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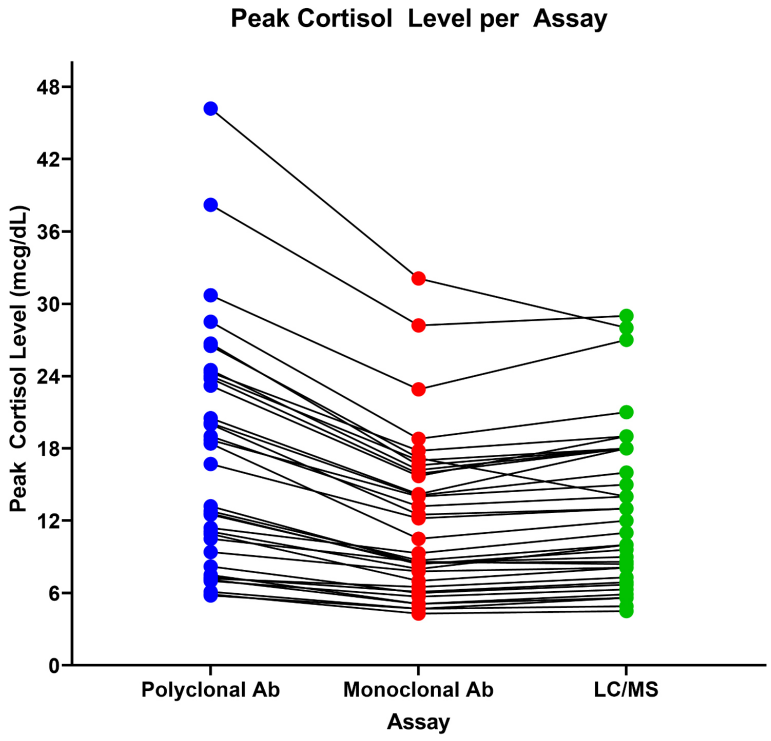

Peak cortisol level (mcg/dL) using polyclonal antibody immunoassay, monoclonal antibody immunoassay, and LC/MS in 36 children undergoing 1 mcg Cosyntropin stimulation test

Peak Serum Cortisol Cutoffs to Diagnose Adrenal Insufficiency Across Different Cortisol Assays in Children

Cortez S et al.

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ORIGINAL ARTICLE

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J Clin Res Pediatr Endocrinol 2023;15(4):338-347

PROKR2 Mutations in Patients with Short Stature Who Have Isolated Growth Hormone Deficiency and Multiple Pituitary Hormone Deficiency

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What is already known on this topic?

Homozygous *PROKR2* mutations have been identifed in Kallmann syndrome and hypogonadotropic hypogonadism. Recently, PROKR2 has been suggested to play a role in pituitary hormone deficiencies. While homozygous *PROKR2* mutations have been reported as pathogenic, the role of heterozygous forms in the mechanism is unknown.

What this study adds?

This study presents strong evidence that heterozygous *PROKR2* mutations play a role in pituitary hormone deficiencies other than Kallmann syndrome. Heterozygous healthy carriers suggest that concomitant oligogenic or digenic inheritance in patients with *PROKR2* mutation is the strongest underlying mechanism of disease causing phenotype.

Abstract

Objective: Recent reports have indicated the role of the prokineticin receptor 2 gene *(PROKR2)* in the etiology of pituitary hormone deficiencies, suggesting a potential role for the PROK2 pathway in pituitary development, in addition to its role in gonadotropin releasing hormone-expressing neuron development. Here, we present the clinical and molecular findings of four patients with *PROKR2* mutations.
Methods: Next-generation targeted sequencing was used to screen 25 genes in 59 unrelated patients with multiple pituitary hormone deficiency (MPHD), isolated growth hormone (GH) deficiency, or idiopathic short stature.
Results: Two different, very rare *PROKR2* missense alterations classified as pathogenic (NM_144773.4:c.518T>G; NP_658986.1:p.(Leu173Arg)) and likely pathogenic (NM_144773.4:c.254G>A; NP_658986.1:p.(Arg85His)) were identified in four patients in heterozygous form. Patient 1 and Patient 2 presented with short stature and were diagnosed as GH deficiency. Patient 3 and Patient 4 presented with central hypothyroidism and cryptorchidism and were diagnosed as MPHD. No other pathogenic alterations were detected in the remaining 24 genes related to short stature, MPHD, and hypogonadotropic hypogonadism. Segregation analysis revealed asymptomatic or mildly affected carriers in the families.
Conclusion: *PROKR2* dominance should be kept in mind as a very rare cause of GH deficiency and MPHD. Expressional variation or lack of penetrance may imply oligogenic inheritance or other environmental modifiers in individuals who are heterozygous carriers.
Keywords: Growth hormone deficiency, multiple pituitary hormone deficiency, *PROKR2*, short stature

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Introduction

The prokineticin system consists of two multifunctional proteins, prokineticin-1 and prokineticin-2, and their G protein-coupled receptors. They were first identified in 2000 by Li et al. (1) as endogenous regulators of the gastrointestinal tract. More recently it has been shown that they have roles in many biological functions, such as circadian rhythm regulation, nociception, angiogenesis, hematopoiesis, immune response, development of the olfactory bulb, and sexual maturation. Expression of prokineticins and their receptors has been reported in various tissues, including the ovary, testis, uterus, adrenal gland, placenta, brain, digestive tract, heart, and bone marrow (2,3,4). As the prokineticin signaling pathway has a critical role in the embryonic development of the olfactory system, it was proposed that both neural and neuroendocrine developmental abnormalities could occur in patients carrying mutations in these genes (5). In *PROK2* and *PROKR2* knockout mice, gonadotropin-releasing hormone (GnRH) secretion was impaired which led to a disruption of sexual development and fertility in both male and female mice, thus making the *PROK2* and *PROKR2* genes strong candidates for human GnRH deficiency (6,7,8).

In recent years the number of patients with Kallmann syndrome who have *PROKR2* mutation has increased (9,10,11). In addition, monoallelic *PROKR2* variants were reported to have a role in multiple pituitary hormone deficiency (MPHD) and septooptic dysplasia (SOD) (12).

Despite this, healthy subjects were reported to have these same variants in heterozygous form (11,13). Therefore, it was proposed that these mutations did not cause major midline defects spontaneously but may contribute as modifier genes or induce the phenotype through digenic or oligogenic inheritance, as previously demonstrated in idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (7,14). Thus, further studies are needed to clarify the role of PROKR2 signaling in the pituitary gland and midline development (12).

In this study, a gene panel was used to screen for the genetic causes of MPHD, growth hormone (GH) deficiency, and idiopathic short stature. We identified four patients with *PROKR2* variants with different phenotypes other than Kallmann syndrome. The role of the PROKR2 gene in the etiology of GH deficiency and MPHD was investigated.

Methods

Patients

Using a candidate gene approach, 59 patients with MPHD, GH deficiency, and idiopathic short stature were screened. Written informed consent was obtained from all patients. The study protocol was approved by the İstanbul University, İstanbul Faculty of Medicine Local Clinical Research Ethics Committee (date: 11.08.2017, approval number: 13).

The data, collected retrospectively, consisted of physical examination, auxological findings, family history, hormone assays, biochemical and radiological findings, surgical and medical treatment, and additional features at follow-up (see below). Anthropometric measurements of the patients and parental heights were measured by the same auxologist and the target height was calculated. Bone age was evaluated by using the Greulich-Pyle method (15). The predicted adult height was calculated according to the Bayley Pinneau method (16). The standard deviation score (SDS) of all auxological measurements was calculated according to national data (17,18). The upper limit for Turkish girls to attain menarche is 14 years old and menarche after 14 years of age was defined as delayed menarche (19).

Molecular Analysis

Chromosomal abnormalities were excluded by using microarray and cytogenetic techniques before the initiation of molecular genetic analysis. Screening of targeted regions for an in-house-designed panel with 25 genes (*BMP4, FGF8, FGFR1, GH1, GHR, GHRH, GHSR, HESX1, HHIP, IGF1, IGF1R, IGFALS, IGFBP3, IGSF1, LHX3, LHX4, OTX2, POU1F1, PROKR2, PROP1, SHH, SHOX, SOX3, STAT5B, WDR11*) were tested using Ion Torrent PGM™ system for next-generation sequencing (ThermoFisher Scientific, Waltham, MA, USA).

Hormonal Assays

Blood samples were collected in the morning after eight hours of fasting. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, cortisol, free thyroxine, and thyroid stimulating hormone were analyzed by electrochemiluminescence immunoassay (Cobas, Roche Diagnostics, Mannheim, Germany). Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP-3) levels were analyzed by immunoradiometric assay (Immunotech, Beckman Coulter Inc, Prague, Czech Republic). GH was determined by radioimmunoassay (Diagnostic System Laboratories Inc., Webster, TX, USA). GH stimulation tests (GHST) were performed with clonidine and L-dopa and GH values less than 10 ng/mL were accepted as GH deficiency (20). GnRH test was performed and serum LH and FSH concentrations were measured at baseline and at 30, 60, 90, and 120 minutes after an intravenous bolus of 0.1 mg gonadorelin acetate. Bone mineral density L1-L4 was evaluated using dual-energy X-ray absorptiometry (Hologic QDR 4500A Fan Beam X-ray Bone Densitometer, Hologic, Bedford, MA, USA) and analyzed using software version 12.3.

Statistical Analysis

The Statistical Package for Social Sciences for Windows 21.0 was used for statistical analysis (IBM Inc., Armonk, NY, USA). Results are reported as median (minimum-maximum) or as number or percentages, where appropriate.

Results

General Results

Genetic analyses revealed two different heterozygous clinical variants in the *PROKR2* gene in four patients. These variants had previously been reported in Kallmann syndrome. Patient 1 and Patient 2 were heterozygous for NM_144773.4:c.254G > A;p.(Arg85His) and Patient 3 and Patient 4 were heterozygous for NM_144773.4:c.518T > G;p.(Leu173Arg) variants. Segregation in families revealed that the mothers of Patients 2 and 3, and the father of Patients 1 and 4 were the carriers of the related variants. Delayed puberty or short stature of carrier parents of three patients were associated with *PROKR2* mutation. However, we could not evaluate the hormone axes because the family members did not consent so that only hypothyroidism and hypogonadism were excluded. The father of Patient 4 could not be evaluated. Pedigrees are shown in Figure 1.

Patient 1

A 12-year-old female patient was referred for short stature. She was born into a consanguineous family at term with low birth weight and had no problems during the prenatal or early postnatal period. Her motor and mental developmental milestones were normal for her age. Family history revealed short stature in her father and delayed menarcheal age in her mother. Physical examination at presentation was normal, except for proportionate short stature. She had a normal sense of smell and no dysmorphic features.

Workup for short stature yielded normal biochemical investigations, thyroid hormone, cortisol, and prolactin levels. IGF-1 and IGFBP-3 levels were in normal ranges but GHSTs were compatible with GH deficiency. Cranial and pituitary magnetic resonance images (MRI) did not reveal any pathology. At 12.75 years of age, growth velocity decreased and GH treatment was started (0.035 mg/kg/day). She was treated with GH until the age of 13.9 years.

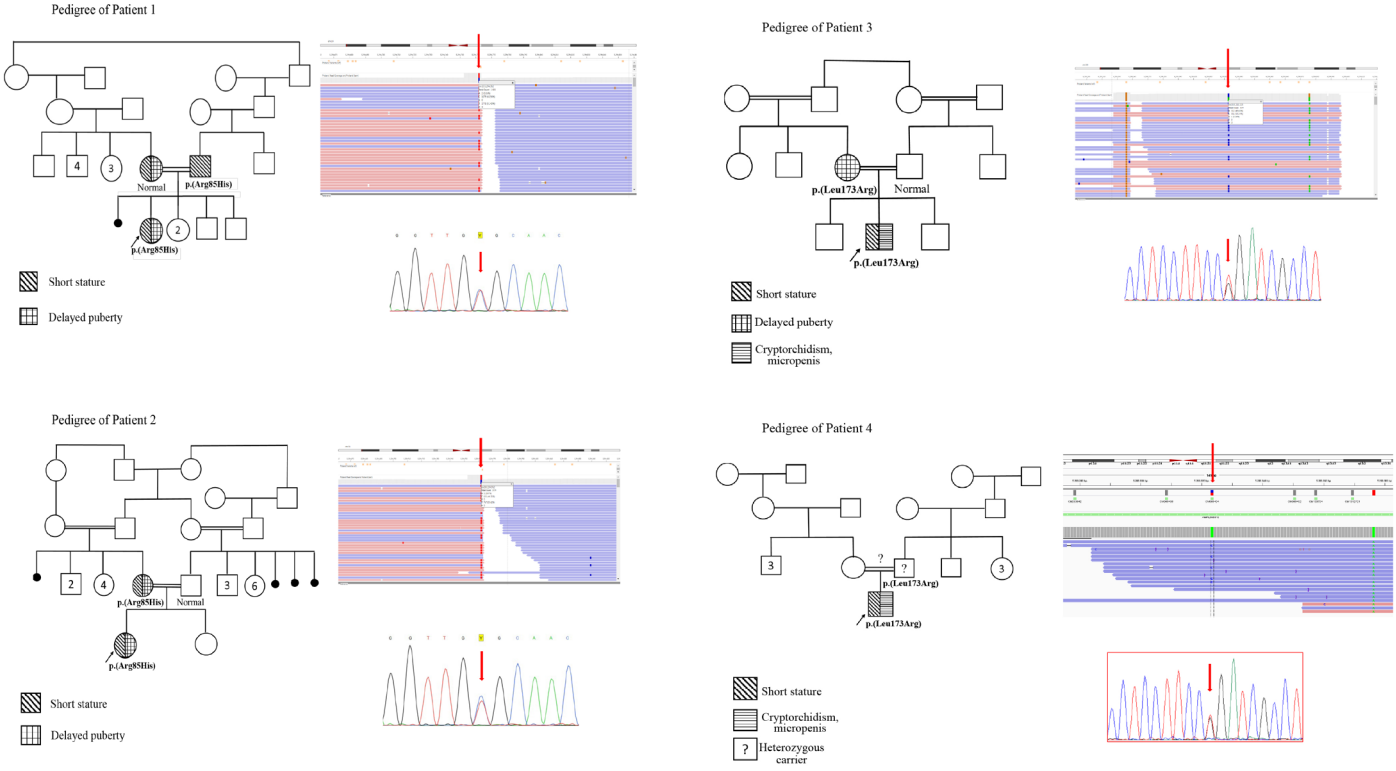

Figure 1. Family pedigrees of the patients with *PROKR2* allelic variants. Arrow points out the probands. The clinical signs that the symbols represent are given by line pattern. The Integrative Genomics Viewer of the variants and the electropherogram of the Sanger sequences of the variant sites are shown

At presentation, the patient was at Tanner stage 2 and menarche occurred at 13.5 years of age. At the last evaluation, the patient was 15.7 years old, pubertal development was complete and she had regular menstruation. GnRH test was performed and basal FSH and LH were 4.47 IU/L and 10.52 IU/L and increased to 10.52 IU/L and 31.77 IU/L, respectively. Urinary tract ultrasonography (USG) and pelvic USG were normal. The clinical and hormonal findings and the molecular results of the patients are shown in Table 1 and Table 2.

Table 1. Clinical and anthropometric findings in the patients

At presentation	Patient 1	Patient 2	Patient 3	Patient 4	Median (min-max)
Age (years)	12	11	0.5	0.5	5.75 (0.5-12)
Gender	F	F	M	M	-
Consanguinity	3rd degree	1st degree	No	3rd degree	-
Presenting features	Short stature	Short stature	Short stature, micropenis, undescended testis	Short stature, micropenis, undescended testis	-
Birth weight g/SDS	2500/-2.0	3600/0.7	3230/-0.6	2100/-2.6	-1.3 (-2.6-0.7)
Height cm/SDS	135/-2.7	128.4/-2.5	59.2/-3.3	51.1/-6.3	-3.0 (-6.3 and -2.5)
Weight kg/SDS	30.9/-2.0	31.4/-1.0	8.3/0.05	4.0/-4.8	-1.5 (-4.8-0.05)
BMI kg/m²/SDS	17/-0.8	19/0.4	23.5/3.3	15.0/-1.7	-0.2 (-1.7-3.3)
HC cm/SDS	52.8/-0.8	51.6/-1.2	43.2/-0.7	35.5/-6.1	-1.0 (-6.1 and -0.7)
SHR/SDS	0.54/0.9	0.53/-0.03	0.69	-	0.69 (-0.03-0.9)
Tanner stage	Ph1B2/2	Ph1B2/2	Ph1T0.5/0.5 mL	Ph1T nonpalpable	-
Bone age (years)	$8^{10/12}$-10	$7^{10/12}$-$8^{10/12}$	1 (at age of 1.6 years)	NA	-
Mother's height SDS	150.6/-1.9	147.4/-2.4	157.5/-0.9	156.5/-1.1	-1.5 (-2.4 and -0.9)
Father's height SDS	158.8/-2.4	167.2/-1.3	168.8/-1.1	NA	-1.3 (-2.4 and -1.1)
Target height cm/SDS	148.2/-2.3	150.8/-1.9	169.7/-0.9	NA	-1.9 (-2.3 and -0.9)
Hormone deficiencies (onset of age-years)	GH (12.3 years)	GH (11.2 years)	GH (14 months) TSH (6 months) PRL (10 months)	GH (22 months) TSH (6 months) PRL (6 months) FSH/LH (6 months) DI (6 months)	-
At most recent evaluation					
Age (years)	15.7	18.4	14.4	1.6	15.1 (1.6-18.4)
Height cm/SDS	146.5/-2.7	153.2/-1.7	173.2/0.8	65/-5.0	-2.2 (-5.0-0.8)
Weight kg/SDS	54.2/-0.3	57.3/-0.1	78/1.6	5.7/-6.1	-0.2 (-6.1-1.6)
SHR/SDS	0.54/0.1	0.52/0.1	0.54/1.4	NA	0.1 (0.1-1.4)
BMI SDS	1.4	1.1	1.4	-2.8	1.25 (-2.8-1.4)
Pubertal stage (Tanner stage)	Ph5B5/5	Ph5B5/5	Ph3T5/5 mL	Ph1T nonpalpabl	-
Bone age (years)	15	16	15	NA	-
Mother's menarcheal age (years)	16	15	14	14	14.5 (14-16)
Menarcheal age (years)	13.5	15.6	-	-	-
Replacement treatment (duration)	GH (until age 13.9)	GH (until age 17)	GH (until age 14.1) L-thyroxine (continue)	GH (started) L-thyroxine (continue) Desmopressin (continue)	-
Zygosity **NM_144773.2** **NP_658986.1** **HGMD id** **dbSNP id**	Heterozygous c.254G > A p.(Arg85His) CM065401 rs74315418	Heterozygous c.254G > A p.(Arg85His) CM065401 rs74315418	Heterozygous c.518T > G p.(Leu173Arg) CM065404 rs74315416	Heterozygous c.518T > G p.(Leu173Arg) CM065404 rs74315416	-
Parental carrier status	Father (+) Mother (-)	Father (-) Mother (+)	Father (-) Mother (+)	Father (+) Mother (-)	-

The median (min-max) of the anthropometric data with the standard deviation score was calculated.
F: female, M: male, SDS: standard deviation score, BMI: body mass index, HC: head circumference, SHR: sitting height ratio, Ph: pubic hair, B: breast, T: testis, NA: not available, GH: growth hormone, TSH: thyroid stimulating hormone, PRL: prolactin, DI: diabetes insipitus, min-max: minimum-maximum

Table 2. Laboratory and radiological results of the patients

	Patient 1	Patient 2	Patient 3	Patient 4	Normal ranges
Na (mmol/L)	139	139	141	147	135-145
K (mmol/L)	4.2	4.8	4.0	5.0	3.5-5.5
Cortisol (nmol/L)	496.8	574	472	265	77.3-635
fT4 (pmol/L)	15.8	16.9	8.3	8.6	11.6-21.5
TSH (mIU/L)	4.6	1.83	0.56	2.39	0.66-4.14
Prolactin (ng/mL)	6.9	19.4	1.9	0.7	4.8-23.3
IGF-1 (ng/mL) (Normal range)	259 (76-542)	67.8 (111-551)	<25 (55-237)	<25 (55-237)	-
IGFBP-3 (ng/mL) (Normal range)	4.67 (2.4-8.4)	2.53 (2.4-8.4)	0.502 (0.7-3.6)	<0.5 (0.7-3.6)	-
FSH (IU/L)	1.7	2.4	0.44	2.3	1.7-7.7
LH (IU/L)	0.6	0.5	0.1	0.2	1-11.4
Estradiol (pg/mL)	5	20.1	-	-	10-100
GHST peak GH (mg/L) Clonidine L-dopa	 1.7 0.14	 8.17 1.9	 0.105 0.08	 0.08 0.31	
MRI	Normal	Anterior pituitary hypoplasia	Normal	Pituitary hypoplasia, diffuse hypomyelination, cerebral atrophy	
Cortisol (nmol/L)	441.6	223.6	447.1	375.4	82.8-579.6
fT4 (pmol/L)	17.2	16.9	14.2	24.1	11.6-21.5
TSH (mIU/L)	3.26	2	0.007	0.005	0.66-4.14
Prolactin (ng/mL)	30.5	19.1	0.49	0.18	4.8-23.3
IGF-1 (ng/mL) (Normal range)	256 (191-496)	80.3 (117-323)	43.3 (120-501)	17.8 (41-225)	
IGFBP-3 (ng/mL) (Normal range)	5.22 (3.3-10)	2.22 (2.9-7.3)	2.47 (3.5-10)	416 (1410-2970)	
FSH (IU/L)	4.5	5.9	8.8	NA	1.7-7.7
LH (IU/L)	2.9	7.4	4.3	NA	1-11.4
Estradiol (pg/mL)	53.7	69.8	16.4	-	10-100
Testosterone (ng/mL)	-	-	2.1** (Tanner 2)	0.02* (Tanner 1)	* <0.02 **0.02-0.58
Bone density Z score BMD g/m^2	-0.1 0.812	-2.3 0.770	1.7 0.903	NA	>-1.0

NA: not available, Na: sodium, K: potassium, fT4: free thyroxine, TSH: thyroid stimulating hormone, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor binding protein 3, FSH: follicle-stimulating hormone, LH: luteinizing hormone, GH: growth hormone, GHST: GH stimulation test, BMD: bone mineral density, MRI: magnetic resonance imaging

Patient 2

Patient 2 was referred for short stature at 11 years of age. She was born into a consanguineous family at term with normal birth weight and had no problems during the prenatal or early postnatal period. Her motor and mental developmental milestones were normal for her age. Family history revealed short stature and delayed menarche in her mother. At presentation, her physical examination was normal except for her short stature. Body proportions were normal. She had a normal sense of smell and no dysmorphic features.

Hormonal evaluation of the pituitary axis yielded normal results for prolactin, thyroid, and adrenal function. IGF-1 level was low and GH deficiency was diagnosed on GHSTs. Cranial and pituitary MRI revealed anterior pituitary gland hypoplasia.

At follow up mild gastrointestinal symptoms started and the patient's weight decreased to -3.0 SDS. A celiac diseaase work-up was negative and the patient was diagnosed with chronic duodenitis. After the correction of malnutrition, growth velocity remained low, therefore GH treatment was started (0.035 mg/kg/day) at the age of 15 years and continued until the age of 17 years.

At presentation, puberty was at Tanner stage 2. Basal serum LH and FSH concentrations were 1.6 IU/L and 2.2 IU/L and increased normally in response to GnRH stimulation (LH increased to 14.2 IU/L and FSH increased to 7.1 IU/L). Spontaneous menarche occurred at the age of 15.6 years

Table 3. The patients with MPHD and isolated GH deficiency reported to have heterozygous *PROKR2* variants

Patient	Ref	Hormone deficiency	Pituitary MRI	Phenotype	*PROKR2* gene NP_658986.1	Additional gene
1	12	GH, TSH, ACTH, LH, FSH	EPP, pituitary stalk agenesis	SOD, MPHD	p.(Arg268Cys)	
2	12	GH, TSH, LH, FSH	Normal	SOD, MPHD	p.(Arg85Gly)	
3	12	GH, TSH, ACTH, LH, FSH	NA	MPHD	p.(Arg85His)	*ANOS1* *NM_000216.4: c.1375C > T p.(His459Tyr)*
4	28	GH, TSH, ACTH, LH, FSH	APH, EPP, absent stalk, thin corpus callosum	PSIS	p.(Leu173Arg)	
5	28	GH, TSH, ACTH, LH, FSH	APH, EPP, interrupted pituitary stalk	PSIS	p.(Arg85His)	*HESX1* *NM_003865.3: c.200G > C p.(Ser67Thr)*
6	28	GH, TSH, ACTH, LH, FSH	EPP, interrupted pituitary stalk, porencephaly	PSIS	p.(Ala51Thr)	
7	28	GH	APH, thin pituitary stalk	Isolated GHD	p.(Ala51Thr)	
8	29	TSH, ACTH, GH	APH, EPP, thin interrupted stalk	PSIS	p.(Arg85Cys)	*WDR11* *NM_018117.12: c.1306A > G; p.(Ile436Val)*
9	30	GH, TSH, ACTH, LH, FSH	Absent anterior pituitary, EPP	MPHD	p.(Arg85Leu)	
10	30	GH, ACTH, TSH, DI	APH, partially descended PP	MPHD	p.(Leu173Arg)	
11	30	GH, ACTH, TSH, DI	Absent septum pellucidum	SOD, MPHD	p.(Leu173Arg)	
12	30	GH, ACTH, TSH	APH, EPP, hypoplastic stalk	MPHD	p.(Leu173Arg)	
13	30	GH, ACTH, TSH	APH, EPP	SOD, MPHD	p.(Leu173Arg)	
14	30	GH	APH	SOD	p.(Ala51Thr)	
15	30	GH, TSH, ACTH, LH, FSH	APH, EPP, hypoplastic stalk	SOD, MPHD	p.(Arg268Cys)	
16	30	GH, TSH, ACTH, LH, FSH	EPP, absent infundibulum	SOD, MPHD	p.(Arg268Cys)	
17	30	GH, TSH	Corpus callosum agenesis	SOD, MPHD	p.(Arg268Cys)	
18	30	GH	APH	SOD	p.(Gly371Arg)	
19	31	GH, ACTH, LH, FSH, DI	Absent posterior pituitary, absent stalk	MPHD	p.(Arg85Cys)	
20	31	GH, TSH, ACTH, LH, FSH	APH, EPP, absent stalk	MPHD	p.(Arg248Glu)	
21	32	ACTH, TSH	Normal	MPHD	p.(Leu173Arg)	
22	32	NA	NA	Hypopituitarism	p.(Arg85Cys)	
23	32	NA	NA	Hypopituitarism	p.(Arg85His)	
24	33	GH, ACTH, TSH, LH, FSH	APH, EPP, absent stalk, optic chiasm asymmetry	MPHD	p.(Glu231Lys)	TGIF1 NM_170695.4: c.90G > A; (p.Trp30Ter)
25	34	GH	Small anterior pituitary	Isolated GHD	p.(Pro12fs*30)	
26	35	GH	NA	Isolated GHD	p.(Trp178Ser)	
27	35	GH	NA	Isolated GHD	p.(Trp178Ser)	
28	35	GH	NA	Isolated GHD	p.(Trp178Ser)	
29	36	GH, LH, FSH	Duplicated pituitary stalk	MPHD, MGS	p.(Arg248Trp)	

GH: growth hormone, TSH: thyroid stimulation hormone, ACTH: adrenocorticotropic hormone, LH: luteinizing hormone, FSH: follicle stimulating hormone, MPHD: multiple pituitary hormone deficiency, PSIS: pituitary stalk interruption syndrome, SOD: septooptic displasia, APH: anterior pituitary hypoplasia, EPP: ectopic posterior pituitary, DI: diabetes insipidus, MGS: morning glory syndrome, NA: not available, GHD: growth hormone deficiency, MRI: magnetic resonance imaging

and the pelvic USG of the patient was normal. At the last evaluation, the patient was 18.4 years old, her height SDS was normal and she had regular menstruation.

Patient 3

Patient 3 was a 0.5-year-old male patient who was referred to the pediatric endocrinology clinic because of central hypothyroidism which was detected during the evaluation of poor height gain. He was born at term with a normal birth weight and had no problems during the prenatal or early postnatal period. His mother and father were not related and there was no history of relevant disease in the family. Physical examination at presentation revealed short stature, nonpalpable testes, and micropenis. Biochemical

investigations confirmed central hypothyroidism accompanied by low prolactin levels. IGF-1, IGFBP-3, and cortisol levels were normal. Treatment was started with L-thyroxine 25 mcg daily. GHSTs, performed after the patient became euthyroid, were compatible with GH deficiency.

MRI scan of the pituitary gland and cranium was normal. Testis USG revealed proximal inguinal located testes with right testis 0.1 mL and left testis 0.2 mL. Renal USG was normal and the patient underwent orchiopexy.

At follow-up at 1.6 years old, growth velocity decreased and GH treatment was started at a dose of 0.03 mg/kg/day. GH induced a remarkable increase in his growth velocity. This patient was suspected to have hypogonadotropic hypogonadism because of low gonadotropin levels, bilateral cryptorchidism, and micropenis at presentation. GnRH stimulation test was performed at the age of 10.5 years and stimulated FSH was 2.27 IU/L, LH was 1.17 IU/L, results which support the diagnosis of hypogonadotropic hypogonadism.

He had a normal sense of smell and no mirror movements of the upper limbs, no abnormal eye movements, no color blindness, and no renal abnormalities or dysmorphic features were noted. At 12.9 years old spontaneous puberty had started and the testis volumes were 4 mL. At the onset of puberty, FSH level was 7.9 IU/L, LH was 1.42 IU/L, and testosterone was 0.112 ng/mL. GH treatment was stopped at 14.1 years old because the patient's height was 173 cm. At final evaluation, the patient was 14.4 years old and the pubertal stage was Tanner 2. Gonadotropin levels, inhibin B (122 pg/mL) and anti-Müllerian hormone levels (7 ng/mL) were in normal ranges. Since GH deficiency continued at retesting, it was decided to continue GH in a dose appropriate for transition.

Patient 4

Patient 4 was referred because of growth retardation, micropenis, cryptorchidism, and hypernatremia at the age of 0.5 years. He was born into a consanguineous family with low birth weight because of oligohydramnios. Physical examination revealed micropenis, nonpalpable testis, scrotal hypoplasia, and short stature. He had severe neuromotor retardation with hypotonia and did not have head control or eye contact. Laboratory evaluation showed hypernatremia with decreased urinary density and increased diuresis which were diagnostic for diabetes insipidus. He had grade 1 pelviectasia on renal USG. Desmopressin treatment was started. Hypophysial axis evaluation revealed prolactin deficiency, hypogonadotropic hypogonadism, and central hypothyroidism. Cortisol response after 1 mcg adrenocorticotropin hormone stimulation test was normal. L-thyroxine treatment was started. On USG, both testes were located inguinally. A human chorionic gonatropin test was performed but testosterone response was inadequate. IGF-1 level was low and GHSTs were compatible with growth hormone deficiency. Cranial and pituitary MRI revealed hypoplasic pituitary and diffuse hypomyelination and cerebral atrophy. GH treatment (0.03 mg/kg/day) was initiated at age 1.9 years old.

Discussion

In this study, we describe four patients with short stature carrying heterozygous variants in the *PROKR2* gene predicted to cause altered function. Patient 1 and Patient 2 had isolated GH deficiency but Patient 3 and Patient 4 had MPHD. Both of the variants p.(Arg85His) and p.(Leu173Arg) have been previously described in patients with IHH, hypothalamic amenorrhea and Kallmann syndrome. However, their role in the etiology of other pituitary hormone deficiencies is unclear.

PROK2 or *PROKR2* variants associated with Kallmann syndrome are usually monoallelic; only a few patients were reported with homozygous or compound heterozygous inheritance (10,13,14). Kallmann syndrome related to heterozygous *PROK2* and *PROKR2* variants is challenging, because knockout mouse models for Kallmann support phenotype in biallelic forms (8). However, functional analyses of monoallelic p.(Leu173Arg) and p.(Arg85His) variants were shown to be deleterious to protein function, supporting a causative role in the clinical outcome (9,10,11,21). Caronia et al. (22) proposed that the monoallelic mutations in *PROKR2* are not sufficient to cause IHH but they could set a lower threshold for functional inhibition of the hypothalamic–pituitary–gonadal axis under adverse hormonal, nutritional, or psychological conditions and thereby lead to hypogonadism. This explanation is compatible with the presence of mutations associated with IHH and hypothalamic amenorrhea in persons who do not have symptoms. For instance, heterozygous *PROKR2* mutations have been reported in patients with IHH, and in many of these patients, the variants were inherited from an asymptomatic parent (10,13,23). An alternative possibility for this variable phenotype of *PROKR2* may be the dominant negative effect of some variants on the normal allele but this mechanism is unlikely to account for the deleterious effect of all missense alterations, as many of them have also been found in healthy individuals (7,11,21,24,25,26). In addition, Monnier et al. (10) reproduced heterozygous *PROKR2* mutations in a recombinant murine *PROKR2* protein and they found that the mutant receptors did not affect cell surface-targeting of the wild-type receptor and did not

properly address the plasma membrane which affects wild-type receptor signaling activity. This finding was evidence against a dominant negative effect of the mutations *in vivo*.

Oligogenic or digenic inheritance has recently been the most plausible explanation for the phenotypes observed in patients with heterozygous mutations in Kallmann syndrome and IHH (12,13,21,24). Few reports of patients carrying mutations in both *PROKR2* and *ANOS1* or in *PROKR2* and *PROK2* supported the digenic inheritance (11,13,23,24,27). Phenotypes resulting from heterozygous *PROKR2* mutations are remarkably variable, ranging from IHH to MPHD with or without abnormalities of the olfactory and optic nerves. Raivio et al. (12) hypothesized that *PROKR2* mutations may underlie both Kallmann syndrome and hypopituitarism because of similar embryonic development and phenotypes of these two entities. They identified patients with MPHD who harbored loss of function variants in the *PROKR2* gene (12). However, the data about the oligogenic inheritance of *PROKR2* in MPHD and isolated GH deficiency is limited.

Additionally, digenic inheritance was shown in some patients as a potential cause of MPHD and pituitary stalk interruption syndrome (12,28,29). If there is incomplete segregation of a heterozygous mutation with the phenotype in a pedigree, digenic inheritance must be considered for the underlying genetic mechanisms (29). To our knowledge and including our cohort, currently 2435 patients with isolated GH deficiency, MPHD, and/or SOD have been investigated for *PROKR2* mutations, and 33 patients (1.4%) harbor 13 different heterozygous *PROKR2* variants (12,28-36). Of these patients, 4 (12%) were reported to have an oligogenic inheritance. Table 3 shows phenotypes of patients with heterozygous *PROKR2* mutations who have MPHD or GH deficiency reported at the time of writing.

In our study, we observed the allele frequency of both variants as 0.017. According to Gnomad database (37,38), the allele frequency of these variants is given as 0.00074 (0.0011-0.00011) for c.254G>A and 0.0023 (0.0063-0.00004) for c.518T>G. In Turkish varioma data (39), which consists mainly of neurological patients, allele frequencies were reported as 0.001 and 0.0036, respectively. In Turkish varioma database, both variants are observed to be 2-10 times higher than the Gnomad frequency but they remain within the frequency ranges of the Gnomad database. In the present study, the allele frequency was found to be dramatically higher than in both databases, although there is a possibility that the frequency will decrease slightly with the increase in the number of patients. The high frequency in our study can be explained by the fact that the phenotype of short stature, which is our patient group, is observed at a higher frequency in the population than in rare diseases.

Study Limitations

The main limitation of this study was the lack of whole exom sequencing, whole genome sequencing, long read sequencing or optical mapping techniques, which are advanced, further step of next generation sequencing and helps to identify underlying additional genes and clarify the etiology. Another limitation was the inability to determine the phenotype-genotype relation and the variability depending on gender because of the small number of patients.

Conclusion

Finally, our data extend previous reports demonstrating that heterozygous *PROKR2* mutations play a role in the etiology of MPHD and isolated GH. Asymptomatic carrier parents and phenotypic variability indicate a yet unknown underlying mechanism of *PROKR2* causing pituitary hormone deficiency. For the mechanisms we have explained in detail above, we concluded that the most likely cause is digenic or oligogenic inheritance in patients with heterozygous *PROKR2* mutations. Although the remaining 24 genes were normal in all patients, we hypothesize our patients carry additional mutations in as-yet-undiscovered Kallmann syndrome or MPHD genes, in the light of all reported data. Besides, the delay in puberty of patients and their relatives may be evidence for *PROKR2* having a role in the constitutional delay of puberty. Further studies are needed to explain in more detail the role of PROKR2 signaling in the reproductive system and pituitary development.

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Ethics

Ethics Committee Approval: The study protocol was approved by the İstanbul University, İstanbul Faculty of Medicine Local Clinical Research Ethics Committee (date: 11.08.2017, approval number: 13).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

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Relative Frequency of Islet Autoimmunity in Children and Adolescents with Autoimmune Thyroid Disease

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What is already known on this topic?

- Two most common autoimmune endocrine diseases, type 1 diabetes mellitus (T1D) and autoimmune thyroid diseases (AITD) - autoimmune thyroiditis (AT) and Graves' disease (GD) - are often found in the same patient and/or within the same families.
- Thyroid autoimmunity was widely studied in T1D patients, but few studies have examined islet autoantibodies (AAbs) and the risk of development of T1D among patients with AITD.

What this study adds?

- This is the first comprehensive study that included children/adolescents with both AITDs (AT and GD) and in which islet cell autoimmunity was estimated by measuring three islet AAbs.
- The observed relative frequency of T1D development in patients with AITD was much higher than in the general Croatian population (3.7% vs. 0.2%).
- This study was the first to evaluate islet autoimmunity and glucose metabolism in family members of patients with AITD and islet AAbs. As five of 20 family members were found to have impaired glucose tolerance/islet cell autoimmunity, we propose that family members of AITD patients have an increased risk of developing T1D. However, these findings should be evaluated and validated in studies with larger sample sizes.

Abstract

Objective: The aim of the present study was to investigate islet autoimmunity and susceptibility to type 1 diabetes (T1D) in children/adolescents with autoimmune thyroid disease (AITD, and in family members of AITD patients with islet autoimmunity.

Methods: Islet-cell cytoplasmic, glutamic-acid decarboxylase, and tyrosine-phosphatase autoantibodies (AAbs) were measured in 161 AITD patients [127 with autoimmune thyroiditis (AT); 34 with Graves' disease (GD)], 20 family members of AITD patients with islet autoimmunity, and 155 age-matched controls.

Results: Islet autoimmunity was found in 10.6% of AITD patients, significantly more frequent than in controls (1.9%; $p = 0.002$). A higher prevalence of islet AAbs was found in females with AITD ($p = 0.011$) but not in males ($p = 0.16$) and in AT ($p = 0.013$) but not in GD patients ($p = 0.19$), compared to corresponding controls. Two or three islet AAbs were found concurrently in six AITD patients with islet autoimmunity. They all developed T1D and had significantly higher islet AAbs titers ($p = 0.01$) than AITD patients with single islet AAbs but normal glucose metabolism. T1D was found in 3.7% of AITD patients compared to 0.2% of the age-matched, general Croatian population. Islet AAbs were found in 5/20 family members of AITD patients with islet autoimmunity, among whom two developed T1D. None of the controls was positive for more than one islet AAb or developed T1D.

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Conclusion: Children/adolescents with AITD, particularly females and patients with AT, appear to represent a risk group for islet autoimmunity and T1D, as do family members of AITD patients with positive islet AAbs. However, these findings should be validated in larger studies.
Keywords: Autoimmune thyroid disease, islet autoimmunity, screening, diabetes mellitus type 1, children

Introduction

Autoimmune endocrine diseases are organ-specific diseases in which the immune response target organs are endocrine glands (1). The most common of these diseases is type 1 diabetes mellitus (T1D) and two autoimmune thyroid diseases (AITD), autoimmune thyroiditis (AT) and Graves' disease (GD). Of all autoimmune endocrinopathies that co-occur, AITD and T1D are far more often found in the same person or family (1,2). This phenotype is classified as a variant of the autoimmune polyglandular syndrome type 3 (3,4).

Thyroid autoimmunity has been widely studied in T1D, and thyroid autoantibodies (AAb) were found in 8-44% of patients with T1D (5), while 50% of these patients developed a clinical form of AITD (5). In contrast, few studies have examined islet autoimmunity and risk of developing T1D among patients with AITD, and only four of these studies were conducted in children and adolescents (6,7,8,9). One study was performed in children with AT and GD (6) and three others in children with AT (7,8,9). Among studies conducted in adults, some were exclusively carried out in patients with AT (10,11) or GD (12,13), while others included patients with both conditions (14,15,16,17,18,19). Islet autoimmunity was assessed by measuring different diabetes-associated AAb as serological markers of β-cell autoimmunity, which included glutamic acid decarboxylase (GAD), islet cell cytoplasmic AAb (ICA), tyrosine-phosphatase (IA2), insulin (IAA), proinsulin and zinc-transporter 8 AAb. These antibodies are age-dependent, with IAA and IA2 more commonly seen in children under ten, while GAD is associated with older age and the female gender (20,21).

In line with growing interest in the early detection of people and groups at risk for T1D development, this study aimed to assess the relative frequency of humoral markers of autoimmunity to islet cells (ICA, GAD, IA2) in children and adolescents with AITD as a group and separately among AT and GD patients. In addition, we wanted to determine the relative frequency of T1D in the same group of patients and family members (parents, siblings) of patients with AITD and islet autoimmunity. In patients with AITD and family members who tested positive for islet AAb, glucose metabolism was assessed to evaluate for T1D.

Methods

Patients

This prospective, observational study included 161 patients with AITD divided into two groups [127 with AT (29 males and 98 females, aged 4.17-19.0 years) and 34 with GD (7 males and 27 females, aged 6.5-21.9 years)]. All patients were treated at the Department of Pediatric Endocrinology and Diabetes, University Hospital Center, Zagreb. Patients were recruited as consecutive patients from the outpatient clinic from June 2012 to December 2014 and followed up until June 2018. Patients affected by any syndromic or another known genetic disease, such as Turner, Down, or Klinefelter syndrome, as well as polyglandular syndromes, were excluded from the study.

The control group consisted of 155 patients (52 males and 103 females, aged 4.0-21.5 years) admitted to the Department of Pediatrics, University Hospital Centre, Zagreb, for evaluation of other non-chronic diseases, whose clinical history was negative for thyroid autoimmunity and other autoimmune disorders, and who had no family history of T1D and AITD. Additionally, 20 family members (18 parents and two siblings) of patients with AITD and positive islet AAb were recruited to the study.

AT diagnosis was based on elevated titers of AAb against thyroid peroxidase (TPO) and/or thyroglobulin (Tg) and thyroid ultrasound examination consistent with this diagnosis. Since measurement of thyroid-stimulating hormone (TSH) receptor antibodies was unavailable, the diagnosis of GD was based on clinical and biochemical findings of hyperthyroidism, thyroid ultrasound, and Doppler examination. Only patients with persistent biochemical results of hyperthyroidism and requiring antithyroid medication during follow-up, ranging 3.5-6 years, were labeled as GD to exclude hyperthyroidism due to AT.

The University Hospital Center Zagreb Ethics Committee and the University of Zagreb Faculty of Medicine Ethics Committee approved the study protocol (no: 641-01/22-02/01, date: 07.12.2022), the study was performed in line with the Declaration of Helsinki, and informed consent was obtained from all participants and/or their parents.

Parameters in the Study

In both patient and control groups, the titers of Tg, TPO AAb, and islet AAb (GAD, IA2, and ICA) were assessed at the time of evaluation.

Islet AAb were also measured in 20 family members (10 mothers, eight fathers, and two sisters) of 10 AITD patients with islet autoimmunity. In 16 patients with AITD and islet autoimmunity, four of their family members with islet autoimmunity, and in three control subjects with islet autoimmunity, glucose metabolism was evaluated with oral glucose tolerance test (OGTT) and glycated hemoglobin (HbA1c) using the immunoassay method on the Siemens A1c Vantage Analyzer (Siemens Healthcare GmbH, Erlangen, Germany) according to ISPAD criteria (22).

Determinations of GAD and IA2 AAb were performed by commercial enzyme-linked immunosorbent assay (ELISA) kits (Euroimmun, Germany). In 2010 the Clinical Institute of Laboratory Diagnosis, University Hospital Merkur, Zagreb, participated in Diabetes Antibody Standardization Program. Sensitivities and specificities were 88% and 94%, respectively, for GAD, and 72% and 99%, respectively, for IA2 AAb. The cut-off for positive results was set at 5 units/mL for GAD and 10 units/mL for IA2 antibodies (23).

Detection of ICA AAbs was performed by indirect immunofluorescence. Scores of fluorescence intensities were then calculated into Juvenile Diabetes Foundation (JDF) units. Results >5 JDF units were considered positive. ICA assays were validated by repeated participation in the immunology of diabetes workshops and proficiency testing programs of the University of Florida (Gainesville, FL, USA) with >95% sensitivity, specificity, consistency, and validity (24). The quality of our performance is validated by continuous yearly participation in Instand EQA schemes. The cut-off values of positivity for TPO and Tg AAb were 20.0 units/mL and 60.0 units/mL, respectively. The manufacturer provided reference ranges and cut-off values for the ELISA (ELISA Brahms GmbH, Henningsdorf, Germany) methods, with results higher than the cut-off values set by the manufacturer considered positive.

Statistical Analysis

Data are presented in tables using descriptive statistics (frequencies, means, and standard deviations). The patient groups were compared using the appropriate tests, depending on the data type and distribution (chi-square test, Fisher's exact test, and the Mann-Whitney U test for unpaired data). Statistical Package for the Social Sciences, version 21.0 (IBM Inc., Armonk, NY, USA) was used for calculations, and a $p < 0.05$ was considered significant.

Results

Islet AAb and Glucose Metabolism in AITD Patients

Islet autoimmunity was significantly more frequent in patients with AITD (10.6%) than in the control group (1.9%; $p = 0.002$). The frequency was significantly higher only in AT patients (11.8%; $p = 0.001$) but no different in GD patients (5.9%, $p = 0.19$), when compared to controls (Table 1). AITD patients with islet autoimmunity were slightly younger at the time of evaluation (median 11.6 years) than those without islet autoimmunity (median 12.8 years), but this was not significant (Mann-Whitney test: $U = 1071$, $z = 1.26$, $p = 0.21$).

Relative frequencies of all islet AAb were significantly higher in AITD patients than in controls (ICA $p = 0.04$; GAD $p = 0.002$; IA2 $p = 0.02$, Table 2).

The clinical and laboratory characteristics of patients with AITD and islet autoimmunity are summarized in Table 3. Three out of 17 AITD patients with islet autoimmunity (patients #1-3) were positive for three islet AAb, and an additional three patients (patients #4-6) were positive for two islet AAb. In contrast, none of the control subjects was positive for more than one islet AAb (Tables 2 and 3).

