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Papers Published in Periodical Journals: Gungor N, Saad R, Janosky J, Arslanian S. Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents. J Pediatr 2004;144:47-55.

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Book Chapters: Darendeliler F. Growth Hormone Treatment in Rare Disorders: The KIGS Experience. In: Ranke MB, Price DA, Reiter EO (eds). Growth Hormone Therapy in Pediatrics: 20 Years of KIGS. Basel, Karger, 2007;213-239.

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Original Articles

Case Reports

Letter to the Editor

CONTENTS

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CONGRESS CALENDAR

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

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

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.3908

J Clin Res Pediatr Endocrinol 2017;9(2):95-100

Hypogonadotropic Hypogonadism due to Novel FGFR1 Mutations

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What is already known on this topic?

Hypogonadotropic hypogonadism is a well-known rare disorder of very variable genetic etiology.

What this study adds?

This study has shown that FGFR1 mutations cause phenotypic variations.

Abstract

Objective: The underlying genetic etiology of hypogonadotropic hypogonadism (HH) is heterogeneous. Fibroblast growth factor signaling is pivotal in the ontogeny of gonadotropin-releasing hormone neurons. Loss-of-function mutations in *FGFR1* gene cause variable HH phenotypes encompassing pubertal delay to idiopathic HH (IHH) or Kallmann syndrome (KS). As FGFR1 mutations are common, recognizing mutations and associated phenotypes may enhance clinical management.
Methods: Using a candidate gene approach, we screened 52 IHH/KS patients.
Results: We identified three novel (IVS3-1G > C and p.W2X, p.R209C) *FGFR1* gene mutations. Despite predictive null protein function, patients from the novel mutation families had normosmic IHH without non-reproductive phenotype.
Conclusion: These findings further emphasize the great variability of FGFR1 mutation phenotypes in IHH/KS.
Keywords: Hypogonadotropic hypogonadism, FGFR1 mutations, Kallmann syndrome, reduced penetrance

Introduction

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare clinical disorder characterized by delayed or absent pubertal development (1). IHH has an incidence of 1-10 cases per 100,000 births and it is more common in males (2). If a patient with IHH has an impaired sense of smell, then the condition is called Kallmann syndrome (KS). To date, at least 17 genes have been associated with KS and these include *KAL1, FGFR1, PROK2, PROKR2, FGF8, HS6ST1, CHD7, WDR11, SEMA3A, FGF17, IL17RD, DUSP6, SPRY4, FLRT3, NELF, FEZF1,* and *CCDC141* (3).

KS is most commonly caused by mutations in anosmin 1 encoded by *KAL1* (2,3,4,5). *FGFR1* encodes a tyrosine kinase receptor that mediates fibroblast growth factor signaling (6). Presence of various congenital anomalies which are not associated with the reproductive system such as defects in kidney and tooth differentiation, ear and palate morphogenesis and development of interhemispheric or cortico-spinal axonal tracts encountered in a proportion of KS patients due to FGFR1 mutations indicates that the fibroblast growth factor signaling plays important roles in many other developmental processes. The precise roles played by *KS* genes in these processes are not known (6). *FGFR1* has been shown to be a key factor for angiogenesis, embryogenic development, and wound healing (7,8). FGFR1 knock-out mice do not have telencephalon and have an altered gonadotropin-releasing hormone (GnRH) migration

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(9). *FGFR1* mutations cause KS/IHH with or without defects in the reproductive system (10).

In this study, aiming to contribute to the genotypic and phenotypic correlation in IHH/KS cases, we report familial cases due to novel FGFR1 mutations.

Methods

We screened 52 IHH/KS patients (36 male, 16 female) in our cohort. The majority of patients (n = 38) were normosmic IHH (nIHH) and the remaining 12 were cases of anosmic or hyposmic IHH (KS). Diagnosis of IHH/KS was based on delayed or absent spontaneous puberty by age 13 in girls (Tanner breast stage 1) and by age 14 in boys (testicular volume < 4 mL). The patients had bone ages of 11.5 years or greater, with concentrations of serum testosterone and estradiol at hypogonadal levels [< 20 ng/dL (714 pmol/L) and < 1.9 ng/dL (73 pmol/L), respectively] in the setting of inappropriately normal or low serum gonadotropins. Serum levels for thyroid-stimulating hormone (TSH) with free thyroxin (fT_4), prolactin, insulin-like growth factor-1, adrenocorticotropic hormone, and cortisol were within normal limits. Exclusion criteria included chronic systemic diseases (impaired renal function, thalassemia, poorly controlled diabetes mellitus), eating disorders (bulimia or anorexia nervosa), or structural anomalies on hypothalamo-pituitary imaging. Sense of smell of the probands was tested while subjecting them to a battery of 10 culturally appropriate odors. This study was approved by the Ethics Committee of Çukurova University Faculty of Medicine. Written informed consents were obtained from all subjects.

Laboratory Methods

Serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone, estradiol, prolactin, fT_4, and TSH were determined by immunofluorometric assays. A GnRH stimulation test (2.5 µ/kg, maximum 100 µ, IV) was performed in all probands. Serum LH and FSH levels were measured at 0, 15, 30, 45, and 60 min after GnRH injection.

For molecular genetic studies, genomic DNAs were isolated from white blood cells. The coding and neighboring intronic regions of the known or strong candidate genes for nIHH (*GNRHR, GNRH1, TACR3, TAC3, FGFR1, KISS1R,* and *KISS1*) or KS (*KAL1, FGFR1, PROK2, PROKR2*) were amplified by polymerase chain reaction (PCR). PCR products were purified and directly sequenced using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif., USA) in an ABI PRISM 3130 automatic sequencer.

Whole Exome Sequencing was performed at Yale Center for Genome Analysis using NimbleGen 2.1M human exome array (Roche NimbleGen, Inc.) according to the manufacturer's protocol with certain modifications, as described previously (11). Sequencing of the library was performed on HiSeq2000. The Illumina pipeline version 1.8 was used for image analysis and base calling.

Case Reports

Family 1

The proband (II-4) was a 17-year-old female patient who was referred for lack of breast development and primary amenorrhea. Her height and weight were 161 cm (25-50th percentile) and 43 kg (< 3th percentile), respectively. She had a normal sense of smell. Her breast development was at Tanner stage 2 at the right and stage 1 at the left breast. Her axillary hair and pubic hair were at stages 3 and 2, respectively.

Her estradiol and gonadotropins were at prepubertal levels. A GnRH stimulation test revealed maximal LH and FSH concentrations of 7.0 and 7.7 mIU/mL, respectively. Chromosome analysis showed a 46,XX karyotype. Her pelvic ultrasonography and cranial magnetic resonance imaging (MRI) results were normal.

One of her sisters (II-2), a 22-year-old female who had complaints of absent breast enlargement and primary amenorrhea and who had been given estrogen treatment in another clinic was also diagnosed as a case of IHH. This patient's height and weight were 165 cm (50-75th percentile) and 55 kg (25-50th percentile), respectively. Her sense of smell was normal. Her pubic and axillary hair were both at stage 3, while her breast Tanner stage was 4. Her karyotype was 46,XX. Her serum plasma estradiol, LH, and FSH levels were prepubertal (Table 1). Their parents were healthy cousins. The family is of Turkish origin.

One of the proband's half-sisters (II-1), a 16-year-old female whose chief complaints were delayed puberty and primary amenorrhea, was also diagnosed to have IHH. This patient was also given estrogen treatment elsewhere. Her height and weight were 158 cm (25th percentile) and 60 kg (50-75th percentile), respectively. Her sense of smell was normal. Her axillary and pubic hair were both at stage 2, while her breast Tanner stage was 3. Her karyotype was 46,XX. Her serum plasma estradiol, LH, and FSH levels were prepubertal (Table 1). Her parents were healthy and unrelated.

Family 2

The proband (II-1) was an 18-year-old female patient who was also referred for lack of breast enlargement and primary amenorrhea. Her height and weight were 159 cm

and 50.5 kg, respectively. Her axillary and pubic hair were at stage 3 while her breast Tanner stage was 2 bilaterally. Her sense of smell was normal. Her serum estradiol, FSH, and LH levels were 10.6 pg/mL, 1.0 mIU/mL, and 0.2 mIU/mL, respectively. Her karyotype was 46,XX. A GnRH stimulation test elicited peak levels of FSH and LH as 2.8 and 2.1 mIU/mL, respectively. Her brain MRI and pelvic ultrasonography results were normal.

One of her siblings (II-2), a 15-year-old boy, was also referred for delayed puberty. His testicular volumes were 2 mL bilaterally. Axillary and pubic hair were at stage 1. He reported a normal sense of smell. His reproductive hormone levels were prepubertal (Table 1). His karyotype was 46,XY. A GnRH stimulation test revealed peak LH and FSH levels of 10.4 and 7.5 mIU/mL, respectively. His cranial MRI was normal. The parents are healthy and of Turkish origin.

Family 3

The proband (II-1) is a 14-year-old male patient who was referred for micropenis and absence of erections. He had a decreased sense of smell. Pubic and axillary hair were at stage 3. Testicular volumes were 2 mL bilaterally. He had no midline anomalies. His height and weight were 54 kg (25-50th percentile) and 160 cm (25-50th percentile), respectively. His basal testosterone and gonadotropin levels were prepubertal. His karyotype was 46,XY. His cranial MRI was normal. A paternal uncle (I-2) of his also reportedly suffers from absent puberty and anosmia. This family is ethnically Arabic.

Results

A Sanger sequence analysis of the entire coding regions of *FGFR1* (HGNC:3688 NM_001174063, NP_001167534) revealed three novel mutations (Figure 1).

A whole exome sequencing on probands confirmed these variants but did not reveal any more potentially contributing variants in other known IHH/KS-associated genes.

The affected three sisters and their unaffected mother from family 1 were found to have the heterozygous IVS3-1G > C (g.G38886C) novel mutation. This splicing mutation in intron 3 is predicted to cause skipping of exon 4, eventually resulting in a totally different protein product as the last nucleotide of the exon 3 forms a codon with the first two nucleotides of exon 5. Stop codon formation occurred after 50 amino acids. Human Splicing Finder (www.umd.be), an

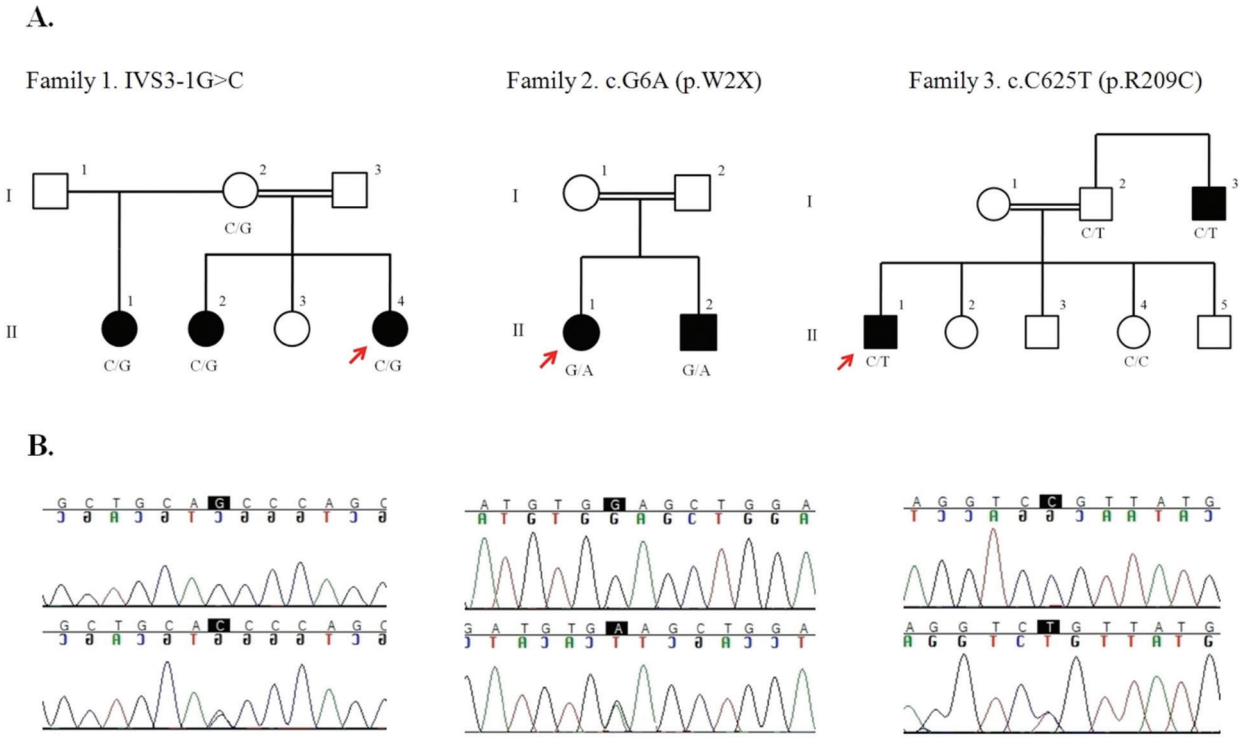

Figure 1. Segregation of the FGFR1 mutations in families with affected individuals. (A) Pedigrees and descriptions in each family are shown. Filled symbols show patients with normosmic idiopathic hypogonadotropic hypogonadism/Kallmann syndrome; open symbols show clinically unaffected individuals. Squares indicate male family members, circles indicate female family members, the double line indicates consanguinity, and arrows point to probands. Genotypes are shown below each tested family members. (B) DNA sequence analysis of patients. The positions of the mutations are marked. The top lines show the homozygous wild-type genotype, and the bottom lines show heterozygous genotype

in silico prediction program for splicing variants, predicted this variant as "alteration of the wild type acceptor site most probably affecting splicing".

In family 2, affected siblings had the p.W2X (c.G6A) mutation in the heterozygous state. Parents were not available for testing.

In family 3, the heterozygous p.R209C (c.C625T) mutation was found in affected individuals as well as in the unaffected father of the proband. One hundred alleles from healthy individuals of Arabic origin did not show this variant.

Severe changes in protein structure and function were predicted in the splice site (IVS3-1G > C) mutation in family 1 and the nonsense mutation (p.W2X) in family 2. The mutation in family 3 (p.R209C) was predicted to be disease causing by Mutation Taster (http:/www.mutationtaster.org/) (probably damaging, PolyPhen-2 score:1.0) by PolyPhen-2 (http:/www.genetics.bwh.harvard.edu) and damaging by SIFT (www.sift.jcvi.org). Conservation analysis showed that the arginine at 209 was highly conserved across species (Table 2).

Discussion

IHH is a term for heterogeneous disorders due to insufficient gonadotropin secretion. Most cases are referred with a delayed sexual maturation as teenagers. To date, more than 30 genes have been reported to be associated with IHH phenotype (3).

Herein, we report the results of screening for known genes associated with IHH/KS in a cohort of 52 patients. We identified three novel FGFR1 mutations including splicing, missense, and nonsense ones. The IVS3-1G > C (g.G38886C) mutation which involves the Ig-like C2-type 1 domain may cause total absence of the *FGFR1* gene product with premature stop codon that results in mRNA decay (12). To date, eight splicing-site mutations were reported in *FGFR1*. Six of the patients with splicing mutations (75 %) were male (10,13,14). All of these patients were anosmic. Similar to our experience, Raivio et al (14) reported IHH patient with splicing mutation (c.C336T) which also affected exon 3. That anosmic patient was male and had a midline defect (corpus callosum agenesis). Our patients with splicing mutation were normosmic females and they had no symptoms other than those pertaining to the reproductive system. Gender differences may account for these remarkable phenotypic differences. Most notably, their heterozygous mother was reproductively healthy as evidenced by giving birth to four children without reproductive assistance. The normal reproductive phenotype in the mother despite having the same genotype as her affected daughters could be explained by reduced penetrance. This is also observed by others in families with IHH/KS due to FGFR1 mutations (15). These intra and inter-familial remarkable disassociations of phenotype and genotype necessitate further studies and probably indicate versatility of fibroblast growth factor signaling in GnRH ontogeny.

Table 1. Clinical and laboratory characteristics of patients with FGFR1 mutations

	Age	Sex	FSH (mIU/mL) M: 1.4-18.1 F: 2.5-10.2	LH (mIU/mL) M: 1.5-9.3 F: 1.9-12.5	E2 (pg/mL) M: 0.8-3.5 F: 6.3-16.5	Testosterone (ng/mL) 175-781	Mutation
Family 1 The proband (II-4)	17	F	1.96	0.88	3.0	N/A	IVS3-1G > C
Sister (II-2)	22	F	1.45	3.0	4.0	N/A	IVS3-1G > C
Sister (II-1)	16	F	1.12	0.7	7.2	N/A	IVS3-1G > C
Family 2 The proband (II-1)	18	F	1.02	0.20	10.6	N/A	p.W2X (c.G6A)
Brother (II-2)	14	M	1.95	0.58	N/A	< 0.02	p.W2X (c.G6A)
Family 3 The proband (II-1)	14	M	0.75	0.1	N/A	17.2	p.R209C (c.C625T)
Uncle (I-3)	35	M	0.54	0.34	N/A	< 0.07	p.R209C (c.C625T)

FSH: follicle-stimulating hormone, LH: luteinizing hormone, F: female, M: male, N/A: non applicable

Table 2. Evolutionary conservation of the mutated (p.R209C) *FGFR1* amino acid across different species

Species	Alignment
Human	DHRIGGYKV**R**YATWSIIMDSVV
G. gallus	DHRIGGYKV**R**YATWSIIMDSVV
P. waltl	DHRIGGYKV**R**YQTWSIIMDSVV
X. laevis	DQRIGGYKV**R**SQTWSLIMDSVV
S. acanthias	EHRIGGYKV**R**SQHWSLIMEGVV

The proband in family 2 and her affected brother had a nonsense mutation (p.W2X, c.G6A) which causes a premature stop codon formation. This mutation is predicted to cause a total absence of protein product with its early occurrence. There are over a hundred different missense and nonsense mutations in the *FGFR1* gene. This novel mutation (p.W2X) affects the signal peptide which is in the initial point of this gene. Laitinen et al (16) reported three nonsense mutations (p.W4X, p.R609X, p.R262X) in the *FGFR1* gene. Their patient with p.W4X was a male who was diagnosed with KS without accompanying non-reproductive comorbidities. The p.W4X mutation is in close proximity to our mutation. Yet, both our patient and her affected brother had normosmic IHH. Different protective mechanisms or ethnic differences may account for the clinical inconsistency in terms of sense of smell.

Proband 3 and his affected uncle had a missense mutation p.R209C (c.C625T). This mutation affects Ig-like C2-type 2 domain which directly interacts with fibroblast growth factors and heparan sulfate proteoglycans (17). Only one patient with the exact nucleotide change resulting in p.R209C mutation has been previously reported by Tommiska et al (17). Their patient had micropenis and hyposmia. In our study, the affected individuals had a very similar phenotype. Laitinen et al (16) found a c.G626A mutation also resulting in the same aminoacid charge (*i.e.* p.R209C). This patient had also KS but no micropenis, suggesting a milder phenotype.

Concomitant whole exome sequencing data on probands did not reveal any more potentially contributing variants in other known IHH/KS-associated genes. In view of the oligogenic inheritance in IHH/KS (18), this finding is remarkable and may point to FGFR1 variants as the sole mediator of the phenotypes. However, the methods employed in this study cannot rule out copy number variations in the etiology as these variants have been shown to be important in KS (19). In conclusion, our results further substantiate great variability of reproductive and non-reproductive phenotype by various FGFR1 mutations. Expanding phenotype genotype catalogue in this pivotal gene may enhance our capability of clinical management as well as understanding *FGF* signaling.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Çukurova University Faculty of Medicine.

Informed Consent: Written informed consents were obtained from all subjects.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Gamze Akkuş, A. Kemal Topaloğlu, Leman Damla Kotan, Design: Gamze Akkuş, A. Kemal Topaloğlu, Data Collection or Processing: Gamze Akkuş, Erdem Durmaz, Eda Mengen, İhsan Turan, Ayça Ulubay, Fatih Gürbüz, Bilgin Yüksel, Tamer Tetiker, Analysis or Interpretation: Leman Damla Kotan, A. Kemal Topaloğlu, Literature Search: Gamze Akkuş, Leman Damla Kotan, İhsan Turan, Writing: Gamze Akkuş, A. Kemal Topaloğlu.

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Basal Serum Neurokinin B Levels in Differentiating Idiopathic Central Precocious Puberty from Premature Thelarche

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What is already known on this topic?

Kisspeptin and neurokinin B have an important role in regulation of puberty. Differentiation of central precocious puberty (CPP) from premature thelarche (PT) is sometime difficult.

What this study adds?

Kisspeptin and neurokinin B levels were higher in the idiopathic CPP and PT groups compared to controls. We demonstrated that serum neurokinin B level can be used as an adjunctive parameter to differentiate idiopathic CCP from PT.

Abstract

Objective: To find out the diagnostic role of kisspeptin and neurokinin B in idiopathic central precocious puberty (ICPP) and premature thelarche (PT).

Methods: The girls who presented with early breast development before the age of 8 years were evaluated. Patients with intracranial pathologies were excluded. Basal and stimulated follicle-stimulating hormone/luteinizing hormone (LH) levels and basal neurokinin B/kisspeptin levels were measured. Patients who had peak value of LH >5 mIU/mL and a bone age (BA)/chronological age (CA) ratio >1.1 were diagnosed as central precocious puberty (CPP), while cases who did not meet these criteria were diagnosed as PT. Healthy age-matched prepubertal girls were included as the control group.

Results: The study group contained 25 girls with ICPP (7 ± 0.8 years), 35 girls with PT (6.8 ± 0.7 years), and 30 controls (6.7 ± 0.7 years). Basal serum kisspeptin and neurokinin B levels were 2.36 ± 0.47 ng/mL and 2.61 ± 0.32 ng/mL, respectively in the ICPP group, 2.23 ± 0.43 ng/mL and 2.24 ± 0.23 ng/mL, respectively in the PT group, and 1.92 ± 0.33 ng/mL and 2.03 ± 0.24 ng/mL, respectively in the controls. Both kisspeptin and neurokinin B levels were higher in the ICPP and PT groups compared to controls ($p < 0.05$). Moreover, basal neurokinin B level was different between ICPP and PT groups ($p < 0.01$). A serum neurokinin B level of 2.42 ng/mL provided the most appropriate level to differentiate ICPP from PT, with a sensitivity of 84% and specificity of 77.1%.

Conclusion: Differentiation of CPP from PT is sometime difficult, and there is a need for a simple method for the differential diagnosis. Our results suggest that basal serum neurokinin B level can be used as an adjunctive parameter to differentiate ICCP from PT.

Keywords: Neurokinin B, kisspeptin, precocious puberty

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Introduction

Puberty is a phase of the maturation process during which secondary sex characteristics develop and reproductive capacity is attained. The activation of pulsatile gonadotropin-releasing hormone (GnRH) secretion from hypothalamic neurons is a major event in the onset of puberty. However, mechanism and timing of GnRH secretion at puberty have not been explained clearly as yet.

Kisspeptin, neurokinin B, and dynorphin A (KNDy) are co-expressed in neurons of the arcuate nucleus of the hypothalamus and they play an important role in regulating pulsatile GnRH secretion (1). Kisspeptin is a neuropeptide encoded by the *KISS1* gene. KISS and G-protein coupled receptor-54 (GPR54) signaling complex have been recognized as essential regulators of pubertal activation (2). In humans, neurokinin B and its receptor are encoded by the *TAC3* and *TAC3R* genes, respectively. Recent studies have shown that neurokinin B can affect GnRH neuronal activity directly and indirectly. Kisspeptin is the most potent secretagogue for GnRH, while neurokinin B stimulates kisspeptin to initiate GnRH pulse (3,4).

Premature activation of GnRH secretion causes early puberty including central precocious puberty (CPP) and premature thelarche (PT). CPP is defined as development of secondary sex characteristics in girls before 8 years of age with an acceleration of linear growth, rapid bone maturation, and axillary and/or pubic hair development. On the other hand, in girls, PT is defined as isolated breast development before age of 8 years with no other signs of puberty (5). Differentiation of CPP from PT is often difficult in the early stage. Therefore, there is a need for a simple method for the differential diagnosis. To date, numerous studies have been published on kisspeptin and/or neurokinin B levels in girls with CPP (6,7,8). In these studies, it has been demonstrated that basal serum kisspeptin and/or neurokinin B levels are higher in girls with CPP and PT as compared to healthy controls. However, none of these studies have found a clear correlation in the differential diagnosis of CPP and PT.

In this study, we compare the basal serum neurokinin B and kisspeptin levels in the differential diagnosis of idiopathic CPP (ICPP) and PT.

Methods

Patients and Methods

Girls who had been referred to our clinic between May 2013 and December 2014 with a complaint of early breast development before the age of 8 years were evaluated. Height and weight were measured using a stadiometer and a calibrated electronic scale, respectively. Height standard deviation scores (H-SDS) and body mass index (BMI) SDS were calculated according to Turkish reference values (9). The same qualified pediatric endocrinologist assessed the pubertal stages in all subjects. An x-ray of the left wrist was taken, and assessment of the bone age (BA) was done by the same person according to the method of Greulich and Pyle.

CPP is defined as development of secondary sex characteristics before age 8 years in conjunction with a BA/chronological age (CA) ratio >1.1 and a peak luteinizing hormone (LH) value >5 mIU/mL after GnRH stimulation. Cranial magnetic resonance imaging (MRI) was performed in all subjects diagnosed as CPP. Those without any organic cranial pathology were classified as ICPP and included in the study. Subjects whose BA/CA ratio was ≤1.1 and/or peak LH level ≤5 mIU/mL were considered as PT (5).

Exclusion criteria were having history of exogenous exposure to estrogen, obesity (BMI-SDS ≥2), organic brain disease, mental retardation, primary gonadal or adrenal diseases, or any other endocrine disease. The control group consisted of age-matched healthy prepubertal girls without obesity.

The written informed consent was obtained from the parents, and the study was approved by the local ethics committee.

Hormonal Evaluation

GnRH stimulation test was performed in all patients with early breast development. GnRH (Gonadorelin acetate, Ferring) was administered intravenously (2.5 mcg/kg, max 100 mcg), and samples were taken at 30, 60, and 90 minutes after injection.

Basal and stimulated levels of serum LH, follicle-stimulating hormone (FSH) and estradiol levels were measured using Beckman Coulter, a two-site immunoenzymatic (sandwich) assay, and an auto analyzer.

Measurement of Neurokinin B and Kisspeptin

The blood samples were drawn in the morning (8-9 AM) from patients and controls. Neurokinin B and kisspeptin levels were measured using a commercially available ELISA kit (Phoenix Pharmaceutical, California, USA, and USCN Life Science, Texas, USA, respectively). Neurokinin B assay range was 0-100 ng/mL, and kisspeptin assay range was 0.06-4 ng/mL (after 1:1 dilution with sample diluent for kisspeptin). For both assays, intrassay coefficient of variation (CV) was $<10\%$, and the interassay CV was $<12\%$. The assays

employed the quantitative sandwich enzyme immunoassay technique. In order to avoid variation within an assay, measurements were performed twice using the same ELISA kit.

Statistical Analysis

The SPSS 20.0 software program was used for statistical analysis. Shapiro-Wilk test was used for testing normality. Groups were compared using ANOVA and Tukey's HSD (Honestly Significant Difference) test or Kruskal-Wallis test, later the groups were compared with one another using Mann-Whitney U-test and Bonferroni correction. A p-value of less than 0.05 was considered as statistically significant. A receiver operating characteristic-area under curve (ROC-AUC) was constructed to obtain neurokinin B levels to differentiate between ICPP and PT groups. The sensitivity and specificity were calculated based on cut-off points obtained by the ROC curve.

Results

The study group contained 25 girls with ICPP (mean age 7.02 ± 0.79 years), 35 girls with PT (mean age 6.85 ± 0.7 years), and 30 healthy prepubertal controls (mean age 6.74 ± 0.73 years). Age, H-SDS, and BMI-SDS values were similar in all three groups ($p > 0.05$). Basal serum LH, and peak serum LH and peak LH/FSH ratio, BA and BA/CA ratio were significantly higher in the ICPP group compared to the PT group ($p < 0.05$).

Serum kisspeptin and neurokinin B levels were higher in girls with ICPP and PT compared to controls. While serum neurokinin B level was higher in the ICPP group compared to PT group ($p = 0.001$), no significant difference was found in serum kisspeptin level between ICPP and PT groups ($p > 0.05$) (Table 1). An ROC-AUC was constructed to obtain neurokinin B levels to differentiate between ICPP and PT groups; a serum neurokinin B level of 2.42 ng/mL provided the most appropriate level with a sensitivity of 84% [95% confidence interval (CI): 63.9-95.5] and a specificity of 77.1% (95% CI: 59.9-89.6) (Figure 1).

Discussion

The control of onset of puberty is regulated by a network which includes KNDy neurons. These sex-steroid responsive neurons communicate via project ipsi- and contralaterally to themselves with neuropeptides (4). KNDy neuropeptides have been described as gatekeepers of puberty in regulating pulsatile GnRH secretion (1,2).

Kisspeptin is the most extensively studied neuropeptide in puberty and precocious puberty (6,7,8). In human, *KISS1* is located on chromosome 1q32 and is detected in brain, pancreas, placenta, testis and genital tract (10). Physiological studies demonstrated that kisspeptin and its receptor GPR54 are essential for GnRH neuron function. Kisspeptin/GPR54 system can directly stimulate GnRH cells in the arcuate nucleus of hypothalamus (11). Kisspeptin is

Table 1. Clinical and laboratory characteristics of the study group

	ICPP (n = 25)	PT (n = 35)	Controls (n = 30)	p-value
CA (years)	7.0 ± 0.8	6.8 ± 0.7	6.7 ± 0.73	0.37*
BA (years)	8.5 ± 1.0	6.8 ± 0.8	-	0.001***
BA/CA	1.21 ± 0.08	0.99 ± 0.10	-	0.001***
Height-SDS	0.91 ± 0.80	0.57 ± 0.91	0.56 ± 0.52	0.26**
BMI-SDS	0.03 ± 0.70	0.31 ± 0.72	0.15 ± 0.36	0.38**
Basal LH (mIU/mL)	0.55 ± 0.49	0.31 ± 0.22	-	0.04***
Basal FSH (mIU/mL)	3.24 ± 2.38	2.18 ± 1.62	-	0.04***
Peak LH (mIU/mL)	9.98 ± 6.37	3.13 ± 1.76	-	0.001***
Peak FSH (mIU/mL)	14.50 ± 4.84	13.8 ± 5.71	-	0.79****
Peak LH/FSH	0.72 ± 0.46	0.25 ± 0.18	-	0.001***
Kisspeptin (ng/mL)	2.36 ± 0.47	2.23 ± 0.43	1.92 ± 0.33	0.02**
Neurokinin B (ng/mL)	2.61 ± 0.32	2.24 ± 0.23	2.03 ± 0.24	0.001*

Data are given as mean ± standard deviation

*Kruskal-Wallis test, **Anova and Tukey's test, ***Mann-Whitney U-test, ****Student's t-test.

Adjusted significance level for kisspeptin: ICPP vs. Controls ($p < 0.001$) and PT vs. Controls ($p < 0.01$), and for neurokinin B: ICPP vs. Controls ($p < 0.001$), PT vs. Controls ($p < 0.002$), and ICPP vs. PT ($p < 0.001$).

ICPP: idiopathic central precocious puberty, PT: premature thelarche, CA: chronological age, BA: bone age, SDS: standard deviation score, BMI: body mass index, LH: luteinizing hormone, FSH: follicle-stimulating hormone

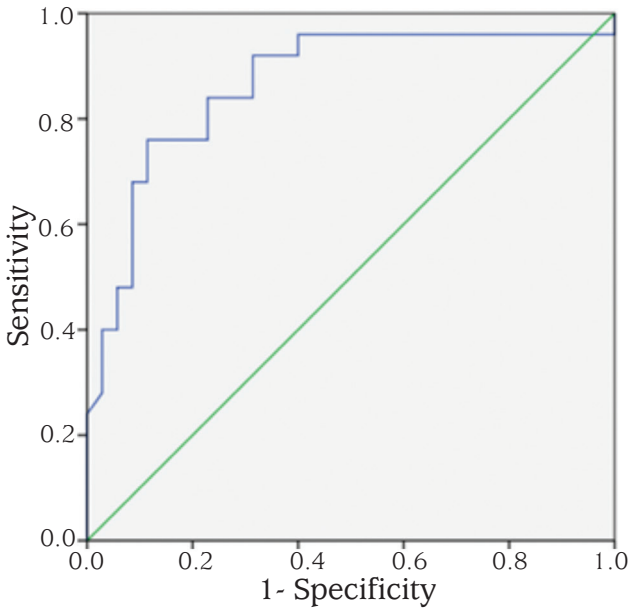

Figure 1. The receiver operating characteristic curve of neurokinin B level. The area under curve was 0.86 [95% confidence interval (CI): 0.75-0.93]. The optimal serum neurokinin B cut-off value to differentiate idiopathic central precocious puberty from premature thelarche was 2.42 ng/mL with a sensitivity of 84% (95% CI: 63.9-95.5) and specificity of 77.1% (95% CI: 59.9-89.6)

a major stimulant for GnRH secretion with GPR54 coupling and has an important role in induction of puberty (12). In some previous studies, serum kisspeptin level has been studied in ICPP and PT cases. De Vries et al (13) investigated the serum kisspeptin level in 31 girls with ICPP and reported significantly higher level of kisspeptin in girls with ICPP than that of age-matched prepubertal girls. In the same study, there was no correlation between kisspeptin and peak LH level in the ICPP group. In another study of 30 girls with ICPP by Rhie et al (7), it was shown that the serum kisspeptin level in ICPP patients was higher than that in prepubertal controls. In both studies, authors suggested that basal kisspeptin level is a useful diagnostic tool in diagnosis of ICPP. Yang et al (6) reported that kisspeptin levels in ICPP patients were higher than those in patient with PT and in prepubertal controls. After 6 months of treatment with GnRHa, kisspeptin level was lower than that prior to treatment. In another study, Abacı et al (8) evaluated serum kisspeptin levels in girls with ICPP and PT compared to healthy prepubertal girls. They detected higher serum kisspeptin levels in ICPP and PT groups than in controls, but there was no significant difference in serum kisspeptin levels between ICPP and PT groups. Similarly, in the present study, kisspeptin level was higher in the girls with ICPP and PT than in controls, while there was no significant difference between ICPP and PT groups. Our study confirms the findings of previous studies, showing that serum kisspeptin level increases in early puberty and is a useful parameter in diagnosis of ICPP but is not helpful to differentiate ICCP from PT.

In recent years, kisspeptin has been accepted as an important indicator of pubertal onset. However, De Croft et al (14) showed that almost all arcuate kisspeptin neurons were directly activated by substance P and neurokinin A and B. True et al (15) suggest that amplification of neurokinin B secretion drives the release of kisspeptin from KNDy neurons. Neurokinin B (TAC3) and TAC3R are known to be expressed mainly in hypothalamic neurons and widely expressed in peripheral tissues (3,16). Additionally, TAC3R mRNA has been identified in GnRH axons (17), and it has been shown that neurokinin B peptides are in direct contact with GnRH axons and directly induce GnRH secretion from TAC3R-expressing GnRH neurons (18,19).

