin
5	*HNF4A*	2.3	Male	+ /3	Hyperglycaemia	6.9	Diet, sulfonylurea, insulin
6	*BLK*	1.5	Male	+ /2	Hyperglycaemia	7.4	Diet, sulfonylurea, insulin
7	*BLK*	10	Female	No	Hyperglycaemia	10.2	Diet, sulfonylurea, insulin
8	*PAX4*	5.5	Female	+ /2	Diabetic ketoacidosis	8.8	Diet, sulfonylurea, insulin
9	*INS*	10.5	Female	+ /2	Diabetic ketoacidosis	9.2	Diet, diet, sulfonylurea, insulin
10	*KLF11*	8.5	Male	+ /2	Diabetic ketosis	7.7	Diet, sulfonylurea, insulin
11	*ABCC8*	2.4	Male	+ /2	Hyperglycaemia	7.1	Sulfonylurea
12	*ABCC8*	4.2	Male	+ /2	Hyperglycaemia	7.3	Sulfonylurea
13	*RFX6*	2.4	Male	No	Diabetic ketosis	7.4	Diet, sulfonylurea, insulin

OADs: oral antidiabetic drugs, NA: not available, HbA1c: glycated hemoglobin

Table 4. Genetic identification of the cases with variants of uncertain significance detected according to According to the American College of Medical Genetics and Genomics

Gene/OMIM ID/MODY type	Variants/types of mutation	dsSNP	Zygosity	*In silico* prediction	Reference
HNF1A 600496 MODY3	c.481G > A p.(Ala161Thr) missense	rs201095611	Heterozygous	pBD/Del/DC	(9,10)
HNF1A	c.517G > A p.(Val173Met) missense	NA	Heterozygous	PD/Del/DC	(11,12)
HNF1B 189907 MODY5	c.1006C > G p.(His336Asp) missense	rs138986885	Heterozygous	B/Tol/DC	(10)
HNF1B	c.1339 + 5G > A p.? splice site (novel)	NA	Heterozygous	NA/NA/DC	NA
HNF4A 125850 MODY1	c.473C > T p.(Ala158Val) missense	rs754143633	Heterozygous	PbD/Tol/DC	NA
BLK 613375 MODY11	c.497delA p.(Asp166ValfsTer8) deletion (novel)	NA	Heterozygous	NA/NA/DC	NA
BLK	c.569C > G p.(Ser190Cys) missense	rs200875749	Heterozygous	B/Del/DC	NA
PAX4 612225 MODY9	c.521G > T p.(Arg174Leu) missense	rs776151854	Heterozygous	PbD/Del/DC	(13)
INS 613370 MODY10	c.2455C > G p.(Arg819Gly) missense	rs1555738952	Heterozygous	B/Del/DC	NA
KLF11 610508 MODY7	c.673A > C p.(Ser225Arg) missense	rs200061013	Heterozygous	B/Tol/Poly	NA

Table 4. Continued

Gene/OMIM ID/Mody type	Variants/types of mutation	dsSNP	Zygosity	*In silico* prediction	Reference
ABCC8 600509 MODY12	c.2395A > G p.(Lys799Glu) missense	rs1336775990	Heterozygous	B/Del/DC	NA
RFX6 NA MODY?	c.1782C > G p.(His594Gln) missense	rs4946206	Heterozygous	B/Tol/Poly	NA

dsSNP: the Single Nucleotide Polymorphism Database, *In silico* prediction: PolyPhen-2, SIFT: mutation tester, respectively, Polyphen predictions: B: bening, PD: possibly damaging, PbD: probably damaging, SIFT predictions: Del: deleterious, Tol: tolerated, mutation tester predictions: DC: disease-causing, Poly: polymorphism ? = unknown effect; NA: not available.

Transcripts: HNF1A(NM_000545.8), HNF4A(NM_000457.5), HNF1B(NM_000458.4), BLK(NM_001715.3), PAX4(NM_001366110.1), INS(NM_000208.4), KLF11(NM_003597.5), ABCC8(NM_000352.6), RFX6(NM_173560.4)

Discussion

MODY is the most common type of monogenic diabetes, accounting for 1-6% of all pediatric diabetic cases (2). Although there are several clinical predictors for diagnosing MODY, such as a positive family history of diabetes mellitus before the age of 25-30 years, negative results for antibodies associated with T1DM, high C-peptide concentrations (14), and high levels of C-reactive protein (15) and lipids, MODY diagnosis is relatively difficult and cases are often misdiagnosed as T1DM (3). A genetic test is the most accurate and cost-effective option for diagnosing the MODY subtypes (16). However, selecting pediatric and adolescent diabetic patients for genetic testing is still controversial. A clinical diagnosis of MODY should be considered in patients who have atypical features of diabetes based on age < 25 years, negative results for antibodies associated with T1DM, the presence of neonatal hypoglycemia, and/or multiple family members with diabetes not characteristic of T1DM or T2DM (1,2,5). Epidemiological studies have shown that 10-15% of children and adolescents diagnosed with T1DM are negative for the three autoantibodies (GADA, IAA, and ICA). In the present study, 49 (12%) of 408 patients diagnosed with T1DM had negative results for these three antibodies. In our study the MODY diagnosis was confirmed in only 14 of 49 (29%) patients. However, in the remaining 35 (71%) patients, there was no P or LP mutations in any of the 14 *MODY* genes. This suggests that the etiology has not yet been elucidated in a significant group of patients diagnosed with T1DM whose autoantibodies are negative. This group of patients may be candidates for genetically inherited diabetes groups that are likely to be identified in the near future. Determining the MODY subtype is important, as the subtypes differ in terms of age of onset, clinical presentation and progression, and response to treatment (2). The current literature shows that the prevalence and frequency of MODY subgroups vary by country. Mutations in *GCK*, *HNF1A*, and *HNF4A* are the most common causes of MODY (17,18,19,20). The most common subtype in European countries (UK, Germany, The Netherlands, Norway, and Poland) is HNF1A-MODY, followed by GCK-, HNF4A-, and HNF1B-MODY (21). The most common subtypes in the UK are HNF1A-, GCK-, HNF4A-, and HNF1B-MODY (5,22). However, Chakera et al. (22) reported that the estimated prevalence of GCK-MODY is about 1 in 1,000 individuals in the UK. GCK-MODY is the most prevalent MODY in Japan (23). In Korea, only 10% of clinical MODY of childhood-onset type 2 diabetes cases harbor known MODY-related genetic defects (*HNF1A*, 5%; *GCK*, 2.5%, and *HNF1B*, 2.5%) (24). The prevalence of MODY in Middle Eastern, Asian, and African populations is unknown. Ağladıoğlu et al. (25) and Gökşen et al. (26) reported that the most prevalent MODY subtypes in Turkish children are GCK-MODY and HNF1A-MODY. Yalçıntepe et al. (27) reported 31 cases of P/LP variants (*GCK*, n = 24; *ABCC8*, n = 3; *KCNJ11*, n = 2; *HNF1A*, n = 1; and *HNF4A*, n = 1) in 61 unrelated cases with clinical diagnosed with MODY in Turkish children. The present study revealed that the most prevalent MODY subgroup in Turkish children pre-diagnosed as T1DM was GCK-MODY, as reported previously (25,26,27). Unfortunately, the number of patients in our cohort diagnosed with MODY is not sufficient to make a realistic comment about the regional MODY frequency or the distribution of MODY subgroups. This would only be possible after national-scale MODY study or studies.

The GCK-MODY phenotype is characterized by lifelong nonprogressive fasting hyperglycemia (2,28,29). Patients are often asymptomatic and diagnosed incidentally during pregnancy or routine examinations, and the majority of patients do not require pharmacotherapy. Although patients with GCK-MODY have long-standing hyperglycemia, they have a low prevalence of micro- and macro-vascular complications (30). However, Kawakita et al. (31) reported that 7 of 55 patients with GCK-MODY were treated with OADs, and the authors concluded that these seven patients required OADs because they consumed carbohydrate-rich foods and had sedentary lifestyles. Insulin treatment is also required in pregnant patients with GCK-MODY to prevent maternal

hyperglycemia and reduce the risk for the development of overweight fetuses (32). In the present study, 11 cases from nine families had nine different mutations in *GCK*. Two cases had novel mutations; one was a splice site type mutation (c45 + 1G > C) and the other was an insertional type and a *de novo* mutation (c.1090_1091insGCTGCGACCCTCGACCACCG, p.Asp364GlyfsTer6). All cases presented with mild hyperglycemia and were followed up with only a low carbohydrate diet.

Heterozygous mutations in *HNF1B* cause HNF1B-MODY (formerly referred to as MODY-5), which is characterized by early-onset diabetes, pancreas hypoplasia, and multicystic kidney dysplasia (33). In patients with diabetes mellitus, the presence of cystic kidneys and elevated liver enzymes may be used as predictors of an *HNF1B* mutation. In the present study, two cases had *HNF1B* mutations and both presented with diabetic ketoacidosis. One of these two mutations was previously reported as a missense mutation (c.1024T > C), and the other was a novel splice site mutation (c.1045 + 1G > A). Both cases were diagnosed with cystic renal disease in the first decade of life. In one of the patients, cystic renal disease was identified by ultrasonography performed in the intensive care unit, where she presented with diabetic ketoacidosis. The other case was followed up by the pediatric nephrology department for polycystic renal disease. The pediatric nephrology department consulted pediatric endocrinology when the patient was diagnosed with fasting hyperglycemia and developed a high HbA1c level. Both patients were followed up with an intensive insulin regimen protocol. Unlike previous studies (13,14,15,21), the present study found that the *HNF1B* mutations were more prevalent in Turkish children than the *HNF1A* or *HNF4A* mutations. However, our cohort size is small so to confirm this hypothesis, more prospective and comprehensive studies are needed with larger cohorts. We suggest that, in the presence of renal abnormalities in young patients with diabetes mellitus, *HNF1B* mutations should be initially suspected and may be screened before the investigation of other MODY subgroups. The major complications in HNF1B-MODY patients have been related to the kidneys, such as chronic kidney disease (34).

The diagnosis of ABCC8-MODY was confirmed in one of the patients initially diagnosed with T1DM but with negative autoantibodies. The ATP-binding cassette transporter subfamily C member 8 (*ABCC8*) gene is expressed in the pancreas where it controls the expression of the sulfonylurea receptor 1 subunit of the ATP-sensitive potassium channel found on the beta cell membrane (35). Heterozygous mutations in the *ABCC8* gene damage the normal function of potassium channels leading to impaired insulin secretion. ABCC8-MODY (formerly referred to as MODY-12) is characterized by congenital hyperinsulinemic hypoglycemia, a transient or permanent form of neonatal diabetes mellitus, or adulthood-onset diabetes mellitus (2). Patients with ABCC8-MODY respond to sulfonylurea treatment (2). Our patient presented with hyperglycemia at 3.5 years old and was managed with sulfonylurea treatment. As seen in this case, by identifying MODY subgroups, correct treatment options can be chosen from younger ages.

In the present study, genetic testing identified 12 different VUS variants in MODY-related genes in 13 of 49 patients diagnosed with autoantibody-negative T1DM, but all patients had a clinical diagnosis of MODY. Two of 12 VUS variants were novel, and the others were reported previously (9,10,11,12,13). A clearer interpretation can be made by comparing the results obtained after segregation analyzes in our patients and previously reported studies related to these VUS variants. Though computational prediction tools and conservation analyses suggest that these VUS variants are not predictive enough to determine pathogenicity, given the current lack of comprehensive MODY-variant classification expertise, current ACMG-based classification should be interpreted cautiously and these VUS variants may be subject to change in the future.

Study Limitations

This study was conducted with a small number of cases, except for patients with GCK-MODY, so the reported frequency of the rare MODY subgroups may be unreliable. Segregation could not be tested in all families for VUS variants. Large-scale prospective studies are needed to allow for a stronger interpretation of the frequencies of rare MODY subgroups in Turkish children.

Conclusion

Clinical evaluation and genetic testing can be used to make a correct MODY diagnosis and subgrouping. The incidence and prevalence of MODY vary between countries. A timely and accurate diagnosis of MODY may prevent some subgroups from unnecessarily treatment with long-term insulin therapy. Therefore, all autoantibody-negative T1DM cases should be screened for known *MODY* genes, and the treatment of patients should be individualized, based on the appropriate identified MODY subgroup. This study established three additional novel mutations in different *MODY* genes. Our study also revealed that the 14 currently known *MODY* genes or *RFX6* were not reported to be P or LP in 71.5% (35/44) autoantibody-negative T1DM cases, indicating that this group may have novel MODY subtypes.

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Ethics

Ethics Committee Approval: This study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the Eskişehir Osmangazi University (approval number: E-2022-263, date: 20.12.2022).

Informed Consent: All patients with autoantibody-negative type 1 diabetes mellitus were routinely screened from the aspects of MODY and a separate consent form was not issued.

Authorship Contributions

Surgical and Medical Practices: Enver Şimşek, Tülay Şimşek, Meliha Demiral, Çiğdem Binay, Concept: Enver Şimşek, Oğuz Çilingir, Sinem Kocagil, Ebru Erzurumluoğlu Gökalp, Design: Enver Şimşek, Data Collection or Processing: Enver Şimşek, Tülay Şimşek, Meliha Demiral, Çiğdem Binay, Analysis or Interpretation: Enver Şimşek, Oğuz Çilingir, Sinem Kocagil, Ebru Erzurumluoğlu Gökalp, Literature Search: Enver Şimşek, Oğuz Çilingir, Sinem Kocagil, Ebru Erzurumluoğlu Gökalp, Meliha Demiral, Çiğdem Binay, Writing: Enver Şimşek, Oğuz Çilingir, Tülay Şimşek.

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Serum Ghrelin and Glucagon-like Peptide 1 Levels in Children with Prader-Willi and Bardet-Biedl Syndromes

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What is already known on this topic?

Ghrelin stimulates appetite and secretion of growth hormone, and induces a positive energy balance and leads to weight gain. Glucagon-like peptide-1 (GLP-1) exerts its central effects through the GLP-1 receptor in the central nervous system, reducing the rate of absorption of food into the blood by suppressing appetite, reducing the rate of gastric emptying and and inhibiting glucagon secretion.

What this study adds?

There was no evidence for a definite role for ghrelin and GLP-1 in the pathogenesis of Prader-Willi syndrome in pediatric patients. There is only one study in which the plasma ghrelin levels do not differ between the patients with Bardet-Biedl syndrome (BBS) and control groups, and there is no study evaluating serum GLP-1 levels in BBS patients. However, similar studies with larger series are needed.

Abstract

Objective: Prader-Willi syndrome (PWS) and Bardet-Biedl syndrome (BBS) are causes of pediatric syndromic obesity. We aimed to investigate a possible role for ghrelin and glucagon-like peptide-1 (GLP-1) in the pathophysiology of PWS and BBS.

Methods: The study included 12 children with PWS, 12 children with BBS, 13 pediatric obese controls (OC) and 12 pediatric lean controls (LC). Fasting serum ghrelin and GLP-1 levels were measured by ELISA.

Results: In the PWS group, no significant difference was detected for median ghrelin levels when compared with OC and LC, which were 0.96 (0.69-1.15), 0.92 (0.72-1.20) and 1.13 (0.84-1.29) ng/mL, respectively. Similarly, there was no difference in PWS median GLP-1 levels when compared with OC and LC; 1.86 (1.5-2.94), 2.24 (1.62-2.78) and 2.06 (1.8-3.41) ng/mL, respectively. In the BBS group, there was no difference in median ghrelin levels when compared with OC and LC; 1.05 (0.87-1.51), 0.92 (0.72-1.20) and 1.13 (0.84-1.29) ng/mL, respectively. Neither was there a significant difference in median GLP-1 levels; 2.46 (1.91-4.17), 2.24 (1.62-2.78) and 2.06 (1.8-3.41) ng/mL for BBS, OC and LC, respectively.

Conclusion: There were no differences in median fasting ghrelin or GLP-1 levels when comparing patients with PWS and BBS with obese or lean peers. However, similar studies with larger series are needed.

Keywords: BBS, PWS, ghrelin, GLP-1

Introduction

Prader-Willi syndrome (PWS) is the most common cause of pediatric syndromic obesity. The clinical characteristics of PWS consist of hyperphagic obesity beginning in early childhood, decreased fetal movement, neonatal hypotonia, developmental delay, cognitive deficits, short stature, and hypogonadotropic hypogonadism (1). Subjects with PWS develope uncontrolled appetite resulting in weight gain, usually starting after two years of age, although there is almost universal poor feeding and appetite in infancy in babies with PWS. PWS is seen in all races, occurring equally in the sexes, with an incidence of approximately 1 in 10,000 to 1 in 15,000 live births, and results mostly from the absence of functionally active paternal inheritance in the 15q11.2-13 chromosome region (2,3).

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Bardet-Biedl syndrome (BBS) may be described as a genetically heterogeneous ciliopathy with an autosomal recessive inheritance. The characteristics of BBS is described by six primary attributes: progressive rod-cone dystrophy, early-onset obesity, postaxial polydactyly, hypogonadism, cognitive impairment, and genitourinary tract malformations with progressive renal dysfunction. Its prevalence is about 1 to 9 in 1,000,000. Mutations in 24 different genes have been described that are associated with BBS (4).

Ghrelin is a 28-amino acid peptide that is the natural ligand for the growth hormone secretagogue-receptor. Ghrelin is produced mainly by the stomach and concentrations increase in fasting. Ghrelin stimulates appetite and secretion of growth hormone, and induces a positive energy balance, leading to weight gain. Ghrelin levels are low after meals or in hyperglycemia, and also in obesity (5). Hyperghrelinemia has been reported in older children and adults with PWS in many studies but the correlation with obesity is still unclear (6). However, there is only one study evaluating ghrelin levels in BBS (7).

Glucagon-like peptide-1 (GLP-1) is mainly synthesized by L-cells in the duodenum, small intestine and in small quantities by the pancreas and hypothalamus. Its secretion in the gastrointestinal tract is modulated by glucose and fatty acid levels after food intake, or as a result of stimulation of the vagus nerve. The main mechanisms of action of GLP-1 include stimulating insulin secretion by β-cells in the islets of Langerhans and inhibiting glucagon secretion by α-cells. GLP-1 also exerts its central effects through the GLP-1 receptor in the central nervous system, reducing the rate of absorption of food into the blood via appetite suppression, and reducing the rate of gastric emptying (8). The relationship between GLP-1 and obesity is not clear and controversial in terms of underlying pathophysiology (9). There are only two studies evaluating fasting plasma GLP-1 levels in PWS; in the first one, fasting GLP-1 concentrations in adults with PWS were similar in individuals with obesity and lean control (LC) groups. In the second one, fasting GLP-1 concentrations in adults with PWS were higher than in the obese and LC groups, but GLP-1 concentrations in the obese and lean groups were similar (10,11). However, there was no study evaluating fasting plasma GLP-1 levels in BBS patients.

In this study, we aimed to investigate the role of ghrelin and GLP-1 in the pathophysiology of pediatric syndromic obesity caused by either PWS or BBS.

Methods

Patients with a diagnosis of BBS or PWS and aged between three and 18 years were selected from patients attending pediatric endocrinology and clinical genetics clinics. The diagnosis in all patients with PWS was genetically confirmed. In the patients with BBS, the majority were confirmed genetically but a quarter of diagnoses had been made clinically. The clinical diagnosis of BBS was made according to criteria published by Beales et al. (12).

The control subjects were matched for age, sex and pubertal stage and were selected from our pediatric clinics. Exclusion criteria were any patient with endocrine (diabetes mellitus, hypothyroidism, and adrenal deficiency), systemic or infectious diseases, and those taking any kind of medication. The study protocol was approved by the University of Health Sciences Turkey, Antalya Training and Research Hospital Local Institutional Review Board (decision no: 10-12, date: 18.05.2022). Informed written consent from the subjects (>8 years old) and their parents were obtained. The study was conducted according to the principles of the Declaration of Helsinki.

Anthropometric Measurements

Body weight of subjects was measured using a pre-calibrated digital scale and height was measured with a 0.1 cm sensitivity in a Harpenden Stadiometer, both produced by Densi Industrial Scale Systems San. and Tic. Ltd. Şti. (Tuzla/İstanbul/Turkey). The obesity was defined as a body mass index (BMI)-standard deviation (SD) score (SDS) greater than or equal to 2 SDS using national BMI data defined according to age and gender (13). Pubertal developmental stage was evaluated using Marshall and Tanner's standards (14).

Assays

Venous blood samples were taken from the subjects in the morning after a 12 hour fast. After centrifugation for 20 min at 2000 RPM, serum samples were stored at -20 °C. Serum ghrelin and GLP-1 levels were measured by the ELISA method [Shanghai Korain Biotech (BT-LAB) Co., Shanghai, China]. The detection range of the ghrelin assay by the competitive inhibition method was 0.05-10 ng/mL with a sensitivity of 0.01 ng/mL. The detection range of the GLP-1 assay by competitive inhibition was 0.05-30 ng/mL with a sensitivity of 0.026 ng/mL. The inter-assay coefficient of variation (CV) and intra-assay CV for both assays was given as <10% and <8%, respectively.

Statistical Analysis

The sample size calculation was performed using the DSS statistical software package (DSS, Locke Ave, Fort

Worth, TX, USA) for research sample size calculations. It was calculated that a minimum of 12 participants in each group would be required to demonstrate a difference of at least 10% for GLP-1 between the groups, with a power of 80% at the 5% significance level. The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 15.0 (SPSS, Chicago, IL, USA). The Shapiro Wilk-test was used to assess continuous variables for normal or abnormal distribution. Continuous variables with a parametric distribution were analyzed using analysis of variance and if the differences were significant, a *post-hoc* Tukey test was performed. Normally distributed data are presented as mean ± SD. The Kruskal-Wallis test was used for comparison of data sets containing at least one abnormally distributed continuous variable. Non-parametrically distributed data is presented as median and IQR [interquartile range (IQR), 25th-75th percentile]. When the Kruskal-Wallis test indicated statistically significant differences, the causes of those differences were determined using a *post-hoc* Dunn's test. The nominal variables were analyzed using the Pearson's chi-square or Fisher's exact test, where applicable. Categorical variables were presented as the number (%) of cases. Statistical significance was set at $p < 0.05$.

Results

The study included 12 patients with PWS (mean age; 10.6 ± 4.8 years), 12 patients with BBS (mean age: 10.3 ± 4.7 years), 13 obese controls (OC), (mean age: 11.1 ± 3.8 years) and 12 LC, (mean age: 10.9 ± 4.2 years). There was no significant difference between PWS, BBS, OC and LC in terms of age, gender or pubertal status. BMI-SDS was similar in the PWS, BBS and OC, while the BMI-SDS of LC was significantly lower than that of the PWS, BBS and OC groups ($p < 0.001$). In the PWS group, three (25%) were on both L-thyroxine and recombinant growth hormone, and one (8.3%) was on both L-thyroxine and hydrocortisone treatment at the time of the study. In BBS group, only one (8.3%) was on L-thyroxine because of central hypothyroidism.

The median (IQR) ghrelin level was 0.96 (0.69-1.15) in PWS group, 0.92 (0.72-1.20) in OC group, and 1.13 (0.84-1.29) ng/mL in LC group. There was no significant difference between the three groups. In addition, the median GLP-1 level was 1.86 (1.5-2.94) in PWS group, 2.24 (1.62-2.78) in OC group, and 2.06 (1.8-3.41) ng/mL in LC group. Again, these values did not differ significantly (Figure 1).

In the BBS group the median ghrelin level was 1.05 (0.87-1.51), while in the OC and LC groups it was 0.92 (0.72-1.20) and 1.13 (0.84-1.29) ng/mL, respectively ($p > 0.05$). In addition, the median GLP-1 level was 2.46 (1.91-4.17) in BBS group, 2.24 (1.62-2.78) in OC group, and 2.06 (1.8-3.41) ng/mL in LC group and did not differ between the groups ($p > 0.05$) (Figure 1). Furthermore, when the PWS and BBS groups were compared in terms of median ghrelin and GLP-1 levels, no significant difference was found ($p > 0.05$). The clinical characteristics and laboratory data of the subjects are shown in Table 1.

Discussion

Early onset obesity and hyperphagia are characteristic features of PWS. In many studies, hyperghrelinemia has been reported in these patients (15). In a large review by Tauber et al. (16), hyperghrelinemia was reported in PWS patients and the authors linked obesity and hyperphagia to

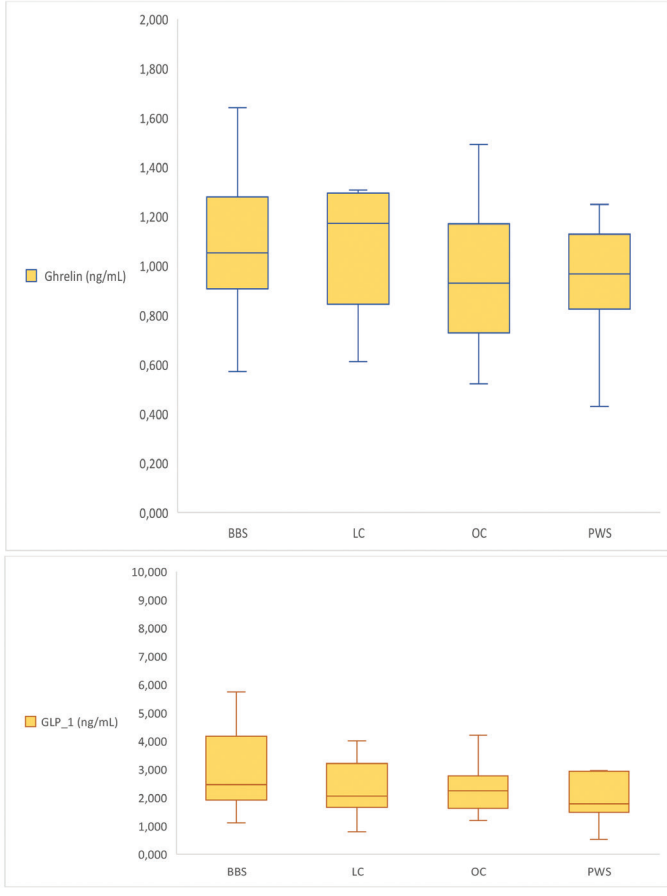

Figure 1. Box-plot presentation of serum ghrelin (ng/mL) and GLP-1 (ng/mL) levels in subjects with PWS and BBS, and in OC and LC. The lower and upper limits of the boxes represent 25th and 75th percentiles, and middle lines in each box represent 50th percentile, while the bottom and top end of the whiskers represent the minimum and maximum values, respectively. Dots represent the outlier data

PWS: Prader-Willi syndrome, BBS: Bardet-Biedl syndrome, GLP-1: glucagon-like peptide-1, OC: obese control, LC: lean control

Table 1. Clinical characteristics and laboratory findings of the patients and the control subjects

	PWS (n = 12)	BBS (n = 12)	OC (n = 13)	LC (n = 12)	p
Age (years) (min-max)	10.6 ± 4.8 (3-17.6)	10.3 ± 4.7 (5.3-19)	11.1 ± 3.8 (6.8-17.2)	10.9 ± 4.2 (2.8-16.4)	0.970
Female/male	6/6	4/8	6/7	8/4	0.412
Pubertal/prepubertal	4/8	6/6	4/8	6/6	0.136
BMI-SDS	2.7 ± 1.6[c]	3.13 ± 0.5[b]	2.81 ± 0.5[a]	1.18 ± 0.9[a,b,c]	**< 0.001**
Ghrelin (ng/mL)	0.96 (0.69-1.15)	1.05 (0.87-1.51)	0.92 (0.72-1.20)	1.13 (0.84-1.29)	0.485
GLP-1 (ng/mL)	1.86 (1.5-2.94)	2.46 (1.91-4.17)	2.24 (1.62-2.78)	2.06 (1.8-3.41)	0.460

Laboratory data are given as median for ghrelin and GLP-1 (interquartile range, 25th-75th percentile). One-way ANOVA (mean ± SD) was used for the age and BMI-SDS, Kruskal-Wallis was used for the rest.

[a]: OC vs LC $p < 0.05$.

[b]: BBS vs LC $p < 0.05$.

[c]: PWS vs LC $p < 0.05$.

PWS: Prader-Willi syndrome, BBS: Bardet-Biedl syndrome, OC: obese controls, LC: lean controls, min-max: minimum-maximum, SD: standard deviation, GLP-1: glucagon-like peptide-1, BMI: body mass index, SDS: standard deviation score

hyperghrelinemia. However, several groups have reported that total ghrelin levels were not elevated in young children with PWS compared to control groups. Among these studies, Haqq et al. (17) found that plasma ghrelin values were similar to the control group in a study with 33 infants with PWS. Butler and Bittel (18) divided PWS patients into groups of patients under and over three years of age, but they did not detect hyperghrelinemia in these groups compared to controls. Lastly, Erdie-Lalena et al. (19) found that ghrelin values were similar in PWS patients under five years of age compared to controls. These studies showed that in obesity in PWS, which develops after the initial phase of poor feeding and usually starts in the second year of life, hyperghrelinemia did not precede or coincide with the development of hyperphagia. An increased number of ghrelin-producing cells in the stomach of PWS patients has been suggested as a cause of the rise in ghrelin levels (20). In the present study, hyperghrelinemia in PWS compared to OC was not found. Therefore, obesity in PWS patients may not be due to hyperghrelinemia. In contrast, in a study by Turkkahraman et al. (21), it was found that the mean α-melanocyte stimulating hormone level in the PWS group was significantly lower than in OC and therefore suggested that obesity in PWS might be due to MC4R upstream pathologies. Thus, there is no consensus regarding the role of ghrelin levels in children with PWS.

There are only two studies in literature investigating serum GLP-1 levels in PWS patients, both performed in adult patients. In the first study, fasting GLP-1 concentrations in PWS subjects were similar in individuals with obesity and LC groups (10). In the second adult study, fasting GLP-1 concentrations in PWS were higher than OC and LC groups, but GLP-1 concentrations in obese and lean group were found to be similar (11). Our results are consistent with the results of the first study. In the present study, we did not find a significant difference in GLP-1 concentrations in children and adolescents with PWS compared to control groups, and in OC compared to LC. Once again, there is no consensus regarding GLP-1 levels, especially in children with PWS, but there is very little published evidence.

The BBSome is a critical regulator of cilia function. Primary cilia are important signaling organelles, including for neuronal trafficking (22). Guo et al. (23) showed that selective disruption of the BBSome via *BBS1* gene deletion led to a significant increase in body weight and adiposity and to leptin resistance and hyperleptinemia. In the literature, there is only one study in which the plasma ghrelin levels are not different between BBS and control groups (7). However, there is no previous study evaluating serum GLP-1 levels in BBS patients. In the present study, both ghrelin and GLP-1 concentrations of BBS patients were compared with obese and LC groups, and no difference was found in median levels between the groups.

Study Limitations

The limitations of our study include the small numbers of PWS and BBS patients, the wide age ranges of the patients across both the child and adolescent ranges and that some patients were receiving additional treatments with recombinant growth hormone, levothyroxine and hydrocortisone.

Conclusion

In conclusion, there are conflicting results regarding ghrelin levels, and not enough data for GLP-1 levels in children with PWS. Similarly, there is scant data regarding ghrelin and GLP-1 levels in BBS patients. Even though no difference was found between median levels of ghrelin and GLP-1 levels in these two syndromes compared with obese and LC, we hope that our study will contribute to the understanding of the pathophysiology of PWS and BBS. However, multi-center studies with larger patient groups will be required.

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Ethics

Ethics Committee Approval: The study protocol was approved by the University of Health Sciences Turkey, Antalya Training and Research Hospital Local Institutional Review Board (decision no: 10-12, date: 18.05.2022).

Informed Consent: Informed written consent from the subjects (>8 years old) and their parents were obtained.

Authorship Contributions

Concept: Doğa Türkkahraman, Suat Tekin, Merve Güllü, Design: Doğa Türkkahraman, Suat Tekin, Merve Güllü, Data Collection or Processing: Suat Tekin, Analysis or Interpretation: Güzin Aykal, Suat Tekin, Merve Güllü, Literature Search: Doğa Türkkahraman, Suat Tekin, Merve Güllü, Writing: Doğa Türkkahraman, Suat Tekin, Merve Güllü, Güzin Aykal.

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Associations of Adipocyte-derived Versican and Macrophage-derived Biglycan with Body Adipose Tissue and Hepatosteatosis in Obese Children

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What is already known on this topic?

In animal models of obesity, adipocyte-derived versican and macrophage-derived biglycan play a crucial role in mediating adipose tissue inflammation. Inhibition of versican in mouse models reduces macrophage accumulation, inflammatory gene expression, and liver inflammation, leading to improved glucose tolerance and insulin sensitivity.

What this study adds?

This is the first study to report elevated levels of versican in obese children and a positive correlation between versican and inflammatory markers, such as interleukin-6 and high sensitivity C-reactive protein. This suggests that attenuating versican release in obese individuals may have the potential to decelerate the inflammatory process, thereby reducing associated complications.

Abstract

Objective: In animal models of obesity, adipocyte-derived versican, and macrophage-derived biglycan play a crucial role in mediating adipose tissue inflammation. The aim was to investigate levels of versican and biglycan in obese children and any potential association with body adipose tissue and hepatosteatosis.

Methods: Serum levels of versican, biglycan, interleukin-6 (IL-6), and high sensitivity C-reactive protein (hsCRP) were measured by ELISA. Fat deposition in the liver, spleen, and subcutaneous adipose tissue was calculated using the IDEAL-IQ sequences in magnetic resonance images. Bioimpedance analysis was performed using the Tanita BC 418 MA device.

Results: The study included 36 obese and 30 healthy children. The age of obese children was 13.6 (7.5-17.9) years, while the age of normal weight children was 13.0 (7.2-17.9) years ($p = 0.693$). Serum levels of versican, hsCRP, and IL-6 were higher in the obese group ($p = 0.044$, $p = 0.039$, $p = 0.024$, respectively), while no significant difference was found in biglycan levels between the groups. There was a positive correlation between versican, biglycan, hsCRP, and IL-6 ($r = 0.381$ $p = 0.002$, $r = 0.281$ $p = 0.036$, rho = 0.426 $p = 0.001$, $r = 0.424$ $p = 0.001$, rho = 0.305 $p = 0.017$, rho = 0.748 $p < 0.001$, respectively). Magnetic resonance imaging revealed higher segmental and global hepatic steatosis in obese children. There was no relationship between hepatic fat content and versican, biglycan, IL-6, and hsCRP. Versican, biglycan, hsCRP, and IL-6 were not predictive of hepatosteatosis. Body fat percentage > 32% provided a predictive sensitivity of 81.8% and a specificity of 70.5% for hepatosteatosis [area under the curve (AUC): 0.819, $p < 0.001$]. Similarly, a body mass index standard deviation score > 1.75 yielded a predictive sensitivity of 81.8% and a specificity of 69.8% for predicting hepatosteatosis (AUC: 0.789, $p < 0.001$).

Conclusion: Obese children have higher levels of versican, hsCRP, and IL-6, and more fatty liver than their healthy peers.

Keywords: Chronic inflammation, biglycan, hepatosteatosis, obesity, versican

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Introduction

Obesity is an epidemic condition affecting all age groups worldwide, and the prevalence is increasing rapidly (1). Adipose tissue serves not only as a primary site of storage for excess energy but may also trigger a chronic inflammatory process through the secretion of autocrine/paracrine molecules and cytokines (2,3). Lymphocytes and macrophages accumulated in adipose tissue release various proinflammatory/anti-inflammatory molecules such as tissue necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-4, IL-6, IL-10. Moreover, the adipocytes also release molecules, such as leptin, adiponectin, visfatin, resistin, and adipsin, thereby initiating a chronic inflammatory process (2,4,5). This process, originating in adipose tissue, culminates in systemic inflammation and may give rise to complications, such as insulin resistance, metabolic syndrome, and type 2 diabetes mellitus (6,7). In addition, increased extracellular matrix (ECM) molecules and their degradation products function as immunomodulators (6,8,9). Identifying ECM components associated with adipose tissue inflammation and metabolic disturbances is important for a better understanding of this process (10).

Versican, released from hypertrophic adipocytes under inflammatory conditions, functions as a proteoglycan, rich in chondroitin sulfate. Versican functions by binding to serum amyloid A in high density lipoproteins (HDL). Versican is known to regulate events associated with adipose tissue inflammation, including lipoprotein retention, lipid uptake, and foam cell formation. Furthermore, versican interacts with molecules, such as chemokines, growth factors, proteases, and immune cell receptors, including CD44, p-selectin glycoprotein-1 and toll-like receptor-2 (TLR2), facilitating the formation of intracellular signals (6,10,11,12).

Another proinflammatory molecule, biglycan, is a small proteoglycan rich in leucine and serves as a structural scaffold by interacting with collagen and elastin molecules in the ECM under physiological conditions. In addition, biglycan production increases in adipose tissue during inflammatory states due to the accumulation of macrophages in the tissues. Elevated biglycan molecules bind to TLR2 and TLR4, inducing the secretion of proinflammatory cytokines such as TNF-α and IL-1β, thus playing a role in adipose tissue inflammation (6,10,13).

Han et al. (6) investigated the effects of adipose tissue proteoglycans on inflammation and insulin resistance. They examined the molecules versican, released from adipocytes, and biglycan released from macrophages. In their experiments with mice, they observed an increased presence of versican and biglycan molecules in the adipose tissue of obese mice. Through targeted deletion of adipocyte-specific versican, the researchers noted a mitigation of macrophage chemotaxis. This intervention was associated with a reduction in the expression of inflammatory genes, attenuation of hepatic inflammation, augmentation of insulin sensitivity, and improvement in glucose tolerance. These findings suggest that versican exerts a regulatory influence on these processes. Furthermore, deletion of macrophage-specific biglycan led to reduced macrophage accumulation and cytokine/chemokine release. However, while a decrease in liver inflammation and an increase in insulin sensitivity were observed with versican deletion, these effects were not evident in mice with biglycan deletion. This study demonstrated the association of elevated biglycan levels with inflammation, obesity, insulin resistance, and type 2 diabetes in mice (6).

An inflammatory process in adipose tissue contributes to the early development of insulin resistance, dyslipidemia and hepatosteatosis in obesity (14). Although ultrasonography (US) is commonly used for detecting non-alcoholic fatty liver disease (NAFLD), which is the most prevalent chronic liver condition, liver biopsy remains the gold standard diagnostic tool (15). However, biopsy, being an invasive procedure, can yield false negatives in patients without diffuse hepatosteatosis. In recent years, a noninvasive method known as "iterative decomposition of water and fat with an echo asymmetry at least-square estimation-iron quantification (IDEAL-IQ) sequence," utilized through magnetic resonance imaging (MRI), has emerged as a reliable means for the detection of NAFLD (16).

A clinical study investigating the relationship between versican, biglycan, and metabolic parameters related to obesity has not been published to date. In the present study, the levels of versican and biglycan, which are believed to play a significant role in the etiopathogenesis and complications of obesity, were investigated in obese children. A further aim was to explore the association of these molecules with adipose tissue, hepatosteatosis, and inflammation in the context of pediatric obesity.

Methods

The study included obese children aged 7-18 years presenting to a single pediatric endocrinology clinic with complaints of weight gain. These children had a body mass index (BMI) ≥95th percentile, based on national data from Turkish children. Gender and age-matched healthy children with BMI <85th percentile were selected as the control group.

Patients underwent detailed physical examinations and laboratory tests were conducted to assess the possibility of underlying endocrine pathologies. Cases with any chronic diseases, a history of medication use, identified endocrine pathologies, and cases suspected of syndromic or monogenic origins of obesity were excluded. Anthropometric measurements were carried out using a Harpenden stadiometer (Crosswell, Crymych, Pembs., SA41 3UF, UK) with a precision of 0.1 cm for height and a SECA scale (Hammer Steindamm 3-25 22089, Hamburg, Germany) with a precision of 0.1 kg for weight. Patients were evaluated after removing all clothing except thin underwear. BMI was calculated by dividing body weight (kg) by the square of measured height (m) and then transformed into standard deviation score (SDS) using national BMI reference data (17).

Blood pressure measurements were conducted by one of the investigators following a validated protocol. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were taken twice from the right arm after a 10-minute rest in the supine position, using a calibrated sphygmomanometer with appropriate cuff size (18). Waist circumference was measured using a flexible but not stretchable tape, positioned midway between the lowest rib and the superior border of the iliac crest (19). The measurement of triceps skinfold thickness was performed using a Holtain skinfold caliper (Crosswell, Crymych, Pembs., SA41 3UF, UK). One investigator performed triceps skinfold thickness measurements by grasping a fold of skin and subcutaneous adipose tissue approximately 2.0 cm above the mid-arm circumference mark. The procedure involved placing the tips of the caliper jaws over the entire skinfold, followed by releasing the caliper handle to apply full tension on the skinfold. The thickness was then read to the closest 0.1 mm (20). Bioelectrical impedance analysis was performed according to standards using the Tanita BC 418 MA device (Maenocho Itabashi-Ku, Tokyo, 174-0063 Japan). The basal metabolic rate was determined through bioimpedance analysis.

Fasting blood samples were collected from a peripheral vein between 08:00 and 09:00 in the morning after a minimum of 12 hours of fasting. Serum fasting glucose, insulin, glycated hemoglobin (HbA1c), liver and thyroid function tests, total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and HDL levels were measured using enzymatic colorimetric methods. Biochemical analyses were performed using original reagents on an auto analyzer with standardized methods at Aydın Adnan Menderes University Faculty of Medicine Hospital. To assess insulin resistance, the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) index was used. Different cut-off values were employed for prepubertal and pubertal subjects to evaluate insulin resistance. The cut-off values for the HOMA-IR index were 2.22 for prepubertal girls, 2.67 for prepubertal boys, 3.82 for pubertal girls, and 5.22 for pubertal boys (21).

Serum levels of versican (cat no: SL1818Hu, detection range: 16-1000 pg/mL, sensitivity: 4.5 pg/mL; Sunlong, Hangzhou, China), biglycan (cat no: SL2244Hu, detection range: 0.08-4.0 ng/mL, sensitivity: 0.01 ng/mL; Sunlong, Hangzhou, China), IL-6 (cat no: 201-12-0091, detection range: 3-600 ng/L, sensitivity: 2.112 ng/L; Sunred, Shanghai, China), and high sensitivity C-reactive protein (hsCRP) (cat no: 201-12-1816, detection range: 0.15-40 ng/L, sensitivity: 0.112 ng/L; Sunred, Shanghai, China) were measured using commercial kits following the manufacturer's directions. These commercial kits used antibody-coated plates and a sandwich ELISA method. Serum samples were applied onto these plates, followed by incubation following the kit procedure to allow the specific binding of the relevant molecules to the specific antibodies. Subsequent washing steps were conducted to remove unbound molecules, a second antibody with a chromogen was added and measurements were taken at 450 nm using an ELISA reader. The results were then calculated based on the standard curve included.