Table 1. The frequency and percentage of islet autoimmunity in patients with AITD (subdivided into groups: AT and GD) and control subjects. The results of chi-square and p value (< 0.05) between the patients positive to islet autoimmunity in all groups compared to controls are presented

Patients	Patients with islet autoimmunity (%)	Chi-square/p value
AITD (n = 161)	17 (10.6%)	**9.91/0.002**
- AT (n = 127)	15 (11.8%)	**11.39/0.0007**
- GD (n = 34)	2 (5.9%)	1.68/0.19
Control group (n = 155)	3 (1.9%)	

AITD: autoimmune thyroid disease, AT: autoimmune thyroiditis, GD: Graves' disease

Table 2. The frequency and percentages of islet autoantibodies in AITD patients and controls are presented. Statistical significance for chi-square or Fisher's exact test between the two groups is presented

Islet AAb n (%)	AITD (n = 161)	Controls (n = 155)	p (chi-square or Fisher's exact*)
ICA	9 (5.6%)	2 (1.3%)	0.04
GAD	10 (6.2%)	0 (0%)	0.002*
IA-2	8 (5.0%)	1 (0.6%)	0.02
One islet AAb (n)			
ICA	3	2	
GAD	5	0	
IA-2	3	1	
Two islet AAb (n)			
ICA + GAD	1	0	
ICA + IA-2	1	0	
GAD + IA-2	1	0	
Three islet AAb (n)			
ICA + GAD + IA-2	3	0	

*Statistical significance for Fisher's exact test.
AAb: autoantibodies, ICA: islets cell cytoplasmic autoantibody, GAD: glutamic acid decarboxylase autoantibody, IA2: tyrosine-phosphatase autoantibody, AITD: autoimmune thyroid disease

There was no significant difference in the frequency of islet autoimmunity between sexes (5.6% males and 12% females with AITD; p = 0.27). A significant difference in proportion with islet autoimmunity was found in females with AITD compared to females in the control group (12% vs. 2.9%, p = 0.011) but not among males with AITD (5.9% vs. 0%, p = 0.16) as compared to males in the control group.

When analyzed separately, significant differences were found in the frequencies of GAD (7.2%) and IA2 AAb (5.6%) in females with AITD compared to females in the control group (0%; p = 0.005 and 1%; p = 0.06, respectively). No differences was found in the frequency of ICA AAb in females with AITD (6.4%) compared to females in the control group (1.9%; p = 0.10), nor was there a difference in the frequencies of any of the three diabetes-associated AAbs in males with AITD (ICA 2.8%; GAD 2.8%; IA2 0%) compared to males in the control group (0%).

At the time of evaluation, T1D was diagnosed in 1/16 AITD patients with islet autoimmunity (patient #1, HbA1c 9.8%; 83.6 mmol/mol) (Table 3), and one patient had impaired glucose tolerance and normal HbAlc (patient #3, Table 3). The remaining 14 patients had normal blood glucose levels on OGTT and normal values of HbA1c. During the 6-year follow-up, another 5/16 AITD patients with islet AAb (4 females and one male, patients #2-6; Table 3) developed T1D. Patient #17 was lost to follow-up, and her glucose metabolism was not investigated. Six patients who developed T1D all tested positive for two or three AAb. All these patients were younger than 15 years at the T1D diagnosis. The relative frequency of T1D in our AITD patient cohort was 3.7%, while in patients younger than 15 years of age, the relative frequency was 4.8%. According to the Croatian registry of diabetes in children and adolescents, the prevalence of T1D in the general age-matched Croatian population is 0.2% (unpublished data).

AITD patients with islet autoimmunity who developed T1D had significantly higher titers of islet AAb in comparison to AITD patients with islet autoimmunity and normal glucose metabolism (Mann-Whitney test, n = 16, Table 3: ICA-U = 6.5; z = -2.50; p = 0.01; GAD-U = 7.0; z = -2.44 p = 0.02; IA2-U = 7.5; z = 2.51; p = 0.01). None of the patients in the control group with positive islet AAb developed T1D during the follow-up.

Islet AAbs and Glucose Metabolism in Family Members of Patients with AITD and Islet Autoimmunity

Positive islet AAb was found in 5/20 family members of patients with AITD and islet autoimmunity (3/9 fathers and 2/10 mothers). The mother of patient #1 was positive for three islets AAb and was diagnosed with T1D at the age of 19 years. The other four parents (#2b, 4b, 11a, and 11b) with positive islet AAb had normal levels of glucose on OGTT and normal values of HbA1c at the time of evaluation. After the first evaluation, HbA1c was measured every 6-8 months. The father of patient #2, with positive GAD and ICA AAb, developed T1D after six years of follow-up at the age of 46 years (Supplement Table 1).

Table 3. Clinical characteristics, thyroid and islet autoantibodies titers, and HbA1c in 17 AITD patients and three control subjects with islet autoimmunity

Patient#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	1	2	3
Dg	**AT**	**AT**	**GD**	**AT**	**AT**	**AT**	AT	AT	AT	AT	AT	AT	AT	AT	GD	AT	AT	Control group		
Age at AITD dg (yr)	**11.8**	**10.1**	**6.5**	**8.0**	**11.4**	**8.1**	13.5	11.3	19.0	11.5	12.3	7.8	13.2	12.2	15.4	13.7	17.3			
Sex	**F**	**F**	**F**	**F**	**F**	**M**	F	F	F	F	F	F	F	F	F	M	F	F	F	F
TPO (U/mL)	**> 2000**	**> 2000**	**> 2000**	**40**	**> 2000**	**> 2000**	269	> 2000	159	> 2000	1191	115	> 2000	96	279	46	1112	0.18	0	0
Tg (U/mL)	**44.2**	**0**	**71.1**	**140**	**25**	**584**	755	147	0	0	28.2	0	177.2	155	27	0	643	1.3	0	0
TSH (µU/L)	**17.3**	**18.4**	**< 0.01**	**11.3**	**34.3**	**> 100**	16.	10.1	4.6	18.8	9.2	3.2	27.4	10.1	< 0.01	1.4	4.0	2.2	1.6	3.6
fT4 (pmol/L)	**10.2**	**11.9**	**44.3**	**9.8**	**9.2**	**3.1**	13.0	14.4	18.7	8.6	12.0	16.6	9.2	11.6	48.6	15.6	10.9	16.3	17.2	14.4
Age at evaluation (yr)	**12.9**	**10.8**	**8.0***	**9.5**	**11.4**	**11.2**	16.8	14.3	20.4	14.2	16.0	11.3	14.9	14.2	16.0	16.6	20.1	12.7	10.5	11.0
ICA (JDF)	**330**	**290**	**370**	**285**	**65**	**50**	45	< 5	< 5	50	< 5	65	95	< 5	60	25	30	< 5	50	35
GAD (U/mL) (IU/mL)	**676**	**859**	**2509**	**0**	**68.6**	**149**	0	22.7	17.7	23.7	0	0	0	27.4	0	0	1193	0	0	0
IA2 (U/mL)	**258.6**	**136.1**	**1882**	**1245**	**226**	**0**	16.7	0	0	0	22.3	0	0	0	0	21.0	0	13.6	0	0
Age at T1D dg (yr)	**12.9**	**12.6**	**10.3**	**12.9**	**14.8**	**13.3**														
HbA1c % (mmol/mol)**	**9.8 (84)**	**8.3 (67)**	**6.8 (51)**	**8.5 (69)**	**7.3 (56)**	**7.8 (62)**	5.5 (37)	5.2 (33)	5.0 (31)	5.2 (33)	5.3 (34)	4.7 (28)	4.8 (29)	5.2 (33)	5.3 (34)	5.0 (31)	NT	4.8 (29)	5.2 (33)	NT

Bold: patients who developed T1D during the investigation period, *impaired glucose tolerance at the time of evaluation, **HbA1c % (mmol/mol) at the time of T1D diagnosis or the last HbA1c measured in patients with normal glucose metabolism.
Dg: diagnosis, yr: year, AT: autoimmune thyroiditis, GD: Graves disease, AITD: autoimmune thyroid disease, M: male, F: female, TPO: autoantibodies against thyroid peroxidase, Tg: autoantibodies against thyroglobulin, TSH: thyroid-stimulating hormone, fT4: free thyroxin, ICA: islet cell cytoplasmic autoantibody, GAD: glutamic acid decarboxylase autoantibody, IA2: tyrosine-phosphatase autoantibody, NT: not tested, HbA1c: glycated hemoglobin

Antithyroid AAb in AITD Patients

Patients with AITD with positive islet autoimmunity had higher TPO and Tg AAb titers compared to AITD patients without islet autoimmunity, but the difference was not significant (Mann-Whitney test: Tg-U = 1126, z = -0.28; p = 0.78; TPO-U = 1768, z = -2.05; p = 0.04).

Discussion

Screening for the risk of T1D is gaining more attention worldwide. The long-term vision for T1D screening programs is to identify individuals at risk of T1D and to offer them interventions to delay or prevent the condition (25). However, other essential and currently achievable clinical benefits drive the current recommendations for screening. It has been shown that screening programs significantly reduce diabetes ketoacidosis (DKA) rates, usually to less than 5%, and reduce hospitalization when coupled with long-term monitoring (26,27,28,29). DKA prevention at diagnosis has potential lifelong benefits, including avoidance of acute morbidity, neurocognitive impairment, and mortality (30,31). Other no less worthy benefits would be to prepare children and families for a smoother transition to insulin therapy and advance preventative treatments through clinical trial recruitment (25).

In the present study, we analyzed three islets AAb (ICA, GAD, and IA2) in children and adolescents with AITD (AT and GD) to assess this group of patients as a possible target for a T1D screening program. All of our patients were positive for thyroid AAb before inclusion in the study, allowing us to select and follow the patients that developed thyroid autoimmunity before the onset of T1D. We found that significantly more AITD patients were positive for one or more islet AAb compared to controls. This difference was entirely due to antibodies found in AT patients, as there was no significant difference in islet autoimmunity between the GD patients and controls. However, due to the small number of patients in the GD group, an association between GD and islet autoimmunity cannot be excluded. When analyzing islet AAb separately, all three AAb were significantly more frequent in AITD

patients compared to the control group and GAD AAb was found only in the AITD patients but not in controls.

Few studies have reported the frequency of ICA autoimmunity in patients with AITD and only four included children and adolescents (6,7,8,9). Bright et al. (6) found ICA AAb in 2.3% of children with AITD compared to 0% of controls. In studies conducted in adult AITD patients, ICA positivity ranged from 0-4.9% (12,13,15,17). Only one study evaluated the frequency of IA2 AAb in children with AT, but excluded GD, and found them to be more common than in control subjects (3.39% vs. 1.16%, $p = 0.012$) (7), as was confirmed in our study. In one study in adult patients, IA2 AAb was found more frequently in patients with AT and GD (18).

GAD positivity was assessed in three studies conducted in children with AT (7,8,9). In two (8,9), GAD AAb was found significantly more often in children with AT than in controls (9.8-10.6% vs. 0-3.3%, $p = 0.003$ and $p = 0.036$, respectively), as was again confirmed in our study. However, Pilia et al. (7), found no significant difference in rates of GAD AAb positivity. Several studies analyzed GAD autoimmunity in adult patients with AT (10,11,14,15,16,19) and GD (12,13,15,16). Relative frequencies of GAD AAb ranged from 2.8-6.6% (10,11,14,15,16,19) in patients with AT. Although a correlation between GAD AAb and AT was found in some studies (15), it was not always significant (16). In adult GD patients, GAD AAb was found in 6.1-13% of patients (12,13,15,16), significantly more common than controls in some studies (15,16). However, the evaluation only sometimes included control subjects (10,11,12,13,14).

We did not find significant differences in islet autoimmunity between males and females with AITD. However, females with AITD were positive for islet AAb (GAD and IA-2 AAb, but not ICA AAb) significantly more often than females in the control group. In contrast in males with AITD, we did not find any difference in islet autoimmunity compared to controls. As thyroid autoimmunity and AITD are more common in females, the female gender *per se* may be a risk factor for the positive association between islet autoimmunity and thyroid autoimmunity (32).

In 16 patients with AITD who were positive for islet AAb, the susceptibility for T1D development was assessed. Upon initial evaluation, one patient was diagnosed with T1D, and five developed T1D during the follow-up period of six years (Table 3). However, we cannot exclude that more patients would develop diabetes if the follow-up were longer. In the study of Bright et al. (6), one of two children with AT and positive ICA AAb developed diabetes after one year, and two children with AT and negative ICA AAb after four and six years, respectively. Pilia et al. (7) reported that over two years of follow-up, 2/19 children with AT and islet autoimmunity developed T1D (one positive to GAD AAb and the other to GAD and IA2 AAb). Lethagen et al. (10) found reduced insulin secretion capacity in GAD-positive AT patients and concluded that GAD AAb might be a marker of subclinical insulitis. During the follow-up of 4 years, 2/15 (13%) of their GAD AAb positive patients compared to 11/426 (2.6%) GAD AAb negative patients were diagnosed with diabetes ($p = 0.08$) (10). Hallengren et al. (13) followed nine GAD AAb-positive patients (two also ICA-positive) for 27-70 months. One patient, who was positive for both islet AAb, developed diabetes. Maugendre et al. (12) found a high frequency of GAD AAb (16/150 GD patients) but a low progression toward diabetes (only one patient). Aksoy et al. (11) studied insulin sensitivity and secretion patterns in GAD AAb positive and GAD AAb negative AT patients. They concluded that it is not likely that the presence of GAD AAb per se is associated with a disturbance in glucose metabolism. A significant relationship between the higher titer of GAD AAb and abnormalities of glucose metabolism was found in the studies by M Marhawa et al. (8) and Moriguchi et al. (16). Kawasaki et al. (15) did not report similar findings. The observed relative frequency of T1D development in our patients with AITD was compared to that in the Croatian general population. In our cohort, T1D was found in 3.7% of AITD patients, much more frequent than in the general population in the same age groups (0.2%) (33, Croatian registry of diabetes in children and adolescents, unpublished data).

In the present study, AITD patients who developed T1D had significantly higher titers of GAD AAb than AITD patients with islet autoimmunity and normal glucose metabolism. Moreover, we noticed significantly higher titers of ICA and IA-2 AAb in this patient group.

We further measured TPO and Tg AAb titer and found higher titers in AITD patients with islet autoimmunity compared to AITD patients without islet autoimmunity. However, the difference was not statistically significant, as was observed in some other studies (15,17). On the other hand, M Marwaha et al. (8) found that GAD AAb levels increased with an increasing titer of TPO AAb.

Islet autoimmunity and susceptibility to T1D were analyzed in 20 first-degree family members of patients with AITD and positive islet AAb (Supplement Table 1). Positive islet AAb was found in 25%, although one of the mothers was already diagnosed with T1D, but interestingly one father developed T1D during follow-up. This suggests that family members of patients affected with AITD and islet autoimmunity might have a higher risk for T1D.

Study Limitations

Study limitations should be noted. The measurement of anti-TSH receptor antibodies were not available in our institution at that time and therefore not performed as part of the diagnosis of GD. However, in a group of GD patients with clear clinical, biochemical, and ultrasound signs of disease, we included only those who did not develop hypothyroidism during follow-up. Moreover, we did not test family members of AITD patients without positive islet AAb for the development of T1D. It would be necessary to confirm the results found in family members of patients with AITD in a much larger number of subjects, including both those with and without islet autoimmunity, to determine the risk for glucose metabolic impairment in relatives of patients with AITD.

Conclusion

In conclusion, AITD patients may be a potentially significant group for targetted T1D screening. Our results suggest that females with AT are at especially high risk for T1D development, as are patients with a higher titer of two or more islet AAb, and family members of AITD with positive AAb. Prospective long-term studies on a larger number of subjects are required to examine the factors responsible for islet destruction, insulin deficiency, and progression toward diabetes in patients with AITD and the correlations with AT or GD separately.

To the best of our knowledge, our study was the first to evaluate islet autoimmunity and glucose metabolism in family members of AITD with islet AAb, indicating their increased risk for developing T1D. However, this observation must be verified in much larger studies.

Ethics

Ethics Committee Approval: The University Hospital Center Zagreb Ethics Committee and the University of Zagreb Faculty of Medicine Ethics Committee approved the study protocol (no: 641-01/22-02/01, date: 07.12.2022).

Informed Consent: Informed consent was obtained from all participants and/or their parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Natasa Rojnic Putarek, Nevena Krnic, Maja Baretic, Miroslav Dumic, Concept: Natasa Rojnic Putarek, Miroslav Dumic, Design: Natasa Rojnic Putarek, Miroslav Dumic, Data Collection or Processing: Natasa Rojnic Putarek, Vesna Kusec, Analysis or Interpretation: Jadranka Kneyevic-Cuca, Vesna Kusec, Literature Search: Natasa Rojnic Putarek, Miroslav Dumic, Nevena Krnic, Writing: Natasa Rojnic Putarek, Miroslav Dumic, Nevena Krnic.

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Click for Supplement Table 1. Distribution of islet AAb in 20 family members of 10 AITD patients with islet autoimmunity

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ORIGINAL ARTICLE

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The Incidence Trend of Type 1 Diabetes among Children and Adolescents 0-14 Years of Age in the West, South, and Tripoli Regions of Libya (2009-2018)

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What is already known on this topic?

- The incidence of type 1 diabetes (T1D) is increasing in Arab countries in the Middle East and North Africa region of the world.
- A study from the Benghazi region of Libya in the period 1991-2000, reported an intermediate incidence of T1D.

What this study adds?

- The age-standardized incidence rate (IR) was 31.7 per 100,000 for the period 2014-2018, indicating more than a trebling of the IR of T1D since 1991-2000.
- There was a higher IR in the 0-4 and 5-9 years age groups compared to the 10-14 years age group.

Abstract

Objective: To estimate the incidence rates (IR) and analyse the trend in type 1 diabetes (T1D) among children aged 0-14 years in the West, South, and Tripoli regions of Libya.

Methods: A retrospective study was conducted on Libyan children aged 0-14 years with a new diagnosis of T1D who were admitted and/or had their follow-up at Tripoli Children's Hospital during the period 2004 to 2018. The data were used to estimate the IR and the age-standardized IR per 100,000 population in the studied region for the years 2009-2018. The IRs by sex and age group (0-4, 5-9, 10-14 years) for every calendar year were assessed.

Results: A total of 1,213 children were diagnosed during the study period (2004-2018), 49.1% were males with a male-to-female ratio of 1:1.03. The mean age (± standard deviation) at diagnosis was 6.3 ± 3.8 years. The distribution of incident cases according to age group 0-4, 5-9, and 10-14 years was 38.2%, 37.8%, and 24.1%, respectively. Poisson regression modelling in the period 2009-2018 revealed an overall trend of a 2.1% increase per annum. In the period 2014-2018, the overall age-adjusted IR was 31.7 (95% confidence interval: 29.2-34.2) per 100,000 population, the IRs of age groups 0-4, 5-9, and 10-14 years were 36.0, 37.4, and 21.6 per 100,000, respectively.

Conclusion: The incidence of T1D in Libyan children in the West, South, and Tripoli regions appears to be rising, with a higher rate in the 0-4 and 5-9 year age groups.

Keywords: Type 1 diabetes, epidemiology, incidence, children, adolescents, registry, Libya, Arabs

Introduction

Diabetes is a common non-communicable disease that has a major impact on the lives of individuals and communities (1). In most countries, the majority of children and adolescents with diabetes have type 1 diabetes (T1D) (2). Type 1 diabetes is caused by an absolute insulin deficiency as a result of the autoimmune destruction of beta cells of the pancreas (3).

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There is wide variation in the incidence of T1D in the world, being very low in most Asian populations and high in European countries (4,5). However, the incidence increased by an estimated 2.8% per year worldwide during the 1990s (6). Furthermore, a recent report estimated the overall annual increase in the incidence rate (IR) of T1D in Europe to be 3.4% (7). Moreover, 42% of all incident cases were estimated to occur in children younger than 15 years of age (8).

Libya is one of the 18 Arab countries in the Middle East and North Africa (MENA) region of the world (9), with an estimated population of 6.69 million in 2018, of which 2.076 million (30.8%) are 0-14 years of age (10). Arabs have one of the highest prevalence of diabetes worldwide (11). Furthermore, three Arab countries (Algeria, Saudi Arabia and Morocco) are in the top ten countries for the estimated number of new cases of T1D in children and adolescents (0-19 years old) per year (11).

Information on recent trends in the incidence and prevalence of childhood T1D in Libya is lacking. This is the result of the absence of a national paediatric diabetes registry and a lack of organization and networking between different diabetes centres/clinics in Libya.

The most recent review of published rates of childhood T1D incidence by the International Diabetes Federation (IDF) (11) relied on a report of rates in Libya in the 1990s (12). Since neighbouring Arab countries that are likely to have similar genetic characteristics to the population in Libya have a high incidence of childhood T1D, this earlier reported incidence in Libya is likely an underestimate of the true IR in the country.

This study aimed to estimate the IR of T1D in children 0-14 years of age in the West, South, and Tripoli regions of Libya from the number of cases seen in a single tertiary referral paediatric diabetes centre, Tripoli Children's Hospital, in the Tripoli health region.

Methods

Health Services in Libya

Public healthcare facilities are present in the six health regions of Libya, with each health region having several health districts. This organization is based on access and referral to the nearest tertiary care facilities. Tertiary healthcare facilities, which are located in the Tripoli health region, also cover the West and South health regions (Figure 1). Tripoli Children's Hospital and Tripoli University Hospital (formerly known as Tripoli Medical Centre until 2018) are tertiary healthcare facilities located in the Tripoli

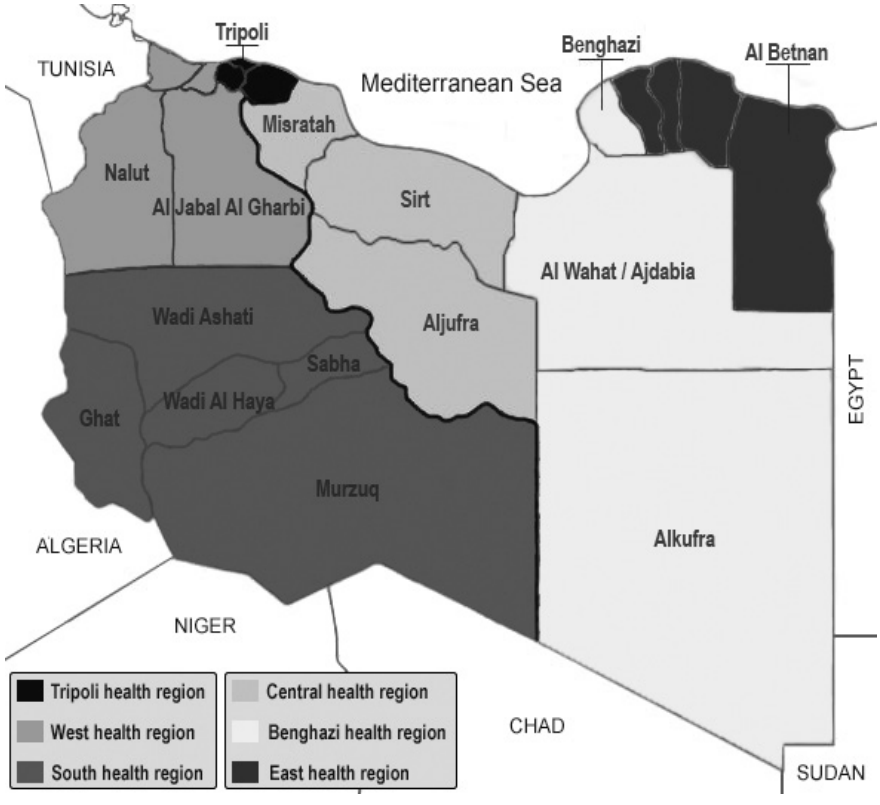

Figure 1. Location of health regions in Libya, modified from (13)

Table 1. Number of new cases of diabetes in children and adolescents seen at TCH and Tripoli University Hospital (2009-2018)

Diagnosis per period	Total number of new cases of diabetes A = (B + C)	Tripoli University Hospital	TCH			
		Number of new cases of diabetes per period (B)	Number of new cases of diabetes per period (C)	Percentage of cases at TCH D = (C ÷ A)*100	Number of other forms of diabetes (E)	Percentage of other forms of diabetes F = (E ÷ C)*100
Period 2 (2009-2013)	1,763	1,281	482	27.3%	34	7.0%
Period 3 (2014-2018)	2,089	1,412	677	32.4%	38	5.6%

With permission from the Head of the Paediatric Endocrinology and Diabetes Department at Tripoli University Hospital.
TCH: Tripoli Children's Hospital

health region (13). The Departments located in these two hospitals cover these three health regions. In the studied geographical area, there are several other cities, however, during the study period, a consultant paediatric endocrinologist or a paediatric consultant/specialist with an interest in diabetes was available at Tripoli Children's Hospital and Tripoli University Hospital, and parents preferred that their children attend follow-up for management and initial diabetes education at these two hospitals.

In the Libyan health care system, all children aged 0-14 years with a suspected diagnosis of diabetes are referred to paediatric hospitals for admission and paediatric diabetes clinics for their follow-up.

Local Background

The Tripoli Children's Hospital Paediatric Diabetes Clinic provides services to over 1000 children and adolescents with diabetes, and the follow-up clinics are run five days a week. There has been an electronic paediatric diabetes registry in use since 2015. The researcher M.S.S. was involved in creating the electronic paediatric diabetes registry and oversees the data entry and running of the registry. Before and after 2015, new patients' details were entered in a handwritten register when opening a clinic file. Data from files of patients already followed up at the clinic were entered into the registry retrospectively in the year 2015. Data from all new cases seen at the clinic have been included in the registry prospectively since then.

At the Tripoli Children's Hospital Paediatric Diabetes Clinic, the majority of children and adolescents younger than 19 years of age have T1D. Other forms of diabetes represent an average of 5.6% of new cases per year (Table 1).

To obtain the proportion of cases of diabetes diagnosed at Tripoli Children's Hospital in the studied geographical area, we compared the list of children with diabetes obtained for the study from Tripoli Children's Hospital with the handwritten paediatric diabetes register from Tripoli University Hospital and removed the duplicate cases that were seen in each hospital. It was noted that 27.3% of cases during the period 2009-2013, and 32.4% of cases during the period 2014-2018 were diagnosed at Tripoli Children's Hospital (Table 1).

Study Population

This retrospective study was conducted to ascertain all cases of T1D in children aged 0-14 years, who were diagnosed during the period between January 1, 2004, to December 31, 2018, in a single paediatric diabetes centre in Tripoli, Libya. The study is based on the number of incident cases of T1D admitted and/or had their follow-up at Tripoli Children's Hospital during the study period. Cases included were Libyan children and adolescents 0-14 years of age at the time of diagnosis, who had been diagnosed with T1D and resided in the West, South, and Tripoli regions of Libya during the study period.

Ethical Approval

The study proposal was approved by the Bioethics Committee at the Biotechnology Research Centre (ref no: BEC-BTRC 17-2019, date: 09.08.2020), which is under the auspices of the Ministry of Higher Education and Scientific Research (14).

Patient Selection

Data were extracted from notes using a specifically designed and adapted form (15,16,17,18). Data collected included full name, date of birth, sex, date of diagnosis, symptoms at presentation, mode of presentation, type of diabetes, treatment at presentation, place of diagnosis, address, nationality, and treatment at the last clinic visit.

The primary data source consisted of cases who were diagnosed as having diabetes by the treating paediatrician and had been identified from admission records with ICD E10 and E11 codes (hospital records from 2011 to 2018 only), hospital discharge letters in Paediatric Diabetes Clinic files (all available clinic files since the year 1999), paediatric diabetes

handwritten clinic files of patients attending the clinic (all notes for patients attending the clinic up to December 31, 2018), the electronic paediatric diabetes registry, and the Paediatric Diabetes Clinic archived handwritten notes (for patients no longer attending the clinic, for example having transitioned to adult services, changed follow-up to another paediatric clinic, or lost to follow-up). Cases that were registered in the handwritten register during the study period (2004-2018), but whose files were not found or were incomplete were excluded from the study (79 cases).

The data collected were reviewed by the researchers (M.S.S. and R.M.K.) to ascertain the completeness of the information. The diagnosis was reviewed by the researchers (M.S.S. and R.M.K.) according to the International Society for Pediatric and Adolescents Diabetes definition of diabetes (19) and was assigned as T1D according to the T1D Exchange criteria (20). In this study, 99.1% of cases (0-14 years of age) met the criteria for definite T1D, which is similar to the T1D Exchange study (100% and 99% for age groups 0-5 and 6-12 years respectively) (20). The date of diagnosis was defined as the date of opening the file with a diagnosis of diabetes (admission/presentation with symptoms and high blood glucose/high glycated haemoglobin) (21).

The data were entered in a Statistical Package for the Social Sciences (SPSS) file by the researcher R.M.K. Duplicate patients were removed using combinations of name, date of birth, date of diagnosis, sex, and address.

We collected data on 1,580 children with diabetes (1999-2018). We compared Tripoli Children's Hospital data with Tripoli University Hospital data and removed duplicate cases from Tripoli Children's Hospital data (143 cases), leaving 1,437 cases eligible to be included in the study.

Exclusion criteria (Figure 2) were age less than six months or 15 years or older at the time of diagnosis, non-Libyan nationality, residency outside the studied region of Libya, and a diagnosis of other forms of diabetes, such as type 2 diabetes, monogenic diabetes, or drug-induced diabetes. In incident cases that had other forms of diabetes, the diagnosis was reviewed by the researchers (M.S.S. and R.M.K.) before exclusion from the study. These exclusions left 1,213 children with T1D to be included in this study.

Since we are investigating incidence trends, we divided the study years into three 5-year periods, period one 2004-2008, period two 2009-2013, and period three 2014-2018.

Ascertainment

We calculated the completeness of case identification for each of the three periods from the primary source only. Completeness of ascertainment was 95% for the whole period (percentage of children with available and complete files to the total number of children registered in the handwritten clinic register). However, ascertainment was very high in periods two and three (95.6% and 99.2%, respectively) compared to the first period (79.3%).

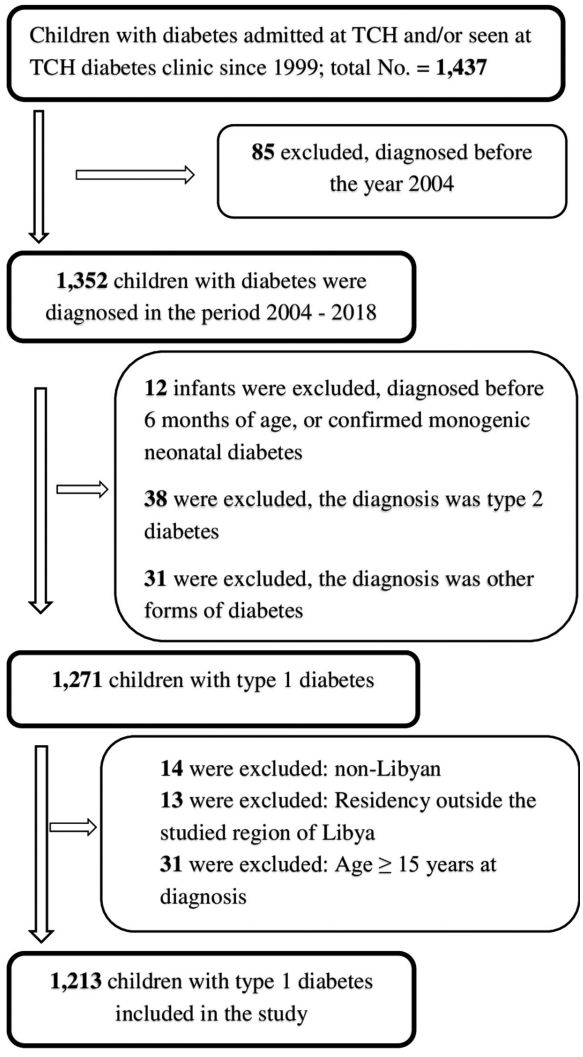

Figure 2. Flow chart of inclusion of cases diagnosed at TCH
TCH: Tripoli Children's Hospital

In Libya, there were no secondary sources to verify the completeness of ascertainment, so our estimates can provide only an imperfect estimate of completeness. However, since all children 0-14 years of age with a diagnosis of diabetes are referred to paediatric hospitals for management, and individuals with T1D are offered treatment free of charge subsidized by the government after being registered in one of the public diabetes services, it is reasonable to assume that data from hospital files and paediatric diabetes clinic records will identify the majority of patients diagnosed in the study period.

Incidence Calculation

We excluded cases diagnosed in period one from the calculation of the IR because of low ascertainment. The incident cases from periods two (2009-2013) and three (2014-2018) were divided into male and female groups as well as three age groups, 0-4, 5-9, and 10-14 years. We used the incident cases seen as a numerator.

The data were used to calculate the crude IR of T1D in children 0-14 years of age in the West, South, and Tripoli regions of Libya. Since the number of incident cases in Tripoli Children's Hospital represents approximately 27.3% and 32.4% of cases seen in the studied geographical region of Libya during periods two and three, respectively (Table 1), we assumed that the proportion of the population of the studied geographical area covered by the Tripoli Children's Hospital Paediatric Diabetes Department is 27.3% and 32.4% in periods two and three respectively.

Age group population data (0-4, 5-9, and 10-14 years) of the West, South, and Tripoli regions of Libya from the last census in the year 2006, were obtained from the Bureau of Statistics and Census Libya (10). We used the projected population growth rate to estimate the population in the years before and after 2006. We used the population percentage covered by Tripoli Children's Hospital (27.3% and 32.4% for periods two and three respectively) to obtain the population numbers for children 0-14 years of age and the six groups (according to age and sex), and these numbers were used as denominators. The estimated IR of T1D was calculated by dividing the number of children with T1D diagnosed (numerator) by the corresponding population (denominator).

The IRs were expressed as new cases per 100,000 population (both sexes) per year for age groups 0-4, 5-9, and 10-14 years as well as for male and female groups. The age-standardized IR was calculated using the direct method (22) and using the World Health Organization (WHO) standard population (23,24).

Statistical Analysis

The data of incident cases included in the study are presented as mean (± standard deviation), or frequency (%). Estimated IRs with 95% confidence intervals (CIs) were calculated assuming a Poisson distribution using MedCalc® Statistical Software version 20.106 (MedCalc Software Ltd., Ostend, Belgium).

Edward's test was used to analyse a sinusoidal (sine/wave) pattern in seasonality.

Poisson regression was used to analyse the rate of increase in IR in the period 2009-2018 and incorporated adjustments for age and sex distribution.

Statistical analysis was done using SPSS for Windows, version 25 (IBM Corp, Armonk, NY, USA), Stata release 14 (Stata Corp, College Station, TX, USA), and the R-4.1.2 programming language.

A $p < 0.05$ was considered significant. P values are not adjusted for multiple tests and should be interpreted exploratorily only.

Results

Demographic Characteristics of Patients and Seasonality

During the period 2004-2018, a total of 1,213 children with T1D were diagnosed at Tripoli Children's Hospital (Table 2). Of these, 595 (49.1%) were males and 618 (50.9%) were females, giving a male-to-female ratio of 1:1.03. The distribution of incident cases according to the age

Table 2. Number of children with type 1 diabetes diagnosed at TCH during the period 2004-2018 and included in the study

Patient's characteristics		Study period (2004-2018)	Period 1 (2004-2008)	Period 2 (2009-2013)	Period 3 (2014-2018)
Total number of cases		1,213	169	434	610
Total group: by sex Number (%)	Male	595 (49.1%)	77 (45.6%)	212 (48.8%)	306 (50.2%)
	Female	618 (50.9%)	92 (54.4%)	222 (51.2%)	304 (49.8%)
Mean age at diagnosis (mean ± SD, years)	Total group	6.3 ± 3.8	6.6 ± 3.8	6.4 ± 3.9	6.2 ± 3.8
	Male	6.1 ± 3.9	5.7 ± 3.6	6.4 ± 4.0	5.9 ± 3.8
	Female	6.5 ± 3.8	7.3 ± 3.7	6.4 ± 3.8	6.4 ± 3.8
	p* (M: F)†	0.03	0.007	0.99	0.11
Total group: by age group Number (%)	0-4 years	463 (38.2%)	56 (33.1%)	162 (37.3%)	245 (40.2%)
	5-9 years	458 (37.8%)	70 (41.4%)	160 (36.9%)	228 (37.4%)
	10-14 years	292 (24.1%)	43 (25.4%)	112 (25.8%)	137 (22.5%)

*Student's t-test for independent samples, †Male-to-female.
SD: standard deviation, TCH: Tripoli Children's Hospital

Table 3. Periods two and three analysis; period IR, and annual increase in the IR of type 1 diabetes by Poisson regression analysis

		Period 2 (2009-2013)	Period 3 (2014-2018)	Periods 2 and 3 (2009-2018)	
		IR (CI, 95%)	IR (CI, 95%)	IR (CI, 95%)	Percentage annual increase in IR§ (CI)
Total (0-14 years)		29.3 (26.6-32.2)	31.7 (29.2-34.3)	30.7 (28.8-32.6)	2.1% (-0.1—4.2)
Total: by sex (0-14 years)	Male	28.1 (24.5-32.2)	31.0 (27.7-34.7)	29.8 (27.3-32.5)	2.7% (-0.4—5.9)
	Female	30.6 (26.7-34.9)	32.4 (28.9-36.3)	31.6 (29.0-34.4)	1.5% (-1.5—4.6)
Total: by age group	0-4 years	31.1 (26.5-63.3)	36.0 (31.7-40.8)	33.9 (30.7-37.4)	3.4% (-0.1—7.0)
	5-9 years	34.0 (28.9-39.7)	37.4 (32.7-42.6)	35.9 (32.4-39.7)	2.6% (-0.9—6.3)
	10-14 years	22.9 (18.9-27.6)	21.6 (18.1-25.5)	22.2 (19.5-25.1)	-0.7% (-4.9—3.7)

§Using Poisson regression analysis.
IR: incidence rate, CI: confidence interval

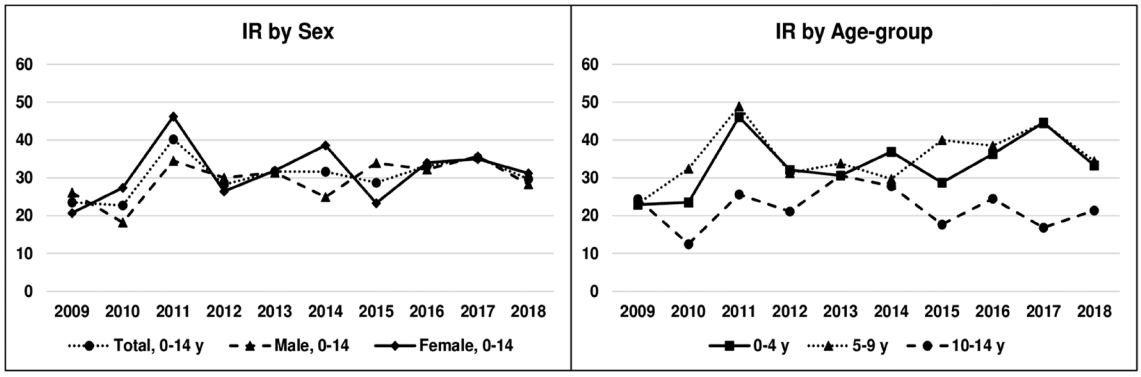

Figure 3. Annual IR of T1D in children aged 0-14 years in the West, South, and Tripoli regions of Libya (periods 2 and 3)
IR: incidence rate, T1D: type 1 diabetes

groups 0-4, 5-9, and 10-14 years was 38.2%, 37.8%, and 24.1%, respectively. The mean age at diagnosis for the total group was 6.3 ± 3.8 years. The mean age at diagnosis was significantly lower in males compared to females over the whole study period. However, on comparing the three periods, this difference was only significant in the first period.

More cases of T1D were diagnosed in autumn and winter (52.0%) than in spring and summer (48.0%). Edward's test was performed on 1044 cases in periods two and three and the analysis showed significant seasonal variation in the total cohort ($p = 0.01$), for males ($p < 0.001$), and the age group 5-9 years ($p < 0.001$), with a fitted peak incidence in January.

Incidence and Trend

The IRs of T1D in the West, South, and Tripoli regions of Libya in periods two and three are shown in Table 3 and Figure 3. Age standardization made minimal difference to the IRs, as the numbers in the three age groups are roughly equal in both the Libyan and the WHO standard populations. The highest IR was noted in the year 2011 in the total cohort, as well as in the female group, while the highest IR in males was in the year 2017. Furthermore, the highest IR in each age group was in the years 2011, 2011, and 2013 for the 0-4, 5-9, and 10-14 years age groups respectively (Figure 3).

Poisson regression modelling of periods two and three together (2009-2018) revealed that the overall trend, adjusted for age group and sex, was an increase of 2.1% per annum. The rates of increase in males were 2.7% and in females 1.5%. The rates of increase in the age groups 0-4, 5-9, and 10-14 years were 3.4%, 2.6%, and -0.7%, respectively.

Period three best represents the recent IR of T1D in the West, South, and Tripoli regions of Libya. The overall IR in this period was 31.7 (CI: 29.2-34.3) per 100,000, and the age-standardized rate was 31.7 (CI: 29.2-34.2). The sex-specific IRs among the male and female populations were 31.0 and 32.4 per 100,000, respectively. The age groups 0-4, 5-9, and 10-14 years had an IR of 36.0, 37.4, and 21.6 per 100,000, respectively (Table 3).

Discussion

This study on the IR of T1D in children aged 0-14 years is the first of its kind in the West, South, and Tripoli regions of Libya. The age-standardized IR for the 2014-2018 period was 31.7 per 100,000. This age-standardized IR for the West, South, and Tripoli regions, if extrapolated to the whole country, places Libya in the top ten countries with the highest age-standardized rates of T1D in this age group in the world, which already includes four other countries from the MENA region of the world, according to the tenth edition of the IDF Diabetes Atlas (11). Furthermore, it is similar to the rates reported recently from Tlemcen city in Algeria [IR: 38.5 (2015-2018)] (25), Oran city in Algeria [IR: 31.1 (2013-2017)] (26), and Qatar [IR: 28.4 (2012-2016)] (27).

The only previous data on the IR of T1D in Libya was produced by Kadiki and Roaeid (12) in children aged 0-14 years from the Benghazi region (1991-2000) who reported an age-standardized rate of 8.3 per 100,000, which is nearly equal to the age-standardized IR (8.6 per 100,000) from Oran city in Algeria (1990-1999) (6), suggesting that the increase in the IR in the two countries has followed a similar trend.

A comparison of the age-standardized IR in Libya from Kadiki and Roaeid (12) with the findings in the present study suggests a more than trebling of the IR of T1D since 1991-2000. Furthermore, in the present study, around 76% of cases (period 2009-2018) were diagnosed before the age of 10 years compared to 44% in the earlier study (12). The higher IRs in 0-4 and 5-9 years age groups in the third period of our study, also indicate an increase in the number of younger children diagnosed with T1D.

This high rate of increase was also seen in Oran city in Algeria (period 2013-2017, nearly double the rate compared to period 2003-2007), (26) in Kuwait (doubled from 1992 to 2013) (28), and in Qatar (nearly doubled from 2006 to 2016) (27,29). The annual rate of increase in the present study was 2.1%, which is lower than the estimated rate of increase worldwide (2.4-3.2%) (6), and in Kuwait (4.1% per annum) (28).

The mean age at diagnosis in our cohort was 6.3 ± 3.9 years, which is younger than the mean age in Tlemcen (7.51 ± 4.12 years) (25), and Kuwait (male 8.7 ± 3.4, female 7.9 ± 3.1 years) (28). This is consistent with the higher IR in the age groups 0-4 and 5-9 years in our study. This contrasts with the reports from the MENA region of the world, which showed a higher IR in 5-9 and 10-14 years compared to the IR in the younger age group [Tlemcen, (25) Oran, (26) and Kuwait (28)]). Our study showed a high IR of T1D in children under 10 years of age.

The high IRs of T1D noted in the year 2011 could be because the year 2011 was the year of a military conflict that resulted in regime change in Libya. This armed conflict might have led to the collapse or disruption of health services in cities around Tripoli, and resulted in the internal displacement of people, thus increasing the number of children diagnosed in the studied geographical area (30).

The seasonality test (periods two and three) demonstrated significant seasonal variation with peaks in January, in the 0-14- and 5-9-years age groups and males. This finding is only partially consistent with the pattern seen in Europe (31).

Study Limitations

This study has certain limitations, it was based on incident cases from a single centre in the West, South, and Tripoli regions of Libya, and an assumption was made that the Tripoli Children's Hospital covers 27.3% and 32.4% of the population of the studied geographical area in periods two and three respectively. However, because of the organization of healthcare facilities in Libya, we are confident that the estimated IR of T1D is reflective of the actual IR in the West, South, and Tripoli regions of Libya. This study was partly retrospective, case ascertainment was from a primary source only, furthermore, some patients from cities near the west border of Libya might be seen in clinics in neighbouring countries (Tunisia and Algeria). Furthermore, some patients might be seen in an adult diabetes clinic in the small cities located in the West and South regions or the adjacent Central region of Libya. As a result, the reported IR in this study could underestimate the true IRs.

Despite its limitations, the strengths of this study should be highlighted. It is based on the population of a well-defined region of Libya, it includes cases diagnosed over 10 years, and is based partly on an electronic paediatric diabetes registry. Furthermore, the diagnosis of T1D was reviewed according to the well-defined criteria of the T1D exchange clinic registry, thus minimizing misdiagnosis, however, if there were adolescents with type 2 diabetes who were not obese and were treated with insulin, they would be considered as having T1D.

Conclusion

In conclusion, the incidence of T1D in Libyan children in the West, South, and Tripoli regions of Libya appears to be rising, with a higher IR in the 0-4 and 5-9 years age groups. We have demonstrated a more than tripling of the IR over 18 years in children aged 0-14 years compared to a previous study from the Benghazi region of Libya. We need to ensure

appropriate planning of services and that resources are in place to meet the needs of increasing numbers of children with T1D.

We hope that this study will stimulate further studies to determine the true incidence of T1D in Libya and encourage healthcare professionals looking after children with diabetes to collaborate in establishing a national paediatric diabetes registry.

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Ethics

Ethics Committee Approval: The study proposal was approved by the Bioethics Committee at the Biotechnology Research Centre (ref no: BEC-BTRC 17-2019, date: 09.08.2020).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Rowida M. Khashebi, Mostafa S. Shebani, Design: Rowida M. Khashebi, Christopher C. Patterson, Mostafa S. Shebani, Data Collection or Processing: Rowida M. Khashebi, Mostafa S. Shebani, Analysis or Interpretation: Rowida M. Khashebi, Christopher C. Patterson, Mostafa S. Shebani, Literature Search: Rowida M. Khashebi, Mostafa S. Shebani, Writing: Rowida M. Khashebi, Christopher C. Patterson, Mostafa S. Shebani.