To our knowledge, there is only one reported study evaluating the serum neurokinin B levels in girls with ICPP and PT compared to prepubertal controls (8). In this study, Abacı et al (8) reported that neurokinin B is significantly higher in ICPP and PT groups than in age-matched prepubertal controls, while there is no difference between the ICPP and PT groups. The limited number of subjects (22 girls with ICPP and 20 with PT) was reported as the limitation of the study.

As a very similar study, we evaluated serum neurokinin B levels in 25 girls with ICPP and in 35 girls with PT compared to 30 healthy prepubertal girls. In contrast to Abacı et al's study (8), we excluded obese patient from the study as obesity by itself can trigger puberty via leptin or other unknown neuropeptides. Additionally, we used a BA/CA ratio >1.1 as a parameter in the differential diagnosis of ICPP. We think that these important criteria make our results more acceptable compared to Abacı et al's study (8). In our study, we detected higher serum neurokinin B levels in girls with ICPP and PT than in controls. Moreover, serum neurokinin B level was higher in the ICPP group compared to the PT group. A serum neurokinin B level of 2.42 ng/mL was found as the most appropriate level in differentiating ICPP from PT, with a sensitivity of 84% and specificity of 77.1%. Although the percentages of sensitivity and specificity are not too high, we think that serum neurokinin B level can be used as an adjunctive parameter, in addition to GnRH stimulation test, to differentiate ICCP from PT. Nevertheless, the assay should be validated in larger cohorts.

In conclusion, in this study, we examined the role of kisspeptin and neurokinin B in ICPP and PT patients. Given the fact that differentiation of CPP from PT is sometime difficult and the need for a simple method for the differential

diagnosis, we suggest that basal serum neurokinin B level can be used as an adjunctive parameter to differentiate ICCP from PT.

Ethics

Ethics Committee Approval: Scientific Research and Ethics Council of Antalya Education and Research Hospital (Grant no: 169-2014).

Informed Consent: Written informed consent was obtained from the parents after being informed about the aim and procedures of the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Mesut Parlak, Design: Doğa Türkkahraman, Mesut Parlak, Data Collection and/or Processing: Mesut Parlak, Ayşe Eda Parlak, Analysis and/or Interpretation: Hamit Yaşar Ellidağ, Necat Yılmaz, Literature Research: Doğa Türkkahraman, Mesut Parlak, Writing: Doğa Türkkahraman.

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Congenital Hypothyroidism and Bone Remodeling Cycle

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What is already known on this topic?

Long-term treatment with LT4 increases bone resorption in patients with congenital hypothyroidism.

What this study adds?

Our results suggest that with effective vitamin D and thyroxin replacement, congenital hypothyroidism is not a deleterious factor for bone turnover.

Abstract

Objective: The present study aimed to evaluate the biochemical markers of bone turnover in children with congenital hypothyroidism during the course of treatment as compared to healthy children selected as controls.

Methods: The study included 31 children with congenital hypothyroidism and 29 healthy children. In both groups, we evaluated serum procollagen type-1 N-terminal propeptide (PINP) and tartrate-resistant acid phosphatase type 5b isoform (TRACP 5b) levels as bone turnover markers.

Results: In both groups, thyroid hormone levels were within normal limits. The levels of vitamin D were significantly higher in the cases with congenital hypothyroidism. Although PINP levels were not found to be different, TRACP 5b levels which are related to osteoclastic activities were significantly higher in the control group.

Conclusion: We did not detect an increase in bone resorption in patients with congenital hypothyroidism, despite long-term treatment with LT4. Our results suggest that with effective vitamin D treatment and thyroxin replacement, congenital hypothyroidism is not a deleterious factor for bone turnover.

Keywords: Congenital hypothyroidism, bone marker, thyroxin

Introduction

Congenital hypothyroidism (CH) is one of the most common preventable causes of intellectual disability (1). Effects of hypothyroidism have decreased in babies administered treatment in accordance with the neonatal screening program (2). Untreated CH results in delayed bone age, growth retardation, and short stature (3).

Thyroid hormones also have important effects on the bone remodeling cycle. However, this effect has been shown to increase in favor of bone resorption, especially in thyrotoxicosis (4). In some studies in adults, long-term treatment with levothyroxine has been reported to decrease bone density, which may result in bone fractures (4,5,6). In contrast, some studies have indicated that long-term treatment would not affect bone density (7,8).

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Both hypothyroidism and hyperthyroidism have been associated with an increased risk of fractures. It has been determined that remodeling increases in adults in the course of hypothyroidism; in this situation, osteoclastic duration is increased twofold, whereas osteoblastic duration is prolonged fourfold. These changes result in low bone turnover and an overall failure to gain bone mass and mineral (3).

In children diagnosed with CH in the neonatal period and treated for a long time, the bone remodeling cycle may indicate the effects of thyroid hormone treatment or those of CH. Additionally, the presence of vitamin D deficiency also affects the bone cycle (9). In the assessment of the bone cycle by serum analysis, the new bone formation may be evaluated by the level of serum procollagen type 1 N-terminal propeptide (PINP), and bone resorption by the level of the enzyme tartrate-resistant acid phosphatase type 5b isoform (TRACP 5b), which is secreted by osteoclasts (10). The present study aimed to evaluate the biochemical markers of bone turnover in children with CH who had been followed up during the course of treatment and in healthy children selected as controls.

Methods

The study included 31 children with CH followed up by the Başkent University, School of Medicine, Pediatric Endocrinology outpatient clinic and 29 healthy children followed up by the General Pediatrics outpatient clinic. All patients and their families were informed about the study, and consent was obtained from the families. Blood samples were withdrawn during the blood sampling for medical purposes.

Of the 31 patients with CH, 14 were cases of hypoplastic thyroid gland, one had agenesis, one had an ectopic thyroid gland, 14 had thyroid dyshormonogenesis, and one had a fetal goiter related to the TPO gene mutation. The initial LT4 treatment was administered at a dose of 7.5-15 µg/kg/day during the neonatal period.

The cases in the control group (n = 29) were selected from those who had been followed up by the General Pediatrics outpatient clinic and had no chronic disease or abnormal neonatal screening result. They were not taking any medication. We did not question the dosage and duration of vitamin D intake in the two groups.

Chronological age, weight, height, and percent of ideal weight for height were evaluated in all patients. All blood samples were withdrawn in the morning, at the same time. The serum levels of calcium, phosphorus, alkaline phosphatase (ALP), magnesium, thyroid-stimulating hormone (TSH), free thyroxine (fT_4), parathyroid hormone (PTH), 25 hydroxycholecalciferol [$25(OH)D_3$-Vitamin D], PINP, TRACP 5b, and urine calcium/creatinine ratio were analyzed in the biochemistry laboratory of Başkent University.

CH and control groups were compared according to vitamin D status, deficient or non-deficient.

Venous blood samples were drawn and sera were stored at -20 ºC after centrifugation until testing. All assays were carried out at the same time. The levels of 25(OH) D were assayed using chemiluminescent microparticle immunoassay (Abbott Architect I2000 analyzer). The architect 25(OH)D assay is designed to have a limit of detection (LoD) of ≤10.0 ng/mL. Serum levels of calcium, phosphorus, ALP, magnesium, TSH, and fT_4 were measured in the blood cell counter using an Abbott Cell-Dyn Ruby System (Abbott Diagnostic, Santa Clara, CA, USA).

Vitamin D levels at or below 15 ng/mL were defined as vitamin D deficiency (9).

PINP was measured by kit which is a sandwich enzyme immunoassay for in vitro quantitative measurement of PINP in human serum incubated for 30 minutes at 37 ºC after covering it with the Plate sealer. The minimum detectable dose of PINP is typically less than 6.2 pg/mL. The intra- and inter-assay coefficients of variation (CV) reported by the manufacturer are < 10 % and < 12 % (Uscn Life Science Inc. Wuhan, Hubei, PRC).

TRACP was assayed by an immunoCapture Enzyme-Activity Assay in serum (BioVendor Research and Diagnostic Products, Czech Republic). In the BioVendor Human TRAP 5 Assay, calibrators, quality control and samples are incubated in microplate wells pre-coated with monoclonal anti-human TRAP 5 antibody. After a thorough wash, TRACP 5b bound to the antibody is allowed to react with the pNPP substrate at pH 5.5. The reaction is stopped by addition of hydroxine solution and absorbance of the resulting yellow color product is measured. The absorbance is proportional to the enzymatic activity of TRACP 5b. A calibration curve is constructed by plotting absorbance values against enzyme activities of recombinant TRACP 5 calibrators, and enzyme activity of unknown samples are determined (U/l) using this calibration curve. LoD is calculated from the real TRAP 5 values in wells and is 0.01 U/l. The intra- and inter-assay CVs reported by manufacturer are 2.4 % and 7.6 %.

This study was approved by the Başkent University Institutional Review Board and Ethics Committee (Project No: KA12/46) and supported by the Başkent University Research Fund.

Statistical Analysis

Data were analyzed using the SPSS 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) statistical software. Values were expressed as mean ± standard deviation, median (maximum-minimum), percentage, and frequency. Variables were evaluated after controlling the normality and homogeneity of variance prerequisites (Shapiro-Wilk and Levene's tests). Data were compared between the two groups using the independent t-test (Student's t-test); the Mann-Whitney U-test was used in the absence of prerequisites. Categorical data were analyzed using the Fisher's exact test and chi-square test. When the expected frequencies were lower than 20%, evaluation by the "Monte Carlo simulation method" was performed in order to include these frequencies in the analysis. A p-value < 0.05 was accepted as statistically significant.

Results

The characteristics of the cases with CH and control subjects are presented in Table 1. The values of chronological age, height-for-age, body weight, height, and percent of ideal weight for height were not different in the CH and control groups. Laboratory results on serum calcium, phosphorus, magnesium, ALP, fT_4, TSH, PTH, and PINP levels were also not statistically different from one another and were also in the normal range. Mean TRACP 5b levels were significantly higher in the control group. The levels of vitamin D were significantly higher in the cases with CH (Table 1).

According to vitamin D status, TRACP 5b levels were significantly higher in controls. PINP levels were not found to be different (Table 2).

Discussion

The acid phosphatases are lysosomal enzymes, and the isoform 5b secreted from the osteoclasts is a marker used to detect bone resorption. On the other hand, procollagen type 1 N-terminal propeptide is a marker used for the evaluation of bone formation (11). In the present study, bone turnover was evaluated in children with and without CH.

Bone is a metabolically active tissue that undergoes continuous remodeling throughout life. In the course of bone remodeling, bone resorption is closely associated with new bone formation. Bone resorption and formation can be affected by some diseases and this process may be evaluated by the serum levels of several markers (10). The effect of congenital non-goiter hypothyroidism on bone is still not completely understood (12). Population studies have demonstrated that both hypothyroidism and hyperthyroidism affect bone remodeling and that they may increase the risk of bone fractures. Studies conducted on mice have shown that TSH affects the bone remodeling cycle

Table 1. The characteristics of the cases with congenital hypothyroidism and the controls

	n	Congenital hypothyroidism	n	Controls	p-values
Male/Female	31	21/11	28	17/11	0.091
Age (years)	31	4.02 ± 2.00	28	4.05 ± 1.45	0.961
Body weight (kilograms)	31	17.56 ± 6.45	28	16.36 ± 3.06	0.373
Height (cm)	31	101.65 ± 10.55	28	104.96 ± 12.47	0.399
Biochemistry					
Calcium (mg/dL)	31	9.89 ± 0.40	28	9.72 ± 0.31	0.075
Phosphorus (mg/dL)	31	5.12 ± 0.35	28	4.71 ± 0.54	0.082
Alkaline phosphatase (U/L)	31	196.38 ± 43.06	28	238.11 ± 28.75	0.442
25-OH-Vit D (ng/mL)	29	29.22 ± 10.54	28	20.87 ± 7.02	0.001*
PINP (pg/mL)	31	16.82 ± 2.21	28	17.83 ± 7.90	0.499
TRACP 5b (U/L)	31	1.88 ± 0.55	28	8.79 ± 0.40	0.001*
TSH (μIU/mL)	31	2.24 ± 2.51	27	1.79 ± 0.94	0.079
fT_4 (ng/dL)	31	1.26 ± 0.19	27	1.18 ± 0.14	0.070
PTH (pg/mL)	27	35.11 ± 2.98	21	23.13 ± 4.38	0.024
Magnesium (mg/dL)	22	2.09 ± 0.12	28	2.07 ± 0.13	0.957
Urine Ca/Cre	20	0.14 ± 0.04	18	0.14 ± 0.03	0.934

TRACP 5b: tartrate-resistant acid phosphatase type 5b, PINP: procollagen type-1 N-terminal propeptide, PTH: parathyroid hormone, fT_4: free thyroxine, TSH: thyroid-stimulating hormone, Ca/Cre: calcium/creatinine ratio, 25-OH-Vit D: 25 hydroxycholecalciferol Vitamin D

Table 2. Comparison of the two groups according to vitamin D status

	Vitamin D levels: 0 to 14.99 ng/mL					Vitamin D levels: ≥15 ng/mL				
	n	Congenital hypothyroidism	n	Controls	p-value	n	Congenital hypothyroidism	n	Controls	p-value
Age (Years)	2	4.95 ± 1.91	5	4.29 ± 1.59	0.655	27	3.90 ± 2.08	23	3.99 ± 1.45	0.874
Height (cm)	2	110.5 ± 7.07	5	108.4 ± 8.2	0.766	27	101.13 ± 17.7	23	104.2 ± 13.2	0.497
Body weight (kg)	2	18.95 ± 0.49	5	16.88 ± 2.72	0.357	27	17.5 ± 6.8	23	16.2 ± 3.1	0.402
Percent of ideal weight for height	2	102.7 ± 8.8	5	99.5 ± 7.37	0.641	27	101.6 ± 10.7	23	95.9 ± 11.5	0.057
Calcium (mg/dL)	2	9.65 ± 0.78	5	9.56 ± 0.19	0.793	27	9.91 ± 0.39	23	9.75 ± 0.31	0.109
Phosphorus (mg/dL)	2	5.1 ± 0.57	5	4.84 ± 0.52	0.584	27	5.11 ± 0.35	23	4.88 ± 0.55	0.065
ALP (U/L)	2	150.5 ± 2.12	5	190.4 ± 26.7	0.414	27	200.2 ± 8.5	23	248.4 ± 65	0.44
PINP (pg/mL)	2	18.25 ± 1.12	5	27.1 ± 7.34	0.503	27	16.73 ± 2.33	23	15.8 ± 1.94	0.136
TRACP 5b (U/L)	2	0.63 ± 0.09	5	9.98 ± 1.21	0.001*	27	1.78 ± 0.58	23	8.5 ± 2.17	0.001*
TSH (µIU/mL)	2	2.35 ± 4.45	5	1.38 ± 0.22	0.073	27	2.12 ± 2.5	22	1.88 ± 0.99	0.081
fT_4 (ng/dL)	2	1.35 ± 0.07	5	1.25 ± 0.13	0.356	27	1.21 ± 1.19	22	1.16 ± 0.14	0.09
PTH (pg/mL)	2	49.25 ± 24.35	4	31.3 ± 10.01	0.062	24	34.57 ± 13.81	17	26.79 ± 4.60	0.145
Magnesium (mg/dL)	2	2.02 ± 0.02	5	1.95 ± 0.13	0.867	20	2.10 ± 0.12	23	2.08 ± 0.01	0.923
Urine Ca/cre	1	0.31	4	0.12 ± 0.05	-	18	0.138 ± 0.039	14	0.146 ± 0.039	0.887

ALP: alkaline phosphatase, TRACP 5b: tartrate-resistant acid phosphatase type 5b, PINP: procollagen type-1 N-terminal propeptide, PTH: parathyroid hormone, fT_4: free thyroxine, TSH: thyroid-stimulating hormone, Ca/Cre: calcium/creatinine ratio

negatively (4,5,6,7,8). Papadimitriou et al (13) determined that the low levels of TSH do not lead to bone loss in mice. In our study, TSH and fT_4 levels were found to be similar in the hypothyroid and control groups, but the level of TRACP 5b, which is an indicator of bone resorption, was found to be lower in the hypothyroid group; in other words, bone resorption was low in hypothyroid cases.

Engler et al (14) showed that the thyroid hormones also affect bone turnover. In this study, the values indicating bone resorption were high in the cases with hyperthyroidism, before the administration of anti-thyroid treatment.

In several studies conducted on adults, TSH was reported to be suppressed by the administration of LT4 in patients with thyroid cancer and non-toxic goiter who did not have thyrotoxicosis, and bone density was reported to be decreased (4,5,6). In contrast to these results, Franklyn et al (7) and Marcocci et al (8) did not detect any change in bone density following treatment with LT4 for as long as 8 to 10 years. Leger et al (4) did not detect a change in bone mineral density in favor of bone resorption, in patients undergoing long-term LT4 treatment. Similarly, in our study, any effect on bone resorption has not been demonstrated in the group with CH.

Vitamin D contributes to bone turnover, and it is used as a supportive treatment, starting in infancy. We determined the level of vitamin D to be significantly higher in children with CH. When the cases were subdivided according to their vitamin D levels, TRACP 5b levels were found to be higher in the controls in both vitamin D sufficiency and deficiency situations. The value of PINP, which indicates the formation of new bone, did not differ significantly between the groups. In this study, we did not detect an increase in bone resorption in patients followed up in our clinic. The children with CH might have received more effective vitamin D replacement due to their close and more frequent follow-ups. The mean duration of follow-up in our hypothyroid patients was four years which may be considered as one of the limitations of the study. We did not question the duration and dose of vitamin D intake in the CH and control groups which is the other limitation.

Reference values for TRACP 5b have been published for Chinese in 2005 and subsequently for Caucasian children, in 2007 and 2012 (15,16,17). Rauchenzauner et al (16) has reported the TRACP 5b values for 50th percentile as 8.1 U/L for boys and 6.8 for girls in the 0-18 age group. Fischer et al

(17) determined the TRACP 5b values for 50th percentile at age 4 years to be 13.8 U/L for boys and 17 U/L for girls (17). The distribution of normal TRACP levels showed a wide range, depending on age, sex, and pubertal stage. Vitamin D levels were not given in the above studies.

Our study included an age-matched healthy prepubertal control group. We measured the vitamin D levels in both groups. We speculate that the main reason for the higher TRACP 5b levels in the controls might be the lower vitamin D levels.

In conclusion, we did not detect an increase in bone resorption in patients with CH, despite long-term treatment with LT4. Our results suggest that with effective vitamin D and thyroxin replacement, CH is not a deleterious factor for bone turnover.

Ethics

Ethics Committee Approval: This study was approved by the Başkent University Institutional Review Board and Ethics Committee (Project No: KA12/46).

Informed Consent: All patients and their families were informed about the study, and consent was obtained from the families.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Sibel Tulgar Kınık, Nazmi Mutlu Karakaş, Design: Sibel Tulgar Kınık, Nazmi Mutlu Karakaş, Data Collection and Processing: Nazmi Mutlu Karakaş, Beril Özdemir, Analysis and Interpretation: Ayşegül Haberal, M. Ağah Tekindal, Literature Research: Nazmi Mutlu Karakaş, Writing: Nazmi Mutlu Karakaş.

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Association between Obesity and Parental Weight Status in Children and Adolescents

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What is already known on this topic?

Parental weight is one of the predictors of obesity in children and adolescents. There is an association between parental body mass index and child birth weight.

What this study adds?

Children from families with obese parents were at a significantly higher risk of obesity compared to children with normal-weight parents. Overweight and/or obesity in children of both genders was significantly associated with parental overweight and/or obesity.

Abstract

Objective: This study aims to assess the relationship between body mass index (BMI) of children and that of their parents in a nationally-representative sample of Iranian population.

Methods: This cross-sectional nationwide study was conducted in 2011-2012 among 6-18-year-old students and their parents living in 30 provinces of Iran. Socio-demographic information was collected. The BMI values of the children/adolescents were categorized according to the World Health Organization reference curves. Association between parental and student weight status was examined using ordinal regression models after adjustment for potential confounders.

Results: Overall, 23043 children and adolescents and one of their parents participated in this study (50.7% boys, 73.4% urban status). Mean age of the subjects was 12.55 ± 3.31 years. Mean BMI values of parents and children/adolescents were 27.0 ± 4.57 and 18.8 ± 4.4 kg/m2, respectively. After adjusting for confounders, overweight and/or obesity in students of both genders was found to be significantly associated with parental overweight and/or obesity. In those students who had obese parents, the odds ratio (OR) of being obese was 2.79 for boys [OR = 2.79; 95% confidence interval (CI) = 2.44-3.20] and 3.46 for girls (OR = 3.46; 95% CI = 3.03-3.94) compared to their peers with normal-weight parents. Boys with overweight parents were 1.7 times more overweight than their counterparts with normal-weight parents (OR = 1.70; 95% CI = 1.15-1.92). Similarly, girls who had overweight parents were more overweight compared to those with normal-weight parents (OR = 2.00; 95% CI = 1.77-2.25).

Conclusion: Our findings highlight the importance of the shared family environment as a multi-factorial contributor to the childhood obesity epidemic and the necessity of implementing family-centered preventive programs.

Keywords: Overweight, obesity, body mass index, children and adolescents, parents

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Introduction

Overweight and obesity in childhood usually track to adulthood. They are associated with several complications and increase the risk of morbidity and mortality later in life (1).

The prevalence of overweight and obesity is increasing in both developed and developing countries (2). The overweight/obesity prevalence has doubled and tripled in pre-school and primary school-aged children, respectively (3). Among developing societies, Eastern Europe and the Middle East have the highest prevalence of childhood overweight (4).

In the Middle East, a high frequency of overweight and/or obesity was documented in adolescents living in Kuwait (5) and Qatar (6). The prevalence of overweight and/or obesity in children and adolescents is on the increase in many developing countries, such as Iran (7). In our previous nationwide study, the prevalence rate of general and abdominal obesity in 6-18 years Iranian students was 11.89% (13.58% of boys vs. 10.15% of girls) and 19.12% (20.41% of boys vs. 17.79% of girls), respectively (8).

Excess weight/obesity is a multi-factorial disorder and derives from two different origins, namely, genetic and environmental factors. However, the relative contributing role of genetic susceptibility and environmental factors to development of obesity is not clear (9). A great number of previous studies have indicated that childhood and adolescent overweight and obesity are linked to obvious familial aggregation, as a result of complex interaction between genetic and environmental effects (10,11).

During recent decades, a number of studies have shown the association between parental body mass index (BMI) and child birth weight (12). In addition, studies indicate the higher impact of parental BMI on the severity of weight gain from childhood to adolescence (13).

Parental weight has been shown as an important predictor for obesity development in children and adolescents. Few studies are available regarding the association between parental and child obesity (14). The present study aims to examine the relationship of parental BMI with overweight and obesity in children/adolescents in a nationally representative sample of Iranian population.

Methods

The Childhood and Adolescence Surveillance and Prevention of Adult Noncommunicable Disease-IV Study was performed in rural and urban regions of 30 provinces of Iran in 2011-2012. The methodology of the study has been published in detail (15). In brief, students were selected from elementary, middle, and high schools by multistage, cluster random sampling method. Stratification was done according to the level of the schools (elementary, middle, and high school) and place of residence (urban, rural). The total child/adolescent sample size was calculated as 25000 students (48 clusters of 10 students in each province) and 23043 students participated in the survey.

Trained health care professionals conducted the physical examination under standard protocols by using calibrated instruments. These professionals were selected from health staff working in the health system in each province (one person in each province, a total of 30 professionals) and attended a 3-day educational workshop on measurement of anthropometric indices according to standard protocols.

Weight was measured with the subject in light clothing, to the nearest 0.1 kg. Standing height was recorded without shoes to the nearest 0.1 cm. BMI was calculated as weight (Kg) divided by height in square meters (m^2). BMI categories were defined according to the World Health Organization (WHO) reference curves for different age and gender groups (16). The subjects were classified as underweight (BMI $<5^{th}$ percentile), normal weight (BMI between 5^{th}-85^{th} percentiles), overweight (BMI between 85^{th}-95^{th} percentiles), and obese (BMI $\geq 85^{th}$-95^{th} percentiles). Parents were asked to report their weight and height. Parental BMI was calculated as underweight (BMI <18.5), normal-weight ($18.5<$ BMI <24.9), overweight (BMI $\geq$25-29.9), and obese (BMI $\geq$30). Socio-demographic information including parental education and occupation, age of the subject, living area (urban vs. rural), and number of people living in the house were also collected.

Statistical Analysis

All analyses were conducted using survey analysis method in STATA software. Categorical and continuous data were presented as numbers (percentages) and means standard deviation (SD), respectively. The weight status of the children/adolescents was analyzed as an ordinal outcome variable. Parental weight status was categorized into four groups (underweight, normal weight, overweight, and obese), which was investigated as an ordinal response variable. Univariate analysis was used to examine the relationship of each independent variable to outcome of interest. Goodman and Kruskal's gamma and Pearson's chi-square statistic were used to determine the association between the weight status and participants' characteristics. Pearson's correlation test was used to investigate the relationship

between continuous variables and BMI values. A variable univariately associated with the outcome was added to the preliminary multivariate model for the outcome.

Ordinal regression models were applied to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) of obesity by parental weight status, adjusted for potential confounders. These models were tested using a full likelihood ratio test comparing the fitted location model to a model varying location parameters. Hence, separate binomial logistic regressions were run on cumulative dependent variables. Boys and girls were analyzed separately due to gender differences in weight status. Two sets of potential confounders were used in the adjusted models. Model 1 was adjusted for some characteristics of children/adolescents including birth weight, gender, living area, and age. Model 2 was further adjusted for potential predictors which were related to their family such as parental education and occupation, type of house and school, and number of the household. All the tests were two-sided and the significance level was set as 0.05.

Results

In total, 23043 students (50.8% boys) completed the study. The mean and SD for age of the subjects was 12.55 (3.31) years. Overall, 73.4% of the study participants were from urban areas and 79.4% of families lived in their private house. Parental mean weight (SD) was 70.9 ± 13.8 kg and that of the students was 42.50 ± 17.0 kg. 17% of fathers and 9.4% of mothers had university degrees. Ninety percent (90.9%) of children/adolescents were students of public schools. About half of fathers (44.38%) were laborers or white collar workers and 88.3% of the mothers were housewives. Nearly half of families in the study owned personal cars and personal computers. The basic and demographic characteristics of the study participants are given in Table 1. Since the weight status was statistically significantly different between boys and girls, simple bivariate analyses were performed for boys (n = 11752) and girls (n = 11364) separately.

The prevalence of overweight/obesity was 15.1% and 65.6% among the students and the parents, respectively. Mean (SD) BMI was18.81 ± 4.43 and 27.0 ± 4.57 kg/m^2 for the children/adolescents and the parents, respectively. Overall, 5.0% of children/adolescents were categorized as obese, 10.1% were overweight, and 5.0% were underweight. There was an association between children/adolescents' weight and parental weight status ($p < 0.001$ for both genders). We found that 0.8% of boys with underweight parents were overweight or obese. 88.8% of children/adolescents with overweight parents and 67.0% of children/adolescents with obese parents were overweight or obese.

Table 2 indicates the multivariate regression models for the association and obesity in children/adolescents and parental weight, adjusted for potential confounders. The analyses were stratified by gender. The prevalence of obesity was high among parents. In models 1-2, childhood and/or adolescent obesity was significantly associated with parental obesity in both genders, after adjusting for confounders. Parental overweight increased the odds of obesity among children and adolescents and strong associations were found between overweight and obese parents and the weight of their offspring ($p < 0.05$). Boys were 2.79 times more likely to be obese if their parents were obese compared to boys with normal weight parents (OR = 2.79; 95% CI = 2.44-3.20). Similarly, girls who had obese parents were more likely to be obese than their peers who had normal-weight parents (OR = 3.46, 95% CI = 3.03-3.94). Boys with overweight parents were 1.7 times more overweight than their counterparts with normal-weight parents (OR = 1.70; 95% CI = 1.15-1.92). In the same way, girls who had overweight parents were more overweight compared to those with normal weight parents (OR = 2.00; 95% CI = 1.77-2.25).

Discussion

The current study is one of the first of its kind to explore the association of the weight status of a large population-based sample of Iranian children with that of their parents at national level. We found that children from families with obese parents were at a significantly higher risk of obesity compared to children of normal-weight parents.

Both genetic and environmental factors contribute to childhood obesity (17,18). Some environmental factors including parental overweight, shared family lifestyle, dietary habits, and socio-economic status (SES) are linked to childhood overweight (7,18,19,20,21). Previous studies have indicated that low SES families have little access to healthy foods; therefore, their consumption of high-calorie, low nutrient foods is higher than that of high SES groups (22,23). Parental education has also been reported to be inversely related to child excess weight and studies have shown a higher prevalence of overweight and/or obesity in children of parents with a low educational level (24,25,26). Previous studies across 11 European countries have indicated that low maternal education could yield a substantial risk of early childhood obesity (27). In another study, it was reported that children of better educated mothers had a more favorable growth pattern, namely, lower overweight and

Table 1. Descriptive characteristics of participants: The weight disorder survey of the CASPIAN-IV Study (Column %)

	Boys (n = 11752)						Girls (n = 11364)					
	Under-weight	Normal	Over-weight	Obese	Total	p-value	Underweight	Normal	Over-weight	Obese	Total	p-value
Age (years)						**< 0.001[a*]**						**< 0.001[a*]**
≤9	74.4	33.7	7.6	8.9	31.7		72.8	31.8	7.0	6.6	30.3	
10 to ≤12	22.5	29.2	26.6	22.3	28.3		24.6	27.2	21.2	20.1	26.0	
13 to ≤14	2.2	15.8	21.3	22.0	16.1		1.4	18.7	26.3	30.5	19.1	
15 to ≤18	0.8	21.2	44.5	46.8	23.9		1.2	22.4	45.5	42.8	24.6	
Level of education						**< 0.001[a*]**						**< 0.001[a*]**
Primary school	91.3	50.2	19.8	17.8	47.4		90.0	46.0	15.6	11.8	43.6	
Middle school	7.5	24.9	28.1	27.0	24.6		9.0	27.7	31.5	37.1	27.5	
High school	1.2	24.9	52.1	55.2	28.1		1.1	26.2	52.9	51.1	28.8	
Type of school						**< 0.001[b*]**						**< 0.001[b*]**
Public	93.2	90.5	82.5	78.9	89.3		95.7	93.2	89.1	87.5	92.6	
Private	6.8	9.5	17.5	21.1	10.7		4.3	6.8	10.9	12.5	7.4	
Birth weight (g)						**< 0.001[a*]**						**< 0.001[a*]**
≤2500	15.0	8.9	6.6	6.3	8.8		15.2	10.2	7.1	8.8	10.1	
2500 to ≤4000	78.2	83.3	81.4	79.8	82.7		80.8	83.2	82.7	79.7	82.8	
> 4000	6.9	7.7	12.0	13.9	8.4		4.0	6.5	10.2	11.4	7.0	
Living area						**< 0.001[b*]**						**< 0.001[b*]**
Urban	64.6	70.8	79.7	83.9	72.0		60.1	73.5	87.8	87.4	74.9	
Rural	35.4	29.2	20.3	16.1	28.0		39.9	26.5	12.2	12.6	25.1	
Type of house						**0.077[b]**						**0.231[b]**
Private	74.6	79.7	80.0	81.9	79.6		75.4	79.2	80.9	78.9	79.1	
Rental	23.8	18.3	18.1	16.1	18.4		21.5	18.4	17.2	18.5	18.4	
Corporate	1.7	2.0	2.0	2.0	2.0		3.1	2.4	1.9	2.6	2.4	
Personal computer						**< 0.001[b*]**						**< 0.001[b*]**
Yes	35.4	47.2	64.6	68.3	49.4		32.9	46.7	61.5	64.2	48.4	
No	64.6	52.8	35.4	31.7	50.6		67.1	53.3	38.5	35.8	51.6	
Personal car						**< 0.001[b*]**						**< 0.001[b*]**
Yes	48.9	56.7	67.8	69.2	58.0		52.1	57.4	68.3	68.3	58.8	
No	51.1	43.3	32.2	30.8	42.0		47.9	42.6	31.7	31.7	41.2	
Number of children						**0.016[a*]**						**0.016[a*]**
1 & 2	32.8	48.0	51.4	47.8	49.1		39.9	48.7	47.8	46.7	47.7	
3 & 4	67.2	52.0	48.6	52.2	50.9		60.1	51.3	52.2	53.3	52.3	

Table 1.Continue

Parental weight status					**< 0.001[a*]**						**< 0.001[a*]**
Underweight	5.1	1.7	0.4	0.4	1.7		3.2	1.5	0.4	0.4	1.4	
Normal	46.2	35.1	21.8	22.2	33.6		47.0	33.8	19.2	11.8	31.9	
Overweight	35.9	42.6	47.1	41.2	42.7		35.0	41.5	44.7	41.2	41.4	
Obese	12.9	20.6	30.8	36.2	22.0		14.9	23.1	35.6	46.6	25.2	
Paternal education					**0.001[a*]**						**0.100[a]**
Literate	11.2	11.0	8.7	7.5	10.6		11.3	11.3	10.7	11.7	11.3	
Primary school	24.1	25.1	23.3	22.8	24.8		25.4	24.3	23.0	22.3	24.1	
Middle school	23.1	23.0	22.9	25.9	23.1		22.6	23.8	22.5	23.1	23.6	
High school	27.2	24.3	27.2	28.3	24.9		22.3	24.1	24.9	26.1	24.2	
College	14.4	16.6	17.9	15.5	16.6		18.3	16.4	18.8	16.8	16.8	
Maternal education					**< 0.001[a*]**						**0.005[a*]**
Literate	19.8	18.3	14.8	14.3	17.8		18.8	18.5	16.0	15.8	18.1	
Primary school	27.3	29.7	28.0	25.2	29.2		28.0	29.0	26.1	29.6	28.7	
Middle school	23.4	20.2	21.0	23.8	20.6		18.0	20.9	22.5	18.6	20.8	
High school	21.6	22.4	25.3	26.9	22.9		25.6	22.5	24.5	24.1	22.9	
College	7.9	9.4	10.9	9.9	9.5		9.6	9.1	10.8	11.9	9.4	
Father's occupation					**< 0.001[b*]**						**< 0.001[b*]**
Unemployed	6.1	4.8	4.5	3.3	4.7		4.8	4.6	4.5	5.2	4.6	
Laborer	28.4	23.6	15.8	13.5	22.6		28.9	21.1	14.0	13.9	20.5	
Employee	18.0	22.2	31.2	29.4	23.2		19.2	22.7	28.3	28.1	23.4	
Farmer	10.8	10.8	6.6	5.5	10.1		7.0	9.7	8.6	6.4	9.2	
Self-employed	35.1	36.0	39.1	44.5	36.7		39.0	38.5	41.1	41.5	39.0	
Other	1.6	2.6	2.8	3.8	2.6		1.1	3.4	3.4	5.0	3.3	
Mother's occupation					**< 0.001[b*]**						**0.002[b*]**
Housekeeper	87.2	89.0	85.8	83.4	88.4		90.5	88.4	85.8	85.3	88.1	
Others	12.8	11.0	14.2	16.6	11.6		9.5	11.6	14.2	14.7	11.9	

[a]Using Goodman and Kruskal's gamma test
[b]Using Pearson's chi-square test
*Significant at level of 5%

Table 2. Adjusted odds ratios (95% confidence intervals) for the association of children/adolescent obesity with parental weight status by gender: The weight disorder survey of the CASPIAN-IV Study

Gender of children/adolescence	Parental weight status	Model 1[a]	p-value	Model 2[b]	p-value
Boys	Normal	1.00 (Reference)	-	1.00 (Reference)	-
	Underweight	0.38 (0.25, 0.57)	< 0.001*	0.44 (0.29, 0.65)	< 0.001*
	Overweight	1.70 (1.15, 1.92)	< 0.001*	1.67 (1.48, 1.88)	< 0.001*
	Obese	2.79 (2.44, 3.20)	< 0.001*	2.55 (2.23, 2.92)	< 0.001*
Girls	Normal	1.00 (Reference)	-	1.00 (Reference)	-
	Underweight	0.71 (0.47, 1.08)	0.112	0.63 (0.41, 0.97)	< 0.001*
	Overweight	2.00 (1.77, 2.25)	< 0.001*	2.02 (1.79, 2.27)	< 0.001*
	Obese	3.46 (3.03, 3.94)	< 0.001*	3.46 (3.04, 3.94)	0.034*

Dependent variable is categorized as obese, overweight, normal, and underweight.