Hepatosteatosis was assessed by both US and MRI. US was performed in the supine position by an experienced radiologist using a Sonostar C5PL portable handheld ultrasound device (Sonostar Technologies Co. Ltd., Guangzhou, China). The definition of hepatosteatosis was based on the increased difference in echogenicity between the liver and kidney. The evaluation was categorized into no steatosis (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3) steatosis using a previously published ultrasound steatosis score (22,23). MRI was conducted using a General Electric 3T Sigma Pioneer SW 29.0_R01_2034.a device (General Electric Company Neumann Way Cincinnati, OH 45215). The IDEAL-IQ sequence, a brief imaging protocol without contrast, was employed to obtain cross-sectional images encompassing the liver, spleen, and subcutaneous adipose tissue within the abdominal region. The acquired images were used to calculate the percentages of fat in the liver, spleen, and subcutaneous adipose tissue using the GE AW 4.7 version work station. The liver parenchyma was divided into nine segments and measurements were taken. In the segmental measurement technique, each segment of the liver was measured separately, and the average of the measurements was taken. In the global measurement technique, the entire liver parenchyma was measured in a single session (16).

Statement of Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Non-interventional Ethics Committee of Aydın Adnan Menderes University (ethics no: 2021/83, date: 24.06.2021). Informed consent in this study was taken from all participants.

Statistical Analysis

The statistical analysis of the data was conducted using the Statistical Package for Social Sciences, version 21 (IBM Corp., Armonk, NY, USA). The normality of continuous variables was assessed through descriptive statistics, skewness, kurtosis coefficients, histograms, and the Shapiro-Wilk test. Descriptive statistics are presented using counts, percentages, means, and standard deviations for normally distributed data, and medians, minimum, and maximum values for non-normally distributed data. For categorical variables, the chi-squared test was used in the statistical analysis. For independent group comparisons, if the data followed a normal distribution, the t-test was applied, and if not, the Mann-Whitney U test was used. Spearman correlation and receiver operator curve (ROC) analysis were used. The type 1 error level was set at 5%, and p values less than 0.05 were considered statistically significant.

Results

In total, 36 obese and 30 healthy children were included in the study. There were no significant differences between the obese and healthy groups in terms of age, gender, height, and DBP. However, obese individuals exhibited higher weight, BMI, SBP, waist circumference, triceps skinfold thickness, basal metabolic rate, and body fat percentage.

In the obese group, serum insulin levels were higher, and the incidence of insulin resistance was greater (Table 1). In addition, TG, LDL cholesterol, HbA1c, alanine aminotransferase (ALT), and white blood cell count were higher among the obese individuals, while HDL cholesterol levels were lower. TC, aspartate aminotransferase, and thyroid function tests showed similar results between the two groups (Table 1).

When compared to the control group, serum levels of versican, IL-6, and hsCRP were higher in obese children, whereas biglycan levels were similar between the two groups (Table 1).

Table 2 shows the associations between versican, biglycan, hsCRP, and IL-6. There was no relationship between the

Table 1. Clinical, demographic, and laboratory characteristics of enrolled cases

	Obese n = 36	Controls n = 30	p*
	Median (min-max)	Median (min-max)	
Age (year)	13.6 (7.5–17.9)	13.0 (7.2-17.9)	0.693
Height (SDS)	0.8 (-2.1–-3.2)	0.3 (-2.2–2.8)	0.114
Weight (SDS)	2.9 (2.0- – -6)	0.1 (-1.9–1.7)	**< 0.001**
BMI (SDS)	2.6 (1.9–3.3)	0.4 (-0.6–0.7)	**< 0.001**
Systolic BP (mmHg)	120 (100-140)	110 (105-134)	**< 0.001**
Diastolic BP (mmHg)	70 (60-91)	70 (55-99)	0.435
Waist circumference (cm)	101 (42-141)	70.6 (55-96.5)	**< 0.001**
Triceps skinfold thickness (mm)	19.5 (8-37)	10.3 (4-18)	**< 0.001**
Glucose (mg/dL)	89.5 (77-146)	88 (76-115)	0.622
Insulin (µU/mL)	17.2 (7.9-42.8)	10.4 (3-19)	**< 0.001**
HOMA-IR index	3.8 (1.7-9.1)	2.2 (0.6-4.3)	**< 0.001**
Triglyceride (mg/dL)	94.5 (26-559)	65 (25-169)	**0.001**
Total cholesterol (mg/dL)	156.5 (117-214)	151.5 (90-207)	0.123
LDL-C (mg/dL)	86.5 (38-149)	78.5 (37-132)	**0.038**
HDL-C (mg/dL)	48.4 (25.2-73.1)	55.3 (38.1-119.9)	**0.005**
HbA1c (%)	5.5 (4.5-6.2)	4.8 (3.9-5.8)	**0.006**
AST (U/L)	19 (9-173)	20 (13-72)	0.111
ALT (U/L)	18.5 (8-311)	15 (10-25)	**0.022**
Free T_4 (ng/dL)	1 (0.8-1.2)	0.9 (0.8-1.2)	0.086
TSH (uIU/mL)	1.8 (0.7-5.1)	1.8 (0.5-9)	0.359

Table 1. Continued

	Obese n = 36	Controls n = 30	p*
	Median (min-max)	Median (min-max)	
WBC (10^3/μL)	9080 (6070-13410)	6520 (3990-12510)	**< 0.001**
Versican (pg/mL)	63.6 (48.3-78.3)	59.1 (44.3-80.2)	**0.044**
Biglycan (ng/mL)	1.2 (0.4-1.7)	1.0 (0.4-1.5)	**0.176**
IL-6 (ng/L)	48.3 (26.1-119.6)	34.4 (8.3-120.4)	**0.024**
hsCRP (ng/L)	5.1 (2.1-9.7)	3.7 (1.3-8.7)	**0.039**
Basal metabolic rate (kcal)	1632.0 (929-3022)	1230.5 (777-2032)	**< 0.001**
Fat mass (kg)	30.0 (9.9-56.1)	10.3 (3.9-26.9)	**< 0.001**
Fat percentage	36.2 (26.9-61.9)	21.9 (10.8-31.6)	**< 0.001**
Liver FQ global method	6 (1.9-25.9)	2.6 (1.3-6.4)	**< 0.001**
Spleen FQ	2 (0.5-7.1)	2.3 (1.3-9.6)	0.143
Subcutaneous fat FQ	93 (88.9-96.3)	92.7 (81.6-98.3)	0.949

*Mann-Whitney U test was used.
min-max: minimum-maximum, BMI: body mass index, BP: blood pressure, HOMA-IR: Homeostasis Model Assessment-Insulin Resistance, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, hsCRP: high sensitive C-reactive protein, IL-6: interleukin-6, FQ: fat quantity, SDS: standard deviation score

Table 2. Correlation table for versican, biglycan, IL-6, and hsCRP

	Biglycan		hsCRP		IL-6	
	r	p	r	p	rho	p*
Versican	0.381	0.002	0.281	0.036	0.426	**0.001**
Biglycan			0.424	0.001	0.305	**0.017**
hsCRP					0.748	**< 0.001**

*Spearman correlation was used.
hsCRP: high sensitive C-reactive protein, IL-6: interleukin-6

degree of hepatosteatosis and serum levels of versican, biglycan, IL-6, and hsCRP. Furthermore, no correlation was found between metabolic parameters (glucose, HbA1c, insulin, HOMA-IR, lipid profile, thyroid-stimulating hormone, sT4, and leukocyte) and serum levels of versican and biglycan.

Similar results were obtained from both US and MRI for the assessment of hepatic fat content. When comparing groups based on MRI findings, obese children had significantly higher liver fat content than the control group, using both the segmental and global measurement techniques ($p < 0.001$). Spleen fat levels were similar in both groups. Liver fat content was positively correlated with TG, LDL, HbA1c, ALT, white blood cell count, basal metabolic rate, and body fat percentage, while it was negatively correlated with HDL ($r = 0.333$ $p = 0,013$, $r = 0.268$ $p = 0,048$, $r = 0.339$ $p = 0.006$, $r = 0.365$ $p = 0.006$, $r = 0.529$ $p < 0.001$, $r = 0.310$ $p = 0.019$, $r = 0.634$ $p < 0.001$, $r = -0.330$ $p = 0.014$, respectively). Patients with hepatosteatosis had higher levels of HbA1c, white blood cells, insulin, HOMA-IR, TG, LDL, ALT, free thyroxine (fT4), body fat percentage, and body fat weight (all $p < 0.05$). Participants with higher body fat percentages exhibited significantly higher serum versican levels ($rho = 0.318$ $p = 0.012$), while those with more subcutaneous adipose tissue had higher IL-6 levels ($rho = 0.255$ $p = 0.047$).

Serum levels of versican, biglycan, hsCRP, and IL-6 were not significantly predictive of hepatosteatosis ($p > 0.05$). A body fat percentage of over 32% had a predictive sensitivity of 81.8% and specificity of 70.5% [area under the curve (AUC): 0.819, $p < 0.001$] for hepatosteatosis. Similarly, a BMI SDS above 1.75 yielded a predictive sensitivity of 81.8% and specificity of 69.8% (AUC: 0.789, $p < 0.001$) for predicting hepatosteatosis (Figure 1, Table 3).

Discussion

This study, which investigated the relationship between serum versican and biglycan levels with metabolic parameters and hepatosteatosis in obese children, revealed that versican levels were higher in obese children and there was a positive correlation between versican and inflammatory markers, such as IL-6 and hsCRP (2,24).

Due to the chronic inflammatory process, inflammatory markers are known to be elevated in obese individuals (2,5,7,25). Furthermore, several studies have demonstrated

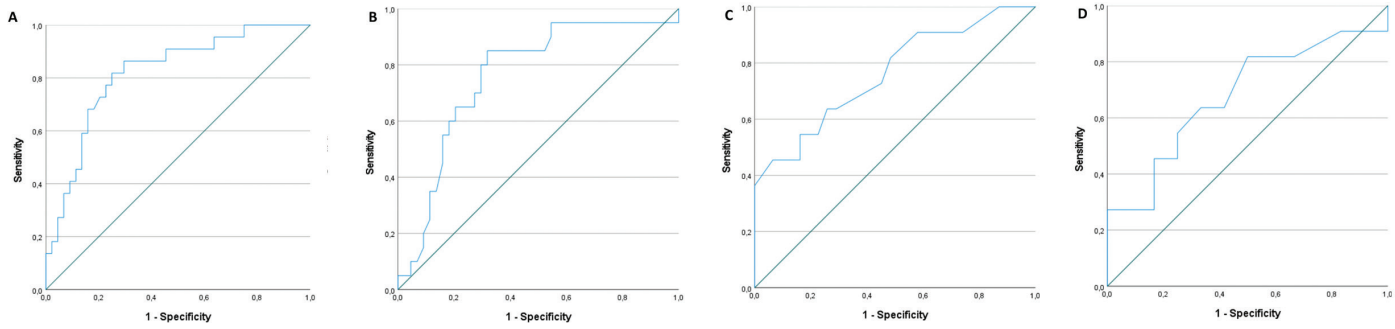

Figure 1. ROC curve of hepatosteatosis for the four parameters: (a) body fat percentage, (b) waist circumference, (c) ALT (girls), (d) ALT (boys)

ROC: receiver operator curve, ALT: alanine aminotransferase

Table 3. Clinical and laboratory predictors of hepatosteatosis

Criteria	Sensitivity	Specificity	AUC	95% CI	p*
Body fat percentage > 32 %	81.8 %	70.5 %	0.819	0.711-0.933	**< 0.001**
BMI SDS > 1.75	81.8 %	69.8 %	0.789	0.659-0.918	**< 0.001**
Waist circumference > 90 cm	70.0 %	70.5 %	0.760	0.631-0.888	**0.001**
ALT > 22 U/L (girls)	45.5 %	83.9 %	0.762	0.590-0.935	**0.010**
ALT > 25 U/L (boys)	45.5 %	75.0 %	0.678	0.451-0.905	0.148

*ROC analysis was used.
BMI: body mass index, SDS: standard deviation score, AUC: area under the curve, CI: confidence interval, ALT: alanine aminotransferase

that complications such as hepatosteatosis, metabolic syndrome, and type 2 diabetes mellitus arise from the chronic inflammation seen in obesity (24,25,26). In our study, inflammatory markers IL-6 and hsCRP were found to be higher in obese children compared to the control group. This finding implies that the origins of complications are established during the early stages of life.

The number of studies focusing on the role of versican in the regulation of inflammation and immunity is steadily increasing. Versican, with five known isoforms, binds to various receptors and components involved in the inflammatory response, playing a pivotal role in both pro- and anti-inflammatory processes (9). In experiments conducted with obese mice, it has been demonstrated that obese mice exhibit increased production of versican from adipocyte cells. Inhibition of versican production from adipocytes has been shown to reduce macrophage accumulation, inflammatory gene expression, and liver inflammation, resulting in improved insulin sensitivity and glucose tolerance (6). In various human studies, the association between versican and inflammation, such as cardiovascular diseases, respiratory diseases, and certain cancer types has been investigated and increased serum versican levels have been reported in these diseases when inflammation is present (9,12,27). However and to the best of our knowledge, there is no published study investigating versican levels in obese humans. In the present study and consistent with published animal studies, serum versican levels in obese children were found to be higher compared to the control group (6). This suggests that interventions aimed at preventing the increased release or accumulation of versican might slow the inflammatory process, thereby reducing the complications caused by chronic inflammation in obese individuals.

High levels of biglycan have been associated with inflammation, obesity, insulin resistance, and type 2 diabetes mellitus (10,28). However, unlike versican, the relationship between biglycan and hepatosteatosis has not been established (6,10). Previous animal studies have shown a link between obesity, insulin resistance, and biglycan levels. Nevertheless, in our study, no significant differences were observed in serum biglycan levels between obese children and the control group. This suggests that the *in vivo* relationship might be different in different species, or this relationship might manifest later in life and may not be evident in the childhood age group. In line with the literature, the present study also found no correlation between serum biglycan levels and liver fat content (6,28).

In obese individuals, the chronic inflammatory process associated with increased adipose tissue, which is both a cause and a consequence of obesity, is known to lead to elevated inflammatory markers, including hsCRP (29,30,31). Furthermore, as previously mentioned, the increased

production and secretion of versican and biglycan due to the expansion of adipose tissue and their relationship with inflammatory cells have been shown to play a role in chronic inflammation (6,8,9). In the present study, a strong positive correlation was observed between hsCRP, versican, and biglycan levels, all of which have functional roles in the chronic inflammatory process. Partial correlation analysis was performed, revealing that the associations between versican, biglycan, IL-6, and hsCRP persisted in a similar manner. The correlation of versican and biglycan levels with hsCRP and IL-6 in obese children suggests a potential role for versican and biglycan in the inflammation process of obesity. Based on insights from animal studies, when evaluating the relationship between serum versican and biglycan levels with metabolic parameters yielded no evident correlation. The lack of correlation between versican and biglycan with metabolic parameters may have been due to the small sample size in this study.

Steatosis involving more than 5% of the weight of hepatocytes or liver tissue is considered abnormal (32). Studies on the accurate detection and grading of NAFLD have been continuing for many years. The gold standard method for the quantitative diagnosis of hepatosteatosis remains biopsy. However, the routine use of biopsy is quite limited due to its invasiveness and sampling error risk (33). Ultrasound is an economical and useful method, but it is highly subjective, and its quantitative and objective criteria are not clear (34). Even though US is relatively easy to perform and interpret, some limitations may be encountered: a quantitative assessment is not performed, when lower than 20% steatosis may not be detected (35). MRI techniques are currently in clinical use for the detection and quantification of hepatic steatosis (36,37). The IDEAL-IQ method of MRI is based on the water and oil separation technique based on chemical change to obtain the proton-dense oil fraction. Many studies have shown that using IDEAL-IQ to test the stability and reproducibility of liver fat is acceptable and has high accuracy (16,38). MRI accurately classifies grades and changes in hepatosteatosis, with 80.0-95.8% sensitivity and 83.6-100% specificity (39,40). However, due to the high cost, time-consuming nature, and limited accessibility of MRI, there is a need for more practical and cost-effective methods to identify hepatosteatosis. Considering this objective, we systematically assessed the relationship between hepatosteatosis identified via MRI, and various biochemical and auxological parameters. In concordance with the existing literature, most of the participants manifesting hepatosteatosis exhibited obesity, with this condition correlating with elevated levels of liver fat accumulation and an augmented ratio of subcutaneous adipose tissue. Similar to previous studies, hepatosteatosis demonstrated positive correlations with TG, LDL, HbA1c, ALT, white blood cell count, basal metabolic rate, and body fat ratio, while exhibiting a negative correlation with HDL (32,40,41). Based on ROC analysis, similar to NASPGHAN, ALT displayed predictive efficacy for hepatosteatosis in females, yielding an AUC of 0.762, 45.5% sensitivity, and 83.9% specificity, utilizing a cutoff of 22 U/L (32). The absence of a significant cut off value in males was most likely due to the smaller numbers involved in our study.

The presence and severity of hepatosteatosis increase with higher waist circumference, BMI SDS, and body fat ratio (42,43). Consistent with these findings, our study identified a relationship between hepatosteatosis and these parameters. Specifically, our results revealed that body fat percentage $>32\%$, BMI SDS >1.75, and waist circumference >90 cm indicating the presence of hepatosteatosis and were in line with previous studies (42,44). However, our study could not establish a significant relationship between hepatosteatosis and IL-6, hsCRP, versican and biglycan, primarily attributed once again to the limited number of participants. Nevertheless, our findings underscored that the most reliable predictors for hepatosteatosis were body fat ratio and BMI SDS.

Study Limitations

The inability to perform a power analysis due to the absence of a similar study in the literature represents a significant limitation of the study. Consequently, the sample size obtained may have been relatively limited as a result of this constraint. Moreover, the patients were not anesthetized during imaging, so movement artifacts occurred in some patients. In the technique we used, the resolution of the liver fat measurement sequence is low,and the presence of fat was not confirmed by biopsy, which is the gold standard method. Additionally, adiposity was measured once by a single radiologist.

Conclusion

This is the first study to report elevated levels of versican in obese children, concomitant with other accepted inflammatory markers. These findings indicated that slowing down the release of versican in obese individuals may mitigate the inflammatory process, as suggested by animal studies, potentially reducing complications. Furthermore, the study showed that waist circumference, BMI SDS and body fat ratio can be used to predict hepatosteatosis identified through the IDEAL-IQ MR sequence. However, further studies with a larger population are needed to identify novel predictive markers for hepatosteatosis.

Ethics

Ethics Committee Approval: The study was approved by the Non-interventional Ethics Committee of Aydın Adnan Menderes University (ethics no: 2021/83, date: 24.06.2021).

Informed Consent: Informed consent in this study was taken from all participants.

Authorship Contributions

Concept: Ahmet Anık, Design: Ahmet Anık, Data Collection or Processing: Reyhan Deveci Sevim, Sebla Güneş, Analysis or Interpretation: Reyhan Deveci Sevim, Mustafa Gök, Özge Çevik, Ömer Erdoğan, Literature Search: Reyhan Deveci Sevim, Tolga Ünüvar, Writing: Reyhan Deveci Sevim, Ahmet Anık.

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Evaluating Postoperative Outcomes and Investigating the Usefulness of EU-TIRADS Scoring in Managing Pediatric Thyroid Nodules Bethesda 3 and 4

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What is already known on this topic?

The European-Thyroid Imaging Reporting and Data System (EU-TIRADS) is a risk stratification system used to evaluate the probability of malignancy in thyroid nodules, based on ultrasound characteristics. This scoring system assists clinicians in determining whether to suggest biopsy or further monitoring of thyroid nodules. The EU-TIRADS scoring system has undergone comprehensive investigation in adult populations, demonstrating acceptable accuracy in the prediction of malignancy. The EU-TIRADS scoring system has the potential to be a useful tool for evaluating thyroid nodules in children, but its accuracy and effectiveness still require confirmation.

What this study adds?

This study is the first comprehensive investigation that assesses the postoperative outcomes and explores the utility of EU-TIRADS scoring in the management of pediatric thyroid nodules categorized as Bethesda 3 and 4. Postoperative pathologies revealed varying EU-TIRADS scores. EU-TIRADS 5 produced a lower percentage of cases with malignancy in Bethesda 3, compared to the low risk and benign group, while in Bethesda 4 cases, EU-TIRADS scores increased as postoperative pathology worsened. These findings highlight the inconsistent results of EU-TIRADS in guiding clinical decision-making for pediatric thyroid nodules.

Abstract

Objective: The aim was to assess postoperative outcomes in pediatric thyroid nodules with atypia of undetermined significance (AUS/FLUS) or suspicious for a follicular neoplasm (SFN) and their respective the European-Thyroid Imaging Reporting and Data System (EU-TIRADS) scores.

Methods: Forty-four pediatric patients at a single center with thyroid nodules classified as AUS/FLUS or SFN from August 2019 to December 2022 were retrospectively reviewed. Data on demographics, thyroid function, nodule size, and ultrasonographic features were collected. Postoperative pathologies were categorized into benign, low-risk, and malignant neoplasms according to the World Health Organization 2022 criteria, and EU-TIRADS was used for retrospective radiological scoring.

Results: Among 21 (47.7%) of patients who had surgical intervention, 72% had Bethesda 3 and 28% had Bethesda 4 thyroid nodules. Post-surgical histopathological classifications were 43% benign, 19% low-risk, and 38% malignant. Of note, EU-TIRADS 3 and 5 scores were present in 44% and 56% of the benign cases, respectively. Malignant cases tended to produce higher EU-TIRADS

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scores, with 64% rated as EU-TIRADS 5. Bethesda category 4 nodules had a 66% malignancy rate, significantly higher than the 27% in category 3.

Conclusion: A substantial proportion of histologically benign cases were classified as EU-TIRADS 5, suggesting that EU-TIRADS may lead to unnecessary biopsies in benign cases. Malignant cases were more likely to have a higher EU-TIRADS score, indicating a positive correlation with malignancy risk, particularly in Bethesda 4 cases. However, the EU-TIRADS system's predictive value for malignancy in Bethesda 3 cases was poorer.

Keywords: Pediatric thyroid nodules, malignancy, AUS/FLUS, Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance, FN/SFN, Follicular Neoplasm or Suspicious for a Follicular Neoplasm, EU-TIRADS, European Thyroid Imaging Reporting and Data System, malignancy

Introduction

Fine-needle aspiration (FNA) is a valuable method for guiding the therapeutic management of patients with thyroid nodules by estimating the risk of malignancy (1,2). The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) includes six diagnostic categories: I) non-diagnostic or unsatisfactory; II) benign; III) atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS); IV) follicular neoplasm or suspicious for a follicular neoplasm (FN/SFN); V) suspicious for malignancy; and VI) malignant (1,3).

In children with thyroid nodules the Bethesda categories 3 and 4 are the two cytopathological diagnoses that present clinicians with the greatest difficulty when making surgical decisions and assessing the risk of malignancy. It has been shown that the malignancy risk for Bethesda category 3 and 4 is higher in children compared to adults (4). For Bethesda category 4 nodules, the risk of malignancy is greater than for category 3 nodules (5). The 2015 American Thyroid Association (ATA) 2015 guidelines recommend surgery for thyroid nodules classified as AUS/FLUS or FN/SFN on the first FNA biopsy (FNAB) in order to definitively establish the diagnosis and provide appropriate treatment if the nodule is malignant (2). The 2022 European Thyroid Association (ETA) pediatric guidelines recommend repeating FNAB after six months for AUS/FLUS and FN/SFN nodules detected on the first FNAB. Surgical management is reserved for cases with significant growth, suspicious ultrasound (US) features, or persistent cytological abnormalities (6). The guidelines note that the risk of malignancy is higher in pediatric thyroid nodules compared to adult nodules, and that surgical management may carry greater risks and consequences in children due to their smaller size and less-developed anatomy. However, the guidelines do acknowledge that careful observation with repeat FNAB at 6 to 12 months may be an option in select cases, such as those with small nodules or those with significant comorbidities that increase the surgical risk (2).

Bethesda category 3 and category 4 are both considered indeterminate categories, meaning that the risk of malignancy is unclear based on cytology alone. Therefore, additional evaluation and clinical correlation are needed to determine the appropriate management. It is important for clinicians to consider the individual patient's clinical and imaging features, as well as the specific cytopathological diagnosis, when deciding on management for Bethesda category 3 and 4 nodules in children. The Thyroid Imaging Reporting and Data System (TIRADS), is a set of risk stratification systems to categorize thyroid nodules based on US features (7,8). The term TIRADS can refer to multiple guidelines, including ACR-TIRADS (American College of Radiology) (9), EU-TIRADS (ETA) (10), and K-TIRADS (Korean Society of Thyroid Radiology) (11,12). The EU-TIRADS is considered to have a more straightforward and potentially less time-consuming approach to nodule classification. The system allows assessment of high specificity US malignancy features, which include marked hypo-echogenicity, irregular shape, irregular margins, and microcalcifications (10). While the EU-TIRADS scoring system has been extensively studied in adult patients and has shown good accuracy in predicting malignancy (13), there needs to be more data on its usefulness in childhood thyroid nodules. Some studies have suggested that the EU-TIRADS scoring system may be helpful in childhood thyroid nodules (14), but further research is needed to confirm its accuracy and usefulness in this population.

In the present study, the aim was to evaluate the histopathologically confirmed postoperative outcomes of cases with AUS/FLUS and SFN detected in thyroid nodules and retrospectively investigate their EU-TIRADS scoring.

Methods

The study was conducted as a single-center, retrospective, cross-sectional analysis. It encompassed patients who presented with findings of AUS/FLUS or SFN on FNAB between August 2019 and December 2022. The follow-up principle for thyroid nodules used by our multidisciplinary team (pediatric endocrinologists, pediatric surgeons, pediatric radiologists, pediatric oncologists and pathologists) in patients with thyroid nodules who are diagnosed as

Bethesda category 3 in their initial biopsy and who do not show pathognomonic findings of malignancy, such as microcalcification, central vascularity or irregular borders on US, is to keep them under observation. These cases were re-evaluated with ultrasonography after 3-6 months, followed by a second FNAB. Our study included 21 of the 44 patients who underwent surgical intervention after being diagnosed with Bethesda categories 3 (AUS/FLUS) and 4 (FN/SFN) on FNAB of the thyroid. Of the remaining 23 patients, ten were lost to follow-up, the second biopsies of six patients were interpreted as benign, and the seven patients had not yet had post-biopsy follow-up. Demographic and clinical data, such as age, gender, serum free thyroxine (fT4), thyroid-stimulating hormone levels, dimensions of thyroid nodules, sonographic characteristics, and histopathological findings post-thyroidectomy were retrieved from the institutional electronic medical records. TBSRTC was employed to categorize the cytopathological findings of the thyroid FNABs. The inclusion criteria encompassed cases classified as AUS or FLUS under category 3, as well as those specified as SFN under category 4. It is noteworthy that our cohort did not consist of any patients classified as follicular neoplasm (FN). All participants underwent a complete thyroidectomy as part of their treatment protocol.

Post-operative pathology results were divided into three categories, as classified by the World Health Organization (WHO) in 2022: benign neoplasms, low-risk neoplasms, and malignant neoplasms. Low-risk neoplasms are non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), thyroid tumors of uncertain malignant potential (UMP), and hyalinized trabecular tumor (HTT) (15,16). In this study, there were cases with NIFTP and UMP. Radiologic features of the nodule were scored according to the EU-TIRADS scoring system (2017) (10) The study was performed in accordance with the Helsinki Declaration of 1975. This study was approved by the Ethics Committee of Ankara City Hospital (approval number: E2-23-3317, date: 01.02.2023).

Statistical Analysis

All data analysis was performed with Statistical Package for Social Sciences, version 26.0 (IBM Inc., Armonk, NY, USA). Descriptive statistics were used to evaluate demographic and clinical characteristics. Data were defined as percent, mean ± standard deviation, and median (minimum-maximum). The chi-square test was used for comparing categorical variables. While investigating the associations between non-normally distributed and/ordinal variables, the correlation coefficients and their significance were calculated using the "Spearman test". Statistically, $p < 0.05$ was considered significant.

Results

In study population consisted of 21 patients who underwent surgical intervention following a diagnosis of Bethesda categories 3 (AUS-FLUS) and 4 (SFN) on FNAB. The median (minimum-maximum) age of the study population was 15.4 (11-17.5) years. Females constituted 86% (n = 18) of the patient population, while males represented 14% (n = 3). A family history of thyroid carcinoma was noted in one. In addition, one patient was under surveillance for an ovarian neoplasm. Thyroid function tests revealed hypothyroidism in 5% (n = 1), subclinical hypothyroidism in 10% (n = 2), and euthyroid status in the majority, 85% (n = 18). Notably, 42% (n = 9) of the patients presented with multiple thyroid nodules. Within this subgroup, 88% (n = 8) exhibited dual nodular formations, whereas 12% (n = 1) had three nodules.

The postoperative pathological analysis reported that 43% (n = 9) were classified as benign, 19% (n = 4) as low-risk neoplasms, and 38% (n = 8) as malignant. Within the low-risk neoplasm category, NIFTP constituted 75% (n = 3) of the cases, while UMP was observed in 25% (n = 1). FNAB cytopathological results and the WHO classification of postoperative pathology are depicted in Figure 1.

On evaluating the postoperative outcomes of thyroid nodules in relation to their EU-TIRADS scores, it was observed that in benign cases, 44% were rated as EU-TIRADS 3 and 56% as EU-TIRADS 5. Low-risk neoplasm cases were equally divided, with 50% being classified as EU-TIRADS 3 and the remaining 50% as EU-TIRADS 5. Among malignant cases, 13% were assessed as EU-TIRADS 3, 25% as EU-TIRADS 4, and 64% as EU-TIRADS 5. The classification

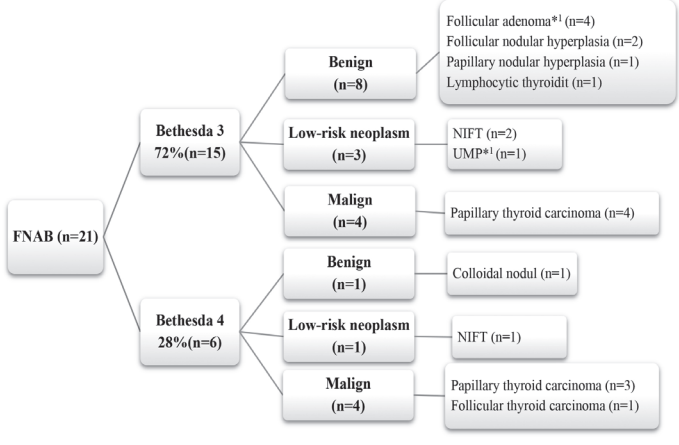

Figure 1. Postoperative pathology and FNAB results of the cases

*NIFT: non-invasive follicular thyroid neoplasm with papillary-like nuclear features, UMP: thyroid tumors of uncertain malignant potential*1 (1 = number of case), AUS: atypia of undetermined significance, FLUS: follicular lesion of undetermined significance, SFN: suspicious for follicular neoplasm, FNAB: fine-needle aspiration biopsy*

and distribution of EU-TIRADS scores with postoperative pathology outcomes are presented in Table 1.

Analysis of Bethesda classifications revealed that 72% (n = 15) of cases fell into category 3, while the remaining 28% (n = 6) were classified under category 4. In our study, the incidence of malignancy among Bethesda 3 cases was lower at 27% (n = 4), with 53% (n = 8) benign and 20% (n = 3) classified as low-risk neoplasms. In contrast, Bethesda 4 cases tended to exhibit a higher malignancy rate of 66% (n = 4), with benign (n = 1) and low-risk neoplasms (n = 1) each constituting 17% of the cases. The data revealed a higher prevalence of malignancy in FNAB for Bethesda category 4 at 66% compared to 27% in Bethesda category 3 (p = 0.20), although not sigificant, as detailed in Table 2. This may be due to low number of cases.

In the Bethesda 3 category (n = 15), TIRADS scoring classified 37% of benign cases as EU-TIRADS 3, and 63% as EU-TIRADS 5. Among the malignant cases (n = 4), 25% were scored as EU-TIRADS 3, 25% as EU-TIRADS 4, and 50% as EU-TIRADS 5. For the low-risk neoplasm group, 33% were assigned EU-TIRADS 3, and 67% EU-TIRADS 5 (Table 3). In the Bethesda 4 category (n = 6), radiological scoring identified EU-TIRADS 3 in one benign case. In addition, one case with a low-risk neoplasm was also scored as EU-TIRADS 3. Among the malignant cases within this group, 25% (n = 1) were classified as EU-TIRADS 4, while the majority, 75%, (n = 3) were classified as EU-TIRADS 5 (Table 4). Notably, there was a significant correlation identified in the Bethesda 4 group, indicating an increase in EU-TIRADS scoring as the postoperative pathology results worsened (r = 0.87, p = 0.02).

The postoperative pathological analysis demonstrated a median (range) nodule size of 12 (5-35) mm in the malignant group, 25 (7-48) mm in the benign group, and 23 (8-32) mm in the low-risk neoplasm group; these sizes did not differ between the classifications (p = 0.33), as illustrated in Figure 2. Lymph node metastasis was found in 24% of the cases overall, affecting 13% of cases in Bethesda category 3 and 60% in Bethesda category 4. There was no correlation between lymph node metastasis and TIRADS.

Table 1. EU-TIRADS scoring of post-op pathology results

	EU-TIRADS 3	EU-TIRADS 4	EU-TIRADS 5	Total
Benign	44% (n = 4)	0% (n = 0)	56% (n = 5)	100% (n = 9)
Low-risk neoplasm	50% (n = 2)	0% (n = 0)	50% (n = 2)	100% (n = 4)
Malign	13% (n = 1)	25% (n = 2)	64% (n = 5)	100% (n = 8)
Total	33% (n = 7)	10% (n = 2)	57% (n = 12)	100% (n = 21)

EU-TIRADS: The European-Thyroid Imaging Reporting and Data System

Table 2. Pathology results of the cases according to post-operative WHO classification

	Post-operative pathology (WHO classification)		
	Benign (n = 9)	Low-risk neoplasm (n = 4)	Malign (n = 8)
Bethesda 3 (n = 15)	53% (n = 8)	20% (n = 3)	27% (n = 4)
Bethesda 4 (n = 6)	17% (n = 1)	17% (n = 1)	66% (n = 4)
Total (n = 21)	43%	19%	38%

WHO: World Health Organization

Table 3. EU-TIRADS scoring of Bethesda 3 and post-op pathology results

Bethesda 3	EU-TIRADS 3	EU-TIRADS 4	EU-TIRADS 5	Total (n = 15)
Benign	37% (n = 3)	0% (n = 0)	63% (n = 5)	100% (n = 8)
Low-risk neoplasm	33% (n = 1)	0% (n = 0)	67% (n = 2)	100% (n = 3)
Malign	25% (n = 1)	25% (n = 1)	50% (n = 2)	100% (n = 4)

EU-TIRADS: The European-Thyroid Imaging Reporting and Data System

Table 4. EU-TIRADS scoring of Bethesda 4 and post-op pathology results

Bethesda 4	EU-TIRADS 3	EU-TIRADS 4	EU-TIRADS 5	Total (n = 6)
Benign	**100% (n = 1)**	0% (n = 0)	0% (n = 0)	100% (n = 1)
Low-risk neoplasm	**100% (n = 1)**	0% (n = 0)	0% (n = 0)	100% (n = 1)
Malign	**0% (n = 0)**	25% (n = 1)	75% (n = 3)	100% (n = 4)

EU-TIRADS: The European-Thyroid Imaging Reporting and Data System

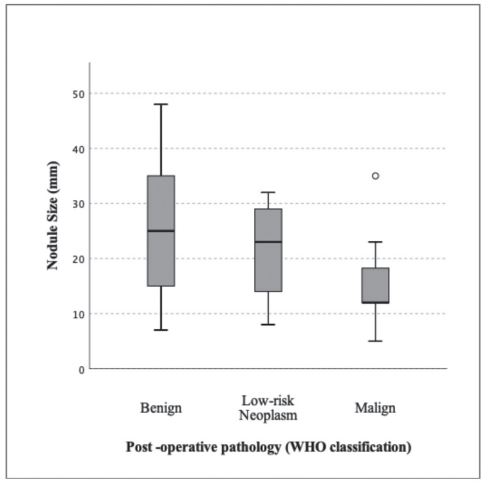

Figure 2. Nodule size (preoperative thyroid ultrasonography) compared to post-op pathology results

*p = 0.33

WHO: World Health Organization

Discussion

Thyroid FNA with US guidance is regarded as the most accurate test for diagnosing malignancy in thyroid nodules and reduces the need for surgery in benign nodules. It is important for clinicians to consider the individual patient's clinical and imaging features, as well as the specific histopathological diagnosis. The optimal management of thyroid nodules with a Bethesda 3 and 4 cytology in pediatric patients is debatable, due to the unpredictability but heightened cancer risk compared to adults. To the best of our knowledge, this study is the first to published study to evaluate postoperative histopathological results and assess the effectiveness of EU-TIRADS in managing pediatric thyroid nodules classified as Bethesda categories 3 and 4. The study evaluated twenty-one pediatric cases with Bethesda categories 3 and 4 and analyzed their EU-TIRADS scoring. The results suggest that EU-TIRADS cannot provide reliable guidance for clinical decisions in children with thyroid nodules. In addition, it was shown that the nodules postoperatively shown to be malignant may have smaller size, and thus, small nodules can also be malignant. In the Bethesda 4 category, there was a significant correlation between higher EU-TIRADS scores and the risk of malignancy development, in SFN cases.

In the context of paediatric EU-TIRADS use age, the evidence remains rather inconclusive. In the study by Yeste Fernández et al. (17), which analysed 31 cases with nodules, there were 5 cases with Bethesda 3 and 2 cases with Bethesda 4 and the results were collected in a very small cohort. Furthermore, a literature review, including the work of Tuli et al. (18) and Scappaticcio et al. (19), which used the SIAPC classification for FNAB pathologies, categorized cases into benign or malignant outcomes. Both studies demonstrated a suboptimal performance in the management of pediatric patients with thyroid nodules. The present study aimed to address the clinical challenges posed by Bethesda 3 and 4 thyroid nodules, with the goal of providing additional empirical evidence specifically targeting these ambiguous categories in the pediatric population.

In our cohort 53% of the nodules were classified as Bethesda 3 and were determined to have benign pathologies postoperatively. This finding is corroborated by the study of Canberk et al. (20) with a substantial cohort (n = 405), which identified 67% of AUS instances as benign. These concurrent findings raise questions about the ATA stance in favor of prompt surgical involvement for AUS/FLUS nodules (2). Since FNAB alone is insufficient for definitive surgical decision-making, supplemental radiological evidence is necessary for guidance.

In the present study, the postoperative results of AUS/FLUS cases showed 27% malignancy rate while SFN cases showed 66% malignancy rate. In 2019, Cherella et al. (4) reported malignancy rates of 44% and 71% for AUS and SFN nodules, respectively. Published pediatric studies show that the malignancy rates for Bethesda 3 nodules range from 8.3% to 44% (4,21,22,23) and for Bethesda 4 nodules range from 35% to 100% (23,24,25). These studies suggest that the rates of malignancy in both groups are different but the risk of malignancy in pediatric patients with Bethesda 4 nodules is relatively high compared to Bethesda 3.

Multiple US scoring systems are available to categorize nodules for fine-needle biopsy (FNB) indications, including fine-needle cytology (10,26). In the study by Borysewicz-Sańczyk et al. (27) conducted in Poland, a 29% malignancy rate was found among 17 pediatric cases with Bethesda categories 3-4-5-6. While all cases classified as Bethesda 5 and 6 were confirmed malignant postoperatively, two cases categorized as Bethesda 4 and labeled as high suspicion according to ATA classification were reported as benign histopathologically. In addition, none of the six Bethesda 3 cases, deemed low suspicion, were found to have malignancy. Conversely, the study of Richman et al. (28) evaluated the ACR TI-RADS against ATA guidelines in pediatric thyroid nodule management. Their findings indicated that while ACR TI-RADS may decrease the biopsy rate in benign nodules, it might also result in a significant number of pediatric cancers not being biopsied (22.1%), suggesting potential inadequacy of ACR TI-RADS in pediatric cases. The high frequency of benign cases classified as EU-TIRADS

5 in the present study may have led to unnecessary FNAB procedures. While most of the malignant cases in our cohort align with EU-TIRADS 4-5, 13% of cases were classified as low-risk, a lower proportion than the 22% reported by Richman et al. (28) using ACR-TIRADS. This suggests that while EU-TIRADS corresponds with the recognized literature in identifying higher-risk cases, there may be a discrepancy in the classification of lower-risk malignancies, indicating a potential area for review or adjustment in classification criteria. Moreover, in the study conducted by Creo et al. (29), malignant nodules were primarily identified within the ATA's high or intermediate suspicion groups. These authors concluded that pediatric radiologists' overall impressions were similarly sensitive but more specific than the ATA risk stratification. They also concluded that no US-based method perfectly separated benign from malignant nodules, affirming the ongoing necessity for FNAB in cases of suspicious nodules.

In the study of Yeste Fernández et al. (17), an evaluation of 31 pediatric thyroid nodules, Bethesda classification was applied, with categories ranging from 1 to 5, and 14 nodules underwent surgery, six of which were malignant. While 16% (n = 5) of the cases were Bethesda 3 without postoperative malignancy, 6.52% (n = 2) were Bethesda 4 with malignancy found. All malignant nodules were categorized as EU-TIRADS 4 or 5. The study highlighted the limitations of the case numbers but found EU-TIRADS classification had a sensitivity of 100%, specificity of 25%, PPV of 44%, and NPV of 100%, making it a reliable diagnostic tool for FNAB decision-making. In our analysis, which exclusively evaluated cases classified as Bethesda 3 and 4, we included a notably larger sample size compared to prior research. In our study, 25% of the 15 cases classified as Bethesda 3 were malignant and exhibited radiological assessments consistent with EU-TIRADS 3-4-5. In the Bethesda 4 group, which comprised six cases, the malignancy rate was 66%, with all cases radiologically assessed as EU-TIRADS 4-5. When evaluating both studies, it appears that EU-TIRADS scoring provides a more dependable guide for FNAB in cases classified as Bethesda 4. However, this level of reliability does not extend to the Bethesda 3 category, where EU-TIRADS scoring does not exhibit the same predictive strength for FNAB decision-making.

Our findings indicate that in Bethesda 3 cases, the presence of EU-TIRADS 5 scores is lower in malignant cases, whereas it is higher in the low-risk and benign groups. In Bethesda 4 cases, a positive correlation was observed between EU-TIRADS scores and the deterioration of postoperative pathology findings. This result emphasizes the intricate relationship between EU-TIRADS scoring, Bethesda categories, and definitive postoperative pathology diagnoses.