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Effects of Blue Light on Puberty and Ovary in Female Rats

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What is already known on this topic?

Blue light is a natural light source. However, in recent years, exposure to blue light has increased with the use of mobile devices and tablets. The severity of the impact of blue light exposure increases as the eye-screen distance decreases. The use of these devices in children has increased, and melatonin suppression by blue light is known to cause disruption in circadian rhythm, increased appetite, and obesity. However, its effects on puberty are unknown.

What this study adds?

Early puberty was observed due to exposure to blue light in the prepubertal period. When exposure to blue light increased, apoptosis and the appearance of polycystic ovary were detected in the ovaries.

Abstract

Objective: This study was designed to examine the effect of blue light exposure and exposure time on puberty in an animal model.
Methods: Eighteen 21-day-old female Sprague Dawley rats were divided into three equal groups which were: control group (CG); blue light-6 hours (BL-6); and blue light-12 hours (BL-12). CG rats were maintained with 12/12-hour light-dark cycles. The animals in BL-6 and BL-12 were exposed to blue light of wavelength 450-470 nm and intensity of 0.03 uW/cm^2 for 6 and 12 hours, respectively. Exposure to blue light continued until the first signs of puberty. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, testosterone, dehydroepiandrosterone sulfate (DHEA-S), leptin and melatonin were measured. Subsequently the ovaries and uterus were examined histomorphologically.
Results: The median day of puberty start was 38, 32 and 30 for the CG, BL-6, and BL-12 groups, respectively ($p = 0.001$). FSH, testosterone, DHEA-S, and leptin concentrations of all groups were similar. However, LH and estradiol concentrations in BL-6 were higher compared to CG ($p = 0.02$). There was a negative correlation between blue light exposure, exposure time, and melatonin concentrations ($r = -0.537$, $p = 0.048$). Ovarian tissue was compatible with puberty in all groups. As blue light exposure time increased, capillary dilatation and edema in the ovarian tissue increased. Prolonged exposure was associated with polycystic ovary-like (PCO) morphological changes and apoptosis in granulosa cells.
Conclusion: These results suggest that exposure to blue light and the duration of exposure induced earlier puberty in female rats. As the duration of blue light exposure increased, PCO-like inflammation, and apoptosis were detected in the ovaries.
Keywords: Blue light (470 nm), early puberty onset, rat, apoptosis, melatonin

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Introduction

Sunlight contains red, orange, yellow, green, and blue light. Light entering the retina produces a stimulus that is transmitted to the suprachiasmatic region of the hypothalamus and regulates circadian rhythm by controlling the body's biological clock. This mediates the timing of functions, such as cortex activity, body temperature, and the sleep-wake cycle. Evening light exposure causes a decrease in the release of the hormone melatonin, leading to disruption of the circadian rhythm and reduction in the antioxidative effects of melatonin (1,2,3). Blue light exposure in daylight during daytime increases alertness and promotes memory and cognitive functions (4,5). However, it is known that exposure to blue light at night has a significant melatonin-suppressing effect (6).

Electronic mobile devices emit high-energy, short-wavelength blue light (7). In the last century, blue light sources such as fluorescent and LED lighting and television became common in daily life. However, over the past ten years, the use of touch-screen devices, such as tablets and smartphones, has increased in all age groups (8). Blue light exposure is more intense with these devices because of the shorter eye-screen distance. In recent years, the age of children using these devices has rapidly decreased (9). Since the Coronavirus disease-2019 (COVID-19) pandemic, screen exposure in children and adolescents has increased substantially due to remote education and more screen time at home during lockdowns (10,11,12). Of note, an increase in the incidence of precocious puberty was observed during the pandemic period compared to the pre-pandemic period (13,14,15).

One of the factors that initiate puberty is a decrease in melatonin. The melatonin hormone has a suppressive effect on gonadotropin-releasing hormone (GnRH) released from the hypothalamus at the onset of puberty. And as melatonin level decreases, GnRH synthesis increase and puberty begins (16). In children living near the equator with lower melatonin concentrations because of the long daylight hours, puberty occurs earlier than in those at higher latitudes (17). It is known that light-exposure at night has a suppressive effect on melatonin and it has been shown that blue light suppressed melatonin production more than any other color such as green, red (7).

However, the impact of this type of blue light exposure on the pubertal process is unclear. The aim of this study was to examine the effects of blue light exposure including duration of exposure in an animal model of puberty.

Methods

Animals

Eighteen prepubertal 21-day-old female Sprague Dawley rats weighing 35-50 g were procured from the Experimental Animal Center of Gazi University (Ankara, Turkey). The study groups were isolated from male rats after postpartum 21 days. The rats were housed in polysulfone cages (42.5 × 26.6 × 18.5 cm in size; three rats per cage) at 21-24 °C and 40-45% humidity at the Laboratory Animals Breeding and Experimental Research Center of the Faculty of Pharmacy, Gazi University (Ankara, Turkey). The animals were fed a standard pellet diet and water *ad libitum* during the experimental period. All the animals were maintained by the Guide for the Care and Use of Laboratory Animals (18), and the experimental procedures were approved by the Experimental Animal Ethics Committee of Gazi University (project no: G.Ü.ET-21.052, date: 09.07.2021).

Light Exposure Protocol

A blue LED strip (FSHI. 1048.B020.6012, HI-LED, FLEX honor-, ILED- İstanbul, Turkey) was the source of the blue light at a wavelength of 450-470 nm was placed approximately 20 cm above the center of each cage in the experimental groups (Figure 1).

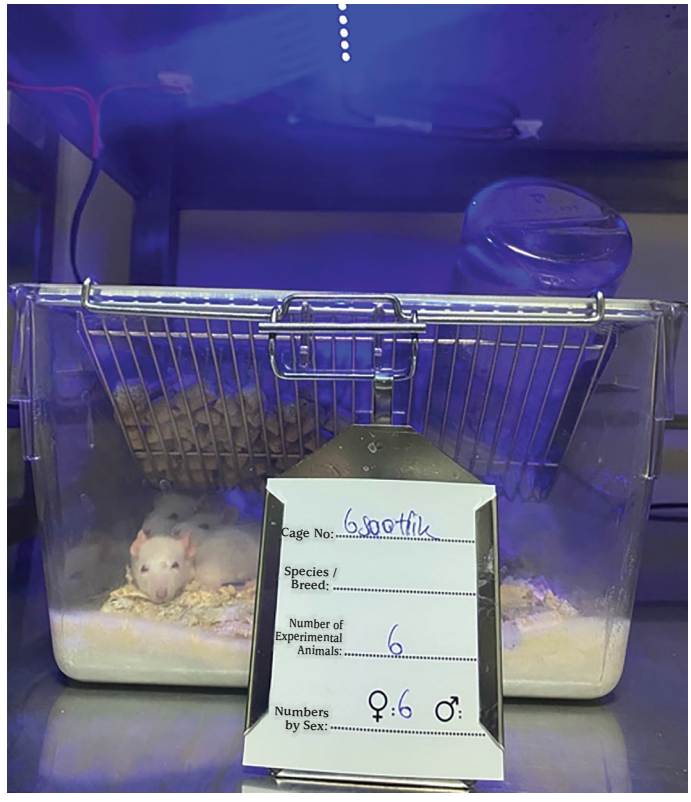

Figure 1. Experimental set-up of the room

In the experimental setup, the blue light source was used at an intensity that lowered rat melatonin concentrations but would not cause retinal damage (19,20,21). This was determined to be an irradiance level of 0.03 uW/cm^2 at the eye level of the animals. The irradiance in the entire area of the cage where the rats were housed was measured with a spectroradiometer and adjusted to the same level.

Experimental Design

The rats were randomly divided into three groups of six rats: the control group (CG), blue light-6 hours (BL-6), and blue light-12 hours (BL-12). CG rats were maintained under standard conditions with 12/12-hour light-dark cycles. The light/dark cycle condition for the BL-6 and BL-12 groups were exposed to blue light (450-470 nm) for 6 hours (light time 6:00 a.m.-6:00 p.m.; blue light time 6:00 p.m.-12:00 p.m.; dark time 12 p.m.-6:00 a.m.); for 12 hours (light time 6:00 a.m.-6:00 p.m.; blue light time 6:00 p.m.-06.00 a.m.), respectively.

The rats were weighed at the beginning and end of the experimental procedure, and the percentage weight gain was calculated with the formula Weight gain (%) = (Last day-First day)/First day x 100.

Vaginal Examination and Cytology

Vaginal opening is one of the external signs of puberty in rodents (22). The rats were examined daily, starting at 22 days of age, to detect vaginal opening. After vaginal opening, vaginal smear samples were collected to determine the estrus stage. To do this, a moistened cotton swab was inserted into the vagina. Cells from the vaginal lumen and walls were gently taken and transferred to a glass slide. After the samples were allowed to air-dry, they were stained with Giemsa stain and examined under a light microscope.

The stages of the estrous cycle were classified as *proestrus* (oval nucleated epithelial cells), *estrus* (irregular-shaped, cornified squamous epithelial cells), *metestrus* (fragmented, cornified epithelial cells and smaller, darker stained leukocytes), and *diestrus* (nucleated epithelial, predominantly leukocytes) (23). Rats were exposed to blue light until the first estrus stage after vaginal opening.

Termination of the Experimental Procedure

At the first estrus stage, all the rats were sacrificed by taking blood from the heart at 8:00 p.m. to determine the peak melatonin rhythm of the rats (24) under general anesthesia (10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride). After the anesthesia procedure, blood samples were obtained by the intracardiac puncture. The blood samples were centrifuged at 3000 rpm (906 x g) for 15 minutes and the serum was separated. The serum samples were stored at -80 °C until analysis. The height of the ovarian tissues was measured by a micrometer, and the uterine and ovarian tissues were dissected and weighed.

Determination of Biochemical Parameters

The collected blood was centrifuged at 3000 rpm for 10 minutes at +4 °C and stored at -80 °C. The serum concentration of the follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, testosterone, dehydroepiandrosterone sulfate (DHEA-S), leptin, and melatonin were evaluated by enzyme-linked immunosorbent assay (ELISA) (Rat-specific ELISA, Bioassay Technology Laboratory, China).

Histopathological Methods

The right and left ovaries and the uterus were weighed after dissection, then the ovaries and the uterus were fixed in Bouin's fixative and embedded in paraffin blocks using standard procedures. Sections of 4-5 micron thickness were taken from the prepared paraffin blocks and stained with hematoxylin and eosin. The samples were examined for histomorphological changes by light microscopy using the Leica DM4000 (Leica, Germany) computer-assisted imaging system, and images were obtained using the Leica-Qwin program.

Statistical Analysis

Statistical Package for the Social Sciences, version 26 was used for statistical analysis (IBM Inc., Armonk, NY, USA). The Kruskal-Wallis test was used when comparing the medians of three independent groups for data that did not fit the normal distribution, and the Mann-Whitney U test were used when comparing the medians of two independent groups. While investigating the associations between non-normally distributed and/ordinal variables, the correlation coefficients and their significances were calculated using the Spearman's test. All data are given as median (minimum-maximum). Bonferroni correction was used in *post hoc* tests. Statistically, $p < 0.05$ was considered significant.

A power analysis was performed using G*Power version 3.1.9.7 to determine the minimum sample size required to test the study hypothesis. Results indicated that a sample size of n = 18 is required to achieve 80 % power for detecting a large effect at a significance of $\alpha = 0.05$.

Results

The mean±stardard deviation (SD) initial weight of the female rats in CG, BL-6, and BL-12 were 42.5±4.7, 42±3.4,

and 42.3±2.7 g, respectively (p = 0.91). The median day of puberty onset was 38th, 32nd, and 30th days in the CG, BL-6 and BL-12 groups, respectively. Puberty onset was significantly earlier in BL-12 compared to the CG (p = 0.001) (Table 1). The age of onset of puberty decreased as the duration of blue light exposure increased (r = -0.910, p < 0.001). The mean±SD weight at onset of puberty were 85.1 ± 9.7 g, 91.6 ± 5.5 g, 80 ± 5 g in CG, BL-6, and BL-12 groups, respectively. The weight at onset of puberty in BL-6 was significantly greater than in BL-12 (p = 0.04).

Median percentage weight gain in CG, BL-6, and BL-12 was 110%, 117%, and 93%, respectively. Percentage weight gain was higher in BL-6 compared to CG, while rats in BL-12 had the least weight gain (Table 1).

Serum concentrations of FSH, estradiol, testosterone and DHEA-S in the blue light-exposed groups were similar to those of controls (p > 0.05) (Table 2). LH concentrations were higher in BL-6 than in CG (p = 0.027). The high concentrations of LH and estradiol in BL-6 can be attributed to the hormonal peak during estrus. The estrus stage of the rats in the BL-12 group was observed in the earlier hours of the day and the time between the estrus stage and time at sacrifice was longer. Therefore, LH and estradiol surges were not detected. On the contrary, we detected LH and estradiol surges in the BL-6 group due to the shorter estrus stage and earlier time of sacrifice.

Serum concentrations of leptin showed no significant difference among the groups (p > 0.05) (Table 1). There was no correlation between percentage weight gain, leptin, and the day of puberty onset (p > 0.05).

Median (minimum-maximum) melatonin levels were 144 (126-197) ng/L in CG, 143.7 (132-152) ng/L in BL-6, and 121 (116-151) ng/L in BL-12 (p > 0.05). Melatonin levels decreased as the duration of exposure to blue light increased (r = -0.537, p = 0.048).

The ovarian size, ovarian weight, or uterine weight of groups were similar (p > 0.05) (Table 3). On histologic examination, ovarian tissue was compatible with puberty in all groups. Primary, antral, and tertiary follicles and corpus luteum were observed in CG (Figure 2) and BL-groups. Edema and congestion in the medulla increased as the exposure time to blue light increased.

When the ovarian tissue of BL-6 was examined, a lower antral and Graafian follicle density and higher preantral follicle density were noted in BL-6 compared to the CG group.

Table 1. Timing of puberty onset, percentage weight gain, and leptin concentrations of the groups

	Control	BL-6	BL-12	p value
Puberty onset (day)	39 (38-40)	33 (30-34)	30 (30-32)	***0.001**
Weight gain (%)	110 (67-133)	117 (98-152)	93 (56-110)	0.09
Leptin (ng/mL)	3.1 (2.6-4.1)	3.2 (2.5-4.4)	3.4 (2.7-11.3)	0.51

Values represent median (minimum-maximum). *Control vs. BL-12.
BL-6: blue light-6 hours, BL-12: blue light-12 hours

Table 2. The hormone and melatonin concentrations of the groups

	Control	BL-6	BL-12	p value
FSH (IU/mL)	16.6 (9.6 -20.2)	19.4 (6.6-24.2)	9.2 (6.3-17.8)	0.07
LH (IU/mL)	67.5 (38.4-99.8)	106.9 (79-153.2)	82 (42.1-101.7)	***0.02**
Estradiol (pmol/L)	92.5 (57.5-166.2)	110.9 (89.6-150.5)	99.6 (46.7-123.1)	0.60
DHEA-S (µmol/L)	1.13 (1.01-1.13)	1.26 (0.90-1.57)	0.99 (0.64-1.54)	0.15
Testosterone (nmol/L)	335 (229-439)	243 (239-534)	261 (134-290)	0.30
Melatonin (ng/mL)	144 (126-197)	143 (132-152)	121 (116-151)	0.11

Values represent median (minimum-maximum). *Control vs. BL-6 p = 0.03.
BL-6: blue light-6 hours, BL-12: blue light-12 hours, FSH: follicle stimulating hormone, LH: luteinizing hormone, DHEA-S: dehydroepiandrosterone sulfate

Table 3. Ovary length and ovary and uterus weights of the groups

	Control	BL-6	BL-12	p value
Right ovary (mm)	4.5 (3.7-5.5)	3.8 (2.3-4.9)	4.9 (4.5-5.5)	0.06
Left ovary (mm)	4.5 (4.4-6.2)	4.3 (2.3-5.6)	4.5 (4.4-6.4)	0.43
Ovary weight (mg)	120 (40-130)	120 (20-140)	110 (60-150)	0.31
Uterus weight (mg)	410 (210-820)	510 (300-650)	640 (250-900)	0.21

Values represent median (minimum-maximum)

Perivascular edema and capillary dilation were prominent in the medulla of the BL-6 group (Figure 3a). At high magnification, extracellular edema in the granulosa cells in the preantral follicles and the presence of apoptotic cells with pyknotic nuclei in the granulosa layer of antral follicles were noted (Figure 3b). The most remarkable finding in the examination of BL-12 ovarian tissue was the presence of the corpus luteum covering most of the organ. In contrast to CG and BL-6, numerous Graafian follicles were noted in BL-12 group (Figure 4a). At high magnification, it was evident that the Graafian follicles had a thinner granulosa layer than in the CG. This appearance was consistent with an appearance of polycystic ovary (PCO). In addition, the extracellular edema in the granulosa cells and the presence of apoptotic cells with pyknotic nuclei in the granulosa layer of the antral follicles were more pronounced in BL-12 than in BL-6 (Figure 4b). Blue light exposure appeared to induced granulosa cell apoptosis, and the number of apoptotic cells increased with longer exposure time.

Histological examination of uterine tissue from control rats was normal (Figure 5). In BL-6, the most remarkable histomorphological finding in the proliferative phase uterus was that the uterine epithelium thickened and became high prismatic epithelium, compared to CG (Figure 6). In BL-12, the uterine and gland epithelium in the proliferative phase uterus had a similar histological appearance to the samples from BL-6. The most prominent change in BL-12 was vessel dilation in the endometrial lamina propria and the capillaries reaching the surface (Figure 7). This finding was consistent with the endometrium entering the secretory phase with changes consistent with ovulation.

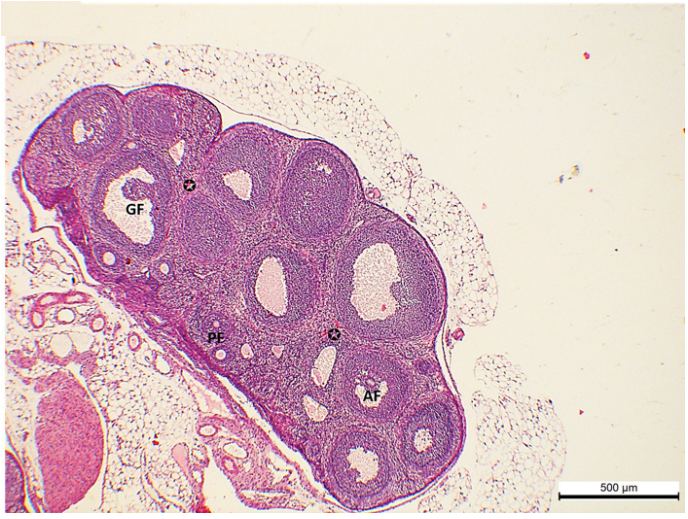

Figure 2. Histological findings of ovary-control group (H&E x40). All developing preantral, antral, and Graafian follicle structures and stroma appeared normal. Normal structure and distribution of blood vessels (✪)

PF: preantral follicle, GF: Graafian follicle, AF: antral follicle

Discussion

To the best of our knowledge, the present study is the first to demonstrate the effects of blue light exposure on puberty in experimental animals. Blue light is the dominant light used for increasing screen brightness of mobile devices. The wavelength of blue light is between 450 and 490 nm and exposure is exacerbated with the reduction of eye-screen distances (7,25). In the last decade, several studies have suggested that night-time exposure to blue light emitted by devices, such as phones and tablets, can impair the normal

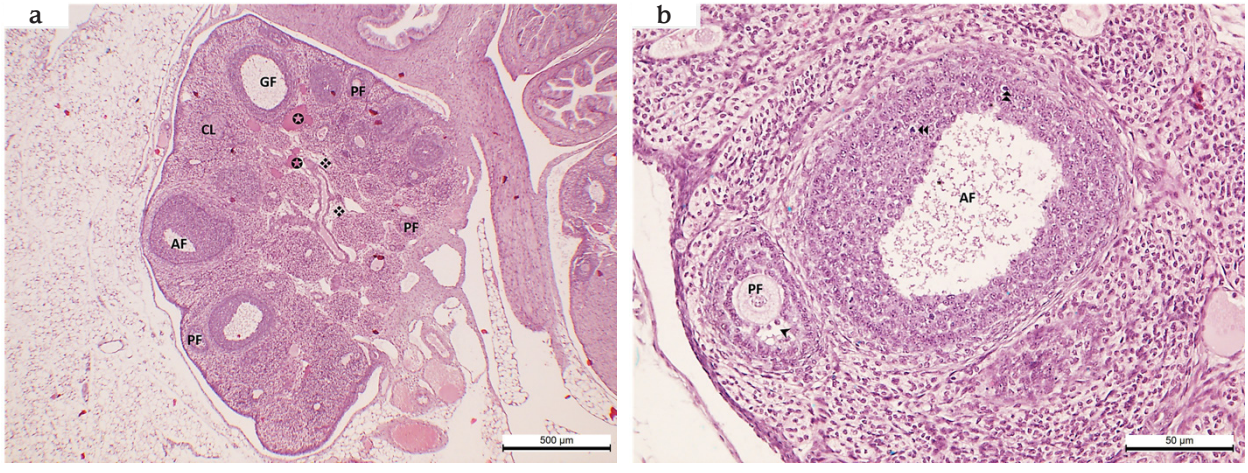

Figure 3. a) Histological findings of ovary-BL-6 group (H&E x40). At low magnification, the lower AF and GF density and higher PF density were noted in the cortex. Small CL structures were observed. There was prominent capillary dilation (✪) and especially perivascular edema (❖). **b)** Histological findings of ovary-BL-6 group (H&E x200). Extracellular edema (➤) in the granulosa cells of the PF and apoptotic cells (⏮) in the granulosa cells of the AF were prominent

PF: preantral follicle, AF: antral follicle, GF: Graafian follicle, CL: corpus luteum, BL-6: blue light-6 hours

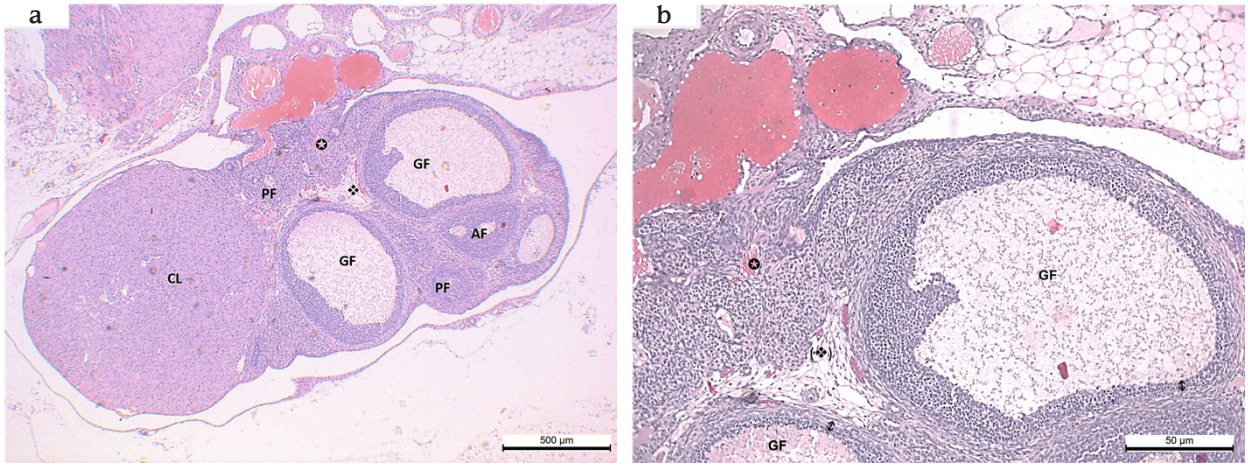

Figure 4. a) Histological findings of ovary-BL-12 group (H&E x40). Pronounced thinning of the granulosa layer of the follicles (↕) was noted. There was increased edema (❖) in the medulla and pronounced capillary dilation (✪). **b)** Histological findings of ovary-BL-12 group (H&E x200). Pronounced thinning of the granulosa layer of the follicles (↕) was noted

✪: capillary dilation, ❖: perivascular edema, PF: preantral follicle, AF: antral follicle, GF: Graafian follicle, CL: corpus luteum, BL-12: blue light-12 hours

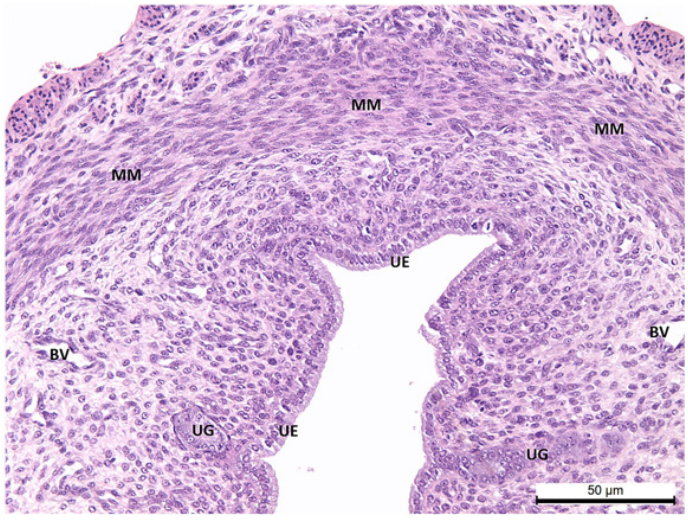

Figure 5. Histological findings of uterus-CG (H&E x40). Uterine tissue from control rats revealed normal UE of proliferative phase endometrium, a small number of UG in the lamina propria, and spiral arterioles that had not yet reached the surface. The myometrial structure was typical

UE: uterine epithelium, UG: uterine glands, BV: blood vessels, MM: myometrium, BL-12: blue light-12 hours

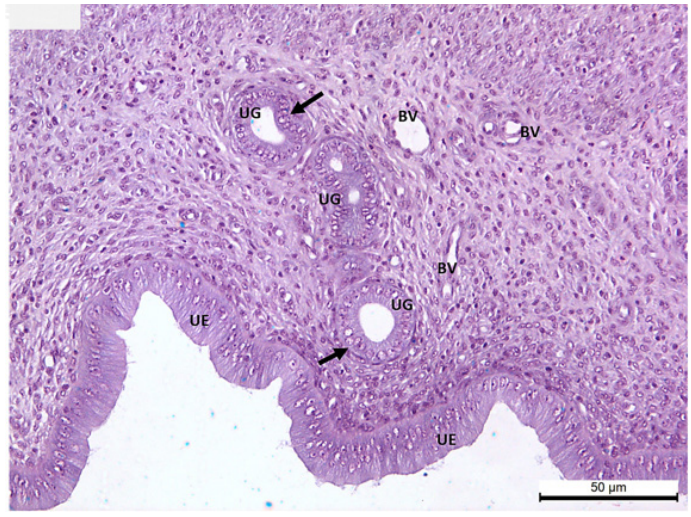

Figure 6. Histological findings of uterus-BL-6 (H&E x40). UE height was relatively increased. UG were more numerous and epithelial height increased. BV advanced towards the surface, the number of UG was also relatively increased in BL-6 group, and UG epithelial cells were larger than in CG. The endometrial vessels spread superficially. All these findings indicated that the uterus was entering the secretory phase

UE: uterine epithelium, UG: uterine glands, BV: blood vessels, BL-6: blue light-6 hours

function of the biological clock, alter sleep-wake cycles, and induce metabolic changes (26,27). The effect of prepubertal exposure to blue light on puberty, however, has not been previously investigated.

The results of the present study suggest that blue light was associated with early puberty in female rats, with blue light exposure and exposure time accelerating the onset of puberty. The levels of FSH, LH, and estradiol in the CG demonstrated that puberty initiated in the hypothalamic-pituitary-gonadal axis. The lack of a difference in hormone levels between the control and both BL groups suggests that puberty also had a central onset in the BL groups. The high concentrations of LH in BL-6 may be attributed to the hormonal peak during estrus. Furthermore, we did not

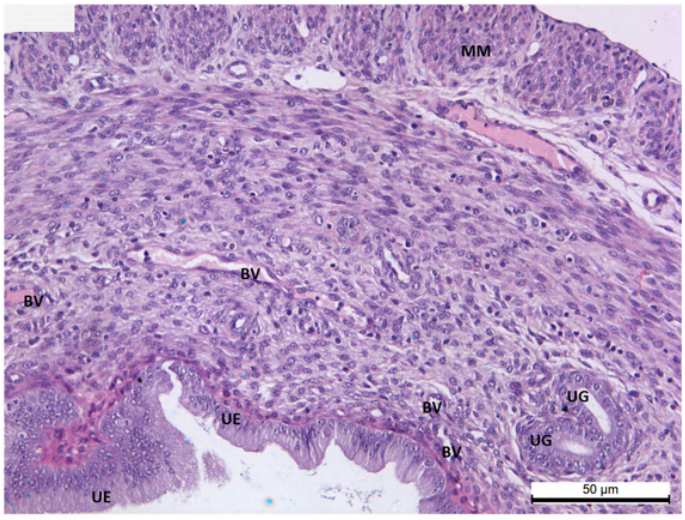

Figure 7. Histological findings of uterus-BL-12 (H&E x40). UE height was relatively increased, UG were more numerous and epithelial height increased. BV extend to the surface and appear dilated

UE: uterine epithelium, UG: uterine glands, BV: blood vessels, MM: myometrium, BL-12: blue light-12 hours

detect LH and estradiol surges in the BL-12 group. On the contrary, we may have detected LH surges in the BL-6 group due to the shorter estrus stage and earlier sacrifice time. The histological findings of the ovarian tissue in all groups were consistent with puberty, providing additional evidence that all the rats had entered puberty.

Through its effects on the hypothalamic center, blue light exposure increases food intake by reducing the release of leptin, which regulates hunger signals (28). Light exposure at night was also shown to increase body weight and body fat in mutant mouse experiments (29), and there are reports that weight gain accelerates puberty onset in rats (29,30). In our study, the group with the longest exposure to blue light had the lowest percentage of weight gain. There was no correlation between percentage weight gain, leptin, and the day of puberty onset. Therefore, this study may rule out weight gain and leptin as factors that accelerate puberty due to blue light.

Another factor contributing to puberty with exposure to blue light could be decreased melatonin secretion. We observed that melatonin levels decreased with increased blue light exposure time and puberty occurred earlier. The melatonin release pattern during the human lifespan involves an increase in melatonin concentrations from the neonatal period to the pubertal period, followed by a decrease at the onset of puberty (31). Neuroendocrine control of the sexual maturation process is influenced by the pattern of melatonin secretion resulting from the light-dark cycle. High melatonin concentrations are thought to have an inhibitory effect on the GnRH (32). *In vitro* studies of cultured prepubertal rat pituitary glands demonstrated that melatonin plays a role in the timing of developmental stages by inhibiting the release of GnRH and, therefore LH (33). A study comparing girls with precocious puberty and age-matched controls found that lower melatonin concentrations were associated with early puberty (32). Lee et al. (34) found that blue light exposure in the evening suppressed melatonin more in children than adults, even if the exposure was shorter. A study examining the effect of light on the circadian system of children in early puberty and mid-puberty showed that children in early puberty were more sensitive to evening light and their melatonin concentrations were more suppressed (35). During the COVID-19 pandemic, online education via electronic mobile devices and an increased time at home resulted in longer screen exposure in the younger age group. Studies have shown an increase in precocious puberty and accelerated puberty during pandemic-related lockdown compared to the pre-pandemic period (13,14). Among these studies, Stagi et al. (13) compared data from the pandemic period and the five years before the pandemic and reported that the incidence of newly diagnosed precocious puberty cases increased, and the rate of puberty was accelerated. They found a significant increase in body mass index (BMI) and pre-sleep screen device usage time among patients diagnosed and followed up during the pandemic.

Similarly, Chioma et al. (14) reported an increase in precocious puberty cases during the pandemic compared to the corresponding months of the previous year. Although there was no difference in BMI between the groups, the authors observed an increase in the duration of electronic device use and a decrease in physical activity. Our study demonstrated the effects of blue light exposure on puberty and the relationship with increased exposure time.

Early-life stress exposure is one of the common risk factors for psychopathology and deviations in pubertal timing. Several studies have demonstrated that stress promotes puberty in girls and female rats (36,37). Exposure to blue light may have induced stress in the rats. Stress may have also contributed to early puberty.

The histological findings in the ovarian tissue samples were striking. There was increased edema and capillary dilation in the ovarian medulla, accompanied by increased granulosa cell apoptosis, and histomorphological changes consistent with PCO in the BL-12 group. PCO syndrome (PCOS) is a common endocrine disorder in adolescence. Genetic abnormalities, lifestyle, prenatal hormonal imbalances, and environmental factors have been implicated in the

etiology of PCOS (38). Simon et al. (39) demonstrated that the effects of circadian rhythm disruption may contribute to PCOS in humans. In a PCOS study in which 6-week-old rats were exposed to continuous light for four weeks, PCOS-like results were discovered in the ovarian tissue of the rats. The authors suggested that constant light exposure appears to induce PCOS-like changes although they detected no differences in serum concentrations of FSH, LH, estradiol, or testosterone (40). In another rat study, 6-week-old rats were exposed to 600 lux light for 16 weeks. The study was conducted to model PCOS in rats and revealed PCOS-like histological findings in the ovaries and increased testosterone concentrations (41). In both studies, it was observed that prolonged light exposure in mature rats appeared to induce PCOS. In our study, BL-12 showed a thin granulosa layer in the Graafian follicles, which was a PCO-like finding (42,43). The absence of other signs of PCO and the lack of difference in androgen concentrations may be related to the duration of blue light exposure, irradiance level, and early sacrifice of the rats. In the present study, increased edema and capillary dilation in the ovarian medulla and apoptosis of the granulosa cells was observed in the rats exposed to blue light. There are no previous publications associating blue light exposure with apoptosis in the granulosa cells of ovarian tissue. However, increased edema and capillary dilation in the ovarian medulla may have been triggered in the experimental groups due to reduced melatonin and the subsequent increase in the pro-inflammatory process (44).

Study Limitations

As one of the limitations of our study, groups exposed to daylight should have been included. In the female rat study in which light-dark, continuous light and continuous darkness were applied, no difference was found in the days of puberty-onset (45). Furthermore, the reason for excluding other wavelengths of light from this investigation arises from the fact that the inhibitory impact of these wavelengths on melatonin was comparatively weaker as compared to blue light. Consequently, we excluded these groups from our study to comply with the 3R rule (46), which pertains to using a minimum number of animals in our research. The high concentrations of LH in BL-6 may be attributed to the hormonal peak during estrus. Furthermore, we did not detect LH and estradiol surges in the BL-12 group. Rats were sacrificed at the time interval when melatonin levels were highest (23). However, at this time, the gonadotropin levels were not at peak in the cycle due to the difference between puberty onset time and the time of sacrifice. We could not show a direct effect of melatonin on Kisspeptin and GnRH. A further limitation was that hormonal measurements were performed with ELISA. Liquid chromatography-mass spectrometry/mass spectrometry or high-performance liquid chromatography methods may have provided a greater degree of accuracy and sensitivity for hormonal measurement.

Conclusion

The blue light exposure and duration of exposure caused earlier onset of puberty. With increased blue light exposure duration, signs of PCO, inflammation, and apoptosis were detected in the ovaries. In the future, human studies are needed to demonstrate that blue light accelerates puberty onset and determine its short- and long-term effects on ovaries.

Ethics

Ethics Committee Approval: The study was approved by the Experimental Animal Ethics Committee of Gazi University (project no: G.Ü.ET-21.052, date: 09.07.2021).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Aylin Kılınç Uğurlu, Aysun Bideci, Design: Aylin Kılınç Uğurlu, Aysun Bideci, Mürşide Ayşe Demirel, Gülnur Take Kaplanoğlu, Duygu Dayanır, Özlem Gülbahar, Tuba Saadet Deveci Bulut, Esra Döğer, Mahmut Orhun Çamurdan, Data Collection or Processing: Aylin Kılınç Uğurlu, Mürşide Ayşe Demirel, Duygu Dayanır, Tuba Saadet Deveci Bulut, Esra Döğer, Analysis or Interpretation: Aylin Kılınç Uğurlu, Aysun Bideci, Mürşide Ayşe Demirel, Gülnur Take Kaplanoğlu, Duygu Dayanır, Özlem Gülbahar, Esra Döğer, Literature Search: Aylin Kılınç Uğurlu, Aysun Bideci, Mürşide Ayşe Demirel, Gülnur Take Kaplanoğlu, Özlem Gülbahar, Esra Döğer, Mahmut Orhun Çamurdan, Writing: Aylin Kılınç Uğurlu, Aysun Bideci, Mürşide Ayşe Demirel, Gülnur Take Kaplanoğlu, Özlem Gülbahar, Esra Döğer.

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ORIGINAL ARTICLE

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Peak Serum Cortisol Cutoffs to Diagnose Adrenal Insufficiency Across Different Cortisol Assays in Children

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What is already known on this topic?

Previous reports suggest that newer and more specific cortisol assays result in lower cortisol values than the traditional polyclonal antibody (pAb) immunoassay. However, no specific peak serum cortisol cutoff value for the diagnosis of adrenal insufficiency (AI) in children has been established for these newer and more specific assays and no information about diagnostic accuracy has been provided.

What this study adds?

This study redefines the biochemical diagnostic cutoff points for AI in children when using a highly specific cortisol monoclonal antibody immunoassay and liquid chromatography tandem mass spectrometry. It also provides information on diagnostic accuracy for each cut off point when compared to the reference pAb immunoassay.

Abstract

Objective: Current peak serum cortisol cutoffs for the diagnosis of adrenal insufficiency (AI) after Cosyntropin stimulation have been established using polyclonal antibody (pAb) immunoassays. However, new and highly specific cortisol monoclonal antibody (mAb) immunoassays are being used more widely, which can potentially yield higher false positive rates. Thus, this study aimed to redefine the biochemical diagnostic cutoff points for AI in children when using a highly specific cortisol mAb immunoassay and liquid chromatography tandem mass spectrometry (LC/MS) to avoid unnecessary steroid use.

Methods: Cortisol levels from 36 children undergoing 1 mcg Cosyntropin stimulation tests to rule out AI were measured using pAb immunoassay (Roche Elecsys Cortisol I), mAB immunoassay (Roche Elecsys Cortisol II), and LC/MS. Logistic regression was used to predict AI using the pAB as the reference standard. A receiver operator characteristic curve, area under the curve (AUC), sensitivity, specificity, and kappa agreement were also calculated.

Results: Using a peak serum cortisol cutoff value of 12.5 µg/dL for the mAb immunoassay provided 99% sensitivity and 94% specificity for diagnosing AI, when compared to the historical pAb immunoassay cutoff of 18 µg/dL (AUC = 0.997). Likewise, a cutoff of value of 14 µg/dL using the LC/MS, provided 99% sensitivity and 88% specificity when compared to the pAb immunoassay (AUC = 0.995).

Conclusion: To prevent overdiagnosis of AI in children undergoing 1 mcg Cosyntropin stimulation test, our data support using a new peak serum cortisol cutoff of 12.5 µg/dL and 14 µg/dL to diagnose AI when using mAb immunoassays and LC/MS in children, respectively.

Keywords: Adrenal insufficiency, cortisol, assays, pediatrics

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Introduction

Adrenal insufficiency (AI) is a common condition characterized by a deficient production of glucocorticoids. The correct diagnosis of AI is of the utmost importance as it may be life-threatening if left untreated. However, the incorrect diagnosis of AI can have a major negative impact on the patient and their family's lives, including medication cost, ongoing medical care, and potential side effects and comorbidity from unnecessary treatment with corticosteroids (1).

Conventionally, the biochemical diagnosis of AI is established through Cosyntropin stimulation test (2). This test assesses the adequacy of cortisol response to stimulation with either a 250 mcg dose of Cosyntropin when primary AI is suspected or 1 mcg for evaluation of central AI or when there is a Cosyntropin shortage. In both instances, a peak serum cortisol cutoff of less than 18 µg/dL (500 nmol/L), using a traditional polyclonal antibody (pAb) immunoassay is considered diagnostic of AI (3).

The pAb immunoassay lacks specificity with varying degrees of antibody cross-reactivity with endogenous proteins. Therefore, in many institutions, this assay has been replaced with other more specific laboratory assays; liquid chromatography tandem mass spectrometry (LC/MS) or monoclonal antibody (mAb) immunoassay. Though cortisol immunoassays are widely used for their high performance and cost-effectiveness (4), LC/MS is considered the gold standard test (5). Previous reports suggest that these newer assays result in lower cortisol values than the traditional pAb immunoassay (6). However, no specific peak serum cortisol cutoff value for the diagnosis of AI has been established for these newer and more specific assays (7,8).

The adoption of these newer cortisol assays while still using historic cortisol cutoffs may lead to an overdiagnosis of AI and unnecessary corticosteroid use. As demonstrated in a previous study, if the cortisol cutoff was not adjusted, the rate of AI diagnosis increased from 26% using a pAb immunoassay to 71% when the mAb immunoassay was adopted (9). Previous studies in adults (10,11,12), report conflicting results when establishing a new cutoff level for the biochemical diagnosis of AI. These studies were conducted in adult population and using an adrenocorticotropic hormone stimulation test with Cosyntropin 250 mcg. Some researchers have proposed a new cortisol cutoff after Cosyntropin stimulation test of 14 to 15 µg/dL for mAb immunoassay and LC/MS (10,11). Others consider the new cutoff to be 12.7 µg/dL when using the mAb immunoassay (12). Moreover, these studies do not provide any information about diagnostic accuracy for the proposed cutoff level or the agreement between the proposed cutoff and the clinically accepted cutoff value when using pAb immunoassay. In addition, there is paucity of data in the pediatric population and when using Cosyntropin 1 mcg for the stimulation test.

Thus, the aim of this study was to establish the optimal peak serum cortisol cutoff when mAb immunoassay and LC/MS are used in pediatric patients undergoing 1 mcg Cosyntropin stimulation test. Further aims were to determine the sensitivity and specificity of these cutoffs to diagnose AI, as well as the probability of agreement between the assays using a kappa statistic.

Methods

De-identified blood samples were prospectively collected from pediatric patients undergoing 1 mcg Cosyntropin stimulation test at St. Louis Children's Hospital from July 1st, 2016, to July 31st, 2017. Samples were analyzed using pAb immunoassay (Roche Elecsys Cortisol I), mAb immunoassay (Roche Elecsys Cortisol II), and LC/MS, which is considered the reference standard.

For the pAb immunoassay, the Roche Elecsys Cortisol I was used and the analysis was completed using a Roche automated system (Cobas e601). This assay has a within-run precision of 1.6% coefficient of variation (CV) at 3.6 µg/dL and 24.2 µg/dL. The between-run precision is 3.0% at 3.7 µg/dL and 1.8% at 24.1 µg/dL levels. All samples were stored at -80 °C until batch analysis at the same time with this assay.

For the mAb immunoassay, the Roche Elecsys Cortisol II was used, which makes use of a competition test principle using a mAb, which is specifically directed against cortisol. The analysis was again completed using a Roche automated system (Cobas e601). The within-run precision is 11.1% CV at 4.0 µg/dL and 22.9 µg/dL, with a between-run precision of 2.4% CV at 4.1 µg/dL and 2.5% CV at 23.3 µg/dL. With this assay, samples were analyzed on the day of collection over the study time.

The LC/MS assay was performed at Mayo Clinic Laboratory in Rochester, Minnesota. Deuterated cortisol (d4-cortisol) is added to each specimen as an internal standard. Cortisol and d4-cortisol are extracted from the samples with methylene chloride and analyzed by LC/MS using multiple reaction monitoring. This assay does not suffer from crossreactivity, as previously published (13).

The study was approved by the Washington University in St. Louis of Institutional Review Board (IRB ID: 202012130, date: 08.09.2022).

Statistical Analysis

Peak serum cortisol level using each different assay was obtained and mean peak serum cortisol level with standard deviation (SD) was calculated. Peak serum cortisol level is defined as the highest cortisol level at any time point during the stimulation test. Samples were collected at 20, 30, and 60 min after Cosyntropin is given. We defined AI as a peak serum cortisol below 18 µg/dL using the pAb immunoassay.

Measurements by LC/MS and mAb immunoassay were individually used in simple logistic regression models to predict AI. For each model, receiver operator characteristic (ROC) curve, area under the curve (AUC), sensitivity, and specificity was used to evaluate the potential of the median values as thresholds for each predictor. In addition, kappa agreement statistic between the new cutoffs and the historic peak serum cortisol cutoff level of 18 µg/dL when using a traditional pAb immunoassay was calculated.

Results

Thirty-six de-identified serum samples from pediatric patients undergoing 1 mcg Cosyntropin stimulation test were collected during the study and compared across the three different laboratory assays. The mean (±SD) serum cortisol level using the pAb immunoassay was 17.1±9.7 µg/dL, while the mean (±SD) serum cortisol level using the mAb immunoassay was 12±6.6 µg/dL. As shown in Figure 1, over 75% of all mAb values were below the historic cutoff of 18 µg/dL, meeting the biochemical diagnosis of AI. The mean difference in serum cortisol level between the mAb immunoassay and the pAb immunoassay was 5.12 µg/dL ($p < 0.001$). The AUC for the mAb immunoassay ROC was 0.997 (Figure 2). Using a cutoff of 12.5 µg/dL for the mAb immunoassay provided a sensitivity of 99% (95% CI: 96-100%) and specificity of 94% (95% CI: 87-100%). Furthermore, a simple kappa agreement between the cutoff for the mAb immunoassay and the pAb immunoassay was calculated to be 0.94 (95% CI: 0.88-1.00).

The mean (±SD) serum cortisol level for the LC/MS assay was 12.9±6.6 µg/dL. As presented in Figure 1, 75% of all the LC/MS values obtained were below the current threshold of 18 µg/dL, meeting the biochemical diagnosis of AI. The mean difference in serum cortisol level between the LC/MS assay and the pAb immunoassay was 4.2 µg/dL ($p < 0.01$) The AUC for the LC/MS ROC was 0.995 (Figure 3). Using a peak serum cortisol cutoff of 14 µg/dL when using LC/MS provided a sensitivity of 99% (95% CI: 96-100%) and specificity of 88% (95% CI: 79-97%). A simple kappa agreement between the cutoff for the pAb immunoassay and the LC/MS assay was calculated to be 0.888 (95% CI: 0.800.97).