[a]Model 1 adjusted for birth weight, gender, living area, and age of children/adolescents.

[b]Model 2 adjusted for parental education and occupation, type of house and school, and number of people living in the house.

*Significant at level of 5%.

obesity rates (in the UK and Sweden), and lower stunting and underweight rates (in rural China) (28). It seems that maternal education has a more substantial effect on child weight status, because mothers spent a longer time with their children than the fathers and are usually the person who prepares the food (27). Also, there is a more direct interaction between the children and their mothers than their fathers (29). The inverse association between parental education and child obesity could possibly affect life-style, dietary intake, as well as the SES position (24,25,30).

One of the most important components of family context is parental weight status, which has been reported to be an important predictor of overweight and obesity in children and adolescents (14,17,18,30). However, to date, the potential association with parental weight has not been extensively investigated.

In the current study, obese parents were more likely to have overweight or obese children, compared to normal-weight parents. These results are consistent with previous studies (14,18,30) and suggest that parental excess weight has an important role on child BMI.

Overweight parents are considered as risk factors for overweight/obesity of their offspring (1,31). The association between overweight children and parental excess weight represents both gene and environment interactions (19). Thus, the increasing risk of childhood or adolescent overweight in individuals with obese parents might be due to their genetics or their living in the same environment. Furthermore, children usually imitate their parents. Therefore, eating habits and family lifestyle could have an influence on child eating behavior. Unfavorable parental eating patterns (including higher consumption of fried, fast foods, sweets) and a sedentary lifestyle such as low physical activity and prolonged TV and computer time might increase the risk of overweight and obesity in both parents and their children (18).

Our study is the first national study conducted on a very large sample to determine the association between an overweight status in children and parental weight status. The study has some limitations which need to be addressed. As a cross-sectional study, we cannot infer cause and effect relationships, thus the association of parental BMI and child weight status needs to be confirmed in prospective studies. The other limitation is the self-reported heights and weights of parents, which may have decreased the accuracy of these data. Estimated obesity prevalence in parents might be affected by underreporting of weight due to the social desirability. However, a number of studies in our community have shown that self-reported anthropometric measurements are reliable (32). Our findings also highlight the importance of asking about family history of overweight and/or obesity by family physicians and primary care practitioners to be able to assess the risk of overweight in children.

In conclusion, our findings highlight the importance of the shared family environment as a multi-factorial contributor to the childhood obesity epidemic as well as the necessity of implementing family-centered preventive programs.

Ethics

Ethics Committee Approval: Cross-sectional study.

Informed Consent: Cross-sectional study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Roya Kelishadi, Mohammad Esmaeil Motlagh, Design: Mostafa Qorbani, Omid Safari, Maryam Bahreynian, Hamid Asayesh, Data Collection and Processing: Bita Moradi Khaniabadi, Analysis and Interpretation: Mostafa Qorbani, Omid Safari, Literature Research: Mostafa Qorbani, Roya Kelishadi, Writing: Mostafa Qorbani, Roya Kelishadi.

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Heart-Type Fatty Acid Binding Protein Level as a Tool in Identification of Early Cardiac Effects of Diabetic Ketoacidosis

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What is already known on this topic?

It is well known that diabetic ketoacidosis (DKA) has acute and severe effects on the myocardium.

What this study adds?

In terms of patient management, heart-type fatty acid binding protein (H-FABP) is superior in showing cardiac involvement in the ischemia phase in DKA and this makes it more valuable than other biomarkers. In our study, the decrease in H-FABP levels noted after improvement of the ketoacidosis state has shown that cardiac ischemia in the early stages of DKA can be reversed with proper treatment without advancing to necrosis. Repetitive attacks of DKA may increase the risk of cardiac necrosis in the early period.

Abstract

Objective: This study aimed to measure the serum levels of heart-type fatty acid binding protein (H-FABP) in patients presenting with diabetic ketoacidosis (DKA) and diabetic ketosis (DK) and to determine its role in identifying early-period cardiac ischemia.
Methods: This prospective study included 35 patients diagnosed with DKA, 20 patients diagnosed with DK, and 20 control subjects. H-FABP, creatine kinase-MB (CK-MB), and troponin I levels were investigated at presentation in patients with DKA and DK and in the control group. H-FABP values were measured again after acidosis correction in the DKA patients.
Results: No statistically significant differences were found with respect to troponin I and CK-MB within the groups. The H-FABP values of DKA patients at presentation were found to be significantly higher than those of DK patients and the control group ($p = 0.015$). The H-FABP value of the DKA group was also found to be significantly higher than the value at hour 36 after acidosis correction ($p = 0.0001$).
Conclusion: We would like to propose H-FABP as a potential marker for indicating myocardial ischemia.
Keywords: Heart-type fatty acid binding protein, child, diabetic ketoacidosis

Introduction

Diabetic ketoacidosis (DKA) is an acute complication of type 1 diabetes mellitus (T1DM) and a significant cause of morbidity and mortality. It is the most frequent reason for hospitalization among children with T1DM. Despite the developments in treatment and management, DKA emerges as the first presentation of newly diagnosed diabetes, as well. This rate may vary among countries and is in the range of 15% and 67% (1). Cerebral edema occurs in 0.5%-0.9% of all episodes of DKA, and the most frequent cause of mortality is brain edema (2,3). Acute respiratory distress, hypo- or hyperkalemia, acute renal failure, and disseminated intravascular coagulation are some of the other life-threatening complications (4).

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Heart-type fatty acid-binding protein (H-FABP) is a novel biomarker shown to be released from the injured myocardium and is detected in blood within 1 hour after the onset of ischemia (5). Several studies have demonstrated that it is a sensitive early marker of myocardial injury (6,7). Recent clinical and experimental studies have suggested that H-FABP is superior to creatine kinase-MB (CK-MB) or cardiac troponins for early detection of ischemic myocardial necrosis (6,8). H-FABP is a low molecular weight (14.5 kDa) protein which contains 132 amino acid residues. Fatty acid-binding proteins bind the long-chained fatty acids reversibly and non-covalently, facilitating the intracellular cytoplasmic transport of the fatty acids and are highly expressed in tissues with active fatty acid metabolism such as the heart muscle. Their concentration in blood reaches the maximum value within 6-8 hours and generally decreases within 24-36 hours (9).

Reports on the cardiac effects of DKA in children and adolescents are limited (10,11). In this study, the serum level of H-FABP, a cardiac-specific marker, was measured in pediatric and adolescent patients presenting with ketoacidosis and ketosis. The role of this protein in indicating early-period cardiac ischemia was demonstrated.

Methods

The study was conducted between 2013 and 2015 at the Pediatric Endocrinology Clinic of the Selçuk University School of Medicine Hospital as a prospective randomized controlled study. The study included 55 patients aged 1-18 years, divided into 35 patients with DKA and 20 patients with diabetic ketosis (DK). The control group comprised 20 volunteer healthy children and adolescents who presented for routine follow-up and who did not have any infectious or chronic diseases. The patients that presented with signs of diabetes received a diagnosis of DKA based on the following findings: plasma glucose level >200 mg/dL, pH <7.35, serum bicarbonate level <15 mEq/L, and presence of ketones in the urine and serum. The patients who had a plasma glucose level of >200 mg/dL and ketonemia or ketonuria but had pH >7.30 in venous blood and blood bicarbonate level >15 mEq/L were diagnosed as DK.

The H-FABP, CK-MB, and troponin I levels of the DKA and DK patients and the control group were determined at the time of presentation. For DKA patients, the H-FABP level was also measured again 36 hours after the correction of acidosis. Blood gases, serum bicarbonate, urea, creatinine, alanine transaminase (ALT), and aspartate aminotransferase (AST) levels, as well as hemograms, were also determined in the patient and control groups. The blood ketones were checked in the patient group.

The electrocardiographs (ECGs) of the patient and control groups were obtained with a Nihon-Kohden device. The assessment was made by a pediatric cardiologist based on the criteria for ischemia of a normal T axis with ST depression, T-wave inversion, and ST elevation in the left precordial leads, and the criteria for infarction of Q-wave longer than 0.03 seconds, ST elevation, and T-wave inversion.

The study was approved by the Ethics Committee of Selçuk University. Informed consent and data release approval forms were obtained from all individuals above 12 years of age and from the parents of all individuals below 12 years of age.

The study excluded patients with type 2 diabetes mellitus or monogenic diabetes, cardiovascular, hepatic, and other chronic diseases, a history of smoking, or a history of drug, substance, or alcohol abuse.

Hycult Biotech (USA) commercial kits were used to analyze H-FABP in a Rayto-2100C Microplate Reader (India) device via the enzyme-linked immunosorbent assay method. CK-MB and troponin I values were analyzed using a Hitachi Cobas 601 (Japan) device through the method of electroluminescence in Roche commercial kits. The hemograms were analyzed in an Abbott CELL-DYN 3700 System (Germany) device. Blood gas was analyzed in a Techno Media Gastat 604 ox (Japan) device. Urea, creatine, and transaminases were analyzed in an Abbott Architect C16000 (Japan) device via the colorimetric method in Abbott commercial kits. Electrolytes were analyzed in an Abbott Architect C16000 (Japan) device via the colorimetric method, using an Ion Selective Electrode (ISE) system in Abbott commercial kits.

Statistical Analysis

For statistical analysis, the SPSS-15 (SPSS Inc., Chicago, Illinois, USA) software pack was used. For the evaluation of nominal distribution, the Kolmogorov-Smirnov test was applied. For data that were compliant with normal distribution, parametric tests, an ANOVA variance analysis, and a Tukey's honest test were performed. For data that were not compliant with normal distribution, non-parametric tests, Kruskal-Wallis variance analysis and Mann-Whitney U-test, were performed. The difference in H-FABP levels at patient's admission and at hour 36 after correction of acidosis in the DKA group was assessed using the paired-samples t-test. The significance level was considered to be $p<0.05$.

Results

Our study included 55 T1DM children and adolescents. In the group presenting with DKA, 17 were female and 18 were male; their mean age was 120.3 ± 60 months. The DK group consisted of 7 female and 13 male subjects; their mean age was 122.1 ± 60 months. The control group included 13 female and 7 male subjects, and their mean age was 108.4 ± 71 months. The groups had a similar distribution in terms of age, sex, height, and weight (Table 1).

In Table 2, the laboratory results of patients with DKA, patients with DK, and the control group are given. A statistically significant difference ($p < 0.0001$) was found among the groups in terms of plasma glucose, venous pH, serum bicarbonate, and blood ketones. There was no significant difference between the group with DKA and the group with DK regarding serum creatinine values ($p > 0.878$). On the other hand, there was a significant difference in serum creatinine values between the DKA/DK groups and the control group ($p < 0.0001$). The H-FABP levels of patients at presentation was found to be significantly higher ($p = 0.015$) in the group with DKA as compared to the other groups. White blood cell count was found to be significantly higher ($p < 0.004$) in DKA and DK patients as compared to the control group. Phosphate and AST levels, not shown in the table, were significantly higher in the control group as compared to other groups (DK and DKA)($p = 0.05$ and $p < 0.0001$, respectively).

No statistical significance ($p = 0.457$) was found with respect to troponin I; the values were 0.06 ± 0.08 (DKA), 0.07 ± 0.04 (DK), and 0.04 ± 0.04 (controls), respectively. No statistically significant difference ($p = 0.229$) was detected among groups with respect to CK-MB values, which were 1.48 ± 0.91 (DKA), 1.55 ± 0.9 (DK), and 2.09 ± 1.37 (controls), respectively. Table 3 shows the H-FABP levels for the patients at the time of presentation and at hour 36 after the correction of acidosis. The value at the time of presentation was found to be significantly higher ($p < 0.0001$). H-FABP level at the time of presentation was statistically significantly higher in the group with DKA as compared to the DK and control groups ($p = 0.015$). H-FABP levels were 1.17 ± 0.79, 0.79 ± 0.5, and 0.69 ± 0.36, respectively in these three groups. The H-FABP value of the DKA group at presentation was found to be higher at a statistically significant level as compared to the value at hour 36 after the correction of acidosis, and these values were 1.17 ± 0.79 and 0.55 ± 0.28, respectively ($p = 0.0001$). The correlation between the H-FABP level at presentation and the demographic and laboratory values of the patients was also checked. H-FABP was identified to be moderately positively correlated with glucose level ($r = 0.335$, $p = 0.013$), corrected serum sodium level ($r = 0.386$, $p = 0.004$), white blood cell count ($r = 0.374$, $p = 0.005$), urea ($r = 0.34$, $p = 0.010$), and blood creatinine ($r = 0.444$, $p = 0.00$) levels.

Abnormal findings such as ischemia, necrosis, arrhythmia, or tachycardia were not detected in the ECGs of neither the patient nor the control groups.

Table 1. The demographic characteristics of patients with diabetic ketoacidosis, diabetic ketosis, and the control group

	DKA (n = 35) (Mean ± SD)	DK (n = 20) (Mean ± SD)	Controls (n = 20) (Mean ± SD)	p-value
Age (months)	120.3 ± 30	122.1 ± 60	108.4 ± 70	NS
Sex (male/female)	17/18	7/13	13/7	NS
Height (cm)	135.6 ± 26.0	138.0 ± 30.0	118.7 ± 36.0	NS
Weight (kg)	34.0 ± 17.0	36.2 ± 21.0	30.0 ± 22.9	NS

DKA: diabetic ketoacidosis, DK: diabetic ketosis, SD: standard deviation, NS: not significant

Table 2. The laboratory results of patients with diabetic ketoacidosis, patients with diabetic ketosis, and the control group

	DKA (n = 35) (Mean ± SD)	DK (n = 20) (Mean ± SD)	Controls (n = 20) (Mean ± SD)	p-value
Glucose (mg/dL)	510.7 ± 145.0	432.0 ± 147.0	87.0 ± 8.0	0.0001
Ph	7.17 ± 0.135	7.36 ± 0.056	7.36 ± 0.01	0.0001
HCO_3 (mEq/L)	8.17 ± 3.61	19.2 ± 4.9	14 ± 2.1	0.0001
Blood ketones	5.72 ± 1.2	3.6 ± 2.1		0.0001
BUN (mg/dL)	26.5 ± 9	24.28	24 ± 8	NS
Creatinine (mg/dL)	0.94 ± 0.33	0.87 ± 0.20	0.56 ± 0.13	0.0001
Troponin I (mg/dL)	0.06 ± 0.08	0.06 ± 0.045	0.04 ± 0.04	NS
CK-MB (mg/dL)	1.48 ± 0.91	1.55 ± 0.9	2.09 ± 1.37	NS
H-FABP 0 (ng/mL)	1.17 ± 0.79	0.79 ± 0.5	0.69 ± 0.36	0.015

DKA: diabetic ketoacidosis, DK: diabetic ketosis, SD: standard deviation, NS: not significant, CK-MB: creatine kinase-MB, BUN: blood urea nitrogen, H-FABP: heart-type fatty acid binding protein, HCO_3: bicarbonate

Table 3. The comparison of heart-type fatty acid binding protein levels at the time of presentation of patients and at hour 36 after the correction of acidosis

	Founded values (mean ± SD)	p-value
H-FABP at the time of presentation (ng/mL)	1.17 ± 0.79	0.0001
H-FABP at hour 36 after the correction of acidosis (ng/mL)	0.55 ± 0.28	

SD: standard deviation, H-FABP: heart-type fatty acid binding protein

Discussion

It is well known that DKA has acute and severe effects on the myocardium. Hyperglycemia and acidosis lead to osmotic diuresis, dehydration, and loss of essential electrolytes (2). Thus, ischemia and myocardial cell damage occur with the disruption of tissue perfusion. Cardiac biomarkers are important in determining the risk of cardiac ischemia and necrosis, and in stopping their progression. The biomarkers which have been defined to show an increase in the blood especially during the course of cardiac ischemia and necrosis are rapidly released into the bloodstream after myocardial injury. The myocardial-specific troponin is used in correlation with CK and myoglobin because it is still used as the golden standard in this fiel d, and it increases 6 hours after the cardiac event. Although CK-MB levels increase significantly in 6-8 hours and myoglobin levels increase significantly 3 hours after myocardial infarction, they are not cardiac-specific markers and cannot distinguish myocardial damage from skeletal muscle injury (12). In studies on the myocardial-specific biomarkers, H-FABP is a new and reliable predictor to be included in the literature because its blood level increases within minutes after the initiation of the event and returns to baseline levels 36 hours later, and is at high levels only in the cytoplasm of the myocardial cells (13).

H-FABP is a cytosolic protein which is found in the cytoplasm of the cardiac myocytes and in the liver. It regulates the gene regulation of intracellular transport of fatty acid in ischemic process and also is considered to protect the cardiac myocytes against detergent-like effects (14). It is known to be excreted unchanged by the kidney (8). The small molecular weight protein, which is involved in intracellular transport of fatty acids, is used as a new marker determining recent myocardial damage. This molecule, which is released into the circulation at the first hour of the damage to the myocardial cell, achieves a peak within an average time of 4 hours (3-8 hours) and returns to normal levels within 12-36 hours (8,9).

The peak times of H-FABP, CK-MB, troponins I and T, which are used as specific markers of myocardial necrosis, are 3-12 hours, 4-12 hours, 12-18 hours and 12-48 hours, respectively (15). Therefore, H-FABP, troponin-I and CK-MB were analyzed together in our study. In a meta-analysis of 16 studies conducted by Azzazy et al (13), it was found that with a combination of H-FABP with other markers, its efficacy as a marker became more significant in the early phase of cardiac ischemia. The levels of H-FABP, CK-MB, and troponin-I were examined in adult patients admitted to the emergency department with carbon monoxide poisoning by Açıkalın et al (16) and they have concluded that H-FABP was a significant biomarker to show cardiac ischemia in the early period. Daly et al (17) emphasized that H-FABP was a valuable marker in the early diagnosis of acute myocardial infarction in 407 adult patients who were admitted to the emergency room with ischemic chest pain and had a negative troponin T result. Bank et al (18) agreed that H-FABP testing improves diagnostic accuracy when used in addition to clinical findings and ECG but stated that it had no additional diagnostic value when hs-cTnT measurements are also available. In the light of several studies performed, it is possible to say that H-FABP is a new and reliable marker in the detection of cardiac ischemia and necrosis.

In our study, H-FABP level was found to be higher at a statistically significant level at admission in patients with DKA compared to the other groups and this finding supports the opinion that acidosis causes cardiac ischemia. In the study performed by Atabek et al (10) to determine the cardiac effects of ketoacidosis in children, troponin I, CK-MB, and myoglobin levels were found to be significantly higher in the group with ketoacidosis. In our study, troponin I level was not higher at a statistically significant level in this group. Nevertheless, H-FABP level being higher at a statistically significant level in the group with DKA suggested that it was a more sensitive marker in ischemia and also that our patients were diagnosed in the early stage prior to the development of necrosis as a result of prolonged acidosis. We think that the possibility to determine patients at the ischemia stage with H-FABP is important in shaping the treatment and management of DKA.

The presence of high H-FABP levels at a stage where troponin I is not affected has demonstrated once again that it is a valuable and usable marker in determining ischemia which has not yet progressed to cause myocardial damage and necrosis. In our study, troponin I level was found to be low.

In the literature, there are studies conducted on adults investigating the effects of DKA on the heart. In a study performed by Al-Mallah et al (19) in 2008, it was reported that troponin I level was high at admission in 26 of 96

patients with ketoacidosis and that patients with a high troponin I level followed for 2 years carried a high risk of cardiovascular disease and mortality. The authors emphasize that it is important to follow such patients for a long term for cardiovascular diseases. Long-term follow-up requirement is also one of the messages of our study which consisted of child and adolescent age groups with a longer life expectancy. Mollera et al (20) found that troponin I and CK-MB levels were high in two adult patients who were admitted with severe ketoacidosis in 2005 and who had no history of serious cardiovascular disease. Myocardial infarction findings were detected in the ECG of both patients. However, these authors have concluded that this situation developed with the release of enzyme-linked membrane instability caused by acidosis and the increased levels of free fatty acids, since the coronary arteriography of these patients was normal (20). George et al (21) made a comparison between patients with DKA and patients with hyperglycemia without ketoacidosis and reported that the mean systolic rate and pre-ejection period/left ventricular ejection times were significantly lower in the group with DKA, but that there was no difference between the two groups in terms of heart rate or arterial pressure.

Hyperglycemia causes abnormalities in calcium homeostasis and lipid metabolism with an increase in the level of free fatty acids and growth factors. Moreover, it is known to cause abnormal gene expression, improper signal transmission, and cardiomyocyte apoptosis due to oxidative stress by increasing the release of reactive oxygen molecules (22). In our study, although the patients with DK were hyperglycemic, their H-FABP level was found to be low. We suggest that while the effect of acidosis on the myocardium is mostly associated with ischemia in the acute phase, hyperglycemia has a cardiomyopathic effect in the chronic phase.

The lack of a statistically significant difference between the groups with and without DKA in terms of creatinine levels was explained by the dehydration present in both groups. The statistically significant difference at admission between the two groups in terms of H-FABP levels has suggested that the molecule is not affected by dehydration and also that ischemia is affected by acidosis rather than glucose elevation and dehydration. The statistically significant difference between the groups in terms of white blood cell counts was explained by the dehydration in the group with and without DKA. The low phosphorus level in the group with DKA was an expected finding. During DKA, the detection of a moderate positive correlation between the H-FABP level at admission and the plasma glucose level and also that between the H-FABP level and the corrected serum sodium levels, white blood cell, urine, blood creatinine, has been associated with prolonged duration of DKA and the severity of ischemia increased by acidosis. The presence of significantly high AST levels in the control group has ruled out the idea that H-FABP was elevated due to the possible hepatic ischemia in the group with ketoacidosis. On the other hand, the lack of need for intensive care and follow-up in our patients who were in a state of clear consciousness has ruled out the idea that the increase in H-FABP is due to cerebral ischemia. Thus, our results support the conclusion that H-FABP may be a biomarker of cardiac ischemia in DKA.

It has been emphasized that ketoacidosis and acidosis can cause a prolonged QT resulting in sudden cardiac death, and that cardiac monitoring is important in patients with DKA (23). In our study, even the ECG tracings of patients with DKA who had high H-FABP levels were normal. The lack of ischemia or infarct signs on ECG taken at the admission in the patient and control groups has suggested that in the early stages of ketoacidosis, cardiac ischemia may be asymptomatic. However, it is important to follow up the possible complications of cardiac ischemia with cardiac monitoring in patients with DKA in the acute phase. The lack of ischemia signs on ECG reveals that checking biochemical markers simultaneously is needed as well.

In conclusion, our findings indicate that H-FABP, which detects cardiac involvement in the ischemia phase in DKA, is an important tool in patient management. In our study, a decrease in H-FABP levels following the improvement of ketoacidosis has shown us that cardiac ischemia in the early stages of DKA can be reversed with proper treatment without progressing to necrosis. Repetitive attacks of DKA may increase the risk of cardiac necrosis in the early period.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Selçuk University.

Informed Consent: Informed consent and data release approval forms were obtained from all individuals above 12 years of age and from the parents of all individuals below 12 years of age.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Sevil Arı Yuca, Fatma Hilal Yılmaz, Design: Yaşar Şen, Data Collection and Processing: İsa Yılmaz, Fatma Hilal Yılmaz, Analysis and Interpretation: Derya Çimen, Hüsamettin Vatansev, Fikret Akyürek, Literature Research:

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Serum Irisin and Oxytocin Levels as Predictors of Metabolic Parameters in Obese Children

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What is already known on this topic?

The relationships among obesity-associated metabolic disturbances, insulin sensitivity, and circulating irisin levels have been investigated in both rats and humans. Oxytocinergic neurons in the paraventricular nucleus of the hypothalamus transmit hypothalamic adiposity signals to the nucleus of the solitary tract, a brain area that integrates satiety signals from the gut and hypothalamus. These signals are recognized to play an important role in body weight regulation and metabolic homeostasis.

What this study adds?

To the best of our knowledge, no study on childhood obesity has investigated the relationship between oxytocin levels and metabolic parameters in children.

Abstract

Objective: Irisin and oxytocin can affect energy homeostasis and it has been suggested that they may play an important role in reducing obesity and diabetes. In this study, we aimed to determine the relationship between metabolic parameters (including irisin and oxytocin levels) and anthropometric parameters in obese children.

Methods: Ninety obese children (mean age, 13.85 ± 1.63 years) and 30 healthy controls (mean age, 14.32 ± 1.58 years) were enrolled in this study. Anthropometric and laboratory parameters (glucose, insulin, lipid, oxytocin, and irisin levels) were analyzed. The serum irisin and oxytocin levels were measured by enzyme-linked immunosorbent assay. Bioelectrical impedance was used to determine body composition.

Results: Irisin level was higher in the patients than in the controls ($p = 0.018$), and this higher irisin level was correlated with increased systolic blood pressure, body mass index, waist/hip ratio, fat percentage, fat mass, glucose level, insulin level, and homeostasis model assessment of insulin resistance. Serum oxytocin level was significantly decreased in obese children compared to the controls ($p = 0.049$). Also, among the 60 obese patients, oxytocin level was significantly lower in patients with than in those without metabolic syndrome (8.65 ± 2.69 vs. 10.87 ± 5.93 ng/L, respectively), while irisin levels were comparable ($p = 0.049$ and $p = 0.104$, respectively). There were no statistically significant relationships between oxytocin or irisin levels and lipid levels ($p > 0.05$).

Conclusion: Obese children had significantly higher irisin levels than the healthy controls. Additionally, this study shows for the first time that oxytocin level is significantly lower in obese compared with non-obese children and also lower in obese children with metabolic syndrome compared to those without.

Keywords: Child obesity, irisin, oxytocin, fat mass

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Introduction

Both adipose and muscle tissues have been in focus during the last few years, because they are recognized to play an important role in body weight regulation and metabolic homeostasis. Interest in brown adipose tissue which increases after cold exposure, hormonal stimulation, expression of key genetic regulatory factors, exercise, and expression of a mitochondrial protein called uncoupling protein 1 has also increased in recent years (1,2). Intracellular signalling pathways regulate transcriptional factors such as peroxisome proliferator-activated receptor-γ co-activator 1-α (PGC1-α) which induces energy expenditure (3).

Irisin is a novel muscle-secreted peptide that is proteolytically processed from the product of fibronectin type III domain-containing 5, a type I membrane protein (2,4). Irisin is regulated by PGC1-α and has been proposed to mediate the beneficial effects of exercise on metabolism, inducing adipocyte browning and thermogenesis by increasing uncoupling protein 1 levels. In this context, stimulation of brown adipose tissue cells by irisin exposure may be a therapeutic method of improving metabolic homeostasis (5).

The relationships among obesity-associated metabolic disturbances, insulin sensitivity, and circulating irisin levels have been investigated in both rats and humans (6,7,8). The research findings have suggested that irisin is secreted by muscle tissue as well as adipose tissue, and that irisin secretion from subcutaneous adipose tissue is more relevant than that from visceral adipose tissue (1). Moreover, in addition to its role in skeletal muscle and adipose tissues, irisin might play a role in neural pathways, because animal studies have shown that irisin is expressed in cerebellar Purkinje cells (9). Whether irisin is expressed and plays a role in other brain areas such as the hypothalamus in obese individuals remains unclear.

Hypothalamic neuropeptides regulate energy intake by affecting the feelings of hunger and satiety (10). Oxytocinergic neurons in the paraventricular nucleus of the hypothalamus transmit hypothalamic adiposity signals to the nucleus of the solitary tract, a brain area that integrates satiety signals from the gut and hypothalamus (11). It has been proposed that oxytocin is regulated by PGC1-α and that neuronal inactivation of both PGC1-α and oxytocin leads to impaired thermoregulation and increased food intake (11,12,13).

Few studies have been performed to determine the circulating oxytocin levels in obese adults (14,15,16,17). Moreover, to the best of our knowledge, no study on childhood obesity has investigated the relationship between oxytocin levels and metabolic parameters in children. The objectives of this study were to determine irisin and oxytocin levels in obese children and identify the associations of oxytocin and irisin levels with metabolic and anthropometric parameters in obese children.

Methods

This prospective study included 90 children and adolescents (41 boys, 49 girls) aged 10 to 18 years. Of these, 60 were referred to the pediatric endocrinology outpatient clinic because of excessive weight gain and constituted the study group. Thirty healthy age- and sex-matched children served as the control group. The exclusion criteria were presence of chronic or hereditary diseases, of endocrinologic disorders including syndromes associated with obesity, and a history of drug use. The pubertal stage was assessed according to the criteria described by Marshall and Tanner (18). All participants were evaluated to be in stage ≥2. A challenge in determining the prevalence of metabolic syndrome (MS) is the multiple definitions and criteria used to identify this condition. In response, the International Diabetes Federation (IDF) released the IDF Consensus Worldwide Definition of MS as a single, universally accepted tool. The IDF defines MS in children and adolescents as the presence of abdominal obesity (waist circumference ≥90th percentile for age and sex) and the presence of two or more of the following clinical features: an elevated triglyceride level (≥1.7 mmol/L), a low high-density lipoprotein cholesterol (HDL-C) level (< 1.03 mmol/L), high blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg and/or a diagnosis of hypertension), and an elevated glucose level (≥5.6 mmol/L and/or a diagnosis of type 2 diabetes) (19). Written informed consent was obtained from the parents after being informed about the aim and procedures of the study. The study protocol was approved by the Clinical Research Committee of Namık Kemal University School of Medicine.

Height was measured while in a standing position without shoes using a wall-mounted stadiometer sensitive to the nearest 0.1 cm (Harpenden, Holtain, Crymych, UK). Weight was measured using a portable calibrated scale sensitive to the nearest 0.1 kg (SECA762; Voge&Hakle, Hamburg, Germany) with the subjects wearing light clothing. Body mass index (BMI) was calculated as weight (kg) divided by height (m)^2. Height, weight, and BMI were expressed as standard deviation scores (SDS) using the updated growth reference percentiles for Turkish children and adolescents (20). The fat mass percentage was obtained via bioelectrical

impedance using the BC-418MA Tanita Segmental Body Composition Analyzer (Tanita Europe BV, Hoofddrop, the Netherlands). Waist circumference was measured around the patient's unclothed abdomen at the narrowest point between the rib cage and superior border of the iliac crest. Hip circumference was measured in patients wearing light clothing at the level of the widest diameter around the buttocks using a nonstretch tape (21). Blood pressure was measured using an automated sphygmomanometer. Elevated blood pressure (≥95th percentile for height) was determined using tables provided by the Task Force Report (22).

In both the study and control groups, peripheral venous blood samples were collected after a 12-hour overnight fast. The serum samples were separated from the complete blood samples by centrifugation at 3000 rpm for 5 min. The serum samples were then stored at −86 °C in the freezer until irisin and oxytocin analysis. Glucose, insulin, free thyroxine (fT_4), thyroid-stimulating hormone, low-density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides, total cholesterol (TC), and alanine aminotransferase levels were determined in these samples using enzymatic methods (Roche Modular DP Automatic Biochemical Analyzer; Roche Diagnostics, Indianapolis, IN, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was used to determine the presence of insulin resistance by employing the following formula: fasting glucose (mmol/L) × fasting insulin (IU/L)/22.5. The HOMA-IR cut-off values for insulin resistance were calculated as 5.22 in boys and 3.82 in girls (23).

Serum irisin and oxytocin analyses were performed by enzyme-linked immunosorbent assay (ELISA). Serum irisin level was measured using an ELISA kit from Hangzhou Eastbiopharm Co., Ltd. (Hangzhou, China). The irisin range of the assay was 0.5 to 300 ng/mL. Serum oxytocin level was measured using the Eastbiopharm ELISA kit, and oxytocin range of the assay was 0.1 to 450 ng/L. The intra- and interassay coefficients of variation were < 7% for oxytocin and < 10% for irisin.

Statistical Analysis

The data were analyzed using SPSS 20.0 Statistical Software (IBM Corp., Armonk, NY, USA). Variables were expressed as means ± standard deviations, medians (maximum-minimum), percentages, and frequencies. All variables were assessed following the preconditional control for normality and homogeneity of variance (Shapiro-Wilk and Levene tests). The groups were compared using an independent t-test or the Mann-Whitney U-test when the precondition was not provided. Associations between two continuous variables were evaluated by Pearson's or Spearman's correlation coefficient analyses when the parametrical test precondition was not provided. Categorical data were analyzed using Fisher's exact test and the chi-square test. If the expected frequency was lower than 20%, the Monte Carlo simulation method was used to include this frequency in the analysis. The sensitivity and specificity of the oxytocin and irisin levels were evaluated using receiver operating curves. Youden's index was then used to determine the cut-off points from the curves. A p-value < 0.05 or < 0.01 was considered statistically significant.

Results

Ninety obese children/adolescents (mean age, 13.85 ± 1.63 years) and 30 healthy controls (mean age, 14.32 ± 1.58 years) were enrolled in this study. The characteristics and baseline laboratory values of the patients and control subjects are shown in Table 1. BMI, BMI SDS, waist circumference, hip

Table 1. Baseline characteristics and metabolic parameters of the obese subjects and controls

	Obese patients (n = 60)	Controls (n = 30)	p-value
Age (years)	13.85 ± 1.63	14.32 ± 1.58	0.195
Height SDS	0.41 ± 1.00	0.02 ± 0.81	0.071
Weight SDS	2.57 ± 0.61	-0.31 ± 0.68	0.001**
BMI SDS	2.49 ± 0.42	-0.34 ± 0.73	0.001**
SBP (mm/Hg)	121.08 ± 14.65	110.33 ± 12.24	0.001**
DBP (mm/Hg)	71.50 ± 12.22	66.73 ± 7.89	0.028
Waist/hip ratio	0.92 ± 0.06	0.84 ± 0.06	0.001**
Fat percentage (%)	37.61 ± 5.32	15.39 ± 7.08	0.001**
Fat mass	30.03 ± 8.17	11.00 ± 2.97	0.001**
FFM	48.31 ± 10.53	43.96 ± 7.53	0.045*
Glucose (mg/dL)	90.57 ± 6.77	85.30 ± 6.17	0.001**
Insulin µU/mL	24.39 ± 10.50	9.54 ± 3.04	0.001**
HOMA-IR	5.43 ± 2.36	1.95 ± 0.68	0.001**
TC (mg/dL)	161.87 ± 37.43	136.93 ± 20.59	0.001**
TG (mg/dL)	122.38 ± 53,38	73.40 ± 27.27	0.001**
HDL (mg/dL)	42.15 ± 9.05	52.47 ± 9.65	0.001**
LDL (mg/dL)	94.48 ± 32.71	81.33 ± 18.87	0.045
Irisin (ng/mL)	64.31 ± 33.77	47.20 ± 26.89	0.018*
Oxytocin (ng/L)	10.17 ± 5.21	12.05 ± 4.43	0.049*

The data are expressed as means ± standard deviations.