Our results suggest that the size of nodules in the malignant group tended to be smaller compared to the benign and low-risk neoplasm groups but this was not significiant. The range of sizes within each group was wide and overlapped. Nodules classified as TBSRTC category IV and V are recommended for surgical resection due to their high risk of malignancy. It has been suggested that for nodules with TI-RADS scores less than or equal to 3, US surveillance instead of FNA can be performed (30). One study retrospectively assessed the effectiveness of three US risk stratification systems (ACR-TIRADS, ATA, and EU-TIRADS) in pediatric patients with thyroid nodules and a history of radiation exposure. With 52 patients, 27% had papillary thyroid cancer (PTC) upon final histology. The systems showed high specificity (95-97%) and negative predictive value (88-93%), but they failed to recommend biopsies in a significant number of PTC cases, often due to nodules being smaller than 1 cm. This study suggested that while these systems are reliable, they could be improved by adjusting the size criteria for biopsy recommendations (31). It's important to note that nodule size alone is not a definitive indicator of malignancy, and other factors such as imaging characteristics and biopsy results must be considered.

Study Limitations

Limitations of this study include the small sample size, data collection from a single center, and the need for postoperative follow-up results. So, there is a need for more in-depth studies with larger sample sizes and results from long-term follow-ups.

Conclusion

The optimal management of AUS/FLUS and FN/SFN thyroid nodules in children is still an area of active research, and it should be individualized based on factors such as the patient's age, the size and characteristics of the nodule, and the results of diagnostic FNAB. The postoperative pathology assessment showed a discernible variability in EU-TIRADS scores. Specifically, within the Bethesda 3 category, instances of malignancy exhibited a comparatively diminished percentage of EU-TIRADS 5, in contrast to its more pronounced occurrence within the low-risk and benign cohorts. Conversely, among Bethesda 4 cases, there emerged a conspicuous ascending trajectory in EU-TIRADS scores concomitant with a worsening trend in postoperative pathology diagnoses. These findings accentuate the nuanced and debatable nature of the EU-TIRADS scoring system's utility in effectively guiding the intricate clinical

decision-making process concerning pediatric thyroid nodules. The EU-TIRADS scoring system has the potential to be a useful tool for evaluating thyroid nodules in children, but its accuracy and effectiveness still require confirmation through well-designed large studies.

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Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Ankara City Hospital (approval number: E2-23-3317, date: 01.02.2023). The study was performed in accordance with the Helsinki Declaration of 1975.

Informed Consent: Retrospective study.

Authorship Contributions

Surgical and Medical Practices: Aylin Kılınç Uğurlu, Abdurrahman Bitkay, Fatih Gürbüz, Esra Karakuş, Gülşah Bayram Ilıkan, Çağrı Damar, Seda Şahin, Merve Meryem Kıran, Nedim Gülaldı, Müjdem Nur Azılı, Emrah Şenel, İnci Ergürhan İlhan, Mehmet Boyraz, Concept: Aylin Kılınç Uğurlu, Esra Karakuş, Müjdem Nur Azılı, Design: Aylin Kılınç Uğurlu, Data Collection or Processing: Aylin Kılınç Uğurlu, Abdurrahman Bitkay, Esra Karakuş, Müjdem Nur Azılı, Analysis or Interpretation: Aylin Kılınç Uğurlu, Esra Karakuş, Literature Search: Aylin Kılınç Uğurlu, Writing: Aylin Kılınç Uğurlu.

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Microcephaly in Infants: A Retrospective Cohort Study from Turkey

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What is already known on this topic?

The definition of microcephaly (MC) varies in the literature. Zika virus is one well-known etiology of MC and the prevalence of this etiology appears to have increased in this era.

What this study adds?

Socio-economic factors, such as low parental age and parental education may be risk factors for MC. A head circumference of -2 standard deviation score and below should be considered as MC. Resolution of MC may occur regardless of the initial severity. Acieving some developmental milestones may be delayed in children with persistent MC.

Abstract

Objective: Microcephaly (MC) is a clinical finding mostly reflecting deficiency of brain growth. The aim of this retrospective cohort study was to assess risk factors and follow-up features of children with MC.

Methods: Children's personal health records (n = 7580) followed between 2002 and 2020 in the Unit of a Well Child Clinic were assessed retrospectively. The case group comprised children with MC. MC was defined as head circumference (HC) standard deviation score (SDS) value ≤-2 SDS. Age and sex-matched children with normal HC were selected as the control group.

Results: Children with MC (n = 49) had more disadvantaged sociodemographic characteristics, such as young maternal and paternal age and low maternal and paternal education. Breastfeeding was more common among controls (n = 98). Resolution of MC was observed in 26 (53.1 %) children with MC, whether it was mild (HC SDS between -2 and -2.9) or severe (HC SDS ≤3). Children with persistent MC had poorer developmental milestones than controls and cases with resolution. Sociodemographic features or developmental milestones in mild and severe MC did not differ.

Conclusion: These results suggest that the use of a definition of MC of ≤-2 SDS would be appropriate in order not to miss cases on follow-up. Greater sociodemographic equality may prevent some cases of MC. Further studies are needed evaluating socioeconomic factors on MC.

Keywords: Microcephaly, child, risk factor, follow-up, definition, epidemiology

Introduction

Microcephaly (MC) is a clinical finding, not a diagnosis. According to the age of onset, MC is classified as primary or secondary (1,2,3,4,5). As there is no agreement on the definition of MC, the frequency is unclear. There are some known acquired and genetic causes for primary and secondary MC, however thee are many cases with unidentified aetiology. In the literature, epidemiological studies of MC are scarce (6,7). Risk factors such as fetal growth retardation, maternal age, and maternal infections

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during pregnancy have been reported (1,6,7,8,9,10). The lack of a clear consensus on the definition, poor understanding of the etiology and unpredictability of the prognosis create clinical uncertainty in children with MC. The aim of the present study was to assess risk factors and follow-up features and outcomes in children with MC.

Methods

This was a retrospective cohort study, conducted in a University Hospital's Well Child Outpatient Clinic. In this Unit, all children are followed up with personal health records created at the time of the first attendance. Follow-up intervals start on the fifteenth day of life and then monthly for the first six months, then at 7-8 months, nine months, then every three months between the ninth and eighteenth months and every six months from the age of two years until six years of age. Subsequent annual follow-ups are planned until the age of 18 years. Medical history, including the prenatal, natal, and postnatal period, feeding types, especially breastfeeding history, family information, including maternal and paternal age and educational levels, and family medical history are obtained at the first visit. All children were measured after delivery. In the unit, anthropometric measurements with weight, height, and head circumference (HC), with HC being recorded until three years of age, were made at each visit by experienced health personnel and recorded on the growth chart. Detailed physical examination was performed, and age-appropriate vaccines according to the national expanded immunization program were administered. In addition, vaccines that are not included in the national program, such as a meningococcal vaccine, rotavirus vaccine, influenza vaccine, and human papilloma virus vaccine may be administered if the family provides them. Age-appropriate developmental status was evaluated. All of this was noted in personal health records. In the unit, a problem-based record system was used, and if any health problem was recorded in the personal record system, multidisciplinary management was started with the co-operation of relevant departments, if necessary. When a patient was noted to have MC, investigations performed included neurological abnormality scanning and screening for genetic, infectious, and metabolic disorders. Extremely preterm infants (less than 28 weeks of gestational age) were not admitted to the unit for well-child visits.

Definitions: MC was defined as HC standard deviation (SD) score (SDS) value ≤-2. Primary MC was defined as MC identified at birth, and secondary MC was identified in the follow-up period. HC SDS values between -2 SDS and -2.99 SDS were designated as mild MC, and ≤-3 SDS as severe MC. HC, weight, and height measurements were evaluated according to Turkish national growth curves (11,12). The online auxology application, created by the Child Endocrine and Diabetes Association of Turkey (official site accessed at: "https://www.ceddcozum.com/Home/Change?LanguageAbbreviation=tr)" was used for calculating SDS of anthropometric values. The resolution of MC was defined as occurring when a HC value increased to normal values. If this did not occur during follow-up, the patient was designated as having persistent MC.

Accompanying signs: Unusual morphological findings, such as hypertelorism, flattened nasal root, long/short philtrum (13), defined by the genetic department after consultation, were accepted as dysmorphic findings. If it was deemed necessary by a genetic specialist, detailed genetic tests were performed. Birth weight below the 10th percentile for gestational week was defined as small for gestational age (SGA) (14). Neurological problems were defined as having pathological findings in the magnetic resonance imaging of brain, the presence of neuromotor retardation, and/or epilepsy. Problems identified by fetal ultrasonography was defined as an identified abnormality during pregnancy. Congenital heart problems were diagnosed by a pediatric cardiologist with echocardiography.

Health records of the children followed up between January 2002 and June 2020 were evaluated. The case group constituted children with MC. For each case, two age and gender-matched controls were selected. The control group was created by choosing gender-matched children with the closest birth date to the case. Sociodemographic characteristics, natal and antenatal history, breastfeeding status, developmental milestones (head holding, sitting without support, independent walking), anthropometric measurements and accompanying signs were extracted retrospectively from the personal health records.

Ethical approval: The study was approved by the İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (number: 2019/738, date: 25.05.2019).

Statistical Analysis

Statistical Package for the Social Sciences, version 17.0 was used for statistical analysis (IBM Inc., Armonk, NY, USA). Descriptive statistics are shown as mean and SD, median and minimum and maximum values. Pearson chi-square and Fisher's exact test were used to compare categorical variables. The Kolmogorov-Smirnov test and histogram graphic were used to examine the compliance of variables with normal distribution. Independent t-test was used for parametric variables, and the Mann-Whitney U test for non-parametric variables. Models were developed using the multivariate binary logistic regression with forward stepwise

selection for analysis of binary dependent variables and independent variables.

Results

Children born at ≥32 gestational weeks and followed up between 2002 and 2020 regularly constituted the global population (n = 7580). The recruitment of children is shown in Figure 1. Of the 7580, 50 (0.66%) had MC. One case with missing data was excluded from the study. Twenty-nine of the remaining 49 (59.2%) were girls. Twice as many children as the case group formed the control group (n = 98). All children with MC were followed up to at least the age of three years.

Clinical and Sociodemographic Characteristics of Children

Sociodemographic characteristics, clinical features, and comparisons between case and control groups are given in Table 1. Children with MC had more disadvantaged sociodemographic characteristics, such as significantly younger parental age and significantly poorer parental education. The rate of breastfeeding was significantly higher among controls. Nine (18.4%) cases of MC had neurological problems, and seventeen (34.7%) had congenital heart problems (Table 1). Of the congenital heart problems, 10.2% were critical heart defects. Only three (3.1%) of the controls had congenital heart problems, these were a ventricular septal defect, bicuspid aorta and secondary atrial septal defect. In the MC group, there was one diagnosis of Di George syndrome and one of Williams syndrome. There was a child with Down syndrome in the control group.

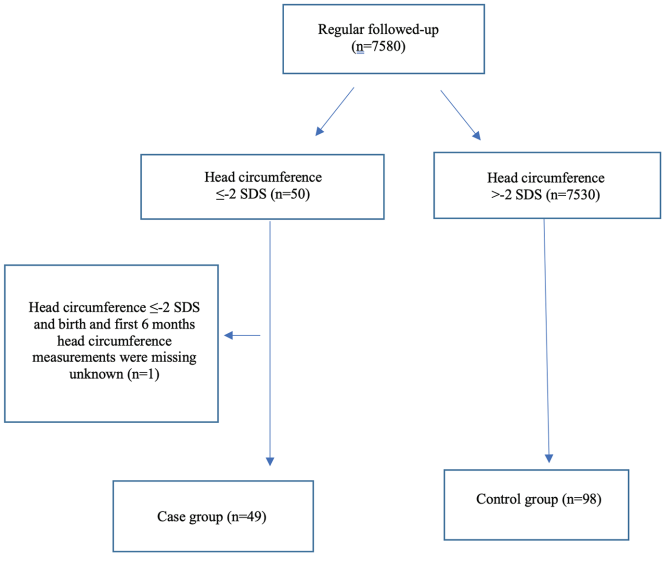

Figure 1. Diagram describing study sample selection

SDS: standard deviation score

No Zika virus (ZV) infection history was noted among the case group. In the mother of one MC case, cytomegalovirus (CMV) infection was diagnosed. None of the mothers of controls had CMV.

Findings of Primary and Secondary Microcephaly

Thirty (61.2%) of the cases were primary MC cases, and 19 (38.8%) were secondary MC. The median (range) age of detection was two (1.0-12.0) months amongst the cases of secondary MC. Of the secondary MC cases, nine were detected in the first month, seven in the second month, one in the third month, and two in the twelfth month of age. No significant differences were found between children with primary and secondary MC in terms of sociodemographic characteristics, clinical features, accompanying signs except for the presence of SGA, or developmental milestones (Supplementary Table 1).

Severity of Microcephaly

In the MC group, forty were classified as mild (81.6%) and nine as severe (18.4%) MC. Of the mild cases, 24 (6%) were primary, and 16 were secondary MC. No significant differences were found between mild and severe cases in terms of sociodemographic characteristics, clinical features, accompanying signs, and developmental milestones (Supplementary Table 2).

Resolution of Microcephaly

The resolution of MC was found in 26 (53%) cases during follow-up. Of these, four were severe, and 22 were mild MC. The distribution of resolution age according to the severity of MC is shown in Figure 2. The median age for resolution was two months. Of these 26 children, 15 had HC in the normal range by two months. No significant difference was found in the median resolution age between children with severe and mild MC (p = 0.72). There were

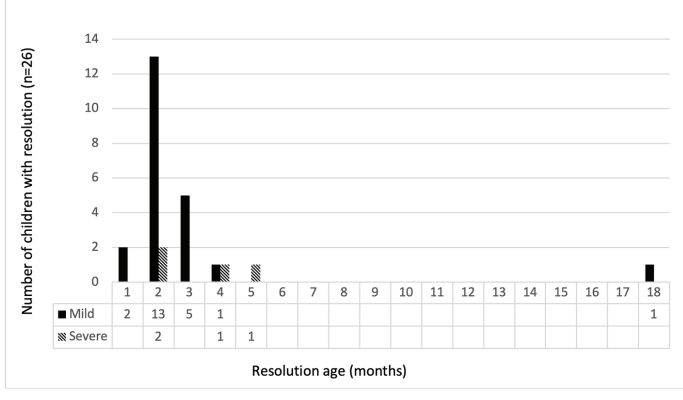

Figure 2. Age distribution of children with resolution according to the onset of age and severity

Table 1. Comparison of sociodemographic and clinical features of children with MC and controls

	Children with MC (n = 49)	Control (n = 98)	p value
Maternal age (years)			
Median (min-max)	28.00 (20.00-46.00)	32.00 (18.00-44.00)	**0.025***
Mean (± SD)	29.57(± 5.81)	31.42 (± 5.26)	
Paternal age (years)			
Median (min-max)	32.00 (24.00-49.00)	34.00 (21.00-52.00)	**0.036***
Mean (± SD)	33.12(± 5.77)	34.52 (± 5.76)	
Maternal education			
≤5 years	12 (24.5%)	14 (14.3%)	**0.013****
6-11 years	27 (55.1%)	40 (40.8%)	
≥12 years	10 (20.41%)	44 (44.9%)	
Paternal education			
≤5 years	14 (28.6%)	11 (11.2%)	**0.008****
6-11 years	22 (44.9%)	40 (40.8%)	
≥12 years	13 (26.5%)	47 (48.0%)	
Birth weight (g)			
Median	2500 (1320-3820)	3265 (1700-4230)	**< 0.001*****
Mean	2504.29 (± 466.22)	3209.41 (± 502.13)	
Birth lenght (cm)			
Median	45.50 (38.00-54.00)	49.00 (42.00-55.00)	**< 0.001***
Mean (± SD)	45.65 (± 2.95)	49.07 (± 2.28)	
Gestational age (week)			
Median (min-max)	38.00 (36.00-41.00)	38.00 (34.00-41.00)	**0.006***
Mean (± SD)	37.84 (± 0.92)	38.39 (± 1.53)	
Presence of SGA [n (%)]			
Yes	36 (73.5%)	11 (11.2%)	**< 0.001****
No	13 (26.5%)	87 (88.8%)	
Fetal ultrasonography [n (%)]			
Normal	38 (77.6%)	92 (93.9%)	**0.008****
Abnormal	11 (22.4%)	6 (6.1%)	
Pre-eclampsia [n (%)]			
Yes	7 (14.29%)	3 (3.1%)	**0.016******
No	42 (85.71%)	95 (96.9%)	
Consanguineous marriage			
Yes	6 (12.2%)	5 (5.1%)	**0.181******
No	43 (87.8%)	93 (94.9%)	
Associated anomalies [n (%)]			
Presence of neurological problems			
Yes	9 (18.4%)	2 (2.0%)	**0.001******
No	40 (81.6%)	96 (98.0%)	
Congenital heart problems			
Yes	17 (34.7%)	3 (3.1%)	**< 0.001******
No	32 (65.3%)	95 (96.9%)	
Dysmorphic findings			
Yes	14 (28.6%)	2 (2.0%)	**< 0.001******
No	35 (71.4%)	96 (98.0%)	
Duration of exclusively breastfeeding			
Median (min-max)	4.00 (0.00-6.00)	5.00 (0.00-6.00)	**0.001***
Mean (± SD)	2.88 (± 2.36)	4.29 (± 2.01)	
Duration of breastfeeding (at least-month)			
Median (min-max)	9.00 (0.00-24.00)	12.00 (1.00-30.00)	**0.002***
Mean (± SD)	9.70 (± 6.46)	13.34 (± 6.66)	
Developmental milestones (months)			
Head holding (n)	44	95	
Median (min-max)	2 (1-12)	1.5 (1-4)	**0.215***
Mean (± SD)	2.32 (± 2.02)	1.74 ± 0.84	
Sitting without support (n)	36	92	
Median (min-max)	7.00 (5.00-12.00)	6.00 (5.00-9.00)	**< 0.001***
Mean (± SD)	7.30 (± 1.48)	6.47 (± 0.82)	
Independent walking (n)	31	89	
Median (min-max)	12 (9-36)	12 (8-18)	**0.134***
Mean (± SD)	14.81 (± 5.87)	12.30 ± 1.52	

*Mann-Whitney U test, **Pearson chi-square test, ***Independent t-test, ****Fisher's exact test.
SGA: small for gestational age, min-max: minimum-maximum, SD: standard deviation, MC: microcephaly

23 (47%) children with persistent MC at the age of three years. Cases with persistent MC were compared to their matched controls (n = 46) and findings are summarized in Table 2. Distributions of socioeconomic disadvantages, such as poorer parental education, and consanguinity were significantly more likely among children with persistent MC. Achievement ages for developmental milestones were late for children with persistent MC. Comparison of the cases with and without resolution is given in Table 3. This showed that there were significant differences in neurodevelopmental ages and parental education between cases with and without resolution.

In the multivariate binary logistic regression model, SGA as independent variable was found to be significant for case-control classification ($p < 0.001$) and the odds ratio (OR) was 26.73. Maternal and paternal age and education, duration of exclusive breastfeeding, fetal ultrasonography, pre-eclampsia, and gestational age were not significant ($p = 0.400$, 0.287, 0.587, 0.871, 0.092, 0.092, 0.824, 0.447, respectively). The model explained 50% of variation

Table 2. Comparison of sociodemographic and clinical features of children with persisent MC and their controls

	Children with persistent MC (n = 23)	Matched controls of the children with persistent MC (n = 46)	p value
Maternal age (years)			
Median (min-max)	29.00 (20.00-40.00)	30.50 (19.00-44.00)	0.561*
Mean (± SD)	29.61 (± 5.10)	30.46 (± 5.49)	
Paternal age (years)			
Median (min-max)	32.00 (26.00-48.00)	34.00 (23.00-51.00)	0.444*
Mean (± SD)	33.22 (± 4.86)	33.80 (± 5.45)	
Maternal education			
≤5 years	10 (43.5%)	8 (17.4%)	**0.003****
6-11 years	12 (52.2%)	19 (41.3%)	
≥12 years	1 (4.3%)	19 (41.3%)	
Paternal education			
≤5 years	10 (43.5%)	7 (15.2%)	**0.018****
6-11 years	10 (43.5%)	22 (47.8%)	
≥12 years	3 (13.0%)	17 (37.0%)	
Birth weight (g)			
Median	2480 (1420-3820)	3075 (1840-4100)	**< 0.001***
Mean	2526.09 (± 544.10)	3136.85 (± 458.28)	
Birth lenght (cm)			
Median	45.00 (40.00-54.00)	49.00 (43.00-55.00)	**< 0.001***
Mean (± SD)	45.78 (± 3.29)	48.80 (± 2.05)	
Gestational age (week)			
Median (min-max)	38.00 (36.00-41.00)	38.00 (34.00-41.00)	0.380*
Mean (± SD)	37.91 (± 1.16)	38.15 (± 1.59)	
Presence of SGA [n (%)]			
Yes	16 (69.60%)	5 (10.90%)	**< 0.001****
No	7 (30.4%)	41 (89.1%)	
Fetal ultrasonography [n (%)]			
Normal	16 (69.6%)	43 (93.5%)	**0.013*****
Abnormal	7 (30.4%)	3 (6.5%)	
Pre-eclampsia [n (%)]			
Yes	2 (8.7%)	1 (2.2%)	0.256***
No	21 (91.3%)	45 (97.8%)	
Consanguineous marriage			
Yes	5 (21.7%)	1 (2.2%)	**0.014*****
No	18 (78.3%)	45 (97.8%)	
Associated anomalies [n (%)]			
Presence of neurological problems			
Yes	8 (34.8%)	2 (4.3%)	**0.002*****
No	15 (65.2%)	44 (95.7%)	
Congenital heart problems			
Yes	14 (60.9%)	1 (2.2%)	**< 0.001****
No	9 (39.1%)	45 (97.8%)	
Dysmorphic findings			
Yes	13 (56.5%)	2 (4.3%)	**< 0.001****
No	10 (43.5%)	44 (95.7%)	

Table 2. Continued

	Children with persistent MC (n = 23)	Matched controls of the children with persistent MC (n = 46)	p value
Duration of exclusively breastfeeding			
Median (min-max)	3.00 (0.00-5.00)	5.00 (0.00-6.00)	**0.001***
Mean (± SD)	2.41 (± 2.29)	4.51 (± 1.90)	
Duration of breastfeeding (month)			
Median (min-max)	10.50 (0.00-18.00)	12.00 (1.00-24.00)	**0.013***
Mean (± SD)	9.22 (± 5.63)	13.60 (± 6.38)	
Developmental milestones (months)			
Head holding (n)	21	43	
Median (min-max)	2.5 (1-12)	2 (1-4)	0.111*
Mean (± SD)	3.12 ± 2.62	2 ± 0.91	
Sitting without support (n)	17	41	
Median (min-max)	8.00 (6.00-12.00)	6.00 (6.00-9.00)	**< 0.001***
Mean (± SD)	7.97 (± 1.72)	6.53 (± 0.80)	
Independent walking (n)	12	40	
Median (min-max)	16.50 (11-36)	12.00 (11-15)	**0.002***
Mean (± SD)	18.58 (± 7.75)	12.40 (± 1.03)	

*Mann-Whitney U test, **Pearson chi-square test, ***Independent t-test, ****Fisher's exact test.
SGA: small for gestational age, min-max: minimum-maximum, SD: standard deviation, MC: microcephaly

according to the Nagelkerke R square and classified 85.1% correctly. Birth weight and being SGA are related in each other, and developmental skills are not risk factors but they are outcomes because of that birth weight, birth length, associated anomalies, and developmental milestone parameters were not included in the model as it was assumed these were not risk factors. In the model for children with resolution of MC, maternal education was found to be significant (p = 0.012); OR was 6.25 for 6-11 years of education and 45 for ≥12 years of education (reference category was ≤5 years of education). Paternal education was not significant (p = 0.630). Nagelkerke R square for this model was 32% with 69.4% classified correctly.

Discussion

The follow-up features and risk factors of children with MC were evaluated in this retrospective cohort study. To the best of our knowledge, this is the first cohort study of children with MC. The results show that resolution may occur in children with MC, regardless of the severity of MC. Furthermore, children with persistent MC had poorer developmental milestones than controls and when compared with children in whom MC resolved. We suggest that poorer socioeconomic status may be a risk factor for MC, and the definition of MC should be re-evaluated.

There are disparities in the definitions of MC. Some have defined MC as HC values of ≤-2 SDS while others use ≤-3 SDS (4,5,15,16). In a study evaluating the prevalence of MC in

Table 3. Comparison of sociodemographic and clinical features of children with MC with resolution and with persistent

	Children with resolution (n = 26)	Children with persistent MC (n = 23)	p value
Maternal age (years)			
Median (min-max)	28.00 (22-46)	29.00 (20-40)	0.588*
Mean (± SD)	29.54 (± 6.48)	29.61 (± 5.10)	
Paternal age (years)			
Median (min-max)	31.00 (24-49)	32.00 (26-48)	0.527*
Mean (± SD)	33.04 (± 6.58)	33.22 (± 4.86)	
Maternal education			
≤5 years	2 (7.7%)	10 (43.5%)	**0.003****
6-11 years	15 (57.7%)	12 (52.2%)	
≥12 years	9 (34.6%)	1 (4.3%)	
Paternal education			
≤5 years	4 (15.4%)	10 (43.5%)	**0.042****
6-11 years	12 (46.1%)	10 (43.5%)	
≥12 years	10 (38.5%)	3 (13.0%)	
Birth weight (g)			
Median	2520 (1320-3120)	2480 (1420-3820)	0.873*
Mean	2485.00 (± 394.95)	2526.09 (± 544.107)	
Birth lenght (cm)			
Median	46.00 (38-51)	45.00 (40-54)	0.724*

Table 3. Continued

	Children with resolution (n = 26)	Children with persistent MC (n = 23)	p value
Mean (± SD)	45.53 (± 2.66)	45.7 (± 3.29)	
Gestational age (week)			
Median (min-max)	38.00 (37-39)	38.00 (36-41)	0.770*
Mean (± SD)	37.77 (± 0.652)	37.91 (± 1.16)	
Presence of SGA [n (%)]			
Yes	20 (76.9%)	16 (69.6%)	0.796**
No	6 (12.2%)	7 (30.4%)	
Fetal ultrasonography [n (%)]			
Normal	22 (84.6 %)	16 (69.6%)	0.359**
Abnormal	4 (15.4%)	7 (30.4%)	
Pre-eclampsia [n (%)]			
Yes	5 (19.2%)	2 (8.7%)	0.424***
No	21 (80.8%)	21 (91.3%)	
Consanguineous marriage			
Yes	1 (3.8%)	5 (21.7%)	0.086***
No	25 (96.2%)	18 (78.3%)	
Associated anomalies [n (%)]			
Presence of neurological problems			
Yes	1 (3.8%)	8 (34.8%)	**0.008*** **
No	25 (96.2%)	15 (65.2%)	
Congenital heart problems			
Yes	3 (11.5%)	14 (60.9%)	**< 0.001****
No	23 (88.5%)	9 (39.1%)	
Dysmorphic findings			
Yes	1 (3.8%)	13 (56.5%)	**< 0.001****
No	25 (96.2%)	10 (43.5%)	
Duration of exclusively breastfeeding			
Median (min-max)	4.00 (0.00-6.00)	3.00 (0.00-5.00)	0.257*
Mean (± SD)	3.19 (± 2.40)	2.41 (± 2.29)	
Duration of breastfeeding (month)			0.835*
Median (min-max)	9.00 (2-24)	10.5 (0-18)	
Mean (± SD)	10.11 (± 7.16)	9.22 (± 5.63)	
Developmental milestones (months)			
Head holding (n)	23	21	
Median (min-max)	1.00 (1-3)	2.50 (1-12)	**0.011***
Mean (± SD)	1.58 (± 0.75)	3.11 (± 2.62)	
Sitting without support (n)	19	17	
Median (min-max)	7.0 (5-8)	8.0 (6-12)	**0.025***
Mean (± SD)	6.7 (± 0.92)	7.97 (± 1.72)	
Independent walking (n)	19	12	
Median (min-max)	12.0 (9-18)	16.5 (11-36)	**0.007***
Mean (± SD)	12.42 (± 2.27)	18.58 (± 7.75)	

*Mann-Whitney U test, **Pearson chi-square test, ***Independent t-test. SGA: small for gestational age, min-max: minimum-maximum, SD: standard deviation, MC: microcephaly

Europe, it was suggested that this use of varying definitions may have been a reason for finding fewer MC cases than expected (17). In the present study, there were no statistical differences regarding sociodemographic characteristics, clinical features, accompanying signs, and developmental milestones between mild and severe MC cases. On the other hand, there were differences between MC and control groups for these variables (Table 1). The severity of MC did not affect the likelihood of resolution. Therefore, based on these it would be prudent to use ≤-2 SDS value for HC as the cut-off value to define MC, so as not to miss children with MC.

Education status is generally used as an indicator of socioeconomic status (18,19). Young maternal and paternal age and low maternal and paternal education may be indicators of low socioeconomic levels that perhaps lead to poor nutrition (20). Malnutrition in pregnancy and poor maternal nutrition has been associated with adverse birth outcomes, such as intrauterine growth retardation (IUGR) (21). Intersetingly, the presence of SGA was the only significant risk factor for MC in the present study. Melo et al. (22) reported that low socioeconomic status may lead to malnutrition, affecting host immunity, and could potentially be a contributing factor to MC when combined with other causes, such as poor environment. Nevertheless, a high proportion of MC has been reported to be idiopathic (23). In the present study, in the MC group, parents tended to be young and poorly educated compared with parents of controls. Moreover, higher maternal education levels were found to be significantly associated with the resolution of MC. This is in keeping with the findings of Nunez et al. (16), who reported the majority of the infants with MC were from disadvantaged parents.

In the present study, the rate of exclusive breastfeeding during the first six months and total breastfeeding duration was shorter in the MC group. As there are growth hormones in breast milk that support brain development, this

finding suggested that breastfeeding should be especially encouraged in infants with MC and that the mother should be supported (24,25,26). We could not find any study on breastfeeding and MC in children. As poor diet should be included in the risk factors for MC with unknown etiology, there is also a need for more detailed studies which evaluate socioeconomic features, such as housing structures, occupation, income, and diet quality.

To the best of our knowledge, there is only one published study on MC resolution (27). In this study examining MC cases in the ZV epidemic, the authors reported that resolution was observed at the age of two months in children with MC. The authors also suggested that molding could mimic primary MC. Molding is defined as overlapping the skull bones due to the pressure applied to the head in the birth canal of a baby who is born in a head-first position (28). Therefore, when assessing HC, serial measurements are important. The World Health Organization suggested that "the most reliable way to assess whether a baby has MC is to measure HC at 24 hours after birth" (29). However, it is not exactly known when molding improves. In the present study, the resolution of MC was observed as late as 18 months of age in one child (Figure 2). The rate of resolution was similar in the severe and mild MC groups. It may be suggested that MC with resolution cannot be true MC. The MC cases with resolution may be due to the transient effects of some infections, and/or poor nutrition during pregnancy. In order to be able to confirm resolution, it is very important to follow HC over the long term, as resolution can be at older ages. Of the MC cases, 46.9% had persistent MC. The milestones of head-holding age, sitting without support age, and independent walking age were delayed in children with persistent MC (Table 3).

There has been an association between critical congenital heart disease and MC in previous studies (1,16,30). Our findings are similar to these earlier studies (Table 1). This highlights the importance of conducting a detailed evaluation to identify other systemic problems in children with MC.

Study Limitations

There are some limitations in our study. The sample was based on data from a single centre, and it was not a prevalence study. Furthermore, there was no available data about teratogen exposure, such as tobacco consumption. No assessment of thyroid function was undertaken in the children with MC. However, being the first retrospective cohort study based on children with MC, follow-up results regarding the prognosis in MC may be considered a strength of the present study.

Conclusion

Socio-economic problems, manifesting as maternal malnutrition, may be a risk factor for MC. Accompanying anomalies and developmental delays should not be missed during the follow-up of children with MC. Considering that there was no difference between accompanying disease, sociodemographic data, and development milestones in children with severe and mild MC in the present study, a cut-off value of ≤-2 SDS for MC definition should be used, in order not to miss children with MC. Further epidemiologic studies are needed on the risk factors associated with MC to develop interventions for prevention.

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Ethics

Ethics Committee Approval: The study was approved by the İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (number: 2019/738, date: 25.05.2019).

Informed Consent: Retrospective cohort study.

Authorship Contributions

Concept: Gonca Keskindemirci, Gülbin Gökçay, Design: Gonca Keskindemirci, Gülbin Gökçay, Data Collection or Processing: Gonca Keskindemirci, Öykü Özbörü Aşkan, Burak Selver, Alev Bakır Kayı, Gülbin Gökçay, Analysis or Interpretation: Gonca Keskindemirci, Öykü Özbörü Aşkan, Alev Bakır Kayı, Gülbin Gökçay, Literature Search: Gonca Keskindemirci, Writing: Gonca Keskindemirci, Gülbin Gökçay.

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ORIGINAL ARTICLE

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Assessment of Executive Function Skills in Children with Isolated Growth Hormone Deficiency: A Cross-sectional Study

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What is already known on this topic?

Growth hormone deficiency (GHD) may be accompanied by sleep problems, mood and behavior problems, impairment of cognitive functions, such as attention and memory, and developmental delay in children. It has been suggested that GH may affect individuals' psychological well-being, memory, and cognitive functions by affecting different areas in the central nervous system through specific receptors. In addition, studies have reported that both GH and insulin-like growth factor-1 receptors, through which GH exerts its effects, are important for cognitive functions such as attention and memory. However, studies on executive function (EF), an important element of cognitive function, in children with GHD are limited.

What this study adds?

EF skills in children with isolated GH deficiency (IGHD) were evaluated. EF skills were significantly compromised in the children with IGHD compared to unaffected peers. As EF may influence academic success, we suggest that children with IGHD may benefit from psychiatric evaluation before and during treatment with GH, which may help to ameliorate the effect on school performance and possibly social development.

Abstract

Objective: The aim of this study was to evaluate executive function (EF), such as inhibition and working memory, in children with isolated growth hormone deficiency (IGHD) using performance-based tests and parent-report scales.

Methods: A total of seventy children between the ages of 7 and 12 years were included in the study. Half (n = 35) had children with IGHD and half were healthy controls. To evaluate the EF performances of the participants, the Visual Aural Digit Span Test-B Form (VADS-B) and Stroop task were applied. EF was also evaluated using the Behavior Rating Inventory of Executive Function (BRIEF).

Results: Children with IGHD scored lower on the VADS-B form for short-term memory ($p < 0.05$) compared to healthy controls. In addition, the completion time for the Stroop-color/word test was significantly longer in children with IGHD ($p < 0.05$). For children with IGHD, their parents reported higher scores on all sub-scales of the BRIEF scale, with statistically significant differences for all sub-scales with the exception of "organization of materials" ($p < 0.05$).

Conclusion: In this study, children with IGHD had poorer EF skills compared to unaffected peers. EF skills may influence academic success by affecting children's language skills, mathematical comprehension, cognitive flexibility, and hypothetical thinking. We believe that psychiatric evaluation of children with IGHD before and during treatment may positively contribute to both their academic performance and social relationships.

Keywords: Executive function, isolated growth hormone deficiency

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Introduction

Executive function (EF) is defined as high-level cognition associated with regulating and controlling cognitive processes. These functions include various cognitive skills that are needed for planning and maintenance of thoughts and behavior in line with a goal, such as orienting attention, inhibition of stimuli irrelevant to the goal, holding processed information in an active state, and switching between information (1). Working memory (WM), inhibition, and cognitive flexibility are among the core components of EF (2).

Growth hormone (GH) deficiency (GHD) is a significant cause of short stature with a prevalence of 1 in 4,000-10,000 births (3). GHD can be isolated or occur with other anterior pituitary hormone deficiencies, and may be congenital or acquired. Clinical findings depend on the specific etiology (3). Congenital causes can be generally classified as isolated GHD (IGHD), anatomical disorders, and genetic pathologies. Acquired causes include trauma, central nervous system tumors, radiation, and infiltrative diseases. However, a large majority of GHD cases are idiopathic (3,4).

GHD may be accompanied by sleep problems, mood and behavioral problems, impairment in cognitive functions, such as attention and memory, as well as failure to thrive (5). It has been suggested that GH can affect different areas in the central nervous system via specific receptors, impacting the psychological well-being, memory, and cognitive functions of individuals (5). Various studies performed in children with IGHD and children born small for gestational age (SGA) have found low GH levels to be associated with poorer memory and cognitive functions (6). A three-year observational study on children with SGA reported that GH replacement resulted in the development of a normal head diameter, as well as an improvement in intelligence and psychosocial functioning (7,8). Studies conducted on adults with GHD showed that GH treatment affected the neuronal signaling pathways associated with attention and memory in the long term (5,9). Furthermore, in a follow-up study performed on children with GHD, an increase in intelligence quotient (IQ) values associated with fluid intelligence was observed after GH replacement (6). GH was reported to affect the secretion of dopamine (DA) and noradrenaline in experimental rodent model studies (10). GH is also found in the cerebrospinal fluid (CSF) as it penetrates the blood-brain and blood-CSF barrier, and is predicted to affect cognitive processes by mechanisms, such as long-term potentiation via its receptors in different areas of the brain [prefrontal cortex (PFC), amygdala, and hippocampus] (11,12). As a result, it is been demonstrated that both GH and the insulin-like growth factor-1 (IGF-1) receptors, through which GH exerts its effects, are important for cognitive function, such as attention and memory (5).

Upon review of the literature, no studies evaluating EF skills in children with IGHD were found, although there are studies evaluating cognitive skills (5,6,7,8,9,10). Therefore, the aim of this study was to evaluate EF skills in children with IGHD in terms of WM, selective attention, and inhibition skills using performance-based tests and compare the findings with unaffected healthy children. In addition, the parents of the children with IGHD were asked to complete parent report scales related to EF in their children.

Methods

Study Sample

The case group of included 35 children, aged between 7 and 12 years, who attended a tertiary care (University of Health Sciences Turkey, Erzurum City Hospital) pediatric endocrinology outpatient clinic and had been diagnosed with IGHD based on neuroimaging, endocrinological and laboratory evaluation. The control group included 35 healthy children, also aged between 7 and 12 years, who had normal height and weight development, did not have any neurological, psychiatric, genetic, metabolic, or endocrine diseases, and had age-appropriate academic performance as reported by their teachers. This study was a cross-sectional study that was approved by the Erzurum Regional Training and Research Hospital, Local Ethics Committee (decision no: 2022/04-28, date: 11.04.2022) and was performed according to the tenets of the Helsinki Declaration.

Procedure

All participants' standing height was measured in quadruplicate using a wall-mounted Harpenden stadiometer accurate to the nearest 1 mm. In addition, a GH stimulation test was performed with L-dopa and clonidine, and IGF-1, and IGF-binding protein-3 levels were measured. The clinical diagnosis of IGHD was defined by height less than the third percentile and peak GH response <10 ng/mL after one of two GH stimulation tests using L-dopa, and clonidine. All participants diagnosed with IGHD underwent a Diagnostic and Statistical Manual of Mental Disorders-5 based clinical interview to determine psychiatric diagnoses and comorbid conditions before GH replacement therapy (13,14). Based on this psychiatric evaluation, the children had normal language and communication skills. Furthermore, following psychometric testing, those with an IQ level of 80 and above were included in the study. Parents of the participants were asked to complete the Sociodemographic Data Form and

the Behavior Rating Inventory of EF (BRIEF)-Parent Form. All children participating in the study underwent the Visual Aural Digit Span-Form-B (VADS-B) and the Stroop test in order. The tests were administered in a quiet and well-lit room without any interaction. The researcher and the child sat on two chairs facing each other during the application of both tests. Time was recorded using a chronometer. The participants had normal or corrected vision and normal hearing. VADS-B was administered starting from the third item of each sub-test. If a successful response was not obtained on the first try, a second sequence of the same length was presented. In case of a correct response, the next sequence was presented. If both trials in the sub-test were answered incorrectly, the sub-test was stopped. Numbers were spoken at 1-second intervals in the auditory sub-test. In the visual sub-test, a booklet that had one number per page was shown to children at 1-second intervals. For sub-tests that needed to be completed by writing, paper and pencil were used and administration time varied between 15-20 minutes. A separate card was used for each of the five parts of the Stroop test. In the first part, individuals were asked to read the names of colors that were written with black ink on a white background. In the second part, individuals were asked to read aloud the names of colors written in a different color on a white background. In the third part, individuals were asked to name the colors of circles printed in different colors. In the fourth part, individuals were asked to name the colors of neutral words written in different colors. In the fifth part, the card used in the second part was re-used, but the individuals were asked to name the colors of the words. The completion time for the relevant task was recorded with a timer for each of the five parts.

Data Collection Tools

Socio-demographic Data Form: The form prepared by the authors was designed and administered to collect information regarding the participants and their family members [age, gender, delivery time, delivery type, developmental steps (walking, talking, toilet training), and settlement]. Socioeconomic status was measured with the Hollingshead-Redich scale (15).

Behavior Rating Inventory of Executive Function-Parent Form: BRIEF was developed by Gioia et al. (16) to assess EF, problem-solving skills, and adaptive behavior in children. BRIEF was standardized and validated in Turkish by Batan et al. (17). Scores on eight different subscales of EF and 2 comprehensive indices [Behavioral Regulation Index (BRI) and Meta-Cognition Index (MCI)] can be calculated from this scale of 86 items. The obtained high scores indicate high executive disorder (16,17). Description of BRIEF subscales is as follows (16,17):

- **Inhibition:** Impulse control and ability to stop one's own behavior at the appropriate time

- **Shifting:** Ability to flexibly transition from one situation, activity, mindset or aspect of a problem to another.

- **Emotional Control:** Ability to modulate emotional response.

- **Initiate:** Ability to start a task or activity and generate ideas independently.

- **WM:** Ability to retain information in mind in order to complete a task or make a response.

- **Plan/Organize:** The ability to predict future events, set goals, develop appropriate steps in advance, perform tasks in a systematic way, understand and communicate main ideas.

- **Organizations of Materials:** Ability to manage current and future-oriented task demands within the situational context.

- **Monitoring:** Ability to control work, evaluate performance, and monitor one's own and others' efforts.

- **Behavioral Regulation Index:** It is the total score of inhibition, shifting, emotional control subscale scores.

- **Meta-Cognition Index:** It is the total score of initiate, WM, plan/organize, organizations of materials and monitoring subscale scores.

- **Global Executive Composite Index:** It is calculated by the sum of the BRI and MCI.