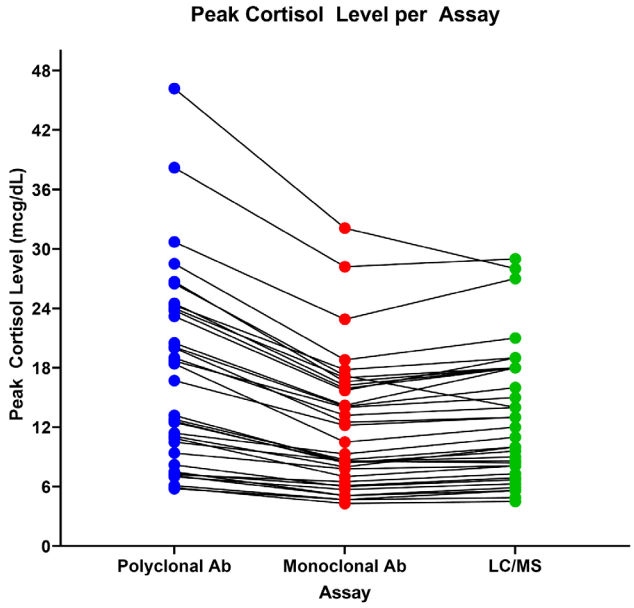

Figure 1. Peak cortisol level (mcg/dL) using polyclonal antibody immunoassay, monoclonal antibody immunoassay, and LC/MS in 36 children undergoing 1 mcg Cosyntropin stimulation test

LC/MS: liquid chromatography mass spectrometry

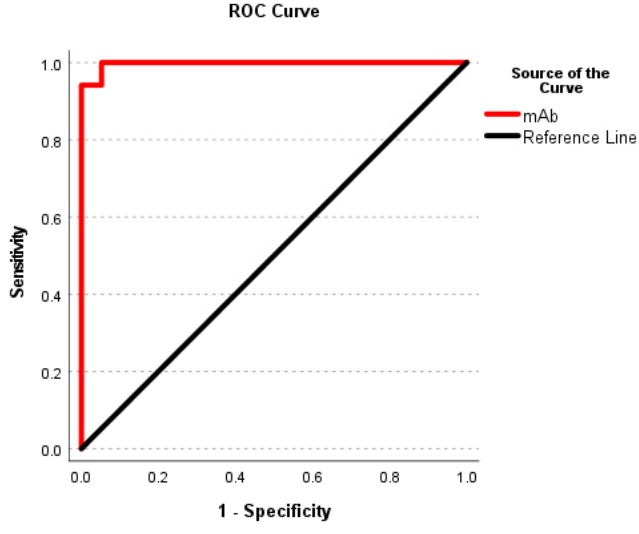

Figure 2. ROC curve for the diagnosis of adrenal insufficiency based on the peak cortisol level during a 1 mcg Cosyntropin stimulation test measured by mAb

mAb: monoclonal antibody immunoassay, ROC: receiver operator characteristic

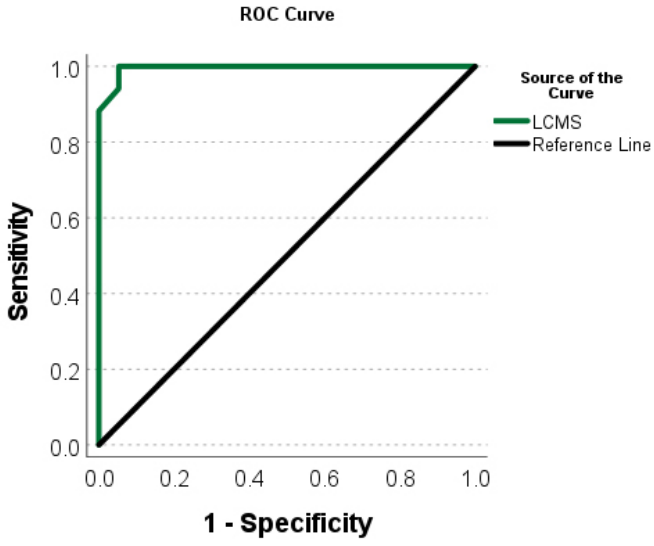

Figure 3. Receiver operating characteristic curve for the diagnosis of adrenal insufficiency based on the peak cortisol level during a 1 mcg Cosyntropin stimulation test measured by LC/MS

LC/MS: liquid chromatography mass spectrometry, ROC: receiver operator characteristic

Discussion

This study found that serum cortisol levels in children using mAb immunoassay and LC/MS were statistically and clinically significantly lower than the traditional pAb immunoassay, which in a clinical setting can potentially translate in an overdiagnosis of AI and increase morbidity for the patient due to unnecessary use of steroids. Therefore, our data supports the need to redefine the biochemical peak serum cortisol cutoffs to diagnose AI during Cosyntropin stimulation tests in children when newer and highly specific cortisol assays are used. We propose a new peak serum cortisol cutoff value of 12.5 µg/dL and 14 µg/dL when using mAb immunoassay or LC/MS, respectively.

This is the first study to do a head to head comparison between the different cortisol assays in a pediatric population undergoing low dose Cosyntropin stimulation test for the biochemical diagnosis of AI. Our monoclonal and LC/MS assay cutoffs demonstrated a high sensitivity and specificity to ensure that the diagnosis of AI is not missed. We demonstrated a strong kappa correlation coefficient between the traditional peak cortisol level cutoff of 18 µg/dL for the diagnosis of AI and the cutoffs proposed in the study (12.5 µg/dL for mAb immunoassay and 14 µg/dL for LC/MS), which to our knowledge have not been calculated in previous studies. These valuable information strengthens the rationale to redefine the cutoff for the diagnosis of AI when using mAb immunoassay and LC/MS.

This study is unique for being performed entirely at a pediatric infusion center, under the same protocol and procedures. Keeping these variables constant adds rigor and reproducibility to the study design and demonstrated that the differences in cortisol levels are assay-specific, leading to cortisol cutoff values that are reliable and applicable in a pediatric population. Moreover, all samples were analyzed using the pAb immunoassay, mAb immunoassay, and LC/MS, which is considered the reference standard when using 1 mcg Cosyntropin stimulation test in pediatric population (14).

Study Limitations

One possible limitation of this study was the use of 1 mcg Cosyntropin stimulation test and the potential variability in results compared to the 250 mcg Cosyntropin stimulation test. Nevertheless, recent studies determined that both 250 mcg and 1 mcg stimulation tests have similar diagnostic accuracy for diagnosing AI, which is also supported by the similar results obtained in our study and results reported from earlier studies (15,16).

Another limitation of this study was that the use of de-identified patient samples did not allow for individual patient or demographic analysis. However, as previously mentioned, the biochemical diagnosis of AI is based on the clinically widely used criteria of peak cortisol levels lower than lower than 18 µg/dL (500 nmol/L) using a traditional pAb immunoassay.

Conclusion

Future research is required to validate these proposed cutoff points using biochemical and clinical information. However, our results agree with previous studies demonstrating that newer and more specific mAb immunoassay yields lower serum cortisol values, potentially leading to the overdiagnosis of AI and unnecessary steroid use. Based on our results and those of previous studies, we recommend a new cutoff value of 12.5 µg/dL when using mAb immunoassay and 14 µg/dL when using LC/MS.

Ethics

Ethics Committee Approval: The study was approved by the Washington University in St. Louis of Institutional Review Board (IRB ID: 202012130, date: 08.09.2022).

Informed Consent: Study was granted a waiver of written informed consent and data collection approved by the IRB/Human Research Protection Office at Washington University in St. Louis (IRB ID #202012130).

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Samuel Cortez, Ana Maria Arbeláez, Kyle McNerney, Design: Samuel Cortez, Ana Maria Arbeláez, Kyle McNerney, Data Collection or Processing: Samuel Cortez, Ana Maria Arbeláez, Michael Wallendorf, Kyle McNerney, Analysis or Interpretation: Samuel Cortez, Ana Maria Arbeláez, Michael Wallendorf, Kyle McNerney, Literature Search: Samuel Cortez, Ana Maria Arbeláez, Kyle McNerney, Writing: Samuel Cortez, Ana Maria Arbeláez, Michael Wallendorf, Kyle McNerney.

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Continuous Glucose Monitoring in Children and Adolescents with Congenital Adrenal Hyperplasia

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What is already known on this topic?

Patients with congenital adrenal hyperplasia (CAH) receive lifelong therapy with steroids. It is known that hypoglycaemia can occur in infancy and early childhood, even at night. This is a life-threatening complication. In adolescence, visceral obesity, hypertension, hyperinsulinism and insulin resistance may occur in these patients. Insulin resistance is a known risk factor for cardiovascular diseases. To date, systematic studies of glucose profiles using continuous glucose monitoring (CGM) technology in patients with CAH are lacking.

What this study adds?

Until now, there has been no systematic study of glucose profiles by CGM in patients with CAH. In this study, we examined children and adolescents with different therapy regimens using a last-generation CGM device in a home-based, blinded setting. We found morning fasting hyperglycaemia, elevated calculated glycosylated haemoglobin and overall elevated average glucose values. In particular, adolescents with reverse circadian therapy showed significantly higher values at night. These results suggest that routine and easily implementable monitoring of even subtle changes in glucose metabolism in patients with CAH should be considered. Thus, it may be possible to detect metabolic complications of the disease and also of the therapy at a very early stage.

Abstract

Objective: Patients with congenital adrenal hyperplasia (CAH) require lifelong therapy with glucocorticoids to suppress androgen excess and substitute for deficient cortisol. An important aspect of care is the prevention of metabolic sequelae. In infants, potentially lethal nocturnal hypoglycaemia has been described. In adolescence, visceral obesity, hypertension, hyperinsulinism and insulin resistance are reported. To date, systematic studies of glucose profiles in this age group with CAH are lacking.

Methods: This was a monocentric, prospective, observational study to determine the glucose profiles under different treatment regimens in a cohort of young patients with CAH. The continuous glucose monitoring device used was the latest generation FreeStyle Libre 3® sensor in blinded mode. Therapeutic and auxological data were obtained.

Results: The cohort consisted of 10 children/adolescents with a mean age of 11 years. Three patients exhibited morning fasting hyperglycaemia. Overall, 6 out of 10 patients had unacceptably few total values in the desired range of 70-120 mg/dL. Tissue glucose values above 140-180 mg/dL were found in 5 of 10 patients. The mean value for glycosylated haemoglobin for the cohort was of 5.8%. All pubertal adolescents with reverse circadian regimens had significantly higher glucose levels at night. Two adolescents showed asymptomatic nocturnal hypoglycaemia.

Conclusion: Most of the patients exhibited abnormalities in glucose metabolism. Two-thirds had elevated total 24h glucose values outside the age-appropriate reference values. Thus, this aspect may need to be addressed early in life by adjusting the doses, treatment regimen or dietary measures. Consequently, reverse circadian therapy regimens should be critically indicated and closely monitored due to the potential metabolic risk.

Keywords: Congenital adrenal hyperplasia, continuous glucose monitoring, hypoglycaemia, hydrocortisone

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Introduction

Patients with classical congenital adrenal hyperplasia (CAH) have a genetically impaired steroid biosynthesis, which in most cases is due to a reduction or lack of function of the enzyme 21-hydroxylase. Impaired steroid synthesis affects glucocorticoids and, in some cases, mineralocorticoids (1,2,3). A distinction is made between two forms, the simple virilizing (SV) and the salt wasting (SW) form (4). If left untreated, CAH can lead to life-threatening SW crises in both sexes and virilization in girls. Thus, the rationale for lifelong treatment with steroids is glucocorticoid substitution and androgen suppression. In case of SW, additional substitution with mineralocorticoids is indicated (1,2,3). When caring for patients with CAH, special attention should be paid to the possible long-term complications, such as obesity, accelerated pubertal development, impaired neurological development, cardiovascular diseases and reduced final height (5).

Continuous glucose monitoring (CGM) is an established method for measuring glucose in the interstitial fluid in children and adults with type 1 diabetes (6,7,8). The advantages of this method are evident, especially in paediatrics. After application of the CGM device, glucose measurement in the interstitial fluid can be performed continuously for up to 14 days without further pricks in the skin, in contrast to the classical self-monitoring of blood glucose that requires multiple finger-sticks per day. There is a good correlation between blood glucose and tissue glucose (9). The last generation sensor from Abbott Diabetes Care (FreeStyle Libre 3®) used in this study showed a very good overall mean absolute relative difference of 7.9% in patients with type 1 diabetes mellitus (10). Very few studies are available on the use of CGM systems in non-diabetic patients (11,12,13,14). However, Shah et al. (15) recently presented a study on normal values of CGM profiles in children and adults. This important work can be used as a basis for evaluating glucose metabolism in hormone-related diseases. However, there are currently no prospective studies on glucose profiles of children and adolescents with CAH. Xiang et al. (16) reported an adult patient with CAH where cortisol dosage adjustment was facilitated by the use of a flash glucose monitoring system.

It is known that hypoglycaemia can occur in patients with CAH, especially under stress in infancy and early childhood (17,18,19,20,21). Episodes of nocturnal asymptomatic hypoglycaemia have also been reported under normal conditions (22). There are numerous reports of lethal outcomes of hypoglycaemic seizures and coma (23). A lack of counter-regulation by means of epinephrines from the adrenal medulla is also likely to be the cause of the hypoglycaemia (24). Standardized recommendations for glucose measurement do not exist for patients with CAH.

In CAH, in adolescence and adulthood, the focus changes to possible metabolic risk factors. Thus, in CAH there is an increased risk for visceral obesity, dyslipidemia, hypertension, venous thromboembolism, hyperinsulinism and insulin resistance. This in turn results in an increased risk for impaired fasting glucose (IFT), impaired glucose tolerance and thus type 2 diabetes (25,26,27). The use of CGM is well established in obesity research and adds to the methodological repertoire. While fasting glucose measurements in the morning, the measurement of glycosylated haemoglobin (HbA1c) and the oral glucose tolerance test provide important clinical data, the use of CGM can provide continuous data from patients' daily lives. Data in children and adolescents is very limited (28,29). Previous studies have specifically demonstrated insulin resistance and fasting hyperglycemia in children and adults with CAH (30,31). Another study in children showed an increased homeostasis model assessment for insulin resistance in children with CAH (32). However, altered parameters for insulin secretion and sensitivity could not be associated with an increased risk of type 2 diabetes (33). In this context, the known unfavorable influence of steroids on glucose metabolism should be noted.

The purpose of the present study was to examine CGM profiles of children and adolescents with CAH under different treatment regimens to investigate the following aspects: evaluating the time in range (TIR) according to time of day and night; occurrence of (asymptomatic) hypoglycaemia; occurrence of hyperglycaemia; or evidence of IFT depended on the treatment regimen.

Methods

Patients

This was a monocentric, prospective, observational study from December 2021 to December 2022 to determine the glucose profiles of patients with CAH undergoing different treatment regimens. All patients had genetically confirmed classical CAH (with and without SW). The exclusion criteria were the use of medication with an influence on glucose metabolism other than steroids and a special nutritional therapy or diet. The age range for the inclusion criteria was 4 to 18 years. As the sensor is approved for use from the age of 4 years, it was not possible to include younger children. Both patients and parents gave written informed consent/assent to participate in the study. All patients were seen at the Division for Paediatric Endocrinology at Dr. von

Hauner Children's Hospital based at the University Hospital Munich (Ludwig Maximilian University). The same team of doctors treated all patients. All study participants received hydrocortisone three times a day at the following times: in the morning between 7 and 9 am; at noon between 1 and 3 pm; in the evening between 8 and 10 pm. The intake times usually coincided with the intake of the three main meals. There was no documentation of physical activity, eating habits or meals.

Investigations

After medical consultation, the sensor was applied to the dorsal upper arm by a study doctor, as instructed by the manufacturer. Only the latest generation FreeStyle Libre 3® sensor was used (Abbott Diabetes Care Ltd., Range Road, Wittney, Oxon, OX29 OYL, United Kingdom). The patient and parents received no feedback from the sensor (blinded). There were no adverse events. All sensor data were complete according to the study protocol. The clinical characteristics were obtained through a review of patients' medical records with the following information extracted: age; sex; CAH type; weight; height; body mass index (BMI); BMI percentile; weight status category; height standard deviation score (HSDS); type of steroid replacement therapy; treatment pattern; waist circumference; pubertal stage; and other medications. The references of BMI-SDS were obtained from Kromeyer-Hauschild et al. (34). The references of HSDS were obtained from Prader et al. (35). The reference ranges for weight status category by percentile range were taken from Barlow (36).

This research related to human participants complied with all the relevant national regulations, institutional policies and was performed in accordance with the tenets of the Helsinki Declaration and was approved by the authors' Institutional Ethical Review Board and with the permission of the patients and their legal guardians. The study was approved in advance by the "Ethikkommission der Medizinischen Fakultaet" at the Ludwig Maximilian University in Munich (approval number: 21-0658, date: 10.09.2021).

Data Processing and Presentation

After completion of the observation phase, the tissue glucose values were extracted using the procedure specified by the manufacturer. This generates a file containing the following data over the entire observation period: continuous 5-minutes documentation of glucose values in mg/dL (blood glucose conversion factor: 1 mg/dL = 0.0555 mmol/L and 1 mmol/L = 18.018 mg/dL) and Glucose Management Indicator in %, that is the calculated value for HbA1c. Tissue glucose values were recorded as mean values with standard deviation over the entire observation period. The sum (n) of the individual measurements was calculated. Measurements were subdivided into day values (8 am to 8 pm) and night values (8 pm to 8 am). In addition, the observation period was divided into 6-hour segments to investigate the influence of different doses of hydrocortisone on tissue glucose values (6 am to 12 am; 12 am to 6 pm; 6 pm to 12 pm; 12 pm and 6 am).

The time for specific ranges of tissue glucose proportionally in % were analyzed, in terms of total, day and night values. Deviation from the reference values, as defined and published by Shah et al. (15) was investigated.

Furthermore, we were able to perform an averaged fasting glucose analysis. Here, the mean value of the 6 am glucose was determined over all examination days. The reference values (normal < 100 mg/dL and elevated > 100 mg/dL) are taken from Speiser et al. (37). Tissue glucose values below 60 mg/dL were considered hypoglycaemia.

Statistical Analysis

Due to the normal distribution of the single values for tissue glucose, the unpaired t-test was calculated to determine statistical significance. Significance was assumed when the p value was below 0.05. Statistical analysis was performed using Statistical Package for the Social Sciences software for Windows version 15.0 (IBM Inc., Armonk, NY, USA).

Results

Clinical-therapeutic Findings

The clinical characteristics of the patients are shown in Table 1. A total of 10 patients (six boys) were included with a median age was 11 years (range 6-15 years). Five patients were still prepubertal. One patient had a SV form of CAH. The median dose of hydrocortisone was 14.16 (7.05-20.98) mg per square metres of body surface per day (mg/m^2 BSA/day). Except for one patient (vitamin D), there was no comedication. The median dose for fludrocortisone was 0.05 mg per day absolute (range 0-0.125 mg/day). Four patients were obese, two were overweight and the remaining four were normal weight according to Barlow (36). The mean absolute values for BMI were 23.32 kg/m^2 (range 16.2-33.8 kg/m^2). As a further marker for the evaluation of weight status, we determined the waist circumferences. Based on the study by Maffeis et al. (38), two patients had normal waist circumferences, one patient had waist circumferences indicating overweight and five patients had waist circumferences indicating obesity. In two patients, the waist circumference was not recorded. The patients

were all treated with hydrocortisone as a glucocorticoid. The distribution of treatment regimens was as follows. No patient with was receiving a circadian regimen, six patients were on the reverse circadian regimen, one patient was receiving equal doses throughout the day, two patients had higher morning and evening doses (which were equal) compared to the afternoon dose, while one patient used another regimen (the lowest dose in the morning and higher and equal doses at noon and in the evening). Severe deviations of the metabolic parameters before and after the measurements shown here or adrenal crises were not observed (data not shown).

Average Fasting Hyperglycaemia and HbA1c

Table 2 shows the mean, total, day and night and every 6-hour period glucose values. The sum (n) of the individual measurements is shown in brackets. Three out of 10 patients showed morning fasting hyperglycaemia, which was defined as a fasting value >100 mg/dL (Table 3). The automatic algorithm of the FreeStyle Libre 3® CGM device allows the specification of a calculated value for HbA1c. The calculated mean value for HbA1c was 5.8% (range 5.7-6.0%).

Tissue Glucose Values by Time of Day

The CGM was conducted without interruptions for a mean of 205 (range 162-335) hours. The mean values including standard deviation for tissue glucose are shown in Table 2. The values are given as mean total values and mean values for the day and for the night. Remarkably, in four patients the mean values from 8 pm in the evening to 8 am in the morning were significantly higher ($p = 0.0001$) than from 8 am in the morning to 8 pm in the evening. All four patients were pubertal and all received reverse circadian glucocorticoid therapy. Two were obese and two were normal weight. In comparison, of the five prepubertal children, only

Table 1. Clinical and auxological characteristics of patients with CAH

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Age (years)		6	7	8	8	11		11	13	15	15	15
Sex (male/female)		M	M	M	F	M		F	M	F	F	M
CAH type (SW/SV)		SW	SW	SW	SW	SW		SW	SW	SW	SW	SV
Weight (kg)		19.8	20.5	32.5	36.0	58.0		39.8	51.8	82.0	79.5	77.0
Weight (Z-score)		-1.14	-0.77	+0.46	+1.4	+1.58		+0.03	-0.19	+2.16	+2.0	+1.29
Height (cm)		112.9	112.4	125.3	133.5	145.0		142.0	161.0	155.8	155.4	170.1
Height (SDS)		-1.6	-1.6	-1.5	+1.0	-0.5		-0.7	-0.2	-1.3	-1.4	0.0
BMI (percentile)		48th	66th	94th	94th	99th		75th	61st	>99th	>99th	96th
Weight status category (N/OW/OB)		N	N	OW	OW	OB		N	N	OB	OB	OB
BMI (kg/m^2)	Prepubertal	15.5	16.2	20.7	20.2	27.59	Pubertal	19.7	20.0	33.8	32.9	26.6
Waist circumference (cm)		54.0	52.5	69.0	Unknown	Unknown		70.0	76.0	115.0	97.0	84.5
Waist circumference (Z-score)		No ref.	No ref.	No ref.	No ref.	No ref.		+0.86	+0.72	+2.67	+2.23	+1.29
Pubertal state (Tanner)		Prepub.	Prepub.	Prepub.	Prepub.	Prepub.		P2, B2	P3, G3	Adult	Adult	Adult
HC regimen (distribution: morning-at noon-evening)		2.5-2.5-5.0	5.0-2.5-5.0	2.5-2.5-2.5	3.0-5.0-5.0	5.0-5.0-7.5		5.0-5.0-10.0	10.0-5.0-12.5	7.5-5.0-7.5	10.0-7.5-15.0	12.5-10.0-17.5
HC dose (mg/m^2 BSA/day)		12.69	15.63	7.05	11.26	11.45		15.96	18.07	10.62	17.55	20.98
FC dose (mg/day)		0.025	0.05	0.05	0.025	0.075		0.05	0.05	0.125	0.075	0
Other medication		None	None	None	None	None		None	None	None	None	Vitamin D
RR		Normal	Normal	Normal	Normal	Normal		Normal	Normal	Normal	Normal	Normal
Clinical signs of hyperinsulinism		None	None	None	None	None		None	None	None	None	None

BSA: body surface area, CAH: congenital adrenal hyperplasia, FC: fludrocortisone, HC: hydrocortisone, N: normal weight status category, OW: overweight status category, OB: obese weight status category, SV: simple virilizing, SW: salt wasting, M: male, F: female

Table 2. Tissue glucose values depending on the time of day and total wearing time of the CGM device in patients with CAH

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Wearing time CGM (h)		203	162	169	173	335		279	195	177	190	163
Glucose mean ± SD total		103 ± 18 (2423)	103 ± 16 (1928)	112 ± 13 (2026)	99 ± 13 (2068)	105 ± 16 (4010)		102 ± 16 (3558)	113 ± 15 (2350)	103 ± 15 (2118)	98 ± 14 (2273)	105 ± 17 (1949)
Glucose mean ± SD 8 am to 8 pm		106 ± 17 (1257)	109 ± 17 (958)	115 ± 12 (1019)	101 ± 15 (1064)	106 ± 18 (1996)		99 ± 15 (1782)	109 ± 14 (1184)	109 ± 17 (1092)	96 ± 13 (1121)	102 ± 19 (941)
Glucose mean ± SD 8 pm to 8 am	Prepubertal	100 ± 19 (1166)	97 ± 12 (970)	110 ± 14 (1007)	96 ± 11 (1004)	104 ± 13 (2015)	Pubertal	106 ± 15 (1776)	116 ± 16 (1166)	104 ± 14 (1026)	100 ± 13 (1152)	108 ± 16 (1008)
Glucose mean ± SD 6 am to 12 am		101 ± 21 (584)	105 ± 16 (434)	112 ± 13 (503)	95 ± 13 (504)	94 ± 12 (1008)		96 ± 12 (864)	108 ± 12 (575)	98 ± 13 (497)	92 ± 11 (545)	96 ± 10 (485)
Glucose mean ± SD 12 am to 6 pm		106 ± 17 (647)	109 ± 17 (500)	117 ± 14 (504)	102 ± 16 (560)	110 ± 17 (1003)		100 ± 17 (894)	111 ± 14 (585)	106 ± 19 (564)	97 ± 16 (576)	105 ± 21 (456)
Glucose mean ± SD 6 pm to 12 pm		109 ± 18 (616)	105 ± 15 (503)	117 ± 12 (504)	101 ± 13 (502)	115 ± 14 (991)		104 ± 16 (936)	115 ± 18 (614)	104 ± 15 (546)	100 ± 14 (576)	109 ± 18 (504)
Glucose mean ± SD 0 am to 6 am		95 ± 12 (576)	94 ± 9 (491)	103 ± 8 (504)	95 ± 9 (502)	102 ± 10 (1008)		108 ± 14 (864)	116 ± 16 (576)	106 ± 13 (504)	102 ± 11 (576)	109 ± 14 (504)

Notice: All values for tissue glucose are given in mg/dL ± SD. The number of measurements (n) is given in brackets.
am: ante meridiem, CGM: continuous glucose measurement, h: hours, pm: post meridiem, SD: standard deviation, CAH: congenital adrenal hyperplasia, CGM: continuous glucose monitoring

Table 3. Average fasting glucose at 6 am and calculated HbA1c in patients with CAH

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5		Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Mean TG	Prepubertal	82	90	101*	86	91	Pubertal	97	110*	90	94	104*
SD		7	10	3	6	9		12	11	11	8	6
n		8	6	7	7	14		13	8	7	8	7
GMI		5.8	5.8	6.0	5.7	5.8		5.8	6.0	5.8	5.7	5.8

Notice: Glucose values expressed in mg/dL, *Elevated fasting glucose value as defined in the method section.
HbA1c: glycosylated haemoglobin, GMI: glucose management indicator (= calculated HbA1c), n: quantity, SD: standard deviation. TG: tissue glucose, CAH: congenital adrenal hyperplasia

two were treated with reverse circadian regimen. These two patients showed no nocturnal abnormalities. As our patients usually take their medication at the same time as their meal, a direct influence of food or medication on tissue glucose levels cannot be differentiated. When the four pubertal patients were examined at 6-hour intervals, the values in the first segment from 6 am to 12 am were lower in all of them than in the evening between 6 pm and 12 pm ($p = 0.0001$) and in the night from 12 pm to 6 am ($p = 0.0001$). In the latter period, the effect of food can no longer be expected.

Comparison Between CAH and Control Population

Table 4 shows a comparison of tissue glucose values (percentage of time within a particular range) of patients with CAH with a healthy reference cohort (15), depending on time of day. No patients showed hypoglycaemia during the day or overall. Two patients (patient 9 and 10) showed nocturnal hypoglycaemia, but no clinical symptoms were reported by the patients or parents. It is worth mentioning that both patients were obese and received a reverse circadian therapy regimen. Overall, 6 out of 10 patients had low TIR (70-120 mg/dL) values. This finding was detected during the day in 8 of 10 patients and at night in 6 of 10 patients. Tissue glucose values above 140 mg/dL, above 160 mg/dL and above 180 mg/dL were found in 5 of 10 patients studied overall and during the day, and in 4 of 10 patients at night.

Table 4. Comparison of tissue glucose values (percentage of time within a particular range) of patients with CAH with a previously reported healthy reference cohort by time of day

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
>180 mg/dL total	0 am-12 pm	0	0	0.1	0	0.1	0.1	0	0	0	0.2
>160 mg/dL total		0.5	0.1	0.7	0	0.2	0.1	0.5	0.4	0	0.4
>140 mg/dL total		3.2	2.4	3.3	1.4	2.0	1.9	4.7	2.5	0.6	2.7
70-140 mg/dL total		95.8	97.1	96.9	98.2	98.0	97.2	95.0	97.1	98.5	96.5
70-120 mg/dL total		81.6	84.6	77.2	93.1	84.6	88.3	71.1	87.4	93.6	81.6
<70 mg/dL total		1.0	0.5	0	0.4	0.2	0.9	0.3	0.4	0.8	0.9
<60 mg/dL total		0.2	0.2	0	0	0	0.3	0.2	0	0.2	0.5
<54 mg/dL total		0	0	0	0	0	0.1	0.1	0	0.2	0.3
>180 mg/dL day	8 am-8 pm	0.1	0	0.1	0	0.2	0.1	0	0	0	0.3
>160 mg/dL day		0.6	0.1	0.9	0	0.2	0.2	0.4	0.3	0	0.6
>140 mg/dL day		4.2	3.2	4.3	1.9	2.6	1.6	4.3	2.7	0.8	3.0
70-140 mg/dL day		94.7	96.7	95.9	98.0	97.2	97.4	95.3	96.8	98.6	96.8
70-120 mg/dL day		76.8	80.0	70.8	91.3	80.2	89.4	74.1	86.4	92.5	82.7
<70 mg/dL day		1.0	0.1	0	0.2	0.2	1.0	0.4	0.6	0.6	0.2
<60 mg/dL day		0.3	0.1	0	0.1	0	0.3	0.2	0.1	0	0.1
<54 mg/dL day		0	0	0	0	0	0.1	0.1	0.1	0	0.1
>180 mg/dL night	8 pm-8 am	0	0	0	0	0	0	0	0	0	0
>160 mg/dL night		0	0	0	0	0	0	0.5	0.6	0	0
>140 mg/dL night		0	0	0	0	0	2.5	5.4	1.8	0	1.8
70-140 mg/dL night		99.1	100	100	98.8	100	97.0	94.6	98.2	98.4	95.4
70-120 mg/dL night		96.7	98.4	96.6	98.8	98.5	84.8	58.5	90.7	97.0	78.6
<70 mg/dL night		0.9	1.6	0	1.2	0	0.5	0	0	1.6	2.8
<60 mg/dL night		0	0.4	0	0	0	0.2	0	0	0.7	1.4
<54 mg/dL night		0	0	0	0	0	0	0	0	0.7	0.8

According to the time of day (day 8 am-8 pm, night 8 pm-8 am or total 0 am-12 pm), ranges of tissue glucose values were given in the first column. All tissue glucose values (indicated as a percentage of the total time within the specific range) that lie within the age-appropriate norm values for the specific range as published by Shah et al. (15) are highlighted in gray. All tissue glucose values outside the above-mentioned norm values are highlighted in light gray.

pm: post meridiem, am: ante meridiem, CAH: congenital adrenal hyperplasia

Discussion

In this study, we were able to use continuous tissue glucose measurement for the first time to systematically evaluate glucose profiles in patients with CAH. The average observation time of 205 hours was fairly long and compensated for the expected fluctuations between individual days (Table 2). A major distinctive feature of this study is the investigation of glucose levels in the context of the patients' regular everyday life. We compared the tissue glucose values overall and divided into day and night with the published reference values of Shah et al. (15). Only these published reference data were available for comparison. However, the reference data of Shah et al. (15) were collected using a different technical system, the Dexcom G6 CGM System (DexCom Inc., 6340 Sequence Drive, San Diego, CA 92121, USA). This may potentially affect the comparability of tissue glucose values. Overall, many subjects showed elevated tissue glucose levels (Table 4). In percentage terms, 60% had too many values outside the desired range of 70-120 mg/dL and even using an extended range (70-140 mg/dL) 40% of patients were still outside the range. In addition, we were also able to diagnose morning fasting hyperglycaemia in 3 out of 10 patients using CGM. Torky et al. (30) demonstrated fasting hyperglycaemia in over 93.0% of cases at one or more visits. In our cohort, fasting hyperglycaemia was present in fewer patients. As in the cohort of Torky et al. (30), one of the patients in our study with fasting hyperglycaemia was prepubertal and only 8 years old. Another study by Metwalley et al. (32) also found increased fasting glucose in children with CAH. It has been reported that the increased fasting hyperglycaemia in young patients with CAH seems to subside in young adulthood (30). However, in a large cohort of adults, impaired fasting blood glucose was found in 6-8% of adults (39). The data on evident diabetes mellitus type 2 in patients with CAH is inconclusive. While one study from Sweden reported an increased prevalence, this was not found in other cohorts (26). However, reduced insulin sensitivity has been repeatedly shown in other studies (40,41,42,43). The contribution of long-standing adrenomedullary hypofunction and intermittent iatrogenic hypercortisolism to insulin resistance has not yet been conclusively clarified (44). Hyperinsulinism is an independent risk factor for cardiovascular disease in non-diabetics (45). CGM technology may identify changes in glucose-insulin metabolism at a very early stage. Subtle abnormalities can also potentially be early warning signs. Thus, in terms of a primary preventive measure, one could probably reduce cardiovascular morbidity through regular screening and early intervention. For instance, the efficacy of insulin sensitizer (piaglitazone) has been shown in adult patients with CAH (46). However, whether insulin resistance is a condition that requires treatment in all patients will have to be investigated further. For example, compensation for insulin resistance via reduced hepatic insulin clearance has been shown in patients with CAH (47). With regard to hyperglycaemia, we identified four patients with significantly higher mean glucose values at night (8 pm to 8 am) than during the day (8 am to 8 pm). Remarkably, all four patients were of pubertal age and all received reverse circadian therapy (Tables 2, 4). It is difficult to separate the influence of hydrocortisone from the influence of food. However, it seems plausible that with the highest hydrocortisone dose in the evening, a tendency to hyperglycaemia may be increased at night. For a more precise distinction between the influence of therapy and eating behaviour, we also performed a 6-hour analysis of the mean values (Table 2). This showed highly significant lower values in the morning (6 am to 12 noon) compared to the evening (6 pm to 12 pm) and night (12 pm to 6 am) for these four patients. Overall, the values at night, which are presumably independent of food intake between 12 pm and 6 am, were still higher than in the morning and in the afternoon. The differences in absolute values must be considered relevant here, as there is no drop in value during the night. In addition, the CGM device gives a calculated value for HbA1c (Table 3), which was 5.8% on average and thus above the age-appropriate norm compared to data reported by Peplies et al. (48). The comparability of a measured and calculated HbA1c value can also be a source of inaccuracy. However, the clinically and biochemically adjusted increased dose of hydrocortisone, which is partly above the international recommendation, may have had a negative influence on glucose homeostasis.

With regard to hypoglycaemia, two patients had relatively frequent glucose levels below 54 mg/dL at night, but not during the day (Table 4). This lower threshold value of 54 mg/dL is internationally regarded as a reasonable lower limit for studies (49). However, it is also known that the sensitivity of all CGM devices decreases and false alarms can occur at low values (50). Since our patient population was blinded to the values, no capillary blood glucose measurement was performed for verification. This is always strongly recommended in an open use of CGM devices. Thus, there is a risk of inaccuracies in the hypoglycaemic range. However, both patients with evidence of hypoglycaemia at night showed increased mean values during the same period. This could be an indication of nocturnal glucose instability. In the context of the known studies on nocturnal hypoglycaemia in patients with CAH, the age of our two patients seems unusually old at 15 years and both patients were obese (17,18,19,20,21). Children younger than 4 years

were not included in the present study as the CGM device is only approved for use from this age onward.

Children and adolescents with CAH in this study had a calculated average HbA1c of 5.8% (Table 3), three children had fasting hyperglycaemia and 6 of 10 children had overall average values above the age-appropriate norm. In particular, all adolescents with reverse circadian therapy had higher tissue glucose values at night compared to during the day. In summary, reversed circadian therapy must be critically indicated in adolescents and may require special monitoring. Furthermore, when caring for patients with CAH, the focus must be on early indications of insulin resistance. However, the different values for BMI, age, gender, and pubertal stages limit comparability and statistical significance.

Study Limitations

The most relevant limitations of this study is the small number of cases, the lack of very young participants (< 4 years) and the technical measurement inaccuracy of the glucose sensors, especially in low ranges.

Conclusion

The use of CGM in patients with CAH may facilitate prompt identification of mean glucose values deviating from the reference population, thus providing an opportunity for early intervention. Based on individual glucose profiles, therapy dose, regimen and dietary habits or drug therapies should be discussed for the most severe cases. Especially for young children, the monitoring of nocturnal hypoglycaemia is potentially of great importance, for which further studies will be required.

We are convinced that this approach is in the best interest of personalised medical care for paediatric patients with CAH.

Ethics

Ethics Committee Approval: The study was approved in advance by the "Ethikkommission der Medizinischen Fakultaet" at the Ludwig Maximilian University in Munich (approval number: 21-0658, date: 10.09.2021).

Informed Consent: All patients and legal guardians provided written informed consent to participate in our study for continuous glucose measurement in congenital adrenal hyperplasia (CGM in CAH-Study).

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ilja Dubinski, Heinrich Schmidt, Design: Ilja Dubinski, Susanne Bechtold-Dalla Pozza, Hannah Franziska Nowotny, Nicole Reisch, Lea Tschaidse, Heinrich Schmidt, Data Collection or Processing: Ilja Dubinski, Susanne Bechtold-Dalla Pozza, Belana Debor, Nicole Reisch, Heinrich Schmidt, Analysis or Interpretation: Ilja Dubinski, Susanne Bechtold-Dalla Pozza, Hannah Franziska Nowotny, Nicole Reisch, Lea Tschaidse, Heinrich Schmidt, Literature Search: Ilja Dubinski, Belana Debor, Hannah Franziska Nowotny, Nicole Reisch, Lea Tschaidse, Heinrich Schmidt, Writing: Ilja Dubinski.

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Comparison of Optical Coherence Tomography Angiography Findings between Healthy Children and Children with Type 1 Diabetes Mellitus and Autoimmune Thyroiditis

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What is already known on this topic?

There are rare studies regarding the impact of type 1 diabetes mellitus (T1DM) and autoimmune thyroiditis (AT) on impairment in retinal microcirculation in children.

What this study adds?

We demonstrated that in children with coexisting AT and T1DM but without clinically detectable diabetic retinopathy (DR), there is impairment in retinal microcirculation and irregularities at the foveal avascular zone margin compared to matched children with isolated T1DM. Impairment in retinal microcirculation and signs of onset of DR proliferative retinopathy can develop independently of AT in children with T1DM and AT.

Abstract

Objective: The aim of this study was to compare the development of early diabetic retinopathy (DR) findings, a microvascular complication, between patients with isolated type 1 diabetes mellitus (T1DM) (Group 1), concurrent T1DM and autoimmune thyroiditis (AT) (Group 2), and healthy controls (Group 3), who were matched for age, sex, number, and body mass index for comparison.

Methods: This was a prospective observational study that included individuals aged 10-20 years, and patients in Groups 1 and 2 had been followed up for ≥5 years. None of them developed clinical DR during the follow-up period. Optical coherence tomography angiography (OCTA) was used to evaluate the foveal avascular zone (FAZ) and parafoveal vascular density (PVD) for the development of early DR. OCTA findings were compared between patients and healthy controls.

Results: Thirty-five individuals were included in each of the groups. The mean FAZ and PVD differed significantly between the three groups (FAZ, $p = 0.016$; PVD, $p = 0.006$). The mean FAZ was higher in Groups 1 and 2 than in Group 3 ($p = 0.013$ and $p = 0.119$, respectively). The mean PVD was lower in Groups 1 and 2 than in Group 3 ($p = 0.007$, respectively). No significant difference was found between Groups 1 and 2 in terms of the mean FAZ and PVD ($p = 0.832$ and $p = 0.653$, respectively). The mean glycated hemoglobin (HbA1c) level was significantly correlated with FAZ and PVD (FAZ: $r = 0.496$, $p < 0.001$; PVD: $r = -0.36$, $p = 0.001$).

Conclusion: In patients with T1DM who did not develop clinical DR, OCTA findings revealed an increase in FAZ, which was associated with higher HbA1c levels. The mean PVD was significantly lower in the group with coexisting AT and T1DM than in the control group. These results suggest that the coexistence of AT and T1DM can contribute to the development of microvascular complications. However, studies with larger patient series are required.

Keywords: Autoimmune thyroiditis, diabetic retinopathy, foveal avascular zone, optical coherence tomography angiography, parafoveal vessel density, type 1 diabetes mellitus

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Introduction

Owing to the prevalence, associated complications, and cost of diabetes, it is recognized as the fastest growing global health issue. In 2021, over 1.2 million children and adolescents had type 1 diabetes mellitus (T1DM) (1). In the first nationwide report on diabetes in Turkey, the prevalence of T1DM was 0.75/1000 from January 2011 to December 2013, and the mean age of patients at diagnosis was 10.6 ± 4.6 years (2). In another study of children aged < 18 years from 2013 to 2015 in Northwest Turkey, 1773 patients (588, 592, and 593 in 2013, 2014, and 2015, respectively) were diagnosed with T1DM. The crude mean incidence was 8.99/100.000 (3).

The life expectancy of patients with diabetes is reduced by 10 years (4,5,6). In the majority of developed societies, DM is identified as a leading cause of blindness, renal failure, and lower limb amputation (4,5,6). Moreover, DM-related complications progress rapidly. Every day, 225 people undergo foot amputation, 120 people undergo dialysis, and 55 people lose their vision. Moreover, diabetic retinopathy (DR) is probably the most characteristic, easily identifiable, and treatable complication of DM but remains an important cause of vision loss in developed countries (7). The prevalence of DR in patients with T1DM ranges from 10.8% to 60.0% in clinic-based populations and from 14.5% to 79.0% in population-based studies (1).

Indirect ophthalmoscopy is known as the primary approach for screening for DR, and it has high diagnostic accuracy (8). Currently, fluorescein angiography is considered the gold standard for the definitive diagnosis and grading of DR, but it needs an intravenous dye injection, which can cause nausea, vomiting, and hypersensitivity (9). Optical coherence tomography angiography (OCTA) is a quick, noninvasive procedure that may be performed without using a dye, making OCTA ideal for use in the pediatric population. Moreover, it is a potentially useful screening and follow-up tool for children with T1DM. Most OCTA studies have focused on adult patients with DR. In these studies, the mean foveal avascular zone (FAZ) in patients with DR was significantly greater than that of healthy controls. In addition, patients with DR had significantly lower parafoveal vascular density (PVD) (10,11,12). Several studies examined children with T1DM to assess FAZ and PVD but no consensus has been achieved to date (13,14,15). Thus, further studies are required to reach clear conclusions on this topic. Moreover, novel parameters such as FAZ and PVD, which can be subjected to automatic quantitative analysis using OCTA software, may help analyze early-onset T1DM without retinopathy screening and disease follow-up.

The aim of this study was to assess the retinal vessel density and FAZ area, as assessed using OCTA, in patients with isolated T1DM and those with concurrent T1DM and autoimmune thyroiditis (AT). Moreover, it aimed to compare potential pathological early changes in this population with those in healthy age-matched controls.

Methods

Patients with isolated T1DM (Group 1), patients with concurrent T1DM and AT (Group 2), and healthy volunteers (Group 3) were included. Participants in these three groups were matched for age, race, sex, number (n = 32), and body mass index. Patients with DM were consecutively enrolled from the outpatient clinic of the Clinic of Pediatric Endocrinology, University of Health Sciences Turkey, İzmir Dr. Behçet Uz Children's Training and Research Hospital, for routine follow-up. Inclusion criteria for patients with DM were aged between 10-20 years, diabetes duration of > 5 years, normotonia, body mass index less than the age- and sex-specific 95th percentile, absence of chronic diseases other than T1DM or AT, no other autoimmune diseases, and no history of smoking. None of the patients with DM took any medication other than insulin or levothyroxine on a daily basis. Moreover, patients with DM were selected based on a similar ratio for poor glycemic control, and they received the same dose of insulin. All patients with DM were undergoing conventional insulin therapy and had T1DM without complications, such as nephropathy or neuropathy. No severe hypoglycemic events that could cause coma and/or seizure were reported in patients with T1DM. Insulin pump or hybrid closed-loop therapy was not used for patients with T1DM. There were no differences between participants and all eligible diabetes hospital populations of the same age in terms of the clinical characteristics. The control group included healthy individuals matched for age, race, sex, number (n = 32), and body mass index.

The healthy controls included in the study were friends of the participants with DM and the participants treated at University of Health Sciences Turkey, İzmir Dr. Behçet Uz Children's Training and Research Hospital.

Written informed consent was obtained from the legal guardians of the participants. The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Clinical Research Ethics Committee of University of Health Sciences Turkey, İzmir Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital (protocol no: 679, date: 07.04.2022).

Blood Pressure Measurements

Blood pressure (BP) was measured in a quiet room during the regular 3-monthly follow-up visits. Notably, BP measurements were obtained using a conventional oscillatory measurement system positioned at the right-upper arm (DINAMAP; GE Healthcare, Munich, Germany). The cuff size was selected according to individual arm circumference, with the cuff bladder covering ≥40% and ≤100% of the arm circumference. Standard deviation (SD) values were calculated by adopting normal values from the study in the relevant literature (16).

Laboratory Methods

Blood samples were obtained at 8:00 a.m. after an overnight fast for ≤12 h during the patients' follow-up visit. Levels of fasting glucose, triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were measured using standard laboratory methods. Each sample was processed immediately after the patient's visit with a maximum delay of one hour. Blood samples were obtained on the day of ocular examination in the DM group for measuring pre- and post-prandial blood glucose and 1-year mean glycated hemoglobin (HbA1c) levels. The duration of T1DM and levels of HbA1c were examined. The blood samples for HbA1c measurement were analyzed using the designated method of National Glycohemoglobin Standardization Program.

OCTA and Ophthalmological Evaluation

Slit-lamp examination and indirect ophthalmoscopy were used to examine the clinical signs of DR in all patients. Patients with retinopathy findings, any ocular diseases, prior ocular surgery, myopia or hypermetropia higher than four diopters (D) were excluded from the study. Avanti RTVue XR AngioVue (Optovue, Inc., Fremont, CA, USA) was used to perform OCTA, and 3 × 3 mm pictures of the retina centered on the fovea were acquired. The AngioVue program was used to calculate the area of FAZ. In particular, the device software used in this study produced a vascular image of the retinal layer to a depth of 10 µm, from the inner limiting membrane to the outer plexiform layer. The FAZ boundary was determined using this method. For assessing PVD, the device automatically assessed the density of blood vessels in a 300-µm wide ring encircling FAZ (Figure 1).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). The mean ± SD or median (range), as appropriate, and percentage values were used to describe the obtained data. Subsequently, a stepwise multivariate linear regression model, including significant variables identified from univariate analyses, was used to determine the factors that independently explained a significant difference ($p < 0.05$) between dependent variables. Quantitative data were expressed as median (minimum-maximum). The Kruskal-Wallis and post-hoc Mann-Whitney U tests were used to compare values among the three groups. Spearman's rank correlation coefficient tests were used for the univariate analysis. A perfect correlation was considered to be indicated by a Spearman's rank correlation coefficient of 1, whereas a negative correlation was indicated by a Spearman's rank correlation coefficient of -1. A p value of < 0.05 was considered significant.