*Correlation is significant at the 0.05 level (two-tailed), **Correlation is significant at the 0.01 level (two-tailed).

BMI SDS: body mass index standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, FFM: fat-free mass, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, TG: triglycerides, HDL: high-density lipoprotein, LDL: low-density lipoprotein

size, waist/hip ratio, fat percentage, fat mass, fat-free mass (FFM), glucose, insulin, HOMA-IR, TC, triglycerides, LDL-C, irisin, systolic blood pressure, and diastolic blood pressure were significantly higher and the oxytocin level lower in the obese patients compared with the controls (Table 1). No significant difference in sex was observed between the two groups ($p > 0.05$). There were significant differences in the presence of acanthosis and insulin resistance between the patient and control groups ($p < 0.01$).

In the patient group, a higher irisin level was correlated with increased systolic blood pressure, weight, weight SDS, BMI, BMI SDS, waist size, hip size, waist/hip ratio, fat percentage, fat mass, glucose, insulin, and HOMA-IR. There were significant relationships for the irisin level with systolic blood pressure at a rate of 31.8%, weight SDS at a rate of 30.8%, BMI SDS at a rate of 34.5%, waist/hip ratio at a rate of 26.1%, fat percentage at a rate of 25.7%, fat mass at a rate of 26.3%, glucose at a rate of 22.9%, insulin at a rate of 21.4%, and HOMA-IR at a rate of 23.2%. The above figures represent the proportion of patients in whom a significant relationship was seen. Additionally, statistically significant relationships were found between oxytocin and waist size at a rate of 21.2%, waist/hip ratio at a rate of 23.6%, the fat percentage at a rate of 24.3%, and fat mass at a rate of 22.9%. Oxytocin level was inversely correlated with these parameters (Table 2). There were no statistically significant relationships between the oxytocin or irisin level and lipid levels ($p > 0.05$).

Of the 60 patients with obesity, 31.7% (n = 19) had MS. The oxytocin levels were significantly lower in patients with than in those without MS (8.65 ± 2.69 vs. 10.87 ± 5.93 ng/L, respectively), while the irisin levels were comparable in the two groups ($p = 0.049$ and $p = 0.104$, respectively).

The irisin level was found to be a statistically significant marker in discriminating the patients from the controls at a rate of 65.5%. The oxytocin level discriminated the patients from the controls at a rate of 35.4%. The cut-off points were 44.75 ng/mL for irisin, with a 70.0% sensitivity and 60.0% selectivity, and 8.30 ng/L for oxytocin, with a 56.7% sensitivity and 60.0% selectivity (Figure 1).

Discussion

Both adipose and muscle tissues secrete cytokines and other peptides such as adipokines and myokines, which are essential for metabolic homeostasis maintenance. Irisin and oxytocin were recently proposed to play important roles in reducing obesity and diabetes and improving life expectancy.

Table 2. Correlation analysis of anthropometric and metabolic parameters

		SBP	DBP	BMI-SDS	Waist/hip	Fat percentage	FFM	HOMA-IR
Weight-SDS	r	0 .403(**)	0.250(*)	-	-	-	-	-
	p	0.000	0.018	-	-	-	-	-
BMI-SDS	r	0.360(**)	0.196	-	-	-	-	-
	p	0.000	0.064	-	-	-	-	
Waist/hip	r	0.233(*)	0.113	0.516(**)	-	-	-	-
	p	0.027	0.290	0.000	-	-	-	-
Fat percentage	r	0.388(**)	0.233(*)	0.878(**)	0.401(**)	-	-	-
	p	0.000	0.027	0.000	0.000	-	-	-
Fat mass	r	0.375(**)	0.200	0.800(**)	0.445(**)	0.864(**)	-	-
	p	0.000	0.058	0.000	0.000	0.000	-	-
FFM	r	0.110	-0.050	0.243(*)	0.225(*)	0.080	-	-
	p	0.303	0.639	0.021	0.033	0.454	-	-
HOMA-IR	r	0.279(**)	0.114	0.669(**)	0.457(**)	0.617(**)	0.172	-
	p	0.008	0.284	0.000	0.000	0.000	0.105	-
Serum irisin level	r	0.318(**)	-0.011	0.345(**)	0.261(*)	0.257(*)	0.075	0.232(*)
	p	0.002	0.915	0.001	0.013	0.014	0.485	0.028
Serum oxytocin level	r	0.039	0.054	-0.172	-0.236(*)	-0.243(*)	0.025	-0.154
	p	0.717	0.612	0.106	0.025	0.021	0.815	0.148

*Correlation is significant at the 0.05 level (two-tailed), **Correlation is significant at the 0.01 level (two-tailed)

FFM: fat-free mass, HOMA-IR: homeostasis model assessment of insulin resistance, BMI: body mass index, SDS: standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure

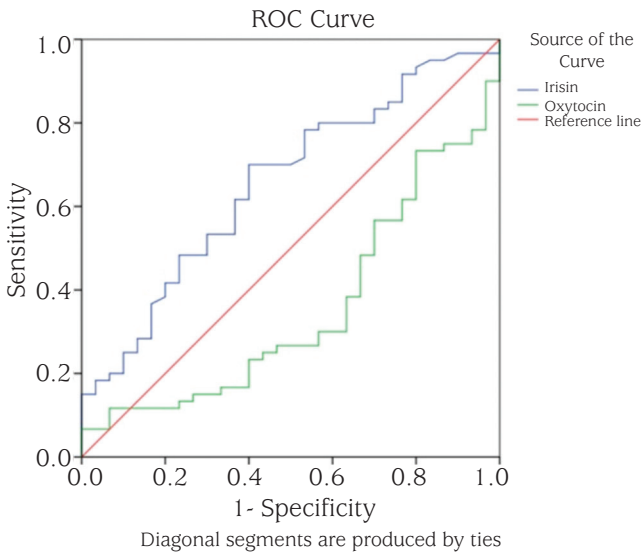

Figure 1. Receiver operating characteristic curve used to evaluate the sensitivity and specificity of serum irisin and oxytocin levels

Previously published results (7,24) have reinforced the concept that a correlation exists between irisin and BMI and suggest that irisin levels reflect the amount of adipose tissue in humans. Importantly, irisin was associated with an increased risk of MS and cardiometabolic variables in humans (25,26). The present study showed that increased irisin levels were correlated with a higher BMI, waist/hip ratio, fat percentage, fat mass, glucose level, insulin level, and HOMA-IR. These results are in agreement with recent reports in which a positive association was observed between circulating irisin levels and fasting insulin levels or HOMA-IR in studies on both adults (27,28) and children (29,30,31).

Crujeiras et al (32) investigated circulating irisin levels in a group of obese adults enrolled in a nutritional program to lose weight, after which some adults then gained weight. They demonstrated that irisin levels reflect net body adiposity. In addition, the authors later showed that irisin levels might predict the onset of insulin resistance in association with weight regain (27). Increased irisin levels have been proposed to serve as an adaptive response that compensates for the decreasing insulin sensitivity and metabolic disturbances associated with obesity (7,33). Irisin is increased in obesity in a manner similar to leptin, which suggests that irisin resistance develops similarly to that of leptin (27,34,35). On the other hand, it could be speculated that in obese individuals, a long-term increase in irisin promotes insulin secretion and insensitivity. Overall, the mechanism underlying increased irisin levels in obese patients remains unclear.

Al-Daghri et al (36) reported that in children, circulating irisin levels were correlated with impaired glucose tolerance and that this relationship was more evident in girls. Fasting blood glucose levels and HOMA-IR were negatively correlated, whereas BMI was positively correlated. However, in this study, the authors focused on sex differences and glucose metabolism rather than obesity and its metabolic consequences. The degree of obesity might be variable, because they found no correlation with BMI, in contrast to HOMA-IR. Palacios-González et al (29) reported that obese children had higher irisin levels which were positively correlated with BMI and leptin levels. Reinehr et al (30) analyzed the relationships among irisin, pubertal stage, obesity, and metabolic parameters in 40 obese children. They found that irisin levels were highest in obese children and were related to the pubertal stage as well as to many MS parameters, such as HOMA-IR, HDL-C, LDL-C, triglycerides, and diastolic blood pressure.

In contrast, some researchers have speculated that irisin levels are significantly decreased in patients with obesity (4), non-alcoholic fatty liver disease (37), and type 2 diabetes (38,39). This might be because of the various degrees of obesity among the patients included in the study groups. Although irisin is also secreted by adipose tissue, the reduced irisin levels in patients with type 2 diabetes may be a result of the decreased fat stores in patients with uncontrolled insulin deficiency, as is the case with leptin (27,40). Another study conducted in 65 obese children revealed no association of irisin levels with sex, age, pubertal stage, weight status, adipokines, or inflammatory markers (41). The different findings in our study may be attributed to the interventional design of the study. However, we did not evaluate the physical activity levels of our subjects.

Swick et al (35) found that irisin action can contribute to and account for differences among individuals whose energy expenditure was predicted by FFM, while FFM can explain approximately 80% of the 24-hour variance in energy expenditure. However, irisin levels were not correlated with the energy expenditure predicted by the FFM equation (35). Notably, in our study, an increased irisin level was correlated with a higher fat percentage and fat mass but was not correlated with FFM. Muscle tissue may be closely associated with changes in irisin levels after exercise training, whereas in pathologic situations such as obesity, adipose tissue is more closely associated with irisin regulation than are other tissues. Irisin showed a significant correlation with excess adiposity and slight correlation with the mass of other tissues (32).

Oxytocin is an anorexigenic neuropeptide that controls metabolic homeostasis not only via an effect on food intake but also by modulating energy expenditure (15). Few studies have been performed to determine the circulating levels of oxytocin in obese adults (14,15,16,17,42). Conflicting findings exist regarding whether oxytocin levels are increased (42), unchanged (43), or decreased (16,17) in obese adults. Variable sample sizes and different study techniques might be the causes of these differences.

In this study, we demonstrated for the first time that the serum oxytocin concentration was significantly decreased in obese children. Furthermore, oxytocin levels were significantly lower in obese children with than in those without MS; the irisin levels were comparable between these groups. Oxytocin level was significantly correlated with waist size, waist/hip ratio, fat percentage, and fat mass. In contrast, no relationship was detected between circulating oxytocin level and HOMA-IR. In our study, the HOMA-IR cut-off values for insulin resistance were 5.22 in boys and 3.82 in girls. However, one study suggested that the HOMA cut-off of 3.16 for insulin resistance is more reliable in adolescents (44). We might have produced different, significant results in our study if we used this cut-off level. We found no previous studies on oxytocin levels in obese children with which to compare our findings. According to our findings, we can state that decreased oxytocin levels may lead to impaired thermoregulation and increased food intake in obese children.

In their study on the influence of oxytocin in adults, Qian et al (16) reported that serum oxytocin levels were decreased in obese adults as well as in adults with type 2 diabetes. In that study, 176 subjects were enrolled, including 88 obese adults and 88 adults with type 2 diabetes; this sample was larger than our study group. In addition, the authors suggested that oxytocin might be involved in lipid metabolism because they found negative correlations with the TC, TG, and LDL-C levels (16). In contrast, we found no significant relationship between lipid levels and oxytocin or irisin levels. Very recently, Yuan et al (17) demonstrated that patients with MS had significantly lower oxytocin levels than did patients without MS, which is consistent with our findings. These reports suggest that pro-inflammatory cytokines may be a key factor in the ability of oxytocin to suppress the inflammation seen in MS.

The mechanism underlying decreased oxytocin levels in obesity remains unclear. Energy expenditure is regulated by many factors, including the transcriptional co-activator PGC-1α and intracellular signalling pathways. In animal studies, oxytocin has been shown to reduce food intake and induce fat weight loss (45,46). Animal studies have revealed that both PGC-1α knock-out and oxytocin receptor-deficient mice exhibit similar abnormalities and impaired thermoregulation and obesity (47,48). Blechman et al (49) showed that PGC-1α was necessary for production of the anorexigenic neuropeptide oxytocin in the zebrafish hypothalamus.

Potential novel clinical uses of oxytocin include treatment of diabetes, insulin resistance, obesity, and cardiovascular disease (50,51). Treatment with oxytocin was shown to reduce the expression of various pro-inflammatory cytokines (tumor necrosis factor-α, IL-1β, and IL-6) (52). These cytokines modulate insulin signalling responses in tissues (53). In addition, according to the results of another study, leptin modulates oxytocin levels and activates oxytocin neurons, thus leptin resistance may be overcome with oxytocin treatment (14).

One limitation of the current study is that our sample size was relatively limited; a larger cohort is needed to investigate this topic more thoroughly. Another limitation is that the physical activity status of the subjects was not evaluated. Finally, our findings did not address the underlying signalling mechanisms and associations between obesity and low oxytocin levels.

In conclusion, in this study, we found that circulating irisin and oxytocin levels were related to obesity in children. In addition, our results show for the first time that the oxytocin level is significantly decreased in obese children and in patients with MS. These findings remain to be confirmed and cannot yet be generalized to all patients. Larger study cohorts are needed to elucidate the mechanism underlying decreased oxytocin levels in obesity.

Ethics

Ethics Committee Approval: The study protocol was approved by the Clinical Research Committee of Namık Kemal University School of Medicine.

Informed Consent: Written informed consent was obtained from the parents after being informed about the aim and procedures of the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Çiğdem Binay, Design: Çiğdem Binay, Data Collection and Processing: Çiğdem Binay, Cem Paketçi, Analysis and Interpretation: Çiğdem Binay, Savaş Güzel, Nedim Samancı, Literature Research: Çiğdem Binay,
Writing: Çiğdem Binay.

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Higher-Than-Conventional Subcutaneous Regular Insulin Doses in Diabetic Ketoacidosis in Children and Adolescents

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What is already known on this topic?

Although dosages of 0.5 U/kg/day to 2 U/kg/day have been used as initial doses in various diabetes centers, little is known about their effect on glycemic control in new-onset type 1 diabetes mellitus (T1DM).

What this study adds?

An initial dose of 1.4-1.5 U/kg/day regular insulin may safely be used after resolution of diabetic ketoacidosis in children with new-onset T1DM without an increased risk of hypoglycemia.

Abstract

Objective: To evaluate the effect of initial insulin dosage on glycemic control in the first 48 hours of subcutaneous regular insulin therapy after resolution of diabetic ketoacidosis (DKA).
Methods: Records of patients with DKA hospitalized in the past 3 years [n = 76, median age = 10.0 (6.0-12.0) years, Male/Female: 44/32] were reviewed. The patients were designated into two groups according to distribution of starting doses of subcutaneous insulin. Group 1 (n = 28) received a median dose of 1.45 U/kg/day (1.41-1.5) and group 2 (n = 48) a median dose of 0.96 U/kg/day (0.89-1). Clinical and laboratory data were analyzed.
Results: Median, minimum, and maximum blood glucose levels of Group 1 in the first 48 hours of treatment were significantly lower than that of Group 2 [213 (171-242) vs. 255 (222-316), p = < 0.001; 102 (85-151) vs. 129 (105-199), p = 0.004; and 335 (290-365) vs. 375 (341-438), p = 0.001, respectively]. The number of patients who experienced hypoglycemia (< 70 mg/dL) were similar [Group 1, 5 (17.9%) vs. Group 2, 4 (8.3%), p = 0.276] and none had severe hypoglycemia. In Group 1, the ratio of blood glucose levels within the target range (100-200 mg/dL) were higher (37.5% vs. 12.5%) and the number of results > 200 mg/dL were lower (50% vs. 81.3%) compared to Group 2 (p = 0.001 and p < 0.001, respectively).
Conclusion: After resolution of DKA, a higher initial dose of 1.4-1.5 U/kg/day regular insulin is associated with better glycemic control in children and adolescents without an increase in risk of hypoglycemia.
Keywords: Type 1 diabetes mellitus, regular insulin, initial doses, children, adolescent

Introduction

Diabetic ketoacidosis (DKA) occurs in 20-40% of children and adolescents with new-onset type 1 diabetes mellitus (T1DM) and after DKA resolves, the therapy is switched to any insulin regimen that aims to control blood glucose (BG) levels. It is known that the required initial daily insulin dose may vary according to many factors including age, body weight, stage of puberty, duration and phase of diabetes (1). The optimal insulin dose for the patient can only be determined empirically (2). An excellent initial insulin dose estimate is one that provides tight BG control and minimizes the risk of hypoglycemia.

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Regular insulin is a soluble crystalline zinc insulin, an essential component of most daily replacement regimens (3). Due to its chemical structure, it has a wide peak and a long tail for bolus insulin, and thus cannot mimic the activity of the β cell but is available to initiate treatment after resolution of DKA and serves to determine the daily insulin dose before basal-bolus insulin regimen. Although guidelines recommend 0.5-1.0 U/kg/day of subcutaneous insulin following resolution of DKA, up to 2 units/kg/d are used in various centers, depending on the preference and experience of the particular diabetes team.

It has previously been reported that intensive insulin therapy would improve endogenous insulin secretion, consequently leading to better metabolic control (4). Thus, one of the aims of therapy following DKA is to control BG levels as early as possible. Higher initial insulin doses could rapidly decrease BG level, but their effect on BG fluctuations have not been extensively investigated (5). This present study aimed to evaluate the effect of the initial insulin dose on glycemic control in the first 48 hours of DKA treatment in children and adolescents with new-onset T1DM and also to compare BG fluctuations with higher and conventional doses of subcutaneous regular insulin therapy.

Methods

The study was conducted in one of the major tertiary hospitals in the region. Hospital records of patients who presented in the last 3 years were reviewed for the study. Diagnosis of T1DM and DKA were made according to the 2014 International Society for Pediatric and Adolescent Diabetes (ISPAD) Clinical Practice Consensus Guidelines for Diabetes in Childhood and Adolescence (6). Newborns, patients who had been treated with any insulin or antihyperglycemic drugs before admission, patients having additional endocrine (hypo-hyperthyroidism, hypo-hypercortisolism, etc.) or non-endocrine diseases (any infectious or inflammatory disease), and those with inadequate hospital records were excluded. Finally, 76 children and adolescents [median age = 10.0 (6.0-12.0) years, Male/Female: 44/32] who presented with DKA due to new-onset T1DM were enrolled as the study group. Age, gender, stage of puberty (patients were noted as pubertal if they had at least Tanner 2 breast development or ≥4 mL testicular volume), body weight and height, glycosylated hemoglobin (HbA1c) levels, BG levels on admission and at the start of regular subcutaneous insulin, insulin dose administered for DKA, and initial dose of regular subcutaneous insulin were recorded. As shown in Figure 1, the patients were designated into two groups according to distribution of starting doses of subcutaneous insulin. Group 1 consisted of patients who received ≥1.25 U/kg/day [n = 28, median dose = 1.45 U/kg/day (1.41-1.5)] and Group 2 consisted of those who were treated with < 1.25 U/kg/day [n = 48, median dose = 0.96 U/kg/day (0.89-1)].

Clinical and laboratory data were collected and analyzed after Behçet Uz Children's Hospital Ethics Committee's approval, in concordance with the principles of Declaration of Helsinki (7).

Treatment Protocol

After resolution of DKA, all patients with new-onset T1DM were started on regular insulin (Humulin R, Lilly, USA) every 6 hours to determine the daily insulin requirement before transition to basal-bolus regimen. As the tissue half-life of insulin is longer than that of intravenous insulin, the first dose of subcutaneous basal insulin was given 30 min before the cessation of intravenous insulin infusion. Premeal BG measurement was performed before each insulin injection 30 minutes before the meals and the insulin dose was determined according to the BG levels: 100-200 mg/dL, same as the previous dose; >200 mg/dL, 110% of the previous dose; <100 mg/dL, 90% of the previous dose (2). The insulin dose was also adjusted according to the consumed amount of the meals. The decision to switch to basal-bolus regimen was made when no significant change was needed in regular insulin doses, generally after 3-4 days. BG levels were measured more frequently in patients who suffered from any symptom of hypo- or hyperglycemia. During hospitalization, the meals of the patients were prepared by dietitians according to the ISPAD Clinical Practice Consensus Guidelines 2014 on nutritional management in children and adolescents with diabetes. The diets contained carbohydrates providing approximately 50-55%, fat up to 30-35%, and protein 10-15% of daily energy requirements (8). Four meals and three snacks were given a day and no additional food was consumed unless hypoglycemic events occurred.

Study Variables

Descriptive characteristics of the patients, treatment information, and every BG measurement during the first 48 hours of subcutaneous regular insulin treatment were recorded. Glycemic variability indices [standard deviation (SD), coefficient of variation (CV), maximum BG, minimum BG, difference between maximum and minimum BG, rate of BG change (the amount of change between consecutive measurements, mg/dL/min)] were calculated. All BG measurements were performed by the same capillary BG monitoring system (Astracheck Plus®, Medisign MM 600, Empecs, Beijing, China), an electrochemical glucometer using the modified glucose oxidase method, calibrated monthly by electronical calibrators.

Statistical Analysis

The data were statistically analyzed using computer software SPSS 15.0 (Chicago, IL, USA). Mann-Whitney U-test and chi-square test were used to compare numerical and categorical variables, respectively, between groups. Univariate correlation analysis was performed between insulin starting dose and median glucose levels during 48 hours. General linear model with repeated measures was applied to assess the differences between the groups regarding the trajectory of glucose levels. Wilcoxon two-related samples test was employed to compare insulin doses at the start and at the 48th hour of treatment among groups. A p-value of <0.05 was chosen to represent statistical significance. Data were presented as median (25p-75p) or n (%).

Results

The study consisted of 76 children and adolescents [median age = 10.0 (6.0-12.0); age range, 1.5-16.0 years; M/F: 44/32] with new onset T1DM admitting with DKA. Thirty six patients (47.4%) were pubertal. The median BG level on admission was 466 mg/dL (383-574) while the median BG level at the start of insulin was 158 mg/dL (123-198). Baseline characteristics of the study group are presented in Table 1.

Group 1 and Group 2 were comparable regarding age, gender, pubertal status, HbA1c, and BG levels both on admission and at the start of subcutaneous insulin treatment. Table 2 presents the descriptive data of the groups.

Both of the groups had similar numbers of BG measurements [Group 1, 8 (8-8) vs. Group 2, 8 (8-8)], p = 0.250). Median BG levels of Group 1 in the first 48 hours were significantly lower than those of Group 2 [213 (171-242) vs. 255 (222-316), p = < 0.001]. Figure 2 shows the trajectory of median BG levels during 48 hours and the difference between the curves was found to be statistically significant (p = < 0.001). All median BG levels at specific time points in Group 1 were lower than those of Group 2, but statistical significance was present at 6th [163 (103-247) vs. 232 (188-294), p = 0.009], 30th [176 (103-219) vs. 258 (178-326), p = 0.001], 36th [184 (139-233) vs. 279 (222-360), p = < 0.001], and 48th [198 (105-223) vs. 277 (205-316), p = < 0.001] hours. Starting insulin dose (U/kg/day) and median glucose levels after starting subcutaneous insulin was found to be mildly correlated (r = -0.489, p = 0.001) (Figure 1).

Rates of BG levels <50 mg/dL and <70 mg/dL were compared between the groups to evaluate the frequency of hypoglycemia, while the frequency of BG levels >200 mg/dL was evaluated in order to assess hyperglycemia (Table 3). Only two patients in Group 1 had experienced

Table 1. Baseline characteristics of the study group

	Total group (n = 76)
Age (years)	10.0 (6.0-12.0)
Male	44 (57.9%)
Pubertal patients	36 (47.4%)
Blood glucose on admission (mg/dL)	466 (383-574)
pH	7.17 (7.05-7.24)
Bicarbonate (mmol/L)	8.85(6.0-12.6)
HbA1c (%)	12.5 (10.8-13.8)
Dose of intravenous insulin (U/h)	0.1 (0.1-0.1)
Dose of subcutaneous insulin (U/kg/day)	1.00 (0.93-1.44)
Blood glucose before start of regular insulin (mg/dL)	158 (123-198)

Table 2. Descriptive data of patients among the two groups

	Group 1 (n = 28)	Group 2 (n = 48)	p
Age (years)	10.5 (5.63-13.6)	9 (6-11.9)	0.205
Male	15 (53.6%)	29 (60.4%)	0.560
Pubertal patients	15 (53.6%)	21 (44%)	0.479
Blood glucose on admission (mg/dL)	465 (366-577)	466 (394-573)	0.635
pH	7.18 (7.06-7.24)	7.16 (7.03-7.24)	0.987
Bicarbonate (mmol/L)	9.6 (7.3-11.7)	8.5 (5.5-13)	0.983
HbA1c (%)	12.8 (11.2-14.6)	12.5 (10.6-13.8)	0.598
Dose of insulin infusion (U/h)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.999
Blood glucose at the start of subcutaneous insulin (mg/dL)	159 (124-194)	157 (123-200)	0.718
Starting insulin dose (U/kg/day)	1.45 (1.41-1.5)	0.96 (0.89-1)	**< 0.001**
Insulin dose on 1st day (U/kg/day)	1.5 (1.41-1.56)	0.99 (0.93-1.03)	**< 0.001**

BG levels < 50 mg/dL and those episodes were treated with oral glucose solutions. Frequency of BG levels < 50 mg/dL, < 70 mg/dL, and < 100 mg/dL were similar in the two groups, while the percentage of BG levels > 200 mg/dL were significantly lower in Group 1 (p = < 0.001). The number of BG measurements in the target range (100-200 mg/dL) were significantly higher in Group 1 than in Group 2 (p = 0.001).

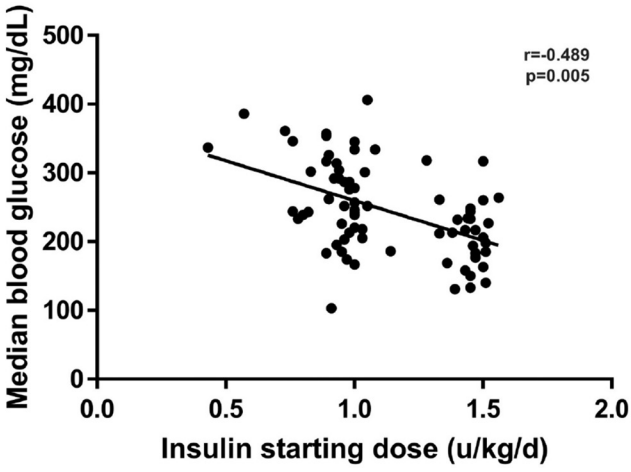

Figure 1. Distribution of the starting subcutaneous insulin doses and its correlation with median blood glucose levels during the first 48 hours of treatment

The minimum and maximum BG levels during 48 hours were significantly lower in Group 1 than in Group 2 (p = 0.004 and p = 0.001) (Table 4). Table 4 presents additional glycemic variability indices of the two groups including BG rate of change, difference between minimum and maximum BG, SD and CV of BG.

During follow-up, subcutaneous insulin doses needed to be increased in order to avoid hyperglycemia. As a result, insulin doses (units/kg/d) administered on the second day in both Group 1 [1.63 (1.47-1.77) and Group 2 [1.07

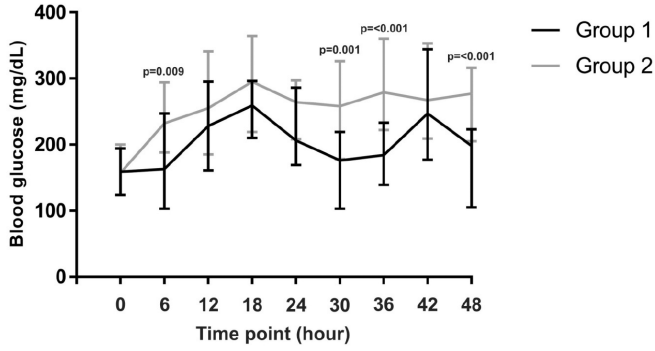

Figure 2. Median, 25th, and 75th percentile values of the two groups at baseline and at specific time points during the first two days of subcutaneous insulin treatment. The p-values are given where statistically significant differences were present between the two groups

Table 3. The characteristics of blood glucose fluctuations in patients with high-dose and conventional-dose subcutaneous regular insulin after resolution of diabetic ketoacidosis

	Group 1 (n = 28)	Group 2 (n = 48)	p
Number of episodes < 50 mg/dL	0 (0-0)	0 (0-0)	0.062
Percentage of episodes < 50 mg/dL	0 (0-0)	0 (0-0)	0.062
Number of patients who experienced episodes < 50 mg/dL	2 (7.1%)	0 (0-0)	0.133
Number of episodes < 70 mg/dL	0 (0-0)	0 (0-0)	0.204
Percentage of episodes < 70 mg/dL	0 (0-0)	0 (0-0)	0.234
Number of patients who experienced episodes < 70 mg/dL	5 (17.9%)	4 (8.3%)	0.276
Percentage of episodes < 100 mg/dL	6.25 (0-25)	0 (0-12.5)	**0.021**
Percentage of episodes between 100-200 mg/dL	37.5 (25-50)	12.5 (3.13-25)	**0.001**
Percentage of episodes > 200 mg/dL	50 (25-75)	81.3 (62.5-87.5)	**0.001**

Table 4. Glycemic variability indices among groups

	Group 1 (n = 28)	Group 2 (n = 48)	p
BG rate of change (mg/dL/min)	0.5 (-0.73-2.13)	2.13 (0.89-3.4)	**0.001**
Minimum BG (mg/dL)	99 (86.3-120)	137 (99-192)	**0.004**
Maximum BG (mg/dL)	335 (290-365)	375 (341-438)	**0.001**
Difference between minimum and maximum BG level (mg/dL)	222 (168-272)	219 (169-304)	0.445
Standard deviation	78.7 (55.9-91.5)	75.8 (60.8-96.1)	0.504
Coefficient of variation	0.36 (0.29-0.44)	0.3 (0.24-0.39)	0.094

BG: blood glucose

(1-1.22)] were significantly higher compared to starting doses ($p < 0.001$ for both).

Discussion

There are different approaches to initiate subcutaneous insulin after resolution of DKA, partly influenced by practice style and health care economics. In some institutions, basal-bolus insulin regimen is initiated immediately after resolution of DKA and the patient is discharged from hospital and further managed as an outpatient. However, many physicians prefer to start with subcutaneous regular insulin prior to discharge and determine the daily insulin requirement for the individual patient. Whichever is preferred, the primary concern is to maintain BG control while avoiding hypoglycemia. Early control of BG with insulin therapy might be associated with improved long-term glycemic control and higher endogenous insulin production (9,10). In practice, total insulin doses of 0.5-0.75 U/kg/day are typically chosen at T1DM onset and the dose is then adjusted on a daily basis to achieve the targeted glycemia. However, transition from 1.2-2.4 U/kg/day during treatment for DKA to much lower doses for subcutaneous insulin treatment often results in increase in BG levels, as was the case in the present study in both groups.

It is well-known that the metabolic abnormalities occurring in the diabetic state, in particular hyperglycemia, cause mitochondrial superoxide overproduction, which leads to activation of major pathways involved in the pathogenesis of complications of diabetes (11). Thus, the primary goal of treatment in children and adolescents with T1DM is to maintain near-normoglycemia as early as possible (12,13). In our study, the ratio of BG levels >200 mg/dL were significantly lower in Group 1 than in Group 2 ($p = < 0.001$), a finding from which we may conclude that regular insulin at a dose of 1.4-1.5 U/kg/day prevents hyperglycemia better than the lower doses in the early period. In a previous study, it was reported that BG fluctuations were also associated with oxidative stress with coexistence of high BG levels and suggested that T1DM treatment should aim at reducing glucose fluctuations as well as achieving the overall control (14). In the present study, the difference between minimum and maximum BG, SD and CV of BG were similar in the two groups.

As a matter of fact, the issue that what is more important in the development of vascular damage is controversial in the literature. Peña et al (15) reported that hypoglycemia rather than BG fluctuation was correlated with vascular dysfunction in a pediatric population with T1DM. Moreover, several studies have shown that the most prominent barrier for tight glycemic control is the fear of hypoglycemia (16,17). In our study, there was no statistically significant difference between the groups with regard to hypoglycemia. In Group 1, only 2 patients had suffered <50 mg/dL hypoglycemia (each with 1 BG episode of <50 mg/dL) which was treated with oral glucose solutions. The number and ratio of BG <70 mg/dL and <100 mg/dL episodes were also similar in the two groups. None of our patients experienced severe hypoglycemia.

Wang et al (5) have recently compared the influence of different subcutaneous insulin infusion doses (0.6 ± 0.2 U/kg/day, 1.0 ± 0.2 U/kg/day, and 1.4 ± 0.2 U/kg/day) on BG dynamics of children and adolescents with newly diagnosed T1DM and reported that approximately 90% of patients tolerated the higher insulin doses (1.4 ± 0.2 U/kg/day) for 2 weeks without showing a significant difference regarding severe hypoglycemia rates. Our results are in line with those results and suggest that higher doses are necessary for better control in the early period. During follow-up, subcutaneous insulin doses needed to be increased in both groups in order to avoid hyperglycemia and the ratio of BG levels in the target range were only 37.5%, even in Group 1. Furthermore, the BG levels of the whole study group were only mildly correlated with initial insulin dose suggesting that higher doses could also be well-tolerated.

Late achievement of glycemic control can also result in increased hospital stay and thus, associated risks of hospitalization. However, due to the retrospective design of our study, we were not able to compare the duration of hospital stays in the two groups. Furthermore, there would be many other confounding factors that could affect hospital stay.

In conclusion, we suggest that an initial dose of 1.4-1.5 U/kg/day regular insulin may safely be used after resolution of DKA in children with new-onset T1DM with no increase in risk of hypoglycemia.

Ethics

Ethics Committee Approval: Clinical and laboratory data were collected and analyzed after Behçet Uz Children's Hospital Ethics Committee's approval, in concordance with the principles of Declaration of Helsinki.

Informed Consent: Not applicable.

Peer-review: Externally and Internally peer-reviewed.

Authorship Contributions

Concept: Özlem Bağ, Korcan Demir, Design: Özlem Bağ, Korcan Demir, Data Collection and Processing: Özlem Bağ, Selma Tunç, Özlem Nalbantoğlu, Çiğdem Ecevit, Aysel

Öztürk, Analysis and Interpretation: Korcan Demir, Behzat Özkan, Literature Research: Özlem Bağ, Çiğdem Ecevit, Aysel Öztürk, Korcan Demir, Writing: Özlem Bağ, Korcan Demir.

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Gene Polymorphisms of Glutathione S-Transferase T1/M1 in Egyptian Children and Adolescents with Type 1 Diabetes Mellitus

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What is already known on this topic?

Oxidative stress plays an important role in the pathogenesis of type 1 diabetes mellitus (T1DM). Control of diabetes throughout childhood and adolescence is mandatory.

What this study adds?

To our knowledge, this is the first study on the association between glutathione genetic polymorphism and T1DM in Egypt. Glutathione S-transferase T1 null/M1 wild genotype can be strongly added to the predictive markers of the disease-related risk and complications.