Visual Aural Digit Span-Form-B: VADS-B is a neuropsychological test developed by Koppitz (18) that assesses WM and short-term memory (19). Its validity in Turkish was established by Karakaş and Yalın (20) in 1993, and revised in 2002 (21). VADS-B consists of number sequences of two to nine digits. The items are presented orally and visually, and the children are asked to repeat the sequence in forward order, orally and in writing. The scoring considers the number of digits in the number with the most digits that could be accurately repeated. Higher scores indicate better performance. The test is composed of four main sub-tests that are auditory-oral, visual-oral, auditory-written, and visual-written sub-tests.

Stroop Test-TUBITAK Basic Sciences Research Group (TBAG) Form: The Stroop test is used to assess selective attention and response inhibition (21). The test focuses on the interference effect and the reaction time associated with incongruence between the color used in the writing of a word and the color name uttered as the word is read, and offers insight into frontal activation (22,23). The present study used the TBAG version of the Stroop test (23). The TBAG

version of the Stroop Test, was developed by Kılıç et al. (24), and was standardized in 2002. In light of earlier published studies, data analysis was performed by taking into account the three sub-sections of the test with the highest reliability: the reading of color names printed in black (Stroop-word), naming of colored circles (Stroop-color), and naming of the colors of colored words, when the color and the meaning may be incongruent for certain words (Stroop-color/word) (25). The critical part, where the interference effect appears in Stroop tests, is the Stroop-color/word section. The test provides completion times for each section, the number of errors, and the corrected number of responses as scores, and comparisons are made based on the completion time for each test (26).

Statistical Analysis

Categorical data are presented as numbers and percentages. The data for continuous variables are presented as mean and standard deviation. The Shapiro-Wilk test was used to determine whether the distributions of continuous variables were normal. The mean differences between two related groups of normally distributed data were compared using the independent sample t-test, while the Mann-Whitney U test was used to compare the non-normally distributed data. The frequencies of categorical variables were compared using the Pearson chi-square, Yates' chi-square, or Fisher's exact test, as appropriate. Statistical significance was considered when $p < 0.05$. Statistical analysis was performed using the Statistical Package for the Social Sciences, version 21 (IBM Corp., Armonk, NY, USA). Differences in mean subscores of VADS-B, Stroop TBAG form, and BRIEF were analyzed using univariate analysis of covariance (ANCOVA) with participant group as factor and VADS-B, Stroop TBAG form and BRIEF subscores as dependent variables; chronological age in years was set as a covariate.

Results

There was no difference between the groups regarding socio-demographic, data including age, gender, delivery time, delivery type, developmental steps (walking, talking, toilet training), family structure, settlement, and socio-economic status (all $p > 0.05$). Characteristics of the case and control groups including age, gender, and height are presented in Table 1.

Children with IGHD scored lower scores on the VADS-B for assessing short-term memory ($p < 0.05$) (Table 2). Although children with IGHD tended to have longer completion times for the Stroop word and Stroop color sections of the Stroop test to assess selective attention and

Table 1. Age, gender, and height distribution of the participants

		IGHD group (mean ± SD)	Control group (mean ± SD)	p value
Male (n = 16)	**Age (years)**	9.6 ± 1.8	9.4 ± 1.6	0.8
	Height (cm)	117.6 (± 3.01)	133.1 (± 0.31)	**< 0.001**
Female (n = 19)	**Age (years)**	9.5 ± 1.7	9.6 ± 1.7	0.8
	Height (cm)	115.7 (± 3.14)	134.3 (± 0.11)	**< 0.001**

IGHD: isolated growth hormone deficiency, p: probability of significance, cm: centimeter, SD: standard deviation

Table 2. Comparison of the VADS-B and Stroop TBAG subscale scores for children with IGHD and controls

Variables		IGHD group (mean ± SD)	Control group (mean ± SD)	p value	ANCOVA	
					F	p*
VADS-B						
Aural-Verbal		5.61 ± 1.27	6.69 ± 1.13	0.001	15.030	**< 0.001**
Visual-Verbal		4.30 ± 0.95	5.31 ± 0.96	< 0.001	21.785	**< 0.001**
Aural-Written		5.33 ± 1.05	6.26 ± 1.12	0.002	17.375	**< 0.001**
Visual-Written		4.82 ± 1.01	5.46 ± 0.82	0.002	9.287	**0.003**
Total		19.47 ± 5.02	23.71 ± 3.34	< 0.001	22.184	**< 0.001**
Stroop TBAG test						
Stroop-Word	Completion time (sec.)	12.69 ± 6.09	11.10 ± 3.47	0.615	2.985	0.09
Stroop-Color	Completion time (sec.)	17.78 ± 6.62	15.87 ± 4.5	0.390	3.346	0.073
Stroop-Color/word	Completion time (sec.)	36.31 ± 10.29	30.83 ± 10.69	**0.011**	9.532	**0.003**

Analysis of covariance (ANCOVA) was used for comparisons between the two groups after adjusting for chronological age (years).
IGHD: isolated growth hormone deficiency, SD: standard deviation, sec: seconds, VADS-B: Visual Aural Digit Span Test-B Form, TBAG: TUBITAK Basic Sciences Research Group

inhibition, the difference was not significant. However, the completion time for the Stroop-color/word test was significantly longer in children with IGHD.

Parents of children with IGHD reported higher scores on all sub-scales of the BRIEF scale. These scores were significantly worse for all subscales except "organization of materials" (Table 3).

Discussion

The mechanisms underlying the relationship between GH and cognitive functions are still not completely clear. To investigate the potential relationship between EF and GH in this study, we assessed the EF skills of children with IGHD, using both performance-based testing and parent-report tests, and found that EF skills were poorer in children with IGHD when compared to their unaffected peers.

In the present study, WM was assessed with both VADS-B and the BRIEF scale and both tasks determined poorer WM in children with IGHD. The effects of GH on cognitive skills, such as learning and memory, have been investigated by various earlier studies, including prospective studies that reported cognitive improvement after GH replacement in adults followed up for GH deficiency (26,27,28). Experimental animal studies observed improvement in spatial memory with GH and replacement with GH secretagogue, ghrelin (29,30). Moreover, an impairment in the Morris water maze performance test was found in spontaneous dwarf rats with an inbred variant of the GH gene that causes GH deficiency (31). In contrast to these studies, an experimental animal study examining the effect of GH on cognitive performance showed that GH excess also had a negative effect and inhibition of GH action had a beneficial effect on spatial learning and memory and thus cognitive performance in male mice (32,33). These different results obtained after GH replacement were attributed to the variable effects of systemic GH on different tissue types (34). However, in another study where 99 prepubertal children (aged 3-11) were monitored for idiopathic short stature and GH deficiency-related short stature, IQ levels were found to significantly increase (6). In recent years, neuroimaging studies have been performed to investigate memory performance in GH deficiency (35,36). Arwert et al. (36) compared the GH/IGF-1 axis and memory performance between two groups with high and low IGF-I among 24 elderly adults and determined that, although error rates on a WM task were similar between the two groups, those with high IGF-I levels had faster memory performance with more blood flow to the task-related prefrontal areas on positron emission tomography. Moreover, the same group using functional magnetic resonance imaging (fMRI) in a study of adults with childhood-onset GH deficiency showed that, although the groups were not different in terms of WM, the imaging results of adults with childhood-onset GH deficiency showed higher activity in dorsolateral/ventrolateral PFC, anterior cingulate cortex, parietal cortex, complementary motor and motor cortex, as well as in the thalamus and precuneus. The authors interpreted these results as GH-deficient patients having a lower-than-normal WM speed, which could be compensated for by dorsal prefrontal regions through different mechanisms with no disruption

Table 3. Comparison of BRIEF Scale scores of children with IGHD and control

Variables	IGHD group (mean ± SD)	Control group (mean ± SD)	p value	ANCOVA	
				F	p*
BRIEF					
Inhibition	22.53 ± 6.46	17 ± 2.86	< 0.001	19.886	**< 0.001**
Shifting	18.94 ± 4.19	15.73 ± 3.56	0.001	11.115	**0.001**
Emotional control	19.91 ± 4.32	14.94 ± 3.01	< 0.001	29.167	**< 0.001**
Initiate	13.82 ± 3.25	11.39 ± 2.79	0.003	10.584	**0.002**
Working memory	19.65 ± 5.31	15.39 ± 4.10	0.001	13.250	**0.001**
Plan	25.29 ± 6.84	19.45 ± 5.33	< 0.001	14.805	**< 0.001**
Organization of materials	12.65 ± 3.94	10.97 ± 3.05	0.090	3.650	0.061
Monitoring	13.53 ± 3.93	10.76 ± 2.96	0.002	10.386	**0.002**
BRI	61.38 ± 12.69	47.67 ± 7.3	< 0.001	28.637	**< 0.001**
MCI	72.29 ± 17.6	57 ± 13.95	< 0.001	15.154	**< 0.001**
GEC	133.68 ± 29.29	104.67 ± 19.64	< 0.001	22.070	**< 0.001**
Total scores	146.35 ± 31.93	115.73 ± 21.58	< 0.001	18.377	**< 0.001**

Analysis of covariance (ANCOVA) was used for comparisons between the two groups after adjusting for chronological age (years).
BRIEF: Behavior Rating Inventory of Executive Function, IGHD: isolated growth hormone deficiency, SD: standard deviation, BRI: Behavioral Regulation Index, MCI: Metacognition Index, GEC: Global Executive Composite

in the quality of memory performance (35). In another study, those with childhood-onset GHD were reported to have more pronounced impairment in cognitive functions compared to those with adult-onset GH deficiency (37). The data obtained in the present study supports the studies that have demonstrated poorer WM in children with IGHD. The difference in the errors on WM tasks, which was not found in adult GHD, was quite prominent in the children with IGHD in our study. It is possible that this may because there is no increased prefrontal blood flow in children that was observed in neuroimaging studies of adults, although there is no empirical evidence to support this as yet.

Another parameter evaluated in the present study was self-regulation or inhibitory control, which is defined as the ability to suppress irrelevant responses. We assessed the inhibitory control ability in children with IGHD using both the Stroop TBAG test and the parent-reported BRIEF scale. The results of both assessments suggested poorer inhibitory control in children with IGHD. Patients with IGHD also had lower scores (poor EF skills) on the components of other EFs, such as shifting, emotional control, initiating, planning/organizing, and monitoring, which were evaluated in our study with the BRIEF sub-scales. In line with the results of our study, a meta-analysis reported that GH deficiency caused impairment in neurocognitive networks associated with attention and EF (38). Moreover, it has been generally reported that cognitive skills and attention improve after GH replacement (5). Further studies employing different neuropsychological tests accompanied by neuroimaging are needed to evaluate the effects of GH deficiency on the EF of children with GHD in more detail.

Lastly, the Stroop TBAG test also provided information regarding selective attention (25). In the present study, we determined poorer selective attention in children with IGHD. GH is thought to enter a mutually excitatory interaction with DA and influence the ventral tegmental area-nucleus accumbens-hippocampus-cingulate cortex axis through the amplification of dopaminergic effects, playing a role in reward and conflict processing (39). It has also been suggested that GHD could affect cognitive functions, such as conflict monitoring, WM, and selective attention, by causing DA deficiency (39). Sartorio et al. (40) reported that children with IGHD had certain academic impairment, especially learning difficulties and attention deficit disorders. Although different studies have used the Stroop test to evaluate the relationship between GH and attention and found no significant difference (39,40,41), studies that used different measurement tools, such as the trail-making test, the divided attention task, and the go/no go task, obtained significant results (37,42,43). In line with the results of our study, a review of GH and selective attention found that GHD was associated with poor selective attention (43). Prospective studies that will evaluate cases of childhood-onset GHD in adulthood are needed to better understand the relationship between GH and selective attention.

Study Limitations

Our study has several limitations. The first of these is that the EF skills of cases with IGHD participating in our study were not evaluated after GH replacement. A further limitation was that the scales used to evaluate EF functions vary between studies. Another important limitation was that the parent-child relationship, attachment, and parental attitudes, which are known to affect EF skills, were not evaluated. Further studies are needed in this context (35). Lastly, the participants were administered neuropsychometric tests, but neuroimaging methods, such as fMRI, were not used. Follow-up studies that will use similar scales, incorporate neuroimaging methods, and encompass the childhood period, the treatment process, and the adulthood period are needed to better understand the relationship between GH deficiency and EF skills.

Despite these limitations, this study is the first that to examine EF in school-age children with IGHD, based on both child performance and parent reports. In this context, we believe that the present study will contribute to the literature on IGHD and potentially stimulate future comprehensive prospective studies.

Conclusion

The results of this study demonstrated poorer EF skills in children with IGHD compared to unaffected peers. EF skills may influence academic success by affecting children's language skills, mathematical comprehension, cognitive flexibility, and hypothetical thinking. We believe that psychiatric evaluation of children with IGHD before and during treatment may positively contribute to both their academic performance and social relationships, although this suggestion requires data from future prospective studies before it can be adopted widely.

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Ethics

Ethics Committee Approval: This study was a cross-sectional study that was approved by the Erzurum Regional Training

and Research Hospital, Local Ethics Committee (decision no: 2022/04-28, date: 11.04.2022) and was performed according to the tenets of the Helsinki Declaration.

Informed Consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Authorship Contributions

Surgical and Medical Practices: Gülsüm Yitik Tonkaz, Atilla Çayır, Concept: Gülsüm Yitik Tonkaz, Design: Gülsüm Yitik Tonkaz, Atilla Çayır, Data Collection or Processing: Gülsüm Yitik Tonkaz, Analysis or Interpretation: Gülsüm Yitik Tonkaz, Atilla Çayır, Literature Search: Gülsüm Yitik Tonkaz, Writing: Gülsüm Yitik Tonkaz, Atilla Çayır.

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Salivary Sex Steroid Levels in Infants and the Relation with Infantile Colic

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What is already known on this topic?

The timings of minipuberty and infantile colic may coincide. There exists no data regarding the relationship between them.

What this study adds?

Sex steroid production may be altered during minipuberty in subjects with infantile colic.

Abstract

Objective: The hypothalamic-pituitary-gonadal axis is active during minipuberty, the timing of which coincides with infantile colic. To the best of our knowledge, the relationship between these entities has not been previously investigated.

Methods: Saliva samples were collected from 15- to 60-day-old term infants (n = 139) between 9 am and 5 pm. Group 1 included infants with infantile colic (n = 68, 54.4% female) while the remaining healthy infants constituted Group 2 (n = 71, 47.9% female). Salivary levels of estradiol (E_{sal}) in females and testosterone (T_{sal}) in males were measured by ELISA in duplicate.

Results: The median (25^{th}-75^{th} centile) age and birth week for all infants were 33 (29-43) days and 39 (38.1-40) weeks, respectively. Levels of T_{sal} in males [Group 1, 73.35 (59.94-117.82) pg/mL vs Group 2, 77.66 (56.49-110.08) pg/mL, p = 0.956] and E_{sal} in females [Group 1, 3.91 (2.76-5.31) pg/mL vs Group 2, 4.03 (1.63-12.1) pg/mL, p = 0.683] were similar. However, in subjects with infantile colic (Group 1), E_{sal} and body mass index (BMI) standard deviation scores of females were slightly correlated (Group 1, r_s = 0.393, p = 0.016 vs. Group 2, r_s = 0.308, p = 0.076) and there was a significant correlation between the sampling time and T_{sal} in males (Group 1, r_s = 0.469, p = 0.009 vs. Group 2, r_s = -0.005, p = 0.976).

Conclusion: Random salivary sex steroid levels were similar in infants with and without infantile colic. However, in subjects with infantile colic, E_{sal} levels in females were positively correlated with BMI and T_{sal} levels were higher later in the day among males. Thus, sex steroid production may be altered during minipuberty in subjects with infantile colic.

Keywords: Gonadal activity, newborn, puberty, fussing

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Introduction

Classical puberty begins in adolescence. However, the hypothalamic-pituitary-gonadal axis is also active during the first months of life resulting in an increase in gonadotropins and sex steroids (minipuberty) (1,2,3,4). In boys, the increase in follicle stimulating hormone (FSH) and luteinizing hormone (LH), primarily LH, reaches its highest value at 4-10 weeks after birth and both decrease to prepubertal levels at around six months. Serum testosterone level, which increases with LH secretion, peaks between 1-3 months and decreases to prepubertal levels between 6-9 months (2,5). Similarly in girls, serum estradiol levels peak between 30-60 days of life, then fall below the prepubertal level towards one year of age (5). In the literature, there are data regarding salivary testosterone levels in male infants, but data on salivary estradiol levels in female infants are limited (6,7,8,9). The importance of minipuberty is not fully understood but minipuberty has been reported to affect genital organ development, body composition, and cognitive functions (3).

Infantile colic was defined by Wessel et al. (10) in 1954 as excessive irritability and crying in the evening for no apparent reason, starting in the first weeks of life. It is seen in 10-40% of otherwise healthy infants aged one to five months. While various hypotheses, including gastrointestinal, neurodevelopmental, and psychosocial causes have been proposed, its etiology is currently unknown (11,12).

Among the few and heterogeneous data regarding the impact of minipuberty on behavior, there are some findings indicating that sex steroid levels might be associated with behavioral patterns in infants as well as in adolescents (3,9,13,14). Since sex steroid levels are elevated during a period when infantile colic is common, the aim was to evaluate the relationship between minipuberty and infantile colic by measuring testosterone and estradiol levels in saliva samples of infants. To the best of our knowledge, there is no similar published study.

Methods

Subjects

This study included term infants aged 15-60 days old without any additional disease who attended outpatient clinics in a single center between March and October 2021. Infants who had any disorder of sex development or a systemic pathology, such as gastrointestinal malformation and gastroesophageal reflux, or who had used antibiotics in the last week were not included in the study. Infantile colic was diagnosed according to the Rome IV criteria (episodes of crying and irritability lasted longer than one week, at least three hours a day, and at least three days in the same week) (11,15). Careful physical examinations of these infants were performed to exclude other systematic causes of irritability. All of the infants were prepubertal and none had an abnormal external genital structure. The parents of the subjects with infantile colic were contacted by phone when they were six months old and it was confirmed that they did not have any other diseases.

Data Collection

All relevant data, including demographic features, family history, anthropometric measurements, and physical examination findings were recorded. Standard deviation scores (SDS) for weight, length, head circumference, and body mass index (BMI) were calculated according to the Turkish child population using child metrics (15,16). Weight for length SDS was calculated according to World Health Organization data (16,17).

Saliva samples were collected using Salimetrics® SalivaBio Oral Swab (Salimetrics, State College, PA, USA) and stored at -80 °C. Measurement was made with Salimetrics® 17 β-estradiol ELISA kit for estradiol (1-3701) in girls and Salimetrics® Testosterone ELISA kit for testosterone (1-2402) in boys and all samples were tested in duplicate. When the first and second results were statistically compared for testosterone and estradiol, p values were 0.922 and 0.347, respectively. An average of the two measurements were used in the study. Both kits are based on a sandwich ELISA method and are read at a wavelength of 450 nm. The sensitivity of the estradiol kit was 0.1 pg/mL, and the testosterone kit was 1 pg/mL. The measurement ranges were 1-32 pg/mL for estradiol and 6.1-600 pg/mL for testosterone.

Ethics

This study was conducted with the approval of Dokuz Eylül University Local Ethical Committee (decision no: 2019/22-22, date: 09.09.2019). Financial support was provided by the Department of Scientific Research Projects of Dokuz Eylül University (2020.KB.SAG.40) and Turkish Society for Pediatric Endocrinology and Diabetes (2020-04). An informed written consent form was obtained from parents before participating the study and it was performed in accordance with the principles of the Declaration of Helsinki.

Statistical Analysis

In order to be able to find a significant difference of 5 pg/mL between the mean of sex steroids of the groups in a situation where the standard deviation values of the groups

were 4 and 8 pg/mL, based on a type 1 error of 0.05 and a power of 0.80, the minimum number of subjects for each gender among the groups was determined as 27. Taking unexpected errors into account, it was planned to include 20% excess cases.

Statistical analysis was performed using IBM Statistical Package for the Social Sciences, version 24 (IBM Inc., Armonk, NY, USA). The distribution of the data was evaluated using the Kolmogorov-Smirnov test. Descriptive statistics are given as numbers and percentages for categorical variables and median (25^{th}-75^{th} centile) for numerical variables. The Mann-Whitney U test was used to compare numeric variables and chi-square test was used for categorical data. The correlation of the parameters was tested with Spearman correlation analysis. A p value < 0.05 was considered significant.

Results

A total of 139 infants (48.9% males) were included in the study. The median age was 33 (29-43) days, and the median gestational age was 39 (38.1-40) weeks. A majority of the newborns (n = 88, 63.3%) were born by caesarean section.

In terms of feeding, 100 (71.9%) were fed with breast milk only, 35 (25.2%) with both breast milk and formula, and four (2.9%) with formula only. The median value of daily stool counts was 3 (2-4; 1-9). Only three (2.2%) were not using any medication, 120 (86.3%) were given vitamin D, 15 (10.8%) were given vitamin D and probiotics and one (0.7%) was only using probiotics.

The subjects were divided into two groups: with infantile colic (Group 1, n = 68); and those without (Group 2, n = 71). The infants in the two groups had similar demographic and anthropometric features, except for gestational age and weight-for-length SDS (Table 1). In addition, the ages, education, health status, drug use, and smoking rates of parents were similar between the groups (data not shown).

Characteristics of the subjects were further analyzed according to gender. Although it was observed that salivary estradiol levels decreased with increasing age in female babies (n = 71), this did not reach statistical significance ($r_s = -0.224$, $p = 0.061$) (Figure 1a). Females with infantile colic (Group 1-F) were born slightly earlier compared to healthy control female subjects (Group 2-F), while the remaining features, including salivary estradiol levels, were similar (Table 2). Correlation analyses in females revealed that saliva estradiol levels showed significant correlation with BMI SDS in Group 1 only (Table 3).

In male infants, salivary testosterone levels decreased with increasing age (Figure 1b). BMI SDS and weight-for-length SDS were found to be significantly higher in the males with infantile colic (Group 1-M); although salivary testosterone levels were similar between the groups (Table 4). Correlation analyses were also done for male babies (Table 5). There was a moderate negative correlation between testosterone and BMI SDS but only in the infantile colic group. When

Table 1. The demographic and anthropometric characteristics regarding presence of infantile colic. Group 1: subjects with infantile colic, Group 2: subjects without infantile colic

	Group 1 (n = 68)	Group 2 (n = 71)	p
Age (in days)	34.5 (29-43.8)	33 (29-43)	0.439
Gender n (%)			
Female	37 (54.4)	34 (47.9)	0.442
Male	31 (45.6)	37 (52.1)	
Mode of delivery n (%)			
SVD	21 (30.9)	30 (42.3)	0.164
C/S	47 (69.1)	41 (57.7)	
Gestational age	38.5 (38-39.6)	39.2 (38.5-40.2)	**< 0.001**
Birth weight SDS	-0.03 [(-0.43)-0.61]	0.01 [(-0.72)-0.52]	0.903
Birth length SDS	0.00 [(-0.38)-0.75]	-0.19 [(-0.66)-0.45]	0.089
Birth head circumference SDS	0.07 [(-0.36)-0.43]	0.07 [(-0.64)-0.79]	0.899
Weight SDS	0.4 [(-0.2)-0.84]	0.15 [(-0.32)-0.62]	0.168
Length SDS	0.16 [(-0.48)-0.77]	0.23 [(-0.34)-0.91]	0.634
BMI SDS	0.13 [(-0.11)-0.75]	0.03 [(-0.67)-0.52]	0.056
Weight for length SDS	0.3 [(-0.16)-1.11]	-0.04 [(-0.87)-0.79]	**0.021**
Head circumference SDS	0.04 [(-0.52)-0.68]	-0.06 [(-0.71)-0.60]	0.607

Data are presented as median (25p-75p) unless otherwise indicated.
SVD: spontaneous vaginal delivery, C/S: cesarean section, SDS: standard deviation score, BMI: body mass index

Table 2. The demographic and anthropometric characteristics regarding presence of infantile colic in females. Group 1-F: female subjects with infantile colic, Group 2-F: females without infantile colic

	Group 1-F (n = 37)	Group 2-F (n = 34)	p
Age (in days)	32 (29-43)	34 (29-47)	0.522
Mode of delivery n (%)			
SVD	11 (29.7)	14 (41.2)	0.313
C/S	26 (70.3)	20 (58.8)	
Gestational age	38.28 (37.71-39.21)	39 (38.42-39.88)	**< 0.001**
Birth weight SDS	-0.14 [(-0.62)-0.46]	-0.11 [(-0.72)-0.52]	0.773
Birth length SDS	-0.19 [(-0.42)-0.75]	-0.19 [(-0.66)-0.75]	0.619
Birth head circumference SDS	0.36 [(-0.36)-0.36]	-0.18 [(-0.36)-0.36]	0.743
Crying frequency (day/week)	5 (4-6)	0 (0-5)	**< 0.001**
Last feeding time (min)	30 (17.5-60)	30 (20-60)	0.902
Sampling time n (%)			
9:00-12:00	21 (56.8)	19 (55.9)	0.941
13:00-17:00	16 (43.2)	15 (44.1)	
Weight SDS	0.41 [(-0.23)-0.82]	0.12 [(-0.29)-0.77]	0.542
Length SDS	0.02 [(-0.5)-0.76]	0.26 [(-0.71)-0.91]	0.936
BMI SDS	0.13 [(-0.09)-0.77]	0.36 [(-0.55)-0.70]	0.881
Weight for length SDS	0.32 [(-0.15)-1.18]	0.06 [(-0.82)-1.02]	0.208
Head circumference SDS	-0.08 [(-0.78)-0.69]	-0.19 [(-0.72)-0.62]	0.995
Weight gain (g/day)	33.75 (27.64-38.44)	32.61 (23.75-40.13)	0.782
Salivary estradiol (pg/mL)	3.91 (2.76-5.31)	4.03 (3.22-5.40)	0.683

Data are presented as median (25p-75p) unless otherwise indicated.
SVD: spontaneous vaginal delivery, C/S: cesarean section, SDS: standard deviation score, BMI: body mass index

Table 3. Correlation of salivary estradiol level with demographic and clinical parameters in females, Group 1-F: females with infantile colic, Group 2-F: females without infantile colic

	All females (n = 71)	Group 1-F (n = 37)	Group 2-F (n = 34)
Age (in days)	r_s = -0.224 (p = 0.061)	r_s = -0.142 (p = 0.4)	r_s = -0.268 (p = 0.125)
Birth length SDS	r_s = -0.11 (p = 0.361)	r_s = -0.007 (p = 0.965)	r_s = -0.211 (p = 0.231)
Sampling time	r_s = 0.017 (p = 0.887)	r_s = -0.003 (p = 0.987)	r_s = 0.042 (p = 0.811)
Weight SDS	r_s = 0.132 (p = 0.274)	r_s = 0.109 (p = 0.521)	r_s = 0.181 (p = 0.305)
BMI SDS	r_s = 0.346 **(p = 0.003)**	r_s = 0.393 **(p = 0.016)**	r_s = 0.308 (p = 0.076)
Weight for length SDS	r_s = 0.241 (p = 0.043)	r_s = 0.287 (p = 0.085)	r_s = 0.215 (p = 0.221)
Crying frequency (day/week)	r_s = -0.078 (p = 0.518)	r_s = -0.216 (p = 0.2)	r_s = -0.009 (p = 0.962)

SDS: standard deviation score, BMI: body mass index

the correlation analysis of the sample collection time and testosterone level was examined, it was found that subjects in the infantile colic group whose samples were taken later in the day had higher salivary testosterone levels (Figure 2). There was no similar association in the control group. In addition, when examined with partial correlation by controlling for age, the relationship between testosterone and sample collection time was found to be stronger (r_s = 0.469, p = 0.009).

Discussion

Many factors have been investigated in studies examining the etiology of infantile colic. Infantile colic is more common in preterm babies (10,18). While term babies were already included in the present study, the median gestational age of the infantile colic group was slightly lower than that of the control group. When the girls and boys were examined separately, this difference was attributable to the girls. We suggest that earlier birth, even when at term, might be associated with infantile colic in girls.

In the present study, there was no significant association between salivary estradiol levels and age in female infants. This situation might be explained by considering the findings of Kuiri-Hänninen et al. (19). They found fluctuating urinary estradiol levels in subjects aged between one week and six months. We observed a positive

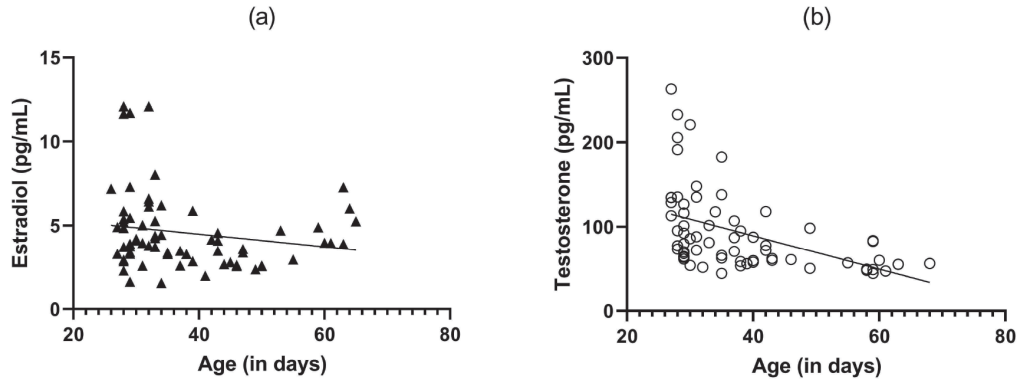

Figure 1. Correlation between salivary sex steroid levels and age in (a) females and (b) males with and without colic

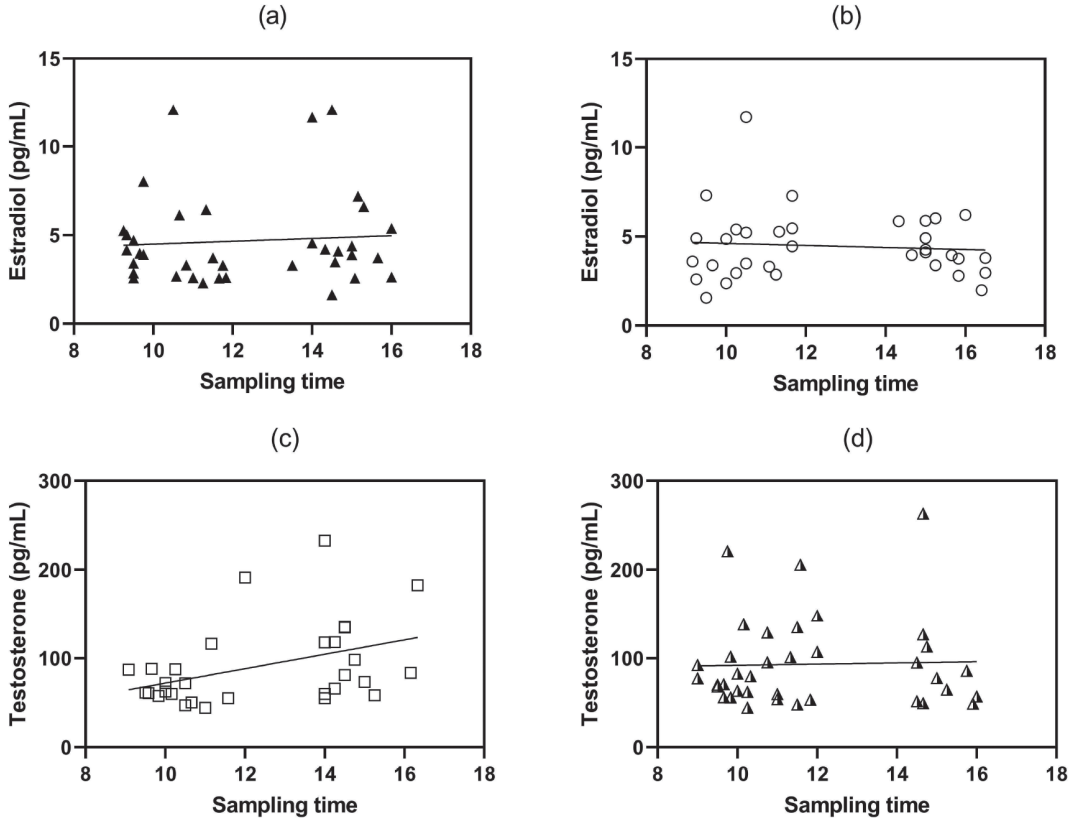

Figure 2. Correlation between salivary sex steroid levels and sampling time in the infantile colic group (a, c) and control group (b, d)

correlation between salivary estradiol levels and BMI SDS both in the whole female cohort and in the infantile colic group. This may be associated with extraglandular estrogen production in increased adipose tissue (20,21). However, we did not observe a significant difference between random salivary estradiol levels of girls with and without infantile colic. Alexander et al. (9) also reported no association between salivary estradiol levels (4.73 ± 0.86 pg/mL) of 3-4 months old female infants with their preferences for various stimuli.

The decrease in salivary testosterone levels with age in male infants in the present study are in line with the previous studies. Testosterone levels in the saliva of male infants aged one to three months, which were measured in duplicate with ELISA method, were reported to be 79.09 ± 22.75 pg/mL, levels which are similar to those found in the present study (6). In older infants, aged between 2.7 and 4.8 months, the mean testosterone level was 40.39 ± 13.39 pg/mL (7). In another study conducted with the same method and studied in duplicate, the salivary testosterone levels of

Table 4. The demographic and anthropometric characteristics regarding presence of infantile colic in males. Group 1-M: male subjects with infantile colic, Group 2-M: males without infantile colic

	Group 1-M (n = 31)	Group 2-M (n = 37)	p
Age (in days)	37 (31-49)	31 (29-39.5)	0.086
Mode of delivery n (%)			
SVD	10 (32.3)	16 (43.2)	0.353
C/S	21 (67.7)	21 (56.8)	
Gestational age	39 (38.28-40)	39.71 (38.7-40.4)	0.139
Birth weight SDS	0.13 [(-0.25)-0.76]	0.09 [(-0.66)-0.50]	0.542
Birth length SDS	0.00 [(-0.45)-0.91]	0.00 [(-0.91)-0.45]	0.072
Birth head circumference SDS	0.07 [(-0.29)-0.79]	0.07 [(-0.64)-0.79]	0.895
Crying frequency (day/week)	5 (4-6)	0 (0-4.5)	**< 0.001**
Last feeding time (min)	60 (30-90)	60 (30-105)	0.369
Sampling time n (%)			
9:00-12:00	16 (51.6)	26 (70.3)	0.115
13:00-17:00	15 (48.4)	11 (29.7)	
Weight SDS	0.38 [(-0.10)-0.86]	0.16 [(-0.36)-0.55]	0.196
Length SDS	0.23 [(-0.49)-0.87]	0.22 [(-0.27)-0.99]	0.649
BMI SDS	0.12 [(-0.15)-0.76]	-0.13 [(-0.68)-0.27]	**0.018**
Weight for length SDS	0.25 [(-0.29)-1.06]	-0.17 [(-0.95)-0.60]	**0.044**
Head circumference SDS	0.22 [(-0.38)-0.68]	0.10 [(-0.67)-0.60]	0.483
Weight gain (g/day)	39.25 (28.52-49.88)	34.13 (26.58-44.44)	0.203
Salivary testosterone (pg/mL)	73.35 (59.94-117.82)	77.66 (56.49-110.08)	0.956

Data are presented as median (25p-75p) unless otherwise indicated.
SDS: standard deviation score, C/S: cesarean section, BMI: body mass index

Table 5. Correlation of salivary testosterone levels with demographic and clinical parameters in males Group 1-M: male subjects with infantile colic, Group 2-M: males without infantile colic

	All males (n = 68)	Group 1-M (n = 31)	Group 2-M (n = 37)
Age (in days)	r_s = -0.622 **(p < 0.001)**	r_s = -0.695 (p < 0.001)	r_s = -0.594 **(p < 0.001)**
Birth length SDS	r_s = -0.288 **(p = 0.017)**	r_s = -0.424 **(p = 0.018)**	r_s = -0.190 (p = 0.260)
Sampling time	r_s = 0.148 (p = 0.229)	r_s = 0.369 **(p = 0.041)**	r_s = -0.005 (p = 0.976)
Weight SDS	r_s = -0.126 (p = 0.307)	r_s = -0.424 **(p = 0.017)**	r_s = 0.132 (p = 0.437)
BMI SDS	r_s = -0.167 (p = 0.172)	r_s = -0.528 **(p = 0.002)**	r_s = 0.057 (p = 0.738)
Weight for length SDS	r_s = 0.043 (p = 0.729)	r_s = -0.200 (p = 0.280)	r_s = 0.168 (p = 0.320)
Crying frequency (day/week)	r_s = 0.175 (p = 0.153)	r_s = 0.120 (p = 0.520)	r_s = 0.256 (p = 0.127)

SDS: standard deviation score, BMI: body mass index

3-6 month-old boys were between 27.51 and 58.13 pg/mL (8). In the present study, random salivary testosterone levels did not differ between males with and without infantile colic, but, in the infantile colic group, there was a significant positive correlation between sample collection time and testosterone level. These higher testosterone levels later in the day may be related with the onset of colic attacks in the evening. Moreover, we observed that boys with infantile colic had higher BMI SDS and weight for length SDS at the time of examination, which may have been related to the fact that parents try to feed restless babies more. A significant relationship between salivary testosterone levels (40.68 ± 10.69 pg/mL) in 3-4 month-old male infants and their behavior was also observed by Alexander et al. (9). They reported that higher salivary testosterone levels were associated with stronger preferences for male-typical stimuli.

Study Limitations

Biochemical demonstration of puberty by measuring serum levels of FSH and LH would be beneficial, but measuring salivary sex steroids in otherwise healthy infants is similarly informative and we successfully provided relevant data by using a noninvasive method. Collecting saliva samples from infants during episodes of experiencing symptoms may be more informative. However, this is not practical in an irritable infant, and the temporal relationship found for testosterone in boys may have been missed if we have done so.

Conclusion

In conclusion, random levels of sex steroids in the saliva of subjects with infantile colic were not different from those of the control infants. However, a significant correlation between salivary estradiol levels and BMI in females and a higher salivary testosterone level later in the day among boys with infantile colic suggest there may be an alteration of sex steroid production in subjects with infantile colic.

Ethics

Ethics Committee Approval: This study was conducted with the approval of Dokuz Eylül University Local Ethical Committee (decision no: 2019/22-22, date: 09.09.2019).

Informed Consent: Informed written consent form was obtained from parents before participating the study.

Authorship Contributions

Concept: Korcan Demir, Design: Fulya Mete Kalaycı, Korcan Demir, Data Collection or Processing: Fulya Mete Kalaycı, Özlem Gürsoy Doruk, İbrahim Mert Erbaş, Osman Tolga İnce, Makbule Neslişah Tan, Adem Aydın, Ayhan Abacı, Ece Böber, Korcan Demir, Analysis or Interpretation: Özlem Gürsoy Doruk, Literature Search: İbrahim Mert Erbaş, Osman Tolga İnce, Makbule Neslişah Tan, Adem Aydın, Ayhan Abacı, Ece Böber, Korcan Demir, Writing: Fulya Mete Kalaycı, İbrahim Mert Erbaş, Osman Tolga İnce, Makbule Neslişah Tan, Adem Aydın, Ayhan Abacı, Ece Böber, Korcan Demir.

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Non-thyroidal Illness in Children with Congestive Heart Failure

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What is already known on this topic?

The sick euthyroid state is seen in critically ill patients and adversely affects outcomes. Thyroid hormones affect cardiac function and contractility.

What this study adds?

The sick euthyroid state was seen in 46% of children with congestive heart failure. Raised brain-natriuretic peptidelevels, a marker of heart failure, significantly affected low free T3 levels. A free T3/reverse T3 ratio of < 1.86 pg/ng predicted mortality.

Abstract

Objective: To estimate the proportion and risk factors of non-thyroidal illness (NTI) in children with congenital heart disease (CHD) with congestive heart failure (CHF).

Methods: This study enrolled children (6 weeks to 60 months age) with CHD and CHF. The clinical profile and disease severity, derived from the Pediatric Early Warning Score (PEWS) was recorded. Baseline blood samples were taken within 24 hours of hospitalization and evaluated for free tri-iodothyronine (fT3), free thyroxine (fT4), thyroid stimulating hormone (TSH), N-terminal pro-brain natriuretic peptide (NT pro-BNP) and reverse T3.

Results: A total of 80 (64 acyanotic CHD) children of median (interquartile range) age 5 (2.5, 8.0) months were enrolled. NTI was seen in 37 (46%) of whom 27 had low fT3 levels. The proportion of NTI was highest in children with severe disease (20/30), than moderate (4/9) or mild disease (13/41) (p = 0.018). Ten (27%) patients with NTI died compared to 2 (4.7%) without NTI with unadjusted odds ratio (OR) [95% confidence interval (CI)] 7.593 (1.54, 37.38); p = 0.006. After adjusting for NTI, shock and NT-pro-BNP levels, PEWS was the only significant predictor of mortality (OR: 1.41, 95% CI: 1.03, 1.92; p = 0.032). Linear regression for fT3 identified a significant relationship with log NT-BNP [beta -3.541, (95% CI: -1.387, -0.388)] and with TSH [beta 2.652 (95% CI: 0.054, 0.383)]. The cutoff (area under the curve, 95% CI) that predicted mortality were fT4 < 14.5 pmol/L (0.737, 0.60, 0.88), fT3/rT3 index < 1.86 pg/ng (0.284, 0.129, 0.438) and NT pro-BNP > 3725 pg/mL (0.702; 0.53, 0.88).

Conclusion: NTI was present in a significant proportion of children with CHD and CHF. fT3 level was significantly associated with NT-BNP levels and thus severity of CHF.

Keywords: Non-thyroidal illness, free T3, reverse T3, NT pro-BNP, Pediatric Early Warning Score

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Introduction

Cardiovascular structure and function in the pediatric age group is affected by changes in body size and metabolic demands, as well as hormonal effects. The effect of thyroid hormones (TH) on the adult cardiovascular system (CVS) has been studied widely. The CVS is a well-known target for TH as is exhibited by its profound effects on cardiac contractility, heart rate, systemic vascular resistance, ventricular mass and body's blood volume (1,2). Cardiomyocytes express thyroid receptors (TRα and TRβ) (3). The genetic transcription of contractile proteins of myosin heavy chains is dependent on TH in fetal and postnatal life suggesting their association (4).

Non-thyroidal illness (NTI), also known as sick euthyroid syndrome, is frequently reported in critically sick patients. It is postulated to be a state of adaptive response where the body adapts to assume a low metabolic state to conserve energy, like calorie restriction in acute illness. During prolonged illness, this state represents the abnormal effect of illness on the hypothalamic-pituitary axis where there is low generation of TH. Low free tri-iodothyronine (fT3) results from reduced enzyme activity of 5' monodeiodinase (type 1 and 2) which is responsible for peripheral conversion of thyroxine (T4) to T3 (1,5). There is an increase in activity of deiodinase type 3 that increases the generation of reverse T3 (3,3',5'-triiodothyronine or rT3), the inactive form, and also suppression of release of thyrotropin releasing hormone (TRH) from the pituitary in prolonged disease states (6,7).