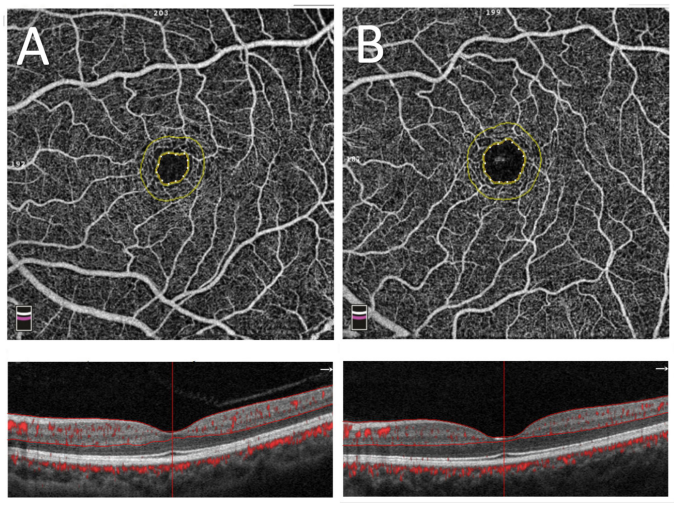

Figure 1. Examples of OCTA images with predicted foveal avascular zone area and parafoveal vessel density (A) a 14-year-old healthy boy's right eye with a foveal avascular zone area of 0.254 mm^2 and a parafoveal vessel density of 55.06, (B) a 15-year-old boy with T1DM has a foveal avascular zone area of 0.456 mm^2 and a parafoveal vessel density of 57.83

OCTA: optical coherence tomography angiography, T1DM: type 1 diabetes mellitus

Results

The characteristics of the study groups are presented in Table 1. The median DM duration was 8 (5.6-10.2) years. In the DM group, the 1-year median HbA1c level was 9.10% (6.3-15.2; reference range, 4.7-5.7%). The median daily insulin dose was 0.96 (0.80-1.10) IU/kg. No significant differences in age, sex, weight, and body mass index were found among the three groups. Groups 1 [172 (148-188) mg/dL] and 2 [168 (149-195) mg/dL] had higher serum

total cholesterol levels than Group 3 [150 (132-172) mg/dL; p = 0.011)], whereas no differences were found in terms of serum triglyceride, LDL, and HDL cholesterol levels among the three groups. Although no differences were found in the systolic BP among the study groups, Groups 1 (60.2 ± 7.69 mmHg) and 2 (65.33 ± 8.06 mmHg) had a higher diastolic BP than the control group (p = 0.020) (Table 1).

In all subjects, the best corrected visual acuity was 20/20. The mean FAZ and PVD were significantly different between the three groups (p = 0.016 and p = 0.006, respectively). The mean FAZ was higher in Groups 1 and 2 than in Group 3 (p = 0.013 and p = 0.119, respectively) (Figure 2). The mean PVD was lower in Groups 1 and 2 than in Group 3 (p = 0.007, respectively) (Figure 3). No significant difference was found between Groups 1 and 2 in terms of the mean FAZ and PVD (p = 0.832 and p = 0.653, respectively).

The correlations between risk factors and FAZ and PVD are shown separately for Groups 1, 2 and 3 in Figures 4 and 5. A significant correlation was found between mean HbA1c levels and FAZ and PVD (FAZ: r = 0.496, p < 0.001; PVD: r = -0.36, p = 0.001), whereas no correlation was found between other parameters and FAZ and PVD. Moreover, no correlation was found between thyroid-stimulating hormone (TSH) levels and FAZ and PVD. The correlations between FAZ and HbA1c and PVD and HbA1c persisted in multivariate linear regression analyses (both p < 0.001).

Discussion

Many researchers have suggested that early DR in T1DM is associated with an increased risk of poor glucose control, high HbA1c level, hypertension, and dyslipidemia (17,18,19,20). However, the effects of T1DM and AT on capillary endothelial structure and retinal microcirculation remain unclear. Based on the results of our study, we suggest that the coexistence of AT and T1DM is associated with a detrimental effects on the capillary endothelial function because of impaired glucose control in patients with T1DM. To the best of our knowledge, this is the first study to assess the potential early pathologic changes of DR in children with isolated T1DM, children with concurrent AT and T1DM, and healthy controls matched for age, race, sex, and body mass index. Notably, no confounding factors such as BP and lipid

Table 1. The characteristics of the study groups

	AT + T1DM (n = 32)	T1DM (n = 32)	Healthy children (n = 32)	p
Age (years)	15.6 (14.0-18.7)	15.4 (13.7-17.2)	15.3 (14.2-18.2)	0.851
Male (n, %)	9 (28.1%)	9 (28.1%)	9 (28.1%)	1
Weight SDS	0.15 (-1.1-1.2)	0.21 (-0.7-0.8)	0.22 (-1.0-1.2)	0.975
Height SDS	-0.47 (-1.0-0.1)[a]	-0.09 (-0.6-0.6)	0.22 (-0.2-1.1)[a]	0.004
BMI SDS	0.41 (-0.6-1.3)	0.46 (-0.3-0.8)	-0.21 (-1.1-1.0)	0.289
SBP (mmHg)	120 (115-128)	120 (111.8-127.8)	119.5 (100.8-128.8)	0.73
DBP (mmHg)	80 (74.3-87)[b]	79.5 (65.8-85)[a]	69 (66.3-75.8)[a,b]	< 0,001
T1DM duration (years)	8.1 (5.9-9.7)	8.0 (5.4-11)	-	0.941*
Insulin dose (IU/kg/day)	0.9 (0.8-1.1)	1.0 (0.9-1.2)	-	0.434*
PGC (n, %)	14 (43.8%)	17 (53.1%)	-	0.617
FBG (mg/dL)	217 (155-289)[b]	263 (177-301)[a]	89 (84-95)[a,b]	< 0.001
Mean HbA1c (%)	9.4 (6.3-15.2)[b]	9.05 (6.8-12.8)[a]	5 (4.9-5.4)[a,b]	< 0.001
Triglyceride (mg/dL)	92 (64-130)	88 (64-110)	85 (62-99)	0.315
HDL (mg/dL)	57 (49-64)	61 (53-69)	56 (47-63)	0.122
LDL (mg/dL)	89 (78-105)	89 (68-102)	84 (65-92)	0.064
TC (mg/dL)	168 (149-195)[b]	172 (148-188)[a]	150 (132-172)[a,b]	0.011
fT4 (ng/dL)	1.24 (1.13-1.35)	1.23 (1.14-1.36)	1.27 (1.17-1.38)	0.621
TSH (μIU/mL)	3.13 (2.20-4.58)[a]	2.34 (1.79-3.46)	2.14 (1.57-2.98)[a]	0.004
Anti-TG (IU/mL)	68 (31-149)[a,b]	16 (14-20)[a]	17 (15-21)[b]	< 0.001
Anti-TPO (IU/mL)	144 (23-271)[a,b]	11 (10-13)[a]	11 (10-13)[b]	< 0.001
FAZ	0.301 ± 0.05	0.303 ± 0.05	0.270 ± 0.03	0.006**
PVD	52.0 ± 3.2	52.9 ± 2.7	54.6 ± 1.2	0.016**

Variables are shown as median (interquartile range), p < 0.05. *Mann-Whitney U test. [a,b]Post-hoc analysis p < 0.0167.
AT: autoimmune thyroiditis, BMI: body mass index, SDS: standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, T1DM: type 1 diabetes mellitus, PGC: poor glucose control, FBG: fasting bood glucose, HbA1c: glycated hemoglobin, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TC: total cholesterol, ft4: free thyroxine, TSH: thyroid stimulating hormone, Anti-TG: thyroglobulin antibody, Anti-TPO: thyroid peroxidase antibody, FAZ: foveal avascular zone, PVD: parafoveal vascular density

abnormalities that may have affected capillary endothelial structure were identified in our study. This cohort was not studied in terms of retinal capillary endothelial dysfunction owing to the fact that diabetes is an endothelial disease.

Impaired capillary endothelial function can be attributed to increasing age, male sex, smoking, BP, body mass index, serum cholesterol and triglyceride levels, and the presence of DM with poor glycemic control (17,18,19,20). In patients with T1DM whose HbA1c level is >7%, microvascular complications, such as retinopathy, nephropathy, and neuropathy are significantly increased (17,18,19,20). Wysocka-Mincewicz et al. (21) compared the FAZ of 84 children with T1DM to that of 20 children with T1DM and AT, and they found no difference between the two groups in terms of FAZ. However, children with T1DM had a greater foveal thickness, global loss volume, and parafoveal thickness than children with T1DM and AT. Notably, Wysocka-Mincewicz et al. (21) did not evaluate confounding factors, such as BP and lipid abnormalities, that could affect the capillary endothelial structure. Ulaş et al. (22) reported a potential association between central serous chorioretinopathy and hypothyroidism, but the effect of the mean arterial pressure and blood glucose abnormalities on capillary endothelial dysfunction were not considered by them. Moreover, they did not consider lipid abnormalities and peroxidation caused by hypothyroidism. Wysocka-Mincewicz et al. (21) did not find a relationship between FAZ and TSH. Similarly, our results did not reveal a correlation between TSH and FAZ or PVD. Onoe et al. (14) compared the FAZ of 29 children with T1DM to that of 24 healthy children and found that FAZ was

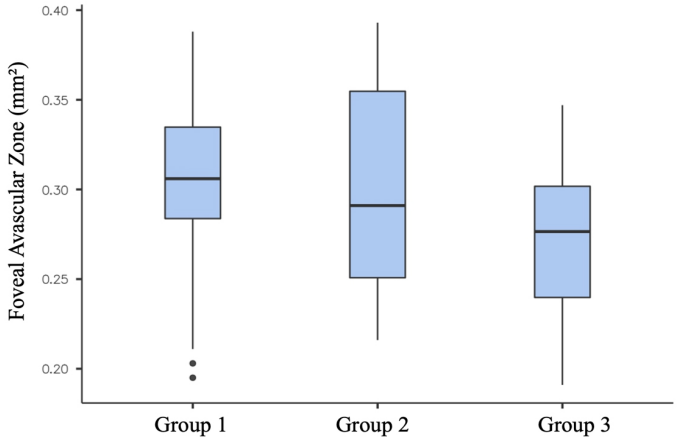

Figure 2. The average foveal avascular zone areas of the three groups

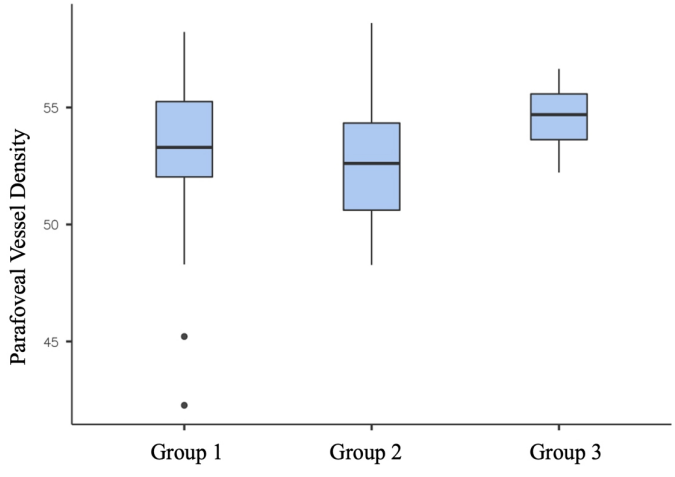

Figure 3. The average parafoveal vessel density of the three groups

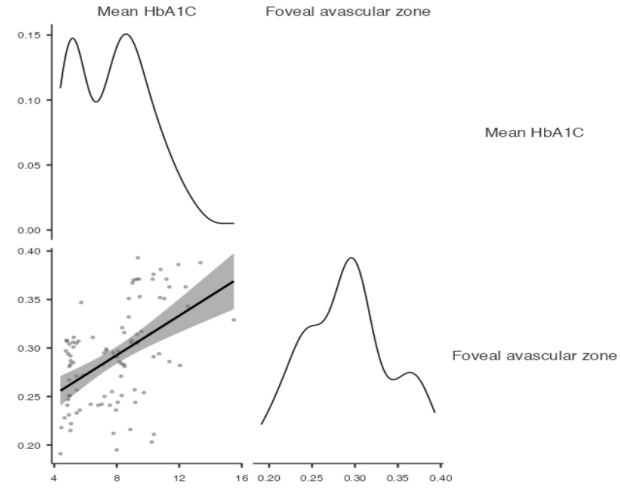

Figure 4. Correlation between mean HbA1c and FAZ

HbA1c: glycated hemoglobin, FAZ: foveal avascular zone

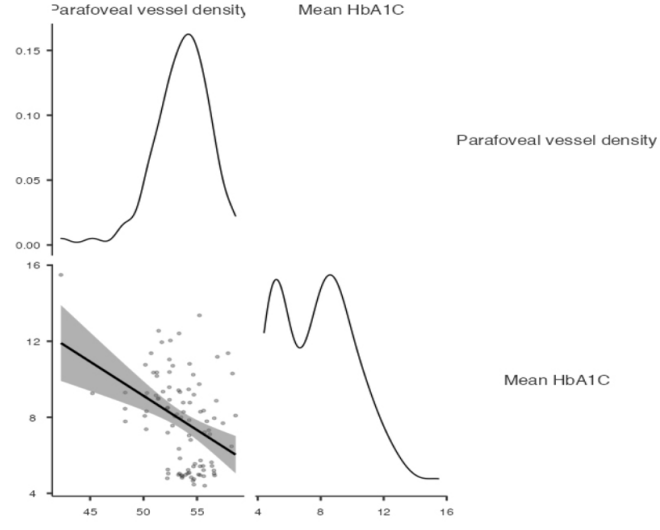

Figure 5. Correlation between mean HbA1c and PVD

HbA1c: glycated hemoglobin, PVD: parafoveal vascular density

greater in patients with T1DM, but there was no difference in terms of PVD. Gołębiewska et al. (23) compared the OCTA parameters of 94 children with T1DM to those of 36 healthy children and found no difference between the two groups. However, elevated HbA1c levels were found to be correlated with reduced parafoveal superficial vessel density and parafoveal thickness. Inanc et al. (13) compared the onset of DR between 60 children with T1DM and 57 age-matched controls, and they found a greater FAZ in children with T1DM. In the present study, the mean FAZ and PVD differed between children with DM and healthy children. Moreover, we found that DR changes started early in children with DM and were related to poor glucose control. These findings suggested that AT had no effect on the development of DR in cases of no lipid abnormalities and peroxidation. Hence, we hypothesized that early DR signs may develop independently of AT in children with T1DM and AT.

HbA1c levels in patients with DM indicate high blood glucose levels during the day. Further, high serum glucose levels are associated with microvascular complications, such as DR (17,18,19,20). Notably, even a 1% reduction in HbA1c levels in patients with DM leads to a 32% reduction in microvascular complications. The increased plasma glucose in patients with T1DM is metabolized via four main metabolic pathways, including polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms, and increased hexosamine pathway flux, as an adaptation mechanism (24,25). In patients with DM, high serum glucose levels increase glycosylation and AGE formation. Further, in the vascular endothelium of all organs, an increase in AGE levels causes binding to receptors for AGEs (RAGEs). As RAGEs are present in eye blood vessels, they bind to the capillary endothelium, which further leads to the development of retinopathy. In patients with hyperglycemia, serum glucose must be metabolized by any available pathway, and this activates the beta and gamma isoforms of PKC via the diacylglycerol (DAG) pathway. Increased PKC levels activate NADPH oxidases and increase reactive oxygen radicals. An excessive increase in reactive oxygen radicals leads to complications. Similarly, increased glucose may be metabolized via the polyol pathway by aldose reductase. The activation of the polyol pathway increases the use of hydrogen. Hydrogen is required for the production of nitric oxide (NO) from arginine. Although hydrogen is not directly involved in the synthesis of NO, it is involved in the polyol pathway as a compensatory mechanism; this decreases NO synthesis. Vasoconstriction and ischemia caused by the reduction in NO and the activation of DAG PKC pathways cause provocations in the bloodstream (24,25). In addition, increasing vascular endothelium hypoxia increases growth factors, such as vascular endothelial growth factor, which in turn leads to an increase in nuclear factor kappa B, which suppresses inflammatory genes and promotes irreversible acceleration. This further leads to vasoconstriction, oxidation, and inflammation. Given that DM is an endothelial disease, defense against oxidation is poor in patients with DM, and signs of early DR are attributed to proliferation, as observed in our cohort. We found a relationship between HbA1c levels and FAZ and PVD but we did not find a relationship between thyroid function tests and FAZ and PVD.

Study Limitations

This study has some limitations. First, this was a single-center study with a relatively small sample, and our post-hoc analysis included children with T1DM and AT. Second, the study was cross-sectional and could not describe the long-term effects of the disease and its treatment. However, we believe that the study is meaningful as it provides data on the retinal microcirculation using OCTA parameters in the analyzed subgroup. Third, all OCTA parameters examined in the study were influenced by age. Therefore, the results should be interpreted taking into account the potential effect of poor glucose control on early DR changes in children with AT and T1DM. Quantitative assessments in OCTA may be impacted by axial length. The fact that the children's axial length was not assessed is one of the study's shortcomings. However, it is feasible to assume that when groups of similar ages and without distinct refractive errors are compared, the difference between the axial lengths of the groups will be limited.

Conclusion

Our data suggest that the coexistence of AT and T1DM in children without clinically detectable DR leads to impaired retinal microcirculation and FAZ margin irregularities, similar to children with T1DM matched for age, race, sex, number, body mass index, BP, and plasma lipid levels. Impairment in retinal microcirculation and signs of DR can develop independently of AT in children with concurrent T1DM and AT. Further studies are needed to evaluate the role of OCTA in early disease detection and treatment counseling in children with both AT and T1DM. Early monitoring of microvascular risk factors may be required.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Turkey, İzmir Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital of Clinical Research Ethics Committee (protocol no: 679, date: 07.04.2022).

Informed Consent: Written informed consent was obtained from the legal guardians of the participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Hüseyin Anıl Korkmaz, Ali Devebacak, Behzat Özkan, Design: Hüseyin Anıl Korkmaz, Ali Devebacak, Behzat Özkan, Data Collection or Processing: İbrahim Mert Erbaş, Cumali Değirmenci, Nilüfer Uyar, Analysis or Interpretation: Ali Devebacak, Cumali Değirmenci, Filiz Afrashi, Literature Search: Ali Devebacak, Cumali Değirmenci, Filiz Afrashi, Writing: Hüseyin Anıl Korkmaz, Behzat Özkan.

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Pulse Wave Analysis in Obese Children with and without Metabolic Syndrome

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What is already known on this topic?

In the latest arterial hypertension guidelines published for adult patients, pulse wave analysis (PWA) is recommended because of its high predictability, easy applicability, and reproducibility when determining cardiovascular risk. Evidence about PWA in children and adolescents with metabolic syndrome (MS) is limited.

What this study adds?

This study showed that additional risk factors other than obesity, which are required for the diagnosis of MS, appear to contribute to an increase in 24-hour and daytime central systolic and diastolic blood pressure. This suggests that PWA may be helpful when determining cardiovascular risk and target organ damage in obese children with MS.

Abstract

Objective: To compare pulse wave analysis (PWA) of obese children with and without metabolic syndrome (MS) with healthy, non-obese children and to evaluate the association between PWA findings and additional risk factors present in children with MS and obesity.

Methods: From the obese patients examined between June 2019 and June 2021, 41 patients with MS, 36 obese patients without MS, and 34 healthy non-obese children of similar age and gender were evaluated retrospectively. Anthropometric measurements, biochemical evaluation, 24-hour ambulatory blood pressure (BP) measurement (ABPM), left ventricular mass index (LVMI) and PWA measurements were compared.

Results: When the three groups were compared, weight standard deviation score (SDS), height SDS and body mass index SDS were all significantly higher in the MS group ($p < 0.05$). The following measurements were significantly higher in both MS and non-MS obese patients compared to the control group: from ABPM measures, the systolic and mean arterial pressure BP SDSs load; from PWA, the night central systolic BP, 24-hour, day and night pulse pressure values and 24-hour, day and night pulse wave velocity (PWV) rates; and from cardiac evaluations, the LVMI and relative wall thickness measurements (all $p < 0.05$). Furthermore, the 24-hour and daytime central systolic (cSBP) and diastolic BP (cDBP) values were significantly different between the three groups, being the highest in the MS group ($p < 0.05$).

Conclusion: Obesity causes higher office, ambulatory and central BP, PWV and LVMI. However our results suggest that additional risk factors associated with MS do not contribute to these parameters, except for 24-hour and daytime cSBP and cDBP values.

Keywords: Children, pulse wave analysis, metabolic syndrome, obesity

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Introduction

Metabolic syndrome (MS) is a cluster of medical problems that put patients at risk for cardiovascular diseases. There are different definitions proposed by different research groups for the definition of MS in children (1). All of these definitions include high body mass index (BMI) and waist circumference measurement, high triglyceride and low high density lipoprotein (HDL) cholesterol levels, high blood pressure (BP) and high fasting blood glucose or high fasting insulin level. The International Diabetes Federation (IDF) recommends that definitions be evaluated separately according to age groups due to age-related variability in children (2). The most important reason for the increase in the frequency of MS in children is the increase in the prevalence of obesity (3). It is known that obesity carries a risk for cardiovascular diseases, and the risk is greater in the presence of MS due to additional components.

The most common target organ damage seen in both adults and children with hypertension (HT) is an increase in left ventricular mass and increased carotid intima-media thickness (4,5). The concept of arterial stiffness (AS) has also emerged in recent years as a strong independent predictor of cardiovascular events. AS is a measure of the viscoelasticity of the vessel wall and is also a strong independent indicator of cardiovascular events (6). Pulse wave analysis (PWA), which evaluates the response and wave reflections created by the pressure on the vessel wall created during the progression of the pulse wave originating from the aortic arch to the periphery, is the most commonly used method to evaluate AS (7,8). In case of increased AS, the central systolic BP (cSBP) increases, central diastolic BP (cDBP) decreases, central pulse pressure (cPP) rises, and left ventricular mass and load increase (9).

PWA can be used to investigate vascular structures by looking at parameters such as pulse wave velocity (PWV) and augmentation index (AIx) (10). PWV is the rate of passage of the pulse wave between two points in the arterial system (11). The most important factors affecting PWV are age and BP. PWV has been shown to be high in chronic diseases, such as kidney failure, obesity and diabetes mellitus (12,13). In cases with increased AS, the back reflection wave reaches the aortic root earlier than diastole since the PWV is high. Adding to the forward wave causes an increase in the amplitude of the wave and the systolic pressure. This percentage of increase is expressed as the AIx (14). High AIx values are associated with increased PWV, in other words with AS. AIx is a normalized index based on 75 beats per minute due to heart rate differences (AIx@75). In the latest arterial HT guidelines published for adult patients, PWA is recommended because of its high predictivity, easy applicability and reproducibility in determining cardiovascular risk (15).

The aim of this study was to compare the PWA of obese children with and without MS with healthy non-obese children and to evaluate the effects of additional MS-associated risk factors in addition to obesity.

Methods

Study Population

The protocol of this single-center, cross-sectional study was approved by the Ethics Committee of University Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/10-09, date: 15.10.2021). The results of ambulatory BP monitoring (ABPM) and PWA performed in obese (BMI ≥95th percentile) children with or without MS and non-obese (BMI <85th percentile) children who attended the pediatric outpatient clinic between June 2019 and June 2021 were analyzed retrospectively. Patients aged between 10 and 18 years (because it is difficult to diagnose MS at <10 years of age) and taller than 120 cm (as ABPM reference data was compiled in children with a height of 120 cm and above) were included. Patients with missing data, or had an additional chronic disease, such as cardiac disease, chronic kidney disease, hyperthyroidism or hypothyroidism, were not included in the study. The data of children whose BP was measured to be high for any reason and who were referred to us for further examination, whose office BP and ABPM were evaluated as normal, who were not hypertensive, healthy, and not obese during follow-up, were evaluated as the control group.

Anthropometric Measurement

Weight measurement was carried out by removing all outer clothing and shoes using a digital scales suitable for adults, sensitive to 100 g. Height measurements accurate to the nearest centimeter were performed using a rigid stadiometer. Height was measured while the child was standing. The measurement was performed on a hard surface, barefoot and without a hat on, with the child's back facing the measuring instrument, with the most protruding part of the head, shoulders, hips and heels in full contact with the measuring instrument, with the arms hanging down, the heels together, and the head straight. The hard and flat and moving head of the measuring instrument was partially touched to the upper part of the head, and this point was read from the scale and the height was determined. Weight and height percentiles were calculated using Turkish national reference data (16). BMI was calculated as the ratio

of body weight (kg) to height in metres squared (m^2). The standard deviation (SD) score (SDS) of BMI was calculated using the Child Metrics program (17). On the BMI reference curve, which was prepared for Turkish children and adjusted for age and gender, those with BMI values between the 85th percentile and the 95th percentile were defined as "overweight", and those above the 95th percentile were defined as "obese" (16).

Waist circumference was measured at the end of expiration from the midpoint between the lower edge of the last rib and the apex of the iliac crest while standing comfortably with the feet approximately 25-30 cm apart. Waist circumference percentiles were evaluated according to percentiles calculated for Turkish children (18). The presence of puberty was evaluated as having physical examination findings compatible with at least Tanner 2 (19,20).

The criteria recommended by the IDF were used for the diagnosis of MS (2).

Biochemical Tests

Fasting glucose, creatinine, uric acid, sodium, potassium, alanine aminotransferase (ALT), free T4, thyroid stimulating hormone (TSH), insulin levels and lipid profiles (total cholesterol, HDL cholesterol and triglycerides) were measured in blood samples taken in the morning after overnight fasting. Blood glucose levels were measured by the glucose oxidase method and serum lipid profiles were measured using routine enzymatic methods. Insulin measurements were made by the immunofluorometric method. An oral glucose tolerance test (OGTT) was performed to detect insulin resistance (IR). The OGTT used 1.75 g/kg (maximum 75 g) glucose administered orally, and blood samples were taken for glucose and insulin measurements at 0, 30, 60, 90 and 120 minutes (21). Homeostasis model assessment (HOMA) of IR was calculated using the Child Metrics program with the formula: fasting plasma glucose (mg/dL) x fasting plasma insulin (μU/mL) / 405 (17). HOMA-IR levels > 2.5 for prepubertal and > 4 for pubertal (Tanner ≥2) participants were defined as IR (22).

Office BP Measurements

Office BP was measured three times at 2-minute intervals on the right arm, after 5 minutes of rest, with aneroid devices calibrated by an experienced nurse, with cuffs appropriate for the child's age, and the last two BPs were averaged for analysis. Office systolic BP (SBP) and diastolic BP (DBP) measurements of all patients were evaluated according to the American Academy of Pediatrics 2017 (AAP-2017) HT guideline (23).

ABPM Measurements and PWA

Office and ABPM and central BP measurement of the patients were evaluated with an oscillometric PWA-ABPM device (Mobil-O-Graph; IEM, Stolberg, Germany) (24).

Daytime measurements were made at 15-minute intervals, and nighttime measurements were performed at 30-minute intervals. The results of the measurement yielded skewness (L), median (M) and coefficient of variation (S), which were converted to SDSs by the LMS method. SDSs were calculated using the Child Metrics program according to the published reference LMS tables for healthy children (17,25). The ratio of BP values above the ambulatory 95th percentile was defined as "BP load". Patients with < 10 % reduction in BP at night compared to the daytime period were defined as "dipper". In ABPM measurements, measurements above the 95th percentile were accepted as HT (23).

The variables measured during PWA included: 24-hour daytime and nighttime SBP (mmHg); 24-hour daytime and nighttime DBP (mmHg); 24-hour daytime and nighttime mean arterial pressure (mmHg) (MAP) [(MAP) = (SBP + 2 DBP) / 3]; daytime and nighttime systolic load (%); daytime and nighttime diastolic load (%); systolic and diastolic dip; 24-hour daytime and nighttime cSBP (mmHg); 24-hour daytime and nighttime cDBP (mmHg); 24-hour daytime and nighttime cPP (mmHg); [cPP = cSBP-cDBP]; 24-hour daytime and nighttime PWV (m/s); and 24-hour daytime and nighttime AIx standardized across 75 heart beats (AIx@75) (10). Central BP indicates BP at the aortic root and is usually lower than the brachial artery measurement (26).

Echocardiographic Assessment

All children included in the study were evaluated by the same pediatric cardiologist with the same echocardiography device. Left ventricular mass index (LVMI) was calculated according to the Devereux formula and indexed to height $(m)^{2.7}$. LVM (grams): 0.8 × 1.04 [(LVEDD + IVST + PWT)-(LVEDD)] + 0.6 where LVEDD is the left ventricular end-diastolic diameter, IVST is the interventricular septum thickness and PWT is the posterior wall thickness (27,28). Left ventricular hypertrophy (LVH) is defined as an LVMI that exceeds the 95th percentile for sex and age in normal children and adolescents (28). The LVH index was obtained by dividing the LVMI by the 95th percentile for that age and gender. The relative wall thickness (RWT) was calculated by the formula: RWT = 2 × PWTd/LVIDed (PWTd: posterior wall thickness diastole, LVIDd: left ventricle internal diameter diastole) (29).

Statistical Analysis

All statistical evaluations were performed using Statistical Package for the Social Sciences for Windows, version

24.0 (IBM Inc., Armonk, NY, USA). Discrete variables are expressed as counts (percentage), continuous variables with normal distribution were calculated as mean ± SD, and continuous variables with non-normal distribution as median (interquartile ranges; 25-75%). The Kolmogorov-Smirnov test was used to evaluate the distribution of continuous variables. Normally distributed variables were evaluated with the ANOVA test and *post-hoc* analyses were performed with the Tukey test in homogeneous groups and with Tamhane's T2 test in heterogeneous groups. For non-parametric distribution, variables were evaluated first with the Kruskal-Wallis test for the three groups and then with the Mann-Whitney U test to determine the group that caused the difference. Depending on the distribution type of the variable, Pearson or Spearman's analysis was performed. A $p < 0.05$ was considered statistically significant for all statistical evaluations.

Results

Of the 111 patients included in the study, 36 (32.4%) were non-MS obese and 41 (36.9%) patients had MS. There were 34 (30.6%) healthy non-obese children in the control group (Figure 1). The mean age and gender were similar between the three groups. The characteristics of the demographic and laboratory findings of the study population are given in Table 1. Height SDS was significantly higher in the MS group compared to the other two groups. Weight SDS and BMI SDS were significantly different between the three groups. Uric acid, total cholesterol, ALT, and TSH levels were similar in the MS and non-MS obese groups, but significantly lower in the control group. Triglyceride levels were significantly higher in the MS group compared to the other two groups. HOMA-IR was significantly higher in the MS group than in the non-MS obese group. All children were euthyroid.

The office BP measurement and 24-hour ABPM results were compared and are shown in Table 2. Office SBP SDS values, 24-hour daytime and nighttime SBP SDS values, 24-hour daytime and nighttime MAP SDS values, daytime and nighttime systolic load, and nighttime diastolic load were similar in the MS and the non-MS obese groups, which were higher than the control group. Office DBP SDS values and daytime DBP load were significantly higher in the MS group than the other two groups. The 24-hour daytime and nighttime DBP SDS and systolic and diastolic dip rates were similar in all groups.

When PWA data were evaluated, 24-hour and daytime cSBP values were higher in the MS group compared to the other two groups. Moreover, 24-hour and daytime cDBP values were higher in the MS group than the control group, but similar between the MS and non-MS obese patients. Nighttime cSBP values, 24-hour daytime and nighttime PP values, and 24-hour daytime and nighttime PWV values were similar in the group with MS and in the non-MS obese group and were higher than the control group (Figure 2). Both 24-hour daytime and nighttime PWV were significantly correlated with BMI SDS, for each group ($r = 0.356$, $p = 0.001$; $r = 0.37$, $p = 0.001$; and $r = 0.315$, $p = 0.005$, respectively).

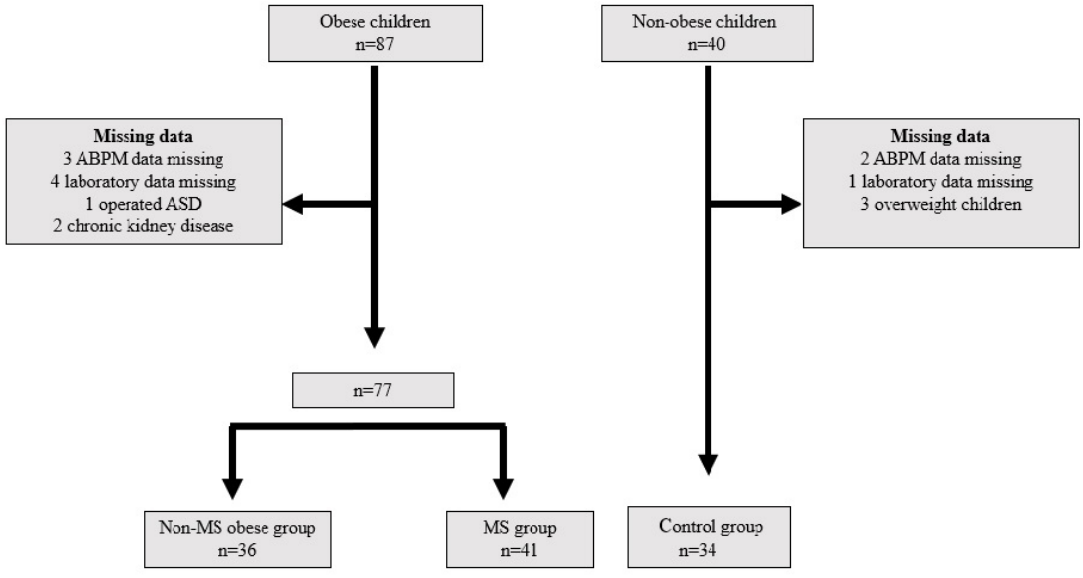

Figure 1. Study sample selection

ABPM: ambulatory blood pressure measurement, MS: metabolic syndrome

LVMI, LVMI/95P and RWT measurements were significantly higher in the MS and non-MS obese groups than the control group (Table 3). LVMI, LVMI/95P and RWT measurements tended to be higher in the MS group compared to the non-MS obese group, but the difference was not significant.

Discussion

The results of this study showed that both 24-hour and daytime cSBP and cDBP were higher in the MS group than the other two groups. In addition, LVH, as an indicator of end organ damage, was more frequent in the non-MS obese

Table 1. Comparison of demographic and laboratory findings

	Non-MS obese group	MS group	Control group	p
Age (years)	12.50 (11.00-15.00)	14.00 (12.00-15.00)	13.00 (11.00-15.25)	0.734
Male, n (%)	14 (38.9)	10 (24.4)	13 (38.2)	0.301
Weight SDS	2.76 ± 1.08	3.60 ± 1.06	-0.13 ± 1.15	**< 0.01***
Height SDS	0.31 (-0.77-1.06)	0.94 (-0.07-2.00)**	-0.13 (-0.88-0.87)	0.002
BMI SDS	2.61 (2.10-3.10)	2.88 (2.64-3.41)	-0.15 (-1.29-0.80)	**< 0.01***
Glucose (mg/dL)	88.20 ± 7.73	88.19 ± 7.90	89.46 ± 9.55	0.770
Creatinine (mg/dL)	0.60 (0.60-0.70)	0.70 (0.60-0.70)	0.60 (0.59-0.70)	0.179
Uric acid (mg/dL)	5.30 (4.50-5.80)	5.60 (5.07-6.82)	4.00 (3.50-4.67)**	< 0.01
Triglyceride (mg/dL)	103.17 ± 27.79	132.86 ± 61.00**	90.77 ± 39.93	0.005
Total cholesterol (mg/dL)	175.30 ± 31.60	171.00 ± 33.80	150.25 ± 38.20**	**0.019**
ALT (IU/L)	23.00 (17.00-34.00)	22.00 (14.50-40.50)	12.00 (10.00-15.00)**	< 0.01
Free T4 (mIU/L)	0.78 (0.71-0.89)	0.81 (0.72-0.94)	0.86 (0.78-0.91)	0.087
TSH (ng/dL)	2.40 (1.95-2.95)	2.32 (1.73-3.40)	1.80 (1.14-2.26)**	0.044
HOMA-IR	3.10 (2.40-4.40)	4.43 (3.07-6.18		**0.008***
Microalbumin/creatinine (mg/g)	5.57 (3.02-21.52)	7.00 (5.00-16.80)	8.49 (4.62-21.95)	0.423

*The results of all groups were statistically different from each other.
**The results were significantly different from the other two groups.
SDS: standard deviation score, BMI: body mass index, ALT: alanine aminotransferase, TSH: thyroid stimulating hormone, HOMA-IR: homeostasis model assessment of insulin resistance, MS: metabolic syndrome

Table 2. Comparison of office and 24-hour blood pressure data

	Non-MS obese group	MS group	Control group	p
Office systolic BP SDS	1.13 [(0.77)-(2.00)]	1.88 [(0.46)-(2.33)]	0.20 [(-0.64)-(1.08)]*	< 0.01
Office diastolic BP SDS	0.95 ± 0.86	1.15 ± 0.91*	0.57 ± 0.68	0.01
24-h systolic BP SDS	-0.02 ± 0.99	0.28 ± 1.17	-1.14 ± 0.74*	< 0.01
Daytime systolic BP SDS	-0.35 ± 1.00	-0.08 ± 1.08	-1.36 ± 0.83*	< 0.01
Nighttime systolic BP SDS	0.69 [(0.20)-(1.53)]	0.76 [(0.11)-(1.79)]	-0.34 [(-0.59)-(0.35)]*	< 0.01
24-h diastolic BP SDS	-0.51 ± 1.02	-0.32 ± 1.10	-0.82 ± 0.82	0.11
Daytime diastolic BP SDS	-0.83 ± 1.00	-0.75 ± 0.96	-1.16 ± 0.82	0.18
Nighttime diastolic BP SDS	-0.67 ± 1.08	0.65 ± 1.10	0.33 ± 1.02	0.34
24-h MAP SDS	0.78 [(0.20)-(1.34)]	1.02 [(0.43)-(1.74)]	-0.05 [(-0.45)-(0.60)]*	< 0.01
Daytime MAP SDS	0.38 ± 1.05	0.71 ± 1.19	-0.30 ± 0.71*	< 0.01
Nighttime MAP SDS	1.55 [(1.07)-(2.49)]	1.66 [(1.06)-(2.83)]	0.84 [(0.32)-(1.43)]*	< 0.01
Daytime systolic load (%)	13.00 [(6.00)-(21.25)]	19.00 [(9.50)-(34.50)]	3.50 [(0.00)-(7.25)]*	< 0.01
Nighttime systolic load (%)	23.00 [(9.75)-(44.25)]	33.00 [(8.00)-(56.50)]	1.00 [(0.00)-(12.75)]*	< 0.01
Daytime diastolic load (%)	8.50 [(4.00)-(16.50)]	14.00 [(6.50)-(22.00)]*	7.00 [(3.50)-(10.50)]	0.01
Nighttime diastolic load (%)	19.50 [(8.25)-(38.00)]	15.00 [(3.00)-(38.00)]	5.50 [(0.00)-(15.50)]*	< 0.01
Systolic dip	7.35 [(0.02)-(10.42)]	6.40 [(3.17)-(10.90)]	5.55 [(3.07)-(10.32)]	0.81
Diastolic dip	12.30 [(6.57)-(14.37)]	11.70 [(5.95)-(18.85)]	13.95 [(6.70)-(20.30)]	0.53

*The results were significantly different from the other two groups.
SDS: standard deviation score, BP: blood pressure, MAP: mean arterial pressure

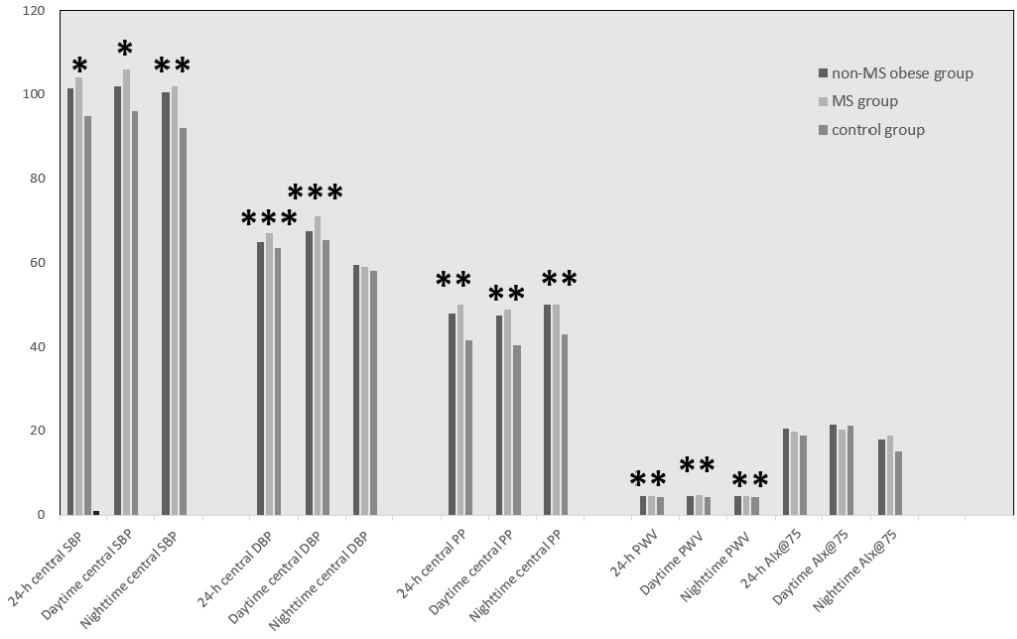

Figure 2. Comparison of PWA data

*The results of all three groups were statistically different from each other.

**Control group was significantly lower than the MS and non-obese MS groups.

***MS group was higher than the control group.

MS: metabolic syndrome, SBP: systolic blood pressure, DBP: diastolic blood pressure, PWA: pulse wave analysis, PP: pulse pressure

Table 3. Comparison of echocardiographic findings between the groups

	Non-MS obese group	MS group	Control group	p
LVMI (g/m$^{2.7}$)	35.85 (33.17-39.62)	37.85 (30.85-44.95)	31.55 (26.30-36.80)*	< 0.01
LVMI/95th percentile	0.95 (0.83-1.05)	0.97 (0.84-1.19)	0.82 (0.70-0.95)*	< 0.01
RWT	0.40 ± 0.70	0.41 ± 0.68	0.34 ± 0.11*	< 0.01

*The results were significantly different from the other two groups.
LVMI: left ventricular mass index, RWT: relative wall thickness, MS: metabolic syndrome

and MS groups compared to the control group, whereas it was similar between the two obese groups. Considering that 17% of the non-MS obese group was hypertensive and 61% of the MS group was hypertensive, we hypothesize that LVH in these patients was not affected by high BP, but by existing obesity status.

High HOMA-IR and triglyceride levels are expected in MS. However, obesity was associated with the difference identified in uric acid, total cholesterol, ALT and TSH values. Free T4 and TSH values were within normal ranges in all participants, and none of our patients had hypothyroidism or hyperthyroidism.

The Mobil-O-Graph device used to assess cSBP in children and adolescents has been shown to perform well compared to simultaneous invasive recordings (30). In recent years, it has been found that central BP measurement is superior in determining cardiovascular risk, especially in young adults (31). Studies in hypertensive adults have shown that cardiovascular mortality and hypertensive target organ damage are better correlated with cSBP and cPP than with brachial arterial BP (32,33). In children, because the brachial artery is more elastic, accumulation of pulse waves results in higher brachial BP measurements, but central BPs are within normal ranges. Therefore, central BP measurement in children and young adults may prevent the detection of more HT with peripheral ABPM measurements. A study by Totaro et al. (34) showed that adults with higher cSBP had higher cIMT, LVMI, PWV, and lower brachial artery dilatation. However, patients with higher cSBP had a significantly higher BMI (mean BMI 38.7), and 42.9% of them had type 2 diabetes mellitus. Few studies have been conducted in childhood. Litwin et al. (35) found significantly higher cIMT, LVMI, and PWV values in adolescents with both younger age and lower BMI and higher cSBP. They also showed that children with primary HT with severe ambulatory HT had

normal central BP values and determined that central BP measurements had the same or higher power as ABPM in predicting end-organ damage. In the present study, 24-hour and daytime cSBP and cDBP measurements were higher in the MS group. LVMI, a marker of target organ damage, was similar in the obese groups with and without MS. However, in both it was higher than the control group.

Since the rate of obesity, IR and cardiac disease is gradually increasing in children and adolescents, it is necessary to screen children in the risk group to identify those at risk of later and atherosclerosis and enable early intervention (36). The literature contains many conflicting reports regarding the change in PWV in children with obesity. In some studies, it has been shown that there is an increase in PWV with obesity (37,38). In contrast, some studies have shown a "paradoxical" decrease in PWV with obesity (39). The paradoxical reduction has been attributed to precocious puberty and increased body size in obese children (40). Another hypothesis is that the decrease in PWV is a short-term adaptation, does not continue in the long term, and increases in time in longitudinal studies (41). In the present study the PWV was similar in the MS and the non-MS obese groups, but in both it was higher than the control group. PWV increase was correlated with BMI SDS increase. Although HOMA-IR was significantly higher in the MS group compared to the non-MS obese group, PWV was similar between these groups. The effects of IR may not be reflected in the PWV yet because of the cross-sectional design of the study.

In a recently published study, it was shown that 24-hour cSBP and 24-hour PWV values in obese children and adolescents were higher in obese subjects, but 24-hour AIx@75 values were not different from non-obese subjects. These results of our study were similar. In obese cases, the total blood volume and thus the stroke volume increases, whereas the total peripheral resistance decreases, the heart rate remains within the normal range or slightly increases to keep the BP at normal levels. As a result of this, augmentation pressure and AIx@75 values remain low in obese cases (42). In the present study, although central and peripheral values were higher in obese subjects than in non-obese subjects, AIx@75 values were similar between the groups.

Study Limitations

We believe that our study was the first in which the PWA of children with and without MS was evaluated using the oscillometric technique. However, it has limitations such as cross-sectional and retrospective design. Considering that PWV increases with age, performing longitudinal studies allows a better evaluation. The relatively small number of patients may also have introduced some degree of error during the analysis of the study results.

Conclusion

ABPM data were similar to the non-MS obese group, with slightly higher values in the MS group. Both 24-hour and daytime central SBP and DBP measurements were higher in the MS group. Obesity was associated with higher office, ambulatory, and central BP, PWV, and LVMI, but the additional MS-associated risk factors beyond obesity did not appear to contribute, with the exception of 24-hour and daytime cSBP and cDBP, which were significantly higher in the MS obese group.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of University Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/10-09, date: 15.10.2021).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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Screening for Anxiety and Depression in Children with Congenital Adrenal Hyperplasia

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What is already known on this topic?