Abstract

Objective: Oxidative stress plays an important role in the pathogenesis of type 1 diabetes mellitus (T1DM). To evaluate the association of glutathione S-transferase mu 1 (GST M1) and glutathione S-transferase theta 1 (GST T1) polymorphisms with development of T1DM and disease-related risk factors.
Methods: Measurement of fasting glucose, serum creatinine, lipid profile, and glycosylated hemoglobin (HbA1c), as well as evaluation of GST T1 and M1 genetic polymorphisms using polymerase chain reaction were done in 64 diabetic children and 41 controls.
Results: The diabetic group had significantly higher fasting glucose, HbA1c, and cholesterol levels. GST T1 null genotype was more frequent in the diabetic than the control group with 4.2-fold increased risk of T1DM (odds ratio = 4.2; 95% confidence interval = 1.6-11.5; p = 0.03). Significant positive associations were found with lipid profile, HbA1c, and duration of illness but not with age, age at onset, and body mass index.
Conclusion: Gene polymorphisms of the enzyme GST are associated with development of T1DM and disease-related risk factors.
Keywords: Glutathione S-transferase T1 and M1, gene polymorphisms, type 1 diabetes mellitus

Introduction

Type 1 diabetes mellitus (T1DM) is the most common metabolic disorder in which both genetic and environmental factors are involved (1). T1DM is considered a chronic immune-mediated disorder. It was hypothesized that whilst children have a genetic predisposition to T1DM, there is likely to be an environmental factor that triggers the development of T1DM. Possible triggers that have been suggested include viral infection, vaccines, low levels of vitamin D, and cow's milk (2). Oxidative stress is one of the important pathways that have been involved in the etiopathogenesis of T1DM (3). Complications of T1DM could be due to the cellular metabolism leading to hyperglycemia and excessive production of reactive oxygen species (ROS). ROS are a substantial threat to the human cells in children with T1DM. Many antioxidants are produced by human cells that counter the effects of these oxidants by decreasing their accumulation. One of the important antioxidants that protects against cellular damage caused by environmental toxins and accumulation of ROS is glutathione (GSH). ROS and xenobiotics are neutralized by GSH via glutathione S-transferase (GST); this enzyme converts these compounds into water-soluble compounds that can be easily eliminated

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(4,5). The human GSTs are a family of enzymes for neutralizing free radicals. They cause detoxification of electrophiles via glutathione conjugation (6). The loci encoding the GST enzymes are located on at least seven chromosomes. This multigene family included seven families (Alpha, Mu, Pi, Theta, Sigma, Zeta, and Omega). There has been substantial interest in studying the associations between particular allelic variants and altered risk of a variety of diseases. Several GST polymorphisms have been associated with an increased or decreased susceptibility to several diseases. Two of the important members of the GST family, named GST mu 1 (GST M1) and GST theta 1 (GST T1) have different polymorphisms. Persons with homozygous deletions of either the GST M1 or the GST T1 locus have no enzymatic activity of the respective enzyme (7,8).

This study aimed to evaluate the association of GST M1 and GST T1 polymorphisms with development of T1DM and disease-related risk factors.

Methods

The study included 64 diabetic children with T1DM with a mean age of 11.7±3.6 years; 26 boys and 38 girls. They were patients attending the Pediatric Genetic and Endocrinology Unit and the Pediatric Outpatient Clinic of Menoufia University Hospitals in Egypt. The study was conducted in the period from January 2015 to March 2016. Diagnosis of T1DM patients was based on the American Diabetes Association (ADA) criteria (9). Patients were followed up, regularly checked and investigated for diabetic complications as well as their current treatment regimens. Cases with type 2 diabetes and those with other chronic diseases such as hypothyroidism, hyperthyroidism, or hypoadrenalism were excluded.

Forty-one apparently healthy children of matched age and sex served as a control group. Written informed consent was obtained from each child included in the study or their participant parents. Ethical clearance was obtained for the research project. The study protocol conforms to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments. Data about the duration of illness and onset of the disease in children with T1DM were obtained from the parents. Body weight, height, and body mass index (BMI) were measured in each child. Biochemical parameters as fasting blood glucose, 2-hour postprandial (2hPP) glucose, serum creatinine, alanine aminotransferase activity, glycosylated hemoglobin (HbA1c), cholesterol, triglyceride levels were determined in each child. The patients were classified as having good or poor glycemic control according to the ADA criteria. The target age-specific HbA1c were as follows: 7.5%-8.5% in <6 year olds, ≤8% in children between 6 and 12 years, and ≤7.5% in those between 13 and 18 years of age (10).

DNA Extraction and Genotype Determination

Genomic DNA was extracted from peripheral venous blood using Bio-spin whole blood genomic DNA extraction kit "Bioflux", according to the recommended protocol. Screening for GST T1 and M1 deletion polymorphisms was done using the polymerase chain reaction (PCR) technique. Details of primers sequence are listed below.

For GST M1 polymorphisms:

5´–GAACTCCCTGAAAAGCTAAAGC–3´ and 5´–GTTGGGCTCAAATATACGGTGG–3´.

For GST T1:

5´–TTCGTTACTGGTCCTCACATCTC–3´ and 5´–TCACGGGATCATGGCCAGCA–3´.

To avoid false-negative readings, internal control was used for amplification of exon 7 of the *CYP1A1* gene, with primers sequence as 5´–AACTTCATCCACGTTCACC–3´and 5´–GAAGAGCCAAGGACAGGTAC–3´.

PCR products were electrophoresed in an agarose gel and visualized after ethidium bromide staining that yield bands of 218 bp, 480 bp, and 315 bp for GST M1, GST T1, and *CYP1A1*, respectively (Figure 1). The GST T1 or GST M1 genotype groups included homozygous and heterozygous states of that functional allele (11).

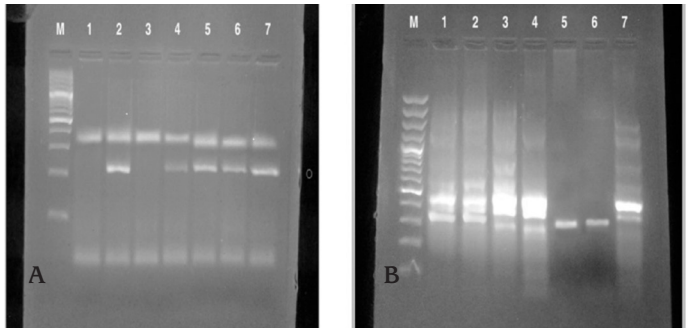

Figure 1. Polymerase chain reaction analysis of glutathione S-transferase M1/T1 gene polymorphism of products visualized in 2% agarose gel electrophoresis.

(A) Lane M is a 100 bp DNA ladder, lanes 1,3 represent glutathione S-transferase M1 null, lanes 2,4,5,6 represent glutathione S-transferase M1 present (218 bp), lane 7 is a positive control sample.

(B) Lane M is a 100 bp DNA ladder, lanes 5,6 represent glutathione S-transferase T1 null, lanes 1,2,3,4 represent glutathione S-transferase T1 present (480 bp), lane 7 is a positive control sample.

Note: Exon7 *CYP1A1* appeared as internal control in all lanes at (315 bp).

Statistical Analysis

Data were collected, processed, and analyzed using SPSS (version 20) software. Continuous variables were presented as mean ± standard eror, and categorical ones were presented in percentage form. Chi-square and Fisher exact tests were used to assess the relationships between various demographic and disease-related risk factors and controls. The strength of association between GST genetic polymorphisms and development of DM was estimated by odds ratio (OR) and 95% confidence interval (CI). Logistic regression analysis was applied for checking calculated OR adjusted for independent variables and risk of T1DM. For all analyses, p-value of < 0.05 was considered statistically significant.

Results

Mean duration of diabetes of the patients was 6.3 ± 4.1 years. Of these diabetic children, 22 were 15-year-old or older; their Tanner stages were 2 or more. 29 of the 64 T1DM patients were in good glycemic control. Demographic and clinical data are shown in Table 1. The frequency rates of GST T1 null genotype were 42.2% and 14.6% in diabetic children and controls, respectively. This demonstrated a significantly increased risk of T1DM among our diabetic patients (OR 4.2, 95% CI 1.6-11.5, p-value = 0.03). The frequency of GST M1 genotypes showed no significant difference. Double analysis of GST genotypes showed that GST T1 null/ M1 *wild* genotype was significantly more frequent in the diabetic group as compared to the control group (29.7% vs. 14.6%, respectively). This combination of alleles 3.2 times increased the risk of T1DM (OR = 3.2; 95% CI = 1.1-9 $\chi^2 = 10.6$; p = 0.014). There were no statistically significant differences regarding GST T1 null/ M1 null genotype and other genotype combinations (Table 2). A significant difference ($p < 0.05$) existed in duration of disease, fasting blood glucose, and HbA1C in relation to GST T1 null genotype, as compared to insignificant difference with age, age at onset, and BMI (Table 3).

Table 4 shows adjusted odd ratios for disease-related characteristics estimated by multivariate logistic regression

Table 1. Demographic and laboratory data in the study and control groups

	Diabetics (n = 64) n (%) Mean ± SE*	Controls (n = 41) n (%) Mean ± SE	X^2	p value
Age (years)	11.7 ± 3.6	8.1 ± 2.9	5.3** U test	< 0.001
Gender			3.2	0.07
Males	26 (40.6%)	24 (58.5%)		
Females	38 (59.4%)	17 (41.5)		
Family history			3.2	0.073
Positive	41 (64.1%)	19 (46.3%)		
Negative	23 (35.9)	22 (53.7%)		
Age at onset		---	---	---
< 5 y	10 (15.6%)			
5-10 y	24 (37.5%)			
> 10 y	30 (46.9%)			
Duration (years)	6.3 ± 4.1	---	---	---
< 5 y	47 (73.4)			
5-10 y	13 (20.3)			
> 10 y	4 (6.3)			
BMI	17.9 ± 4.3	16.7 ± 0.9	1.7 t test	0.08
FBG mg/dL	185.8 ± 64.1	77.2 ± 2.7	10.8 U test	< 0.001
2hpp mg/dL	199.4 ± 74.3	103.6 ± 10.9	8.2 U test	< 0.001
HbA1c	8.9 ± 2	4.9 ± 0.3	12.5 U test	< 0.001

*SE: standard error, **U test, Mann-Whitney U test

BMI: body mass index, FBG: fasting blood glucose, 2hpp: 2 hours postprandial glucose, HbAlc: glycosylated Hb

analysis, which showed that patients with disease duration 5 y or more were more vulnerable to poor glycemic control (OR = 1.07, p = 0.004). This was also noticed among patients with raised serum TG level (OR = 2.7, p = 0.001). Those with GST T1 null/M1 present genotype had 2.2 times increased risk of impaired control of T1DM. Sex, age, and BMI were not significant factors of glycemic control.

Discussion

DM is associated with a high endogenous inflammatory load and oxidative stress (12). Environmental toxicants and oxidative stress are important in the development of T1DM. GSTs can enhance or decrease the toxicity of several compounds and robust GST activity depletes GSH, which can lead to the disruption of cellular homeostasis and cell death. GST T1 and M1 polymorphisms were supposed to be associated with many disorders like hypertension, ischemic heart disease, cancer, and allergic conditions (13). GSTs are involved in the detoxification of ROS and in the synthesis of different inflammatory mediators. Both mechanisms can lead to pancreatic beta cell damage. So, it is hypothesized that GST polymorphisms may have a role in the pathogenesis of T1DM. In our study, the associated risk between GST and T1DM was investigated. Patients with T1DM had a higher frequency of GST T1 null genotype than controls. This represented a significantly increased risk of T1DM (4.2-fold). The combination of GST T1 null/ M1 *wild* genotype was significantly more frequent in diabetic patients than controls. This represented a significantly increased risk of T1DM (3.2-fold). Our findings are concordant with many previous studies on GST T1/M1 gene polymorphisms that reported an increased risk of T1DM with null genotype (14,15,16,17). This can be attributed to the fact that carriers of GST null genotype have significantly lower antioxidant enzymatic activity (18). However, in 2005, Bekris et al (4) reported that null genotype is a protective gene regarding the risk of T1DM. The difference from our study can be explained by several possible mechanisms such as up-regulation of other antioxidant genes like superoxide dismutase gene that follows the depletion of GST activity. However, these interpretations require confirmation by further research. Previous studies reported that GST T1 or M1 gene polymorphisms can be risk factors for the

Table 2. Association of glutathione S-transferase T1 and M1 genotypes with type 1 diabetes mellitus

Genotype	T1DM	Controls	OR	95% CI	X^2	p-value
GST T1						
Wild	37 (57.8)	35 (85.4)	4.2	1.6-11.5	8.8	0.003
Null	27 (42.2)	6 (14.6)				
GST M1						
Wild	50(78.1)	37 (90.2)	2.6	0.8-8.5	2.6	0.108
Null	14(21.9)	4 (9.8)				
*						
T1 + /M1 +	31 (48.4)	31 (75.6)	--	--		
T1 + /M1-	6 (9.4)	4 (9.8)	1.5	0.4-5.8	10.6	0.014
T1-/M1 +	19 (29.7)	6 (14.6)	3.2	1.1-9		
T1-/M1-	8 (12.5)	0 (0)	---	---		

*Double analysis of glutathione S-transferase T1, M1 genotypes and the risk of type 1 diabetes mellitus

GST: glutathione S-transferase, T1DM: type 1 diabetes mellitus, OR: odds ratio, CI: confidence interval

Table 3. Relationships between glutathione S-transferase genotype and disease-related characters in diabetic patients

	Diabetes					
	GST T1		p-value	GST M1		p-value
	Wild	Null		Wild	Null	
Age at onset	7.9 ± 3.3	9.6 ± 2.9	0.038	8.1 ± 3.1	10.3 ± 2.9	0.026
BMI	18.3 ± 2.6	17.4 ± 5.8	0.449	18 ± 4.8	17.6 ± 1.6	0.785
FBG mg/dL	165 ± 66.1	214.4 ± 49.4	0.002	185.4 ± 67.7	187.3 ± 51.6	0.922
HbA1c %	8.5 ± 2.1	9.3 ± 1.8	0.143	9.1 ± 2.1	7.9 ± 1.3	0.035
TC mg/dL	152.2 ± 6.7	155.9 ± 9.6	0.07	155.3 ± 8.6	148.5 ± 3.1	0.005
TG mg/dL	79 ± 9.1	85.8 ± 13.9	0.02	84.7 ± 11.8	71.6 ± 1.9	< 0.001

GST: glutathione S-transferase, BMI: body mass index, FBG: fasting blood glucose, HbAlc: glycosylated Hb, TC: total cholesterol, TG: triglyceride

Table 4. Adjusted odds ratio for disease-related characters among poorly controlled diabetic patients

	Value	OR	p-value	95% CI
Age				
<15 y	1.04	3.2	0.07	1.13-6.12
≥15 y	0.98	2.5	0.002	1.48-4.83
Duration of illness (>5 y)	1.02	1.07	0.004	(1.02-1.68)
Lipid profile				
Raised TG	1.06	2.7	0.001	1.84-3.24
BMI	1.5	1.28	0.562	0.6-2.97
Genotype				
GST M1 (wild)	1.5	1.28	0.72	0.56-2.8
GST T1 (null)	2.9	2.167	0.001	1.42-3.41

Using multivariate logistic regression analysis

GST: glutathione S-transferase, BMI: body mass index, TG: triglyceride, OR: odds ratio, CI: confidence interval

development of diabetes mellitus and chronic diabetic complications (11,19). However, the reported results differ according to the authors, population, and the locality. Most of the reported data pertain to adult subjects with T2DM (14,20). Very limited studies were conducted on GST polymorphisms and diabetes susceptibility in patients with T1DM. Regarding GST T1 genotype, no association was found in a young Swedish population. However, M1 wild genotype was associated with a higher risk of T1DM in a 14-20-year-old group (6). In a Slovakian population, GST T1 null/M1 wild genotype was shown to increase the risk of T1DM (17). The differences in the results between the two populations can be attributed to ethnic background differences in the two populations. It is possible that, as noted in our study group of diabetic children, decreased antioxidant enzymatic activity due to GST T1 null allele, together with one of the tentative potentials due to GST M1 wild allele, constitutes a risk factor for T1DM. Results of other studies investigating the associations of GSTM1 and GSTT1 polymorphisms with T1DM suggest that the GST M1 null genotype is associated with T1DM protection and T1DM age-at-onset and that susceptibility to T1DM may involve GST conjugation (6). Also in our study, it was found that clinical and biochemical parameters showed a significant association with lipid profile, duration of illness, and glycosylated hemoglobin but not with age, age of onset, or BMI. Our results were in concordance with those reported by Vats et al (21). Studies on the impact of genotype on T1DM progression and development of its complications are rare. Hovnik et al (22), considering that the earliest signs of diabetes complications occur after 5-10 years and the highest incidence (between 75-95%) is observed after 10 years, stated that the duration of diabetic state is an important risk factor in development of microangiopathic complications in most patients with T1DM. However, a limited period of poor glycemic control can have a prolonged effect on disease complications. This effect, known as metabolic memory, has been demonstrated in the Epidemiology of Diabetes Interventions and Complications cohort follow-up, a study which showed that the GST T1 deletion, disease duration, and raised triglycerides independently show the risk of T1DM progression and glycemic control above the target level for age (23).

To the best of our knowledge, this is the first study on GST genetic polymorphisms and their relationship to T1DM risk among Egyptian children. Our results show the association of GSTT1 null/M1 present genotype with T1DM and indicate that testing of genetic markers in the glutathione family alone or in combination with other disease-associated factors can be used as a marker to predict the risk of T1DM. For the implementing of measures towards effective diabetes control, there is a need for larger scale studies which also involve interaction of complex pathologic effects.

Ethics

Ethics Committee Approval: Ethical clearance was obtained for the research project. The study protocol conforms to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments.

Informed Consent: Written informed consent was obtained from each child included in the study or their participant parents.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Naglaa Barseem, Mona Elsamalehy, Design: Naglaa Barseem, Mona Elsamalehy, Data Collection and Processing: Naglaa Barseem, Mona Elsamalehy, Analysis and Interpretation: Naglaa Barseem, Mona Elsamalehy, Literature Research: Naglaa Barseem, Mona Elsamalehy, Writing: Naglaa Barseem, Mona Elsamalehy.

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Association of Subclinical Hypothyroidism with Dyslipidemia and Increased Carotid Intima-Media Thickness in Children

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What is already known on this topic?

Subclinical hypothyroidism (SH) increases the intima-media thickness (CIMT) of the carotid artery. SH in children is also associated with increased total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels as well as increased TC/high-density lipoprotein (HDL) and LDL/HDL ratios.

What this study adds?

Our study is of prime value since there are very few studies reporting on lipid profile abnormalities and increased CIMT in children with SH, as opposed to a large number of studies in adults. The present study is the first study in the literature to show increased TC and LDL-C as well as increased TC/HDL and LDL/HDL ratios in non-obese children with SH.

Abstract

Objective: Subclinical hypothyroidism (SH) is defined as an elevated serum thyroid-stimulating hormone (TSH) level with free thyroxine (fT_4) level in the normal range. There are very few studies in the literature reporting on the effect of SH on lipid metabolism and carotid intima-media thickness (CIMT) in children.

Methods: The study included 38 children diagnosed with SH and a control group comprising 38 healthy, euthyroid children. SH was diagnosed based on an elevated TSH level (4.2-20 mIU/L) and normal fT_4 level measured in two morning fasting blood samples obtained at an interval of 2 to 6 weeks. Blood samples were collected by venipuncture in the morning after an overnight fast.

Results: The patient group included 38 children (16 male, 22 female) with SH and the control group -38 healthy, euthyroid children (20 male, 18 female). Mean age was 8.1 ± 3.6 (range, 3.5-15) years in the patient group and 8.9 ± 2.4 (range, 4.5-15) years in the control group. In the patient group, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), TC/high-density lipoprotein cholesterol (HDL-C), and LDL-C/HDL-C were higher compared to the control group ($p = 0.049$, $p = 0.014$, $p = 0.002$, and 0.003, respectively). In the patient group, CIMT was also significantly higher compared to the control group ($p = 0.001$). The patient group was further divided into two subgroups based on their serum TSH level: (I) patients with mildly elevated TSH (TSH = 4.2 ± 10 mIU/L) (n = 33) and (II) patients with high TSH (TSH≥10 mIU/L) (n = 5). However, no significant difference was found between the patients with mild and severe SH with regard to TC, LDL-C, HDL-C, triglyceride level and CIMT levels ($p = 0.635$, $p = 0.424$, $p = 0.310$, $p = 0.342$, and 0.610, respectively).

Conclusion: Subclinical hypothyroidism leads to increased dyslipidemia (increased TC and LDL) and increased CIMT, which leads to increased risk of cardiovascular disease. Further studies are needed to substantiate these findings in children with SH.

Keywords: Subclinical hypothyroidism, carotid intima-media thickness, dyslipidemia, childhood

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Introduction

Subclinical hypothyroidism (SH) is defined as a state of elevated serum thyroid-stimulating hormone (TSH) level with a free thyroxine (fT_4) level in the normal range (1). Depending on the level of serum TSH, SH is divided into mild (TSH = 4.2-9.9 mIU/L) and severe (TSH≥10 mIU/L). Mild SH constitutes almost 75% of the patients with SH (2). SH affects 3-18% of the adult population and this prevalence increases with age (3). The prevalence of SH in children is reported to range between 1.7-9.5% (4,5). The most common cause of SH in children, as in adults, is Hashimoto's thyroiditis (4). There is no consensus on an ideal treatment for the management of SH. Moreover, whether dyslipidemia and increased carotid intima-media thickness (CIMT) in SH should be treated remains controversial.

Available data from adult studies and from few pediatric studies indicate that SH is associated with an alteration in lipid profile (3,6,7). In addition, SH has also been shown to have impact on carbohydrate metabolism, the neuromuscular system, and on cognitive functions (8,9). However, SH has its major effects on the cardiovascular system. Atherosclerosis is an important factor affecting the incidence of cardiovascular disease. Although there is no consensus on the association between SH and atherosclerosis, atherosclerosis is considered to be triggered by subintimal lipoprotein deposition and endothelial dysfunction. CIMT is used as a marker of atherosclerosis (2). Literature reviews show that the studies reporting on the effect of SH on lipid metabolism and CIMT have been mainly conducted in adults and that studies in children are sparse (8,10). In this study, we evaluated lipid profile and CIMT in pediatric patients with SH.

Methods

The study included a patient group comprising 38 children diagnosed with SH and a control group comprising 38 healthy children with normal thyroid functions [serum TSF, free triiodothyronine (fT_3), fT_4] who presented to the Pediatric Endocrinology Department of Dicle University Faculty of Medicine, in Diyarbakır, Turkey, between April-August 2016. Normal ranges of our laboratory were as follows: TSH 0.27-4.2 mIU/L, fT_3 3.69-9.85 pmol/L, and fT_4 12-22.8 pmol/L. The diagnosis of SH was based on an elevated TSH level (4.2-20 mIU/L) and normal fT_4 level measured in two morning fasting blood samples obtained at an interval of 2 to 6 weeks.

No subject in the study or control group had any sign or symptom of hypertension, liver and kidney dysfunction, lung disease, systemic infection, or any chronic disease. Patients with diabetes mellitus, obesity, and a history of drug use were excluded from the study. In both groups, no participant was using any medication during the study. In all subjects, the electrocardiogram (ECG) showed normal sinus rhythm and conventional transthoracic echocardiography findings were normal. Prior to the study, a written consent was obtained from the parents of each subject and an ethical approval was received from the local ethics committee.

Serum TSH, fT_3, and fT_4 levels were determined by using a Cobos e601 analyzer (Roche HITACHI Germany) with electrochemiluminescence immunoassay (ECLIA) method. Blood samples were collected by venipuncture in the morning after an overnight fast. Serum levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were determined by using a photometric method (Abbott diagnostics C16000 chemistry analyzer, Illinois, USA). Calculation of the value of low-density lipoprotein cholesterol (LDL-C) was performed using the Friedewald formula (11).

Carotid Intima-Media Thickness Measurements

CIMT was determined by ultrasonographic images of the right carotid artery which were recorded with a 12 MHz linear array transducer (Vivid S5 Pro, GE, Horten, Norway). The patient was placed in the supine position with the neck slightly extended and the head rotated 45° to the opposite direction. The M-mode cursor was positioned 1.0 cm proximal to the right carotid artery bulb during end diastole. CIMT was accepted as the distance between the lumen-intima and the media-adventitia interfaces. The CIMT on the frozen frame of a suitable longitudinal image was manually measured off-line. The value of CIMT was determined based on the mean value of a minimum of three measurements. All the CIMT evaluations were performed by an experienced pediatric cardiologist blinded to the clinical and biochemical characteristics of the patients.

Statistical Analysis

Data were analyzed using IBM SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as mean ± standard deviation (SD) and categorical variables were presented as count and percentage (%). Binary variables were compared by using independent samples t-test for normally distributed variables and by using Mann-Whitney U-test for non-normally distributed variables. Qualitative variables were compared by using chi-square test and the relationship among numerical variables was analyzed by using Pearson's correlation coefficient. The hypotheses were two-tailed and a p-value of ≤0.05 was accepted statistically significant.

Results

The patient group included 38 children (16 male, 22 female) with SH and the control group included 38 healthy, euthyroid children (20 male, 18 female). Mean age was 8.1 ± 3.6 (range, 3.5-15) years in the patient group and 8.9 ± 2.4 (range, 4.5-15) years in the control group. No significant difference was found between the groups with regard to age, gender, body weight, and SD score (SDS) values for body mass indices. Table 1 presents the demographic profiles of the subjects in the study and control groups.

In the patient group, TSH level was significantly higher ($p < 0.001$) and the fT_3 and fT_4 levels were similar compared to the control group. Total TC, LDL, TC/HDL, and LDL/HDL were also higher in the patient group compared to the control group ($p = 0.049$, $p = 0.014$, $p = 0.002$, and $p = 0.003$, respectively). In the patient group, 9 children were detected as having high lipid levels. Of these, 3 children had high TC levels (max. 225 mg/dL), 5-high TG levels (max. 275 mg/dL) and one patient had both high TG and TC levels. In the control group, 3 children had slightly high TG levels (max. 140 mg/dL). CIMT was significantly higher in the patient group compared to the control group ($p = 0.001$). Table 2 presents the laboratory parameters and the CIMT values for both groups. There was no relationship between CIMT and lipid levels, and no correlations were detected between increasing lipid level and CIMT.

The patient group was further divided into two subgroups depending on serum TSH level: (I) patients with mildly elevated TSH (TSH = 4.2 ± 9.9 mIU/L) (n = 33) and (II) patients with high TSH (TSH ≥10 mIU/L) (n = 5). No significant difference was found between the two subgroups with regard to TC, LDL-C, HDL-C, TG, and CIMT ($p = 0.635$, $p = 0.424$, $p = 0.310$, $p = 0.342$, and $p = 0.610$, respectively) (Table 3).

Discussion

The association between SH and lipid profile alteration has been reported in numerous studies (6,7,12). Moreover, TSH

Table 1. Demographic characteristics of the study and control groups

	SH group (n = 38)	Control group (n = 38)	p
Gender (Female/Male)	22/16	18/20	0.818
Age (years)	8.1 ± 3.6 6.3 (3.5-15)	8.9 ± 2.4 8.2 (4.5-15)	0269
Weight (kg)	25.6 ± 11.8 19.5 (12.8-63)	28.2 ± 8.3 26.6 (16-57)	0.277
SDS-BMI	-0.43 ± 0.94 -0.66 [-2.24- (1.56)]	-0.39 ± 0.90 -0.10 [-2.08- (1.18)]	0.988

Data are given as mean ± standard deviation and median (range)

SH: subclinical hypothyroidism, SDS-BMI: standard deviation score of body mass index

Table 2. Biochemical characteristics and carotid intima-media thickness of the study groups

	SH group (n = 38)	Control group (n = 38)	p
TSH (mIU/L)*	7.48 ± 2.37 6.4 (5-14.5)	2.35 ± 0.82 2.2 (0.82-3.96)	**< 0.001**
fT_4 (pmol/L)	16.40 ± 1.80	16.91 ± 2.18	0.269
fT_3 (pmol/L)	6.71 ± 0.68	6.75 ± 0.73	0.811
TC (mmol/L)	163.7 ± 27.8	153.6 ± 20.6	**0.049**
LDL-C (mmol/L)	92.8 ± 27.1	79.7 ± 17.0	**0.014**
HDL-C (mmol/L)	54.2 ± 9.6	57.8 ± 9.3	0.103
TG (mmol/L)	82.7 ± 48.9	74.9 ± 28.6	0.382
TC/HDL-C	1.76 ± 0.60	1.40 ± 0.36	**0.002**
LDL/HDL-C	10.83 ± 3.60	10.04 ± 4.01	**0.003**
CIMT	0.05 ± 0.019	0.04 ± 0.00	**0.001**

Data are given as mean ± standard deviation

Data are given as mean ± standard deviation and median (range)

CIMT: carotid intima-media thickness, fT_3: free triiodothyronine, fT_4: free thyroxine, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, SH: subclinical hypothyroidism, TC: total cholesterol, TG: triglycerides, TSH: thyroid-stimulating hormone

Table 3. Demographic characteristics, laboratory parameters, and carotid intima-media thickness values of the patients with thyroid-stimulating hormone < 10 mIU/L and thyroid-stimulating hormone ≥10 mIU/L

	TSH 4.2-9.9 mIU/L (n = 33)	TSH ≥10 mIU/L (n = 5)	p
Sex (Female/Male)	19/14	3/2	0.818
Age*	7.9 ± 3.5 6.5 (3.8-15)	9.2 ± 4.1 8.8 (3.5-13.5)	0.531
Weight (kg)*	25.1 ± 11.8 21 (12.8-63)	28.6 ± 12.1 35 (13.5-40)	0.545
Height*	121.2 ± 19.4 120 (94-163)	127.5 ± 21.7 140 (97-148)	0.449
SDS-BMI*	-0.58 ± 1.24 -0.43 [-2.04- (1.47)]	-0.32 ± 1.27 -0.96 [-1.63- (1.56)]	0.897
TSH*	6.76 ± 1.45 6.30 (5-9.6)	12.22 ± 1.70 11.20 (10.8-14.5)	< 0.001
TC	164.97 ± 27.19	155.8 ± 33.98	0.635
LDL-C	95.14 ± 25.95	78.02 ± 33.35	0.424
HDL-C	53.66 ± 9.84	57.86 ± 7.69	0.310
TG	80.18 ± 47.28	99.6 ± 51.84	0.342
CIMT	0.049 ± 0.019	0.051 ± 0.005	0.610

Data are given as mean ± standard deviation

Data are given as mean ± standard deviation and median (range)

HDL: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, SDS-BMI: standard deviation score of body mass index, TC: total cholesterol, TG: triglycerides, CIMT: carotid intima-media thickness, TSH: thyroid-stimulating hormone

has been shown to induce the production of the hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase, which is a rate-limiting enzyme in cholesterol biosynthesis. Studies have also indicated that thyroid hormones may affect HDL metabolism by increasing the cholesteryl ester transfer protein activity and that they may also stimulate lipoprotein lipase (12).

The studies investigating the effect of SH on lipid profile alteration have reported contradictory findings. Although some studies found significant lipid profile changes in patients with SH (6,7,12,13), some others did not (14). In most of the studies that found significant lipid profile changes, increased TC, TG, and LDL levels, and decreased HDL levels have been reported (6,13,15,16,17,18).

Literature reviews indicate that studies investigating lipid profile changes in children with SH are few, as opposed to the large number of studies conducted in adults (7,19,20,21). A previous study evaluated both children and adults with SH and found no lipid abnormality in children with TSH levels < 10 mIU/L and reported that the only abnormality was low HDL levels in children with TSH > 10 mIU/L compared to controls. The study also reported low HDL levels in adults with TSH > 10 mIU/L and, unlike in children, increased TC and LDL in adults (19). In our study on the other hand, no significant difference was found between the patients with TSH < 10 mIU/L and those with TSH ≥10 mIU/L in terms of lipid profile. However, the small number of patients with TSH ≥10 mIU/L (n = 5) included in this comparison was a disadvantage. Çatlı et al (20) evaluated 27 children with SH and found no significant difference in TG, HDL, and LDL levels in these patients compared to controls and suggested that SH is not associated with dyslipidemia in children with SH. Paoli-Valeri et al (21) evaluated 17 children with SH aged between 2-9 years and found significantly low HDL-C levels in these patients. Sert et al (8) compared obese children with and without nonalcoholic fatty liver disease (NAFLD) and found increased TC and LDL and decreased HDL levels in patients with NAFLD compared to children without NAFLD. Contrariwise, in our study, we evaluated non-obese children with SH and, to our knowledge, there has been no study reporting on increased TC and LDL in non-obese children with SH in the literature. However, there are reports of several studies similar to our study suggesting that SH causes no significant difference in HDL-C level but may increase the TC/HDL-C or LDL-C/HDL-C ratios (6).

A correlation between CIMT and cardiovascular disease has been frequently reported in epidemiological studies, indicating that increased CIMT is a reliable marker for subclinical atherosclerosis (22). However, although the frequency of hypertension and dyslipidemia is remarkably high in patients with SH, the association between SH

and CIMT has been shown to be independent from these two conditions (8). In addition, another study showed that SH leads to increased risk of myocardial infarct and atherosclerosis, independent of serum cholesterol levels (23).

The correlation between SH and CIMT has mostly been reported in adult patients (8,24,25,26). To our knowledge, there are only two publications in the literature reporting an increased CIMT in children with SH (7,10). In one of these, Isik-Balci et al (11) evaluated 53 children with SH and reported that CIMT was significantly increased in children with SH compared to controls (0.48 ± 0.04 mm *vs.* 0.43 ± 0.03, respectively). The other study, which was conducted by Sert et al (8), found that CIMT was significantly increased in obese children with NAFLD compared to obese children without NAFLD and these authors have also reported that CIMT had a positive correlation with TSH. A meta-analysis investigating the correlation between SH and CIMT in adults concluded that CIMT was more prevalent in SH patients with TSH >10 mIU/L and suggested that CIMT was increased in patients with TSH <10 mIU/L as well, though slightly (8). Delitala et al (27) evaluated subclinical thyroid disorders (subclinical hypo- and hyperthyroidism) in 5,815 individuals aged between 14-102 years and reported that there was no association between these disorders and CIMT. However, the study had an important shortcoming since the TSH and fT_4 measurements were performed only once; instead, these measurements should be repeated for a second time, since slightly increased TSH levels have been shown to return to normal in the subsequent measurement in almost 70% of the patients (28). To the best of our knowledge, the present study is the third study in the literature reporting a significantly increased CIMT in children with SH.

In conclusion, our study revealed increased TC, LDL-C, TC/HDL and LDL/HDL levels and a significant increase in CIMT in non-obese children with SH, findings which have been scarcely reported in the literature. Since dyslipidemia and increased CIMT are accepted as risk factors for cardiovascular diseases, L-thyroxine could be considered for the treatment of SH. Future studies with larger sample sizes and longer periods of follow-up are needed to further substantiate the importance of L-thyroxine treatment in SH patients to decrease the risk of cardiovascular diseases.

Ethics

Ethics Committee Approval: Ethical approval was received from the local ethics committee.

Informed Consent: Prior to the study, a written consent was obtained from the parents of each subject.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Edip Unal, Alper Akın, Design: Alper Akın, Edip Unal, Data Collection and Processing: Edip Unal, Alper Akın, Ruken Yıldırım, Vasfiye Demir, Yusuf Kenan Haspolat, İsmail Yıldız, Analysis and Interpretation: Alper Akın, Edip Unal, Ruken Yıldırım, Yusuf Kenan Haspolat, İsmail Yıldız, Literature Research: Ruken Yıldırım, Alper Akın, Edip Unal, Vasfiye Demir, Writing: Alper Akın, Edip Unal, Vasfiye Demir.