Generally, NTI is associated with poor disease outcomes and mortality. An association between lower fT3 level, raised rT3 level and lower cardiac ejection fraction has been seen in patients with acute myocardial infarction, heart failure and after coronary artery bypass surgery (1). A few studies have reported the occurrence of NTI in critically sick children, which was associated with a worse prognosis (5,8,9,10). Congenital heart diseases (CHD) are one of the most common cardiovascular diseases in children that usually present with congestive heart failure (CHF). Children with CHF are usually sick at presentation with an increased risk of repeated hospitalizations and require follow-up. The associated hepatic congestion in CHF may cause an inhibition of hepatic deiodinase type 1 with resultant lower T3 levels (9). A suboptimal thyroid status in children with CHD and CHF can inturn compromise the cardiac function further. In animal models, poorly controlled CHF with decompensated left ventricular hypertrophy and low cardiac output can further decrease the TH compared to animals with preserved cardiac output (11).

The primary objective of this study was to evaluate the proportion of children with CHD and CHF with NTI. We also aimed to study the association of TH with cardiac contractility (ejection fraction) and clinical outcomes, including duration of hospitalization and mortality.

Methods

This observational study was conducted at the Department of Pediatrics and Cardiology in a large tertiary care public hospital from July 2021 until June 2022 after approval of the Institutional Ethics Committee Maulana Azad Medical College, New Delhi (letter F No.1/IEC/MAMC/82/10/2020/no. 125 dated: 14 Jan 2021). Written parental consent was taken before enrollment. The study protocol was in agreement with the Declaration of Helsinki for conduct of research and registered under clinical trials registry with registration number: CTRI/2021/03/032417.

Children in the age group of six weeks to 60 months and diagnosed with CHD (cyanotic or acyanotic heart disease) with CHF were eligible for inclusion. Children with a past history of thyroid disease (congenital hypothyroidism) or history of intake of thyroxine in the preceding three months, congenital anomalies such as Down syndrome, Turner syndrome or Williams-Beuren syndrome, and children with a history of birth asphyxia, cardiomyopathy or myocarditis were excluded. All eligible children were enrolled within 24 hours of hospitalization.

Sample Size

Sample size calculation was done using Open Epi software with a proportion of NTI as 24.5% in patients with underlying heart failure (1). A sample size of 72 was calculated with 5% alpha error and power of 80%. A total of 80 children were recruited for the study.

The demographic details, birth and developmental history and feeding history were elicited and recorded. The age of diagnosis of CHD, disease progression, details of prior hospitalization (if any) and treatment history for decongestants or other drugs were noted. Children with CHF who were controlled on medications and those who presented in decompensated CHF were both enrolled. The vital sign data, including temperature, heart rate, respiratory rate and blood pressure were measured, followed by physical examination including anthropometry. The WHO 2006 standards were used to interpret anthropometry in terms of standard deviation (SD) scores (SDS) (12). Non-invasive blood pressure was measured as per standard protocol and interpreted as per the American Academy of Pediatrics (AAP) (13). Cardiac lesions were further classified as acyanotic CHD (ACHD) or cyanotic CHD (CCHD), based on clinical examination and confirmed on echocardiography.

A prognostic clinical scoring was done at hospitalization using the Pediatric Early Warning Score (PEWS) (14). The score recorded a value from 0 to 3 (0 for best and 3 for worst) for each of three variables: behaviour;cardiovascular; and respiratory. As per PEWS, patients were categorized as mild, moderate or severe sickness for a score of 0-3, 4-6 or 7-9, respectively. The outcome of all children was recorded until discharge or death. The duration of hospitalization and oxygen need were also recorded in total days. A venous blood sample was collected and processed for biochemical investigations. One serum aliquot was separated and stored at -80 °C for thyroid functions. Liver function test, serum creatinine and C-reactive protein (CRP) were measured at the time of enrolment. The liver function tests and serum creatinine was measured on an autoanalyzer (Vitros 5600, Quidel Ortho, USA). CRP was measured using ELISA with normal range < 5 mg/L. A three-fold rise in CRP was taken to be suggestive of infection and affected patients were evaluated further for sepsis.

The thyroid stimulating hormone (TSH), fT3, and fT4 were processed on a weekly basis and reverseT3 (rT3) level was processed as a single batch by electrochemiluminescence (ECLIA). NTI (or sick euthyroid illness) was defined as low T3 and/or low T4 with normal TSH values and elevated rT3 levels. Hypothyroidism was defined as elevated TSH (> 10 mIU/L) with low fT4. Subclinical hypothyroidism was defined as elevated TSH between 5-10 mIU/L with normal fT4 values.The normal ranges were: TSH 0.46-4.68 mIU/L; fT3 4.26-8.10 pmol/L; fT4 10-22.8 pmol/L; and rT3 0.06-0.76 ng/mL (< 250 pg/mL).The fT3/rT3 index (in pg/ng) was mathematically derived, by converting fT3 pmol/L to pg/mL (multiply by 0.651) and the ratio was calculated versus rT3 (in ng/mL). The intra and inter-assay coefficient of variation (CV) for fT4 was 1.7-5.7% and 3-10.7%, for fT3 1.1-3% and 1.9-8.2% and for TSH 1.5-4.2% and 2.4-6.3%, respectively.

One mL of blood sample for serum N-terminal pro-brain natriuretic peptide (NT pro-BNP) was measured within 24 hours of collection by ECLIA with a maximum storage limit at 2-8 °C of 3 days. The normal range for serum NT pro-BNP (children > one-month age) was 20-40 pg/mL (15). NTI was classified as NTI-1 or Low T3 syndrome, in which there is decrease total or free T3 with normal T4 and TSH level and NTI-2 or low T4 syndrome, with significant fall in both T3 and T4 with normal or low TSH level (16).

A plain chest radiograph was done at enrolment to document the cardiac size and any other lung abnormality. A standard electrocardiogram was done at the bedside of the patients after standard calibration and abnormalities recorded. Echocardiography was done using a EPIQ CVX ultrasound machine (Philips, Netherlands) at a frequency of 7-12 Hz in M mode to measure ejection fraction (EF) once the child was stable for transport. EF was defined as percentage of blood volume ejected per cardiac cycle. EF was calculated using Simpson's biplane method and the formula used was EF = (EDV-ESV)/EDV or EF = SV/EDV where EF = ejection fraction, EDV = end diastolic volume, ESV = end systolic volume, and SV = stroke volume.

Statistical Analysis

Data were entered in Microsoft Excel and were analyzed using Statistical Package for the Social Sciences, version 25.0 (IBM Inc., Armonk, NY, USA). The normality of continuous variables was checked using the Kolmogorov-Smironov test. Categorical variables were described as frequencies and proportions, median (interquartile range-IQR) and mean (SD) were calculated for baseline characteristics, such as age, growth parameters, and biochemical parameters. Continuous variables were compared using t-test or Mann-Whitney U test, as appropriate. Parameters between three categories of PEWS were compared using ANOVA or Kruskal-Wallis test (for non-parametric data). Proportions for those with and without NTI were compared by chi-square test or Fisher's exact test. Odds ratio (OR) [95% confidence interval (CI)] was calculated to predict mortality with categorical risk factors (sex, type of CHD, previous hospitalization, failure to thrive, NTI, shock and PEWS category). Binary logistic regression analysis was used, using the enter and forward selection method, to estimate the risk of mortality based on covariates. NT pro-BNP levels were transformed logarithmically for regression analysis.The correlation between continuous variables wasperformed using Spearman's rank correlation coefficient (r) for non-parametric variables. Stepwise multiple linear regression was used, with fT3 as the dependent variable and other variables being independent (TSH, NT pro-BNP, PEWS score); fT4 and rT3 were not considered independent for fT3 levels and not included. Receiver operating curve was constructed to measure cutoff points for serum NT pro-BNP, fT4 and fT3/rT3 index to predict mortality. A p value less than 0.05 was taken as significant.

Results

A total of 122 patients were screened during the study period, out of whom 42 patients were excluded (26 had Down syndrome, 8 Down phenotype, 1 Turner syndrome, 3 dysmorphism with renal abnormalities, and 4 with primary hypothyroidism who were on thyroxine treatment). A total of 80 children were enrolled, including 49 (61.25%) males, and 64 (80%) had acyanotic heart disease. The median (IQR) age was 5 (2.5, 8) months and age at diagnosis was

2.25 (1.5, 4) months. Feeding difficulty and poor weight gain were seen in 57 (71.3%) and 52 (65%), respectively, with a median (IQR) weight-for-age Z score of 3.3 (-4.56, -2.21) and weight-for-length Z score of -3.02 (-4.03, -1.38). Sixteen children with CCHD and three children with ACHD and severe pulmonary hypertension had cyanosis. As per PEWS, 41 (50%) children had mild, 9 (11.3%) had moderate and 30 (38.7%) had severe disease at hospitalization, with shock in 12 (15%) children. Elevated CRP (more than three times upper limit of normal) was seen in 12 children, one of whom also had septic shock.

The comparison of biochemical parameters as per disease severity is shown in Table 1. A total of 37 (46.25%) patients had NTI; 27 (33.8%) had NTI-1 and 10 (12.4%) had NTI-2. The dispersion of fT3, fT4, rT3 and TSH levels as per disease severity in those with and without sick euthyroid syndrome is shown in Figure 1A, 1B, 1C and 1D respectively.

Table 2 shows the comparison of clinical and laboratory parameters in children with or without NTI. Only 3/12 children with elevated CRP had NTI. Similarly, 9/12 children with shock had NTI with an OR of 4.286 (95% CI: 1.06, 17.23) of developing NTI (p = 0.031). Logistic regression was performed for predicting mortality with a model based on variables including disease severity (PEWS), presence or absence of shock, rT3 levels, log NT pro-BNP levels, and presence of NTI. The model (using enter method) explained 44.3% variation in mortality and correctly classified 86.5% of patients with only PEWS as a statistically significant variable (adjusted OR: 1.41, 95% CI: 1.03, 1.92; p = 0.032). PEWS remained the only significant variable with adjusted OR: 1.63 (95% CI: 1.25, 2.13; p < 0.001) in the second model (forward conditioning) explaining 37.2% of variation.

The fT3 levels showed significant correlation with TSH (r = 0.461, p < 0.001), fT4 (r = 0.373, p < 0.001), rT3 (r = -0.488, p < 0.001), NT pro-BNP (r = -0.430, p < 0.001), and insignificant weak correlation with PEWS (r = -0.162, p = 0.154). A significant positive correlation was observed between rT3 level and serum NT pro-BNP (r = 0.311, p = 0.007), and a negative correlation with ejection fraction (r = -0.233, p = 0.044). Likewise, serum NT pro-

Table 1. Clinical and Biochemical parameters by Pediatric Early Warning Score (PEWS)

Parameters	PEWS mild (n = 41)	PEWS moderate (n = 9)	PEWS severe (n = 30)	p
Age (mo)	5 (2.5, 11)	6 (2.5, 23)	4.5 (2.8, 7.0)	0.541
Duration of stay (d)	8 (7, 10.75)	9 (7.5, 12.5)	14 (10.75, 20.75)	**< 0.001**
Duration oxy (d)	4 (2, 7)	4 (1.5, 8)	11 (8, 19.25)	**< 0.001**
WAZ score	-3.36 (-4.56, -2.35)	-2.65 (-4.03, -1.63)	-3.65 (-4.68, -2.74)	0.356
H/LAZ score	-1.85 (-2.95, -0.71)	-2.18 (-2.88, -0.36)	-1.92 (-3.02, -0.98)	0.939
WHZ/WLZ score	-2.96 (-3.82, -1.48)	-2.23 (-3.91, -0.47)	-3.3 (-4.33, -1.40)	0.677
Male*	26 (63.4%)	4 (44.4%)	19 (63.3%)	0.566
ACHD*	34 (82.9%)	9 (100%)	21 (70%)	0.12
Expired*	2 (4.8%)	0	10 (33.3%)	**0.002**
Past hospitalisation*	33 (80.4%)	8 (88.9%)	25 (83.3%)	0.896
Shock*	1 (2.4%)	0	11 (30.55%)	**< 0.001**
Sick euthyroid illness*	13 (32%)	4 (44.4%)	20 (66.6%)	**0.018**
fT3 (pmol/L)	4.76 (3.75, 6.16)	4.9 (2.55, 6.77)	4.07 (3.13, 5.43)	0.275
fT4 (pmol/L)	15.3 (12.65, 23.02)	18.5 (17.85, 21.75)	14.35 (12.17, 15.8)	0.059
TSH (IU/mL)	2.38 (0.91, 3.85)	1.62 (0.96, 2.26)	1.61 (0.43, 3.43)	0.453
rT3 (ng/mL)	0.7 (0.61, 0.91)	0.88 (0.72, 1.24)	0.85 (0.65, 1.15)	0.086
fT3/rT3 index (pg/ng)	5.36 (2.74, 6.88)	4.13 (1.91, 5.99)	2.54 (1.51, 3.44)	**0.019**
NT-pro BNP (pg/mL)	2966 (1099, 14008)	8396 (672, 26517)	9690 (2721, 23515)	0.277
Creatinine (mg/dL)	0.2 (0.2, 0.3)	0.3 (0.25, 0.44)	0.3 (0.2, 0.38)	0.226
CRP (mg/dL)	2 (0.61, 4.51)	2.13 (0.5, 5)	3 (0.68, 9)	0.915
AST (IU/mL)	40.5 (34.5, 56.75)	37 (30, 53)	67 (38, 99.2)	**0.040**
ALT (IU/mL)	33.5 (18, 50.5)	37 (23.5, 43)	50 (30.25, 78.5)	**0.037**
Ejection fraction (%)	60 (55.25, 60)	60 (55.5, 60)	55 (50, 60)	**0.042**

Values shown in *numbers (%) for categorical variables or median (IQR) for continuous variables; comparison by *chi-square test or Kruskal-Wallis test.
AST: aspartate transaminase, ALT: alanine transaminase, BNP: brain natriuretic peptide, CRP: C-reactive protein, fT3: free tri-iodothyronine, fT4: free thyroxine, HAZ/LAZ: height or length for age Z score, Duration oxy: duration of oxygenation, rT3: reverse T3, TSH: thyroid stimulating hormone, WHZ/WLZ: weight-for-height/length Z score, fT3/rT3 index: units as pg/mL for fT3 and ng/mL for rT3

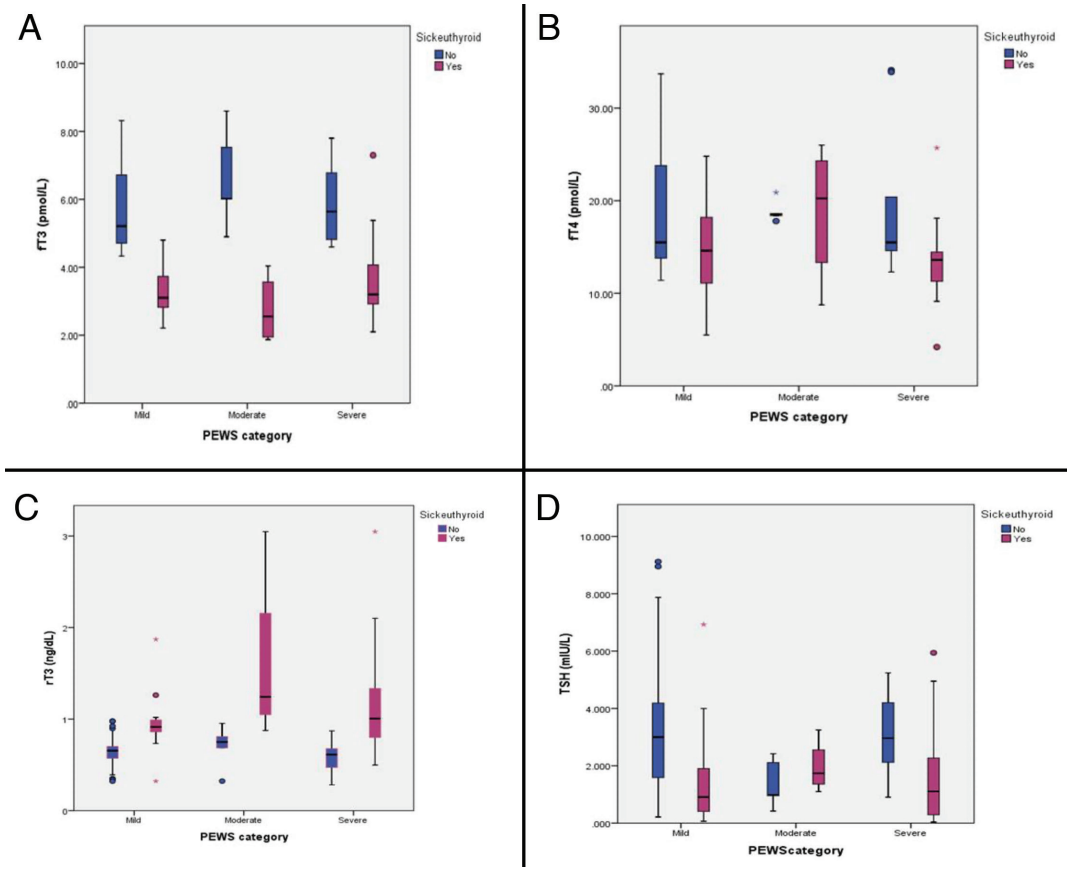

Figure 1. Box and Whisker plot showing (A) fT3 levels, (B) fT4 levels, (C) rT3 and (D) TSH levels in non-thyroidal illness versus severity of PEWS score

PEWS: Pediatric Early Warning Score, TSH: thyroid stimulating hormone

Table 2. Comparison of clinical and laboratory parameters as per sick euthyroid status

Parameters	Non-SES (n = 43)	SES (n = 37)	p
Age (mo)	6 (3, 11)	4.5 (2, 6)	0.096
Duration of Stay (d)	9 (7, 12.25)	11 (7.5, 15.5)	0.099
Duration of oxygenation (d)	5 (2, 10)	8 (4.5, 12.5)	**0.012**
PEWS	2 (1.75, 3.5)	5 (2, 9)	**0.003**
WAZ	-3.25 (-4.59, -2.68)	-3.35 (-4.47, -2.01)	0.563
HAZ/LAZ	-1.68 (-2.75, -0.57)	-1.97 (-2.99, -0.91)	0.311
WHZ/WLZ	-3.26 (-4.05, -2.04)	-2.57 (-3.92, -0.83)	0.108
fT3 (pmol/L)	5.7 (4.8, 6.8)	3.2 (2.7, 3.9)	**< 0.001**
fT4 (pmol/L)	17.1 (14.4, 23.6)	14.2 (10.95, 17)	**0.001**
TSH (mIU/L)	2.66 (1.4, 4.0)	1.1 (0.32, 2.2)	**< 0.001**
rT3 (ng/mL)	0.66 (0.47, 0.71)	0.97 (0.85, 1.26)	**< 0.001**
fT3/rT3 index (pg/ng)	6.19 (5.11, 7.72)	2.08 (1.42, 2.72)	
NT-pro BNP (pg/mL)	2786 (725, 8893)	14900 (3345, 26392)	**0.001**
Creatinine (mg/dL)	0.2 (0.2, 0.3)	0.3 (0.2, 0.39)	0.165
Ejection fraction (%)	50 (55, 60)	58 (50, 60)	0.053

Values shown in median (IQR) for continuous variables, comparison by Mann-Whitney U test.
AST: aspartate transaminase, ALT: alanine transaminase, BNP: brain natriuretic peptide, CRP: C-reactive protein, fT3: free tri-iodothyronine, fT4: free thyroxine, HAZ/LAZ: height or length for age Z score, PEWS: Pediatric Early Warning Score, rT3: reverse T3, SES: Sick euthyroid syndrome, TSH: thyroid stimulating hormone, WHZ/WLZ: weight-for-height/length Z score, fT3/rT3 index: units as pg/mL for fT3 and ng/mL for rT3

BNP had a significant negative correlation with TSH (r = -0.392, p < 0.001), ejection fraction (r = -0.307, p = 0.006) and a weaker insignificant correlation with fT4 (r = -0.082, p = 0.475). Multiple linear regression model for fT3 with independent variables PEWS, TSH, and log NT pro-BNP showed statistical significance with TSH and log NT pro-BNP levels as shown in Table 3. The statistical values to predict mortality as per the TH and serum NT pro-BNP are shown in Table 4.

Discussion

The present study showed a high proportion of NTI in children with CHD and CHF that manifested as low fT3 levels in the majority. The presence of severe disease predicted NTI and increased risk of mortality.

NTI has been reported in intensive care settings in pediatric studies (8,9,16) and adult studies (17,18). The pooled prevalence of NTI in adults with CVD was 21.7% (95% CI: 18.4-25.3); and was highest in patients with CHF (24.5%; 95% CI: 18.5-31.7), followed by acute myocardial infarction (18.9%; 95% CI: 10.4-31.9) and acute coronary syndrome (17.1%; 95% CI: 8.5-31.3) (1). NTI has also been reported in children who were critically ill or had undergone surgical repair of CHD (19). The present study also reported NTI in a significant proportion of enrolled children with CHF that corroborated with disease severity.

The overall median values of TH (fT4 and fT3) and TSH were normal in this study, in contrast to median rT3 levels that were higher than the normal range, suggesting that rT3 is the earliest affected thyroid parameter in acute sickness. The TSH levels appeared within normal range, but the loss of pulsatile fraction, decreased pulse amplitude, and absence of response to TRH has been reported earlier (20). These parameters were not assessed in the present study but it is likely,although unproven, that these were also affected.

Among natriuretic peptide neurohormones that are secreted as a result of myocardial stretch, NT pro-BNP is a biochemically stable molecule with a longer half-life than BNP, though biologically inactive (21). The levels increase in CHF and higher levels are associated with poor outcome (15,22). A few studies have reported in patients with surgical repair for CHD and higher serum BNP level that there were poorer outcomes (22,23,24). However, there is limited data on NT pro-BNP levels in children with CHF and the association with thyroid function that was measured in this study. The median NT pro-BNP levels were clearly more elevated than normal and higher in those with severe condition, as shown by the PEWS score association. Serum NT pro-BNP levels showed a significant correlation with fT3, TSH and rT3 in this study, as has been reported previously (25,26).

Low fT3 levels have been associated with poor outcomes in children and adults with illness in earlier studies (26,27,28). The presence of low T3 and raised BNP levels strongly predicted one-year all-cause mortality in acute decompensated CHF in adults, and weakly for in-hospital mortality (29). Serial trends in TH levels in 40 sick children with shock showed a decrease in the proportion of low fT3 levels as the sickness improved (82.5% at baseline to 72.5% five days after shock reversal) (30). The fT3 levels were significantly affected by raised BNP levels in this study, although PEWS disease severity remained the only significant predictor of mortality.

Table 3. Linear regression analysis for low free T3 levels

Model	R square change	Model	Beta	95% CI	p
1	0.207	Log NT-BNP	-0.455	-1.59, -0.61	< 0.001
2	0.275	Log NT-BNP TSH	-3.541 2.652	-1.387, -0.388 0.054, 0.383	0.001 0.010

PEWS excluded during stepwise regression analysis in the mode.
BNP: brain natriuretic peptide, fT3: free tri-iodothyronine, PEWS: Pediatric Early Warning Score, TSH: thyroid stimulating hormone, CI: confidence interval

Table 4. Laboratory cutoffs of thyroid hormones to predict mortality

Parameter	Value	Sensitivity	Specificity	AUC	95% CI	p
fT4 (pmol/L)	< 14.5	75%	64.2%	0.737	0.60, 0.88	**0.009**
fT3 (pmol/L)	< 4.15	66.7%	63.2%	0.600	0.41, 0.79	0.269
rT3 (ng/mL)	> 0.77	83.3%	52.4%	0.741	0.60, 0.88	**0.009**
NT-BNP (pg/mL)	> 3725	75%	49.3%	0.702	0.53, 0.88	**0.026**
fT3/rT3 (pg/ng)	< 1.861	87.3%	50%	0.284	0.129, 0.438	**0.018**

BNP: brain natriuretic peptide, fT3: free tri-iodothyronine, fT4: free thyroxine, rT3: reverse T3, fT3/rT3 index: units as pg/mL for fT3 and ng/mL for rT3, CI: confidence interval, AUC: area under the curve

Critical illness, such as infection and inflammation, are associated with NTI. A small proportion of children in this study had associated sepsis, and fewer among them had NTI, suggesting the etiology of NTI in the majority was cardiac in origin and less likely to be due to infection. A study in critically sick children with haemato-oncological and multisystemic disease reported that a fT4 cutoff of 16.6 pmol/L and fT3/rT3 ratio of 11.61 pg/ng predicted mortality (31). Our study had a lower threshold of fT4 (14.5 pmol/L) and fT3/rT3 ratio (1.86 pg/ng) to predict mortality, suggesting a lower threshold to monitor sick children with an underlying heart disease.

A few studies have analysed the cardiac response to thyroid supplementation in NTI and have reported contentious results. A meta-analysis did not conclude any beneficial role of either levo-thyroxine or triiodothyronine in NTI (32). An experimental study with the addition of levothyroxine preoperatively in children undergoing cardiac surgery showed a decrease in the inotrope requirement and post-operative cardiac injury (33). Similar results were also concluded in a meta-analysis of nineteen studies on children undergoing cardiac surgery for CHD with NTI that evaluated the role of preoperative levothyroxine. However, there were no significant improvements in clinical parameters, such as duration of hospitalisation, duration of oxygen supplementation, mechanical ventilation support, cardiac index or mortality (34). There is a lack of similar evidence of thyroxine supplementation in non-operative critical illnesses with NTI and this should be evaluated in the future.

Study Limitations

This is one of the few studies that have evaluated the TH axis in children with CHD during hospitalization with consideration to various clinical outcomes, taking into account the level of sickness as represented by the PEWS score. The limited observational period without a prospective follow-up until resolution of NTI was a limitation in this study. The intense severity scoring scales, like PRISM/sequential organ failure assessment were not performed due to logistic reasons.

Conclusion

NTI was seen in a significant proportion of children with CHD presenting with CHF. The severity of illness and CHF predicted poorer thyroid function status that was associated with mortality. This study highlights the need to monitor thyroid functions in children with CHD and CHF during acute sickness.

Ethics

Ethics Committee Approval: This observational study was conducted at the Department of Pediatrics and Cardiology in a large tertiary care public hospital from July 2021 until June 2022 after approval of the Institutional Ethics Committee Maulana Azad Medical College, New Delhi (letter F No.1/IEC/MAMC/82/10/2020/no. 125 dated: 14 Jan 2021).

Informed Consent: Written parental consent was taken before enrollment.

Authorship Contributions

Concept: Biswajit Sahoo, Aashima Dabas, Binita Goswami, Design: Aashima Dabas, Anurag Agarwal, Sumod Kurian, Data Collection or Processing: Biswajit Sahoo, Aashima Dabas, Binita Goswami, Anurag Agarwal, Sumod Kurian, Analysis or Interpretation: Biswajit Sahoo, Aashima Dabas, Sumod Kurian, Literature Search: Aashima Dabas, Binita Goswami, Anurag Agarwal, Writing: Biswajit Sahoo, Aashima Dabas, Binita Goswami, Anurag Agarwal, Sumod Kurian.

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CASE REPORT

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Juvenile Granulosa Cell Tumor Mimicking HAIR-AN in a 4-year-old: A Case Report

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What is already known on this topic?

Juvenile granulosa cell tumors typically present with pain, bloating, and a palpable mass on physical exam. They may also present with symptoms of precocious puberty and elevated serum estrogen or, less commonly, testosterone. Hyperandrogenism and hyperinsulinism are interrelated and may be seen in ovarian pathology but the exact mechanism of this relationship is not fully understood.

What this study adds?

This is a case of an unusual androgen secreting juvenile granulosa cell tumors accompanied by hyperinsulinemia in a prepubertal patient. Tumor removal resulted in resolution of hyperinsulinemia, which suggests that elevated testosterone may affect insulin levels.

Abstract

Predominantly androgen secreting juvenile granulosa cell tumors (JGCT) are uncommon and few reports have been published. We present a case of a JGCT that presented with signs of prepubertal hyperandrogenism and insulin resistance to highlight the possible interaction between hyperandrogenemia and hyperinsulinism. A 4-year-old girl presented with acanthosis nigricans and hyperinsulinism, mimicking the hyperandrogenism, insulin resistance and acanthosis nigricans syndrome at an age much younger than is typical for this diagnosis. Laboratory studies revealed elevated insulin, inhibin A and B, and total testosterone. All laboratory results normalized after unilateral salpingo-oophorectomy. The final diagnosis was Stage 1A JGCT. This case highlights the importance of including ovarian tumors in the differential diagnosis when considering causes of virilization and insulin resistance. This case also suggests a potential relationship between excess testosterone secretion and hyperinsulinemia and strengthens evidence that hyperandrogenemia may promote hyperinsulinism in ovarian disease.

Keywords: Juvenile granulosa cell tumor, hyperandrogenism, insulin resistance

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Introduction

Ten to twenty percent of all pediatric ovarian tumors are ovarian sex cord stromal tumors. A subtype of sex cord stromal tumors, which account for 70% of ovarian tumors in patients under the age of 20 years, includes granulosa cell tumors (1). A distinguishing feature of granulosa cell tumors is the secretion of estrogen (up to 97-98%), progesterone, and testosterone (2-3%) (2). Patients often present with symptoms of pain and bloating, with a palpable mass on physical exam and those with juvenile granulosa cell tumor (JGCT) may present with symptoms of precocious puberty and elevated serum estrogen (1). Increased expression of inhibin B may help distinguish JGCT from other causes of precocious puberty (3).

Type A insulin resistance syndrome is characterized by extreme insulin resistance, acanthosis nigricans, hirsutism and other features of polycystic ovarian syndrome (PCOS) in a non-obese patient (4). This syndrome has been described in females of all ages, with virilization present in infants and toddlers. Most cases are caused by insulin receptor mutations that reduce insulin affinity or insulin receptor tyrosine kinase activity (4). Treatment for insulin resistance syndrome is high doses of insulin. The clinically similar hyperandrogenism, insulin resistance and acanthosis nigricans (HAIR-AN) syndrome typically presents in adolescence or adulthood and includes obesity, hyperandrogenism and is characterized by the lack of insulin receptor mutations (5).

Here we present an illustrative case of a 4-year-old presenting with features associated with hyperinsulinemia and hyperandrogenism, which was caused by a rare, virilizing JGCT.

Case Report

The patient presented at 4-years and 1-month of age with a 6-month history of abnormal hair growth on her arms, legs, and pubic area, greasy scalp hair, facial acne, and intermittent mood swings. Her parents noted increased irritability that accompanied changes to her appearance. The patient's birth history, past medical and developmental history were unremarkable. Of note, no genital ambiguity was noted at birth. The patient's mother had a history of hemochromatosis and PCOS, and her maternal great-aunt and paternal great-grandmother had a history of ovarian cancer at advanced ages. There was no family history of type 2 diabetes mellitus or of precocious puberty, and the parents were not consanguineous.

Physical exam revealed a height of 110.4 cm [97.3% / +1.9 standard deviation (SD)], weight 29 kg (99.9% / +3.2 SD), and body mass index (BMI) 23.79 kg/m^2 (99.9% / +3.1 SD). The patient had extensive acanthosis nigricans on the back of her neck and in skinfold creases. She also had acne on her nose and chin, Tanner Stage 3 pubic hair, and thickened hair on her upper and lower extremities bilaterally. Lipomastia in the chest was consistent with her BMI, and there were no distinct breast buds. The patient

Table 1. Pre-operative and post-operative laboratory values

	Pre-operative	Within 2 weeks post-operative	Six months-1 year post-operative	Normal reference	Units
LDH	363	261	-	94-250	IU/L
AMH	6.75	4.306	2.98	0.256-6.345	ng/mL
Inhibin A	43.7	1.4	-	<7-14	pg/mL
Inhibin B	1715	25	15.2	<73.0	pg/mL
AFP	4	-	-	<8.0	ng/mL
CA125	13.6	-	-	0.0-38.0	ug/dL
β-hCG	<1	-	-	0-5	mIU/mL
FSH	<0.017	<0.1	-	1.0-4.2	mIU/mL
LH	<0.005	-	-	0.02-0.3	mIU/mL
Estradiol	11.8	-	<5	0-14.9	pg/mL
17-OH-progesterone	145	<10.00	-	0-90	ng/dL
DHEA-S	21.4	-	-	1.8-97.2	ug/dL
Testosterone, total	205.6	<1	4	≤20	ng/dL
Insulin	45.2	2	17	2.6-24.9	uIU/mL

LDH: lactate dehydrogenase, AMH: anti-Mullerian hormone, AFP: alpha-fetoprotein, FSH: follicle stimulating hormone, LH: luteinizing hormone, DHEA-S: dehydroepiandrosterone sulfate

had no clitoromegaly and the rest of her physical exam was within normal limits.

Initial laboratory evaluations were notable for an elevated free testosterone, lactate dehydrogenase, anti-Mullerian hormone, 17-hydroxyprogesterone (17-OH-P), insulin, inhibin B, and inhibin A (Table 1). β-hCG, α-fetoprotein, and CA-125 were all within normal limits. Follicle stimulating hormone level was low, and the remainder of her laboratory results were unremarkable (Table 1). Her bone age was 6-years and 4-months (chronological age 4-years and 1-month) as determined by interdisciplinary collaboration through evaluation by radiology and pediatric endocrinology using the method of Greulich and Pyle.

Pelvic ultrasound demonstrated a heterogeneous, hypoechoic, solid, vascular mass measuring 6.3 cm x 3.4 cm x 7.1 cm in the left adnexa with a normal right ovary with no cysts noted. A computed tomography investigation of the pelvis demonstrated a heterogeneous, solid, left-sided ovarian mass measuring 4.4 cm x 4.6 cm x 4.2 cm without intense enhancement and no evidence of metastatic disease or lymphadenopathy. The uterus was noted to be large for her age with the fundus larger than the cervix (Figure 1).

After unilateral salpingo-oophorectomy was performed, inhibin A, inhibin B, 17-OH-P, testosterone, anti-Mullerian hormone, and insulin levels normalized (Table 1). Most recently in follow-up at the age of 6 years 3 months, her height was 129.5 cm (99.1 % / +2.4 SD), weight 48.8 kg (99.9 % / +3.4 SD), and BMI 29.1 kg/m^2 (99.8 % / +2.9 SD). The acanthosis nigricans had substantially regressed. Her acne and hirsutism had completely resolved. Ultrasound showed a normal uterus and right ovary with no residual adnexal mass. Her bone age was 7-years and 10-months at the chronological age of 6-years.

Discussion

Our patient's extensive acanthosis nigricans and virilization was observed at an age typical for onset of type A insulin resistance, but too young to qualify as HAIR-AN (5). However, her obesity and modestly elevated insulin levels (significantly lower than values generally reported in type A insulin resistance) made a diagnosis of type A less likely. As neither of these diagnoses fitted the patient's presentation (obesity, moderate insulin levels, advanced bone age), laboratory and radiological studies were obtained which revealed a testosterone secreting JGCT with accompanying hyperinsulinemia. There is evidence that some JGCTs are associated with a heritable *DICER1* mutation, however, our patient did not fit the clinical picture for DICER syndrome (6,7). The distant family history of ovarian cancer was

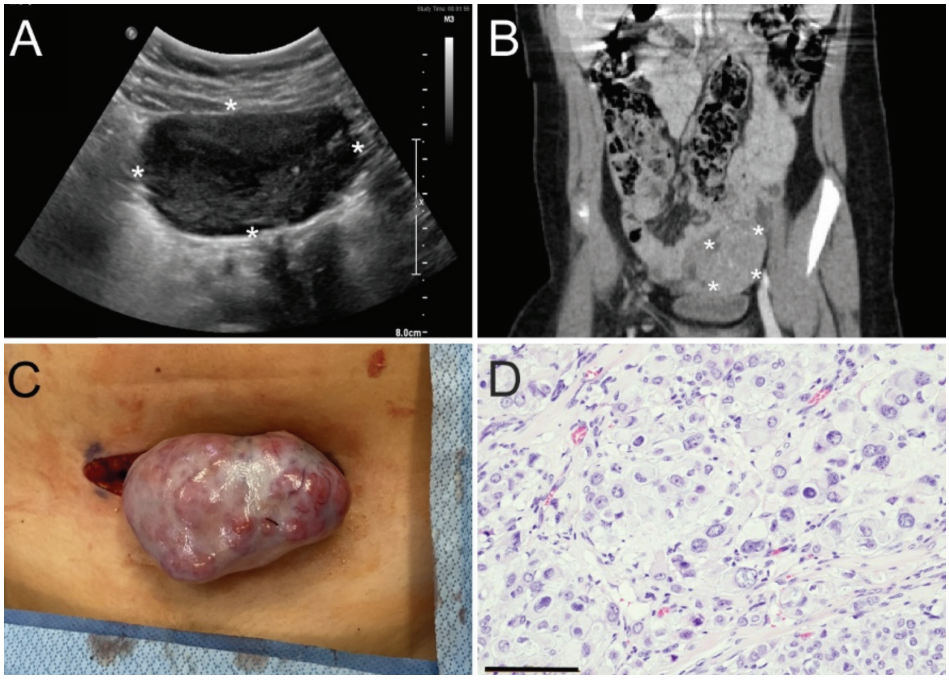

Figure 1. A) Sagittal ultrasound view of left ovarian lesion (outlined with *), **B)** Coronal view of CT abdomen-pelvis demonstrating solid left adnexal lesion (outlined with *), **C)** Left adnexal mass delivered through Pfannenstiel incision, **D)** Hematoxylin and eosin staining of JGCT demonstrating pleomorphic cells. Immunohistochemistry (data not shown) was positive for inhibin, and negative for pancytokeratin, WT1 and calretinin

CT: computed tomography, JGCT: juvenile granulosa cell tumors

associated with advanced age and is unlikely to represent genetic predisposition in our patient.

Granulosa cell tumors are rare, accounting for about 5% of pediatric ovarian tumors with an incidence of 2.6 per 100,000 females every year (1). Hormonally active JGCTs most often secrete estrogen, which leads to premature breast development, but they can also secrete androgens, which results in virilization. Kalfa et al. (3) identified six patients with JGCT associated with hyperandrogenism and found that all tumors had either absent or significantly decreased intra-tumoral expression of aromatase, suggesting that increased testosterone production may be attributed to decreased conversion of androgens into estrogens within these tumors.

We speculate that in this patient, testosterone secretion led to the hyperinsulinism and substantial acanthosis nigricans, because her fasting insulin declined and her acanthosis nigricans improved after complete resection of the tumor. Her increased weight may have also contributed to her initial hyperinsulinemia, however on follow up her insulin levels had normalized despite her BMI increasing. Additionally, clinical findings of hyperinsulinemia are not typically seen in overweight children of this age. We found only three reports of JGCT that presented with increased insulin, all of which were accompanied by hyperandrogenism. Larizza et al. (8) described a 16-year-old female presenting with secondary amenorrhea, high dehydroepiandrosterone (DHEA), 17-OH-P, and insulin who was found to have a JGCT. Ovulation resumed ten days after the tumor was removed, and DHEA, 17-OH-P, and insulin returned to normal levels at post-operative follow up. Kwiatkowska et al. (9) report a case of JGCT in a 17-year-old female who presented with elevated testosterone and insulin. Testosterone levels subsequently decreased after tumor excision. Post-operative insulin levels were not reported. Brisigotti et al. (10) identified a 7-week-old patient with Donohue syndrome who had clinical hyperandrogenism, hyperinsulinemia, and bilateral JGCTs. Unfortunately, the patient died two days after resection of the JGCTs, so no data is available as to whether hyperandrogenism and hyperinsulinemia resolved following the surgery. The present case did not meet clinical criteria for Donohue syndrome.

The extent to which hyperandrogenemia and hyperinsulinemia reciprocally influence each other is an area of active research. Although testosterone has been shown to cause insulin hypersecretion through androgen receptor signaling (11), others have hypothesized that increased insulin production induces ovarian hyperandrogenism in conditions such as PCOS (12). Mishra et al. (11) demonstrated that increased exposure to androgens resulted in hyperinsulinemia in female rats through dose-dependent upregulation of the insulin gene (*Ins*). They identified an androgen receptor binding site on the promoter region of *Ins,* which they hypothesize facilitates this response. Huang-Doran et al. (12) found that gonadotropin releasing hormone agonists reduced androgen levels in patients with severe insulin resistance without affecting insulin sensitivity, suggesting that hyperinsulinism is the main driver of hyperandrogenism in individuals with insulin resistance. In contrast to our patient's age (4-years), their study focused on patients over the age of 10-years with a presumed mature hypothalamic-pituitary-gonadal axis (HPGA) (12). The presented case provides insight into the relationship between hyperandrogenism and hyperinsulinemia because the patient presented at a young age with an immature HPGA, allowing a unique perspective on the relationship between androgens and hyperinsulinism in the absence of gonadotropins. This case report suggests that excess androgen production in a prepubertal female may result in increased insulin production.

Conclusion

We report a case of an androgen secreting JGCT, which mimicked HAIR-AN/type 1A insulin resistance. This case emphasizes the importance of a broad differential when considering potential causes of virilization and insulin resistance and highlights the possible relationship between testosterone secretion and hyperinsulinemia.

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Ethics

Informed Consent: Informed written consent form was obtained from parents before participating the study.

Authorship Contributions

Data Collection or Processing: Rachel Choe Kim, Hamama Tul-Bushra, Rebecca Batiste, Andrew H. Lane, Helen Hsieh, Analysis or Interpretation: Rachel Choe Kim, Andrew H. Lane, Helen Hsieh, Literature Search: Rachel Choe Kim, Ilya Goldberg, Andrew H. Lane, Helen Hsieh, Writing: Rachel Choe Kim, Ilya Goldberg, Trevor Van Brunt, Andrew H. Lane, Helen Hsieh.

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CASE REPORT

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A Case of Diabetes Mellitus Type MODY5 as a Feature of 17q12 Deletion Syndrome

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What is already known on this topic?

The hepatocyte nuclear factor-1-beta (*HNF1B*) gene, is important in the regulation of tissue-specific gene expression so that mutations in *HNF1B* may lead to organ abnormalities. However, *HNF1B* mutations may be difficult to diagnose as there is a large phenotypic variation. The 17q12 microdeletion syndrome, also termed 17q12 deletion syndrome, is a rare chromosomal anomaly caused by the deletion of a small amount of material from a region in the long arm of chromosome 17. It is typified by deletion of more than 15 genes, including *HNF1B*, resulting in organ abnormalities and neurodevelopmental disorders.

What this study adds?

In case of clinical suspicion of *HNF1B* variants, further genetic examination using other techniques such as MLPA and CGH array may be required to detect the variant. This is because deletions and duplications may not be detected using next generation screening panel techniques.