Congenital adrenal hyperplasia (CAH) is a chronic genetic condition that has been associated with behavioral mental health changes due to its chronic nature and exposure to elevated androgen levels.

What this study adds?

After undergoing validated mental health screening, children and adolescents with CAH may not present with an increased prevalence of anxiety and depression as prior studies may suggest.

Abstract

Objective: Congenital adrenal hyperplasia (CAH) is an inherited condition in which individuals require multiple daily doses of medication and are at risk for life-threatening adrenal crisis. The chronic nature and severity of CAH place children at risk for psychiatric morbidity. The aim was to assess the degree of anxiety and depressive symptoms in children with CAH.

Methods: A cross-sectional cohort study of children (7-17 years) with CAH and their caregivers were recruited between May and December 2021. Children with hypothyroidism (HT) and their caregivers served as unaffected controls. Validated mental health questionnaires [Children's Depression Inventory 2 Self Report-Short (CDI-2), Screen for Child Anxiety Related Disorders (SCARED), Patient Health Questionnaire modified for Adolescents (PHQ-A); self and proxy] were completed by participants at one clinic visit. Higher scores indicated greater symptoms of anxiety and depression.

Results: A total of 60 children and 56 parents participated. Among the children 34 had CAH (68% female, mean age 11.41 ± 2.5, CAH duration 8.5 ± 4.1) and 26 had HT (73% female, mean age 12.7 ± 2.9 years, HT duration 6.0 ± 4.2 years). There was no increase in anxiety and depression symptoms in children with CAH compared to controls. In sub-analyses, children with CAH and controls reported a greater number of anxiety and depression symptoms than their caregivers on the SCARED and CDI-2, respectively. There was no association between adrenal control and the degree of anxiety or depression symptoms.

Conclusion: Children with CAH do not have more symptoms of anxiety or depression compared to controls. Child and caregiver-proxy responses lack agreement, suggesting that children with CAH may continue to benefit from routine mental health evaluation, regardless of voiced caregiver concern.

Keywords: Anxiety, depression, congenital adrenal hyperplasia

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Introduction

Mental health has become a greater focus in the management of pediatric chronic illness in the last decade, especially as children with chronic conditions are living longer lives and there is an emphasis on improving quality of life (1,2). There has subsequently been a move to integrate mental health assessment and treatment into routine care. Studies thus far have suggested that children with chronic illness present with higher levels of depressive symptoms than healthy peers and that the degree of depressive symptoms can differ between different chronic illnesses (3). Similarly, anxiety is prevalent in those with chronic illness, and children with anxiety and a physical illness may present with more emotional and functional impairment than children with anxiety who do not have a physical illness (4). Research groups have explored the relationship between mental health and pediatric chronic disease in many conditions, such as juvenile idiopathic arthritis and inflammatory bowel disease (5,6). However, there has been limited research into mental health in the pediatric congenital adrenal hyperplasia (CAH) population (7,8,9).

CAH is an inherited, life-long condition that is most commonly caused by a deficiency in the 21-hydroxylase enzyme. This enzyme deficiency disrupts the steroid biosynthesis pathway by decreasing cortisol production and upregulating levels of 17-hydroxyprogesterone (17-OHP). Elevations in 17-OHP shift the pathway towards increased androgen synthesis. Classical and non-classical CAH differ in the degree of enzyme deficiency. Classical CAH presents as either salt-wasting or simple virilizing types. Salt-wasting CAH, accounting for approximately 75% of classical cases, is the most severe form of CAH as it is also due to suboptimal aldosterone production which, without treatment, leads to life-threatening hyponatremia and hyperkalemia (10). Simple virilizing CAH does not present with a significant degree of mineralocorticoid deficiency but does present with considerable elevations in androgen synthesis, leading to genital atypia in biological females. Non-classical CAH is the least severe form because of a milder enzyme deficiency and can present in childhood with premature adrenarche.

CAH often involves the administration of multiple daily medications, in some cases from birth. Since individuals with CAH have glucocorticoid deficiency and a degree of mineralocorticoid deficiency, they remain at risk for life-threatening adrenal crisis if medication is not taken as directed. CAH, more commonly classical CAH, leads to excess androgen production and, therefore, infant girls may present with varying degrees of genital atypia. Moreover, as children with CAH develop, growth acceleration and pubertal advancement may occur at earlier stages, especially if they are not in optimal adrenal control. Given the chronic nature, severity and physical stigmata of CAH, individuals are at risk for psychiatric morbidity.

Prior studies have largely been limited to the adult CAH population. Men with CAH have been found to have increased rates of psychiatric disorders and suicidality, especially in those who experienced a delayed diagnosis of CAH (11). Women have been noted to have increased anxiety, and women with classical CAH (particularly, simple virilizing CAH) have double the risk of having a psychiatric diagnosis compared to age-matched controls (7). Review of the literature has found few dedicated pediatric studies evaluating mental health in children with CAH and conclusions have been variable (7,8,9). As such, there remains a need to further characterize mental health concerns in the pediatric CAH population.

The aim of this study was to assess the degree of anxiety and depression symptoms in children and adolescents with CAH using validated mental health questionnaires during a routine follow-up visit.

Methods

Participants

Children diagnosed with CAH or hypothyroidism (HT) and who were between the ages of 7-17 years and their respective caregivers were eligible for this single-center, prospective, observational cohort study. All the prospective participants with CAH had 21-hydroxylase deficiency. Sex of children was specified by karyotype analysis in those found to have atypical genitalia only. To the best of our knowledge, all children were raised in accordance with their chromosomal sex. A non-CAH control group was deemed necessary given the baseline mental health concerns stemming from the Coronavirus disease-2019 (COVID-19) pandemic, which was concurrent with the study period.

Children with HT (congenital or autoimmune) were chosen as the control group as they also have a chronic illness, require daily administration of medication, and have frequent medical visits and laboratory assessments, similar to children with CAH. In addition, children with HT have a bimodal age of presentation similar to CAH; congenital HT and classical CAH are often diagnosed in the newborn period and autoimmune HT and non-classical CAH are diagnosed in later childhood. However, unlike the CAH population, children with HT do not share the added stressors associated with physical stigmata (from hyperandrogenism or precocious puberty) and adrenal crisis

(requiring glucocorticoid stress-dosing and hospitalization). All children with HT were biochemically euthyroid at the time of questionnaire administration.

Participants were recruited from the pediatric endocrinology and urology clinics at NewYork-Presbyterian Hospital/Weill Cornell Medical Center (NYPH/WCMC). NYPH/WCMC is one of eight CAH Comprehensive Care Centers in the United States. Study assessments were completed over seven months from May 2021 to December 2021. Child and caregiver participants were excluded if unable to read English and if child participants had another chronic illness (diabetes or nephropathy) or malignancy.

A total of 116 participants (60 children and 56 caregivers) completed questionnaires on anxiety and depression symptoms. Two caregivers completed separate proxy forms as they had two children with CAH. One caregiver did not complete proxy forms for their two children with CAH.

Data Collection

Written consent was obtained from caregivers and written assent from child participants. All caregivers completed a demographic questionnaire. One-time questionnaires were completed at a single routine clinic visit. Children completed questionnaires privately, and all children ages 7-11 years were offered help in reading and clarifying questions. Intelligence levels were not separately measured in child participants but all children, to the best of our knowledge, were in his or her appropriate grade level, on discussion with the caregivers. Children were told that if there were concerns of harm to self or others, confidentiality would be broken and parents and appropriate individuals would be notified.

Upon completion, child questionnaires were scored immediately. Higher scores indicated a greater number of symptoms of anxiety or depression. A risk assessment flowsheet, including need for emergency room assessment, was used if acute safety risk (acute suicidality) was a concern. Appropriate community resources were given to children and caregivers for further mental health evaluation. All child participants received a $10 electronic gift card. This study was approved by the Weill Cornell Medicine Institutional Review Board, approval #20-04021748, date: 22.02.2022.

Measures

Children completed age-appropriate questionnaires on anxiety and depression. All caregivers completed an associated proxy questionnaire.

Children's Depression Inventory 2 Self Report-Short (CDI-2) (12): A 12-item self-report assessing signs of depression and validated for ages 7-17 years and takes approximately five minutes to complete. A modified T-score of ≥60 ("at-risk") may indicate the presence of a depressive disorder.

Screen for Child Anxiety Related Disorders (SCARED) (13): A 41-item self-report screening for anxiety disorders and validated for ages 8-18 years, which takes approximately 10 minutes to complete. It includes five subscales: *panic disorder or significant somatic symptoms, generalized anxiety disorder, separate anxiety disorder, social anxiety disorder* and *significant school avoidance*. A total score of ≥25 ("at-risk") may indicate the presence of an anxiety disorder.

Patient Health Questionnaire modified for Adolescents (PHQ-A) (14): A 9-item measure assessing for symptoms of depressive disorders, validated for ages 11-17, which takes approximately two minutes to complete. A total score of ≥10 ("at-risk") has good sensitivity for major depressive disorder. Suicide risk is screened with Yes/No questions.

Caregivers were given proxy forms of all the child questionnaires.

Pre-pandemic General Population Normative Data: The data was used and available for the SCARED (13) and CDI-2 (12).

SCARED Normative Data: Participants included 635 healthy young people (7 to 18 years old) and parent dyads and questionnaires were completed before 2019.

CDI-2 Normative Data: Participants included up to 1,100 healthy young people (7 to 17 years old) and parent dyads. Data based on participant age and gender were available.

PHQ-A normative data was also available but our study's sample size was insufficient to draw meaningful conclusions.

Adrenal Control

Adrenal control was based on 17-OHP values obtained over the prior 12-month period. Levels were drawn 1-2 hours after a morning hydrocortisone dose (normal practice at this center). Adrenal control was determined if participants had at least four 17-OHP levels in the prior twelve-month period. "Good" control was defined as 17-OHP < 1.000 ng/dL greater than or equal to 75% of the time, "moderate" if 17-OHP < 1.000 ng/dL more than 25% and less than 75% of the time, and "poor" if 17-OHP < 1.000 ng/dL less than or equal to 25% of the time."

Statistical Analysis

Descriptive statistics were used to describe the cohort of patients using n (%) and mean, standard deviation, median, interquartile range for categorical and continuous factors. Chi-square test or Fisher's exact test was used to compare the proportion of "at-risk" individuals between CAH and HT children. Wilcoxon rank sum test was used to compare raw scores from each questionnaire (SCARED, CDI-2, and PHQ-A) between children with CAH and HT, as well as their caregivers. Kruskal-Wallis test was used to compare raw scores of each questionnaire across different disease classifications (CAH: salt-wasting, simple virilizing, non-classical) or adrenal control (poor, fair, good). When comparing the general population to our cohort of CAH and HT children, a t-test was used. Linear mixed modelling was used to determine difference in scores on the questionnaires between children and caregivers.

All p-values were two-sided with statistical significance evaluated at the 0.05 alpha level. Ninety-five percent confidence intervals for all parameter estimates of interest were calculated to assess the precision of the obtained estimates. All analyses were performed in R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria).

For analyses pertaining to adrenal control, patients included had four 17-OHP completed at NYPH/WCMC. Patients who completed 17-OHP levels at a different hospital were removed.

Results

Baseline Characteristics

Baseline characteristics are summarized in Table 1. Sixty children, ages 7-17 years, completed an anxiety and/or depression questionnaire based on age eligibility. Fifty-eight parent participants completed an anxiety and/or depression proxy questionnaire (Table 2).

Participant Anxiety

Of the children who completed the SCARED (CAH n = 30, HT n = 25), there was no difference in total scores between the CAH and HT groups (p = 0.2). Similarly, there was no difference in at-risk scores between the CAH and HT groups (p = 0.4), scoring "at risk" in 33% and 44%, respectively.

Seven children in each group had a history of anxiety based on caregiver report, and the difference in social anxiety scores was not significant (p = 0.6). There was no difference in scores among the other subscale categories.

Table 1. Child participant characteristics

Child characteristic	CAH (n = 34)	Hypothyroidism (n = 26)
Disease classification, n (%)	Classical: 23 (68) Non-classical: 11 (32)	Congenital: 7 (27) Autoimmune: 19 (73)
Sex, %		
Male	32	27
Female	68	73
Mean ± SD age at assessment, years (SD)	11.41 ± 2.54	12.68 ± 2.91)
Median age at diagnosis, years (IQR)	0.04 (0.00, 7.16)	6.9 (0.4, 11.9)
Mean ± SD duration of condition, years	8.48 ± 4.12	5.99 ± 4.21
Race/ethnicity, %		
Non-hispanic white	67.6	53.8
Hispanic	14.7	23.1
Asian	8.8	15.4
Multiracial	2.9	3.8
Other	5.9	3.8
Insurance type, %		
Private	79	96
Public	21	4
Family income, %		
< $100,000	26.5	19
≥$100,000	58.8	73
Declined	8.8	8
Unknown	5.9	0
Reported prior history of anxiety or depression, n (%)	7 (22)	5 (19)

SD: standard deviation, IQR: interquartile range, CAH: congenital adrenal hyperplasia

Participant Depression

Sixty children completed the CDI-2 (CAH n = 36, HT n = 26) and there was no difference in total T-scores (p = 0.4) or at-risk scores (p = 0.5). Caregivers identified three children with CAH and two children with HT as having a history of depression; there was no difference in total T-scores in this subset (p > 0.9).

Twenty-nine children completed the PHQ-A (CAH n = 13, HT n = 16). Children in the CAH group were found to have lower mean total scores than the HT group (p = 0.038) but had no difference in at-risk scores (p = 0.4). Of note, 87.5% (14 of 16) children in the HT group had a history of autoimmune HT. One child was found to have suicidal ideation in the HT group.

Child Versus Caregiver Proxy Scores

As seen in Figure 1, differences were noted between child and caregiver scores on all questionnaires. After evaluating SCARED mean total scores, both the CAH (p < 0.001) and HT (p < 0.001) groups showed that children reported a greater number of anxiety symptoms than reported by their respective caregivers. Similarly, children in both groups reported a greater number of depression symptoms than reported by their respective caregivers (CAH p = 0.002 and HT p < 0.001). With PHQ-A scores, there was no significant

Table 2. Parent participant characteristics

Parent characteristic	CAH (n = 32)	Hypothyroidism (n = 26)
Sex, %		
Male	19	23
Female	81	77
Mean age at assessment, years (SD)	45 (6)	47 (6)
Highest level of education, n (%)		
High school or less	5 (16)	1 (3)
Associate or bachelor	16 (50)	10 (39)
Master/professional	11 (34)	15 (58)
Marital status, n (%)		
Divorced/separated/widowed	4 (13)	1 (4)
Married	27 (84)	21 (81)
Single	1 (3)	4 (15)
Reported prior history of anxiety or depression, n (%)	15 (47)	9 (35)

SD: standard deviation, CAH: congenital adrenal hyperplasia

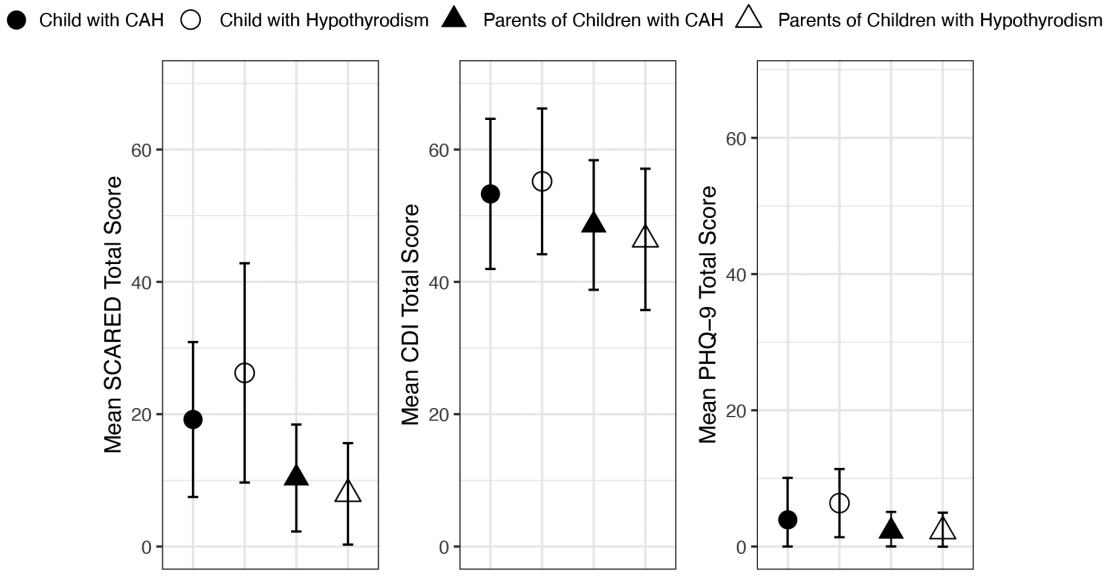

Figure 1. Child and parent scores on the CDI-2, SCARED, and PHQ-A

CAH: congenital adrenal hyperplasia, CDI-2: Children's Depression Inventory 2 Self Report-Short, SCARED: Screen for Child Anxiety Related Disorders, PHQ-A:

Patient Health Questionnaire modified for Adolescents

difference in scores between children with CAH and their caregivers (p = 0.300). However, children with HT reported a greater number of depression symptoms than their caregivers reported (p = 0.005). After controlling for age, a difference remained between children and caregivers in both their SCARED and CDI-2 scores (p < 0.001).

Adrenal Control and Mental Health

Adrenal control was determined in a subset of 23 children as only 23 children had at least four 17-OHP levels in the prior 12-month period. Table 3 includes the number of participants deemed to be in good, fair or poor adrenal control. There was no difference in adrenal control between the salt-wasting, simple virilizing and non-classical CAH groups (p = 0.11). Moreover, there was no difference in adrenal control when comparing classical (salt-wasting and simple virilizing) and non-classical CAH groups (p = 0.2). Seventy-eight percent of participants were considered to have fair or good control (22% poor, 43% fair, 35% good).

For children with CAH who scored as at-risk on the SCARED and CDI-2, there was no difference in adrenal control (p > 0.999). Of children who scored at-risk on the SCARED (n = 10) and had available 17-OHP levels (n = 7), 86% (6 of 7) had 17-OHP levels that were considered fair or good. Similarly, 86% (6 of 7) of those who scored at risk on the CDI-2 had 17-OHP levels that were considered fair or good. Adequate analysis for the PHQ-A would have been unreliable due to small sample size so was not performed.

Our results, albeit in a small sample, suggest that children with CAH have no difference in the number of anxiety symptom scores comparing those aged 7-11 years with 12-17 years (p = 0.500), illustrated in Table 4. Interestingly, there was a difference in the depression symptom scores comparing the same age groupings, with the 7-11 year group having higher CDI-2 scores (p = 0.016). The HT group showed no difference in scores with regards to anxiety and depression symptoms between the two age groups (p = 0.300).

COVID-19 Pandemic Comparisons

Anxiety

As seen in Table 5, when the CAH and HT groups were separately compared to the pre-pandemic general population, both the CAH and HT groups had significantly higher anxiety symptom scores (p = 0.0051 and p = 0.0004, respectively).

Depression-Gender

With regards to depression based on the CDI-2, CAH boys were found to have lower depression scores compared to both HT boys and pre-pandemic population boys (p = 0.026 and p = 0.031, respectively). There was no difference in

Table 3. Adrenal control among CAH child participants

	Adrenal control		
	Poor (25% or less), n = 5[1]	Fair (25 to 74.99%), n = 9[1]	Good (75% or more), n = 8[1]
CAH diagnosis			
Simple-wasting	2 (40)	7 (78)	4 (50)
Simple virilizing	2 (40)	1 (11)	0 (0)
Non-classical	1 (20)	1 (11)	4 (50)
Prior genitoplasty			
No	0 (0)	2 (33)	1 (33)
Yes	1 (50)	4 (67)	2 (67)
Pending	1 (50)	0 (0)	0 (0)
Unknown	3	3	5

[1]n (%).
CAH: congenital adrenal hyperplasia

Table 4. Median scores in CDI-2 and SCARED comparing younger and older age groups

Characteristic	7-11 years, n = 21[1]	12-17 years, n = 13[1]	p value[2]
CDI-2 total score			
CAH	52 (48, 60)	44 (43, 50)	**0.016**
HT	48 (45, 59)	55 (48, 65)	0.400
SCARED total score			
CAH	22 (15, 29)	11 (7, 28)	0.500
HT	17 (14, 29)	25 (15, 40)	0.300

[1]Median (IQR).
[2]Wilcoxon rank sum test.
CAH: congenital adrenal hyperplasia, HT: hypothyroidism, CDI-2: Children's Depression Inventory 2 Self Report-Short, SCARED: Screen for Child Anxiety Related Disorders

Table 5. General child population vs. child participant scores

Mean total calculated scores (SD)	CAH	HT	General	p
SCARED[1]	19.2 (11.71)	26.24 (16.57)	12.65 (9.37)	CAH vs. HT p = 0.2 CAH vs. Gen **p = 0.0051** HT vs. Gen **p = 0.0004**
CDI-2				
Age[2]				
7-12 years	3.8 (3)	4.17 (3.33)	2.7 (2.82)	CAH vs. HT p = 0.8 CAH vs. Gen p = 0.0977 HT vs. Gen p = 0.1567
13-17 years	3.44 (3.97)	5.71 (4.48)	3.48 (3.42)	CAH vs. HT p = 0.14 CAH vs. Gen p = 0.976 HT vs. Gen p = 0.0866
Gender[3]				
Female	4.78 (3.18)	5.32 (4.36)	3.09 (3.36)	CAH vs. HT p > 0.900 CAH vs. Gen **p = 0.02** HT vs. Gen **p = 0.04**
Male	1.45 (1.97)	4.14 (2.85)	2.95 (2.99)	CAH vs. HT **p = 0.035** CAH vs. Gen **p = 0.0313** HT vs. Gen p = 0.3137

[1]CAH n = 30, HT n = 25, General population n = 635.
[2]7-12 years: CAH n = 25, HT n = 12, General population n = 600; 13-17 years: CAH n = 9, HT n = 14, General population n = 500.
[3]Female: CAH n = 23, HT n = 19, General population n = 524; Male: CAH n = 11, HT n = 7, General population n = 522.
SD: standard deviation, CAH: congenital adrenal hyperplasia, HT: hypothyroidism, CDI-2: Children's Depression Inventory 2 Self Report-Short, SCARED: Screen for Child Anxiety Related Disorders

scores between HT boys and pre-pandemic population boys (p = 0.31). CAH and HT girls had higher depression scores compared to pre-pandemic population girls (p = 0.02 and p = 0.04, respectively). There was no difference in scores between CAH and HT girls (p = 0.5).

Depression-Age

There was no significant difference in depression scores with regards to age (7-12 years and 13-17 years groups) between the CAH and HT groups (7-12y p > 0.9 and 13-17y p = 0.140). There was no difference in scores with regards to the same age groups between the CAH and pre-pandemic general population (7-12y p = 0.1 and 13-17y p = 0.98) and between the HT and pre-pandemic general population (7-12y p = 0.16 and 13-17y p = 0.09).

Discussion

Current standard of care guidelines for CAH management recommend that individuals undergo mental health evaluations given the psychological and physical stressors that may surround a diagnosis of CAH (10). Contributing factors towards this recommendation include CAH being a chronic disease, risk of severe electrolyte imbalance, presence of genital atypia, signs of hyperandrogenism and caregiver distress. Our findings demonstrate that children and adolescents with CAH do not have a greater degree of anxiety or depression symptoms as compared to controls unaffected by CAH. These results challenge the notion that individuals with CAH, especially children and adolescents, may be at higher risk for having mental health concerns, specifically anxiety and depression. Findings from earlier CAH studies that included children with CAH have reported varying conclusions with regards to mental health.

A large, Swedish retrospective cohort study of women and girls with CAH (7) identified girls less than 12 years of age as having an increased risk of a psychotic disorder (p < 0.05) but this conclusion was limited by low statistical power. Risk of any psychiatric disorder, including anxiety, increased in those greater than or equal to 18 years of age. The authors note that the odds of having a psychiatric disorder was higher in those born before 1986, which was the year in which newborn screening for CAH was just introduced. Children who participated in our study were diagnosed with CAH after the advent of CAH newborn screening in the United States, which may have allowed for more prompt recognition of classical CAH and initiation of hormone treatment, lessening the continued exposure to elevated androgens that has been associated with increased rates of psychopathology (8).

Mueller et al. (8) conducted a prospective cohort study from 2002-2009 to characterize psychiatric morbidity in children (ages 8-18 years) with genetic etiologies of hyperandrogenism, including classical CAH. Their results revealed that 19% of females and 21% of males with classical CAH had anxiety disorders. Major depression was within the category of "mood disorder" and was evident in 0% of CAH females and 3% of CAH males. A limitation of this study was its lack of a control group, as the authors argued a healthy control group could not control for both the experiences of a chronic medical condition and genetic contributions to a psychiatric disorder. Our study selected children with HT as control participants. HT is a chronic condition that has a similar bi-modal age of diagnosis distribution as CAH. Pediatric HT management involves chronic medical therapy but neither has the additional concerns for serious illness and hospitalization nor the physical stigmata related to hyperandrogenism. Our data suggest there was no difference in at-risk anxiety and depression symptom scores between children with CAH and HT. Among those with classical CAH, at-risk scores for anxiety and depression symptoms were 26% (5 of 19) and 22% (5 of 23), respectively. Additionally, parents of children were asked to self-report their own history of anxiety and depression. In children with CAH who scored at-risk for anxiety, 38% (3 of 8) of parent participants also had a history of anxiety and 57% (4 of 7) of depression. Similarly, of children with HT who scored at-risk for anxiety, 45% (5 of 11) of parents also had a history of anxiety and 12% (1 of 8) of depression. We therefore did not find a statistical difference between the parents of children with CAH and HT. Previous studies have suggested that carriers of CAH may have psychological vulnerability to stress (15,16). None of our parent participants were carriers of CAH. If there were parent participants found to be carriers, it would be difficult to determine whether it is the carrier status itself or the responsibility of caring for a child with CAH that may cause the vulnerability to stress.

Our findings agree with a recent retrospective review of behavioral health diagnoses from a large pediatric database (PEDSnet) that abstracted at least one outpatient visit from 2009-2019 (CAH n = 1647, controls n = 6588). Their results found that children with CAH, when compared to controls, did not have a statistically significant increase in anxiety or depressive disorder diagnoses (9). The authors argue that there may be a higher risk of developing and diagnosing mental health disorders during adolescence. Our results, although from a small sample, suggest that children with CAH have no difference in the number of anxiety symptoms between 7-11 years and 12-17 years. However, there was a difference with regards to depression symptoms with the 7-11 year-old group having higher CDI-2 scores. The HT group showed no difference in anxiety and depression symptom scores between the two age groups. Younger age at CAH diagnosis and a diagnosis of classical CAH may have contributed to the younger age group having higher CDI-2 scores. In our cohort, the median age at CAH diagnosis was 0.00 years versus 2.25 years between the 7-11 and 12-17 year old age groups, respectively. A larger percentage of the younger age group compared to the older age group had classical CAH (71% vs. 61.5%). Children with classical CAH, as compared to non-classical CAH, have a younger age at diagnosis, higher rate of genital atypia and may have a more significant concern for adrenal crisis given the greater degree of enzyme deficiency. Larger studies could help investigate and further delineate these possible contributing factors. Of note, prior studies have shown that individuals with classical CAH may also have decreased adrenomedullary function that can potentially affect their ability to cope with psychological stressors (17,18). Our data did not assess adrenomedullary function in our patients to challenge this conclusion.

In adult men with CAH, Falhammar et al. (11) found that the risk of psychiatric morbidity increased in men born before the introduction of CAH newborn screening, possibly due to prolonged androgen exposure. Elevated androgens are thought to play a role in behavior in children with CAH (8,19). However, associations with anxiety and depression have not been as clear. Our study is one of the first to examine whether a child's degree of adrenal control correlates with anxiety or depression. Eighty percent were found to be in fair or good control. Though we acknowledge that the sample size for this sub-analysis was very small, there was no significant increase in anxiety or depression symptom scores in those with poor control.

In March 2020, the COVID-19 pandemic was at its peak. Our study recruitment began approximately one year after this time, in May 2021, and continued until December 2021. During this recruitment period, several studies reported increased rates of anxiety and depression in the pediatric population (20,21,22). Given the influence of the pandemic on mental health, it was important for our study to include a control group of children since they, too, similarly experienced the potential mental health consequences of the pandemic. Our study participants, both CAH and HT controls, had overall higher anxiety symptom scores compared to the pre-pandemic pediatric population. With regards to depression, girls from both the CAH and control groups, had higher depression symptom scores when compared to the pre-pandemic population; this finding is similar to what has been found in recent studies (21,22).

Interestingly, boys with CAH were found to have lower depression symptoms scores compared to both HT controls and the pre-pandemic population, although as our sample size was small, it is difficult to deduce reasons for this.

It is important to note that child scores for anxiety and depression (CDI-2) were higher than reported by parents in both CAH and HT groups. Internalizing symptoms include feelings of anxiety, loneliness and sadness (23). Angold et al. (24) found that by late adolescence, at least 20% of females and 7% of males exhibit internalizing symptoms. Children also often report internalizing symptoms at higher rates than their parents consider (25). Our results suggest that children with CAH may benefit from routine mental health evaluations, regardless of voiced caregiver concern, given the lack of agreement between child and parent proxy responses.

A few theories emerged as to why our cohort of children with CAH were not found to have an increased predominance of anxiety or depression symptoms when compared to HT controls. Our institution is considered a Center for Excellence in CAH, the first of eight designated centers by CARES Foundation. It was our experience after engaging with children and their families during visits that parents expressed a confidence in the care provided to their children. Patients are seen by a pediatric endocrinologist with extensive experience with CAH treatment and management. They are expected to follow up every three months in order to allow for careful dose adjustments that help optimize growth and development. As previously mentioned, nearly half of our CAH participants were found to be in good adrenal control. Androgens play a role in human behavior (26) and elevated androgens have been associated with an increase in severe behavioral symptoms in girls with CAH (27). In our cohort of females with classical CAH, 69% (n = 11) underwent genitoplasty and 6% (n = 1) were planning to undergo later in the year. Prior studies have suggested that adults with an XX karyotype and classical CAH supported genitoplasty within the first year of life (28). Whether completion and timing of genitoplasty is truly correlated with mental health outcomes is not clear, as no long-term observational studies have been completed. Our results show that 67% (6 out of 9) of children with a history of genitoplasty did not score at-risk for anxiety and 73% (8 out of 11) did not score at-risk for depression.

Study Limitations

There were several limitations with our study. Our study recruitment was at a single institution and completed over a seven-month period. As CAH is a rare condition with an incidence of approximately 1:14,000 to 1:18,000 births (10), the sample size of a single institution obtained during this time period was fairly significant. However, expanding the study to include multiple institutions over a longer study interval would potentially change or strengthen our findings. Moreover, our study highlights the pediatric CAH population at a center of excellence in which children are given specific expert care, and we recognize that the study results may not be generalizable. Therefore, it would be valuable to expand the study to patients who are both at centers of excellence and not at centers of excellence. Our study used validated mental health questionnaires that were free (SCARED, PHQ-A) or low-cost (CDI-2), and of a short time-commitment. The main limitation of these questionnaires was that these are self-report measures, which may introduce bias compared to an independent evaluator. However, these questionnaires are the most commonly used research self-report measures for youth depression and anxiety, as well as the most frequently used screening tools in pediatric clinical practice. Though the questionnaires used were reliable general screeners for anxiety and depression, they were not specific to CAH-related concerns. A questionnaire including topics on gender identity, genital surgery and body image may provide a more comprehensive view and insight into anxiety and depression symptoms in the CAH population. This study included parent participants with a history of anxiety and/or depression. As children may have a genetic predisposition to anxiety and depression (29), our study is limited by the absence of exclusion of these few parent participants who reported such history. Our study included participants with both classical and non-classical CAH. There have been reports regarding anxiety in adults with non-classical CAH that has suggested that having non-classical CAH may contribute to anxiety (30) and that females with non-classic CAH can have higher anxiety scores as compared to age and sex-matched controls (31). However, we acknowledge that those with non-classical CAH versus classical CAH may have different degrees of mental health concerns. Additionally, elevated 17-OHP levels were used as a biochemical marker for anxiety in our study but elevated dehydroepiandrosterone sulfate and pregnenolone sulfate levels may also have an effect on anxiety (30). With regards to our control group, the majority of HT participants did not have congenital HT so comparisons between those with classical CAH were affected by sample size. We recognize there was a female predominance in our sample. Therefore, our results cannot necessarily be extrapolated to males. Lastly, this study was completed one year after the start of the COVID-19 pandemic, a time during which there was evidence of increasing rates of anxiety and depression among youth. We attempted to control for the overall increase in mental health concerns by the inclusion

of the HT control group. Although our findings on anxiety and depression were similar to what had been found in the general youth population during this time, it would be important to replicate this study several years removed from the pandemic to ascertain whether the increased mental health concerns were related to the pandemic or are inherent to the HT population.

Conclusion

Our study suggests that children with CAH do not have a greater degree of anxiety or depression symptoms compared to controls with HT, despite having more unique risk factors for increased psychiatric morbidity. Expertise in care, frequent patient follow-up and good adrenal control may have played a role in alleviating anxiety and depression symptoms. Our study also illustrates the ease and benefit of mental health questionnaire administration at routine visits, especially as mental health evaluations by trained providers are currently difficult to obtain due to resource availability, scheduling difficulties and insurance barriers. Moreover, such screenings at routine visits can highlight any differences in patient-caregiver perspective with regards to internalizing symptoms of anxiety and depression. Future multicenter studies that are several years removed from the COVID-19 pandemic will better aid in the understanding of mental health in children with CAH and whether further measures should be considered in optimizing CAH care.

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Ethics

Ethics Committee Approval: This study was approved by the Weill Cornell Medicine Institutional Review Board, approval #20-04021748, date: 22.02.2022.

Informed Consent: Written consent was obtained from caregivers and written assent from child participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Corinne Catarozoli, Karen Lin-Su, Marianne Jacob, Oksana Lekarev, Design: Karen Lin-Su, Marianne Jacob, Oksana Lekarev, Data Collection or Processing: Charlene Thomas, Karen Lin-Su, Marianne Jacob, Oksana Lekarev, Analysis or Interpretation: Charlene Thomas, Karen Lin-Su, Marianne Jacob, Oksana Lekarev, Dix Poppas, Literature Search: Karen Lin-Su, Marianne Jacob, Oksana Lekarev, Dix Poppas, Writing: Corinne Catarozoli, Charlene Thomas, Karen Lin-Su, Marianne Jacob, Oksana Lekarev.

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CASE REPORT

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Hemolytic Anemia due to Glucose 6 Phosphate Dehydrogenase Deficiency Triggered by Type 1 Diabetes Mellitus

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What is already known on this topic?

Glucose 6 phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in humans and is among the important causes of hemolytic anemia. Severe hemolytic anemia due to G6PD deficiency has rarely been reported in association with newly diagnosed diabetes. No genetic analysis of the *G6PD* gene has been published in these cases.

What this study adds?

Rapid correction of blood sugar in diabetic individuals may induce severe hemolytic anemia due to G6PD deficiency. In the present case a previously reported missense pathogenic variant (c.653C > T; p.Ser218Phe) in the *G6PD* gene was found.

Abstract

Glucose 6 phosphate dehydrogenase (G6PD) is expressed in all tissues and is necessary to maintain oxidant stress capacity of cells. G6PD deficiency is the most common enzymopathy in humans and is among the important causes of hemolytic anemia. It has been reported that severe hemolytic anemia due to G6PD deficiency may develop in newly diagnosed diabetes, especially during the correction of hyperglycemia. To date, nine cases have been published. Genetic analysis was not performed for G6PD deficiency in these published patients. We present a case of hemolytic anemia due to G6PD deficiency secondary to newly diagnosed type 1 diabetes mellitus. Genetic testing was performed for the index patient and revealed a previously reported missense pathogenic variant (c.653C > T; p.Ser218Phe) in the *G6PD* gene.

Keywords: Diabetes mellitus, G6PD, anemia

Introduction

Glucose 6 phosphate dehydrogenase (G6PD) is expressed in all tissues and is necessary to maintain the oxidant stress capacity of cells. G6PD is a cytoplasmic enzyme involved in the hexose monophosphate pathway and protects erythrocytes against oxidative damage by producing NADPH. G6PD deficiency is the most common enzymopathy in humans and is among the important causes of hemolytic anemia (1). Medications, certain foods (such as broad beans) and acute infections can cause hemolysis in individuals with G6PD deficiency (2). In G6PD deficiency,

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the severity of hemolysis is variable, and this hemolysis resolves when the normal metabolic balance is restored (3). It has been reported that severe hemolytic anemia due to G6PD deficiency may develop in newly diagnosed diabetes, especially during the correction of hyperglycemia, but this is very rare (4,5).

Case Report

A 4-year-old male patient was admitted with polyuria and polydipsia and was hospitalized with a diagnosis of diabetes when his blood glucose (BG) level was 750 mg/dL. He was not taking any medication. He was tachypneic and weak at admission. Body temperature was 36.8 °C. Height was 105 cm [-0.18 standard deviation score (SDS)], body weight was 13 kg (-2.29 SDS), body mass index was 12.7 kg/m^2 (-2.68 SDS). Other system findings were normal for age. Blood results were: hemoglobin (Hb) 13.1 g/dL (NR 9.6-13.5); white blood cell count (WBC) 6310/mm^3 (5-14.8); platelet count 341,000/mm^3 (150-400,000); C-reactive protein 2.98 mg/L (< 3); blood urea nitrogen 15 mg/dL (0-23); creatinine 0.76 mg/dL (0.3-1.2); BG 750 mg/dL; sodium (Na) 126 meq/L (135-143); corrected Na 137.7 meq/L; potassium 4.55 meq/L (3.1-5.5); aspartate aminotransferase 23 U/L (< 48); and alanine aminotransferase 12 U/L (0-39). The blood tests performed to investigate diabetic ketoacidosis (DKA) showed: pH 7.29 (7.35-7.45); bicarbonate 12.4 mmol/L (18-23); PCO_2 25.8 mmHg (32-46); and base excess -12.3. Urine ketone test was positive. With these results, the patient was diagnosed with DKA. The patient was given intravenous insulin and hydration therapy. Then, intensive insulin therapy was started. Further investigations to confirm the diagnosis showed: HbA1c 15.3%; BG 750 mg/dL (60-110); insulin 2.2 mIU/mL (2-23); C peptide 0.3 ng/mL (0.9-7.1); islet cell antibody positive at 3.38 U/mL (< 1); glutamic acid decarboxylase antibody positive at 1.03 U/mL (< 1); and anti-insulin antibody negative at 4.63% (0-8.2). On the tenth day of admission, while receiving 0.96 U/kg/day subcutaneous insulin treatment, he developed tachycardia with hypoglycemia (heart rate was 160 beats/min while BG was 45 mg/dL). The patient had not previously developed hypoglycemia. The celiac test performed at the time of diagnosis of the patient was negative. Due to hypoglycemia, the dose of insulin was reduced and hypoglycemia did not recur thereafter. The patient, whose tachycardia continued after the hypoglycemia resolved, was examined. There was no jaundice and organomegaly on physical examination. Test results for tachycardia were: Hb 8.3 g/dL (9.6-13.5); mean corpuscular volume 92.8 fL (72.3-90.1); red cell distribution width 23.1% (11.8-15.1); red blood cells 3.06x10^6/L (3.3-5.4); hematocrit 28.4% (28.5-39.5); platelet count 639,000/mm^3 (150,000-400,000); WBC 13,170/mm^3 (5000-14,800). Test results for anemia were: ferritin 136 ng/mL (6-24) and vitamin B12 was 594 pg/mL (200-1080). There were anisocytosis and normoblast in the peripheral smear. There were no atypical cells. The reticulocyte count was 10% (0.2-2). Other results were: prothrombin time 11.7 sec (10-14.7); INR 0.98 (0.8-1.2); activated partial thromboplastin time 23 sec (22-34); total bilirubin 1 mg/dL (0-2) (indirect bilirubin 0.8, direct bilirubin 0.2); and lactate dehydrogenase (LDH) 286 IU/L (140-304). The abdominal ultrasound was normal. The patient was subsequently diagnosed with acute hemolytic anemia. Hb electrophoresis was normal, direct Coombs test was negative, Epstein-Barr virus, cytomegalovirus, brucella and mycoplasma IgM were negative and the G6PD level was low at 0.56 U/g Hb (6.97-20.5). During follow-up, Hb was 7.1 g/dL and erythrocyte suspension was given to the patient whose tachycardia and hemolysis continued. His tachycardia and anemia improved during follow-up. The patient, whose blood sugar regulation was provided, was discharged with intensive insulin therapy. The Hb value measured two weeks after discharge was 12.4 g/dL, and three months after discharge was 12.2 g/dL. Hemogram results are given in Table 1. Genetic examination was performed and a hemizygous mutation (c.653C > T; p.Ser218Phe) was detected in the *G6PD* gene. Genetic analysis was not performed on the patient's parents. Written informed consent for publication of the case was obtained from the parents of the child.

Table 1. Hemogram results of the patient in the follow-up

	First application	In hemolytic crisis	In recovery period
Hb (g/dL)	13.1	7.1	12.2
RBC (10^6/L)	5.53	2.61	4.47
MCV (fL)	71.1	93.5	81
RDW (%)	13.8	23.9	12.3
Hemotocrit (%)	39.3	24.4	36.2
Platelet (10^5/mm^3)	346	499	348
WBC (10^5/mm^3)	6.31	11.83	7.69

Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume, RDW: red cell distribution width, WBC: white blood cell

Discussion

G6PD protects cells from oxidative damage and is a cytoplasmic enzyme involved in the hexose monophosphate pathway. It reduces glutathione and increases the detoxification of free radicals. G6PD deficiency is inherited in an X-linked fashion and is the most common enzymopathy. G6PD deficiency causes hemolytic anemia in homozygous women and hemizygous men (1). Hemolysis due to G6PD deficiency is a very rare condition in individuals with diabetes. In general, hemolysis occurs while hyperglycemia improves. NADPH production in the hexose monophosphate pathway, the rate-limiting step of this pathway, is G6PD dependent. NADPH is required for reduced glutathione production. In hyperglycemia, erythrocytes shift to the sorbitol pathway. Sorbitol and NADP production increases in erythrocytes. The NADPH/NADP ratio and reduced (GSH) / oxide (GSSG) glutathione ratio decreases. With rapid correction of long-standing hyperglycemia in newly diagnosed diabetes, there is no glucose flow from the sorbitol pathway to the hexose monophosphate pathway, NADPH production decreases, and there is stress in the erythrocyte. Another possible mechanism for hemolysis is increased volume reduction due to hyperglycemia, increased reactive oxygen species (ROS) and oxidative damage, damage to the erythrocyte cell membrane because of increased ROS, and the formation of fragile erythrocytes prone to hemolysis (6). It has been reported that G6PD activity and expression are decreased in islet cells in severe hyperglycemia (7). It has been suggested that the frequency of G6PD deficiency in diabetic patients may be higher than the normal population; similarly, impaired fasting glucose and diabetes frequency may increase in individuals with G6PD deficiency (8). Nine pediatric diabetes cases with hemolysis due to G6PD deficiency have previously been reported. The clinical and laboratory characteristics of the patients are given in Table 2. These cases were between 3.5 and 12 years old. Eight of the cases were male, and two were female. Seven cases were diagnosed with ketoacidosis and one case with hyperglycemia. Hemolytic anemia developed in the first 10 days. In one case, blood transfusion was required, and other cases resolved spontaneously. The distribution of cases were; two Greek, two Egyptian, four Italian and one African American. Most cases were reported from Mediterranean countries and in keeping with this, the presented case was Syrian. Genetic analysis was not performed for G6PD deficiency in previously published patients. Genetic testing was performed for the index patient and revealed a previously reported missense hemizygote pathogenic variant in the *G6PD* gene. The distribution of G6PD activities depended on the type of mutation patterns and genders. Hemizygote, homozygote, and compound heterozygote were predominantly associated with severe G6PD deficiency, whereas heterozygotes with single mutation usually present with moderate enzyme deficiency (9). Thus the hemizygous missense mutation found in the presented case may explain the severe clinical condition.

In addition, an adult case with type 1 diabetes mellitus who was found to have hypoglycemia and hemolytic crisis due to G6PD deficiency has been reported (10). Hypoglycemia was detected in the follow-up of the presented patient and he had tachycardia secondary to hypoglycemia and was diagnosed with G6PD deficiency. In this case, it is not clear whether hemolysis was due to hyperglycemia or a hypoglycemic attack triggered the crisis.

Table 2. Clinical and laboratory characteristics of G6PD deficiency in children with diabetes mellitus

Age	Sex	Country	Presentation at diabetes diagnosis	Time of hemolysis	Hb (g/dL)	Treatment
10	M	African American	Ketoacidosis	9th day	9	Spontaneous
3.5 Twin1	M	Greek	Ketoacidosis	6th day	10.2	Spontaneous
3.5 Twin2	M	Greek	Ketoacidosis	6th day	8.3	Spontaneous
8	M	Sicilian	Hyperglycemia	4th day	7.1	Erythrocyte transfusion
4 Twin 1	M	Egypt	Ketoacidosis	7th day	10.3	Spontaneous
4 Twin 2	M	Egypt	Ketoacidosis	7th day	8.1	Spontaneous
12	F	Sardinia	Ketoacidosis	1st day	Unknown	Unknown
9	F	Sardinia	Ketoacidosis	4th day	Unknown	Unknown
4	M	Italian	Unknown	5th day	Unknown	Unknown
4 Presented case	M	Syria	Ketoacidosis	10th day	7.1	Erythrocyte transfusion

Hb: hemoglobin, G6PD: glucose 6 phosphate dehydrogenase

Conclusion

This case was diagnosed with type 1 diabetes with DKA. The patient had hypoglycemia and tachycardia that developed after his blood sugar was regulated, and his tachycardia continued after hypoglycemia improved. Anemia due to G6PD deficiency was found in the patient who was examined for tachycardia. It should be kept in mind that diabetic individuals may develop severe anemia due to G6PD deficiency when treated for blood sugar regulation.

Ethics

Informed Consent: Written informed consent was provided from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Burçe Orman, Şenay Savaş Erdeve, Design: Burçe Orman, Şenay Savaş Erdeve, Data Collection or Processing: Burçe Orman, Şenay Savaş Erdeve, Semra Çetinkaya, Meltem Akçaboy, Ali Fettah, Nergiz Öner, Naz Güleray Lafcı, Analysis or Interpretation: Burçe Orman, Şenay Savaş Erdeve, Literature Search: Burçe Orman, Şenay Savaş Erdeve, Writing: Burçe Orman, Şenay Savaş Erdeve.

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CASE REPORT

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Tumor-induced Osteomalacia in a Boy with Maxillary Ossifying Fibroma

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What is already known on this topic?