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Can Stoss Therapy Be Used in Children with Vitamin D Deficiency or Insufficiency without Rickets?

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What is already known on this topic?

Due to the proven benefits of vitamin D outside the skeletal system, physicians use stoss therapy in their adult patients with vitamin D deficiency/insufficiency.

What this study adds?

This study shows that stoss therapy regimens (10 000 IU/kg and 300 000 IU vitamin D_3) can be used safely in children with vitamin D deficiency/insufficiency without rickets.

Abstract

Objective: Stoss vitamin D treatment has been recommended for its non-skeletal benefits in adults, but there is a lack of data on the optimal dose of vitamin D stoss therapy in children with vitamin D deficiency/insufficiency without rickets. This study aimed to compare efficiency/side effects of two different stoss therapy regimens (10 000 IU/kg and 300 000 IU vitamin D_3) administered in children with vitamin D deficiency/insufficiency without rickets.

Methods: Sixty-four children who had vitamin D deficiency/insufficiency were studied. A serum 25-hydroxyvitamin-D (25-OH-D) level of 15-20 ng/mL was considered as vitamin D insufficient and <15 ng/mL was considered as vitamin D deficient. The patients were divided into two groups according to the stoss therapy doses they received. Serum calcium, phosphate, alkaline phosphatase, 25-OH-D, parathyroid hormone levels, and spot urine calcium/creatinine ratios before/after treatment were recorded. Wrist radiography and renal ultrasonography were performed.

Results: The mean age of the subjects was 10.6 ± 4.4 years. Thirty-two children were treated with a single vitamin D_3 dose of 10 000 IU/kg and 32 patients received 300 000 IU. No difference was found in 25-OH-D levels between the two groups at presentation. The mean level of 25-OH-D was higher in the 10 000 IU/kg group at the second week of therapy. There was no difference between the groups at post-treatment weeks 4 and 12. The 25-OH-D was found to be below optimal levels (≥30 ng/mL) in 66.5% and <20 ng/mL in 21.8% of patients at the third month in both groups. None developed hypercalcemia and/or hypercalciuria. Nephrolithiasis was not detected in any patient.

Conclusion: This study showed that both doses of stoss therapy used in the treatment of vitamin D insufficiency/deficiency are effective and safe. However, an optimal level of 25-OH-D cannot be maintained for more than three months.

Keywords: Vitamin D deficiency, rickets, stoss therapy

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Introduction

Vitamin D is essential for mineralization of bones, for calcium and phosphate homeostasis, and neuromuscular conduction (1). Low vitamin D levels lead not only to rickets in children and osteomalacia in adults but also to muscle weakness and predisposition to respiratory infections, hyperparathyroidism, inability to acquire peak bone mass, and increased risk of fracture (2). Many clinicians now measure vitamin D levels as part of routine laboratory workup and recommend vitamin D supplements often at high doses to their patients for the possible prevention of cancer, cardiovascular disease, diabetes, autoimmune disorders, and other conditions since observational studies support the speculation that there is a relationship between low vitamin D levels and an increased risk for these situations (3). Stoss treatment, namely, the administration of vitamin D in a high dose (300 000-600 000 IU) has also been suggested in adults due to the non-skeletal effects of vitamin D, but no sufficient data are present for children with vitamin D deficiency/insufficiency without rickets. Thus, an optimal treatment dose of vitamin D is not well known in patients with vitamin D deficiency or insufficiency who do not show marked signs of rickets. Despite the worldwide programs of vitamin D supplementation as a public health campaign, a considerable number of children are still at high risk because of the poor adherence of the parents to supplementation regimens. Because the signs and symptoms of vitamin D deficiency without rickets are insidious or nonspecific, it often goes unrecognized and untreated (4).

In this retrospective study, the efficacy and side effects of two different doses of stoss therapy (10 000 IU/kg and 300 000 IU, oral, single-dose vitamin D_3) in vitamin D deficient or insufficient children without marked signs of rickets were compared.

Methods

The children and adolescents who had been referred to the pediatric endocrinology department due to vitamin D deficiency or insufficiency between January 2014 and January 2015 and who received stoss therapy in two different doses (10 000 IU/kg and 300 000 IU, oral, single-dose vitamin D_3) were studied retrospectively (5). Patients with chronic diseases such as malabsorption, liver disease, renal disease, gastrointestinal, hematologic and rheumatologic diseases, and those using drugs which may influence vitamin D metabolism were excluded from the study. Age, gender, anthropometric measurements, season of admission, and complaints at presentation of the patients were recorded. A serum level of 25-hydroxyvitamin D (25-OH-D) between 15-20 ng/mL was accepted as vitamin D insufficiency, <15 ng/mL as vitamin D deficiency, and <5 ng/mL as severe vitamin D deficiency. The serum levels of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), 25-OH-D, parathyroid hormone (PTH), spot urine calcium/creatinine (UCa/UCr) ratio before and after treatment (weeks 2, 4 and 12), and renal ultrasonography (USG) outcomes in the three groups were compared. Clinical complaints that brought patients to the clinics were recorded and information about whether these complaints continued two weeks after vitamin D therapy was obtained from the patient records. Laboratory reference values based on age groups were used for defining hypocalcemia, hypophosphatemia, elevated ALP, and hyperparathyroidism. UCa/UCr >0.2 in spot urine was considered as hypercalciuria. Serum Ca, P, and ALP levels were measured using the calorimetric method. Serum PTH and 25-OH-D levels were measured by electrochemiluminescense enzyme immunoassay method (ADVIA Centaur, USADPC Co., USA).

Statistical Analysis

Statistical analysis was performed using SPPS 21.0 (SPSS Inc., Chicago, IL, USA) software. All data were given as mean ± standard deviation scores (SDS). Homogeneity of the data was assessed using the Kolmogorov-Smirnov test. Differences in the mean level of vitamin D between the two treatment groups (10 000 IU/kg and 300 000 IU) and between patients with or without symptoms were tested using the student's t-test for data with normal distribution and the Mann-Whitney U test for data without normal distribution. The chi-square test was used to compare the number of patients with complaints before and after treatment. The results were expressed with a 95% confidence interval and a p-value of less than 0.05 was considered statistically significant.

Results

Sixty-four patients of a mean age of 10.6 ± 4.4 years were included in the study. Age, gender, anthropometric measurements, season of admission, and laboratory data of the patients are shown in Table 1. Of the patients, 32 were treated with 10 000 IU/kg (max. 600 000 IU) and the remaining 32 patients received a single 300 000 IU dose oral vitamin D_3. Severe vitamin D deficiency was determined in 13, vitamin D deficiency in 42, and vitamin D insufficiency in 9 patients. Ca and P levels were in normal ranges in all patients, while the level of ALP was high in

12 and PTH was high in 8 patients. The mean 25-OH-D levels of the groups (10 000 IU/kg and 300 000 IU) were not significantly different before treatment (10.8 ± 4.9 and 8.8 ± 3.6 ng/mL, respectively, p > 0.05). Of the patients, 26.6% (n = 17) were asymptomatic. The most common symptoms at presentation were weakness (40.6%), lower back pain (40.6%), hair loss (37.5%), numbness in hands and feet (28.1%), constipation (20.3%), excessive sweating (15.6%), and frequent respiratory tract infection (12.5%). None of the patients had a history of a clinically significant fracture. More than one complaint was observed in 56% of patients. When the patients were re-evaluated two weeks after treatment, the number of patients with symptoms was found to be significantly reduced (p < 0.05) (Figure 1). The mean level of 25-OH-D was significantly higher in the 10 000 IU/kg group at the second week after treatment (76.6 ± 30.6 vs. 57.4 ± 18.1 ng/mL, p < 0.05), but there were no statistically significant differences between the groups in the levels of vitamin D at the 4th and 12th weeks after treatment (p > 0.05) (Figure 2). The level of 25-OH-D was reduced below optimal levels (≥30 ng/mL) in 66.5% and below 20 ng/mL in 21.8% of the patients at 12 weeks post-treatment. None of the patients in either group developed hypercalcemia, hypercalciuria, or vitamin D intoxication. Nephrolithiasis was not detected in any of the patients at the third month of the treatment.

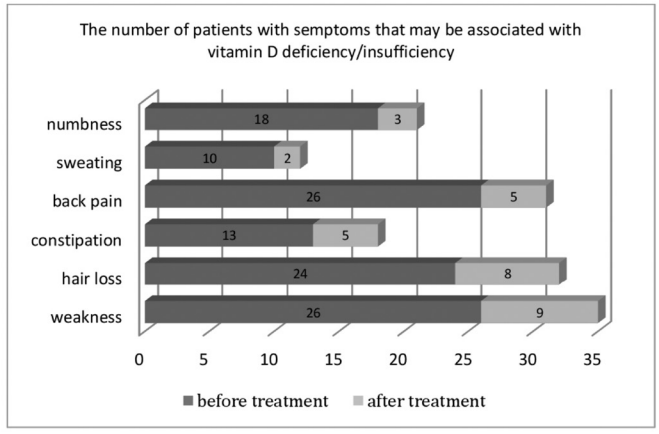

Figure 1. Numbers of patients with symptoms that may be associated with vitamin D status before and two weeks after treatment

Table 1. Clinical and laboratory characteristics of the patients before and two weeks after treatment

Patients	10 000 IU/kg	300 000 IU	p
Age (years) (mean, range)	8.7 ± 4.3 SDS, (3-17)	9.4 ± 3.8 SDS, (3-17)	> 0.05
Female/Male	19/13	22/10	> 0.05
Weight SDS	0.06 ± 0.9	0.12 ± 0.9	> 0.05
Weight range (kg)	13-72	16-76	
Height SDS	-0.21 ± 0.84 SDS	0.18 ± 1.0	> 0.05
Body mass index (kg/m^2)	0.29 ± 1.1 SDS	0.40 ± 0.8 SDS	> 0.05
Admission			
Spring	10%	12%	
Summer	6%	4%	> 0.05
Autumn	8%	4%	
Winter	76%	80%	
Calcium (mg/dL)	Before treatment 9.6 ± 0.6	Before treatment 9.5 ± 0.3	> 0.05
	After treatment 10.0 ± 0.3	After treatment 9.7 ± 0.4	> 0.05
Phosphorus (mg/dL)	Before treatment 4.7 ± 0.6	Before treatment 4.4 ± 0.6	> 0.05
	After treatment 4.8 ± 0.6	After treatment 4.5 ± 0.5	> 0.05
ALP (IU/L)	Before treatment 260 ± 125	Before treatment 223 ± 158	> 0.05
	After treatment 235 ± 93	After treatment 214 ± 150	> 0.05
PTH (pg/mL)	Before treatment 56 ± 40	Before treatment 50.1 ± 28.6	> 0.05
	After treatment 34.2 ± 12	After treatment 34.5 ± 22	> 0.05

Date are given as mean ± SDS

ALP: alkaline phosphatase, PTH: parathyroid hormone, SDS: standard deviation score

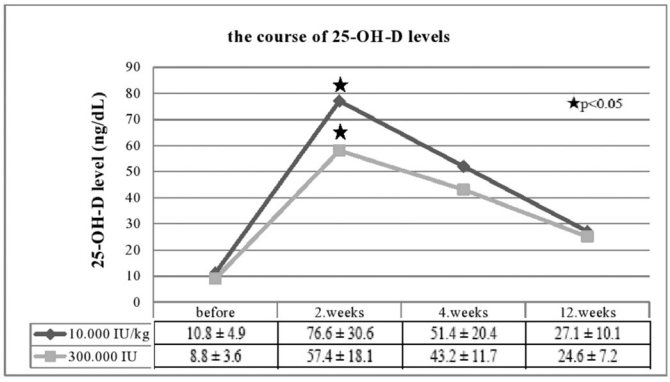

Figure 2. The course of 25-hydroxyvitamin-D levels over time in patients on the two different high-dose treatment protocols

Discussion

The blood level of 25-OH-D defined as vitamin D deficiency remains somewhat controversial. As determined by the measurement of serum concentrations of calcidiol (25-OH-D), vitamin D deficiency is accepted to be present when values are below 15 ng/mL. In children, calcidiol concentrations between 15 and 20 ng/mL indicate vitamin D insufficiency, whereas those >20 ng/mL are adequate or sufficient (6,7). However, these guidelines, based on the recommendations of the Institute of Medicine report, are not accepted by all authorities and remain the subject of ongoing investigations. Alternate guidelines state that a normal 25-OH-D concentration be defined as greater than 30 ng/mL with values of 20 to 30 ng/mL used to define insufficiency and values of less than 20 ng/mL considered as vitamin D deficiency, especially for adults (8). Vitamin D deficiency in the infant may be managed by administration of vitamin D 1000 to 5000 IU/day. For the child who is one year of age or older, or an adolescent, vitamin-deficient rickets may be treated with 5000 to 10000 IU/day of vitamin D (9). Alternative therapeutic regimens for treatment of rickets include 50 000 IU orally weekly for 8 weeks, the administration of a single oral (or intramuscular) dose of 150 000 to 600 000 units or 10 000 IU/kg of vitamin D_3 (5,10). Our clinical experience has shown us that there is poor patient compliance to long-term low-dose vitamin D applications, which may be insufficient to achieve the desired level of vitamin D. We use a 25-OH-D level below 20 ng/mL as a threshold value for pharmacological treatment in our clinic. We prefer stoss therapy rather than long-term low-dose vitamin D administration because of better patient compliance. Indeed, in a previous study, half of 42 infants and children aged between 5 months and 3 years with a 25-OH-D level of <20 ng/mL had been treated with stoss therapy (150 000 IU, single dose, oral) and the remaining were given low-dose long-term vitamin D_3 therapy (2000 IU/day for 6 weeks) and a better vitamin D level was provided in the stoss therapy group without any side effects observed (11).

The clinical signs of vitamin D deficiency and insufficiency can be variable; the patient may be asymptomatic, have nonspecific clinical findings, or obvious rickets signs (4). Studies have shown that the risk of fracture is increased in children with low levels of vitamin D and that these children are also sensitive to respiratory tract infections (12,13). In the present study, none of the patients had a history of clinically significant fracture. According to the parents' self-reports, 12.5% of the patients had experienced frequent respiratory tract infections. Additionally, there were some nonspecific clinical symptoms in the majority of the patients that could not be explained by any illness at admission, and these symptoms showed a decrease after vitamin D treatment (Figure 2). However, no relationship was found between these symptoms and the levels of vitamin D. In a previous study, Voloc et al (14) have reported poor correlation between clinical features and serum 25-OH-D levels.

All available evidence suggests that children and adults should maintain a blood level of 25-OH-D above 20 ng/mL to prevent rickets and osteomalacia. However, to maximize the effects of vitamin D on calcium level, bone, and muscle metabolism, it has been reported that the 25-OH-D blood levels should be above 30 ng/mL. Numerous epidemiological studies suggest that a 25-OH-D serum level above 30 ng/mL may have additional health benefits such as reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases (8,15). In the present study, both treatment protocols were effective in providing a sufficient level of vitamin D, which remained higher in the 10 000 IU/kg treatment protocol group compared to the other protocol at the second week. However, the levels of vitamin D were decreased below the optimal level (≥30 ng/mL) at week 12 of the treatment in the majority of patients in both groups. Similar to our findings, in a randomized controlled study, half of the patients with rickets (mean age 12 months) received a 300 000 IU dose (Group 1) and the other half received a 600 000 IU dose (Group 2) of oral vitamin D_3 therapy. The levels of 25-OH-D studied 12 weeks after initiation of the treatment were found to be below 20 ng/ mL in 62.5% of the patients in Group 1 and 64.3% of the patients in Group 2. In that study, the authors reported that both regimens failed to optimize the level of vitamin D for more than 3 months, but that no side effects were observed with either regimen (16). Although studies on this topic are limited, both our results and the data provided by Mittal et al (16) suggest that stoss therapy protocols should be repeated once every three months,

especially in patients at risk for vitamin D deficiency with a poor compliance to vitamin D supplementation.

There is a lack of consensus on the dose of vitamin D_3 in stoss therapy because of conflicting results from numerous studies. Stoss therapy can lead to side effects such as hypercalcemia, hypercalciuria, and nephrocalcinosis if vitamin D deficiency is not documented before therapy. Studies that compared low- and high-dose stoss therapy in nutritional rickets showed that 150 000 and 300 000 IU of vitamin D were adequate as treatment, but a 600 000 IU dose of vitamin D carried a risk of hypercalcemia (17,18). However, there are also studies reporting the use of a dose of 600 000 IU in the treatment of rickets due to vitamin D deficiency without any side effects (19,20). Lubani et al (19) showed that intramuscular vitamin D in a dose of 600 000 IU was safe and effective. In Shah and Finberg's study (20), a single dose (600 000 IU) of vitamin D was given to 42 vitamin D-deficient children aged between 5 and 19 months; biochemical improvement was detected in 4-7 days and radiological improvement was detected in 10-14 days. Side effects such as hypercalcemia or hypercalciuria were not observed. In a recent meta-analysis, no risk was found for hypercalcemia or hypercalciuria in stoss therapy with a single oral dose below 400 000 IU, whereas doses above 400 000 IU created a risk for hypercalcemia (5). In the present study, none of the patients in either group developed hypercalcemia, hypercalciuria, vitamin D intoxication, or nephrolithiasis.

There are some limitations of this study that need to be acknowledged. Because of its retrospective design, we do not know about the calcium and vitamin D content of the patients' diets and we have no evidence regarding the cause and effect relationship between the clinical symptoms and vitamin D deficiency at admission.

Unlike other studies in literature, this study has been performed in subjects who had no obvious clinical or radiological signs of rickets. This study shows that stoss therapy could be used in these groups of patients safely without any serious side effects.

In conclusion, our study demonstrated that the two stoss therapy protocols, namely 10 000 IU/kg and 300 000 IU doses, used in vitamin D insufficiency or deficiency patients who showed no marked signs of rickets seem to be similar in terms of efficacy and side effects. The study also showed that an optimal serum level of 25-OH-D cannot be maintained for more than three months and the treatment dose should be repeated at the 12th week. We believe that these stoss therapy protocols can be used safely, especially in cases with poor patient compliance to vitamin D supplementation and in cases in which the optimal level of vitamin D cannot be achieved despite supplementation complemented by adequate nutritional approaches and encouragement of sunbathing.

Ethics

Ethics Committee Approval: Tepecik Education and Research Hospital Local Research Ethics Committee (Decision No:23).

Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Bumin Nuri Dündar, Design: Gönül Çatlı, Bumin Nuri Dündar, Data Collection and Processing: Cemil Koçyiğit, Gülberat İnce, Analysis and Interpretation: Gönül Çatlı, Cemil Koçyiğit, Literature Research: Cemil Koçyiğit, Writing: Cemil Koçyiğit, Elif Büşra Özkan.

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Menarcheal Age and Risk of Type 2 Diabetes: A Community-Based Cohort Study

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What is already known on this topic?

Results indicate that early menarche is a potential risk factor for type 2 diabetes and pre-diabetes.

What this study adds?

Results on a large cohort-supported previous findings indicating that early menarche is a potential risk factor for type 2 diabetes and pre-diabetes.

Abstract

Objective: It has been reported that early menarche is associated with higher risk for type 2 diabetes. We aimed to explore the association between age at menarche and risk of type 2 diabetes in a population-based cohort study.

Methods: For the purpose of the present study, 5191 subjects of reproductive age who were participants of the Tehran Lipid and Glucose Study and also met the eligibility criteria were selected. Demographic, lifestyle, reproductive, and anthropometric data as well as risk factors for metabolic diseases were collected. Menarcheal age was categorized into five categories, as < 11 years, 11-12 years, 13-14 years, 15-16 years, and > 17 years. Diabetes and pre-diabetes were defined according to the American Diabetes Association criteria. Logistic regression analysis was used to assess the risk of the menarcheal age group for type 2 diabetes and pre-diabetes.

Results: Of 5625 participants, 673 women had pre-diabetes and 187 had diabetes. Early menarche was associated with higher risk of diabetes and pre-diabetes, compared to the reference group (13-14 years), (OR = 3.55, 95% CI: 1.6-7.8 and OR = 2.55, 95% CI:1.4-4.8, respectively), an association which remained after further adjustment for potential confounders including family history of diabetes, parity, education, age, body mass index, waist circumference, smoking history, physical activity, and duration of oral contraceptives use.

Conclusion: Results showed early menarche to be a potential risk factor for type 2 diabetes and pre-diabetes.

Keywords: Menarcheal age, blood glucose, reproductive age, noncommunicable disease

Introduction

Puberty is considered to be a reproductive milestone in a woman's life (1,2). The initiation of puberty is strongly regulated by genes, and early puberty may be associated with an increased risk for various poor health outcomes, including obesity, type 2 diabetes, cardio-vascular disease (3,4,5).

Assessment of menarcheal age, namely, age at the initiation of menstruation, enables researchers, using a noninvasive approach, to investigate the association between developmental timing and future disease states (6). Recent studies demonstrate that obesity, type 2 diabetes, and even cardiovascular mortality may all be related to early pubertal maturation (7).

Results of studies show a relationship between prevalence of type 2 diabetes and pubertal timing, an association, which is attenuated when adjusting for adulthood body mass index (BMI) (4,5,8).

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The prevalence of type 2 diabetes has increased rapidly worldwide during the recent decades (4,5). At the time of their diagnosis for type 2 diabetes, a number of individuals have been shown to have already developed important complications, a finding making it increasingly important to identify those at risk in early life, especially when there is a possibility of early intervention (4,9).

Individuals with a pre-diabetes status, namely, those with an impaired fasting glucose and/or impaired glucose tolerance, are at the highest risk of developing type 2 diabetes (10). Unfortunately, data on the effects of lifestyle or therapeutic interventions for the primary prevention of pre-diabetes are still in an inadequate stage (11).

On the other hand, studies have suggested that earlier menarche is related to poorer glycemic control in later life, to increased risk factors for diabetes such as excessive adiposity in childhood (12,13,14) and adulthood (15,16,17), as well as to elevated blood glucose levels (18) and insulin resistance (19), independent of adiposity (4).

Concurrent with the rising prevalence of obesity, menarcheal age in Iranian girls has been declining during recent decades (20,21,22).

This study was conducted to investigate the association between early menarcheal age and risk of type 2 diabetes and pre-diabetes in a population-based cohort study of the Tehran Lipid and Glucose Study (TLGS).

Methods

TLGS is an ongoing prospective study initiated in 1998 with the aim of determining the prevalence of non-communicable disease risk factors. The project is conducted on a representative sample of residents of District 13 of Tehran, the capital of Iran. Details of the rationale of the study have been published elsewhere (23). The participants in the study consisted of 15 005 individuals (male and female) aged ≥3 years. Of the 7718 females (aged 10-50 years), 5625 met our eligibility criteria (having complete data and not having yet reached menopause), after exclusion of those with pathological late-onset puberty such as in case of hypothyroidism (n = 3), Cushing's syndrome (n = 0), hypopituitarism (n = 0), chronic renal failure (n = 1), type one diabetes (n = 2).

The ethical committee of the Research Institute for Endocrine Sciences approved this study. Written informed consent was obtained from all study participants.

For all participants, demographic and lifestyle variables as well as information on various risk factors for non-communicable diseases and their medical and reproductive histories were collected by trained interviewers, during face-to-face interviews.

A modifiable activity questionnaire was used to assess the achieved physical activity pattern (24); the subjects were asked to report the physical activities in which they had participated during the past 12 months. "Leisure time physical activity" was defined as performing three or more days of vigorous-intensity activity of at least 20 minutes, or five or more days of moderate-intensity activity or walking at least 30 minutes, or five or more days of any combination of walking, moderate or vigorous-intensity activities, achieving a minimum of at least 600 metabolic equivalent task minutes per week (25,26).

Height was measured with a measuring tape with a 0.5-cm accuracy, in a standing position against a wall, without shoes, and with shoulders in a normal position. Weight was measured by an electronic digital weighing scale with 100-g accuracy while the subject was minimally clothed and without shoes. BMI was calculated by dividing weight in kilograms by height in meters squared. Waist circumference (WC) was measured midway between the lower rib margin and the iliac crest at the end of a gentle expiration with 0.5-cm accuracy and without any compulsory pressure. Blood samples were taken after a 12-h overnight fast for biochemical measurements.

Definitions

Menarcheal age was defined as the age at the first menstrual bleeding, based on data obtained during interviews with participants. For our analysis, menarcheal age was categorized into five categories including < 11 years, 11-12 years, 13-14 years, 15-16 years, and > 17 years.

Diabetes was defined according to the American Diabetes Association (2013), namely as a fasting blood glucose ≥126 mg/dL (7.0 mmol/L) or a 2 h plasma glucose ≥200 mg/dL (11.1 mmol/L) (27).

Pre-diabetes was defined according to the American Diabetes Association (2013) as a fasting plasma glucose of 100 mg/dL (5.6 mmol/L) to 125 mg/dL (6.9 mmol/L) or a impaired fasting glucose or 2-h PG of 140 mg/dL (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) (impaired glucose tolerance) (27) in the 75-g oral glucose tolerance test or drug treatment.

Laboratory Tests

All blood analyses were done at the TLGS research laboratory on the day of blood collection. Plasma glucose was measured by the enzymatic colorimetric method, using a glucose oxidase kit (Pars Azmoon Inc., Tehran, Iran); inter- and intra-assay coefficients of variation were both < 2.2%.

Details of Ethics Approval

The ethical review board of the Research Institute for Endocrine Sciences approved the study proposal, and written informed consent was obtained from all subjects.

Data Analysis

Demographic and reproductive characteristics were compared by menarcheal age in participants, using ANOVA test for continuous variables and Dunnett post-hoc test and χ^2 test for categorical variables.

The logistic regression method was used to assess the risk of the menarcheal age group (independent variable) for type 2 diabetes and pre-diabetes (dependent variables), before and after adjustment for confounding variables including family history of diabetes, parity, education, age, BMI, WC, smoking history, physical activity, and duration of oral contraceptives (OCP) use. The reference was women with a menarcheal age of >13 and ≤14 years, as this group constituted 49.4% of all participants.

Data were analyzed using SPSS 15 (SPSS Inc., Chicago, IL, USA).

Results

Among 5625 participants, 673 women had pre-diabetes and 187 had diabetes. The demographic and reproductive characteristics of the participants stratified by five menarcheal age groups are presented in Table 1. The number of women in each menarcheal age group were 109, 1603, 2779, 1027, and 107, respectively.

Results showed that the mean menarcheal age was 13.3 ± 1.5 years; mean age (p = 0.001), BMI (p = 0.046), and WC (p = 0.001) differed significantly between these groups.

Logistic regression analysis demonstrated that there was a statistically significant difference in the risk of pre-diabetes type 2 between women with earlier menarche [<11 years (group 1)] and women with menarcheal ages of >13 and ≤14 years [reference (group 3)], before and after adjustment for covariates (Table 2); a statistically significant difference in the risk of diabetes type 2 between women with earlier menarche [<11 years (group 1)] and women with menarcheal ages of >13 years and <14 years [reference (group 3)] was also demonstrated before and after adjustment for covariates (Table 3). The reference group was women with menarcheal ages of >13 years and ≤14 years as it constituted 49.4% of all participants.

Table 1. Characteristics of the study participants according to their menarcheal age

Characteristics	Sub-groups					
Menarcheal age	**<11years n = 109**	**11-12 years n = 1603**	**13-14 years n = 2779**	**15-16 years n = 1027**	**17-19 years n = 107**	**p-value**
δPresent age (years)	28.1 ± 12.7	29.5 ± 11.3	33.6 ± 10.4	35.3 ± 9.1	36.9 ± 9.0	<0.0001
δBMI (kg/m^2)	27.1 ± 6.1	25.7 ± 5.3	25.8 ± 5.4	25.4 ± 5.0	26.4 ± 5.6	0.046
δWC (cm)	88.3 ± 14.3	84.9 ± 13.1	86.3 ± 13.1	85.8 ± 11.9	88.5 ± 13.7	0.001
δParity (n)	0.8 ± 1.4	0.8 ± 1.3	1.2 ± 1.5	1.2 ± 1.5	1.3 ± 1.5	<0.0001
φDiabetes family history	17.5	10.3	12.1	9.9	17.6	0.03
φEducation						
High school & lower	46.5	37.3	41.2	31.3	35.3	<0.0001
Diploma	52.3	61.4	58.8	66.6	63.5	
University degree	1.1	1.3	0.0	2.1	1.2	
δPhysical activity	837.6 ± 1019.6	1466.2 ± 2358.2	1537.4 ± 2625.3	1693.0 ± 2478.0	1544.5 ± 2066.8	0.15
φSmoking history, yes	3.7	2.6	3.0	2.5	1.2	0.78
δDuration of OCP use (months)	0.4 ± 2.4	0.3 ± 1.3	0.5 ± 2.8	0.5 ± 2.7	0.6 ± 1.8	0.7
φDiabetes type 2	11.4	3.3	4.3	3.7	2.9	0.1
φPre-diabetes	22.7	12.4	11.6	10.2	14.3	0.09

BMI: body mass index, WC: waist circumference, OCP: oral contraceptive pill

δMean ± standard deviation

φ%

Table 2. Risk ratios (and 95% confidence intervals) for pre-diabetes by menarcheal age

Sub-groups	< 11 years	11-12 years	13-14 years	15-16 years	17-19 years
Total subjects in groups	n = 109	n = 1603	n = 2779	n = 1027	n = 107
Model 1	2.74 (1.5-4.9)*	1.10 (0.9-1.3)	1.0 (Ref)	0.82 (0.6-1.03)	0.87 (0.5-1.6)
Model 2	2.55 (1.4-4.8)*	1.13 (0.9-1. 4)	1.0 (Ref)	0.82 (0.6-1.03)	0.88 (0.5-1.6)
Model 3	2.70 (1.4-5.2)*	1.15 (0.9-1.4)	1.0 (Ref)	0.8 (0.6-1.0)	0.8 (0.4-1.5)
Model 4	3.28 (1.5-7.1)*	1.09 (0.8-1.4)	1.0 (Ref)	0.9 (0.7-1. 2)	1.15 (0.5-2.4)
Model 5	2.06 (1.04-4.1)*	1.11 (0.9-1. 4)	1.0 (Ref)	0.88 (0.7-1.1)	0.80 (0.4-1.5)
Model 6	2.7 (1.1-6.6)*	1.06 (0.8-1.4)	1.0 (Ref)	0.9 (0.6-1.2)	1.1 (0.5-2.5)

*Statistically significant.

Model 1: No adjustment; Model 2: Adjusted for family history of diabetes, parity, and education; Model 3: Adjusted for variables in model 2 plus age; Model 4: Adjusted for variables in model 3 plus body mass index. Model 5: Adjusted for variables in model 3 plus waist circumference. Model 6: Adjusted for smoking history, physical activity, and duration of oral contraceptives use.

Table 3. Risk ratios (and 95% confidence intervals) for type 2 diabetes by menarcheal age groups

Sub-groups	< 11 years	11-12 years	13-14 years	15-16 years	17-19 years
Total subjects in groups	n = 109	n = 1603	n = 2779	n = 1027	n = 107
Model 1	3.55 (1.6-7.8)*	0.83 (0.6-1.2)	1.0 (Ref)	0.88 (0.6-1.3)	1.20 (0.5-3.0)
Model 2	3.64 (1.6-8.2)*	0.91 (0.6-1.3)	1.0 (Ref)	0.9 (0.6-1.3)	1.33 (0.5-3.3)
Model 3	3.74 (1.6-8.6)*	0.92 (0.6-1.3)	1.0 (Ref)	0.89 (0.6-1.3)	1.13 (0.4-2. 9)
Model 4	3.56 (1.2-10.2)*	0.62 (0.4-1.1)	1.0 (Ref)	0.97 (0.6-1.7)	0.76 (0.2-3.3)
Model 5	3.0 (1.3-7.1)*	0.83 (0.5-1.2)	1.0 (Ref)	1.06 (0.7-1.6)	0.17 (0.4-3.1)
Model 6	3.6 (1.2-10.7)*	0.6 (0.2-1.8)	1.0 (Ref)	0.8 (0.5-1.39)	0.7 (0.2-3.3)

*Statistically significant.

Model 1: No adjustment; Model 2: Adjusted for family history of diabetes, parity and education; Model 3: Adjusted for variables in model 2 plus age; Model 4: Adjusted for variables in model 3 plus body mass index. Model 5: Adjusted for variables in model 3 plus waist circumference. Model 6: Adjusted for smoking history, physical activity, and duration of oral contraceptives use.

Discussion

In this population-based study, we found that early menarche (< 11 years) was significantly associated with increased risk of type 2 diabetes and pre-diabetes before and after adjustment for potential confounders (Tables 2, 3). The potential confounders including family history of diabetes, parity, education, age, BMI, WC, smoking history, physical activity, and duration of OCPs use were adjusted in regression models.

The decreasing age at menarche among Iranian women (22) noted in recent decades is an issue of concern for future risk of type 2 diabetes and pre-diabetes. On the other hand, the main pathways explaining the association between early menarche with type 2 diabetes have not been well described. It has been shown that lower serum concentration of insulin-like growth factor-1 (IGF-1) is associated with an increase in type 2 diabetes (28).

Estrogen modulates growth hormone secretory activity in a biphasic manner; low levels of estrogen stimulate secretion of IGF-1 through growth hormone release, whereas high levels inhibit IGF-1 production resulting in menarche (29,30), indicating that early exposure to higher levels of estrogens may lead to diabetes in the future (31). Also the growth spurt and the beginning of menstruation during puberty are caused by estrogen secretion. The results of a study conducted among 329 girls showed that earlier menarche was related to lower levels of both IGF-binding protein-I and sex hormone-binding globulin (SHBG) and also to higher levels of IGF-I, androstenedione, dehydroepiandrosterone sulfate (DHEAS), leptin, and fasting insulin at the age of 8 years (32). Also, the associations between high levels of IGF-I, androstenedione, and DHEAS to earlier menarche continue after adjustment for BMI (32). Insulin resistance manifesting early in life could be an important pathologic disorder caused by association between earlier age at menarche and higher risk of diabetes (33).

There are limited studies on the association between menarcheal age and risk of type 2 diabetes and pre-diabetes and results of those available are controversial (2,34,35).

Results of the Stöckl et al (2) study, conducted on 1503 women aged 18-32 years, showed an adverse association between menarcheal age and diabetes/pre-diabetes, before and after adjusting for potential confounders. Gambineri

et al (34), in their study conducted on 121 women with polycystic ovary syndrome, reported that early menarcheal age was associated with type 2 diabetes. On the other hand, the Rancho Bernardo Study of 997 post-menopausal women aged 50-92 years, showed that menarcheal age was not associated with abnormal glucose tolerance or type 2 diabetes, whereas late menarcheal age was inversely associated with fasting and post challenge glycemic levels (35). The Shanghai Women Health Study, conducted on 69385 women aged 40-70 years, reported that older age at menarche was significantly associated with reduced risk of diabetes after adjustment for birth cohort, education, and household income, a significance that disappeared after adjustment for baseline BMI (36). Another study, conducted on 34022 Chinese women aged 45-74 years, reported that older menarcheal age was related to lower prevalence of diabetes after adjusting for several confounders. Baseline BMI and menarcheal age of <12 years (compared to 13-14 years) was related to an 18% higher risk of diabetes, even after adjustment for BMI (33). Results of another study from China on postmenopausal women showed that early menarche was not associated with diabetes. Differences in the results obtained in these studies have been attributed to differences in menarcheal age grouping and participant recruitment (37). The results of the cohort study of the European Prospective Investigation into Cancer and Nutrition (the EPIC-Norfolk study) conducted on 13308 women aged 40-75years showed that menarcheal age was inversely related to diabetes, an effect totally induced by adult adiposity (38).