Abstract

Maturity onset diabetes of the young (MODY) is characterized by noninsulin-dependent diabetes diagnosed before the age of 25 years with an autosomal dominant inheritance. Rare mutations in the hepatocyte nuclear factor-1-beta (*HNF1B*) gene produce a syndrome that resembles MODY. About half of patients diagnosed with MODY type 5 due to *HNF1B* variants, carry a whole gene deletion, known as 17q12 deletion syndrome. 17q12 deletion syndrome is a rare chromosomal anomaly and is typified by deletion of more than 15 genes, including *HNF1B* resulting in kidney abnormalities and renal cysts, a diabetes syndrome and neurodevelopmental or neuropsychiatric disorders. A 12-year-old girl was referred after high blood sugar was detected in the hospital where she presented with polyuria and polydipsia, which had persisted for one month. Her serum magnesium (Mg) level was low at 1.5 mg/dL (normal value 1.6-2.6) and glycated hemoglobin was 14% (normal value 3.6-5.8) concurrent with a c-peptide of 1.54 ng/mL (normal value 0.8-4). MODY5 was suspected but the NGS gene panel (*ABCC8, BLK, CEL, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11, KLF11, NEUROD1, PAX4, PDX1, RFX6, ZFP57, GLIS3, FOXP3, NEUROG3, G6PC2*) did not identify any abnormality. During follow-up, her serum Mg remained low (1.2 mg/dL) together with elevated urinary Mg excretion at 172.5 mg/day. An *HNF1B* variant was again suspected in a patient with chronic hypomagnesemia with normal basal C peptide level. Abdominal computed tomography and magnetic resonance imaging revealed a 43 mm diameter, cystic lesion in the head of the pancreas, with agenesis of the pancreatic neck, trunk and tail. Genetic testing using a microarray analysis was subsequently performed and a heterozygous deletion at 17q12, including *HNF1B*, was detected. In case of clinical suspicion of *HNF1B* variants, further genetic examination using other techniques such as MLPA and CGH array may be required to detect the variant. This is because deletions and duplications may not be detected using next generation screening panel techniques.

Keywords: MODY5, 17q12 deletion, diabetes mellitus, hypomagnesemia

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Introduction

Maturity onset diabetes of the young (MODY) is a clinically heterogeneous disorder characterized by noninsulin-dependent diabetes diagnosed at a young age (<25 years) with an autosomal dominant inheritance. MODY results from heterozygous mutations in various transcription factors acting in the development and function of pancreatic beta cells (1). Mutations in hepatocyte nuclear factor-1-alpha (*HNF1A*) and the glucokinase (*GCK*) gene are most commonly identified (2). Rare mutations in the hepatocyte nuclear factor-1-beta (*HNF1B*) gene produce a syndrome that resembles MODY and has been termed MODY5 (3).

HNF1B has an important role in the regulation of tissue-specific gene expression and mutations in *HNF1B* may lead to organ abnormality in both pancreas and kidney. Affected patients can develop a variety of manifestations in addition to early-onset diabetes. These include pancreatic atrophy, which may be evident on computed tomography (CT) scan, and abnormal renal development, which may be visible as renal dysplasia on ultrasonography in the fetus. There may be single or multiple renal cysts, glomerulocystic disease, oligomeganephronia (a form of renal hypoplasia) and slowly progressive renal insufficiency. Other abnormalities may include hypomagnesemia, elevated serum aminotransferases, and genital abnormalities, such as epididymal cysts, atresia of vas deferens, and bicornuate uterus (4). Almost half of patients diagnosed with MODY5 (*HNF1B* mutation) harbor a whole gene deletion (5). 17q12 microdeletion syndrome, also known as 17q12 deletion syndrome, is a rare chromosomal anomaly caused by the deletion of a small amount of material from a region in the long arm of chromosome 17. It is typified by deletion of more than 15 genes, including *HNF1B*, resulting in kidney abnormalities and the renal cysts and diabetes syndrome, with neurodevelopmental or neuropsychiatric disorders (6).

Here, we report a patient presenting with MODY5 diabetes who was eventually diagnosed with 17q12 deletion syndrome identified by microarray analysis.

Case Report

A 12-year-old girl was referred to our clinic, after high blood sugar was detected in the hospital where she attended because of polyuria and polydipsia of one month standing. She was born at term weighing 2250 g [-2.5 standard deviation (SD)] by normal spontaneous vaginal delivery. There was no family history of diabetes mellitus (DM). On physical examination, her body weight was 45 kg [0.29 SD score (SDS)], height 149 cm (-0.81 SDS), body mass index (BMI) 20.0 kg/m^2 (0.18 SDS), and vital signs were stable. There was no acanthosis nigrigans. On admission, random blood glucose level was 429 mg/dL, blood ketone was 2.2 mmol/L with normal blood pH. Of note, serum magnesium (Mg) level was low at 1.5 mg/dL (normal value 1.6-2.6 mg/dL). Her hemoglobin A1c was 14% (normal value 3.6-5.8%) and her C-peptide was within the normal range at 1.54 ng/mL (normal value 0.8-4 ng/mL). She was initially treated with basal-bolus insulin regimen and oral Mg treatment. Subsequently, her Mg level increased to 1.9 mg/dL. Type 2 DM was not considered because the patient did not have obesity and there were no signs of insulin resistance, such as acanthosis nigricans, hypertension, or hirsutism. Early-onset DM or MODY were considered in the differential diagnosis. Anti-GAD antibody was negative. Although there was no family history of diabetes in three generations, based on the other findings, MODY5 was suspected. Her DNA was extracted and tested on a next generation sequencing (NGS) gene panel including *ABCC8, BLK, CEL, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11, KLF11, NEURODD1, PAX4, PDX1, RFX6, ZFP57, GLIS3, FOXP3, NEUROG3,* and *G6PC2*. However, no abnormality was detected on this screening panel. The patient was followed up with 1 U/kg/day basal bolus insulin therapy. When she was 16 years old, she developed morbid obesity (BMI 39.1 kg/m^2). Physical examination revealed clinical signs of insulin resistance, including acanthosis nigricans and hypertension. Her C-peptide level remained normal at 3.04 ng/mL when postprandial blood sugar level was 391 mg/dL. Her mother had been diagnosed with type 2 DM one year previously. Therefore, metformin was started (500 mg/day) considering type 2 DM, but she could not tolerate the treatment due to abdominal cramps. When she was 17 years old, the patient complained of numbness in the hands and feet. Her serum Mg level was found to be low (1.2 mg/dL) and her urinary Mg excretion was high at 172.5 mg/day. *HNF1B* gene mutation was again considered in this patient with chronic hypomagnesemia with increased basal C-peptide level. Targeted diagnostic work up with abdominal CT and magnetic resonance imaging (MRI) revealed a 43 mm diameter, cystic lesion in the head of the pancreas, accompanied by agenesis of the pancreatic neck, trunk and tail (Figure 1). There were no renal or urinary anomalies on imaging and liver function tests were normal. Although fecal elastase was not available, there were no symptoms of malabsorption. As the earlier NGS screening panel had not detected any variant in the *HNF1B* gene, microarray analysis was performed and a heterozygous deletion of 1.63 Mb of DNA at chromosomal location 17q12, including *HNF1B*, was detected (Figure 2). No mutation was detected in her parents. In terms of accompanying anomalies, an arcuate uterus anomaly was found on pelvic MRI. When she was evaluated

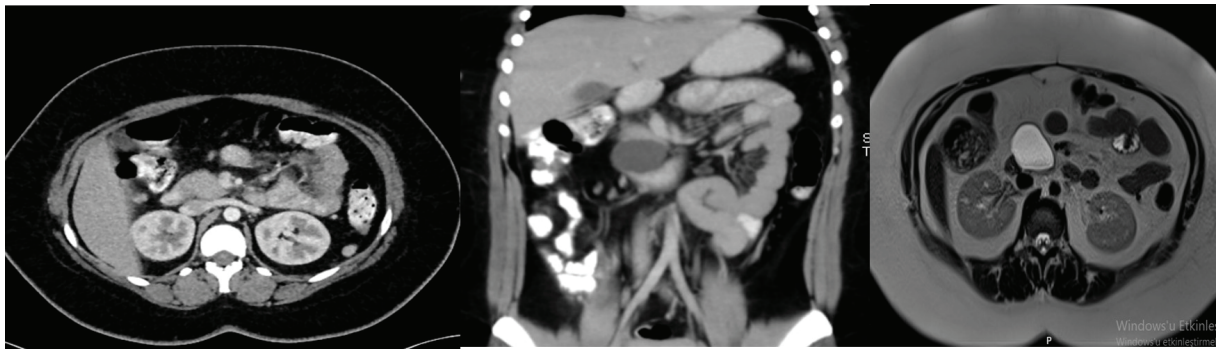

Figure 1. Abdominal computed tomography and magnetic resonance images demonstrating a cystic lesion in the head of the pancreas, and agenesis of the pancreatic neck, trunk and tail

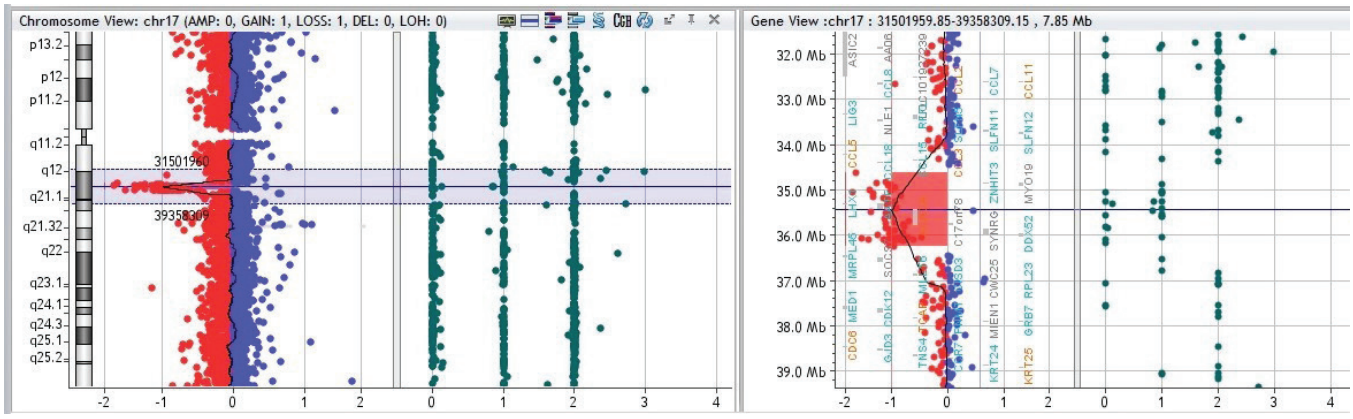

Figure 2. Microarray analysis demonstrated a prominent, heterozygous deletion of a 1.63 Mb-spanning DNA sequence at chromosomal location 17q12, which included the *HNF1B* gene

in terms of neuropsychiatric disorders that may accompany 17q12 deletion, the pediatric psychiatry clinic diagnosed anxiety disorder and obsessive compulsive disorder.

Informed consent was granted by the parents of the patient for publication.

Discussion

The presented patient was eventually found to have 17q12 microdeletion, harboring more than 15 genes, one of which was *HNF1B*. The 17q12 deletion syndrome consists of MODY5 type DM, renal malformation, impaired renal function, pancreatic malformations and neurodevelopmental/neuropsychiatric disorders. HNF1B is known to play an important role in the development of the kidney, liver, pancreas and urogenital tract during the embryonic period (7).

Renal dysfunction and anatomical malformations frequently accompany *HNF1B* mutation (8). Multicystic dysplastic kidney is the most common renal cystic disease. Apart from cystic renal disease, other renal abnormalities reported include solitary kidney, renal hypoplasia, and horse-shoe kidney (9). In the present case there were no structural renal anomalies evident. However, serum electrolyte imbalances, including hypokalemia, hyperuricemia, and hypomagnesemia are also common in patients with *HNF1B* mutations. *HNF1B* is essential for the expression of *FXYD2*, a subunit of Na^+/K^+-ATPase and is involved in the reabsorption of Mg in the distal convoluted tubule (10). These electrolyte imbalances develop with age and became apparent in late childhood. The present case had hypomagnesemia, hyperuricemia (uric acid 7,9) (normal value 2,6-6,0) with normal serum potassium levels. The low Mg level only became evident in late adolescence.

HNF1B mutation-related MODY5 DM has been reported in 63% of patients with 17q12 deletion syndrome (9). Since *HNF1B* is related to pancreatic organogenesis, variants in this gene are often associated with pancreatic malformations (11). In these cases, DM is caused by insulin deficiency due to pancreatic hypoplasia (12). However, hepatic insulin resistance also plays a role in the pathogenesis. Patients are diagnosed in adolescence and early adulthood. In a UK study, it was reported that around 24% of patients with *HNF1B* gene mutations developed DM at a median age of 12 (10,11,12,14) years (13). The presented patient was not

diagnosed with diabetes until the age of 12.5 years. In a study, it was reported that patients with 17q12 deletion syndrome had lower BMI and a greater insulin requirement at diagnosis compared to patients with intragenic *HNF1B* mutations (14). In the present case, her BMI was in the normal range at the time diagnosis, but morbid obesity developed during follow-up despite receiving high-dose insulin treatment (1 U/kg/day basal-bolus insulin at the time of diagnosis). Although diagnostic imaging demonstrated pancreatic neck, trunk and tail agenesis, there was no clinical sign or symptoms of exocrine pancreas disorder. Unfortunately, fecal elastase testing was not available.

Liver function test abnormalities including elevation of transaminases and mild hyperbilirubinemia may be seen in patients with *HNF1B* gene mutation or 17q12 deletion syndrome (15). However, the mechanism causing these abnormalities is not completely clear. Liver biopsy histology has reported increased steatosis, periportal fibrosis and hypoplastic bile duct (16). In the presented patient, there were no remarkable liver function test abnormalities.

Autism, cognitive disorders, neuropsychiatric and neurodevelopmental disorders have been reported in patients with 17q12 deletion syndrome, in contrast to patients with *HNF1B* intragenic mutations (17). Clissold et al. (17) detected neurodevelopmental disorders in patients with 17q12 deletion/without *HNF1B* mutation. On the other hand, Lim et al. (7) reported that three (21%) patients had neurologic findings of 14 patients with intragenic *HNF1B* mutations, one of which was a whole gene deletion, and the other two had missense mutations.

Of note, 17q12 deletion includes the *LHX1* and *ACACA* genes. *LHX1* is expressed in the brain in early development and therefore represents a candidate gene for the neurocognitive phenotype. *LHX1* variants have been reported to be associated with epilepsy, autism, and mental retardation (18). However, Loirat et al. (19) reported that three patients with 17q12 deletion in a large cohort of 86 patients with *HNF1B* gene abnormalities had developed autism, growth retardation and social interaction impairment over time. These patients had negative genetic tests for autism. In these three patients and 32 control patients with autism, no mutation was found in the *LHX1* gene and it was considered that autism might be an additional finding to the *HNF1B* deletion (19). The presented patient had a deletion in the *LHX1* gene and was being followed up by a child psychiatrist with diagnosis of anxiety disorder and obsessive-compulsive disorder. It has been reported that facial dysmorphism may be present in some patients with 17q12 deletion (9). However, there was no facial dysmorphism in the presented case. 17q12 deletion can be inherited as autosomal dominant or *de novo*, with 70% developing as a result of *de novo* mutation. Therefore, the absence of diabetes and other clinical features in the family does not exclude 17q12 deletion syndrome. In the presented patient, because there was no history of diabetes in three generations and no mutation was found on genetic screening panel for analysis of the parents, we strongly suspect that the mutation developed *de novo*.

HNF1B variants may be difficult to diagnose and the resulting syndrome has a large phenotypic variation. Furthermore, most of these mutations occurs *de novo*. Faguer et al. (20) developed an *HNF1B* scoring system to select patients for *HNF1B* gene analysis, based on clinical, imaging and biological variables. This scoring system consists of 17 parameters and a total score of >8 increases the probability of an *HNF1B* variant, in which case genetic analysis is recommended for these patients (20). The presented patient scored 6 points using this scoring system. As has been previously reported and when considering the presented case too, the specificity of the scoring system is low (21). In patients with 17q12 deletion syndrome, genital malformations due to unsuccessful fusion of the Mullerian ducts can be seen. It has been reported to be a risk factor for Mayer Rokitansky-Küster Hauser syndrome (22). It has been suggested that this may be related to *LHX1* mutation (23). Bernardini et al. (22) did not detect a mutation in the *LHX1* gene in the chromosome 17 on CGH array analysis of 20 patients with Mayer Rokitansky-Küster Hauser syndrome. It was reported that genital anomalies might only be seen in patients with *HNF1B* point mutations, and thus an intact *LHX1* gene. Interestingly, despite our patient having no *HNF1B* point mutation, she had arcuate uterus anomaly and an *LHX1* gene mutation.

Finally, it has been reported that *HNF1B* is required in the expression of parathyroid hormone and that *HNF1B* mutation may result in hyperparathyroidism without renal failure (24). There were no findings of hyperparathyroidism in our patient.

Conclusion

In conclusion, findings accompanying diabetes in children and should be carefully evaluated. Since 17q12 deletion is often *de novo*, monogenic diabetes should be considered in the presence of clinical findings, even if there is no family history of diabetes. In case of clinical suspicion, further genetic examination using techniques such as MLPA or CGH array, may be required since deletions and duplications may be missed on NGS panels including the *HNF1B* gene. In addition, patients diagnosed with MODY5 should be screened for 17q12 deletion sydrome, when neurological

developmental delay and/or psychiatric disorders are present.

Ethics

Informed Consent: Informed consent was granted by the parents of the patient for publication.

Authorship Contributions

Concept: Hümeyra Yaşar Köstek, Filiz Tütüncüler Kökenli, Design: Hümeyra Yaşar Köstek, Fatma Özgüç Çömlek, Emine Neşe Özkayın, Filiz Tütüncüler Kökenli, Data Collection or Processing: Fatma Özgüç Çömlek, Hakan Gürkan, Filiz Tütüncüler Kökenli, Analysis or Interpretation: Hümeyra Yaşar Köstek, Fatma Özgüç Çömlek, Hakan Gürkan, Emine Neşe Özkayın, Filiz Tütüncüler Kökenli, Literature Search: Emine Neşe Özkayın, Filiz Tütüncüler Kökenli, Writing: Filiz Tütüncüler Kökenli.

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CASE REPORT

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Endocrine Evaluation and Homeostatic Model Assessment in Patients with Cornelia de Lange Syndrome

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What is already known on this topic?

Cornelia de Lange syndrome (CdLS) is a rare developmental genetic disorder associated with short stature and delayed puberty. However, research on other hormonal assessments in this condition, such as high homeostatic model assessments of insulin resistance (HOMA-IR), is scarce.

What this study adds?

Three of seven prepubertal patients with CdLS had high HOMA-IR values but no metabolic risk factors, suggesting insulin resistance in this population. Two of the 17 postpubescent patients had altered HOMA-IR values associated with increased body mass index, which to the best of our knowledge, has not been published before. These findings highlight the importance of endocrine follow-up in these patients.

Abstract

The aim of this study was to expand knowledge about endocrine disorders in individuals with Cornelia de Lange syndrome (CdLS), a rare developmental genetic disorder with anomalies in multiple organs and systems. Hormone levels, clinical scores, anthropometric measurements, and molecular analysis were assessed in 24 individuals with CdLS. Hyperprolactinemia was the most common endocrine disorder. Three patients showed subclinical hypothyroidism. Concerning the gonadotropic axis, mildly delayed puberty was observed, as well as genital anomalies, such as cryptorchidism. Despite short stature, levels of insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 tended to be normal. Three prepubertal individuals without risk factors had higher than normal values for the homeostatic model assessment of insulin resistance (HOMA-IR) and for insulinemia, suggesting insulin resistance. Furthermore, two adults had elevated body mass indexes associated with HOMA-IR values over the cut-off values. CdLS may lead to dysregulation of the endocrine system, particularly in patients with high HOMA-IR values and insulinemia who are at risk of insulin resistance. Therefore, clinical follow-up with comprehensive hormonal assessment appears warranted in individuals with CdLS.

Keywords: Cornelia de Lange syndrome, HOMA-index, insulin resistance, endocrine evaluation and hypothalamic-pituitary axis

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Introduction

Cornelia de Lange syndrome (CdLS) [(OMIM) #122470, 300590, 300882, 610759 and 614701)] is a congenital malformation syndrome characterized by distinctive facial features, microcephaly, growth retardation, and anomalies in multiple organs and systems. The prevalence of CdLS is estimated to be below 1:30000 live births (1). The onset of this syndrome has been linked to mutations involving proteins associated with the cohesin complex, which is a basic regulator of chromosomal biology. Eight causal genes (*NIPBL, SMC1A, SMC3, RAD21, BRD4, HDAC8, ANKRD11,* and *MAU2*) (2,3,4,5) and several candidate genes have been identified. Mosaicism and splicing mutations are relatively frequent (6,7,8). Thus, a broad phenotypic spectrum (9) has been described, and a clinical score (2) has been developed.

Recently, small-fibre neuropathy and changes in body composition have been associated with CdLS (10,11). Abraham and Schlesinger (12,13) performed the first endocrine studies, Kline et al. (14) reported CdLS-specific growth charts, and Schwartz et al. (15) published a pituitary study of five patients. In 2007, an extensive study including comprehensive endocrinological work-ups on 49 patients with CdLS reported mildly delayed puberty (1), and these findings were included in a CdLS consensus paper published in 2018 (2). However, a thorough endocrine evaluation of patients with CdLS has rarely been reported. Thus, the aim of this study was to expand the knowledge of endocrine findings in patients with CdLS.

Patients

A descriptive study of 24 Spanish individuals aged 2-37 years, seven (29.2%) of whom were prepubescent and the remaining 17 postpubescent patients, with CdLS was performed. All subjects were evaluated by a paediatrician or a clinical geneticist with experience in CdLS. After comprehensive and detailed clinical and auxologic evaluations, patients were classified based on recently published CdLS consensus criteria (2). Pubertal development in children was assessed by an expert endocrinologist using Tanner staging (16). Adult participants completed a questionnaire reporting the time of onset of main secondary sexual characteristics. All data were confirmed by checking levels of gonadotropic and sex steroid hormones. Informed consent from participants, their parents, or legal guardians was obtained before entry into the study.

Hormonal Studies

Venous blood samples were drawn and centrifuged, and plasma or serum was separated. Levels of insulin, thyrotropin, thyroxine, prolactin, adrenocorticotropic hormone, cortisol, luteinizing hormone, follicle-stimulating hormone, 17-beta-estradiol, and total testosterone were measured using specific electrochemiluminescence assays in a Cobas e601 autoanalyzer (Roche Diagnostic, Mannheim, Germany). Serum glucose was analysed using an enzymatic spectrophotometric method with the Cobas 8000 autoanalyzer (Roche Diagnostic). Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-binding protein-3 (IGFBP-3) levels were measured using chemiluminescent immunometric assays (Immulite 2000Xpi, Siemens Healthcare Diagnostics, Los Angeles, CA, USA). After ruling out comorbidities, analyses were performed while participants were fasting and in a resting state for 20-30 minutes.

Weight was measured in kilograms (kg) using an AMGI-IMSA model and height in centimetres (cm) using a Harpenden Tallimeter. Body mass index (BMI) was calculated as weight (kg) divided by height in metres squared (m^2). The obtained value was expressed as a Z-score, according to the reference graphs in a Carrascosa Lezcano et al. (17) (2010) Spanish Growth Study. Insulin resistance was calculated by means of the homeostatic model assessment of insulin resistance (HOMA-IR), defined according to the following equation: HOMA-IR = fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5 (18). All molecular analyses for each individual were performed using a custom, targeted gene panel including *NIPBL, SMC1A, SMC3, RAD21, HDAC8, BRD4, ANKRD11,* and *MAU2* (19). Chromosomal studies were performed using standard methods.

Ethics Committee Approval: The ethical guidelines for human research outlined by the Declaration of Helsinki and revised by Fortaleza (2013) were followed. The Ethics Committee of Clinical Research from the Government of Aragón (CEICA; PI15/00707) and the subjects approved the study protocol.

Results

Study participants totalled 24 Spanish patients with CdLS, of whom nine (37.5%) were male. The patients were aged 2-37 years and seven (29.2%) were prepubescent with the remaining 17 being postpubescent. Most had mild involvement of most endocrine axes. In the thyroid axis, three participants had normal or low thyrotropin level and low thyroxine level. Hyperprolactinemia was the most common endocrine disorder, affecting half of the participants (P1, P2, P4, P6, P9, P13, P14, P15, P16, P18, P20, and P22). Regarding pubertal and gonadal function, two male and one female patient had mildly delayed puberty, and four of the nine males had bilateral cryptorchidism.

Four females (P15, P16, P19, and P21) reported having irregular menstrual cycles; a 16-year-old participant (P17) showed absence of menarche and no breast development. Chromosomal studies were normal in all cases. Among all 24 participants, 63% had prenatal growth retardation, and 80% had postnatal growth retardation. Finally, the HOMA-IR values in three prepubertal (P5, P7, and P8) and two adult participants (P21 and P22) exceeded the cut-off points. Tables 1 and 2 summarize these results.

Discussion

Although individuals with CdLS rarely develop severe endocrine disorders, the endocrinological work-ups reported here suggest a mild involvement of most axes. Decreased thyroxine values and normal thyrotropin levels might suggest a central subclinical hypothyroidism; however, no abnormalities were detected on brain imaging. Hyperprolactinemia was the most frequent endocrine disorder, occurring in 50% of participants, among whom P9, P13, P14, P18, P20, and P22 were taking antipsychotic drugs that may explain this increase, but P1, P2, P4, P6, P15, and P16 were not undergoing any treatment. Furthermore, six had normal adrenocorticotropic hormone and cortisol levels, thus ruling out acute stress as a cause of their hyperprolactinemia.

Regarding gonadal function, cryptorchidism (14) was observed in two prepubertal participants (P1 and P6) and two pubertal ones (P14 and P18), suggesting that dysfunction of the pituitary-gonadal axis could be present in early gestation. In addition, P14 and P18 had delayed puberty, as diagnosed by the absence of testicular development at age 14 years and no progression of secondary sexual characteristics at more than two years after pubertal onset. Seven female participants reported irregular menstruation and one (P17) reported delayed puberty and lack of breast development at age 13 years. In these patients, gonadotropins were not elevated, suggesting a possible central origin of the disorder. Regardless of LH levels, clinically there is often a pubertal delay, and LH values suggest that central hypogonadism may be transitory or permanent depending on the evolution, thus requiring close clinical follow-up.

Prenatal and postnatal growth retardation is a common feature in individuals with CdLS (14,15,20). In the present study, 20 of the 24 patients had heights >2 standard deviation (SD) below the means for age and sex, and 15 were born small for gestational age (>2 SD below means for birth weight or birth length), indicating a prenatal origin. In addition, 80% did not show catch-up growth at four years of age. However, levels of IGF-1 and IGFBP-3 were normal for all, except in P5, whose low BMI could indicate malnutrition associated with secondary IGF-1 deficiency.

One research objective of this study was to evaluate carbohydrate metabolism in these patients. Although the euglycemic-hyperinsulinemic clamp method is considered the gold standard technique to estimate insulin sensitivity, this approach can be invasive for patients with intellectual disabilities and behavioural disorders. Therefore, it is more appropriate to use a simple and indirect method, such as the HOMA-IR index (21), which estimates insulin resistance using a simple equation. However, the cut-off point for insulin resistance on this index remains a matter of controversy. In adults, more invasive techniques have confirmed a cut-off point of 3.8 (22). In children, such studies are more difficult and ethically controversial. However, a previous study in a Spanish cohort of 372 individuals established 3.42 as the cut-off point (23), and a recent meta-analysis of populations of various ethnicities indicated cut-off points between 2.30 and 3.54 (24).

Five participants, or approximately 21% of the study population, had HOMA-IR levels exceeding the respective 3.54 cut-off for insulin resistance in children (25) and the 3.8 cut-off for adults. Prepubertal participants P5, P7, and P8 had HOMA-IR values of 4.53, 5.54, and 7.2, respectively. Adults P21 and P22 had HOMA-IR values of 6.23 and 10.7, respectively. These results could be related to obesity and increased BMI, particularly in adults. However, the three prepubertal patients all had normal BMI and no family history or risk factors, such as hypertension or obesity, suggesting that CdLS may be associated with increased insulin resistance. Notably, a fasting insulin value above 16 µU/mL in children and adults is considered suggestive of hyperinsulinemia (22,25). Thus, all participants with elevated HOMA-IR values also had high blood insulin levels.

To the best of our knowledge, this is the first study to associate CdLS with elevated insulin and HOMA-IR values. It therefore seems reasonable to recommend follow-up assessments of carbohydrate metabolism in these patients. Close endocrinological follow-up is also necessary to assess nutritional status, growth, and pubertal development in patients with CdLS so that severe endocrinological alterations of central origin, such as hypothyroidism and/or hypogonadism, can receive appropriate treatment as soon as possible.

Limitations of this study include the low incidence of this rare disease and the absence of elderly participants. The method of measuring insulin resistance may also be considered a limitation although HOMA-IR is widely used as it is more practical. In most cases, hyperglycaemic/euglycemic clamp is the gold standard for quantifying *in vivo* insulin action, secretion, and disposal, but clamp studies are expensive to conduct and invasive, which raises ethical concerns

Table 1. Anthropometric values, clinical score and affected gene in individuals with CdLS patients

Patient	*P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Age	2	2	3	3	3	4	5	5	7	8
Gender	M	M	M	M	F	M	F	F	M	F
Gene*	*H*	*N*	*N*	*N*	*N*	*N*	*R*	*S*	*N*	*N*
Clinical score*	11	14	7	14	15	13	8	5	6	13
Pubertal stage (Tanner)	I	I	I	I	I	I	I	I	I	I
Birth weight (SDS)	-1.24	-1.15	-1.08	-1.09	**-2.31**	**-2.17**	0.44	-1.04	-0.69	-1.9
Birth length (SDS)	**-2.57**	**-2.39**	-0.97	**-2.18**	-1.27	**-3.45**	-0.28	0.57	1.69	-1.96
Weight (kg)	10.2	9.9	14	9.8	6.63	12.1	11.5	20	19.2	16.8
Weight (SDS)	-2.12	**-2.66**	-1.29	**-2.6**	**-4.02**	**-2.23**	**-2.47**	-0.11	-1.3	-2.09
Height (cm)	78.2	81	95.2	87.2	78	93.3	98	108.8	115.2	111.1
Height (SDS)	**-3.93**	**-3.82**	**-2.09**	-3.47	**-5.49**	**-4.8**	**-2.98**	-0.98	-1.58	-3.61
BMI (kg/m^2)	16.6	15.09	15.45	12.89	10.8	13.9	11.9	16.9	14.47	13.6
BMI (SDS)	0.09	-1	-0.33	-1.75	**-2.88**	-1.04	-1.91	0.49	-0.89	-1.34
Waist circumference (cm)	-	-	-	39.7	-	-	43.2	57.7	-	45.4
Waist circumference (SDS)	-	-	-	**-2.66**	-	-	-3.15	0.39	-	-1.66

*Genes: *H (HDC8). N (NIPBL). R (RAD21). S (SMC1A). A (ANKRD11).*

Reference levels have been used according to age. sex and pubertal stage. Abnormal values are highlighted in bold. *HDC8, RAD21, SMC1A* and *ANKRD11* genes are highlighted in gray. *Clinical Score: According to Kline et al. (2) (2018) Diagnosis and management of Cornelia de Lange syndrome: first international consensus statement. Nat Rev Genet; 19:649-666. Anthropometric values have been expressed in Z-scores according to the reference graphs [Spanish Growth Study 2010, Carrascosa Lezcano et al. (17)].

Abnormal values are highlighted in bold. *P: patient abbreviation.

CH: carbohydrate, IUGR: intrauterine growth restriction, BMI: body mass index, CdLS: Cornelia de Lange syndrome, SDS: standard deviation score

Table 2. Endocrine assessment in twenty four individuals with CdLS

Patient		*P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Age		2	2	3	3	3	4	5	5	7	8
Gender		M	M	M	M	F	M	F	F	M	F
Thyroid axis/ prolactin	Thyrotropin	**5.94**	2.04	1.1	1.83	**0.26**	1.4	1.74	2.76	2.89	1.57
	Free thyroxine	1.23	1.41	0.99	1.28	1.28	1.24	1.6	1.07	1.22	1.25
	Prolactin	**38**	**90.6**	13.7	27	4.37	**28.9**	15.4	12.3	37.6	16.7
	Neuroleptic tx	-	-	-	-	-	-	-	-	+	-
Adrenal axis	Adreno-corticotropic hormone	50.4	-	17	32.9	19.3	46.9	17.4	30.7	21	16.8
	Cortisol	7.47	-	-	13.7	11.9	< 0.3	7.53	12.3	-	10.2
Gonadal axis	Luteinizing hormone	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
	Follicle-stimulating hormone	1.35	2.1	0.87	0.56	0.75	0.48	1.97	1.58	0.4	1.48
	17-beta-estradiol	-	-	-	-	< 20	-	< 20	< 20	-	-
	Total testosterone	< 0.2	< 0.2	< 0.2	< 0.2	-	< 0.2	-	-	< 0.2	-
Growth axis	IGF-1	87.8	94.8	53.4	66.6	< 15	151	78.1	116	145	119
	Insulin-like growth factor-binding protein 3	2.71	3.2	2.32	2.15	1.85	4.61	3.08	3.6	3.95	4.67
Carbohydrate metabolism	Glucose	71	87	105	84	90	82	107	90	93	72
	Insulin	**15.2**	2	7.5	**14.3**	**20.4**	2.1	**17.2**	**32.4**	7.48	2
	HOMA-IR	2.66	0.42	1.95	2.96	**4.53**	0.42	**4.54**	**7.2**	1.71	0.36

Normal values: Glucose (60-100 mg/dL); insulin in children/adolescents (Tanner I: 0.62-11.57 µU/mL. Tanner II: 0.69-13.75 µU/mL. Tanner III: 3.42-16.28 µU/mL. Tanner IV-V: 2.02-20.76 µU/mL). Insulin < 15 µU/mL; thyrotropin (0.6-4.84 mU/L); thyroxine libre (0.97-1.67 ng/dL); prolactin: Male (4.04-15.2 ng/mL). Female (4.79-23.2 ng/mL); IGF-1 Tanner I (53-332 ng/mL). Tanner II (84-431 ng/mL). Tanner III (114-773 ng/mL) Tanner IV (217-843 ng/mL). Tanner V (147-842 ng/mL). adults 20-30 years (116-358 ng/mL). 30-40 years (109-307 ng/mL); IGF-BP3: Tanner I (1.3-6.3 microgr/mL). Tanner II (2.4-6.7 µg/mL). Tanner III (3.3-9.1 µg/mL). Tanner IV (3.5-8.6 µg/mL). Tanner V (2.7-8.9 µg/mL). Adults 20-30 years (3.4-7.8 µg/mL). 30-40 years (3.5-7 µg/mL); adrenocorticotropic hormone (0-46 pg/mL); cortisol (5-25 mcg/dL); luteinizing hormone prepubertal < 0.3 UI/L. pubertal: Male (1.7-8.6) UI/L. Female: follicular phase (2.4-12.6 UI/L). Ovulation phase (14.0-95.6 UI/L), luteal phase (1-11.4 UI/L); follicle-stimulating hormone prepubertal: Male (< 0.3-3) UI/L. Female (< 0.3-4) UI/L; pubertal: Male (15-12.4) UI/L. Female: follicular phase (3.5-12.5 UI/L). Ovulation phase (4.7-21.5 UI/L). Luteal phase (1.7-7.7 UI/L); 17-beta-estradiol: male (0-56) pg/mL. Female: prepuberal < 20 pg/mL puberal > 20 pg/mL; total testosterone: prepuberal < 0.2 ng/mL. Puberal > 0.2 ng/mL. Abnormal values are highlighted in bold. Tx: treatment abbreviation. *P: patient abbreviation

M: male, F: female, IGF-1: insulin-like growth factor-1, HOMA-IR: homeostatic model assessments of insulin resistance, CdLS: Cornelia de Lange syndrome

Table 1. Continued

Patient	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
Age	9	11	11	14	15	15	16	17	20	23	25	30	31	37
Gender	F	F	F	M	F	F	F	M	F	M	F	F	F	F
Gene*	*N*	*N*	*S*	*N*	*N*	-	*N*	*N*	*N*	*N*	*N*	*A*	*N*	*N*
Clinical score*	-	14	14	14	7	11	15	14	16	13	-	9	9	13
Pubertal stage (Tanner)	II	II	III	I	V	V	III	III	V	V	V	V	V	V
Birth weight (SDS)	-0.07	-1.15	-1.98	**-2.45**	0.34	-0.87	**-3.44**	**-2.03**	**-3.78**	**-2.91**	**-2.1**	**-3.61**	**-0.83**	**-2.06**
Birth length (SDS)	**-5.11**	-1.61	-2.21	**-4.48**	-0.77	-0.44	-1.7	**-2.04**	**-5.73**	**-3.84**	**-2.9**	**-5.73**	-1.25	**-5.2**
Weight (kg)	20.6	18.8	26.5	43	58.8	40.2	24	50.5	22.8	43.3	96.2	54.8	36	62.8
Weight (SDS)	-1.83	**-2.42**	-1.91	-1.23	0.24	-1.5	-3.47	-1.65	-	-	-	-	-	-
Height (cm)	114.8	114	135	134	155.8	148.8	118	166.1	107	153.6	140.3	138.4	142.8	148.3
Height (SDS)	**-3.75**	**-4.8**	**-2.28**	**-3.79**	-1.01	**-2.04**	**-6.98**	1.44	-	-	-	-	-	-
BMI (kg/m^2)	15.6	14.4	14.54	23.95	24.22	18.16	17.2	18.3	19.9	18.31	**48.87**	**28.61**	17.65	**28.55**
BMI (SDS)	-0.82	-1.35	-1.47	0.74	0.87	-0.86	-1.39	-1.25	-	-	-	-	-	-
Waist circumference (cm)	56.4	49.8	-	76.8	73.8	63.5	-	70.4	-	67.2	-	76.6	57.8	86.5
Waist circumference (SDS)	-1.19	**-2.14**	-	0.14	1.39	-0.66	-	-0.35	-	**-2.11**	-	0.73	-1.79	**3.92**

Table 2. Continued

Patient		P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
Age		9	11	11	14	15	15	16	17	20	23	25	30	31	37
Gender		F	F	F	M	F	F	F	M	F	M	F	F	F	F
Thyroid axis/ prolactin	Thyrotropin	2.23	2.39	0.94	1.18	2.09	2.07	0.78	1.57	1.33	0.74	0.69	1.57	1.29	1.04
	Free thyroxine	1.08	1.44	1.2	1.2	0.92	1.21	1.13	1.21	**0.84**	1.18	1.59	**0.94**	1.27	0.98
	Prolactin	20.8	5.37	**33.6**	**40.4**	**26.5**	**29.6**	9.06	**34.6**	12.4	60	12.5	**29.6**	14	11.3
	Neuroleptic tx	-	-	+	+	-	-	-	+	-	+	+	+	-	-
Adrenal axis	Adreno-corticotropic hormone	**182**	13.3	10.6	60	13.7	35.7	10.9	28.9	-	11	16.9	9.6	6.33	7.61
	Cortisol	13	7.66	9.16	16.6	8.2	9.89	7.35	14.7	-	7.28	9.27	3.46	6.44	9.15
Gonadal axis	Luteinizing hormone	1.82	**0.58**	13.1	4.74	11.3	3.05	3.68	5.52	9.82	4.31	10.2	4.78	12.6	2.66
	Follicle-stimulating hormone	3.87	6.95	4.08	8.76	5.3	2.14	4.65	7.48	4.44	5.58	6.45	5.71	6.96	4.25
	17-beta-estradiol	61.4	41.6	73.7	-	112	143	32.1	-	-	-	54.5	35.5	108	73.9
	Total testosterone	-	0.58	-	0.4	-	-	-	3.22	-	3.98	-	-	-	-
Growth axis	IGF-1	245	164	266	213	279	282	194	259	-	157	214	118	124	164
	Insulin-like growth factor-binding protein 3	6.34	4.6	4.81	5.89	6.62	6.36	5.56	5.6	-	4.81	4.77	3.68	3.5	5.94
Carbohydrate metabolism	Glucose	94	86	91	90	82	79	75	87	86	**102**	85	**115**	80	87
	Insulin	9.32	8.57	7.85	8.36	9.38	14.6	14.4	9.3	-	14.7	**29.7**	**37.8**	16.6	9.36
	HOMA-IR	2.16	1.82	1.76	1.85	1.89	2.84	2.66	1.99	-	3.7	**6.23**	**10.7**	3.27	2.01

for populations with physical, cognitive, and medical challenges, such as those with CdLS. Less invasive methods were used to evaluate axis integrity, including the luteinizing hormone-releasing hormone response test and thyrotropin-releasing hormone test, and the HOMA-IR index was used as a surrogate marker of insulin resistance.

Conclusion

Individuals on the CdLS spectrum may have dysregulation of the endocrine system, especially altered prolactin levels and also mildly delayed puberty, cryptorchidism, and short stature. In addition, some of the HOMA-IR assessments of patients in this study suggest early development of insulin resistance. Therefore, clinical follow-up with comprehensive hormonal assessment appears warranted in individuals with CdLS.

Ethics

Informed Consent: Informed consent from participants, their parents, or legal guardians was obtained before entry into the study.

Authorship Contributions

Surgical and Medical Practices: Ángela Ascaso, Ana Latorre-Pellicer, Beatriz Puisac, María Arnedo, Ilaria Parenti, Elena Llorente, Juan José Puente-Lanzarote, Ángel Matute-Llorente, Ariadna Ayerza-Casas, Frank J. Kaiser, Concept: Feliciano J. Ramos, Juan Pié Juste, Gloria Bueno-Lozano, Design: Feliciano J. Ramos, Juan Pié Juste, Gloria Bueno-Lozano, Data Collection or Processing: Ángela Ascaso, Laura Trujillano, María Arnedo, Juan José Puente-Lanzarote, Ángel Matute-Llorente, Analysis or Interpretation: Ángela Ascaso, Ana Latorre-Pellicer, Beatriz Puisac, Laura Trujillano, Juan Pié Juste, Gloria Bueno-Lozano, Literature Search: Ángela Ascaso, Ana Latorre-Pellicer, Juan Pié Juste, Gloria Bueno-Lozano, Writing: Ángela Ascaso, Beatriz Puisac, Laura Trujillano, María Arnedo, Juan José Puente-Lanzarote, Ariadna Ayerza-Casas, Frank J. Kaiser, Feliciano J. Ramos, Juan Pié Juste, Gloria Bueno-Lozano.