Tumor-induced osteomalacia (TIO) is a rare syndrome characterized by severe hypophosphatemia and osteomalacia. Very few cases have been reported in children.

What this study adds?

We describe an adolescent diagnosed with TIO and we locate his tumor in the right maxilla, which is quite a rare site. We review five pediatric TIO patients with tumors in the oral/maxillary region. These findings suggest the importance of head and neck examination and suitable imaging for patients in whom TIO is suspected.

Abstract

Tumor-induced osteomalacia (TIO) is a rare, paraneoplastic disorder of hypophosphatemia associated with elevated tumor-produced fibroblast growth factor 23 (FGF23). Maxillofacial tumors are rarely involved in TIO, especially maxillary TIO in children. We present a 14-year-old boy with osteomalacia and high serum levels of FGF23, a hormone associated with decreased phosphate resorption, due to a maxillary tumor. The patient was treated with oral phosphorus and calcitriol, and surgical removal of the tumor was performed. After 21 months follow-up, he was pain free and had returned to full activity. We review the reported pediatric cases of TIO in the maxillofacial and oral region and discuss the management of these patients considering the published evidence.

Keywords: Tumor-induced osteomalacia, fibroblast growth factor-23, maxilla, children

Introduction

Tumor-induced osteomalacia (TIO) is a rare paraneoplastic syndrome, first described in 1947 by McCance (1) but in 1959 Prader et al. (2) first highlighted the relation between the neoplasm and the disease in an 11-year-old girl who presented with rickets in association with tumor of a rib. TIO is characterized by progessive bone pain, proximal muscle weakness, gait disturbance and multiple fractures, which are a consequence of severe hypophosphatemia. The key pathogenetic mechanism of TIO involves tumor-driven secretion of phosphatonins, most frequently fibroblast growth factor 23 (FGF23). FGF23 binds to the fibroblast growth factor receptor 1-Klotho complex in the renal proximal tubule to stimulate the excretion of phosphorus from the kidney. High FGF23 levels reduce the expression of type IIa sodium phosphate contransporters leading to renal phosphorus wasting (3). FGF23 is also a regulatory hormone for 1,25 dihydroxyvitamin D [1,25$(OH)_2$D] and

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leads to a decreased concentration of the vitamin in blood. The majority of tumors associated with TIO are located in the extremites (skin, muscles, bones) or around the head, but they may occur in almost any part of the body. Few pediatric patients with TIO have been reported; in a 2015 review of TIO in pediatrics, the authors reported 26 children in the literature (4). Pharmacotherapy is initiated with oral phosphorus and calcitriol supplementation, but surgical removal of the tumor is the definitive treatment for TIO (5).

We present a 14-year-old boy who developed hypophosphatemia and urinary phosphate wasting, muscle weakness, bone pain and he had been using a wheelchair for 18 months. This is the first adolescent with TIO reported from Vietnam.

Case Report

We report a previously healthy, 14-year-old adolescent who presented with 18 months of progressive, bilateral knee pain and ankle pain, as well as muscle weakness. He was able to walk slowly with crutches but was nearly wheelchair-bound. He had no fever and no weight loss. He was prescribed non-steroid anti-inflammatory drugs, methotrexate and physical therapy because of a misdiagnosis of juvenile idiopathic arthritis at a provincial hospital. However, his condition did not improve over the subsequent six months and he was referred to our hospital in October 2019. At the time of presentation, his weight was at the 50th percentile for age, his height was at the 7th percentile and he had no overt skeletal deformities. He had bilateral proximal weakness in upper and lower extremities. Although he was unable to walk due to pain, his joints were not swollen. The maxilla was neither painful nor enlarged. His family did not have history of hormonal, skeletal or metabolic problems.

Investigations

Laboratory test showed low serum ionized calcium [1.08 mmol/L, (reference range, 1.17-1.29 mmol/L)], hypophosphatemia [0.32 mmol/L, (reference range, 0.87-1.45 mmol/L)], elevated alkaline phosphatase [1138 U/L, (reference range, 40-129 U/L)], low $1,25(OH)_2D$ (11.4 pg/mL, [reference range 16-65 pg/mL]), nearly normal 25-hydroxyvitamin D [19.6 ng/mL, (reference range, >20 ng/mL)], and normal parathyroid hormone [59.85 ng/L, (reference range, 11-79 ng/L)]. The percent tubular reabsorption of phosphate was 85% (reference range, >90%); the maximal tubular renal phosphate reabsorption normalized for glomerular filtration rate was 0.42 mmol/L (reference range, 1.15-2.44 mmol/L). Serum FGF23 level was elevated at 159.3 pg/mL (reference range 23.2-95.4 pg/mL measured at Cerba Laboratories, Paris, France).

Radiographs of skull and bones in upper and lower of both legs demonstrated generalized demineralization with decreased bone density, cortical thinning and trabecular blurring; fatigue fracture also seen in the cortex of the right radius diaphysis accompanied by bilateral widening of the proximal humerus physis (Figure 1). A dual-energy X-ray absorptiometry (DXA) revealed a severe loss of bone mineral density (BMD) at L1-L4 (0.498 g/cm^2, Z-score, -4.0) and the femoral neck (0.448 g/cm^2; Z-score, -3.6). Positron emission tomography with ^{18}fluorodeoxyglucose (^{18}FDG-PET) demonstrated a metabolically active lesion with increased standardized uptake values in the right maxilla (Figure 2). Once the lesion was identified by ^{18}FDG-PET, magnetic resonance imaging and computed tomography (CT) scans showed a tumor measuring 23x14 mm in the right maxilla (Figure 2).

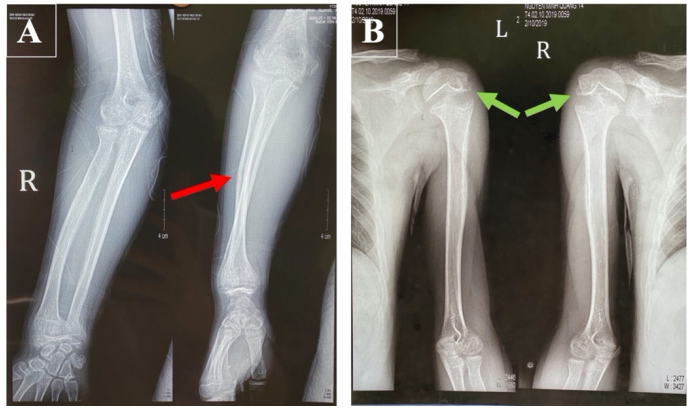

Figure 1. Radiograph of the left forearm **(A)** and arms bilateral **(B)** shows diffuse demineralization of bones, fatigue fracture (looser zones) of diaphysis in left radius (red arrow), widening of bilateral proximal humeral physics (green arrows)

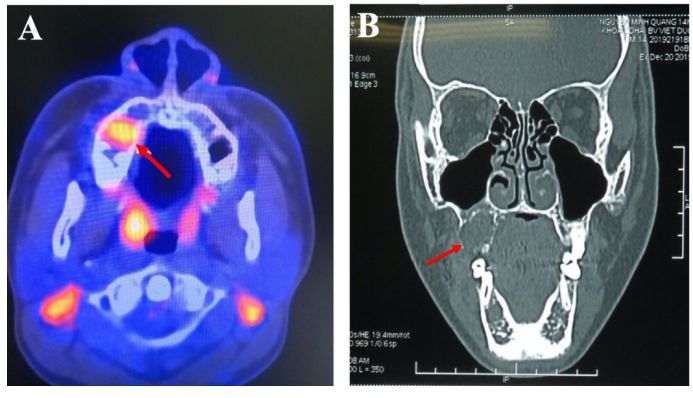

Figure 2. (A) Positron emission tomography/computed tomography (CT) with ^{18}fluorodeoxyglucose, **(B)** CT scanner, T1W images show a osteolytic lesion in the right maxilla, intense FDG uptake, hypointense on T1W and non-homogenous gadolinium enhancement (red arrows)

Differential Diagnosis

The differential diagnosis of hypophosphatemia due to urinary phosphate wasting can be divided in to genetic and acquired causes. Inherited conditions include X-linked hyphophosphatemia, autosomal dominant hypophosphatemia rickets, autosomal recessive hypophosphatemic rickets, and hereditary hypophosphatemic rickets with hypercalciuria (6). These diseases are often associated with short stature in early childhood, fractures, deformities in the legs and tooth findings. However, the presented patient had rapid onset of symptoms and a negative family history that ruled out these causes.

Acquired causes include TIO, renal tubular damage from heavy metal exposure or drugs such as aminoglycoside antibiotics, vitamin D deficiency, and primary or secondary hyperparathyroidism. In this case, the patient had a normal serum PTH level and he did not use such drugs. Besides, his parents said that he did not eat or drink something tainted with lead or mercury.

Treatment

The patient was managed medically with oral phosphorus (60-80 mg/kg/day) and calcitriol (40 ng/kg/day), which improved but did not normalize his serum phosphorus. He underwent surgical resection of the tumor three months after diagnosis. Histopathological examination indicated that the tumor was an ossifying fibroma (OF) (Figure 3). One month after removal of the tumor, the patient's serum phosphorus level was 1.57 mmol/L. Three months later, his serum FGF23 level was normal (34.3 pg/mL) and he was asymptomatic. One year later, DXA showed that his BMD in the lumbar spine had recovered with a normal Z-score of 1.58. Twenty-one months after diagnosis, he was pain free and returned to full activity. He has returned to school and at the time of writing has no evidence of tumor recurrence.

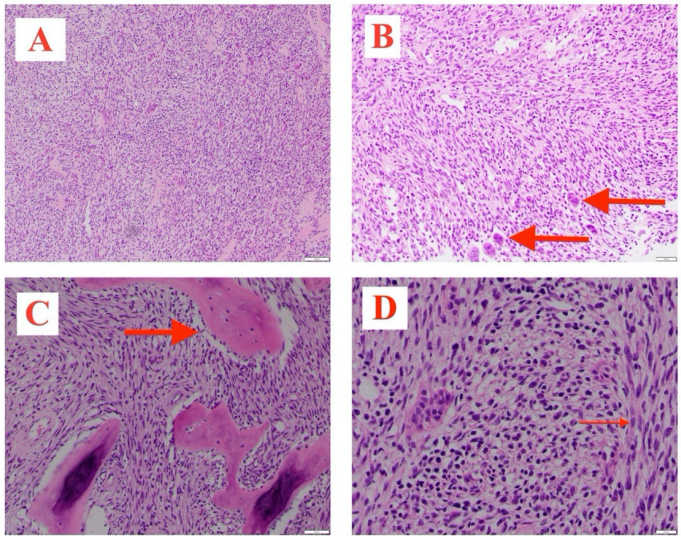

Figure 3. (A, B) Low power (x40, x100) photomicrographs showing diffuse neoplastic cells arranging in fascicular pattern infiltrates with osteoid, scattered clusters of multinucleated giant cell are noted (arrow), **(C)** low power (x100) photomicrograph showing trabecular bone with osteoblastic rimming (arrow), **(D)** intermediate power (x200) photomicrograph showing spindle tumor cells with mild to moderate nuclear pleomorphism, oval nuclei and eosinophilic cytoplasm (arrow). Mitosis is very rare

Discussion

Through this report, we aim to highlight our experience with maxillary TIO in a child and provide a review of published literature. Tumors associated with TIO may occur in the lower extremity (>40%) or craniofacial locations (>20%) (7). Generally, the maxillofacial region is rarely involved in TIO, and maxillary TIO is rare, comprising around 9% of such tumors in the neck and head (8).

We searched for all publications in English in PubMed up to August 2021 with key words "Tumor-induced osteomalacia", "Oncogenic Osteomalacia" and "Child". Only publications reporting pediatric TIO in the oral and maxillofacial area were included. Five cases were identified, of whom two were boys and three were girls, with ages from 3-18 years (Table 1). Associated tumors were found in the mandible in two cases, the maxilla in two cases and both mandible and maxilla in one case. The diagnosis of these pediatric TIO cases averaged 21.9 months and ranged from 1.5 to 72 months. The presented case took about 18 months from onset to identification of the associated tumor but it is still shorter than the average time. The delay in diagnosis may be due to several reasons. Firstly, the bone pain is just dismissed initially since this symptom may not yet be associated with radiographic abnormalities or obvious deformities. Secondly, serum phosphate is not a routine laboratory test. Lastly, TIOs tend to be quite small, hidden and clinically silent. Looking at Table 1, the managing clinicians located tumor related TIO based on physical examination for patients 3, 4 and 5 while we had to perform ^{18}FDG PET/CT to find the tumor. This difference may be explained that in our case, the tumor was not only asymptomatic but also invisible at its location. Besides, some articles reporting adult TIO showed that tumors without local symptoms were seen in over half of the patients with oral region lesions (55.9%). On admission, we did not examine the maxillary alveolus because he had no symptoms at this location. After the tumor was found by functional imaging tests (^{18}FDG-PET/CT), physical oral examinations were performed, none of which revealed any notable findings. This highlights the

role of PET/CT in diagnosis of occult tumors and it can be helpful, as seen in this case.

The presented case had uncommon microscopic findings in tumor samples, while the majority of the tumors that cause TIO are phosphaturic mesenchymal tumors (4). OF is a slow-growing, benign lesion of bone, most commonly associated with the jaws (14). The literature search performed uncovered no publications describing OF tumor as a cause of osteomalacia in children. Diagnosis of TIO was made in this case based on hypophosphatemia, hyperphosphaturia, high serum FGF23 level, and an inappropriately low $1,25(OH)_2D$. The improvement of serum phosphate level, serum FGF23 level, and clinical symptoms after tumor removal of the OF provided strong clinical evidence that this tumor was the etiology of the TIO. In the presented case, histophathological examination showed lesion-related peripheral fibroma. Mesenchymal tumors can be divided in to osteomas, chondromas, mixed connective tissue tumors and fibroma, and all may produce a phosphatonin, now termed FGF23 (15). However, a limitation in this case was not being able to obtain immunohistochemical confirmation of FGF23 production in the resected tumor tissue.

The present case was initially treated medically for around three months before surgery, due mainly to the delay in measuring serum FGF23 level for diagnosis. Moreover, this was the first TIO case from our country so there were extensive clinical consultations. After tumor removal, clinical symptoms improved remarkably and there have been no signs of tumor recurrence. The primary treatment for TIO in the literature is surgical resection but data on alternative therapies for TIO are limited, especially for children. Besides, it is necessary to clinically assess muscular strength, serum phosphate levels and alkaline phosphatase concentrations because these problems may complicate postsurgical management, for example the requirement for prolonged mechanical ventilation and delayed recovery (16). Additionally, intralesional corticosteroid injection after surgical removal has been reported in patients 3 and 5 with central giant cell tumor (Table 1). One was a 3-year-old boy with tumors in the mandible and maxilla. Surgical removal was performed but CT imaging showed growth of tumor four months later, so the patient was treated by injection of triamcinolone in maxillary and mandibular sites. This resulted in serum FGF23 and phosphorus levels normalizing two months later (13). The other patient was a 3-year-old boy and total removal of his tumor was impossible because its extension. Also, local instillation of triamcinolone resulted in disappearance of tumor 2 months after (11).

Table 1. Review of clinical, laboratory, and outcome of head and neck pediatric TIO cases

Case no.	First author (references)	Age/sex (years)	Duration of osteomalacia (months)	Symptoms at location	Serum phosphate (mmol/L)	Serum FGF23 (pg/mL)	Localization methods	Tumor region	Histopathology results	Treatment	Outcome
1	Reyes-Múgica et al. (9)	9 F	1.5	Normal	0.52	NA	MRI	Left mandible	PMT-MCT	Surgery	Recover
2	Wu et al. (10)	15 F	24	NA	NA	NA	NA	Right mandible	PMT	Surgery	Recover
3	Fernández-Cooke et al. (11)	3 M	6	Visible tumor	0.84	395.1	PE	Maxilla	CGCG	Surgery, local steroid infiltration	Recover
4	Emodi et al. (12)	18 F	72	Soft tissue	0.42	NA	PE	Left maxilla	Fibro-osseous lesions	Surgery	Recover
5	Crossen et al. (13)	3 M	6	Ulcerated lesions, thick, irregular to palpation	0.58	450	PE	Maxilla and mandible	CGCG	Surgery, local triamcinolone injection	Recover
	Present case	14 M	18	Normal	0.32	159.3	18FDG-PET	Right maxilla	OF	Surgery	Recover

F: female, M: male, MRI: magnetic resonance imaging, NA: not available, OF: ossifying fibroma, PE: physical examination, PMT-MCT: phosphaturic mesenchymal tumor mixed connective tissue type, PMT: phosphaturic mesenchymal tumor, CGCG: central giant cell granuloma,18FDG-PET: positron emission tomography with ^{18}fluorodeoxyglucose, FGF23: fibroblast growth factor 23, TIO: tumor-induced osteomalacia

Conclusion

In conclusion, we report a case with a rare cause, TIO, of osteomalacia in childhood. Diagnosis is often delayed due to the slow progression of tumor and rarity in childhood, thus physicians should perform careful investigation, extensive imaging, laboratory test, particularly serum phosphate and FGF23 plasma levels and histopathological examination of resected tissue samples. This case highlights that head and neck imaging play an important role in evaluating pediatric patients with suspected TIO.

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Ethics

Informed Consent: Written informed consent was obtained from the patient's father.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ha Nguyen Thi, Cuong Pham Manh, Linh To Tuan, Concept: Ha Nguyen Thi, Cuong Pham Manh, Linh To Tuan, Lan Anh Le Thi, Nam Nguyen Thanh, Soamarat Vilaiyuk, Design: Ha Nguyen Thi, Cuong Pham Manh, Linh To Tuan, Lan Anh Le Thi, Nam Nguyen Thanh, Soamarat Vilaiyuk, Data Collection or Processing: Ha Nguyen Thi, Cuong Pham Manh, Lan Anh Le Thi, Nam Nguyen Thanh, Analysis or Interpretation: Ha Nguyen Thi, Cuong Pham Manh, Linh To Tuan, Lan Anh Le Thi, Nam Nguyen Thanh, Soamarat Vilaiyuk, Literature Search: Ha Nguyen Thi, Cuong Pham Manh, Writing: Ha Nguyen Thi, Cuong Pham Manh, Linh To Tuan, Lan Anh Le Thi, Nam Nguyen Thanh, Soamarat Vilaiyuk.

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CASE REPORT

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Neonatal Diabetes, Congenital Hypothyroidism, and Congenital Glaucoma Coexistence: A Case of *GLIS3* Mutation

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What is already known on this topic?

Neonatal diabetes and congenital hypothyroidism syndrome is a rare condition caused by homozygous or compound heterozygous mutations in the *GLIS3* gene, with 22 patients reported so far. Small for gestational age infant, congenital glaucoma, polycystic kidney disease, cholestatic hepatic fibrosis, pancreatic exocrine insufficiency, developmental delay, dysmorphic facial findings, sensorineural deafness, osteopenia, and skeletal anomalies are accompanying findings in these patients.

What this study adds?

Herein, one of the oldest surviving *GLIS3* mutation cases reported to date, with both cardinal findings of neonatal diabetes and congenital hypothyroidism syndrome is presented. The patient is the second case with a homozygous exon 10-11 deletion and is also the second known Turkish case.

Abstract

Neonatal diabetes and congenital hypothyroidism (CH) syndrome is a rare condition caused by homozygous or compound heterozygous mutations in the *GLIS3* gene. Small for gestational age, congenital glaucoma, polycystic kidney disease, cholestatic hepatic fibrosis, pancreatic exocrine insufficiency, developmental delay, dysmorphic facial features, sensorineural deafness, osteopenia, and skeletal anomalies are other accompanying phenotypic features in the 22 cases described so far. We present a male patient with neonatal diabetes, CH, congenital glaucoma, developmental delay, and facial dysmorphism. During the patient's 17-year follow-up, no signs of exocrine pancreatic insufficiency, liver and kidney diseases, deafness, osteopenia, and bone fracture were observed. A homozygous exon 10-11 deletion was detected in the *GLIS3* gene. We report one of the oldest surviving *GLIS3* mutation case with main findings of neonatal diabetes and CH syndrome to contribute to the characterization of the genotypic and phenotypic spectra of the syndrome.
Keywords: *GLIS3*, neonatal diabetes, congenital hypothyroidism, congenital glaucoma

Introduction

Neonatal diabetes mellitus (NDM) is an extremely rare cause of monogenic diabetes in which persistent hyperglycemia usually occurs in the first six months of life. Although the frequency is reported to be 1 in 90,000-300,000 in different studies, it is suggested that it may be at least 3-10 times more common in the Middle East region, where consanguineous marriage is more common (1,2). Syndromic NDM constitutes 10% of this rare patient group (1). NDM with congenital hypothyroidism (CH) (MIM#610199) is a rare condition caused by homozygous or compound heterozygous mutations in the GLI-similar 3 (*GLIS3*) gene. The *GLIS3* transcription factor was first identified in 2003 (3). In the same year, the association of NDM, CH, congenital glaucoma, hepatic fibrosis, and polycystic kidney disease

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were reported in two siblings with the hypothesis that it might constitute a new syndrome (4). *GLIS3* mutations (9p24.2, OMIM*610192) were first associated as the cause of the coexistence of persistent NDM and CH in six cases in 2006 (5).

GLIS3 encodes the zinc finger protein GLI-like protein 3 (*GLIS3*) (6). The protein is expressed early in embryogenesis and tissue expression plays a critical role in the development of pancreatic β cells, the thyroid gland, eyes, liver, and kidneys, and to a lesser extent heart, skeletal muscles, stomach, brain, lungs, adrenal glands, testes, ovaries, uterus, and bones (3,5,6). To date, only 22 patients with *GLIS3* variants variability have been reported and express significant phenotypic variability. The phenotypic features described so far include small for gestational age (SGA) infant, congenital glaucoma, polycystic kidney disease, cholestatic hepatic fibrosis, pancreatic exocrine insufficiency, developmental delay, dysmorphic facial features, sensorineural deafness, osteopenia, and skeletal anomalies (2,7,8).

Genetic evaluation was performed in a 17-year-old patient, who has been followed up in our clinic since infancy, because of the coexistence of permanent NDM, CH, glaucoma, developmental delay, and facial dysmorphism. A significant mutation was detected in the *GLIS3* gene. Since *GLIS3* mutations are a very rare cause of persistent neonatal syndromic diabetes, the case is presented to expand the published information about this rare condition.

Case Report

A forty-day-old male infant presented with complaints of feeding difficulties and fatigue. He was born at term by spontaneous vaginal delivery with a birth weight of 2800 g. The parents were first-degree cousins. In the follow-up after emergency intervention, high blood sugar (>200 mg/dL) and primary hypothyroidism [elevated thyroid stimulating hormone (TSH) and low free T4] were detected and insulin (0.4-0.6 IU/kg/day) and levothyroxine (10 mcg/kg/day) treatments were started. Glaucoma was detected during eye examination, triggered by the finding of corneal clouding. Hearing evaluation was normal. When the patient was four months old, the thyroid gland was found to be normal in size [+1.5 standard deviation (SD)] and *in situ* on ultrasound (USG). Serum thyroglobulin level was 707 ng/mL (3.5-77), urinary iodine level was 10 µg/dL (normal range 10-46). He was operated at the age of two years for unilateral undescended testis. At the age of 6 years and 10 months, his psychometric assessment was compatible with the age of 4.5-5 years. Brain magnetic resonance imaging (MRI) performed at the age of seven years was normal, and bone age was consistent with chronological age. Proteinuria was detected during diabetes follow-up at the age of eight. Abdominal USG evaluations at different periods were normal. There were no signs of hepatic fibrosis, cholestasis, or polycystic kidney disease. Thyroid stimulating hormone (TSH), free thyroxine (fT4), thyroid autoantibody levels, and anti-endomysium IgA concentration during follow-ups were normal. In the last seven years of follow-up, glycosylated hemoglobin levels were between 8.2-10.6%. Multiple dose subcutaneous insulin therapy with insulin detemir and insulin aspart continues at a dose of 1.1 u/kg/day. The most recent levothyroxine treatment dose was 175 mcg/day. Treatment and follow-up for glaucoma continues. The spot urine microalbumin/creatinine ratio in the follow-up of proteinuria has been <30 mg/g for the last year and he is followed without treatment. On the last physical examination, the 17-year-old patient's weight was 56.9 kg (-1.3 SD), height was 172.1 cm (-0.45 SD), and body mass index was 19.2 kg/m² (-1.3 SD). Pubertal assessment was consistent with Tanner stage 5. The patient had a long facial appearance, bilateral low-set ears, a long philtrum with a thin vermilion border of the upper lip, and multiple nevi smaller than 1 cm on the face and body (Figure 1). Dual-energy X-ray absorptiometry evaluation of patient to exclude osteopenia was normal and he had no history of fracture. His school performance was poor. In the last psychometric evaluation made with the Kent EGY test, the mental age was found to be 9.5 years, and the intelligence score was 69, while his chronological age was 17 years. Echocardiographic and repeated hearing examinations were normal. Genetic evaluation was performed in the patient due to the coexistence of permanent NDM, CH, congenital glaucoma, developmental delay, and facial dysmorphic findings. A homozygous exon 10-11 deletion of the *GLIS3* gene was detected. Furthermore, it was confirmed that both parents were heterozygous for this mutation. The genetic evaluation findings are thought to be compatible with the phenotypic characteristics of the patient.

Molecular Analysis

DNA isolation from the peripheral blood samples was performed using the MagNAPure LC DNA isolation kit (Roche Diagnostic GmbH, Mannheim, Germany) and the MagNAPure LC 2.0 (Roche Diagnostic Ltd., Rotkreuz, Switzerland) device according to the manufacturer's instructions. Quantification of DNA concentration and enriched library was performed with a Qubit® 3.0 Fluorometer (Invitrogen, Life Technologies Holdings Pte Ltd., Malaysia).

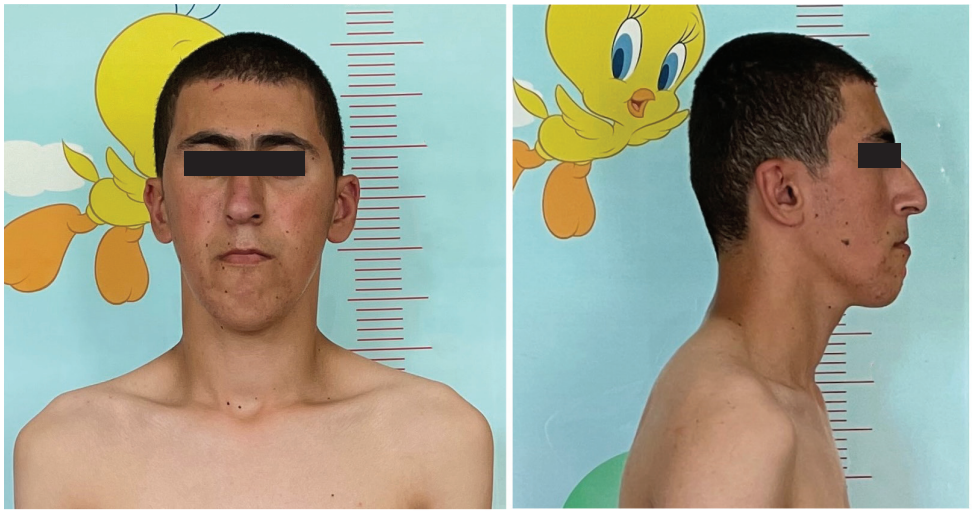

Figure 1. Facial dysmorphic findings in the patient

Table 1. Comparison of the phenotype and genotype characteristics of the presented patient with the previously reported Turkish patient and the other patient from the literature with the same mutation

	Previously reported Turkish patient	Previously reported patient with the same mutation	The presented patient
Sex	Male	Female	Male
Origin	Turkish	Yemeni	Turkish
Birth weight, g	1520	1235	2800
Gestation week	30	36	39
Age at diagnosis of ND	21 days	3 days	40 days
Hypothyroidism	+	+	+
Congenital glaucoma	-	+	+
Kidney disease	+	+	-
Exocrine pancreas insufficiency	-	+	-
Liver disease	+	+	-
Facial dysmorphism	+	+	+
Developmental delay	+	+	+
Age at death	6 months	NA	Alive (17 years)
Mutation type	Exons 3-4 del/exons 3-4 del	Exons 10-11 del/exons 10-11 del	Exons 10-11 del/exons 10-11 del

ND: neonatal diabetes, NA: not available

Library size distribution was measured with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Clinical Exome Solution kit (SOPHiA GENETICS, Saint-Sulpice, Switzerland) was used for library preparation and exome enrichment. The DNA sequencing was performed on Illumina NextSeq 500 instrument (Illumina, Inc., San Diego, CA, USA). Bioinformatic analysis was carried out via Sophia DDM version 5.10.8 (SOPHiA GENETICS, Saint Sulpice, Switzerland). Using dose analysis, this test may be able to detect copy number variation. In the presented patient a homozygous *GLIS3* exon 10 and 11 deletion was identified (NM_001042413.1). This deletion was also found to be heterozygous in both parents.

Discussion

NDM and CH syndrome are associated with mutations in the *GLIS3* gene (5). Most of the patients reported to date had ≥1 exon deletion in this gene (2,7,8). We present a case with coexisting NDM, CH, and congenital glaucoma and homozygous *GLIS3* exon 10-11 deletions. In the literature, the same deletion was described in a patient with NDM, CH, SGA, developmental delay, fibrotic liver cholestasis, polycystic kidney disease, and pancreatic exocrine insufficiency (Table 1). During the presented patient's 17-year follow-up, no symptoms of polycystic kidney disease, hepatic fibrosis, cholestasis, or exocrine pancreatic

insufficiency occurred. Furthermore, less common clinical findings of the condition, such as deafness, osteopenia, bone fractures, skeletal dysplasia, hernia, and cardiac illness, were not present in this case (7,8). Most of the previously described patients had a history of SGA or preterm birth (2,7,8). Although the presented patient had a history of normal delivery at term, birth weight was at the lower limit of normal. The association of hypospadias and bilateral undescended testis was reported in only one case previously, and coincidence was considered because extensive genetic evaluation was not performed (9). In the presented case, there was unilateral undescended testis but no hypospadias. Unlike the previously reported patients, the presented patient had many nevi over his body, all of which were less than 1 cm in diameter (7,8).

The present case is one of the oldest living patients described with a *GLIS3* variant as he has reached young adulthood. Interestingly, the presented case and the two other oldest reported patients had no liver, exocrine pancreas abnormalities, or kidney disease (2,5,7). We hypothesize that the absence of parenchymatous organ disease allows these patients to reach early adulthood. Of the other two older patients, the French patient who lived to adulthood and had a homozygous 149kb del and did not have congenital glaucoma (5) while in the other oldest patient, with p.Arg589Trp/exons 1-11 del, neonatal diabetes was not accompanied by CH, congenital glaucoma, or facial dysmorphism (7).

Although fT4 was within the normal range with treatment, TSH elevation, fluctuation, and TSH resistance, as well as thyroid agenesis and hypoplasia were frequently reported conditions (2,8). The thyroid gland of the presented patient was of normal size and in its normal location. Although fT4 and TSH were within normal limits during follow-up, levothyroxine treatment was maintained at > 3 mcg/kg/day (> 100 mcg/m²/day), indicating TSH resistance. Elevated serum thyroglobulin, which was reported in previously described cases, was also present in our patient (8,9).

The presented case is the second Turkish patient reported with a *GLIS3* variant. The previously reported Turkish patient had facial dysmorphism, developmental delay, and liver and kidney disease, but he did not have SGA, congenital glaucoma, exocrine pancreatic insufficiency, or skeletal disease (Table 1). In that case exon 3-4 deletions were detected, and the child died at the age of six months (7,9).

Conclusion

We present a case with homozygous exon 10-11 deletion in the *GLIS3* gene to contribute to the genotypic and phenotypic characterization of patients with neonatal diabetes and CH syndrome, a rare cause of neonatal diabetes. Furthermore, the identification of other accompanying clinical features in these cases will aid understanding of the disorder, and facilitate early diagnosis and appropriate treatment of patients with this condition.

Ethics

Informed Consent: Consent form was filled out by a participant.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Emre Sarıkaya, Mustafa Kendirci, Concept: Emre Sarıkaya, Mustafa Kendirci, Munis Dündar, Design: Emre Sarıkaya, Mustafa Kendirci, Mikail Demir, Data Collection or Processing: Emre Sarıkaya, Mikail Demir, Analysis or Interpretation: Emre Sarıkaya, Mikail Demir, Munis Dündar, Literature Search: Emre Sarıkaya, Writing: Emre Sarıkaya, Mustafa Kendirci, Mikail Demir.

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CASE REPORT

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A Novel Pathogenic IGSF1 Variant in a Patient with GH and TSH Deficiency Diagnosed by High *IGF-I* Values at Transition to Adult Care

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What is already known on this topic?

IGSF1 deficiency is a recently discovered disorder and data on long-term follow-up are scarce. Patients are known to have central hypothyroidism (CeH), prolactin deficiency, disharmonious pubertal development, and adult macroorchidism. Some of the patients exhibit short stature and growth hormone (GH) deficiency. However, GH deficiency is transient, and adult patients may have increased GH secretion and acromegaly-like features.

What this study adds?

Genetic analysis revealed a novel c.3559C > T (p.Q1187*) variant causing a stop codon in *IGSF1*. We suggest genetic analysis of *IGSF1* in patients with CeH, especially when accompanied by the clinical and laboratory features associated with the syndrome. Notably, children with CeH and GHD who show high insulin-like growth factor 1 levels at transition to adult care, should prompt analysis of *IGSF1*.

Abstract

IGSF1 deficiency is a rare X-linked condition characterized by central hypothyroidism and a wide variety of other clinical features with variable prevalence, including a delayed pubertal testosterone rise and growth spurt in the context of normal or accelerated testicular growth, and adult macroorchidism with relatively low serum testosterone concentrations. Other features include increased waist circumference, attention deficit, prolactin deficiency and transient partial growth hormone (GH) deficiency in childhood, contrasting with an increased GH secretion in adulthood. Patients with this disorder are not detected shortly after birth if neonatal screening programs are based on thyroid-stimulating hormone (TSH) concentrations. A 13.2-year-old male patient was referred to pediatric endocrinology for evaluation of short stature. He was born large for gestational age into a nonconsanguineous family. During work-up for short stature, deficiencies of TSH, prolactin and GH were detected, leading to treatment with levothyroxine and GH. At 16.9 years, GH treatment was stopped and during transition to adult care, his insulin-like growth factor 1 level was above the normal range. This prompted an analysis of IGSF1, in which a novel hemizygous variant causing a stop codon at c.3559C > T (p.Q1187*) was found, confirming the diagnosis of *IGSF1* deficiency syndrome. In this report, we describe his clinical and hormonal characteristics at presentation and during long-term follow-up.

Keywords: *IGSF1*, central hypothyroidism, short stature, large for gestational age, growth hormone deficiency, prolactin deficiency

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Introduction

Immunoglobulin superfamily member 1 (IGSF1) is a plasma membrane glycoprotein encoded by *IGSF1*. It consists of 20 exons and is located in the Xq26.1 region (1,2). Pathogenic *IGSF1* variants cause the X-linked IGSF1 deficiency syndrome (MIM #300888) and characteristic features in hemizygous male patients include central hypothyroidism (CeH), delayed pubertal testosterone rise and growth spurt in the context of normal or accelerated testicular growth (disharmonious pubertal development) and adult macroorchidism with relatively low serum testosterone concentrations. A variable proportion of affected males show prolactin deficiency, increased waist circumference, decreased attentional control, with short stature and in 16 % there is growth hormone (GH) deficiency (3). However, GH deficiency is transient, and adult patients may exhibit increased GH secretion (4). The degree and age at presentation of CeH are variable, with some patients presenting in infancy or childhood and some patients being diagnosed with mild hypothyroidism at older age based on family studies (2,3,5,6). Given the rarity of this syndrome, the full spectrum of its phenotype and pathophysiology remain subjects of investigation (7).

Here we report a patient who presented with short stature, CeH and GH deficiency treated with levothyroxine and GH, in whom high plasma insulin-like growth factor-1 (IGF-1) levels during transition to adult care ultimately led to the identification of a novel *IGSF1* variant. We present longitudinal data of the patient with respect to pubertal development and anthropometric measurements, as well as clinical and laboratory features from his parents and brother.

Case Report

A 13.2-year-old male patient was referred to the endocrinology clinic for evaluation of short stature. He was born as the second child into a non-consanguineous family at 38 gestational weeks by Caesarean section, with a birth weight of 4400 g [3.0 standard deviation score (SDS)] (8). The first child had died one day after birth due to respiratory distress. Neonatal screening for hypothyroidism based on thyroid-stimulating hormone (TSH) measurement was normal. Neuromotor development was normal and, except for allergic bronchiolitis, the medical history revealed no abnormalities.

At presentation, the initial physical examination was normal (Table 1). His height was 1.44 m (-1.9 SDS), considerably shorter than target height (0.0 SDS). Body proportions and weight were normal. Bilateral testicular volume assessed by the Prader orchidometer was 5 mL [-1.1 SDS, (9)] and pubic hair was at Tanner stage 2 (-0.7 SDS according to Dutch references) (10).

During workup for short stature (Table 2), his total blood count and biochemistry including electrolytes and lipids were within reference ranges. Plasma free thyroxine (fT4) concentration was below the normal range, while plasma TSH concentration was normal, leading to the diagnosis of CeH. The adrenocorticotropic hormone stimulation test showed a normal peak cortisol of 19.5 mcg/dL. Levothyroxine treatment was started and led to normalisation of fT4. Low levels of plasma IGF-1 (180 ng/mL, -2.0 SDS) (11), and prolactin were detected. Low IGF-1 levels of 168 ng/mL (-2.3 SDS) persisted after levothyroxine treatment had started. Cranial and pituitary magnetic resonance images did not reveal any pathology.

At follow-up his growth velocity decreased (4.3 cm/year, -1.1 SDS) and GH stimulation tests were performed, showing a peak GH level of 6.87 ng/mL after insulin and 2.67 ng/mL after clonidine. Serum IGF-1 was 168 ng/mL (-2.3 SDS). GH treatment was started and consequently growth velocity increased to 8.9 cm/year (1.5 SDS) in the first year. Molecular analysis for *PROP1* and *POU1F1* genes was normal.

During pubertal evaluation, orchidometric testicular volume was 6 mL (-1.5 SDS) at 14 years, 15 mL (-1.7 SDS) at 16 years and 25 mL (0.0 SDS) at 17 years. At 15 years of age patient's follicle stimulating hormone (FSH) level was 5.5 mIU/L, luteinizing hormone (LH) level was 2.4 mIU/mL and testosterone level was 4.96 ng/mL. At 17.9 years testicular volume was at least 25 mL bilaterally (25 mL being the largest bead on the orchidometer) and measured 6 cm in length. Pubic hair was at Tanner stage 5 and testosterone level was 16.9 nmol/L. A testis ultrasound, performed at 22.8 years and using a similar formula to calculate testicular volume as used for the reference population, resulted in a normal testicular volume [right testis 16.2 mL (0.9 SDS), left testis 12.9 mL (-0.1 SDS)] (9). We performed a dual-energy X-ray absorptiometry scan and L1-L4 bone mineral density was 0.891 g/cm^2 (Z-score -1.8 SDS). GH was stopped at 16.9 years. Anthropometric measurements and pubertal stages of the patient are presented in Table 1.

At transition to adult care, six years after the cessation of GH therapy, plasma IGF-1 level had increased to 2.6 SDS (396 ng/mL) (Table 2), raising the suspicion of a possible *IGSF1* variant. Genetic analysis was performed and a novel hemizygous variant causing a premature stop codon (c.3559C > T, p.Gln1187*) was found (Figure 1). We established the diagnosis of the IGSF1 deficiency syndrome,

Table 1. Anthropometric measurements and pubertal stage of the patient

Age (years)	13.2	15.9	16.9	22.8
Height, m (SDS)	1.44 (-1.9)	1.63 (-1.5)	1.69 (-0.9)	1.77 (0.2)
Weight, kg (SDS)	46.5 (0.4)	60.5 (-0.6)	60.7 (-0.9)	86.3 (1.3)
BMI, kg/m^2 (SDS)	22.4 (0.7)	22.7 (0.3)	21.3 (-0.4)	27.5 (1.2)
SH/height (SDS)	0.54 (1.1)	0.52 (-0.6)	0.52 (-0.8)	0.51 (-1.2)
Arm span, cm	NA	NA	NA	180.5
Bone age, years	12.5	14	14	NA
PAH, m	1.69	1.76	1.82	NA
Tanner stage	T 5/5 mL PH 2 SPL 4 cm	T 15/15 mL PH 4 SPL 8 cm	T 25/25 mL PH 5 SPL 8 cm	T 30/25 mL PH 5 SPL 9 cm

BMI: body mass index, NA: not available, PAH: predicted adult height, PH: pubic hair, SH: sitting height, SDS: standard deviation score, SPL: stretched penile length, T: testis volume by orchidometer

Table 2. Hormonal evaluation of the patient

	At presentation (13.2 years)	Last evaluation (22.8 years)	Reference range
Prolactin (ng/mL)	2.3	2.9	4.8-23.3
TSH (mIU/L)	3.2	< 0.015*	0.66-4.14
fT4 (pmol/L)	10.1	13.3*	11.6-21.5
Cortisol (µg/dL)	11.5	11.8	2.8-23
DHEAS (µmol/L)	287	NA	114-296 at 13 years
IGF-1 (ng/mL)	180[a]	396[b]	[a]183-850 [b]116-358
IGFBP-3 (µg/mL)	10.6[a]	7.46[b]	[a]3.1-9.5 [b]3.4-7.8
FSH (IU/L)	NA	5.8	2.6-11
LH (IU/L)	NA	2.8	0.4-7.0
Testosterone (nmol/L)	NA	23.6	6.1-27.1
SHBG (nmol/L)	NA	40.5	14.5-48.4
AMH (ng/mL)	NA	7.78	0.73-16.05

*During suppletion with 150 µg levothyroxine (1.7 µg/kg) once daily.
AMH: anti-mullerian hormone, DHEAS: dehidroepiandrostenedione sulfate, FSH: follicle-stimulating hormone, fT4: free thyroxine, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor binding protein 3, LH: luteinizing hormone, SHBG: sex hormone binding globulin, TSH: thyroid stimulating hormone

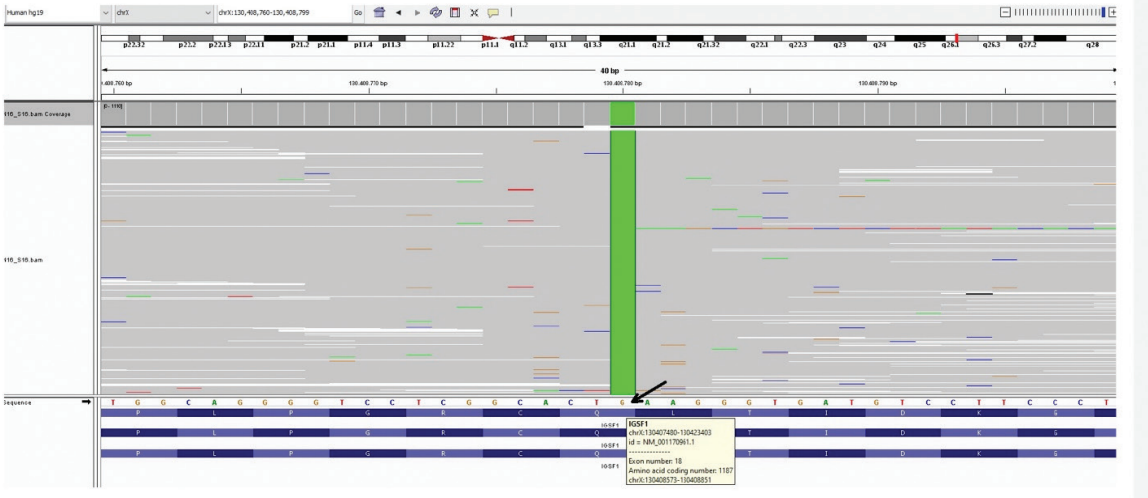

Figure 1. Next gene sequencing image of the novel hemizygous c.3559C > T (p.Q1187*) change in *IGSF1* gene of the patient

consistent with the combination of CeH, hypoprolactinemia, transient GH deficiency, short stature, large for gestational age at birth, and high IGF-1 levels in adulthood. The patient complained of clumsiness but did not report any attention problems, and currently works as a teacher. He had subtle facial coarsening without facial puffiness. At 22.8 years his waist circumference was 1.9 SDS (12) and body mass index (BMI) was 1.2 SDS (13).

The patient's mother is a heterozygous carrier for the *IGSF1* variant and her serum prolactin and fT4 levels were in the lower end of the reference range, while lipid profile was normal. Her age at menarche was 13 years. Her height was 1.58 m (-0.9 SDS) and weight was 94.8 kg (3.8 SDS). His younger brother did not consent to genetic analysis, but his fT4 was at the lower end of the reference range. His prolactin and lipid profile were in the normal ranges. His height was 1.89 m (2.0 SDS) and weight was 120 kg (3.3 SDS). The proband's father's thyroid function tests, prolactin levels and lipid profile did not reveal any pathology and he had a normal weight and height.

Molecular Analysis

Written informed consent was obtained from the patient and parents. Genomic DNA was extracted according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). *IGSF1* gene coding exon sequencing was performed on the MiSeq platform (Illumina inc, San Diego, CA, USA) with 150-bp pair-end reads.

Discussion

We identified a novel hemizygous *IGSF1* mutation variant in a male patient who initially presented with borderline short stature and a history of a high birth weight, in whom further investigations revealed CeH, GH deficiency and low serum prolactin. At that time, the IGSF1 deficiency syndrome had not yet been clearly defined (2), and only recently information became available about the surprisingly broad phenotypic spectrum of IGSF1 deficiency syndrome (3). If similar patients presented today, the combination of CeH (100%), prolactin deficiency (61%), increased birth weight (26%), and partial/transient GH deficiency (16%) would probably have led to genetic testing for *IGSF1*. In our case, the suspicion of IGSF1 deficiency syndrome was triggered by a relatively novel and unusual observation, that of elevated plasma IGF-1 in adulthood, which has been described in 20% of patients with this syndrome (3,4).

Interestingly, several relatively frequent features of the syndrome were absent in this patient. The most prominent of these is the normal testicular development in terms of testicular size and serum testosterone, while in a cohort of 69 males (3) low-normal testosterone concentrations and macroorchidism in adulthood were reported in 88%, and a delayed pubertal testosterone rise and early/normal timing of testicular growth in 75%. Other features that were absent included mild problems with attentional control (reported in 75%), a small thyroid gland (74%), increased waist circumference in adults (59%) and decreased dehidroepiandrostenedione sulfate (40-50%).

Regarding the reported mild problems with attentional control in the IGSF1 deficiency syndrome (14), it has been hypothesized that this may be related to inadequate prenatal T4 concentrations during critical periods of brain maturation (15,16). *IGSF1* is expressed (although in low quantity) in the adult rat cerebral cortex, striatum, subfornical organ, amygdala and glial cells of the hypothalamus, possibly having a direct effect on executive functioning (17). Our patient did not mention attention deficits. However, he did complain of clumsiness, which has also been described previously (3).