Also, the Atherosclerosis Risk Communities Study (ARIC), conducted among 8491 women aged 45-65 years (39) reported that age at menarche was inversely related to diabetes after adjusting for potential confounders; although these associations were partially reduced by adult adiposity, the association remained significant. On the other hand, in the Nurses' Health Study conducted among 100547 younger women (26-46 years), those with earlier menarcheal age had raised risk of diabetes even after adjusting for adiposity, findings consistent with ours (4).

Several studies report that early menarche is associated with an increased risk of diabetes even after adjusting for potential confounders (33,37,38,39). Also, results of other studies showed an association between early menarcheal age and elevated fasting insulin, insulin resistance (HOMAIR), and A-cell function (HOMA-A), compared with usual menarcheal age (31,40,41). However, our results and those of some other studies yield additional evidence that early menarcheal age is related to risk of diabetes, although the mechanism(s) of this association are unclear (31).

Based on results of studies in US, Europe, China, and Iran, the secular trend of menarcheal age has decreased during the recent decades (22,42,43). Moreover, the prevalence of diabetes mellitus has increased worldwide sharply during these years (4,5) indicating that earlier menarcheal age is related to a diabetes risk although the fundamental mechanism of this association is still unknown (31).

In this population-based study, we found that early menarche (≤11 years) was significantly associated with increased risk of type 2 diabetes and pre-diabetes, a result which persisted after adjustment for potential confounders including family history of diabetes, parity, education, age, BMI, WC, physical activity, smoking history, and duration of OCPs use; risk of pre-diabetes increases even after further adjustment for BMI. This finding may be partly explained by the dual role of obesity in both early menarche and diabetes type 2. It has been shown that obesity is associated with early onset of puberty (40,44,45) as well as with an elevated risk of insulin resistance and type 2 diabetes (46). Fredriks et al (43) demonstrated that women with a history of early menarche have an increased risk of type 2 diabetes and that this is mainly due to the direct effect of early onset of puberty on the risk of diabetes; BMI has a limited effect on this association.

In this study, the duration of OCPs use was adjusted as a potential confounder because some studies have reported changes in carbohydrate metabolism in OCP users that are related to both estrogen and progesterone which are components of OCPs (47). Existing studies on the effects of OCPs on type 2 diabetes have demonstrated controversial results. There are studies reporting an increase in insulin resistance and fasting blood glucose (FBG) among OCPs users (47,48) In contrast, the results of another study showed that women with prolonged OCP use had FBG levels lower than never users (49). There are also studies reporting that the risk of type 2 diabetes did not differ between long-term OCP users and never users (50,51).

Regarding strengths and limitations, this is the first study demonstrating a significant association between earlier menarcheal age and raised risk of diabetes and pre-diabetes in a large Middle East population (n=5625); most studies reporting a relationship on such issues have been conducted in Western countries (31). Our study has the advantage of using an ongoing population-based cohort. The amount of intra-assay variability in our data is also likely to be minimal because all laboratory measurements were done simultaneously at the same laboratory, by the same person. However, the limitations of our study were that pre-pubertal information on our participants as well as measurements of HA_1C were not available. We therefore added any treatment

for diabetes as a criterion of presence of diabetes. Recall bias might be a problem with self-reporting of menarcheal age; however, in the TLGS cohort, menarcheal age was assessed four times (once every three years) and the findings showed good confirmation.

In conclusion, it can be stated that early menarche is associated with an increase in prevalence of type 2 diabetes. This risk factor needs to be considered in screening programs of diabetes conducted at a community level. Identification of individuals at higher risk and implementing adequate prevention programs may decrease the adverse consequences of diabetes resulting from micro- and macro-vascular complications (52,53).

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Ethics

Ethics Committee Approval: The ethical committee of the Research Institute for Endocrine Sciences approved this study.

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Maryam Farahmand, Fahimeh Ramezani Tehrani, Design: Maryam Farahmand, Fahimeh Ramezani Tehrani, Data Collection and Processing: Maryam Farahmand, Fahimeh Ramezani Tehrani, Analysis and Interpretation: Maryam Farahmand, Fahimeh Ramezani Tehrani, Fereidoun Azizi, Literature Research: Maryam Farahmand, Fahimeh Ramezani Tehrani, Marzieh Rostami Dovom, Writing: Maryam Farahmand.

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CASE REPORT

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Delayed Diagnosis of a 17-Hydroxylase/17,20-Lyase Deficient Patient Presenting as a 46,XY Female: A Low Normal Potassium Level Can Be an Alerting Diagnostic Sign

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What is already known on this topic?

17-hydroxylase deficiency (17-OHD), a rare cause of congenital adrenal hyperplasia, is associated with hypertension and remarkable hypokalemia.

What this study adds?

We emphasized that near low levels of potassium should suggest 17-OHD in a 46,XY disorder of sex development patient, thus may prevent diagnostic delay. We also compared phenotypes of literature cases that have the same mutation as our case.

Abstract

17-hydroxylase/17,20-lyase deficiency (17-OHD), a rare autosomal recessive defect in adrenal and gonadal steroidogenesis, causes absence of secondary sexual characteristics and frequently associated with hypertension and hypokalemia. Here, we report a 46,XY case who had normal potassium levels and no hypertension. Our patient was a 2.5-year-old female admitted with female external genitalia and inguinal swelling. Pathology of biopsy revealed that this gonad was a testis. Karyotype was 46,XY. She had no hypertension and no hypokalemia. Serum luteinizing hormone and follicle-stimulating hormone levels were high; testosterone, dehydroepiandrosterone sulfate, and androstenedione were low. Human chorionic gonadotrophin stimulation resulted in partial testosterone response. She was initially diagnosed as partial gonadal dysgenesis or testosterone synthesis defect. In her follow-up after noticing low normal potassium levels at age 9 years, progesterone level was measured and detected to be high. Adrenocorticotropic hormone-stimulated steroid measurements were consistent with 17-OHD. Genetic analyses revealed p. R96Q (c.287G > A) homozygous mutation on exon 1 of *CYP17A1* gene. In conclusion, evaluation of 46,XY disorder of sex development patients must include serum potassium levels, and near low levels of potassium levels should also suggest 17-OHD despite absence of hypertension or remarkable hypokalemia. Testosterone synthesis defects must be excluded before establishing the diagnosis of partial gonadal dysgenesis.

Keywords: 17-hydroxylase deficiency, 46,XY disorder of sex development, diagnose, potassium

Introduction

17-hydroxylase/17,20-lyase deficiency (17-OHD) results from *CYP17A1* gene mutations. These mutations, disrupt steroidogenesis both in adrenals and gonads thus causing decreased production of glucocorticoids and sex steroids but increased mineralocorticoid precursors (1). CYP17A1 loss-of-function mutations can result in 17-OH or 17,20-lyase or combined enzyme deficiencies partially or completely (2). Over 100 mutations in the *CYP17A1* gene have been associated with combined 17-OH/17,20-lyase deficiency (OMIM 202110), including point mutations, small insertions or deletions, splice site alterations, and rarely large deletions (1). Isolated 17,20-lyase deficiency is rare and characterized with sex hormone deficiency without mineralocorticoid excess (3). P450 oxidoreductase (POR) and cytochrome b5

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are also important for 17-OH/17,20-lyase enzyme activity. POR and cytochrome b5 deficiencies are among important differential diagnoses of 17-OHD (1).

The most common presentation of a 46,XX patient is an adolescent girl without secondary sexual characteristics or menses and showing a varying degree of low-renin hypertension and hypokalemia (1,4). Early diagnosis is easier in 46,XY patients who present with ambiguous genitalia or apparent female genitalia and an inguinal hernia/mass associated with hypertension and hypokalemia (1,4). Patients with an apparent female genitalia and who are not associated with the above features may go undiagnosed until adolescence or young adulthood and eventually present with lack of secondary sexual characteristics, hypertension and hypokalemia (2). The steroid profile of these patients shows low androgen, estrogen levels with high gonadotrophins and adrenocorticotropic hormone (ACTH). Cosyntropin stimulation testing results show low cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S) and 17-OH progesterone but high, DOC and corticosterone levels and reveal the adrenal steroidogenic defect (1). Progesterone is also high in 17-OHD (5). Hypertensive patients have low renin and potassium levels, but 10%-15% of 17-OHD patients are normotensive at diagnosis (2).

Here we report a case with a p. R96Q (c.287G>A) mutation on exon 1 of *CYP17A1* gene who presented as a 46,XY disorder of sex development (DSD) with no hypertension and hypokalemia and who was misdiagnosed first as partial gonadal dysgenesis and subsequently diagnosed as 17-OHD via low normal levels of potassium and high levels of progesterone.

Case Report

This girl patient presented to our Pediatric Endocrinology Outpatient Unit at age 2.5 years for investigation of inguinal testis tissue on the right side. Her history revealed that she was born at term with a weight of 2670 grams and was registered as a girl. Her parents first noticed inguinal swelling while she was crying at age 5 months. The patient was admitted to a pediatric surgery unit in another hospital at age 2 years. When she was operated on for inguinal hernia, it was noticed that there was a gonad that seemed to be a testis. A gonadal biopsy was performed and pathologic evaluation revealed that the tissue was consistent with immature testis. The patient was referred to our hospital. She was the only child of non-consanguineous healthy parents. Her family history was unremarkable. On physical examination, her external genitalia had a female appearance and a vaginal opening was observed. Her height SDS was -0.81, blood pressure was normal (90/60 mmHg). Laboratory analyses revealed serum Na: 140 mEq/L (N: 135-145), K: 3.8 mEq/L (N: 3.5-5.5), follicle-stimulating hormone: 13.99 mIU/mL, luteinizing hormone: 2.19 mIU/mL, total testosterone (TT): 9.28 ng/dL, DHEA-S: 5.59 mcg/dL (N: <40), E2: 11.89 pg/mL, androstenedione: <0.03 ng/mL, dihydrotestosterone (DHT): 30.12 pg/mL. Human mammotropic gonadotropin (hMG)-stimulated estradiol level was 17.5 pg/mL, human chorionic gonadotropin (hCG)-stimulated testosterone was 50.04 ng/dL (partial response). Stimulated TT/DHT ratio was 2.05 (<12). Pelvic ultrasound revealed a 15x7.6x5 mm testis-like gonad in the right inguinal channel, and a 15x5x7 mm testis-like gonad in the left abdominal cavity. There were structures like prostate and seminal vesicles, but no Müllerian structures were seen. The patient's karyotype was 46,XY and SRY was (+). Pathologic evaluation of the gonadal biopsy material in our hospital revealed that it was a testis tissue which included seminiferous tubules but no germ cell. The patient was diagnosed as having a partial gonadal dysgenesis or testosterone synthesis defect. At follow-up, psychiatric evaluation was compatible with female sexual identity and the local ethics committee decided that the patient be raised as a girl, with removal of the gonads because of potential malignancy. A gonadectomy was performed. On her follow-up, she had no hypertension. Her serum K levels were near low limits (between 3.7-3.9 mEq/L). At age 9 years, her serum Na was 142 mEq/L, K 3.7 mEq/L, plasma renin activity was 0.06 ng/mL/h (N: <17), progesterone level was high (9.57 ng/mL) (N: 0.07-0.52), and ACTH level was 40.78 pg/mL (N: 10-60). High-dose synacthen stimulation test (HDSST) revealed: basal cortisol: <0.4 mcg/dL (N: 8-21), DHEAS: 2.5 mcg/dL (N: 13-115), progesterone: 9 ng/mL (N: 0.07-0.52), 17-OH progesterone: 0.4 ng/dL (N: 0.03-0.9). Stimulated levels were 0.53 mcg/dL, 2.9 mcg/dL, 18.15 ng/mL, and 0.46 µg/dL, respectively. Low stimulated cortisol, DHEA-S, 17-0H progesterone, and high progesterone levels were consistent with 17-OH/17,20-lyase deficiency. Genetic analyses revealed p. R96Q (c.287G>A) homozygous mutation on exon 1 of *CYP17A1* gene which was previously reported as a cause of complete 17-hydroxylase/17,20-lyase deficiency. The patient was prescribed oral hydrocortisone (10 mg/m^2/day) to prevent hypertension. On the last visit when she was 12.8 years old, her height SD was -1.75 and bone age was 8 years and 10 months. She had no hypertension. Her serum Na was 138 mEq/L and K was 4.5 mEq/L. Estrogen replacement therapy was planned on her follow-up.

Discussion

17-OHD is a rare form of congenital adrenal hyperplasia caused by mutations in the *CYP17A1* gene. 46,XX patients present with lack of secondary sexual characteristics or menses with hypertension and hypokalemia. 46,XY patients present with ambiguous genitalia or apparent female genitalia and inguinal hernia/mass associated with hypertension and hypokalemia. Müllerian structures (fallopian tubes, uterus, and upper third of vagina) are absent because Müllerian duct regression occurs due to normal production of Müllerian inhibitory factor from the testes. 17-OH and 17,20-lyase deficiencies can be diagnosed early and easily if hypertension and hypokalemia are associated with ambiguous genitalia or female external genitalia and an inguinal gonad in a 46,XY patient (1). However, hypertension and hypokalemia may not be seen in 10-15% of these patients and diagnosis may present difficulties in this group (2).

We reported here a case presenting as a 46,XY DSD patient with a low normal potassium level and no hypertension. Absence of hypertension and hypokalemia resulted in a delay in diagnosis until the patient reached the age of 9 years. At that time, noticing low normal levels of potassium levels brought to mind the correct diagnosis. Presence of a low stimulated cortisol, DHEA-S, 17-OH progesterone, and a high progesterone was consistent with a diagnosis of 17-OH/17,20-lyase deficiency. Genetic analyses revealed p. R96Q (c.287G > A) homozygous mutation on exon 1 of *CYP17A1* gene. p. R96Q mutation was reported before as associated with alterations in the steroid binding domain leading to complete 17α-OHD or combined 17-OH/17,20-lyase deficiency (2), but not reported from Turkey.

At the same amino acid site, first Laflamme et al (6) reported a homozygous novel missense mutation R96W caused by a C to T transition converting codon Arg96 (CGG) into a Trp (TGG) in exon 1 in two siblings with 46,XY DSD (14 and 9 years old). Both parents were heterozygous for this mutation. They showed that presence of R96W substitution almost completely abolished the activity of the mutant 17α-OH/17,20-lyase protein (6). These patients, similar to our patient, had no hypertension and hypokalemia.

Brooke et al (7) reported a novel missense homozygous R96Q mutation (same mutation of our case) in a 17-year-old 46,XX female patient who had presented with primary amenorrhea, sexual infantilism, and a malignant germ cell tumor. She had palmar and buccal hyperpigmentation, hypertension, and hypokalemia. Biochemical findings showed complete loss of 17-OH activity (7).

Athanasoulia et al (8) reported the same missense mutation in a 17-year-old 46,XY DSD patient who had presented with amenorrhea and no breast development and with mild diastolic hypertension. She was normokalemic. This patient also showed no breast development despite adequate estrogen replacement treatment for three years. The authors stated that the lack of breast development could be due to irreversible breast tissue alterations following high serum progesterone levels (8).

In a recent report, 4 affected XX siblings in an Arab family who had the same missense mutation were reported (9). The first sibling (17 years old) presented with abdominal pain and was diagnosed as a case of retroperitoneal malignant mixed germ cell tumor. She also had hypertension, primary amenorrhea, and lack of secondary sexual characteristics. One sibling (14 years old) presented with headache due to hypertension and pubertal delay. Two siblings (14 and 8 years old) were diagnosed with hypertension on a routine school check. All four patients had hypokalemia. They were treated with glucocorticoids and antihypertensive agents; three were also given estradiol for pubertal induction. Breast development in these patients was poor as also reported by Athanasoulia et al (8).

The phenotypic severity of combined 17α-OH/17,20-lyase deficiency varies depending on whether the activities of these enzymes are completely or partially lost according to the type and localization of the mutation. Alterations in the redox-partner binding site (e.g., p. R347H, p.R358Q, and p.E305G) lead to isolated 17,20-lyase deficiency (10), whereas mutations in the heme-binding site (e.g., p.R440C) or substrate binding pocket (e.g., p.S106P, D487_F489 deletion, duplication of I112, and p.R96Q) lead to complete 17α-OHD (2). Missense mutations in the steroid-binding domain, such as p.H373L, p. S106P, and p.R96Q, also result in combined 17α-OH /17,20-lyase deficiency (2).

The correlation between the CYP17A1 genotype and phenotype remains unclear. Patients who have the same mutations can have different presentations. Our case had no hypertension and no hypokalemia. The patient reported by Athanasoulia et al (8) had mild diastolic hypertension and normokalemia. On the other hand, the four siblings reported by Deeb and the case reported by Brooke et al (7) had notable hypertension and hypokalemia (9). Two patients with p. R96Q (c.287G > A) mutation were reported to have malignant germ cell tumor (7,9). This mutation may be associated with malignant germ cell tumor development in 46,XX patients. It is difficult to speculate that in CYP17A1 deficiency, the testes bear a malignant potential unless a dysgenesis exists. So, the decision for early gonadectomy should be cautious and better avoided. Gonadectomy should

be postponed until pubertal ages so that the patient`s own consent and tumor surveillance can be suggested.

Some mutations were suggested as founder mutations for some populations: p.H373L (exon 6), p. Y329fs (exon 6), and D487_F489del (exon 8) mutations in Asian populations; pW406 and p.R362C (exon 6) mutations in Brazil; 4-bp duplication following Ile479 (exon 8) in Canadian Mennonites and Dutch Frieslanders; and p53(or54) del (exon1) in Japan (2).

CYP17A1 gene mutations associated with 17-OHD previously reported from Turkey are: large deletions exons 1-6 (eight patients from two different families) (11,12), stop codon mutation p.Y27*(c.81C > A) in exon 1 (two patients from different families) (13,14), R239Q(G > A) exon not-known (one patient) (15), and a point mutation c.1307G > A (one patient) (16).

Patients who were diagnosed late showed poor breast development. It is suggested that high progesterone levels during pubertal development have irreversible effects on breast tissue. Spontaneous full breast development in patients who have normal progesterone levels and poor breast development in patients who have high progesterone levels support this suggestion (15). Accordingly, as a hypothesis, early diagnosis and treatment in these patients can prevent long-time exposure to high progesterone and breast development may be better in these cases. We will follow the breast development in our patient after estrogen replacement therapy. Deeb et al speculated that this particular mutation can have a specific adverse effect on breast development (9). Turan et al (15) also reported no improvement in breast development after estrogen replacement therapy in a 17-OHD patient who had another mutation (R239Q) in *CYP17A1* gene.

In conclusion, 17-OHD is a rare cause of 46,XY DSD. Although hypokalemic hypertension is a major component of 17-OHD, it is not seen in 10-15% of patients and due to this fact, diagnosis may be difficult and delayed. All 46,XY females should be first investigated for ACTH, PRA, HDSST, electrolyte status, and adrenal steroid profile before hCG test is performed. Low/near low levels of potassium and high progesterone levels should suggest 17-OHD despite absence of hypertension or remarkable hypokalemia. Early diagnosis and early treatment allow the induction of puberty at the appropriate time and can prevent hypertension and its complications.

Ethics

Informed Consent: Written informed consent was obtained from the patient's parents to participate in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu, Pınar Kocaay, Design: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu, Pınar Kocaay, Data Collection or Processing: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu, Pınar Kocaay, Analysis or Interpretation: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu, Serdar Ceylaner, Pınar Kocaay, Literature Search: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu, Serdar Ceylaner, Writing: Emine Çamtosun, Zeynep Şıklar, Merih Berberoğlu.

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CASE REPORT

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Response to Anastrozole Treatment in a Case with Peutz-Jeghers Syndrome and a Large Cell Calcifying Sertoli Cell Tumor

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What is already known on this topic?

Large-cell calcifying Sertoli cell tumor (LCCSCT) is a kind of sex cord-stromal tumor and can be encountered in Peutz-Jeghers syndrome (PJS) patients.

What this study adds?

Without diagnostic biopsy, gynecomastia due to LCCSCT in PJS can be treated successfully with anastrozole treatment.

Abstract

Peutz-Jeghers syndrome (PJS) is inherited as an autosomal dominant trait characterized by multiple gastrointestinal hamartomatous polyps, mucocutaneous pigmentation, and an increased risk of neoplasm. Large-cell calcifying Sertoli cell tumor (LCCSCT) is a kind of sex cord-stromal tumor which may co-exist with PJS and which is characterized radiologically by calcification foci within the testes. Surgical treatment options for this tumor range from testis-preserving surgery to radical orchiectomy. Not with standing this invasive approach, recently, there are some case reports demonstrating the efficacy of aromatase inhibitors in avoiding orchiectomy and its associated complications. In this paper, we have presented a LCCSCT case diagnosed in a boy with PJS and his response to anastrozole treatment.
Keywords: Peutz-Jeghers syndrome, large-cell calcifying Sertoli cell tumor, prepubertal gynecomastia, anastrozole

Introduction

Peutz-Jeghers syndrome (PJS), inherited as an autosomal dominant trait, is characterized by multiple gastrointestinal hamartomatous polyps, mucocutaneous pigmentation, and an increased risk of neoplasm (1,2).

PJS may be accompanied by endocrine conditions such as precocious puberty, gynecomastia, adrenocortical hyperplasia, and pituitary adenoma. Gynecomastia may refer to a clinical manifestation of a large-cell calcifying Sertoli cell tumor (LCCSCT) in PJS patients (2).

The exact mechanism that underlies the development of the LCCSCT remains unknown. LCCSCT accounts for 0.4-1.5% of all testicular tumors (3). Oftentimes, it appears in a bilateral and multifocal manner prior to, in the course of, or in the late phase of puberty. LCCSCT is radiologically characterized as a sex cord-stromal tumor by the calcification foci within the testes.

The polymorphism in the gene encoding a key enzyme in the estrogen biosynthesis, namely CYP 19 (P450 19), stimulates the increased levels of estrogen and is held responsible for the clinical findings associated with PJS. In approximately 60% of patients, gene mutation is identified in the LKB1/STK 11 tumor suppressor gene. Other genes, undefined for the time being, may also be responsible for the pathogenesis (3,4,5,6).

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LCCSCTs develop in a minority of PJS cases. The first finding of PJS, on the other hand, might be related to LCCSCT. Under physiological conditions, Leydig cells represent the source of aromatase enzyme production in the testes, while in LCCSCT patients, they are expressed particularly by neoplastic Sertoli cells. Elevated expression of aromatase enzyme clinically causes gynecomastia in males. The estrogen level, however, is not necessarily found to be increased (7).

The surgical treatment options for this tumor vary in a range of techniques from testis-preserving surgery to radical orchiectomy. However, today, medical treatment is opted as the first choice. Treatment with aromatase inhibitors (anastrozole) is among the options (1,2,3,7,8,9). Anastrozole treatment is effective in controlling LCCSCT (1). Aromatase inhibitors may help in avoiding orchiectomy and its associated complications. There is a limited number of cases reported in the literature (1,2,3,7,8,9).

Case Report

A 9.5-year-old male patient was brought to our department with a complaint of enlargement of the breast tissue noted in the past 6 months. The history of the patient revealed that a circumoral pigmentation had been detected at the age of 2.5 years and that the patient was referred to a department of pediatric gastroenterology when he was 4 years old at which time he was diagnosed to have PSJ upon findings during endoscopic investigation which revealed presence of three rectal hamartomatous polyps and ultrasonographic (USG) findings which showed multiple paraaortic, mesenteric, hypoechoic lymph nodes.

Results of body measurements revealed a height standard deviation score (SDS) of 0.34 and a body mass index (BMI) of 82.48%. Hyperpigmented lesions were noted around the mouth, on the lower lip, and on the buccal mucosa. On pubertal examination, testicular volumes were 3 mL and 3 mL, and pubic hair was at stage P1. The patient had breast growth, with a diameter of 2 cm on both sides, in compliance with bilateral gynecomastia. Bone age was assessed as 9 years.

Laboratory testing resulted as follows: Luteinizing hormone (LH): 0.31 mIU/L (N: <0.3 for prepubertal), follicle-stimulating hormone (FSH): 0.4 mIU/L (N: 0.21-4.33), estradiol <20 pg/mL (N: <20 pg/mL) (sensitivity for ADVIA® is between 11.8 to 3000 pg/mL), estrone <0.04 nmol/L (N: 0.03-0.22), beta human chorionic gonadotropin: 0.11 mIU/L (N: <5), total testosterone <10 ng/dL (N: <20 for prepubertal), 17-hydroxylase progesterone: 0.6 ng/mL (N: ≤90), dehydroepiandrosterone sulfate: 55.5 mg/dL (N: ≤91), AFP: 1.57 ng/mL (N: <6), inhibin B: 200 pg/mL (N: 35-170), inhibin α: 0.4 pg/mL (N: <2), and prolactin: 18.53 ng/mL (N: <20 ng/mL). No hormonal pathology was detected to explain the gynecomastia. Unfortunately, we did not have the means to investigate presence of polymorphism in the *CYP19* gene and mutations in the LKB1/STK 11 tumor suppressor gene.

According to scrotal USG imaging results, the size and dimensions of the right testis were 22.4x14.1x9.8 mm (1.6 mL) and those of the left testis 21.9x13.3x11.2 mm (1.5 mL). In addition, coarse calcification of a size of 1.4-1.8 mm in compliance with microlithiasis and a relatively well-circumscribed hypoechoic nodular formation of 3 mm at the right testis were noted. No typical vascularity implying a neoplasia was detected.

As a result of our assessment in collaboration with the department of pediatric urology, based on the past diagnosis of PJS, detection of gynecomastia, bilateral coarse calcification of the testes as imaged by scrotal USG, the patient was considered most likely to have a LCCSCT. The department of pediatric urology did not recommend a biopsy to be performed taking into account the technical difficulty arising from the small size of the lesion and the potential risk of alteration of lymphatic drainage due to transscrotal biopsy which could also lead to testicular damage and eventually orchiectomy. Joint decision was established to initiate treatment with anastrozole (1 mg/day). After six months of treatment, gynecomastia was observed to have remarkably regressed. After one year of treatment (calendar age: 10.8 years), the height SDS of the patient was 0.11 and his BMI was 82%. On pubertal examination, bilateral testicular volumes were 4 mL, penile length was measured to be 6 cm, and pubic hair appearance was at pubarche stage P1. On the left side, gynecomastia had totally regressed, while its size was reduced to 0.5 cm on the right side. Throughout the follow-up, the patient's somatic development, height and weight curves remained within the age-matched interval with a parallel course to the percentile line. Laboratory testing resulted as follows: LH: <0.2 mIU/L, FSH: <0.2 mIU/L, total testosterone: <10 ng/dL, E2: <20 pg/mL, and was evaluated to be normal. No change was viewed on scrotal USG imaging of the solid lesion in the right testis. After one year of treatment, it was decided to discontinue anastrozole treatment, since the clinical findings were found to be improved.

No gynecomastia increase occurred during the 6-month follow-up after treatment discontinuation. During the 1.5 years of follow-up without treatment, the growth of the patient showed a trend in accordance with his age.

Discussion

In males with PJS, presence of Sertoli cell tumor may manifest as gynecomastia and feminization-related findings (1,2,7). The first PJS case with Sertoli cell tumor-associated feminization findings was described by Cantú et al in 1980 (8). Until today, 30 comparable cases have been reported (1,3,9). In PJS patients, the functioning of Sertoli cells varies, consequently leading to clinical variance. While gynecomastia may appear as the first finding of the Sertoli cell tumors, it may not appear at all, despite the existence of the tumor.

Testicular tumors are quite rare in the prepubertal period. In patients with PJS, however, endocrinological findings such as prepubertal gynecomastia might be indicative of a testicular tumor, namely, LCCSCT. Malignancy is found in approximately 17% of patients with LCCSCT but is rare in young patients with bilateral tumors or in association with a genetic syndrome including PJS (10). Despite the low risk of malignancy in our patient, we planned biannual USG examination of the testes in addition to clinical and laboratory follow-up.

The polymorphism in the gene encoding a key enzyme in the estrogen biosynthesis, namely CYP 19 (P450 19), stimulates the increase in estrogen levels and is held responsible for the clinical findings associated with PJS. Elevated aromatase activity not only causes gynecomastia but also leads to other undesired effects such as increased rates of linear growth and bone maturation (1,2,3,7,8,9).

Local estrogen biosynthesis within the breast can be highly variable. It has been suggested that small amounts of estrogen may be sufficient to induce breast enlargement. In addition, increased bioavailability, local biosynthesis, or tissue responsiveness might be the factors responsible for normal estrogen levels despite the probable elevated aromatase activity (9).

In our patient, a prior diagnosis of PJS, presence of prepubertal gynecomastia of recent onset, and appearance of bilateral coarse calcifications in scrotal USG were the factors suggestive of a diagnosis of LCCSCT.

When the department of pediatric urology was consulted, we have been notified that despite a suspected malignancy, implementation of a biopsy procedure might result in an altered lymphatic drainage and lead to testicular damage and complications which may ultimately require orchiectomy. For the purpose of avoiding such complications, anastrozole treatment was initiated for LCCSCT without performing a biopsy. Although biopsy is an intervention which allows establishment of a definite diagnosis (4,11), LCCSCT diagnosis based on clinical and laboratory findings should be kept in mind especially for patients with PJS.

Anastrozole treatment was reported to be effective in achieving control over clinical findings of LCCSCT cases with gynecomastia (2,9,12). Treatment was initiated for our patient. After six months of treatment with anastrozole (1 mg/day), gynecomastia was observed to have remarkably regressed. No gynecomastia increase occurred during the 6-month follow-up after treatment discontinuation.

PJS was diagnosed in a 7.5-year-old patient showing painless gynecomastia when buccal pigmentation was noticed (2). In this patient, multiple parenchymal calcifications were noted in scrotal USG. Unlike our case, estradiol level was high in that patient. Testicular biopsy results were in compliance with a Sertoli cell tumor and aromatase treatment was initiated. After one year of treatment, initially with testolactone, followed by anastrozole, the breast diameter of the patient decreased from 7 cm to 3 cm, and, in parallel, testicular volume receded from 5 mL to < 4 mL. Although elevated estradiol levels may be helpful in the diagnosis, these levels are not necessarily increased in all LCCSCT cases, as was the case in our patient (9).

In cases with PJS who develop LCCSCT, elevated aromatase activity not only causes gynecomastia but also leads to other undesired effects such as increased rates of linear growth and bone maturation (1,2,3,7,8,9). After 2 years of anastrozole treatment, 9-year-old twins with PJS who were diagnosed to have LCCSCT upon occurrence of prepubertal gynecomastia, were found to have a decrease in rate of growth (1,13). Somatic growth of our case was followed-up due to the risk of potential growth retardation; he was found to have normal height and weight and his growth curve followed a normal course. Also in our patient, no increase was noted in gynecomastia after 6 months without treatment. However, new studies are needed to assess the probability of gynecomastia relapse beyond the cessation of treatment. No side effect was reported following anastrozole treatment in a dose of 1mg/day for two years (1). Long-term effects constitute another subject field which needs to be investigated. Anastrozole treatment is more commonly administered in adult breast cancer patients. There are a plethora of studies in the above-mentioned field, but the number of studies regarding its adverse effects in patients with LCCSCT and prepubertal gynecomastia is limited (1,2,3,7,8,9,13,14). Therefore, considering the reported side effects of these drugs, follow-up of growth and of bone density should be conducted in patients treated with aromatase inhibitors.

In conclusion, LCCSCT diagnosis based on the clinical and laboratory findings is feasible. This is particularly important for patients who are susceptible to the risks of a biopsy procedure. Aromatase inhibitors are effective in treatment of gynecomastia induced by LCCSCT accompanying PJS. Anastozole treatment lasting for one year does not have a negative impact on growth. Medical treatment may help in avoiding orchiectomy and its associated complications. However, there is need for longer follow-up studies and more extensive information regarding the efficacy of medical treatment and its long-term effects.

Ethics

Informed Consent: Informed consent was given.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Merve Koç Yekedüz, Zeynep Şıklar, Berk Burgu, Zarife Kuloğlu, Pınar Kocaay, Emine Çamtosun, Mehmet İsakoca, Aydan Kansu, Tarkan Soygür, Merih Berberoğlu, Design: Merih Berberoğlu, Zeynep Şıklar, Merve Koç Yekedüz, Data Collection or Processing: Merve Koç Yekedüz, Zeynep Şıklar, Berk Burgu, Zarife Kuloğlu, Pınar Kocaay, Emine Çamtosun, Mehmet İsakoca, Aydan Kansu, Tarkan Soygür, Merih Berberoğlu, Analysis or Interpretation: Merih Berberoğlu, Zeynep Şıklar, Merve Koç Yekedüz, Literature Search: Merih Berberoğlu, Zeynep Şıklar, Merve Koç Yekedüz, Writing: Merih Berberoğlu, Zeynep Şıklar, Merve Koç Yekedüz.

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CASE REPORT

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The Effectiveness of Cinacalcet as an Adjunctive Therapy for Hereditary 1,25 Dihydroxyvitamin D_3-Resistant Rickets

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What is already known on this topic?

Hereditary vitamin D-resistant rickets (HVDRR) is a rare autosomal recessive disease characterized by early-onset severe rickets, alopecia, hypocalcemia, and secondary hyperparathyroidism in the face of an elevated serum 1,25 dihydroxyvitamin D_3 [1,25(OH)2D_3] level. Secondary hyperparathyroidism from inadequate calcium absorption in the gut is the underlying pathophysiology for the rachitic changes in HVDRR. The recent availability of the calcimimetic agent cinacalcet enables the suppression of parathyroid hormone secretion through activation of the calcium-sensing receptors.

What this study adds?

We observed that cinacalcet is effective in the management of secondary hyperparathyroidism and that it improves the biochemical and radiological findings in HVDRR. The calcimimetic agent, cinacalcet, may be considered as an adjunct to high-dose 1,25(OH)2D_3 (calcitriol) and calcium therapy in the management of children with HVDRR.

Abstract

High doses of oral calcium or long-term calcium infusions are recommended to correct the hypocalcemia and secondary hyperparathyroidism in patients with hereditary 1,25 dihydroxyvitamin D_3-resistant rickets (HVDRR). Preliminary studies revealed that calcimimetics may be a safe and effective therapeutic choice in children with secondary hyperparathyroidism. Our aim was to observe the efficacy of cinacalcet in the normalization of secondary hyperparathyroidism and hypophosphatemia in two siblings aged 2.5 years and 6 months with HVDRR who did not respond to traditional treatment regimes. Both patients were admitted to the hospital with severe hypocalcemia. They were treated with high doses of calcitriol and calcium infusions intravenously. Secondary hyperparathyroidism was normalized temporarily, but did not improve completely. Cinacalcet (0.25 mg/kg) once a day along with the high doses of oral calcium and calcitriol was added to the treatment schedule. After 3 months, biochemical and radiologic findings reverted to normal. Our findings indicate that cinacalcet is effective in normalizing the hyperparathyroidism and hypophosphatemia in these cases and in improving the bone pathology.