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CASE REPORT

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Stress Induced Hyperglycemia in Early Childhood as a Clue for the Diagnosis of NEUROD1-MODY

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What is already known on this topic?

The clinical features of NEUROD1-MODY vary widely in terms of age and body mas index at diagnosis and in response to oral hypoglycemic agents.

What this study adds?

Here, to the best of our knowledge, we report the youngest patient with a heterozygous *NEUROD1* variant in whom stress-induced hyperglycemia during a febrile illness led to the diagnosis. Obtaining a careful and detailed family history for diabetes could help to identify children who are at risk of monogenic diabetes.

Abstract

Maturity-onset diabetes of young 'MODY' type 6 is a rare form of monogenic diabetes caused by mutations in *neuronal differentiation 1* (*NEUROD1*). Clinical features vary in a large spectrum in terms of age and body mass index (BMI) at diagnosis. Here, we reported the youngest patient with a *NEUROD1* variant to the best of our knowledge. A 2.1-year-old girl was referred to pediatric endocrinology clinic for elevated fasting BG (104 mg/dL) which was detected at another center where she had been evaluated for loss of appetite. Her maternal aunt and uncle had been diagnosed with type 2 diabetes mellitus (DM) at the age of 40 and 45 years; they were obese (BMI: 30.2 and 30.6 kg/m^2). At the age of 3.7 years old, she was hospitalized for buccal cellulitis and plasma glucose concentration was 239 mg/dL at admission. Targeted next-generation sequencing (NGS) was performed considering the stress induced hyperglycemia without serious illness, negative islet cell antibodies and insulin autoantibodies, age at the presentation, and family history of DM. NGS analysis revealed a previously reported heterozygous missense variant in *NEUROD1*. Segregation studies showed that the identified variant was inherited from her 44-year-old mother with a BMI of 27.2 kg/m^2 and a normal oral glucose tolerance test. Heterozygous *NEUROD1* mutations cause low-penetrant diabetes that is heterogeneous in terms of clinical features as some patients fulfill the classic MODY definition and others are mimicking type 2 DM. Clinical manifestations and family history should be carefully evaluated in patients with stress induced hyperglycemia to identify candidate cases for molecular testing, and proper follow-up should be initiated in affected individuals.

Keywords: MODY, NEUROD1, stress induced hyperglycemia, early childhood

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Introduction

Maturity-onset diabetes of the young (MODY) is an inherited disorder of non-autoimmune diabetes mellitus (DM) with a young age of onset. It accounts for 1-5% of all patients with diabetes (1), and 1-6% of pediatric patients with diabetes (2,3,4). However due to existence of overlapping features with other types of diabetes, its prevalence could be higher than estimated (5). As the correct diagnosis prevents unnecessary therapies such as insulin in some types of MODY like *HNF1A*-MODY, clinical manifestations and family history should be evaluated properly. Nevertheless, most cases need confirmatory genetic testing for the exact diagnosis. Mutations in *GCK*, *HNF1A*, *HNF4A*, and *HNF1B* are the most common causes of MODY (6). With the recent identification of novel genes, there are a total of 14 genes including *neuronal differentiation 1 (NEUROD1)* that cause MODY (7,8). NEUROD1 is a helix-loop-helix (bHLH) transcription factor that is expressed in pancreatic islet cells, intestine, and neurons in the central and peripheral nervous system (9). NEUROD1 dimerizes with E47, a ubiquitous bHLH transcription factor, and regulates insulin gene expression (9). Autosomal dominantly inherited *NEUROD1* mutations were first reported by Malecki et al. (10) in two families including both obese and non-obese individuals with type 2 DM whose aged 17 to 59 at the time of diagnosis. Afterward, *NEUROD1* mutations were classified as MODY6, considering the clinical features (11). Since then, patients with *NEUROD1* mutations whose clinical features vary in a large clinical spectrum in terms of age and body mass index (BMI) at diagnosis, and response to oral hypoglycemic agents have been reported (12,13).

Stress induced hyperglycemia is a transient condition associated with insulin resistance and relative insulin deficiency (14). It is often considered as a physiologic response to stress. However, stress induced hyperglycemia could be important in terms of uncovering the underlying islet cell dysfunction. Previous studies demonstrated a higher risk of future insulin-dependent diabetes in the case of positive islet cell antibodies, insulin autoantibodies and stress induced hyperglycemia without serious illnesses (15). Also, positive family history of diabetes could enable the patients with stress induced hyperglycemia to be diagnosed with monogenic diabetes (16).

Here, to the best of our knowledge, we reported the youngest patient with a heterozygous *NEUROD1* variant in whom stress induced hyperglycemia during a febrile illness led to the clinical diagnosis. Obtaining a careful and detailed family history for diabetes could help to identify children who are at risk of monogenic diabetes.

Case Report

A 2.1-year-old girl was referred to pediatric endocrinology clinic for elevated fasting blood glucose (BG) (104 mg/dL) which was detected at another center where she had been evaluated for loss of appetite. There was no history of weight loss or polyuria-polydipsia. She was born at term to healthy non-consanguineous parents with a birth weight of 2800 g (-0.7 standard deviation score (SDS)]. Her mother's pregnancy was uncomplicated including a normal oral glucose tolerance test (OGTT). Developmental milestones were normal and the mental and psychomotor developmental index of Bayley Scales of Infant Development-2, which was performed at the age of 13 months, was compatible with 14 months and 13 months of age, respectively. Her maternal aunt and uncle was diagnosed with type 2 DM. The age at diagnosis of diabetes in these subjects was 40 and 45 year; they were obese (BMI: 30.2 and 30.6 kg/m^2) and on metformin therapy (Figure 1). Maternal grandmother and grandfather were died of unknown causes. Index patient

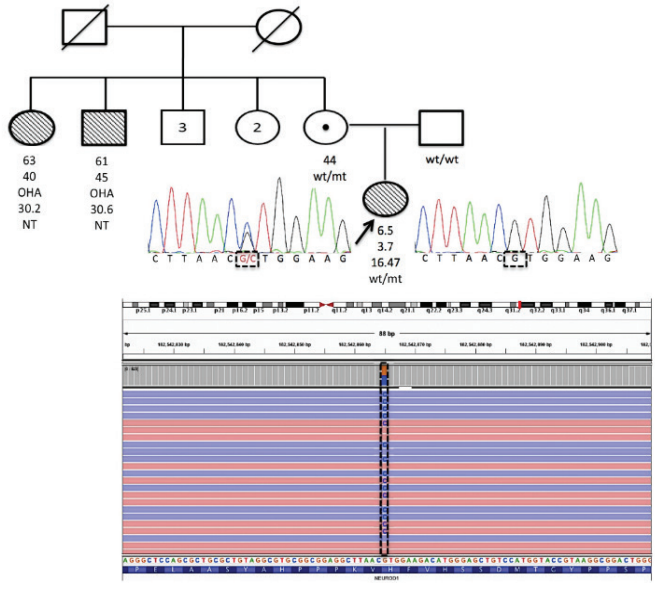

Figure 1. Pedigree, clinical characteristics, and genotype of the family. Filled symbols and empty symbols represent diabetic patients and healthy individuals with normal or unknown genotype, respectively. Dot filled symbol represents healthy individual the alternate allele. The present age of the individuals is shown below the symbols, followed by the age at diagnosis, the most recent treatment, body mass index (kg/m^2) and genotype interpretation. OHA, oral hypoglycemic agents; Genotypes are expressed by normal allele (wt) and alternate allele (at); NT, not tested. An arrow indicates the index case. *NEUROD1 (NM_002500)* is located on the reverse strand. IGV browser visualization of the identified heterozygous variant g.182542865G > C (c.723C > G;p.His241Gln) in the index patient is shown at the bottom of the figure. Sanger sequencing chromatograms of both parents, showing the heterozygous variant in the mother and the wild type sequence in the father

presented with a height of 87.7 cm (-0.17 SDS), a weight of 12.7 kg (0.25 SDS), a BMI of 16.47 kg/m^2 (0.41 SDS) at the age of 2.1 years old. She was prepubertal, and other systemic examination was unremarkable. Biochemical analysis revealed normal fasting BG concentration at the time of admission (plasma glucose 95 mg/dL, insulin 25.1 pmol/L, C-peptide 0.92 ng/mL, HbA1c 5.7%). There was no glucosuria. Anti-glutamic acid decarboxylase, islet cell and insulin antibody were all negative. Dietary interventions were performed maintaining age appropriate calories and reducing simple carbohydrates. At the age of 3.7 years old, she was hospitalized for buccal cellulitis. She was hemodynamically stable and had low grade fever. She had elevated acute phase reactant [C-reactive protein 0.86 mg/dL (N: 0-0.5)], leukocytosis 28,700/µL (N: 4,000-12,000) and plasma glucose concentration was 239 mg/dL at admission. She had glucosuria and no ketonemia. Other biochemical parameters were all normal, and blood culture test was negative. Normoglycemia was provided spontaneously without insulin treatment while she was administered intravenous antibiotics. An OGTT was performed later to evaluate the insulin secretory capacity (fasting BG 87 mg/dL, insulin 71.7 pmol/L; 2-hour BG 98 mg/dL, insulin 276.9 pmol/L).

Considering the stress induced hyperglycemia without serious illness, negative islet cell antibodies and insulin autoantibodies, age at the presentation, and family history of DM, genetic studies were conducted after obtaining written informed consent from the patient's parents. Genomic DNA of peripheral blood leukocytes was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Targeted next-generation sequencing (NGS) was performed with VariFind™ Diabetes assay (Parseq Lab, Saint Petersburg, Russia) on a MiSeq platform (Illumina, San Diego, CA, USA) and then analyzed by VariFind software. This targeted assay covers a total of 24 genes (Supplementary Table 1) associated with various types of glucose intolerance. Target regions include exon-intron boundaries and coding sequences for 12 genes and only hot spots regions for the remaining genes (Supplementary Table 1). After the filtering steps, targeted NGS analysis revealed a previously reported heterozygous missense variant c.723C>G (p.His241Gln) in *NEUROD1* (NM_002500). Parental segregation studies were performed by Sanger sequencing using an ABI 3500 Genetic Analyzer (Thermofisher Scientific, Waltham, MA, USA). Identified variant was found to be inherited from her mother (Figure 1). Her mother was 44 years old, her BMI was 27.2 kg/m^2 and OGTT revealed no abnormalities of glucose metabolism (fasting BG 100 mg/dL, insulin 51.6 pmol/L, C-peptide 1.66 ng/mL; 2-hour BG 128 mg/dL, insulin 669.4 pmol/L, C-peptide 12.4 ng/mL, HbA1c 5.7%). Other family members including the two individuals with DM did not accept the genetic testing.

Discussion

Maturity-onset diabetes of the young is an autosomal dominantly inherited non-autoimmune diabetes classically presenting before the age of 25 years (a more liberal definition is before the age of 35 years). Based on this definition of MODY, the frequency of *NEUROD1* mutations were reported to be between 0.64% and 7.14% (13). However, segregation analysis of families revealed a wide range of phenotypic features of *NEUROD1* mutations. Contrary to the classical MODY definition of presentation in adolescence or young adulthood, the age of diagnosis can vary in a wide range up to the seventh decade (8). Here we reported the youngest patient who presented with stress hyperglycemia, and underwent genetic analysis based on the family history of diabetes and a previously reported *NEUROD1* variant was detected.

Stress induced hyperglycemia is a disorder of glucose metabolism that develops during an acute physiological stress. Combination of increased counterregulatory hormones and overproduction of cytokines cause insulin resistance and impairment of insulin secretion (17). Although it is a physiological response to stress and associated with greater illness severity, it could be the earliest clinical manifestation of islet cell dysfunction (15,18). Besides the presence of markers of autoimmunity and no serious illness, a family history of diabetes could help to identify patients who are at risk of development of any type of diabetes. Detection of hyperglycemia during a relatively mild infection and a positive family history of diabetes prompted us to the molecular testing. So far, impaired fasting glucose, impaired glucose tolerance, overt diabetes and gestational diabetes have been reported in patients with *NEUROD1* mutations (10,12,19). Also, Szopa et al. (12) reported a newborn with neonatal hypoglycemia and macrosomia born to a mother with well-controlled gestational diabetes, and suggested *NEUROD1* mutations as a cause of biphasic diabetes, like *HNF1A* and *HNF4A* mutations.

Diabetic and non-diabetic individuals within the same family represent the intra-familial variability of *NEUROD1* mutations (20). Despite carrying the same variant, index patient had stress induced hyperglycemia in childhood while mother had normal glucose metabolism and did not have gestational diabetes. More than half of the patients harboring *NEUROD1* variants with overt diabetes reported so far had obesity. The presence of obesity within the

families irrespective of carrying a *NEUROD1* variant and a higher frequency of diabetes in individuals with a high BMI suggests that obesity is not a hallmark phenotypic feature of the *NEUROD1* mutations, however, it could be a facilitating factor for the development of diabetes. So, the normal glucose metabolism of the mother could be explained by her not being obese. Intriguingly, approximately three-quarters of the MODY6 families, manifested with diabetes at an earlier age compared to the previous generation, suggesting there may be other factors modifying the phenotypic expression. Also, a parent-of-origin effect was suggested based on the observation of higher proportion of individuals who inherited variants from the mother developed disorder of glucose metabolism compared to ones who inherited variants from the father, as also observed in the presented family. Horikawa et al. (21) reported four MODY6 families in whom the probands with normal BMI manifested with ketosis or ketoacidosis at the age of 10 to 14 years (21), and authors thought that more severe phenotype, regarding younger age of diagnosis with ketosis or ketoacidosis despite normal BMI, was related to less insulin secretory capacity of Japanese population (22). So, one of the factors affecting the manifestation of the clinical and laboratory findings could be the ethnic background as complexities of genetic background can affect their occurrence (23).

NEUROD1 protein consists of two domains; bHLH domain and transactivation domain (7). Most of the reported variants of *NEUROD1* affected transactivation domain of the protein as did the identified variant (7). p.His241Gln was reported previously in individuals diagnosed with MODY whose unaffected family members were also heterozygous for the variant (20,24). In one of these studies, this variant was detected after the sequencing of *NEUROD1* and *PDX1* in all individuals who had previously tested negative for *HNF1A*, *GCK* and *HNF4A* mutations (20), while, in the other after using a targeted NGS panel for known MODY genes (24). Clinical details of those previously reported individuals carrying the same variant were also recently summarized by Horikawa et and Enya (25) and Abreu et al. (26) in 2019. Furthermore, this variant was classified as a rare coding variant of potential interest in RaDio study which included DNA samples from 2,178 people with type 2 diabetes and 4,170 control individuals (27).

Additionally, this variant was interpreted as having conflicting interpretations of pathogenicity in ClinVar database (28). *In silico* bioinformatics analyses, including MutationTaster and MutationAssessor, supports a deleterious effect in addition to a CADD score of 23.1, whereas SIFT sets a benign computational verdict on the variant. Moreover, in the gnoMAD database (v2.1.1), while we expect individuals with severe early onset disease to be heavily depleted and conditions with reduced penetrance such as MODY6 are likely included, this variant is observed mostly in South Asians with an allele frequency (AF) of 0.006467, while allele frequencies in other populations such as Europeans were much more lower (AF <0.0001) (29).

There is no clear genotype-phenotype correlation considering the phenotypic heterogeneity of the patients who carry the same genetic variant in the same family (21). The median age at the time of diabetes diagnosis in patients with p.His241Gln was 25 years (range between 19 and 65). Except for one patient with a digenic inheritance (*NEUROD1* and *PDX1* variants) (23), all of the other patients with p.His241Gln were obese (20). Despite being rare this variant is observed in healthy appearing South Asian population and since obesity or a digenic inheritance is observed in all patients with this variant for the manifestation of diabetes (30,31), it could be speculated that the His241Gln variant causes a minor impairment in insulin secretion capacity. In addition, we could not rule out the presence of other genetic variants contributing to phenotype throughout the genome or within the whole coding region of *NEUROD1* since the utilized targeted panel did not cover all known MODY genes or all coding regions of some MODY genes including *NEUROD1*. Other limitations of the present study were that we did not evaluate copy number variants of the known 14 MODY genes and were not able to extend segregation studies since samples from maternal relatives with diabetes were not available for testing.

It has been shown that a considerable number of euglycemic individuals harbor pathogenic variants in monogenic diabetes genes (27). Pathogenic variants in *HNF1A*, for example, do not always cause MODY phenotype and may contribute to type 2 diabetes predisposition or may be found harmlessly in the genomes of healthy appearing individuals partially explained by reduced penetrance that may occur by the functional effects of regulatory variants (27,32). These studies, which expose putative disease-causing alleles in the genome of healthy appearing individuals, complicate variant interpretation and precise pathogenicity assignments as in the present case (32).

Taking all of the abovementioned findings into consideration including the knowledge that NEUROD1 deficient diabetes appears to be low penetrant and possibly occur in combination with other environmental and genetic factors, it is currently unclear whether the identified *NEUROD1* heterozygous variant definitely contributes to the phenotypic manifestations in the proband. Therefore, further functional testing is required to elucidate the precise role of the identified missense variant on the development of diabetes.

The treatment modalities varied among patients that reported so far. The most common treatment is oral hypoglycemic agents (more than one third of patients), and combination of insulin and oral hypoglycemic agents. Some of the patients had to switch the treatment from oral hypoglycemic agents to insulin. We could not perform molecular analysis for maternal aunt and uncle whose diabetes had been regulated with oral hypoglycemic agents. Our case is not obese, however there are other factors that could modify the development of diabetes. Since most of the cases reported to date have been manifested with diabetes at an earlier age compared to the previous generations, we consider diabetes could develop at an earlier age compared to other family members in our case. Also, parent-of-origin effect could increase the probability of development of diabetes in our case. In this case, the treatments and treatment responses of other family members can be used as a guide to determine the use of oral antidiabetic drugs or insulin.

Conclusion

In conclusion, to the best of our knowledge, we reported the youngest patient with a heterozygous *NEUROD1* variant in whom stress induced hyperglycemia during a febrile illness led to the clinical diagnosis. Heterozygous *NEUROD1* mutations cause low-penetrant diabetes that is heterogeneous in terms of clinical features as some patients fulfill the classic MODY definition and others are mimicking type 2 diabetes mellitus. Hyperglycemia during a relatively mild infection with a family history of diabetes should prompt clinicians to investigate monogenic diabetes with molecular test, and proper follow-up should be initiated in affected individuals.

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Ethics

Informed Consent: Written informed consent was collected from the patient.

Authorship Contributions

Concept: Semra Çetinkaya, Design: Nur Berna Çelik, Analysis or Interpretation: Nur Berna Çelik, Naz Güleray Lafcı, Şenay Savaş-Erdeve, Semra Çetinkaya, Literature Search: Nur Berna Çelik, Semra Çetinkaya, Writing: Nur Berna Çelik, Semra Çetinkaya.

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CASE REPORT

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Successful Management of Severe Hypercalcemia with Zoledronic Acid: A Report of Two Pediatric Cases

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What is already known on this topic?

Vitamin D intoxication and malignancies should be considered in the differential diagnosis of children presenting with hypercalcemia. Treatment of pediatric hypercalcemia with conventional bisphosphonates, such as pamidronate, can take many treatment cycles. There is a paucity of published evidence concerning the use of Zoledronic acid (ZA) in the treatment of pediatric severe hypercalcemia.

What this study adds?

ZA was an effective treatment in cases of severe hypercalcemia in children, due to two different etiologies, vitamin D intoxication and malignancy-associated hypercalcemia. Only one dose of ZA was sufficient to achieve normocalcemia within 48 hours in both cases. Patients should be followed closely after ZA infusion due to the risk of subsequent hypocalcemia.

Abstract

Severe hypercalcemia associated with vitamin D intoxication or malignancy in children is a rare and life-threatening condition. There is little published experience with Zoledronic acid (ZA) in the treatment of pediatric severe hypercalcemia. Here, we present two pediatric cases of severe hypercalcemia, one due to vitamin D intoxication and the second to malignancy, in which ZA was used as the first-line bisphosphonate in the treatment. While both cases responded well to a single dose of ZA, the second case experienced hypocalcemia requiring calcium treatment after ZA infusion. Our report shows that ZA may be an effective option in the treatment of severe pediatric hypercalcemia, although patients should be followed closely after infusion due to the risk of hypocalcemia. We provide additional published evidence for the effectiveness of ZA in correcting severe pediatric hypercalcemia and hope this will encourage future studies with larger numbers of patients.

Keywords: Hypercalcemia of malignancy, hypocalcemia, pamidronate, vitamin D intoxication, zoledronic acid

Introduction

Hypercalcemia in children is a rare condition that may present with a variety of signs and symptoms. Vitamin D intoxication and malignancy are among the known etiologies (1).

Although there is no formal classification to define the severity of hypercalcemia, serum calcium values greater than 14 mg/dL are considered severe hypercalcemia and require urgent treatment (1). Bisphosphonates are effective in the treatment of hypercalcemia, and pamidronate is the most widely used bisphosphonate in children (2). However, data on the use of Zoledronic acid (ZA) in childhood are limited.

Herein, we present our experience with ZA as a first line treatment in two pediatric cases of severe hypercalcemia, the first secondary to vitamin D intoxication and the second case associated with malignancy.

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Case Reports

Case 1

A 22-month-old girl with known diagnoses of hydrocephalus and epilepsy was admitted to the emergency department with complaints of loss of appetite, constipation and polyuria. On examination, she was dehydrated and had hypotonicity. Vital signs were within normal limits. When the parents were questioned, it was learned that the girl had received a vitamin D supplement (cholecalciferol) with a dose of 300,000 IU daily for 20 days.

Initial laboratory investigations revealed severe hypercalcemia with an albumin-corrected serum calcium level of 17.8 mg/dL (normal: 8.8-10.6), ionized calcium level of 2.1 mmol/L (normal: 1.15-1.29), phosphorus level of 3.4 mg/dL (normal: 3.8-6.5), alkaline phosphatase level of 124 U/L (normal: 55-377), and creatinine level of 0.6 mg/dL (normal: 0.5-0.9). Complete blood count, other biochemical markers, and electrolytes were normal. Her serum intact-parathyroid hormone (PTH) level was 18 pg/mL (normal: 12-88), 25-hydroxyvitamin D level was 160 ng/mL (normal: 20-100), and 1,25-dihydroxy vitamin D level was 44.7 pg/mL (normal: 28-81). Spot urine analysis revealed a calcium/creatinine ratio of 1.1 (normal: <0.2) (Table 1). Renal ultrasonography showed increased bilateral medullar echogenicity, suggestive of nephrocalcinosis.

Then, intravenous (IV) fluids containing normal saline and dextrose at a dose of 150 mL/kg/day were started for the treatment of severe hypercalcemia due to vitamin D intoxication. IV furosemide with a dose of 2 mg/kg and methylprednisolone with a dose of 1 mg/kg were also administered for three days. ZA at a dose of 0.0125 mg/kg was administered IV, since a sufficient decrease in serum calcium values had not been achieved at that point. After 24 hours of ZA infusion, the calcium value decreased to 11.3 mg/dL from 14.2 mg/dL. There were no serious side effects or need for calcium supplementation after the ZA infusion. The patient was discharged when the calcium value was 10.1 mg/dL, five days after the ZA infusion (Figure 1). While serum 25-hydroxyvitamin D values remained high or at the upper limit of normal for three months (Figure 2), calcium values 1 week, 1 month, 3 months and 1 year after discharge were within normal limits at 9.4 mg/dL, 9.6 mg/dL, 8.9 and 9.3 mg/dL, respectively.

Case 2

A previously healthy, 3-year-old boy presented with a 1-week history of difficulty in walking. On physical examination, he was mildly dehydrated and his vital signs were within normal range. His lower limbs were painful, but there was no sign of inflammation. Laboratory investigations revealed hypercalcemia, with an albumin-corrected serum calcium level of 13 mg/dL, ionized calcium level of 1.5 mmol/L, phosphorus level of 5.6 mg/dL, alkaline phosphatase level of 149 U/L, and creatinine level of 0.3 mg/dL. The rest of the biochemical markers, and electrolytes were normal. Complete blood count was also normal. His serum PTH

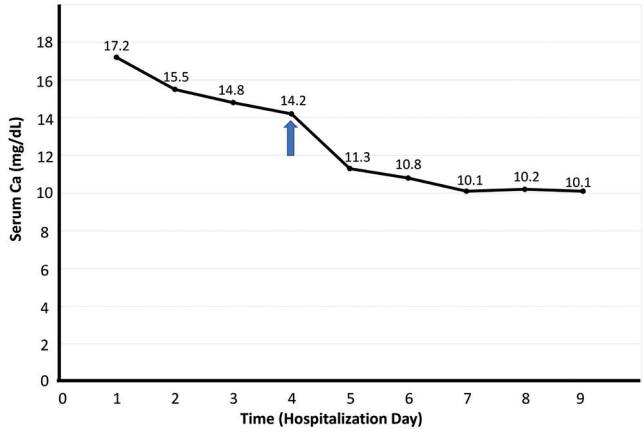

Figure 1. Serum calcium levels of Case 1 during hospitalization and response to Zoledronic acid. Zoledronic acid infusion is highlighted with a blue up-arrow

Ca: calcium

Table 1. Laboratory data of patients on admission

Parameter	Case 1	Case 2	Normal
Albumin-corrected serum calcium (mg/dL)	17.8	13	8.8-10.6
Ionized calcium (mmol/L)	2.1	1.5	1.15-1.29
Serum albumin (g/L)	43.3	44.6	35-52
Serum phosphorus (mg/dL)	3.4	5.6	3.8-6.5
Serum alkaline phosphatase (U/L)	124	149	55-377
Serum creatinine (mg/dL)	0.6	0.3	0.5-0.9
Serum intact-parathyroid hormone (pg/mL)	18	<1.2	12-88
Serum 25-hydroxyvitamin D (ng/mL)	160	17.2	20-100
Serum 1,25-dihydroxy vitamin D (pg/mL)	44.7	16.5	28-81
Spot urine calcium/creatinine ratio (mg/mg)	1.1	1.1	<0.2

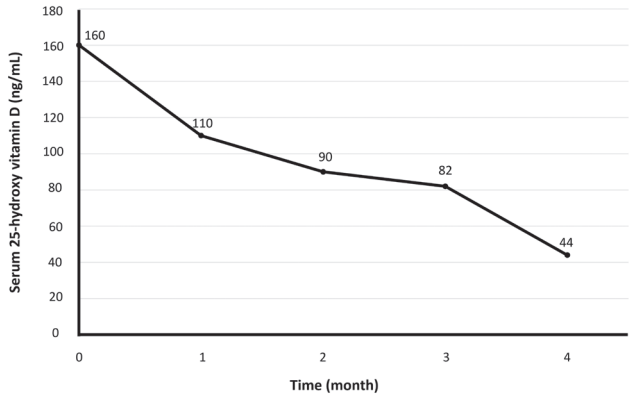

Figure 2. Time course of serum 25-hydroxyvitamin D levels of Case 1 after Zoledronic acid treatment

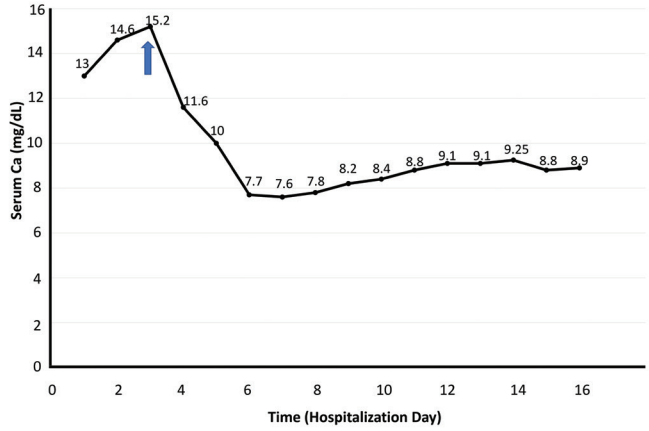

Figure 3. Serum calcium levels of Case 2 during hospitalization and response to Zoledronic acid. Zoledronic acid infusion is highlighted with a blue up-arrow

level was low at < 1.2 pg/mL, 25-hydroxyvitamin D level was low at 17.2 ng/mL, and 1,25-dihydroxyvitamin D level was in the lower limit of normal at 16.5 pg/mL. Spot urine analysis revealed a calcium/creatinine ratio of 1.1 (Table 1). Serum PTH-related peptide (PTHrP) could not be performed. Radiologic examinations of bones with X-rays and scintigraphy did not reveal any lytic lesions. Abdominal and renal ultrasound results were normal. Peripheral blood smears revealed blasts suggesting malignancy. Therefore, hematology was consulted. While further hematological investigations continued, normal saline and dextrose at a dose of 150 mL/kg/day were started to correct the hypercalcemia. Then, IV furosemide at a dose of 2 mg/kg/day was added. Despite fluid and furosemide, serum calcium increased to 15.2 mg/dL. Subsequently, ZA was administered with a dose of 0.0125 mg/kg. Serum calcium levels progressively decreased and normalized within 48 hours after the ZA infusion. Bone marrow examination and flow cytometry results were consistent with the diagnosis of acute lymphoblastic leukemia (ALL). Therefore, induction chemotherapy (vincristine, prednisolone, asparaginase and daunorubicin) was started. Three days after the ZA infusion serum calcium was 7.7 mg/dL and so calcium and vitamin D supplementations were provided (Figure 3). The patient is currently on his fourth month of chemotherapy treatment and there has been no recurrence of hypercalcemia.

Discussion

This case series describes two pediatric cases of severe hypercalcemia, one secondary to vitamin D intoxication and the other associated with malignancy, in which serum calcium levels were normalized shortly after ZA administration.

Causes of hypercalcemia in children can be classified as PTH-dependent or PTH-independent, based on serum PTH levels. In the presence of low PTH levels during severe hypercalcemia, vitamin D intoxication and malignancies are among the diagnoses that should be considered (1).

Administration of high doses of vitamin D due to incorrect prescription of vitamin D, formulation errors in drugs or unconscious use of vitamin D may lead to vitamin D intoxication and hypercalcemia. In intoxication caused by 25-hydroxyvitamin D, serum 25-hydroxy vitamin D levels are high, while 1,25-dihydroxy vitamin D levels are usually normal. This suggests that 1,25-hydroxyvitamin D is not responsible for the development of hypercalcemia. 25-hydroxyvitamin D competes with calcitriol, binds to the vitamin D receptor and exerts biological effects resulting in hypercalcemia (3).

Hypercalcemia of malignancy (HCM) is extremely rare in the pediatric cancer population. The underlying mechanisms of HCM are secretion of PTHrP, local calcium release from osteolytic metastatic tissue, unregulated, extrarenal production of 1,25-dihydroxy vitamin D, and, rarely, ectopic PTH secretion (1). Although our patient did not have PTHrP measured, the low PTH and 1,25 dihydroxy vitamin D levels, as well as the absence of lytic lesions in radiological examinations, suggested the cause of HCM was most likely due to PTHrP secretion.

The standard approach for the management of hypercalcemia includes establishing the underlying diagnosis, discontinuing medications that may cause hypercalcemia, IV fluids, and antiresorptive therapy. Bisphosphonates are effective in the treatment of severe hypercalcemia, acting by reducing osteoclastic activity. Although there are studies showing

that pamidronate is effective and safe in the treatment of hypercalcemia due to both vitamin D intoxication and malignancy in childhood (4,5), definitive studies on the use of ZA in childhood hypercalcemia are lacking.

ZA has been shown to be superior to pamidronate in the treatment of HCM in adults. ZA normalized serum calcium levels earlier than pamidronate, while patients treated with pamidronate experienced an earlier recurrence of hypercalcemia than those treated with ZA (6). In a study evaluating the short-term safety of ZA in 81 young patients with various bone disorders, it was stated that acute side effects related to ZA infusion are common, occurring mainly after the first ZA infusion in bisphosphonate-naive patients, and are easily managed (7). Hypocalcemia is one of the side effects of ZA. It has been noted that low vitamin D level is a strong risk factor for hypocalcemia, and it has been suggested that the vitamin D level should be >30 mg/dL before ZA infusion (8).

There are a few cases in the pediatric literature reporting that ZA is successful in the treatment of severe hypercalcemia due to both vitamin D intoxication and malignancy. Nimesh et al. (9) reported that they achieved normocalcemia with ZA in an infant with severe hypercalcemia due to vitamin D intoxication. Our case with vitamin D intoxication was first treated with fluid, furosemide, and methylprednisolone without good effect. However, normocalcemia was achieved within 48 hours after a single dose of ZA given because the calcium level continued to increase. Although serum calcium values remained within normal limits at one-year follow-up, late morbidities may develop due to the long half-life of 25-hydroxyvitamin D in serum and its accumulation in adipose tissues (3). It was noted that despite 19 repeated cycles of pamidronate in an infant who experienced severe hypercalcemia secondary to vitamin D intoxication, hypercalcemia could not be improved, and alendronate treatment was required for six weeks afterwards (10). Therefore, it should be kept in mind that the treatment and follow-up period may be long in intoxications caused by 25-hydroxyvitamin D. The persistence of hypervitaminosis for three months in the presented case may support the use of ZA in this situation. In addition, the rapid action, the lack of need for several repeated doses and shorter hospitalization time, unlike pamidronate, may further support the superiority of ZA to pamidronate in these cases.

In four children with ALL, published in four separate articles, who experienced HCM, it was reported that after a single dose of ZA, hypercalcemia resolved within 24-48 hours, and calcium replacement was required due to hypocalcemia (11,12,13,14). Similar to these reports, in the presented case with HCM, serum calcium returned to normal limits within 48 hours after a single dose of ZA and calcium replacement was required due to hypocalcemia. The low 25-hydroxyvitamin D level of our case before ZA infusion may have contributed to hypocalcemia. We also believe that induction chemotherapy containing prednisolone may also predispose to hypocalcemia. Therefore, after ZA infusion, patients should be followed closely due to this risk of hypocalcemia.

A case of pediatric HCM unresponsive to pamidronate but successfully treated with ZA has also recently been published (15). In this report, three repeated doses of pamidronate were tried as the first-choice bisphosphonate, but since no response was obtained, a single dose of ZA was then administered. Hypercalcemia resolved 24 hours after the ZA administration and no further ZA doses were required. The authors indicated that ZA should be considered as a first line of treatment when bisphosphonates are required in the treatment of HCM.

Conclusion

Our experience in cases of severe hypercalcemia due to two different etiologies showed that ZA was effective in the treatment of severe hypercalcemia in childhood. Although it is not possible to report that ZA should be the first choice of bisphosphonate in childhood on the basis of only two cases, we hope that we have provided some evidence to encourage others to try ZA in pediatric cases, especially if recalcitrant to pamidronate therapy. We believe that as the evidence builds, the use of ZA in pediatric cases with severe hypercalcemia, may become more widespread, if it proves to be as effective as we have found.

Ethics

Informed Consent: Written informed consent was obtained from the parents on behalf of the children for treatment protocol, and publication of the case report.

Authorship Contributions

Surgical and Medical Practices: Fatih Kilci, Filiz Mine Çizmecioğlu-Jones, Concept: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones, Design: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones, Data Collection or Processing: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones, Analysis or Interpretation: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones, Literature Search: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones, Writing: Fatih Kilci, Jeremy Huw Jones, Filiz Mine Çizmecioğlu-Jones.

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CASE REPORT

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Severe Growth Hormone Deficiency in an Indian Boy Caused by a Novel 6 kb Homozygous Deletion Spanning the *GH1* Gene

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What is already known on this topic?

Mutations in *GH1* genes are associated with a rare condition called isolated growth hormone deficiency. The most common *GH1* deletions reported are 6.7 and 7.6 kb in size. The largest reported deletion is 45 kb in size.

What this study adds?

We report a 3-year old boy with extreme short stature with a deletion in the *GH1* gene. The deletion was 6 kb in size which has not been reported before. The proband is homozygous for the deletion and the parents, who are also short, each have a heterozygous deletion.

Abstract

Growth disorders resulting in extreme short stature (ESS) are often a result of deficiency in growth hormone (GH) released from the pituitary gland or a defective GH releasing receptor. Genetic defects in the *GH1* and *GHRHR* genes account for around 11.1-20% of ESS cases, resulting in a rare condition called isolated GH deficiency (IGHD). We describe the characterization of a *GH1* genetic defect discovered in a 3-year-old male patient with ESS, developmental failure and undetectable serum levels of GH. There was a family history of short stature, with both parents being short. Whole genome sequencing of the patient DNA revealed a large, novel 6 kb homozygous deletion spanning the entire *GH1* gene in the patient. While the deletion was homozygous in the proband, it was present in the heterozygous state in the parents. Thus, we report a novel homozygous deletion including the *GH1* gene leading to IGHD-type 1A associated with ESS.

Keywords: *GH* gene deletion, short stature, familial short stature

Introduction

Growth disorders resulting in extreme short stature (ESS) are often a result of deficiency in growth hormone (GH) released from the pituitary gland, coded for by the *GH1* gene, located on chromosome 17q23, or defective GH releasing receptor, which is coded for by the *GHRHR* gene, located on chromosome 7p14.3. Genetic defects in the *GH1* and *GHRHR* genes account for around 11.1-20% of ESS cases, resulting in a rare condition called isolated GH deficiency (IGHD). This frequency is reported to be 18.6% higher in familial cases of IGHD (1).

IGHD is a disorder with varying prevalence in different populations, ranging from 1:1800 in Sri-Lanka to 1:30,000 in the United Kingdom (2). Familial IGHD has been grouped into four main subtypes: type 1A, type 1B, type 2 and type 3 (3). These subtypes have a wide range of phenotype, including ESS, doll-like facies, central obesity, high pitched voice and puberty that is often delayed (4). Type 1A and 1B often manifest as ESS (3,5) and follow an autosomal recessive or compound heterozygous inheritance pattern (6).

GH is a peptide hormone that contains two active sites for GH receptor (GHR) binding, a class 1 cytokine

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receptor. GHRs are expressed in a broad range of tissue cellular membranes, including kidney cells, hepatocytes, adipocytes, myocytes, and many others. One GH molecule binds with two GHRs causing dimerization and this tertiary complex activates Janus kinase 2 (JAK-2) bound to GHR (7). Then JAK phosphorylates STAT5, a signal transducer and transcriptional activator, which enters the nucleus to induce GH-mediated genes expression. The mode of action of GH relies on the secretion of insulin-like growth factor-1 (IGF-1) from cells and stimulation of chondrocytes (8) leading to their differentiation. IGF-1 has an important role in stimulating growth at the end/growth plates of bones as well as in muscle cells. In addition to the JAK-STAT pathway, the dimerization of GHR further causes the initiation of other cascades, including the mitogen activating protein-kinase (MAP-K) pathway and the phosphoinositide 3 kinase (PI3K) pathways. Thus, the deficiency of endogenous GH is directly linked to perturbation of these pathways leading to short stature.

We report a 3-year-old male patient with ESS and failure to thrive with a large, novel, 6 kb homozygous deletion spanning the entire *GH1* gene in the patient. We have also review all cases of *GH1* deletion previously reported and compared with the presented case.

Case Report

A 3-year-old male child at presentation and of Indian origin was born at full term via C-section with a birth weight of 2.8 kg. The neonatal period was uneventful. At six months of age his parents noticed that he was not gaining weight. He was referred to Sidra Medicine at the age of 16 months for investigations of short stature. His weight was 6.80 kg [0th percentile, -5.03 standard deviation score (SDS), Figure 1] and his height was at 71 cm (0th percentile, -5.4 SDS, Figure 1). On examination he had severe frontal bossing, a pointed chin and a central incisor. There was no skeletal dysplasia, intellectual or developmental delay.

He underwent a glucagon GH stimulation test, which revealed undetectable serum GH levels. This suggests severe GH deficiency most likely due to a defect in the *GH1* gene. His free thyroxine level was 11.8 pmol/L (normal: 9.5-17.8 pmol/L) indicated normal thyroid functioning. Pituitary magnetic resonance imaging scan was structurally normal. The rest of the pituitary function tests were also normal. Table 1 shows the results of biochemical tests done.

Family History

His parents also have short stature, the father in particular (Figure 2) with a height of 152 cm (-3 SDS), while the mother's height was 151 cm. The mid-parental height of the child is 158 cm.

Follow-up and Management

The patient is being treated with recombinant human GH at 0.029 mg/kg/day with a growth velocity of 10 cm/year. His current height at age 3 years is 81.5 cm (<-2 SDS) and weight is 8.4 kg (<1 SDS).

Genetic Testing Methodology

Informed consent was obtained from parents. DNA samples were extracted from peripheral blood specimens of subject

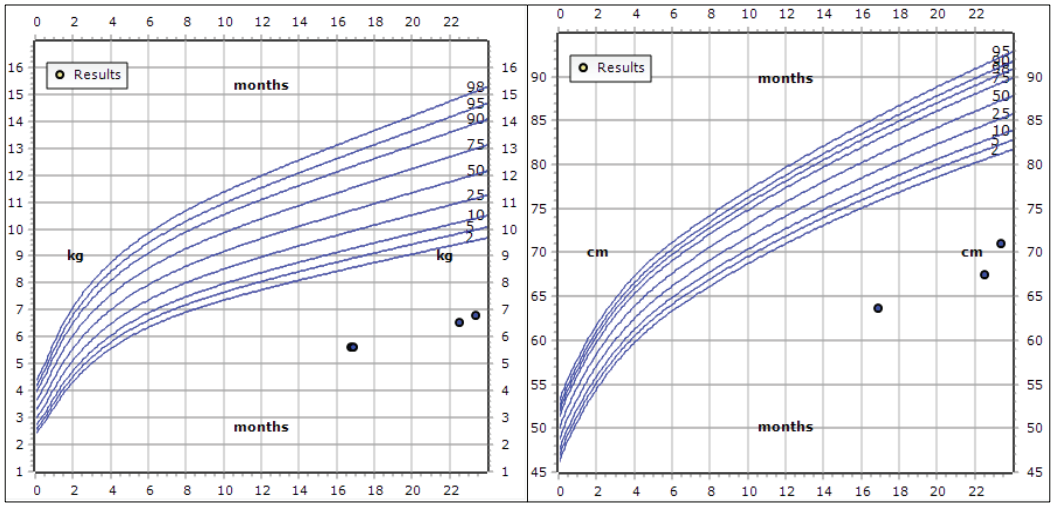

Figure 1. Weight-for-age and height-for-age growth chart for males aged 0-2 years. At the time of recruitment, the patient's weight was 6.80 kg (0th percentile, -5.03 SDS) indicating that he was severely underweight. The patient has severe short stature with a height of 71 cm (0th percentile, -5.4 SDS). The mid parental height is 158 cm

SDS: standard deviation score

and parents. Whole genome sequencing (WGS) was performed on an Illumina HiSeq platform using a 150-base paired-end single-index-read format. Reads in FASTQ files were then mapped to the NCBI human reference genome GRGh37/hg19 using Burrows-Wheeler Aligner (BWA-MEM) version 0.7.8. All subjects underwent variant calling using GATK (v3.6) and annotation was performed using SNPEff. The variants file was normalized and decomposed using vt. Additionally, vcfanno was used to annotate VCF file with extensive available data resources, including gnomad, exomes.r2.0.2, gnomad.genomes.r2.0.2.sites, 1K genome, and Exac. Genomic variants belonging to genes already known to be implicated in familial short stature were extracted.