CeH is a rare condition and can result from dysfunctioning of the hypothalamus or pituitary gland. It can be part of multiple pituitary hormone deficiencies or be isolated. Isolated CeH is very rare and genetic causes include variants in *TSHβ, TRHR, TBL1X* or *IGSF1*, the latter being the most common. The combination of CeH and prolactin deficiency are particularly suspicious for IGSF1 deficiency, but are also observed in *TRH* receptor variants and may be a part of multiple hormone deficiency caused by a variant in *POU1F1, PROP1, HESX1* or *LHX3* (18). IGSF1 deficiency is the most frequent inherited cause of CeH (18). The incidence of CeH caused by IGSF1 deficiency is estimated to be 1:100,000 in the Netherlands and 1:80,000 in Japan (5,19). We therefore suggest performing genetic analysis for this gene in any case of non-acquired CeH, but especially if there are additional suggestive features of this syndrome (3). Detection of CeH by neonatal screening programs requires both fT4 and TSH determinations (20). In the Netherlands, neonatal screening for congenital hypothyroidism consists of fT4, TSH, and thyroxine-binding globulin since 1995. Our patient demonstrates the importance of determining both fT4 and TSH in screening and diagnosing all forms of hypothyroidism.

Growth of patients with the IGSF1 deficiency syndrome resembles the growth pattern of constitutional delay of growth and puberty (CDGP), as characterized by growth retardation in early adolescence and a late pubertal growth spurt (3,6); in both conditions circulating testosterone levels are low. However, in IGSF1 deficiency syndrome there is a

normal or even early start of testicular growth, while this is delayed in CDGP. It is unclear whether the slow growth in adolescence of males with IGSF1 deficiency can be completely explained by the low circulating testosterone concentrations, or whether the partial GH deficiency also plays a role. Using current criteria for GHD, this was reported in 16% of children in IGSF1 deficiency (3,4), but in all cases GHD was transient. Similarly, a recently published Turkish patient with IGSF1 deficiency was treated with GH for two years, but during adolescence his GHD proved transient (21). Remarkably, in adult IGSF1 deficient patients IGF-1 levels are generally in the upper half or even above the reference range, with increased GH secretion and variable mild acromegalic features in late adulthood (3,4,6). During childhood, our patient had short stature and low GH responses to pharmacological stimuli, but in adulthood plasma IGF-1 level was high. He had minimal facial coarsening however he did not have other acromegalic features. The underlying mechanism of transient GH deficiency and somatotrope hyperfunction in adulthood is unknown. As IGSF1 is expressed in rat hypothalamus, including ~19% of Ghrh-expressing neurons, as well as in pituitary somatotropes, it may be implicated either in hypothalamic regulation of GH or play a somatotrope-specific role in GH production or secretion (4). As pathogenic *IGSF1* variants were not prevalent in a cohort of patients with CDGP without CeH or macroorchidism, testing for *IGSF1* variants in the absence of those symptoms is not recommended (22).

Delayed testosterone production and macroorchidism after adolescence are characteristic features of IGSF1 deficiency (2). On ultrasonographic examination, most pediatric patients had testicular volumes in the upper half of the reference range, and 87% of adults showed macroorchidism (3,6). Our patient was at an early stage of puberty at presentation and it took about 2.5 years for his testes to enlarge from 4-5 mL to at least 25 mL at 22.8 years (an increase from -1.1 to +1.5 SDS). Thus, true macroorchidism was not diagnosed with the orchidometer in this patient. However, the upper limit of normal testicular volume in adults is 32 mL (9), and as the largest bead of a typical orchidometer is 25 mL, this device is not suitable for diagnosing adult macroorchidism. At physical examination, the length of the patient's adult testis was 6 cm. When assuming a corresponding width of 3.5 cm, the orchidometric volume would be estimated at 38.5 mL (π/6 x length x width^2 (9), thus above the reference range. The reasons for a lower Z-score measured with ultrasound is unclear.

It is not known why macroorchidism occurs in IGSF1 deficient patients. It has been suggested that an increased FSH to LH ratio plays a role (23), although both are generally within normal ranges, as was the case in our patient. An alternative explanation may be that FSH action is increased, either through increased sensitivity of gonadotropins to TRH (24) or by hypothyroidism, which lengthens the period of Sertoli cell proliferation and leads to an increase in Sertoli cell number and testes size (25). However, macroorchidism is also observed in IGSF1 deficient patients treated with levothyroxine since birth. Although IGSF1 deficient patients without macroorchidism have been reported (26), testicular growth in this condition typically starts at a normal or even advanced age, and shows a faster and longer growth period. Remarkably, testosterone secretion, pubic hair development and growth spurt start late. In all patients fertility was preserved, except one patient who was diagnosed with azoospermia and was resistant to treatment with FSH and LH (3).

Increased waist circumference and obesity are common in patients with IGSF1 deficiency (3). In a previous report, an untreated patient with IGSF1 deficiency showed profound hypercholesterolemia, with improvement after starting levothyroxine (27). Also, seven out of 69 (10.1%) male *IGSF1* patients were diagnosed with dyslipidemia, two of whom were on levothyroxine treatment (3). Our patient had a normal BMI and lipid profile before and after the levothyroxine was started.

Heterozygous female carriers are reported to have milder phentotypes, with low fT4, mild prolactin deficiency, increased BMI, and increased waist circumferenc. No fertility issues were reported among the female carriers (3). The mother of the index case was a carrier, and her prolactin and fT4 levels were at the lower tertile of the reference range and she did not have problems while lactating.

Since IGSF1 deficiency has only been recognized for 10 years, data on long-term follow-up are scarce. Phenotype and genotype relation of patients with IGSF1 deficiency has not been determined yet and clinical findings varied with the same mutation within families (3). Recently, Roche et al. (28) reported the largest family of IGSF1 deficiency (c.2318T>C, p.L773P) with ten hemizygous males and 11 heterozygous females. Patients had classical endocrine manifestations of IGSF1 deficiency however the penetrance of throid dysfunction (mild to moderate) and prolactin deficiency were variable.

Conclusion

In summary, we report a patient with a novel, pathogenic IGSF1 variant who presented with CeH, hypoprolactinemia, borderline short stature and normal pubertal development

with an increasing testicular volume SDS over time, as well as a striking reversal of GH deficiency in adolescence to increased IGF-1 levels in adulthood. We suggest genetic analysis of *IGSF1* in any patient with CeH, especially when accompanied by any of the clinical and laboratory features associated with the IGSF1 deficiency syndrome. Clinicians should keep in mind that these patients may exhibit high IGF-1 levels, acromegaly-like features, and macroorchidism in adulthood.

Acknowledgments

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Ethics

Informed Consent: Written informed consent was obtained from the patient and the family.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Aslı Derya Kardelen, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler, Design: Aslı Derya Kardelen, Feyza Darendeliler, Data Collection or Processing: Aslı Derya Kardelen, Esin Karakılıç Özturan, Firdevs Baş, Serdar Ceylaner, Analysis or Interpretation: Aslı Derya Kardelen, Sjoerd D. Joustra, Jan M. Wit, Feyza Darendeliler, Literature Search: Aslı Derya Kardelen, Sjoerd D. Joustra, Jan M. Wit, Writing: Aslı Derya Kardelen, Sjoerd D. Joustra, Jan M. Wit.

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CASE REPORT

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A Novel Heterozygous *NF1* Variant in a Neurofibromatosis-Noonan Syndrome Patient with Growth Hormone Deficiency: A Case Report

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What is already known on this topic?

Neurofibromatosis-Noonan syndrome (NFNS) is a rare autosomal-dominant hereditary disease characterized by clinical manifestations of both neurofibromatosis type 1 (NF1) and NS. Several *NF1* gene mutations have been reported to be associated with NFNS, such as a *de novo* heterozygous deletion of exons 1-58 and a heterozygous mutation c.7549 C > T in exon 51.

What this study adds?

A novel heterozygous mutation in the *NF1* gene was identified in an NFNS patient, who showed phenotypic features of both NF1 and NS. Short stature, a common feature of NFNS, can be caused by growth hormone (GH) deficiency. There is no consensus as to whether NFNS patients should be treated with recombinant GH.

Abstract

Neurofibromatosis-Noonan syndrome (NFNS), a rare autosomal-dominant hereditary disease, is characterized by clinical manifestations of both neurofibromatosis type 1 (NF1) and NS. We present a case of NFNS with short stature caused by a heterozygous nonsense variant of the *NF1* gene. A 12-year-old boy was admitted because of short stature, numerous café-au-lait spots, low-set and posteriorly rotated ears, sparse eyebrows, broad forehead, and inverted triangular face. Cranial and spinal magnetic resonance imaging showed abnormal nodular lesions. Molecular analysis revealed a novel heterozygous c.6189 C > G (p.(Tyr2063*)) variant in the *NF1* gene. The patient was not prescribed recombinant growth hormone (GH) therapy because exogenous GH may have enlarged the abnormal skeletal lesions. During follow-up, Lisch nodules were found in the ophthalmologic examination. NFNS, a variant form of NF1, is caused by heterozygous mutations in the *NF1* gene. The mechanism of GH deficiency caused by NF1 is still unclear. Whether NFNS patients should be treated with exogenous GH remains controversial.

Keywords: Neurofibromatosis-Noonan syndrome, growth hormone deficiency, *NF1* gene

Introduction

Neurofibromatosis-Noonan syndrome (NFNS) (OMIM #601321), a rare autosomal-dominant hereditary disease, was first reported in 1985 by Allanson and colleagues. Patients with NFNS have clinical manifestations of both neurofibromatosis type 1 (NF1) (OMIM #162200) and NS (OMIM #163950) (1,2). NFNS, NF1, and NS belong to the RASopathies caused by dysregulation of the RAS-mitogen-activated protein kinase (MAPK) signaling pathway (3). Several *NF1* gene mutations have been reported to be associated with NFNS (3,4,5,6,7), such as a *de novo* heterozygous deletion of exons 1-58 (7) and a heterozygous mutation c.7549 C > T in exon 51 (6). However, the heterozygous nonsense variant c.6189 C > G, (p.(Tyr2063*)) in the *NF1* gene has not previously been reported. Here, we report a novel *NF1* variant detected in a 12-year-old patient with NFNS who had short stature.

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Case Report

A 12-year-old boy was referred for evaluation of short stature. His parents were not consanguineous and there was no family history of genetic disease. At birth, café-au-lait spots of different sizes had been observed. He was born of full-term, vaginal birth and his birth length and weight were 52 cm and 3.8 kg, respectively.

On physical examination, the height and weight of the child were 136.5 cm [-2 standard deviation (SD) to -3 SD] and 25.5 kg (-2 SD to -3 SD), according to the height- and weight-standardized growth charts for Chinese children and adolescents aged 0-18 years (8). Upper/lower segment ratio was 0.94, and the arm span was 130 cm, suggesting no skeletal deformity. The dysmorphic facial features, including low-set and posteriorly rotated ears, sparse eyebrows, broad forehead, and inverted triangular face, were suggestive of NS. Unfortunately, the patient's parents declined to provide clinical photographs for publication. Relative macrocephaly, axillary freckling, and more than six café-au-lait spots (Figure 1A) with the largest measuring 2.5 cm × 4.2 cm (Figure 1B) suggested NF1. No abnormalities were detected on neurological and cardiovascular examination. His developmental milestones were normal for his age and he had no cutaneous neurofibroma. No Lisch nodules were observed on ophthalmological examination during the initial diagnosis. The pubertal stage was classified as Tanner stage 1. Serum insulin-like growth factor-1 (IGF-1) levels were approximately -1 SD for his age. Peak growth hormone (GH) response to pyridostigmine bromide and L-dopa was 4.38 ng/mL. Male tumor marker levels as well as thyroid and adrenal gland function tests were normal. The detailed auxological parameters and hormone levels are shown in Table 1.

Chest radiography showed thoracolumbar scoliosis (Figure 2A), and the bone age lagged 2 years behind the chronological age. Cranial magnetic resonance imaging (MRI) showed abnormal nodular signals bilaterally in the basal ganglia-thalamus region (Figure 2B). Spinal cord MRI revealed slight localized thickening and nodular appearance of the C8 nerve originating from the left brachial plexus nerve (Figure 2C); thoracic spinal cord showed no obvious abnormality, while the anterior branch of the fifth lumbar nerve on the left was slightly thicker than the contralateral at the L5/S1 level (Figure 2D).

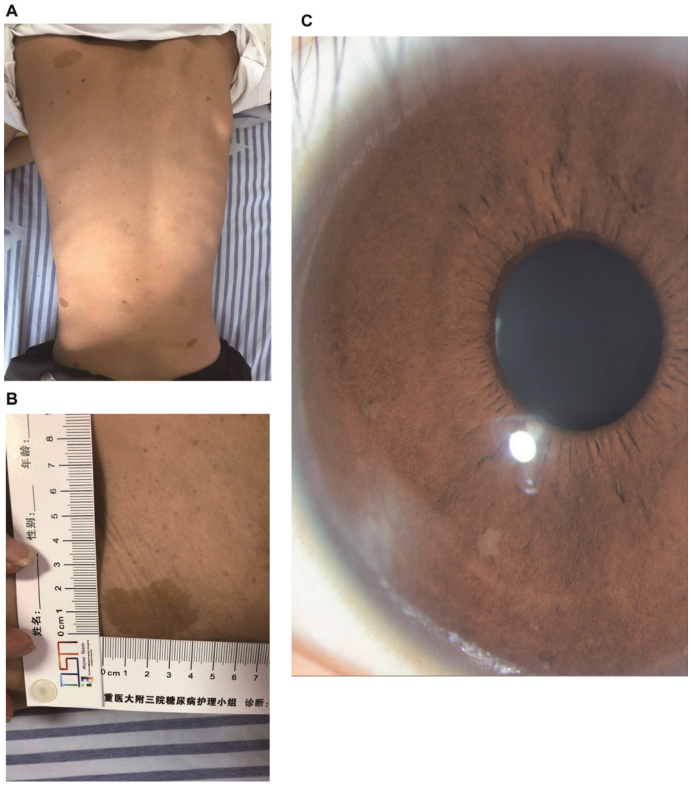

Figure 1. Phenotypic findings of the patient. A, B) Café-au-lait spots on the skin. C) Ophthalmologic findings: Lisch nodules

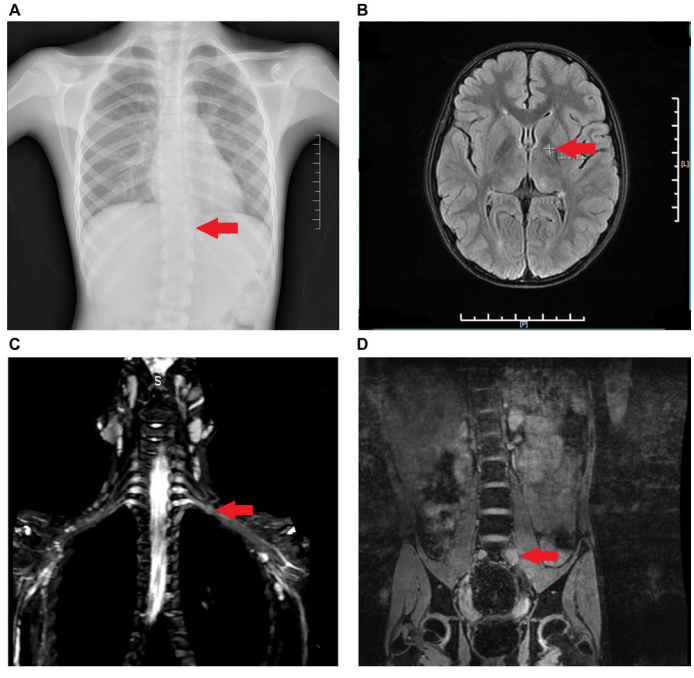

Figure 2. A) Chest radiograph showing thoracolumbar scoliosis. Magnetic resonance imaging of brain and spinal cord. B) Abnormal nodular signals in bilateral basal ganglia-thalamus region. C) Slight thickening of C8 (the left brachial plexus nerve). D) Slight thickening of the anterior branch of the fifth left lumbar nerve

Genetic analyses were conducted for the patient and his parents. Whole-exome sequencing of the peripheral blood DNA was performed. Molecular analysis revealed a novel heterozygous c.6189 C > G (p.(Tyr2063*)) variant in the *NF1* (NM_000267.3) gene (Figure 3). No mutation was found in the *PTPN11* gene. This novel variant is predicted as likely pathogenic according to American College of Medical Genetics consensus recommendations (null variant, variant not found in public databases) (9). However, his parents did not carry the same gene mutation.

Although the boy had GH deficiency (GHD), he was not treated with recombinant GH replacement therapy as the

Table 1. Auxological parameters and hormone levels of the patient

Age (years)	12	13	14
Bone age (years)	10	12.8	13.1
Height (cm)	136.5	143	150
Weight (kg)	25.5	27.8	33.2
BMI (kg/m^2)	13.7	13.6	14.8
Upper/lower segment ratio	0.94	0.93	0.92
Arm span (cm)	130	139	144
Father's height (cm)	172		
Mother's height (cm)	155		
Predicted adult height (cm)	170		
fT4 (pmol/L)	7.31 (4.27-6.96)		
fT3 (pmol/L)	10.18 (7.95-14.79)		
TSH (µIU/mL)	0.95 (0.670-6.060)		
ACTH (pg/mL)	17.20 (7.2-63.3)		
Blood plasma cortisol (µg/dL) (8:00 am)	8.38 (am: 6.71-22.54; pm: < 10.00)		
IGF-1 (µg/L)	202.00 (143-693)	330.00 (183-850)	
Peak GH level in L-dopa test (ng/mL)	4.38 (≥10)		
Peak GH level in pyridostigmine bromide test (ng/mL)	4.38 (≥10)		

BMI: body mass index, fT4: free thyroxine, fT3: free tri-iodothyronine, TSH: thyroid-stimulating hormone, ACTH: adrenocorticotropic hormone, IGF-1: insulin-like growth factor-1, GH: growth hormone

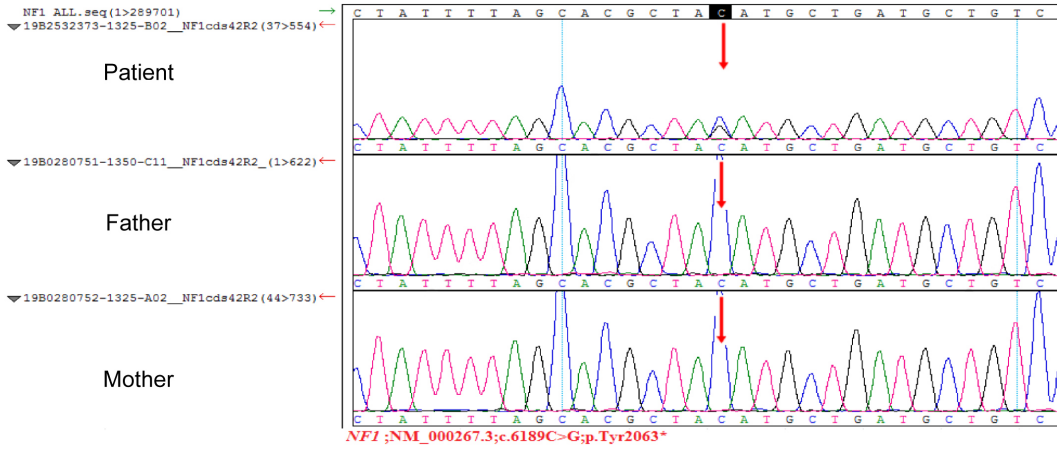

Figure 3. Results of *NF1* Sanger sequencing. NM_000267.3: c.6189 C > G, (p.(Tyr2063*)), heterozygote, nonsense

use of exogenous GH may enlarge the nodular lesions in the brain and spinal cord.

One year after the diagnosis, his height and weight were still 2 to 3 SD below the Chinese reference standards. At the age of 14 years, his height and weight were 150 cm (-2 SD to -3 SD) and 33.2 kg (-2 SD to -3 SD), respectively. Anthropometric follow-up data are shown in Table 1. At the time of writing the child has still not been treated with recombinant GH. However, several Lisch nodules were observed on ophthalmologic examination at a one-year follow-up visit (Figure 1C).

Discussion

NF1 and NS are both related to abnormalities in the RAS-MAPK signaling pathway, but have distinct differences at the genetic level. In patients with NF1, neurofibromin, encoded by the *NF1* gene and acting as a negative regulator in the Ras-MAPK pathway (6), can inactivate or deregulate Ras-GTPase. However, NS is genetically heterogeneous. *PTPN11*, *RAF1, SOS1, KRAS, BRAF, SOS2* and 14 other genes are related to NS; in particular, the *PTPN11* gene has been implicated in the etiology of more than 50% of NS cases (10). In NS patients, SHP2 protein, encoded by the *PTPN11* gene and acting as a positive regulator of Ras-mediated signaling transduction, can activate Ras signaling pathway (6).

Several NFNS patients have been reported to date. Among these, several cases showed the co-occurrence of *NF1* and *PTPN11* mutations (4,11); however, the majority of genetic studies only identified mutations in the *NF1* gene (7,12). Investigations have been performed to determine whether NFNS represents a variable manifestation of either NS or NF1, or is an independent disease. These investigations (3,13) have found that NFNS, a variant form of NF1, is caused by heterozygous mutations in the *NF1* gene. *NF1* was also the only pathogenic variant gene causing NFNS in the presented case.

Genetically, the variants related to NF1 and NFNS are associated with 17p11.2 (5,10,13,14,15,16,17). Moreover, the combination of a mutation in the *NF1* gene and an environmental epigenetic factor resulting in muscle hypotonia may cause the NFNS phenotype (18,19). Ekvall et al. (3) reviewed different *NF1* mutations reported in patients with NF1 and NFNS, and identified peculiar characteristics of the variants associated with NFNS with respect to type and location. Firstly, compared to NF1, a higher prevalence of in-frame deletions and missense mutations were found in NFNS. Secondly, small insertions, splicing mutations, and small indels were more common in NF1 compared to NFNS. Thirdly, NFNS showed a tendency for clustering of in-frame mutations in the GAP related domain (exon 20-27a). Finally, small deletions seemed to be clustered in the cysteine/serine-rich domain (exon 11-17). A subsequent literature search related to NFNS found three novel mutations: a *de novo* heterozygous deletion including exons 1-58 of the *NF1* gene (7), a novel heterozygous c.3052_3056delTTAGT (p.(L1018*)) variant (12), and a truncating mutation c.7846 C > T (p.(Arg2616*)) (20). In our case, the novel nonsense variant failed to conform to these characteristics. Although *de novo* occurrence is the likely explanation for this patient's illness, gonadal mosaicism in one of his parents could not be excluded.

Moreover, the pathogenicity of this nonsense variant was predicted by PolyPhen, Sorting Intolerant From Tolerant (SIFT), the loss of function (LoF) tool (21), and Combined Annotation Dependant Depletion (CADD) phred scores (22). On *in silico* analysis, the LoF tool score was 0.116, and CADD phred score for the same variant was 36.0; however, PolyPhen and SIFT failed to predict this variant. A low LoF tool score implies a damaging effect and a CADD score above 10 indicates a deleterious effect of the variant on the gene (23). Therefore, this novel variant may impair both the gene structure and protein function.

GHD is common in patients with NF1, NS, and NFNS. There is a plausible explanation for the relationship between GHD and NF1. First, the *NF1* gene may affect pituitary development. Hegedus et al. (24) showed a reduced size of the anterior pituitary gland and decreased body weight in mice with deactivated *NF1* gene; in addition, the reduced anterior pituitary gland size could present decreased expression of neurofibromin in the hypothalamus, resulting in diminishing production of GH-releasing hormone, GH, and IGF-1. Therefore, a common cause of short stature in these diseases is suprasellar lesions, but GHD can be found in some patients lacking a fundamental suprasellar lesion (25). Second, neurofibromin may regulate body growth by acting on the hypothalamic-pituitary axis (24) but the pathophysiological mechanisms and related signaling pathways are still unclear. This may explain why our patient had GHD in the absence of pituitary lesions. Further high-quality studies are required to uncover the underlying signaling pathways.

Recombinant GH replacement is the canonical treatment for GHD (26) but there is no consensus as to whether NFNS patients should be treated with exogenous GH. To date, only two case reports (20,27) have described GH treatment in NFNS with GHD. Use of recombinant GH may lead to the enlargement of nodular abnormal lesions and so recombinant GH replacement therapy was not used in the

presented case because of these probable contraindications. Selumetinib, an oral, specific inhibitor of MAPK kinase 1 and 2, has been used in NF1 children with inoperable plexiform neurofibromas (28). However, this drug was not available for our case in China.

Conclusion

Owing to the rarity of NFNS and difficulty in clinical diagnosis, there is limited experience in managing NFNS patients with GHD. The presented case showed clinical manifestations of NFNS with GHD and had a novel heterozygous mutation in the *NF1* gene. However, due to the limited number of related trials, further research on the effectiveness and safety of recombinant GH or selumetinib in treating NFNS patients with GHD would be helpful.

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Ethics

Informed Consent: Written informed consent was obtained from the patient's father.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Si Qin, Yinxing Ni, Jian Zhong, Concept: Si Qin, Yinxing Ni, Jian Zhong, Design: Si Qin, Yinxing Ni, Jian Zhong, Data Collection or Processing: Si Qin, Yindi Zhang, Fadong Yu, Analysis or Interpretation: Si Qin, Yindi Zhang, Fadong Yu, Literature Search: Si Qin, Yindi Zhang, Fadong Yu, Writing: Si Qin, Yinxing Ni, Jian Zhong.

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CASE REPORT

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Unfavorable Effects of Low-carbonhydrate Diet in a Pediatric Patient with Type 1 Diabetes Mellitus

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What is already known on this topic?

Low-carbohydrate diet is used in adults who are obese or have type 2 diabetes. Its use in children is limited to resistant epilepsy.

What this study adds?

Low-carbohydrate diet is not recommended for glycemic control in children with type 1 diabetes mellitus (T1DM), as it causes growth retardation, increased blood lipid levels, and risk of cardiovascular disease. Nutritional therapy for children and adolescents with T1DM should be based on widely accepted clinical guidelines.

Abstract

A balanced and healthy diet is very important in type 1 diabetes mellitus (T1DM) in childhood. In addition to regulating blood glucose with diet, diet should also support optimal growth. Low-carbohydrate diet aims to provide daily energy from fats and was originally used for childhood epilepsy. We present a patient with T1DM who experienced unfavorable effects when on a low-carbohydrate diet.

Keywords: Low-carbohydrate diet, type 1 diabetes mellitus, nutrition therapy, childhood

Introduction

Nutritional therapy is one of the basic mainstays of type 1 diabetes mellitus (T1DM) management and should include macro- and micro-nutrients that are based on universally accepted clinical guidelines. In addition, nutritional therapy should support the normal growth and development of children while also aiding in metabolic control, and should comply with healthy eating principles and the nutritional habits of the individual (1,2). In the guidelines of the International Pediatric and Adolescent Diabetes Association it has been suggested that for energy needs 45-55% be met from carbohydrates, 30-35% from fats (< 10% saturated fat + trans fatty acids), and 15-20% from proteins (1).

The ketogenic diet (KD) is a low-carbohydrate (low-carb), protein-limited and high-fat diet that aims to provide daily energy from fats. Low-carb diets have recently been popularized in social media, by showing healthy benefits. Here, we present the clinical and laboratory findings of a patient who has been on a low-carb diet for two years after the diagnosis of T1DM and the negative effects of this nutrition model in childhood will be discussed.

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Case Report

A 6.42 year-old female patient attended our clinic because of being overweight. She had been overweight from the age of three. She was born with a birth weight of 2.950 g at term. There were no unusual features in her prenatal and natal history. Her mother had Hashimoto thyroiditis (HT), father had multiple sclerosis, and 25-year-old sister had T1DM and had been using insulin therapy for 18 years. Her height was 121 cm [0.49 standard deviation score (SDS)], body weight (BW) was 31 kg (1.78 SDS) and body mass index (BMI) was 21.17 kg/m^2 (1.87 SDS). Her blood pressure was 120/70 mmHg, thyroid gland was non-palpable, and pubertal stage was Tanner stage 1. Laboratory examinations revealed a glucose 90 mg/dL, normal liver transaminases (SGOT 34 U/L NR <47 and SGPT 24 U/L NR 0-39), cholesterol 217 mg/dL (NR 96-211), low-density lipoprotein (LDL) cholesterol 140 mg/dL (NR 38-140), high-density lipoprotein (HDL) cholesterol 57 mg/dL (NR >35), triglycerides 102 mg/dL (NR 35-110), a thyroid stimulating hormone (TSH) of 3.67 µIU/mL (NR 0.6-5.5), and a free thyroxine (fT4) of 1.26 ng/dL (NR 0.8-1.9). No fatty liver was detected in the abdominal ultrasonography (USG). The patient, who was overweight, was followed-up by making recommendations for a healthy diet with appropriate calories for her age and exercise.

In the first year of her follow-up, when she was 7.33 years old, she attended with complaints of increased appetite and not gaining weight. Her height was 127 cm (0.74 SDS), BW was 34 kg (1.98 SDS) and BMI was 21.08 kg/m^2 (1.94 SDS), blood pressure was 110/70 mmHg, pubertal stage was Tanner stage 1 and other system examinations were normal. Her blood sugar was 280 mg/dL (NR <100 mg/dL), hemoglobin A1c (HbA1c) 7.8%, and c-peptide 0.925 ng/mL (NR 1.1-4.4). The patient had positive diabetes autoantibodies with islet antibody 67.05 U/mL (<1), anti-GAD 41.45 U/mL (<1) and was diagnosed with T1DM and an intensive insulin regimen (1 U/kg/day; 60% insulin lispro, 40% insulin glargin) was started. A diabetic diet was recommended to her providing 1711 kcal, made up of 53% carbohydrates, 19% protein, and 28% fat (5.8% saturated fatty acids, 3.5% polyunsaturated fatty acids, 16.4% monounsaturated fatty acids). Diabetes education was given to the family and the patient and she was discharged.

The patient, who continued her follow-up in a private endocrinology clinic for a while, was found to have a TSH of 27.4 µIU/mL (0.6-5.5) and fT4 1.42 ng/dL (0.8-1.9) during this period and sodium levothyroxine (LT4) treatment was started for supposed HT. Thyroid antibodies were positive. She then left the pediatric endocrinology clinic and was followed in a private clinic. In this private clinic, the patient was given a low-carb diet consisting of 1342 kcal, 23% carbohydrates, 23% protein, 54% fat (15.8% saturated fatty acids, 11.8% polyunsaturated fatty acids, and 22.8% monounsaturated fatty acids). She was fed with a low-carb diet for two years, and the LT4 treatment was discontinued.

After two years on a low-carb diet, the patient re-attended our department when she was 9.25 years old because her blood sugar was high while she was taking the low-carb diet. At that time, height was 131.7 cm (-0.35 SDS), BW was 26.35 kg (-0.71 SDS), BMI was 15.19 kg/m^2 (-0.69 SDS), goiter stage 1b, puberty Tanner stage 1 and other system examinations were normal. The growth rate during feeding with the KD was 4.7 cm over two years. The patient's complete blood count, serum electrolytes, and kidney function tests were normal. However, liver function tests and lipid profile were SGOT 111 U/L (<47), SGPT 168 U/L (0-39), cholesterol 1029 mg/dL, LDL cholesterol 826 mg/dL, HDL cholesterol 136.2 mg/dL, and triglycerides 334 mg/dL. Thyroid function tests were TSH 10.275 µIU/mL and fT4 1.18 ng/dL. Her HbA1c was 7.78% and c-peptide was 0.41 ng/mL. Stage 1 hepatosteatosis was detected on abdominal USG. Carotid color Doppler USG and echocardiographic examinations were normal. No diabetic retinopathy was detected in the eye examination. The patient was started on intensive insulin regimen (multiple daily injection treatment, 1 U/kg/day; 60% insulin lispro and 40% insulin glargine treatment) and 2 mcg/kg/day LT4 therapy. Her nutrition therapy was revised again to 1908 kcal, provided by 56% carbohydrates, 18% protein, and 26% fat (5.5% saturated fatty acids, 3.4% polyunsaturated fatty acids, and 15% monounsaturated fatty acids). Atorvastatin treatment was started with the recommendation of the metabolism department until the blood lipid parameters decreased to acceptable levels, and ursodeoxycholic acid and vitamin E treatments were started with the recommendation of the gastroenterology department for elevated transaminases. Structured diabetes and diet education was given to the patient and her family again. The mother was reluctant to adhere to the healthy eating plan. In the follow-up, it was observed that there was an improvement in growth rate (5.04 cm/year), liver function tests returned to normal, and cholesterol levels decreased. The follow-up data of the patient is given in Table 1. Dietary contents at diagnosis and follow-up are given in Table 2.

Table 1. Clinical and laboratory findings of our patient with type 1 diabetes who applied low-carb diet

	First presentation	At diagnosis of T1DM	First presentation after low-carb diet	Third month follow-up after a healthy diet plan and intensive insulin therapy	One year after a healthy diet and intensive insulin therapy
Age (years)	6.42	7.33	9.25	9.5	10.42
Height (SDS), cm	121 (0.49)	127 (0.74)	131.7 (-0.35)	132.5 (-0.45)	136.7 cm (-0.65)
Weight (SDS), kg	31 (1.78)	34 (1.98)	26.35 (-0.7)	28.25 (-0.48)	32.85 (-0.31)
Body mass index (SDS), kg/m²	21.17 (1.87)	21.08 (1.94)	15.19 (-0.69)	16.09 (0.31)	17.5 (0.04)
Puberty (Tanner stage)	1	1	1	1	2
Growth rate	-	6 cm/year	2.35 cm/year	3.2 cm/year	5.04 cm/year
HbA1c (%)	-	7.1	7.78	6.19	7.8
C-peptide (ng/mL)	-	1.1	0.41		
Cholesterol (mg/dL)	217	203	1029	348.0	351
LDL cholesterol (mg/dL)	140	125	826	249	255
HDL cholesterol (mg/dl)	57	53	136.2	88.9	80,5
Triglyceride (mg/dL)	102	125	334	50	80
SGOT (U/L)	34	33	111	52	28
SGPT (U/L)	24	22	168	53	17
Abdominal USG	Normal	-	Stage 1 hepatosteatosis	-	
TSH (µIU/mL)	3.675	2.876	10.275	1.091	1.8
fT4 (ng/dL)	1.26	1.07	1.18	1.59	1.5
Anti TPO (IU/mL)	287.6	538.1	1300	-	-
Anti Tg (IU/mL)	29.5	223.2	289.4	-	-

SDS: standard deviation score, HbA1c: hemoglobin A1c, LDL: low-density lipoprotein, HDL: high-density lipoprotein, USG: ultrasonography, TSH: thyroid stimulating hormone, fT4: free thyroxine

Table 2. Composition of the three dietary interventions

	Reference diabetic diet	Carbohyrate-restricted diet	Reference diabetic diet
Age (years)	7.33	9.25	9.5
Total energy intake (kcal)	1711	1342	1908
Carbohydrates (g)	222	75.9	260
Carbohydrates (%)	53	23	56
Fibre (g)	41.4	12.8	46.1
Protein (g)	77.0	75.1	83.2
Protein (%)	19	23	18
Fat (g)	53.6	81.1	55.6
Fat (%)	28	54	26
SFA (g)	11.2	23.6	11.7
SFA (%)	5.8	15.8	5.5
PUFA (g)	6.7	17.7	7.3
PUFA (%)	3.5	11.8	3.4
MUFA (g)	31.3	34.0	31.9
MUFA (%)	16.4	22.8	15.0
Cholesterol (mg)	252.3	319.1	291.9
Trans fatty acids (g)	0	0	0

SFA: saturated fatty acids, PUFA: polyunsaturated fatty acids, MUFA: monounsaturated fatty acids

Discussion

Low-carb diets, which have recently become one of the trendy diets, have been popularized as promoting good health in the social media, individual internet blogs, on television and in nutrition magazines. If daily energy is provided by more than 55% carbohydrate intake, it is called a high carbohydrate diet, while approximately 45% energy provision is average carbohydrate, while less than 26% intake is low-carb, and if there is less than 10% intake, it is called very low-carbdiet (3). The KD is a low-carb, protein-limited, high-fat, long-chain triglyceride-rich diet that aims to meet daily energy from fats. Protein intake is kept at the lower limit of daily requirement and carbohydrate intake is severely limited. The use of KD in the pediatric population is limited to epilepsy treatment (4). While the side effects of the KD in the acute period are vomiting and fatigue, in the chronic period, side effects such as stagnation in growth and development, impaired lipid profile, vitamin-mineral deficiencies, pancreatitis, kidney stones, arrhythmia, cardiomyopathy, and osteopenia have been reported (3,5,6,7).

The use of KD has come to the fore in individuals with diabetes, with the thought that it reduces both glycemic fluctuations and insulin need with less carbohydrates, but studies investigating its place in T1DM treatment are mostly studies with a small sample, conducted in the adult age group (5,6). Childhood diabetes differs from adult diabetes in that it has longer sleep duration, frequent infections, unpredictable physical activity and eating patterns, non-adherence to treatment in adolescence, concerns about appearance, variable metabolic status, and insulin requirement (1). Therefore, effective treatment and management of pediatric and adult T1DM will differ.

Since nutrition with KD cannot provide enough energy in growing children, the most striking feature is the slowdown in growth rate. The present case only grew 4.7 cm in height over the two years that she used a KD, which was abnormally low for her age. After a healthy diet and multi-dose insulin therapy, the annual growth rate returned to normal. There is a misconception that less carbohydrate consumption and therefore reduced insulin requirement are better in KD nutrition, but insulin is directly and indirectly effective in cell growth and proliferation. Insulin acts by binding directly to the insulin-like growth factor-1 (IGF-1) receptor, indirectly increasing the hepatic production of IGF-1, and is in a synergistic relationship with growth hormone and other growth stimulating factors (3). Thyroid hormones are also effective in growth. Our patient's thyroid mismanagement may have contributed to the poor linear growth. As in KD, high-fat diets have also been shown to blunt pituitary growth hormone secretion. Growth arrest has been reported in the literature in patients with T1DM fed low-carb diets (2,7). It has also been shown that children with T1DM who receive intensive insulin therapy and are fed low-carb and high fat have worse glycemic control and higher HbA1c values (8,9). There are also studies showing that feeding with KD increases the risk of hypoglycemia and impairs the effectiveness of glucagon used in the treatment of hypoglycemia (10). In nutritional studies, it has been observed that as the amount of carbohydrates in the daily diet decreases, children tend to consume lower quality foods (11).

Another negative aspect of KD is that it increases blood lipids. Since the blood lipid results in our patient were above the values seen in familial homozygous hypercholesterolemia, which is the most severe form of hypercholesterolemia, and the lipid profile measured at the age of 6.42 years was close to normal, increased lipid levels may be considered as a serious complication of KD in this case. This dyslipidemia after feeding with KD have been reported previously (2,3,4,5,6,7). In a series in which six children with T1DM who followed a low-carb diet were reported, there was a case with dyslipidemia (2). The development of dyslipidemia is associated with excessive consumption of saturated fats instead of carbohydrates and, worryingly, increases the risk of cardiovascular disease (11).

Consumption of more than one type of food group and making restrictions in nutrition in childhood may also result in psychological comorbidities. This type of diet may cause social isolation and the related psychosocial burden in children. In addition, this restricted eating pattern may lead to eating disorders in the future (2).

Conclusion

In summary, in T1DM the nutrition plan should include sufficient energy and micro- and macro-nutrients to ensure the growth of the child. Nutritional therapy with KD is not recommended for glycemic control in children with T1DM, as it causes growth retardation, increased blood lipid levels, and increased risk of cardiovascular disease. Nutritional therapy for children and adolescents with T1DM should be based on universally accepted clinical guidelines.

Ethics

Informed Consent: Written informed consent was obtained from the parents of the patient.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ceren Güleryüz, Ece Eker, Gülin Karacan Küçükali, Merve Şakar, Fatma Nur Genç, Nursel Muratoğlu Şahin, Selin Elmaoğulları, Semra Çetinkaya, Şenay Savaş Erdeve, Concept: Gülin Karacan Küçükali, Nursel Muratoğlu Şahin, Semra Çetinkaya, Şenay Savaş Erdeve, Design: Gülin Karacan Küçükali, Nursel Muratoğlu Şahin, Semra Çetinkaya, Şenay Savaş Erdeve, Data Collection or Processing: Ceren Güleryüz, Ece Eker, Merve Şakar, Fatma Nur Genç, Selin Elmaoğulları, Analysis or Interpretation: Gülin Karacan Küçükali, Fatma Nur Genç, Semra Çetinkaya, Şenay Savaş Erdeve, Literature Search: Ceren Güleryüz, Ece Eker, Gülin Karacan Küçükali, Semra Çetinkaya, Şenay Savaş Erdeve, Writing: Ceren Güleryüz, Ece Eker, Gülin Karacan Küçükali, Semra Çetinkaya, Şenay Savaş Erdeve.

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Letter to: Endocrinological Approach to Adolescents with Gender Dysphoria: Experience of a Pediatric Endocrinology Department in a Tertiary Center in Turkey

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Keywords: Gender dysphoria, GnRH agonists, puberty blockade

Dear Editor,

There are some contradictions, ambiguities, and even ethical and legal violations in this study published in your journal, which performed pubertal suppression (PS) in 22 adolescents and gender reassignment surgeries (bilateral mastectomy, voice and facial feminization, and breast augmentation) in 7 adolescents under the age of 18. This is against the law in Turkey.

In the conclusion/abstract, it is stated that "no significant side effects were observed". However, there is no evidence to support this conclusion in the results (moreover, a decrease in BMD-z scores was detected). Also in the results, the findings are not presented, only some limited information describing the sample is presented. Similarly, the methods are not explained in the method.

"All physical examinations were performed by the same physician at each visit (ECO)". So who did the psychiatric evaluation? This was not explicitly stated, instead using the general phrase "made by a gender identity expert". This situation raises the suspicion that the psychiatric evaluation is not performed by the appropriate person(s).

Although genital examination was not performed in most of the adolescents, the authors state, *"No signs of a difference/disorder of sex development were detected, and all subjects had appropriate sex characteristics of the sex assigned at birth".* How was this result obtained without genital examination? How were possible genital pathologies/intersex ruled out? (cannot be done). Also, how can gonadotropin-releasing hormone (GnRHa)/CSH be used without genital examination?

The authors report that gender dysphoria (GD) begins in adolescence (>10 years) in 28.3% of cases. There is no indication for PS in these adolescents according to the original criteria of the Dutch model (1,2). Also, in how many adolescents did GD flare up with puberty? What were the psychiatric comorbidities in these adolescents? Was adequate psychological and social support provided during PS? These criteria were not taken into account in the study, meaning that a significant portion of these adolescents did not meet the eligibility criteria of the Dutch model.

The guidelines cited in the study were also not followed (in terms of principles such as adequate psychiatric follow-up, detection of permanent GD, detection/treatment of psychiatric comorbidities, eligibility criteria for GnRHa/CSH, confirmation of mental competence for detailed consent, and psychosocial support) (3).

The authors not only ignore the side effects they detected, but also do not mention the side effects and dilemmas of PS known in the literature (4). They ignore the current literature. PS has many physical and mental side effects, and its long-term consequences are also unknown (4,5). In addition, the expected benefit cannot be achieved with PS. A team from

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the Tavistock clinic in the UK detected in 2021 showing no detectable improvement in mental health among young people who were administered puberty-blocking drugs and were followed for up to three years (5).

In conclusion, the study contains methodological and presentational problems and ethical and legal violations. The authors even ignore the side effects they detected and state in the main message that "no significant side effects were observed". This shows that the authors focused on their intended results and were biased.

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J Clin Res Pediatr Endocrinol 2023;15(4):451-452

In response to: "Letter to: Endocrinological Approach to Adolescents with Gender Dysphoria: Experience of a Pediatric Endocrinology Department in a Tertiary Center in Turkey"

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Keywords: Gender dysphoria, adolescent, pubertal suppression

Response to the Letter,

The accusations of Dr. Bayraktar are relying on a false narrative about the article published in Journal of Clinical Research in Pediatric Endocrinology. Medical treatment of transgender patients is evidence based, and it is not against the law in Turkey. The surgeries mentioned in the article are not gender-affirming genital surgeries and it has been clearly stated that these surgeries are done without our initiative and referral.

There were no significant and unexpected adverse effects of the medical treatment as has been stated clearly in the paper.

Disorders of sex development were excluded in all cases, and this was also confirmed through medical history, physical and hormonal evaluations. The psychiatric examinations have been performed by psychiatrists who are experts in the field. All evaluations, including the psychiatric assessments, were documented. Pediatric endocrinologists only provide treatment to transgender youth after the diagnosis has been made by psychiatrists and referred to endocrinologists. The focus of the article is endocrinological approach to transgender people and experience of an endocrinology department, and this is clear all through the article. That explains why the accompanying psychiatric process/treatment has been presented in a sufficient context, but has not become the focus of the paper. All cases met the criteria for gonadotropin-releasing hormone (GnRHa) therapy after being referred to us by the diagnosis of gender dysphoria and pubertal suppression may be administered (1). All the cases continued their psychological treatment as recommended by international guidelines (2).

This study has consent from the Ethical Committee of the İstanbul Faculty of Medicine that found no ethical violation, in contrast to what Dr. Bayraktar claims, without any evidence.

Although prospective cohort studies focusing on long-term effects are needed (which is currently under research in various centers) for optimizing the medical and mental healthcare for transgender youth (5), the up-to-date international and national clinical practice guidelines relying on expert opinions and based on the best available evidence, recommend the use of treatments to suppress the rise in sex hormones in adolescents experiencing gender dysphoria during puberty (1,2,3,4).

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A recent study cited by the author of the Letter detecting a slight change in the psychological functioning, contrary to the findings of the majority of existing literature, still calls for larger and longer-term prospective studies to fully quantify the benefits and harms of GnRHa treatment (6). However, this study also notes positive overall patient experience of changes on GnRHa treatment, as well as predominantly positive or neutral changes in family and peer relationships (6). Together with the psychological functioning, these reversible treatments are used mainly "to prevent the development of secondary sex characteristics and provide time up until 16 years of age for the individual and the family to explore gender identity, access psychosocial supports, develop coping skills, and further define appropriate treatment goals" (4).

Furthermore GnRHa's have been used for early puberty for nearly two decades and long term studies have shown that the effects are reversible with no major adverse events.

The study meets the rules of scientific research and makes an important contribution to the literature on the scientific medical approach to adolescents with gender dysphoria.

Feyza Darendeliler, on behalf of all authors

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The mistake has been made inadvertently by the author.

The institution information of Ekrem Akbulut (institution number 2), one of the authors on the first page of the article, has been corrected by the author as follows.

Incorrect institution information of Ekrem Akbulut (institution number 2).

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