Table 1. Results of biochemical tests done on the patient at the time of recruitment

Test	Patient results	Reference level
Growth hormone at 0, 60, 120, 180 mins (mcg/L)	Undetectable	>10 mcg/L at any time point
IGF-1 (ug/dL)	Undetectable	0.06-0.57
IGF binding protein-3 (mcg/mL)	<0.5	0.7-3.6
Morning ACTH (pg/mL)	146	7.2-63.3
Random blood glucose (mg/dL)	82.8	70-110
Total calcium (mg/dL)	10.2	8.8-10.8
TSH (mIU/L)	3.61	0.76-4.64
Free T4 (ng/dL)	0.92	0.74-1.38
Prolactin (mIU/L)	302	70-390
Cortisol (ug/dL)	7.94	2.17-12.79

IGF-1: insulin-like growth factor-1, ACTH: adrenocorticotropic hormone, TSH: thyroid-stimulating hormone

Copy number variation of the WGS analysis detected a novel, homozygous, 6 kb deletion on the long arm of chromosome 17, 17q23.3 with coordinates (GRCh37/hg19 17:61993713-62000168) in the proband. However, the parents were heterozygotes for the deletion. This deletion was manually identified using integrated genome viewer and spans the entirety of *GH1*, which is the main candidate gene and consistent with the phenotype of the patient (Figure 3a). We further confirmed the deletion using Samplot by visualizing and comparing the coverage of the structural variant with the surrounding regions (Figure 3b).

Discussion

IGHD Type IA is the second most commonly reported type of IGHD and these cases often have a variety of mutations

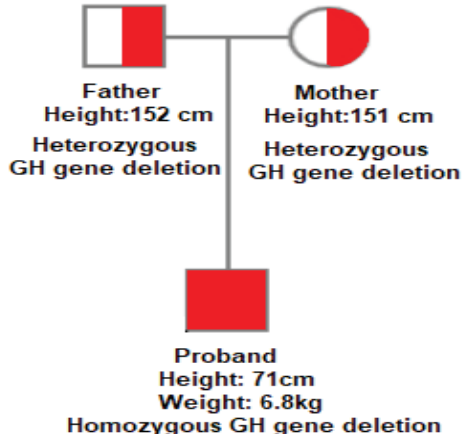

Figure 2. Family pedigree for this patient with IGHD type 1A

IGHD: isolated growth hormone deficiency

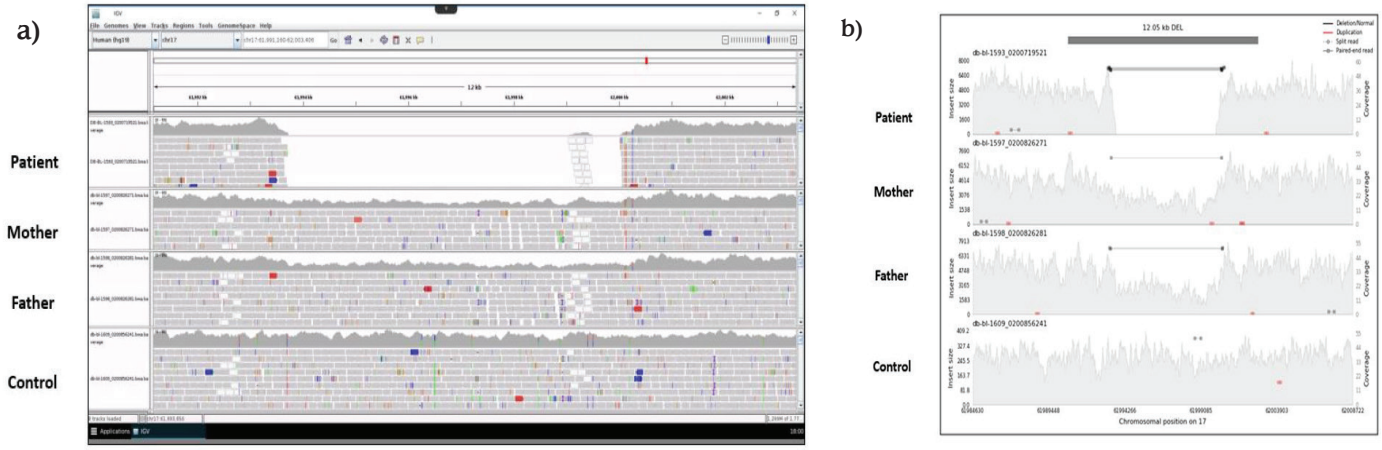

Figure 3. Results of genetic analysis for the patient and parents. a) Integrated genome viewer track showing homozygous 6 kb deletion in the proband (top), heterozygous deletion in both parents (middle) and wild type control (bottom) from the bam-files of the samples. b) Samplot analysis of whole genome sequencing showing structural variant. A 6 kb deletion spanning the entire *GH1* gene on chromosome 17 (GRCh37/hg19 17:61993713-62000168)

Table 2. Deletion size and phenotype of all reported subjects with GH deletion causing short stature

	Deletion size	Deletion zygosity	Phenotype
1	7.5 kb [10]	Homozygous	- Reported four patients from three families with severe IGHD. - Extreme short stature. - Absence of GH production. - Formation of anti-hGH-antibodies in high titers after hGH therapy.
2	7.5 kb [13]	Homozygous	- Reported four unrelated Jewish patients with IGHD. - All patients carry 7.5 kb deletion. - All four patients showed good response for hGH therapy.
3	6.7 kb and 7.6 kb [14]	Compound heterozygous	- Severe GH deficiency, truncal obesity, acromicria, low IGF-1 and IGFBP-3 and severe anterior pituitary hypoplasia.
4	6.7 kb and 2 bp [15]	Compound heterozygous	- Severe growth deficiency. - GH therapy resulted in catch-up growth at 9 years and 2 months with development of anti-GH antibodies.
5	7.6 kb [16]	Homozygous	- Two sibling patients with short stature. - Responded well to GH substitution. - No formation of blocking antibodies occurred. - *GH1* and *CSHL1* gene affected by the deletion.
6	6.7 kb and 7.6 kb [17]	Homozygous	- A patient with IGHD. - Very low response of GH secretion. - Undetectable levels of IGF-1 and IGFBP-3. - MRI showed a severe anterior pituitary hypoplasia.
7	6.7 kb or 7.6 kb [9]	Homozygous	- Ten patients with either 6.7 kb (8/10) or 7.6 kb (2/10) deletions. - 3 North European, 3 Mediterranean and 4 Turkish patients. - All the patients presented with severe growth retardation, decreased growth rate, and retarded bone age.
8	45 kb [18]	Homozygous	- An Italian family 3 affected IGHD patients. - These three patients showed heterogeneity in growth response and antibody formation on hGH replacement therapy.
9	45 kb [19]	Homozygous	- A Turkish family with three affected boys presenting with growth retardation. - The deletion involved GH and somatotropin gene clusters.
10	40 kb [20]	Homozygous	- A French family with two affected siblings, with severe congenital growth deficiency. - Both patients developed antibody after the hGH therapy.
11	6.7 kb [21]	Homozygous	- A girl with short stature and cystic fibrosis. - Developed anti hGH antibodies after 2 months of hGH replacement.
12	7.1 kb [22]	Homozygous	- A Chilean patient with short stature and repeated hypoglycemic episodes (neonatal period). - hGH treatment discontinued after the patient developed anti hGh antibodies and switched to IGF-1 treatment.
13	3.8 kb [23]	Homozygous	- A patient with severe congenital GH deficiency. - Non-detectable plasma GH levels in response to pharmacological stimulation tests. - A round face, small nose with depressed nasal bridge. - Treated with biosynthetic methionyl (met) GH but developed antibody after 5 years.
14	6.7 kb [24]	Homozygous	- Three Indian siblings with short stature. - Patients carried exactly same deletion but showed heterogeneity in rhGH treatment response.
15	22 kb and c.10 + 1G > T [25]	Compound heterozygous	- A 1 year and 9 months patient with growth retardation. - Auxiliary examinations showed low GH, low IGF-1 and elevated TSH.
16	6.7 kb and/or 7.6 kb [26]	Homozygous and compound heterozygous	- 12 patients with IGHD: - 10/12 (homozygous 6.7 kb del). - 1/12 (homozygous 7.6 kb del). - 1/12 (compound HT 6.7 and 7.6 kb del). - All patients had growth failure, very low GH, IGF-1 and IGFBP-3. - MRI showed hypoplastic adenohypophysis.
17	7.6 kb [27]	Homozygous	- Two Hispanic sisters with short stature and high body fat. - Developed antibodies against rhGH exposure.
18	c.1G > T 7.6 kb [28]	Compound Heterozygous	- A Japanese patient with growth retardation confirmed after provocative GH testing. - Responded well to hGH therapy. - Did not develop anti-hGH antibody.
19	6.7 kb or 7.6 kb [29]	Homozygous	- 3 Brazilian patients with GH deficiency. - 2 patients had 6.7 kb del (developed anti-GH antibodies after therapy) and 1 patient with 7.6 kb del (no anti-GH antibodies developed). - All the 3 patients had common phenotype: large forehead, low nasal bridge, increased subcutaneous fat, thin hair, and a high-pitched voice.

hGH: human growth hormone, GH: growth hormone, IGHD: isolated growth hormone deficiency, IGF-1: insulin-like growth factor-1, IGFBP-3: IGF binding protein-3, MRI: magnetic resonance imaging, TSH: thyroid-stimulating hormone, rhGH: recombinant human growth hormone

involving the *GH1* gene (3). Specifically, 66.7% of familial cases of IGHD type 1A involve *GH1* aberrations (6). Eighty percent of reported *GH1* deletions are of the 6.7 kbs size, while many others are of 7.6 kbs in length (9,10). These deletions are caused by imbalanced recombination between 98% homologous 454-592bp flanking regions of the *GH1* gene (9,11). Due to these mutations, IGHD type 1A patients will often have undetectable serum levels of GH due to a lack of endogenous GH and rhGH treatment may frequently lead to an antibody response against GH (3,10,12). Affected proportionate short stature patients will have heights ranging from -3 to -9.6 SDS (6). In keeping with this, undetectable serum GH levels were reported in the proband on stimulation testing.

Genetic testing also revealed that both parents, who are both affected with short stature, even though they were heterozygous with only one identical copy of the deletion. This suggested that a heterozygous deletion with this variant in *GH1* may also contribute to the defects in short stature. However, we could not find any previous reports of heterozygous deletions of *GH1* causing short stature.

We performed an electronic literature review of the PubMed database to identify relevant articles about *GH1* that lead to short stature, written in English and published up to March 2022. The following terms were used to search the database: "growth hormone 1 gene deletion", "*GH1* deletion", "pituitary growth hormone gene deletion", "IGHD1A and deletion", and "short stature". Twenty articles were identified with *GH1* deletion. Table 2 shows full list of deletions with clinical phenotype and size of deletions. These studies included different ancestries (Europeans, Asians, South/North Americans, and Arabs), however, most of the studies were conducted in European populations. The majority of the patients were diagnosed with severe short stature in early infancy. Our analysis showed that the most common size of *GH1* deletion reported is 6.7 or 7.6 kb and the largest deletion reported was 45 kb in size. The majority of subjects displayed a similar phenotype with development of GH antibodies after hGH therapy after varying durations of therapy. Most of the patients with 6.7 kb deletion developed GH antibodies in response to GH therapy while patients who carry the 7.6 kb deletion tend to exhibit more immunological tolerance when treated with exogenous GH. However, there is heterogeneity in the development of GH antibodies, even between members of the same family. The proband in this case report is still young and has been on GH therapy for two years, with a promising increase in height but not in weight gain. A limitation of our study was that GH antibodies were not measured.

Conclusion

We report a 3-year-old male patient of Indian origin with ESS, failure to thrive and a family history of short stature. GH levels were undetectable on stimulation testing. WGS revealed a large, novel, 6 kb homozygous deletion spanning the entire *GH1* gene in the patient leading to IGHD-type 1A associated with ESS. Both parents were heterozygous for the same variant and were also of short stature.

Ethics

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

Authorship Contributions

Surgical and Medical Practices: Basma Haris, Khalid Hussain, Concept: Basma Haris, Diksha Shirodkar, Khalid Hussain, Design: Basma Haris, Diksha Shirodkar, Khalid Hussain, Data Collection or Processing: Basma Haris, Diksha Shirodkar, Idris Mohammed, Analysis or Interpretation: Basma Haris, Diksha Shirodkar, Idris Mohammed, Umm-Kulthum Ismail Umlai, Khalid Hussain, Literature Search: Basma Haris, Diksha Shirodkar, Idris Mohammed, Khalid Hussain, Writing: Basma Haris, Diksha Shirodkar, Idris Mohammed, Umm-Kulthum Ismail Umlai, Khalid Hussain.

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CASE REPORT

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Short Adult Height After Rapid-tempo Puberty: When is it too Late to Treat?

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What is already known on this topic?

Treatment of growth disorders in puberty involves: gonadotropin-releasing hormone analogues to halt progression of puberty. Growth hormone (GH) to promote linear growth during puberty. Aromatase inhibitors block conversion of androgen, slowing epiphyseal maturation and providing more time for growth. These therapies have been studied in short pubertal individuals having GH deficiency, small for gestational age, idiopathic short stature, SHOX deficiency and central precocious puberty.

What this study adds?

There is a rare unreported phenomenon in which puberty, usually not precocious, advances very rapidly so that testosterone levels rise much more rapidly than is typical. This results in rapid advancement of skeletal age leading to growth being completed early, resulting in short stature. This occurs among brothers and as a consequence may be recognized in a younger brother after occurring in an older brother. This report of five such males using the standard therapies among all but the oldest, and found for the youngest that early therapy preserved or reclaimed adult height (AH) potential. Data also suggest that therapy begun before growth potential is already complete may improve AH.

Abstract

A rarely reported phenomenon of rapid-tempo puberty in which the physical changes of puberty and testosterone levels increase very rapidly has not been reported outside apart from in two reviews. The resulting rapid advancement of skeletal age causes early completion of growth with shorter adult stature than expected. This appears to be genetic given its occurrence in the present report in two families, one with three brothers, one with two. We also describe potential treatments and found for the youngest that early initiation of standard therapy preserved or reclaimed adult height (AH) potential. The foreshortened AH in this situation involves rapidly advancing puberty resulting from high circulating testosterone levels leading to rapid advance in skeletal age. This was recognized earlier among younger brothers and treatment with gonadotropin-releasing analogues, growth hormone (GH) and/or aromatase inhibitor therapy (AIT) was tried. Two brothers in family A and family B were treated. Case 5 started treatment early enough so his AH was within target height (mid-parental height) range. Cases 2, 3, 4 were tried on GH and/or AIT with outcomes suggesting benefit. The prevalence and mechanism of rapid-tempo puberty requires further study. Furthermore, as illustrated by two of the current cases, this phenomenon may have a heightened prevalence, or at least may occur, in children previously diagnosed with constitutional delay of growth, underscoring the need to be cautious in assurance of a normal AH outcomes in this population, based on data from a single assessment.

Keywords: Short adult height, rapid-tempo puberty, male-limited genetic pubertal trait, gonadotropin-releasing hormone analogue, growth hormone, aromatase inhibitor therapy

Introduction

The cases reported here illustrate a phenomenon previously mentioned in only two review articles, which has been called “rapid-tempo puberty” (1,2). This involves an unusual accelerated advance of physical puberty with a rapid maturation of bone age (BA) resulting in foreshortening of adult height (AH) (1,2). Rapid-tempo puberty may be associated with familial patterns, being born small for

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gestational age (SGA), overweight/obesity accelerated early childhood growth, adoption, endocrine disrupters, and delayed treatment of chronic primary hypothyroidism (1). These children have a growth rate along the lower percentiles before puberty and commonly have been evaluated by pediatric endocrinologists who have assured them of normal AH because of BA delay. The physical changes of puberty occur early within the normal age range and progress rapidly with an early growth spurt. However, nothing raises concern until linear growth slows at a height (HT) shorter than expected. By the time this is brought to medical attention, not only is physical development complete, but BA is so advanced that linear growth is also essentially complete and therefore, it is too late for any therapeutic interventions. However, since this may be genetic, younger siblings, especially brothers, may also be at risk and should be evaluated soon after onset of puberty to determine it they may be similarly affected.

A recent review of treatment of short stature during puberty discussed the use of gonadotropin-releasing analogue (GnRHa), growth hormone (GH) and aromatase inhibitor therapy (AIT) among a range of diagnoses, including GH deficiency, SGA, idiopathic short stature (ISS), *SHOX* gene deficiency and central precocious puberty (CPP) (2). An opinion article regarding treatments to delay skeletal maturation to gain AH noted that its effect in short children with normal pubertal timing is equivocal (3). GnRHa therapy is well described for CPP (4) although it has frequently been used during the normal years of puberty (5). A retrospective assessment of growth in ISS patients having early/normal puberty and short adolescents not previously treated with GH found a beneficial effect of combined GH/GnRHa therapy on AH (6,7). A 2016 report of SGA girls treated for two years with GnRHa or GH had similar AHs, if there were adequate growth during GnRHa therapy (7,8). However, a 2023 review which included two of the same authors, found that GH-treated SGA children were still short at the onset of puberty with expected AHs less than -2.5 standard deviation (SD) and have a mean improvement of AH of 6.6 cm (8,9). GnRHa therapy in early pubertal girls with poor predicted AH (PAH) (10) has been reported to result in statistically significant greater AH among a cohort of 16 girls compared with historical controls (11). A publication discussing the use of GH and GnRHa noted that puberty may begin at an appropriate age “but precocious for height age” indicating there may be a positive effect on AH (12). The author found no reports of the use of such therapies, specifically in males with rapid tempo puberty. There is also no reason to assume that the same phenomenon cannot occur in girls who may suffer from referral ascertainment bias. The five cases reported below suggest the genetic nature of rapid-tempo puberty and potential therapeutic approaches to improve AH.

Case Reports

Case 1, presented at 15 years of age, fully mature. He was 167 cm tall and by history had not grown in more than 2.5 years. His father is 178 cm tall and mother 160 cm. Based on his target/mid-parental height (MPH), AH was predicted to be 172.5 cm with a range from 164 and 181 cm.

Case 2, the second of these three brothers, was seen at 12.3 years of age and his BAs, HTs, PAH, weights and treatment are shown in Figure 1. He was then begun on an AIT at 12.6 years of age. It was assumed that the AIT would be effective and he was not fully evaluated until the author first saw him at 15.3 years of age. At this time, by history his voice had changed two years previously at age 13 years. At 15.3 years, his HT was 169.7 cm, considered to be 99% complete. Because knee epiphyses were not yet fused, he continued on anastrozole until he was 16 years old. His near AH at 15.8 years was 170.5 cm.

Case 3, the youngest of the three brothers, was seen elsewhere at 11.6 years with early pubertal development and his HTs, BAs, PAH, and weights are shown in Figure 1. Anastrozole was begun at 12.9 years and GH at 14.1 years when he was first seen by the authors, after potential treatments were discussed. GnRHa was not prescribed, since his BA was considered to be too advanced for this therapy to be potentially helpful. GH was given at 3.2 mg daily (0.37 mg/kg/week), and AIT was changed to letrozole. At 15.3 years, he had grown another 3 cm in 8 months, at which time his family chose to continue the GH and AIT. At 15.8 years, his HT was 170.5 cm, an increase of only 0.8 cm in six months, at which time he was considered to be near AH. GH and AIT were discontinued at this time. AH at 29 years of age, reported separately by the patient and his mother, was 172.5 cm.

Case 4 was healthy as a child, had grown along the 25th percentile for HT and by history had documented delayed BAs. When he was 11.3 years, it was noticed that he had Tanner stage 3 genital development. At 12.3 years, his BA had advanced to 15.2 years (Figure 2) and he was Tanner stage 5. When seen together with his younger brother by the author, further advance in BA and full sexual maturity with testicular volume of 18 cc was noted. Random LH was 2.54 mIU/mL and follicle stimulating hormone (FSH) 1.79 mIU/mL. He was started on GH at an adolescent dosage of 4.5 mg/day (0.5 mg/kg/week) and AIT because of his compromised HT potential. He and his parents understood that his BA age was too advanced for GnRHa therapy to be

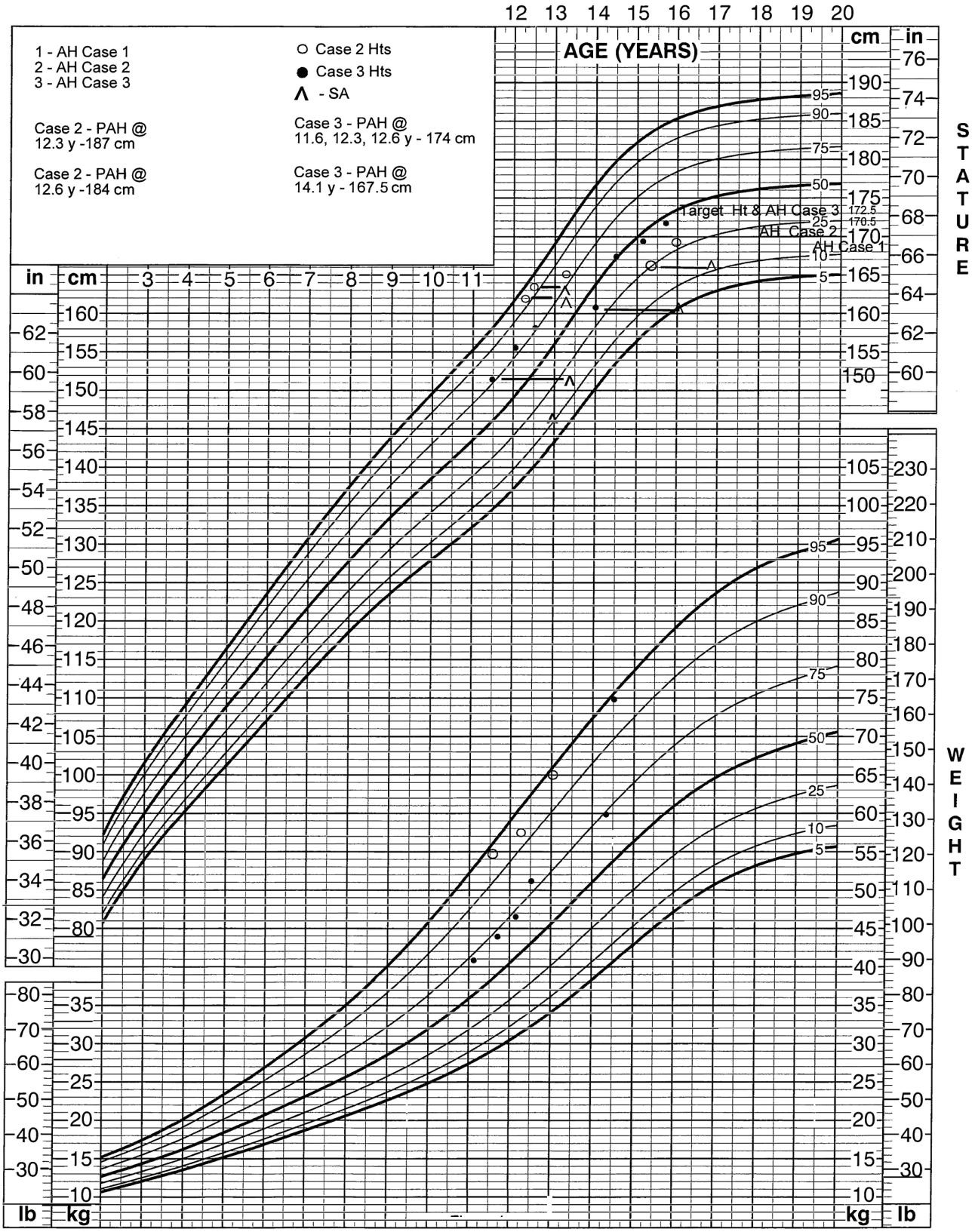

Figure 1. Shows HTs, bone age/SA B weights and PAH for age for Cases 2 and 3. It is apparent that PAHs are not accurate in rapid-tempo puberty. AH of 167 cm is indicated for Case 1. AH for Case 2 and 3 are 170.0 and 172.5 cm respectively

SA: skeletal age, AH: adult height, PAH: predicted adult height

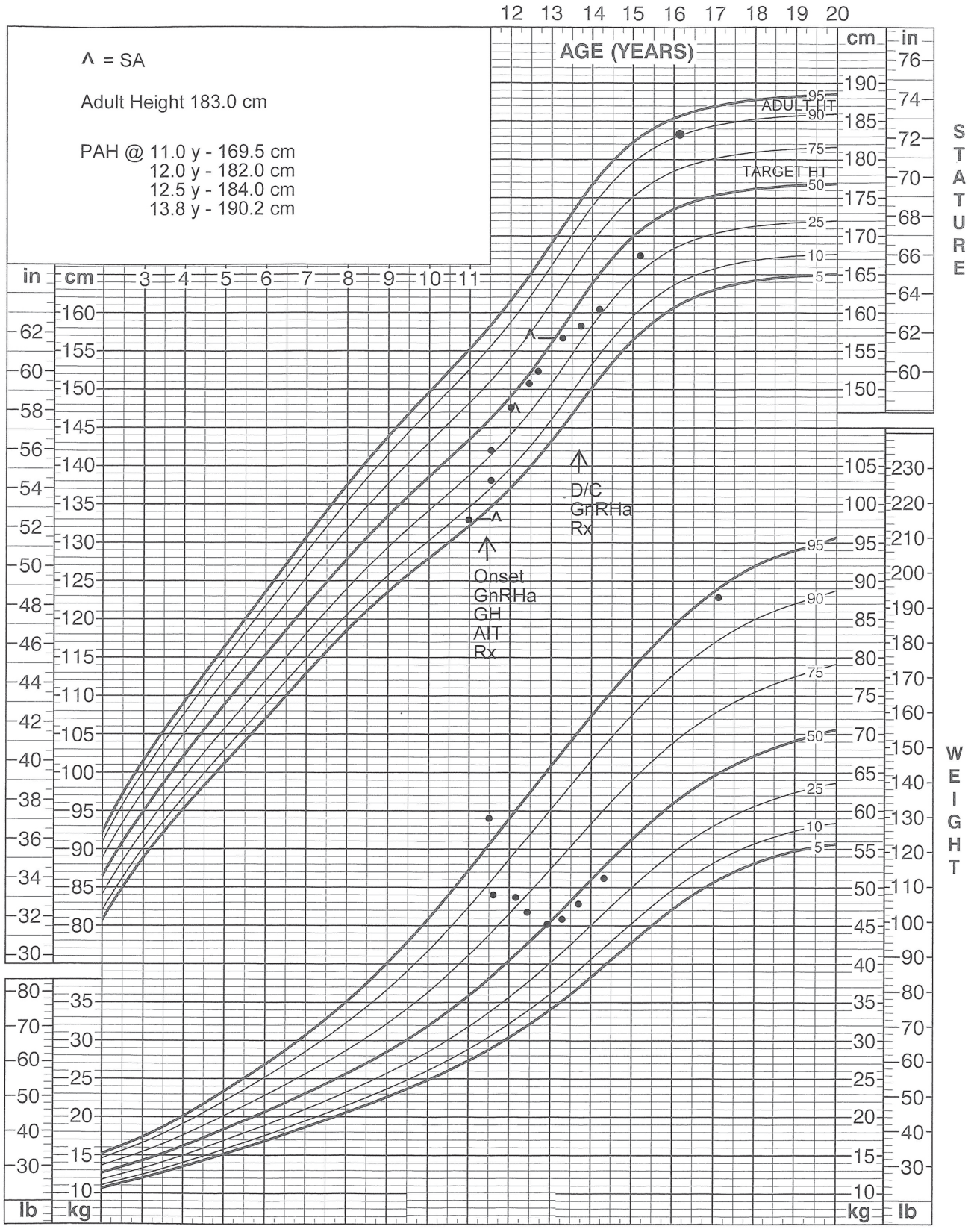

Figure 2. Depicts weight and HT for age, bone age (SA) B of age, treatment, AH, Target/MPH and AH for Case 2. Note that PAH are inaccurate

HT: height, SA: skeletal age, AH: adult height, PAH: predicted adult height, MPH: mid-parental height

helpful. Four months later, at 14.9 years, total testosterone was 1000 ng/dL, IGF1 907 ng/mL and IGFBP3 4.9 mg/L. His HTs and weights were documented four times from age 14.9 to 16.4 years (Figure 2). It is noteworthy that he grew 6.6 cm over the next 1.8 years while receiving GH and AIT. His near AH attained on his 17th birthday was 170 cm, at which time GH and AIT were discontinued. Currently he is 172.5 cm tall at 40 years of age, is married with a healthy child and works as a recruiter for one of the largest companies in the world.

Case 5 had normal birth, neonatal and early developmental histories. From age 3 onward he grew along the 5th percentile for HT; had had BA interpreted as 5 years at 5.8 years of chronological age and again at 8.5 years of age when his BA was read as 8 years. He was seen and evaluated by the author, together with his older brother (Case 4) because he experienced rapid onset of puberty beginning at 11 years and because of his brother's growth history. By history, at 9 years of age, he had developed initial penile growth and body odor, which was followed by recent onset of pubic hair. An accelerated growth rate had been noted within the past year. At 11.0 years, he had another BA which had advanced to 11.5 years with a total testosterone of 158 ng/dL, LH of 0.5 mIU/mL and FSH of 1.4 mIU/mL. His chronological ages, BAs, HTs and weights are shown in Figure 3, together with PAH. When seen at 11.3 years, he had Tanner stage 3 genital development with testicular volumes of 12 cm bilaterally and pubic hair Tanner stage 2. He was begun on three therapies: a) an AIT, using letrozole (2.5 mg/day); b) GnRH analogue therapy (15 mg of leuprolide 4 weeks) to suppress testosterone; and c) GH (2.0 mg/day or 0.22 mg/kg/week).

Four months later, pubic hair had increased to Tanner stage 3. Testosterone had decreased to 15 ng/dL while IGF1 was 341 ng/mL His GH dosage was increased to 2.5 mg daily or 0.24 mg/kg/week). Five months later, his growth in HT continued (Figure 3) while weight decreased further and his testicular volumes had decreased to 10 cc. His BA had advanced by only 6 months to 12 years, consistent with his chronological age, over the 9 months since therapies had begun. IGF1 was repeated and found to be 459 ng/mL. Fifteen months after starting treatment, at 12.4 years, a further decrease was noted in weight to 45.9 kg and in testicular volumes to 8 cc. Testosterone had dropped to <15 ng/dL. Four months later at 12.8 years, his BA was 12.6 years and IGF1 was 481 ng/mL at 13.4 years, his HT and weight had begun to increase. Almost 6 months later at 13.8 years, his age exceeded his BA, which remained at 12.6 years, and there was no increase in testicular volumes. GnRHa therapy was discontinued. Six months later his HT and weight both had further increased, Tanner genital and pubic hair stages were 4 and testicular volumes had slightly increased, providing evidence of resumption of puberty. At age 15 years he weighed 84 kg, and was 167 cm tall, but GH and letrozole were continued as he was still growing at 16.5 years of age. Currently at 26 years of age he is 183 cm tall, healthy and working in cybersecurity tech sales. His AH exceeded his PAH at all times after presentation at 13.0 years, although all data suggest that PAH is often not helpful, since it is based upon the expectation of normal progression of puberty. His AH in relation to his Target/MPH suggests that interruption with appropriate therapies of rapid-tempo puberty can result in AH within the range of Target/MPH, even if well above the mean.

Discussion and Conclusion

It can be assumed that the foreshortened adult HT that occurs with rapid-tempo puberty is the result of elevated circulating testosterone levels resulting in BA advancement, as can be seen with the risk factors that preclude full potential growth in HT that accompany the typical pace of bone maturity. In addition, those caring for prepubertal children with a diagnosis of constitutional delay of growth should be aware of this infrequent entity of rapid-tempo puberty and follow these children closely to catch any accelerated puberty and BA maturation that could have a major adverse effect on ultimate AH. Potential therapy for patients with rapid-tempo puberty recognized before growth is complete, include GnRHa, GH and AIT. Case 1 illustrated that, without intervention, AH is compromised, being at the lower limit (-2 SD) of the range of his Target/MPH. It is not possible to determine if Case 2 benefitted from AIT since his AH is also within the range of his Target/MPH at 169.9 cm, approximately at -1.2 SD. Case 3 appeared to have benefitted from AIT and GH therapy, since his near AH of 170.5 cm was approximately -1.0 SD. Case 4 at 14 years had a PAH of 166 cm and MPH of 175 cm when GH and AIT were begun and reached an AH of 172.5, almost at the mean Target/MPH, strongly suggesting benefit from his therapy (Table 1). Case 5 clearly benefitted from GnRHa therapy until chronological and BA matched, with continuation of GH and AIT, because his AH approached +2 SD.

Case 5, who was started on therapy before his HT potential diminished, even though rapid puberty had begun, suggested that interrupting this process with treatment before high testosterone levels lead to advanced BA and can result in expected AH. The other cases suggest, but do not prove, that both AIT and GH therapy result in greater AH in patients with rapid tempo puberty. Hence, if recognized early, this condition can be successfully treated.

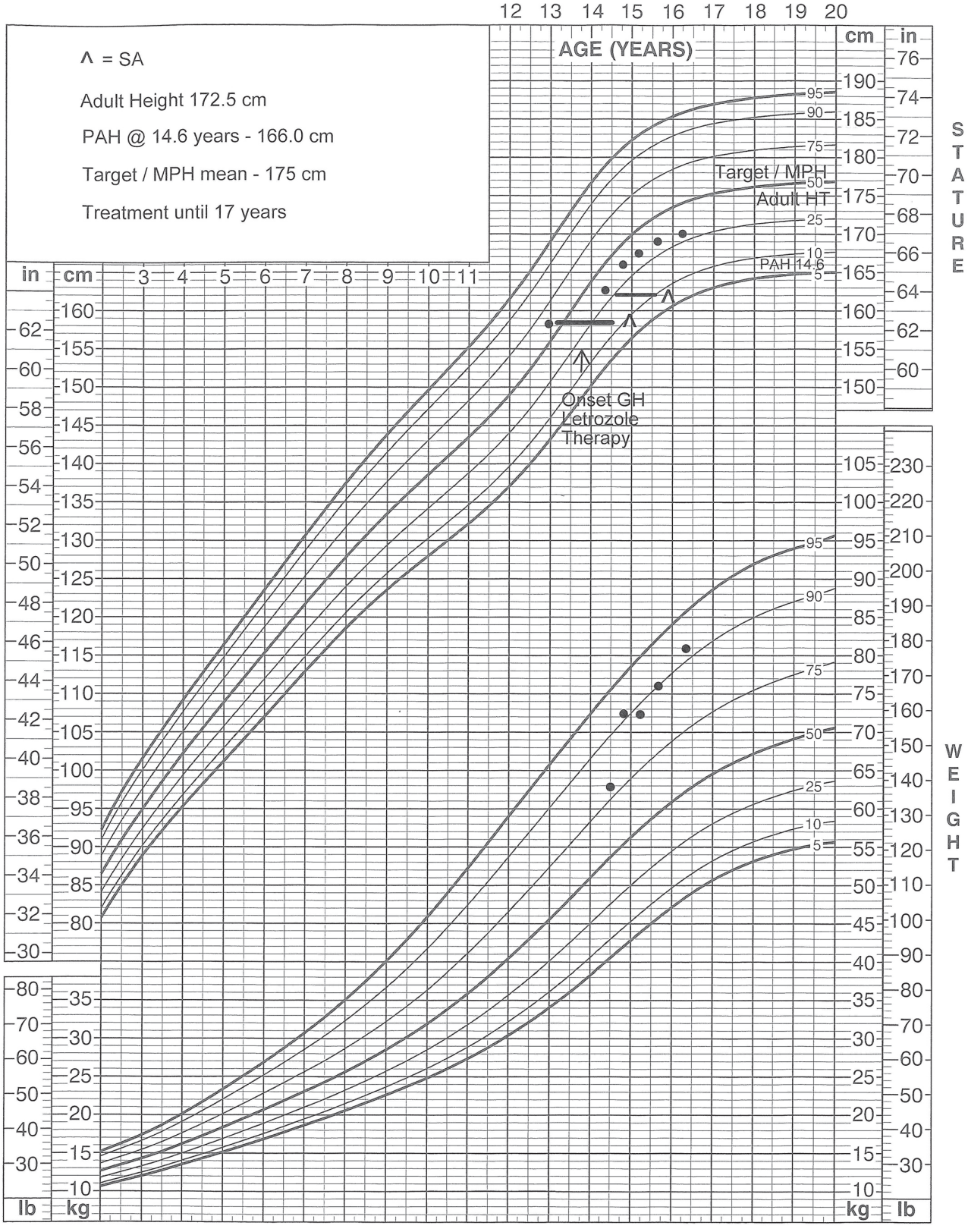

Figure 3. Shows weight and HT for age, bone age or SA for age, treatment, and AH. Note that during GnRHa therapy that SA remained at 12.5 to 13 years during which time HT for age advanced reclaiming lost HT potential. Note also that weight dropped over this interval

HT: height, SA: skeletal age, AH: adult height, GnRHa: gonadotropin-releasing hormone analogue

Table 1. Data from two brothers in family B showing age, skeletal age, height, predicted adult heights, weight and treatment

Family B	Chronologic age (y)	Bone age (y)	Height (cm)	Predicted adult height (cm)	Weight (kg)	Treatment
Case 4	12.3	13.5				None
	13.1	15.2	159.0			
	14.6	16.3	163.4	166.0	63.1	GH and letrozole
	14.9		166.4		71.3	
	15.3		167.5		72.4	
	15.8		168.9		76.6	
	16.4		170.0		79.9	
	40		172.5			None
Case 5	11.0	11.5	133.0	169.5		None
	11.3		138.8		58.8	GH, GnRHa letrozole
	11.6		143.0		48.9	
	12.0	12.0	147.0	182.0	48.00	
	12.4		150.4		45.9	
	12.8	12.5	152.4	184.0	44.9	
	13.4		155.6		46.1	
	13.8	12.5	157.8	190.2	46.1	
	14.3		160.4		50.7	
	15.0		167.0			
	16.5		170.0		84.0	
	26		183.0			None

That the median mid-parental height for sons was 175 cm, with the range from 183.5 to 166.5.
GnRHa: gonadotropin-releasing hormone analogue, GH: growth hormone

Acknowledgments

I appreciate these individuals and their parents for being willing to discuss their situation over many years as well as currently in the hope of helping others. I also am thankful for my administrative associate, Lydia Hibshman, for help preparing this manuscript including the figures.

Note: The author, who diagnosed and cared for these patients until late teens and has maintained contact since, wrote this manuscript from that knowledge, medical records and current discussions with the patients and their parents.

Ethics

Informed Consent: Informed consent has been obtained from the patients and they indicated permission to discuss their outcome with their parents.

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

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J Clin Res Pediatr Endocrinol 2024;16(2):243

Vitamin D Receptor Gene Polymorphisms with Type 1 Diabetes Risk: Correspondence

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Keywords: Diabetes, genetic, risk

Dear Editor,

We would like to share ideas on the publication "Vitamin D Deficiency and Vitamin D Receptor Gene Polymorphisms with Type 1 Diabetes Risk: A South Indian Familial Study (1)." The purpose of this study was to determine the importance of vitamin D status and vitamin D receptor gene polymorphisms in connection to the risk of type 1 diabetes (T1D) in the South Indian population. One hundred-twenty T1D patients and 214 unaffected first-degree relatives (FDRs) participated in the study. The amplification refractory mutation system-polymerase chain technique was used to genotype VDR polymorphisms at four distinct loci (FokI, BsmI, TaqI, and ApaI). A group of 98 T1D patients and 75 age- and sex-matched siblings had their serum vitamin D levels checked using the ELISA technique.

The sample size is one potential weakness of this study. Despite the fact that the study comprised 120 T1D patients and 214 FDRs, a higher sample size would yield more solid and accurate results. Furthermore, the study was conducted on a specific demographic (South Indians) and may not be applicable to other ethnic groups. Another problem is that the study only looked at the relationship between *VDR* gene polymorphisms and T1D risk, not the relationship with vitamin D levels. The researchers did not look into the functional significance of these polymorphisms or the underlying processes that relate vitamin D insufficiency to the development of T1D. More research is required to investigate these features and provide a more comprehensive grasp of the subject.

At the very least, certain genetic variants may be related to the pathophysiological mechanisms generating the reported clinical appearance. SNPs in the *SIRT1* gene are one illustration of this genetic variation (2). To ascertain the effect of any potential confounding polymorphisms, more investigation is required.

Ethics

Authorship Contributions

Concept: Hinpetch Daungsupawong, Viroj Wiwanitkit, Design: Hinpetch Daungsupawong, Viroj Wiwanitkit, Analysis or Interpretation: Hinpetch Daungsupawong, Viroj Wiwanitkit, Literature Search: Hinpetch Daungsupawong, Viroj Wiwanitkit, Writing: Hinpetch Daungsupawong.

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

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In response to: "Letter to: Vitamin D Receptor Gene Polymorphisms with Type 1 Diabetes Risk: Correspondence"

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Response to the letter,

Our study focused on exploring the significance of vitamin D levels and vitamin D receptor (VDR) gene polymorphisms with the risk of developing type 1 diabetes (T1D) in the South Indian population comprising 334 participants (120 T1D patients and 214 controls) belonging to 120 families (comprising parents, first degree relatives and 36 trios).

The study found that vitamin D deficiency was more common in patients with T1D than their unaffected relatives. The study limitations were highlighted as given below in the article published,

"As this study was structured for a family-based approach, the small sample size is the foremost limitation. A comprehensive familial study with large sample size, DNA sequencing, and gene expression evaluations are necessary to clarify the role of the VDR gene variants on T1D in the future. Furthermore, factors possibly influencing serum vitamin D synthesis, such as intake of supplements, obesity, liver and kidney diseases, and cutaneous factors, were not investigated" (1).

Thus, the comment is a replica of the study limitations provided.

Mariakuttikan Jayalakshmi, on behalf of all authors.

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1. Thirunavukkarasu R, Chitra A, Asirvatham A, Jayalakshmi M. Association of Vitamin D Deficiency and Vitamin D Receptor Gene Polymorphisms with Type 1 Diabetes Risk: A South Indian Familial Study. J Clin Res Pediatr Endocrinol 2024;16:21-30. Epub 2023 Aug 10

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