Keywords: Cinacalcet, hereditary vitamin D-resistant rickets, secondary hyperparathyroidism

Introduction

Hereditary vitamin D-resistant rickets (HVDRR) is a rare autosomal recessive disorder caused by mutations in the *vitamin D receptor* (*VDR*) gene. It is characterized by severe rickets, hypocalcemia, hypophosphatemia, secondary hyperparathyroidism (hPTH), increased serum alkaline phosphatase (ALP) and 1α,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] levels (1,2). Total or partial alopecia and dermal cysts are encountered in a subset of these pediatric patients. The *VDR* gene is expressed in most tissues of the body, including intestines, kidney, bones, and keratinocyte of hair follicles (3). Alopecia due to the defective *VDR* gene activity within keratinocytes appears in approximately two-thirds of the cases and is considered as a marker of disease severity and response to therapy. There is no standard therapy protocol for patients with HVDRR, and patients with alopecia are usually more resistant to

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treatment and require high doses of $1,25(OH)_2D_3$ and also large doses of calcium, either administered orally or intravenously (4,5,6). Most patients with HVDRR require prolonged hospitalization for intravenous calcium therapy in order to maintain normocalcemia. Especially in infants, prolonged hospitalization and high infection risk related to catheter application are a few of the difficulties of this treatment regime. Another risk is unsustained parathyroid hormone (PTH) suppression and related hypophosphatemia and poor healing of the skeletal findings. It is possible to suppress the development of secondary hPTH, normalize serum phosphate levels, and resolve the rachitic changes with cinacalcet, which is a calcimimetic (7). We report the effectiveness of cinacalcet on the normalization of metabolic parameters and radiological healing of rickets in two sisters with HVDRR who had inadequately controlled secondary hPTH despite treatment with oral/or intermittent i.v. calcium infusion and calcitriol in high doses.

Case Reports

Case 1

The proband was a 2.5-year-old female child who was admitted to our hospital for evaluation of her seizures, deformities of lower extremities, failure to thrive, and alopecia. She was born to consanguineous parents as a full-term infant by normal spontaneous vaginal delivery, with a birth weight of 3250 g and a birth length of 50 cm. Her mother had received prenatal care and had taken vitamins regularly. The patient had been almost exclusively breastfed since birth up to 15 months. In addition, she had received 25-hydroxyvitamin D_3 [$25(OH)D_3$] 400 IU per day for one year. At presentation, her height was 83 cm (3-10% p), her weight was 10.7 kg (weight for height: 25% p), and head circumstance was 47 cm (3-97% p). She had rachitic rosary over the chest wall, widening of wrists and ankles, and "X" deformity of lower extremities. Her head appeared disproportionately large, but head circumference was within the normal range with an open anterior fontanel and frontal bossing. She was able to sit without support, but her ability to stand and walk were restricted due to the "X" deformity of the legs. She had frontal bossing, near total alopecia, and sparse eyebrows and eyelashes (Figure 1). Laboratory results revealed normal levels for serum electrolytes, serum albumin, blood urea nitrogen (BUN), and creatinine. Other laboratory results were as follows: serum calcium (Ca): 7.5 mg/dL (8.8-10.8), serum phosphorus (P): 2.3 mg/dL (4.5-5.5), ALP: 2278 U/L (80-220), intact PTH: 1194 pg/mL (10-71 pg/mL), $25(OH)D_3$: 25.2 ng/mL (10-44 ng/mL), $1,25(OH)_2D_3$: 59 pg/mL (16-65 pg/mL). Serum Ca, P, and ALP were determined by spectrophotometric method (Abbott, Architect C 16.000, IL, USA), intact PTH by chemiluminescent method (Beckman Caulter DxI800), and $25(OH)D_3$ by electro chemiluminescent method (Roche, Cobas e-411). Serum electrolytes, albumin, BUN, and creatinine levels were within normal ranges. Serum $1,25(OH)_2D_3$ level was in the upper limit of the normal range. The skeletal survey showed generalized osteopenia with advanced features of rickets manifested by cupping and fraying at the metaphyseal ends of long bones of upper and lower extremities (Figure 1a). *VDR* gene sequence analysis was performed by using MiSeq next-generation sequencing (NGS) platform, a Food and Drug Administration-approved diagnostic system (IIIumina, San Diego, CA, USA), and it was shown as a homozygote stop-codon mutation (c.148C > T) with mutation number: NM-001017535 (Figure 2). This mutation was described previously (8). Parents were heterozygous for the same mutation.

As shown in Table 1, the patient was initially treated with high doses of oral calcium (elemental Ca: 2 g/day), phosphate (1 g/day), and calcitriol [1α-$25(OH)D_3$] (2 µg/day). Elemental calcium and calcitriol were subsequently increased to 4 g/day and 6 µg/day, respectively. After 8 weeks of this protocol, serum Ca concentration became close to normal levels, but PTH was not suppressed and ALP was still high, and no radiological improvement was observed. Treatment was continued with high-dose oral calcium and calcitriol. After 6 weeks, the patient was admitted to hospital again with hypocalcemic seizures. During this period, HVDRR was confirmed by *VDR* gene mutation and intravenous calcium infusion was initiated. Elemental calcium was administrated at 150 mg/kg/day, infused via central line over a period of 10 hours for five days in a month. She

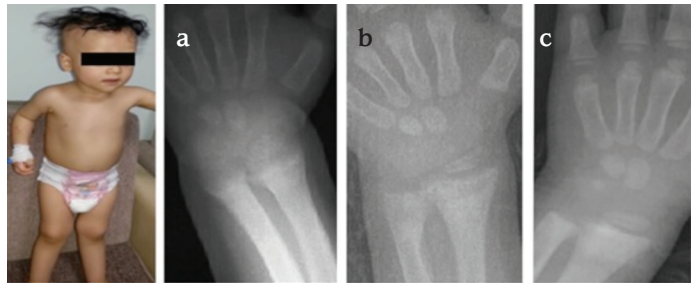

Figure 1. Alopecia and rickets in case 1

1a. Before treatment: Anteroposterior radiography of the patient's hand demonstrating cupping of the metaphyseal region

1b. After treatment with high dosages of i.v. calcium infusion, wrist x-ray showing partial healing of the rickets findings

1c. The x-ray of the wrist showing progressive healing of rickets while the child was receiving high-dose oral calcium, calcitriol, and cinacalcet

has been on this treatment for 4 months. We observed a decrease in PTH and ALP levels and normalization of serum P levels only during the periods of intravenous calcium infusion administration. After 4 steps of i.v. calcium infusion, partial healing in skeletal bone rickets findings were observed (Figure 1b); however, when the calcium infusion was stopped, secondary hPTH, increased ALP levels, and hypophosphatemia developed again and rachitic

Table 1. Effects of treatment with elemental calcium (oral/i.v.), calcitriol, and cinacalcet on serum calcium, phosphorus, alkaline phosphatase, and parathyroid hormone levels in case 1

Date	Ca (mg/dL)	P (mg/dL)	ALP U/L	PTH pg/mL	Treatment
First admission	7.2	2.3	2278	1194	Elemental Ca oral: 2 g/day Calcitriol: 2 µg/day P: 1 g/day
4th week (second visit)	7.8	2.4	2127	1078	Elemental Ca oral: 4 g/day Calcitriol: 4 µg/day P: 1.5 g/day
8th week (third visit)	8.5	2.8	2047	1036	Continued with same protocol no radiological changes
After 6 weeks of 3rd visit First-step i.v. Ca infusion for 5 days in a month	6.8	1.8	1427	940	Elemental Ca i.v.: 150 µg/kg/day P: 2 g/day Calcitriol: 6 µg/day
After first step of i.v. Ca therapy for 5 days	9.4	2.7	1540	95	Elemental Ca oral: 4 g/day Calcitriol: 6 µg/day P: 2 g/day
After 4 weeks of i.v. Ca infusion therapy	7.9	3	1887	887	Elemental Ca oral: 4 g/day Calcitriol: 6 µg/day P: 2 g/day
After oral drugs for 2 months, second-step i.v. Ca therapy applied	7.2	1.5	1651	1085	Elemental Ca i.v.: 150 µg/kg/day Calcitriol: 6 µg/day P: 2 g/day
After 5 days of i.v. elemental Ca infusion	9.4	2.1	1433	90	Elemental Ca oral: 4 g/day Calcitriol: 6 µg/day P: 2 g/day
After 4 weeks of i.v. Ca infusion off	7.1	2.4	1638	681	Elemental Ca i.v.: 150 mg/kg/day Calcitriol: 6 µg/day P: 2 g/day
After 4 steps of i.v. Ca infusion	7.5	2.6	1480	716	Partial radiological healing Cinacalcet: 0.25 mg/kg/day
After 15 days of cinacalcet	7.9	2.7	1335	503	Elemental Ca oral: 4 g/day Calcitriol: 6 µg/day P: 1 g/day Cinecalcet: 0.4 mg/kg/mg/day
After 1 month of cinacalcet	8.9	3	1100	184	Elemental Ca oral: 2 g/day Calcitriol: 4 µg/day Cinacalcet: 0.4 mg/kg/day
After 4 months of cinacalcet	9	3.1	200	64	Elemental Ca oral: 2 g/day Calcitriol: 2 µg/day Cinacalcet: 0.25 mg/kg/day Radiological healing observed

Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, PTH: parathyroid hormone

bone features became more apparent in the radiograms. After 4 months of intermittent calcium infusion, serum PTH and ALP levels decreased to near normal levels and radiological improvements were detected, secondary hPTH was normalized temporarily but not improved completely. After these improvements, intravenous calcium treatment was stopped and oral calcium and calcitriol treatment in high doses was administered again. However, after a while, serum PTH and ALP levels started rising again, serum Ca and P levels tended to decrease. We started cinacalcet (0.25 mg/kg) once a day along with high doses of oral calcium (4 g/day) and calcitriol (6 µg/day) and the cinacalcet dose was incrementally increased based on serum calcium and PTH levels, reaching 0.4 mg/kg/day over the next 2 weeks. We observed a temporary hypocalcemia during cinacalcet therapy. After 4 months of cinacalcet, serum Ca and P levels were within normal limits and we also observed sustained control of serum PTH and ALP levels and healing of the radiological rickets findings (Figure 1c). Treatment has been continued with a decreased dose of calcitriol (2 µg/day) and oral Ca (2 g/day) along with low-dose cinacalcet.

Case 2

The proband's sister was a 4-month-old girl with nearly total alopecia since birth (Figure 3). She had no history of hypocalcemic symptoms or seizures. She was taking daily 25(OH)D_3 (400 IU). At first visit, we have not observed any pathological features at upper and lower extremities. Her length was 63 cm (50% p), weight was 6.5 kg (25% p), and she had no radiological findings of rickets. Laboratory examinations showed normal serum Ca and P levels; however, serum PTH and ALP levels were high. The same mutation of VDR gene which was previously detected in her sister was also found in this patient. In her next visit after 2 months, serum Ca and P levels tended to decrease (Ca: 7.9 mg/dL and P: 2.8 mg/dL), but PTH (480 pg/mL) and ALP (689 U/L) levels were increased (Table 2). Rickets findings were observed radiologically in her elbow joint (Figure 3a). The same treatment protocol which was applied to her sister was also applied to this patient. At first, she was treated with high-dose calcitriol and intermittent calcium infusion; a temporary metabolic and radiological improvement was detected, as in her sister (Table 2). After adding cinacalcet to the treatment schedule, PTH and ALP levels decreased, serum Ca and P levels were sustained within normal levels, and complete radiological healing of rickets was observed (Figure 3b).

Informed consent forms were obtained from the parents of the patients for publication of the cases, including images.

Discussion

HVDRR results from loss of *VDR* gene function leading to target-organ resistance to 1,25$(OH)_2D_3$ which regulates Ca/P metabolism and bone mineralization. It is associated with severe rickets, secondary hPTH due to hypocalcemia, and hypophosphatemia with or without alopecia. Serum

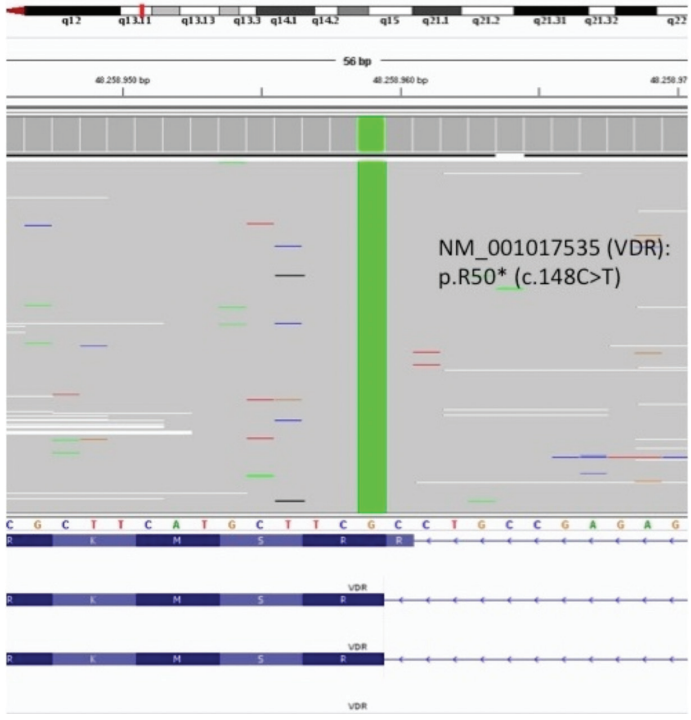

Figure 2. *VDR* gene analysis performed by using MiSeq next-generation sequencing platform, a Food and Drug Administration-approved diagnostic system (Illumina, San Diego, CA, USA). The gee has 11 exons and NM–001017535(VDR): p.R50* (c.148 C > T) is causing a premature stop codon in exon 5. This variation causes a truncated protein and severe damage on protein function

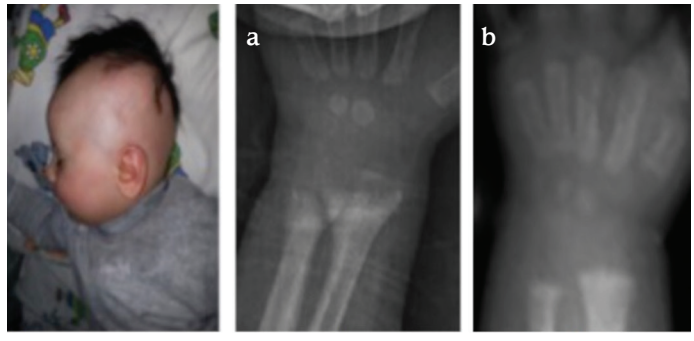

Figure 3. Alopecia and rickets in case 2

3a. Baseline x-rays at the age of 4 months showing changes consistent with rickets in case 2

3b. Bone roentgenogram showing markedly improved signs of rickets with high-dose calcitriol, oral calcium, and cinacalcet

$1,25(OH)_2D_3$ level is usually elevated. While $25(OH)D_3$ levels were within normal limits in our patients, $1,25(OH)_2D_3$ levels were in the upper limits of the normal ranges (1,2,3).

Alopecia has been considered as one of the indicators of severe hormone resistance in patients with HVDRR. Though there is genotype-phenotype variability, the severity of alopecia and hormone resistance seems mostly to depend on the different types of VDR mutation in these patients. Several mutations in the *VDR* gene have been identified as the cause of HVDRR. It has been revealed that most of the patients with mutations in the DNA-binding domain have alopecia, whereas patients with mutations in the ligand-binding domain have variable degrees of vitamin D resistance usually not associated with alopecia (1,9,10). In our patients, we identified a stop-codon mutation in the *VDR* gene (c.148C>T) in both sisters as previously described in two sisters with alopecia and HVDRR (8). In these patients, the mutation was transmitted in an

Table 2. Effects of treatment with elemental calcium (oral/i.v.), calcitriol, and cinacalcet on serum calcium, phosphorus, alkaline phosphatase, and parathyroid hormone levels in case 2

Date	Ca (mg/dL)	P (mg/dL)	ALP U/L	PTH pg/mL	Treatment
First visit	9.2	4.1	522	234	-
After 1 month of first visit	7.8	3.5	847	555	Calcitriol:1.5 µg/day Oral Ca: 1 g/day
Two months after first visit Radiological findings of rickets were apparent	7.2	1.8	1427	940	First step of elemental Ca i.v.: 150 mg/kg/day for 5 days Oral P: 1.5 g/day Calcitriol: 2 µg/day
After 5 days of first-step i.v. Ca infusion	9.4	2.7	1540	115	Oral elemental Ca: 1 g/day Calcitriol: 4 µg/day Oral P: 1.5 g/day
After one-week i.v. Ca infusion off	7.8	3	1887	887	Oral elemental Ca: 2 g/kg/day Calcitriol: 4 µg/day Oral P: 2 g/day
After 1-month first-step i.v. Ca infusion off	6.9	1.5	1651	785	Second-step elemental Ca i.v.: 150 mg/kg/day for 5 days Calcitriol: 4 µg/day P: 2 g/day
After second-step Ca infusion for 5 days	9.6	3.1	1433	165	Oral elemental Ca: 2 g/day P: 2 g/day Calcitriol: 4 µg/ day
After 1-month second-step Ca infusion off	7.1	2.4	1638	681	Elemental Ca i.v.: 150 mg/kg/day for 5 days Calcitriol: 4 µg/day P: 1.5 g/day Cinacalcet: 0.25 mg/kg/day
After 2 months of cinacalcet applied	9.2	3.7	215	95	Oral elemental Ca: 1 g/day Calcitriol: 2 µg/day Cinacalcet: 0.25 mg/kg/day
After 3 months of cinacalcet applied	8.9	3.2	220	62	Same treatment protocol continued Radiological healing observed

Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, PTH: parathyroid hormone

autosomal recessive inheritance, and the parents who had the same mutation in heterozygous form were asymptomatic, with no features of metabolic bone disease or alopecia**.** Our patients had near total alopecia and severe rickets findings on physical examination and X-ray findings similar to those of the two sisters carrying the same VDR mutation, previously reported (8).

In HVDRR, the main metabolic characteristics are severe hypocalcemia-induced secondary hPTH and renal phosphate wasting which prolongs bone healing. Traditional therapy with high-dose oral calcium and calcitriol aims to correct serum Ca concentration to normal levels and to radiological healing of rickets. However, these treatment protocols are usually not able to suppress the secondarily increased hPTH and ALP levels. Sustained hPTH prolongs the bone healing process by stimulating bone turnover and hypophosphatemia. Actually, serum PTH level is suppressed by ionized serum Ca concentrations. But it is difficult to maintain normocalcemia and to suppress PTH level in patients with HVDRR, especially patients with alopecia. For these reasons, some authors proposed HVDRR to be a form of PTH-dependent rickets. High doses of calcium and calcitriol treatment which are required to maintain normocalcemia may lead to vitamin D intoxication, nephrocalcinosis, and kidney damage. Moreover, tertiary hPTH and osteitis cystica fibrosa may develop in some patients with HVDRR (11,12). Some authors recommend parathyroidectomy in patients with HVDRR who have unsuppressed secondary hPTH. It has also been proposed that parathyroidectomy can be considered not only for correction of hypocalcemia but also for PTH suppression to maintain metabolic and radiological improvement in HVDRR. In Case 1, during 2.5 months of high-dose oral calcium and calcitriol therapy, serum Ca concentration reached a near normal level from time to time, but serum P level was lower than normal limits despite phosphorus supplementation, and serum PTH and ALP levels were still extremely elevated. During this period, radiological healing was not observed. Enteral or parenteral calcium infusions are reported to suppress PTH level with metabolic and radiological healing in HVDRR patients with or without alopecia (4,5,6). In our patients, long-term normocalcemia and normalization of hypophosphatemia secondary to unsustained hPTH suppression could not be achieved with high-dose oral calcium and calcitriol treatment. Therefore, we tried treatment with intermittent parenteral calcium infusions. As previously reported, two siblings who had the same mutations in *VDR* gene were successfully treated with parenteral calcium infusion (8). We observed a decrease in PTH and ALP levels and normalization of serum P levels only during the periods of intravenous calcium infusion. After four-step intermittent calcium infusion (each step covering five days of i.v. calcium infusion in a month), metabolic improvement and partial healing in bone findings was observed; but when the calcium infusions were stopped, secondary hPTH and hypophosphatemia occurred again and rachitic bone features became more apparent in X-rays. Intravenous calcium treatment for a long time is difficult especially in infants because of prolonged hospitalization and increased risk of infection due to central catheter application. For these reasons, alternative therapeutic approaches have been tried to suppress PTH. The recent availability of the calcimimetic agent cinacalcet enables the suppression of PTH secretion through activation of the calcium-sensing receptors. Cinacalcet has been used in adults with primary hPTH or secondary hPTH due to chronic renal disease (13,14,15). The safety and efficacy of cinacalcet in children have been shown in a few reports and in cases of secondary hPTH with disorders such as x-linked hypophosphatemia, HVDRR, and renal failure (15,16). We can only reach the suppressed PTH levels with low-dose cinacalcet combined with high-dose oral calcium and calcitriol therapy. After 4 months of this combination therapy, long-term normocalcemia and PTH suppression with normalization of serum phosphorus level were achieved in addition to radiological healing, but, as expected, there was no recovery in alopecia. Treatment with cinacalcet, as a side effect, may cause hypercalciuria due to activation of calcium-sensing receptors in the thick ascending limb of the loop of Henle, and for this reason, administration of thiazide diuretics may be beneficial (16). Another potential risk of cinacalcet therapy is hypocalcemia due to hypercalciuria. In Case 1, we have observed a temporary hypocalcemia but not hypercalciuria. At the beginning, Case 2 was also treated with high-dose oral calcium and calcitriol, but this proband did not respond to this therapy protocol. We observed only temporary PTH suppression, metabolic and radiological healing with intermittent i.v. calcium infusion (Table 2). Therefore, to achieve long-term metabolic and radiological healing as in Case 1, low-dose cinacalcet was added to the high-dose oral calcium and calcitriol therapy. Both two siblings are at present continuing treatment under the same protocol.

In this report, we described two siblings with HVDDR from parents with consanguinity. Heterozygous mutations in *VDR* gene were detected in the parents while the children were homozygous for the same mutation. Our patients did not respond to traditional treatment with calcium and calcitriol in high doses, and responded partially to intravenous calcium infusions. In addition to high-dose calcium and calcitriol, by administrating cinacalcet we were able to obtain a positive effect in maintaining the biochemical and radiological

healing. Thus, we have shown that cinacalcet improves the biochemical and radiological findings in HVDDR and we therefore recommend its use in the management of secondary hPTH.

Ethics

Informed Consent: Informed consent forms were obtained from the parents of the patients for publication of the cases, including images.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ayşehan Akıncı, Design: Ayşehan Akıncı, Data Collection and Processing: İsmail Dündar, Analysis and Interpretation: Ayşehan Akıncı, Literature Research: Meltem Kıvılcım, Writing: Ayşehan Akıncı.

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CASE REPORT

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Persistent Müllerian Duct Syndrome with Transverse Testicular Ectopia: A Novel Anti-Müllerian Hormone Receptor Mutation

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What is already known on this topic?

Persistent Müllerian duct syndrome (PMDS) is a disorder of sexual differentiation characterised by the persistence of Müllerian derivatives in males with an XY karyotype and normal virilization. PMDS are caused by mutations in the anti-Müllerian hormone gene, which lead to defects in its secretion or activity, or to mutations in the gene for the type II receptor for anti-Müllerian hormone, which results in a clinical picture of hormonal resistance.

What this study adds?

We report a previously undescribed homozygous c.24G > A (p.W8X) mutation determined at *AMHR2* gene analysis.

Abstract

Persistent Müllerian duct syndrome is the result of either anti-Müllerian hormone (AMH) deficiency or AMH receptor resistance. A long tubular structure was palpated during the physical examination of a 13-month-old male patient who had presented with bilateral undescended testes. At physical examination, the testes were not palpable. The patient's karyotype was XY, SRY (+), and his AMH level was 22 ng/mol. Structures suggestive of ovaries, a uterus, and fallopian tubes were observed during the laparoscopic examination of the ectopic testis. *AMHR2* gene sequence analysis performed with a preliminary diagnosis of AMH receptor resistance revealed a previously unreported homozygous c.24G > A (p.W8X) mutation. The patient was assessed as a case of AMH receptor resistance. Orchiopexy was performed.

Keywords: Undescended testis, anti-Müllerian hormone receptor mutation, anti-Müllerian hormone receptor resistance

Introduction

Persistent Müllerian duct syndrome (PMDS) is a rare disorder of 46,XY sex development. It occurs as a result of anti-Müllerian hormone (AMH) deficiency or AMH receptor resistance, conditions which arise due to mutations in the AMH gene or the *AMH type 2 receptor (AMHR2)* gene. In these patients, the external genital structure is that of a normal virilized male, while fallopian tubes and a uterus are observed in the internal genital structure (1,2). Here, we report a case of PMDS in a 13-month-old male presenting with bilateral cryptorchidism and a novel homozygous mutation in the *AMHR2* gene.

Case Report

A 13-month-old male presented to the pediatric surgery department with a complaint of bilateral undescended

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testis. A long, tubular structure (testis?) was palpated within the canal. Ultrasonography revealed absence of a testicular structure in the left inguinal canal or scrotum. On the right side, two structures measuring 12x8 mm and 11x7 mm and thought to be testes were observed in the proximal and middle parts of the inguinal canal. These findings were interpreted as transverse testicular ectopia on the right. During laparoscopic examination of the ectopic testis, tissues suggestive of a uterus, fallopian structures, and ovaries were observed inside the abdomen. Gonad biopsy was performed and the patient was referred to the pediatric endocrinology department with a preliminary diagnosis of sexual development disorder. At physical examination, weight was 10.2 kg [standard deviation score (SDS): -1.06] and height was 81 cm (SDS: -0.39). Blood pressure was 98/60 mm/Hg, Quickly score 2. The testes were not palpable and the phallus was 3.5 cm in length. The mother and father were first-degree relatives. Karyotype analysis revealed a XY, SRY (+) karyotype. Serum follicle-stimulating hormone level was 0.44 mIU/L (0.26-3 mIU/L), luteinizing hormone was 0.27 mIU/L (0.02-0.3 mIU/L), free testosterone 0.3 pg/mL (0.15-0.6 pg/mL), total testosterone 0.1 ng/dL (0.2-1.3 ng/dL), E_2 <20 ng/mL (<15 ng/mL), and AMH >22 ng/mL (4.9-264.5 ng/mL). Immature seminiferous tubular structures were observed in gonad biopsy specimens sent from the pediatric surgery department. *AMHR2* gene sequence analysis performed with a preliminary diagnosis of AMH receptor resistance revealed a previously undescribed homozygous c.24G>A (p.W8X) mutation. The parents had the same mutation in heterozygous form. The patient was evaluated as a case of AMH receptor resistance and presented to our "Sex Development Disorders Council". Upon their decision, orchiopexy was performed.

Discussion

AMH is secreted from immature Sertoli cells in males and from ovarian granulosa cells in females and is responsible for the regression of the Müllerian ducts in the male fetus. The clinical picture that appears in the 46,XY genotype in AMH synthesis and effect deficiency is known as PMDS. This occurs as a result of mutations in the *AMH* gene or in the *AMHR2* gene. Serum AMH levels are low or undetectable in *AMH* gene mutations, while they are normal or elevated in *AMHR2* gene mutations (1,2).

Most cases of PMDS are diagnosed following a virilized XY patient presenting with bilateral or unilateral undescended testis or inguinal hernia. Three anatomic variants of PMDS have been described. In group 1, bilateral testicles are located intraabdominally. In group 2, one testis is located in a hernia sac or along with the Müllerian structures (hernial uterus inguinalis). In group 3, both testes are found in the same hernia sac, along with uterus and tubes (crossed or transverse testicular ectopia) (3,4). Given that the genotype of AMH and AMHR2 is not related to the phenotypes, the phenotype of our index case was consistent with group 3 (5,6). Our patient presented to the Pediatric Surgery Department with a complaint of bilateral undescended testes and was referred to us when female internal genital structures were observed at laparoscopy. It has been reported that the undescended testes in PMDS may undergo malignant transformation. The prevalence of germ cell tumors such as seminoma, most commonly, as well as embryonal carcinoma, yolk sac tumor and, more rarely, clear cell adenocarcinoma in such patients varies from 15-40%, a frequency no higher than that reported for abdominal testes in general (7,8). Orchiopexy is therefore recommended as early as possible in these cases (9). In our case, too, orchiopexy was performed on both testes seen on the same side inside the abdomen, and both right and left were enabled to descend into the scrotum.

AMH is a member of the TGF-beta family. In males, serum levels of AMH remain high until 2 years of age and persist in measurable levels until puberty, before decreasing to undetectable levels at puberty (10,11). Low or undetectable AMH levels in cases with PMDS indicate AMH mutation, whereas high AMH levels may indicate mutations in the *AMHR2* gene (12). The *AMH* gene is located on the short arm of the 19th chromosome and was first cloned in 1986 by Cate et al (13). It consists of five exons and is 2.8 kbp in length. PMDS exhibits an autosomal recessive pattern. Mutation in the *AMH* gene or *AMHR2* gene has been reported in 84% of cases. *AMHR2* gene mutations are located in the long arm 13.13 (12q.13.13) region of the 12th chromosome. *AMHR2* contains 11 exons and more than 27 mutations have already been described in this gene (14,15). The *AMHR2* gene contains four intronic polymorphisms, located in t276a intron 1, c1280t intron 3, c1827t intron 5, and a6503g intron 10 (16). In a study of 32 families with PMDS, Imbeaud et al (17) determined mutations in the *AMH* gene in 16 families and in the *AMHR2* gene in the other 16. Deletion 27-bp in size was observed in the 10th exon in 10 of the 16 patients with mutation in the *AMHH2* gene. This mutation has been reported as the cause of 25% of cases of PMDS (17). AMH receptor resistance was primarily considered in our case due to AMH level. At mutation analysis, a previously undefined homozygous c.24G>A (p.W8X) mutation was determined at *AMHR2* gene analysis. Mutation screening revealed that the mother and father bore the same mutation in heterozygous form.

In conclusion, in cases with bilateral cryptorchidism, the clinicians should be aware of the possibility of PMDS. The condition should be considered when persistent Müllerian structures are observed, particularly in virilized males with a normal external genital structure.

Ethics

Informed Consent: After getting informed consent from the parents, genetic analysis was performed.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Samim Özen, Şükran Darcan, Design: Özlem Korkmaz, Samim Özen, Data Collection and Processing: Özlem Korkmaz, Analysis and Interpretation: Özlem Korkmaz, Samim Özen, Nurhan Özcan, Petek Bayındır, Sait Şen, Hüseyin Onay, Damla Gökşen, Ali Avanoğlu, Ferda Özkınay, Şükran Darcan, Literature Research: Özlem Korkmaz, Writing: Özlem Korkmaz, Samim Özen.

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A Comprehensive Online Calculator for Pediatric Endocrinologists: ÇEDD Çözüm/TPEDS Metrics

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To the Editor,

Pediatricians need to make many medical calculations during follow-up of both healthy and sick children. This is particularly relevant for pediatric endocrinologists. The World Health Organization (WHO) provides different tools for offline use in order to assess various anthropometric variables (1). Calculators for many specialties are available in Up To Date which requires subscription (2). There have also been a number of promising and inspiring attempts in our country to make such calculations using Excel or other programming language, however, they could not be widely used. We have recently launched an online, freely accessible, computerized, user-friendly, and scientific tool set containing a wide array of formulae in order to meet the needs of pediatric endocrinologists: ÇEDD ÇÖZÜM (www.ceddcozum.com). ÇEDD stands for Çocuk Endokrinolojisi ve Diyabet Derneği, the official society of pediatric endocrinologists in Turkey and ÇÖZÜM means solution. TPEDS stands for Turkish Pediatric Endocrinology and Diabetes Society and METRICS denotes the nature of the online tool. For international use, we chose a simpler and memorable name for the website: www.childmetrics.org.

ÇEDD ÇÖZÜM (TPEDS METRICS) mainly provides assessment of various physical growth variables. On the main page (Auxology), standard deviation (SD) scores and percentile values can be calculated for weight, height, body mass index, and head circumference of children using one of the three reference data: Centers for Disease Control (CDC), Neyzi et al, and the WHO (weight data are present only for children < 10 years of age) (3,4,5,6). Body surface area is calculated according to Costeff method (7). For target height, firstly, mean of paternal and maternal height was calculated; next, 6.5 cm was added to and subtracted from that mean value for boys and girls, respectively (8). Estimation of target height SD scores is done by analyzing the calculated target height using the data of the oldest age group available in the selected reference data (WHO: 18 years, Neyzi et al: 19 years, and CDC: 20 years) (3,4,5,6). At the bottom of the results, there are four links to related pages.

- Growth charts: The input can be seen on the relevant charts derived from the selected reference data.

- Results can be seen in text format.

- Further anthropometric assessments: SD scores of upper/lower segment ratio (for girls between 3-18 years of age and for boys between 2-18 years of age), waist circumference (< 17 years of age), and sitting height/height ratio (6-17 years of age) are calculated according to Turkish references (9,10,11,12).

- Calculation of predicted adult height can be made using Bayley-Pinneau (for children with ≥6 years of bone age but the extent of difference between chronological and bone age might prevent calculation for some age groups, especially in boys) and Roche-Wainer-Thissen methods (from 1 year of chronological age to 16 years for boys and 14 years for girls) (13,14).

Growth velocity rates and SD scores can be calculated on another page using four reference data including Turkish

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(unpublished Neyzi et al data for boys between 10 and 15 years of age and girls between 8 and 13.5 years of age), American [Baumgartner et al (15): 6-month to 15.5-year-old boys and 6-month to 13.5-year-old girls; Kelly et al (16): 5.5-year to 18.5-year-old boys and 5.5-year to 17.5-year-old girls] and WHO data (for children < 24 months) (17). Growth section also includes a page for calculation of SD scores of insulin-like growth factor 1 (IGF-1) and IGF-binding protein-3 levels (generated with chemiluminescence method) using the data from 1-17-year-old healthy Turkish children (18). A tool to estimate growth hormone dose is also available.

Bone section provides an opportunity to calculate SD scores for total L1-L4 areal bone mineral density using the data obtained with dual X-ray absorptiometry from healthy Turkish children between 2 and 18 years of age (19). Tubular excretion of phosphate and calcium can be estimated as well.

Calculations of thyroid volume SD score are made according the two Turkish studies. Data from newborns and older children up to 19 years of age are derived from the reports by Mutlu et al (20) and Aydıner et al (21), respectively. Ovarian volume is estimated according to the following formula: x*y*z*0.523 (22,23).

Glucose/insulin ratio, homeostatic model assessment for insulin resistance, and Quick index can be calculated on the Obesity section. Testosterone/dihydrotestosterone and testosterone/androstenedione ratios can be computed on the human chorionic gonadotropin test section. Unit converter is a simple tool for commonly used laboratory variables (24).

SD scores for a given measurement (x) are mainly calculated using LMS data with following formulae: L≠0, SD score = [(x/M)**L–1]/LS or L = 0, SD score = ln(x/M)/S (3,4,5,6,10,11,12,16,17,25). Interpolation by weighted mean is used to obtain L, M, and S values at finer intervals that are not provided in the relevant references (26). When no LMS data are present for a variable, SD scores for a given measurement (x) are obtained by the following formula: SD score = (x–mean)/SD (9,15,18,19,20,21). Percentile values corresponding to calculated SD scores are obtained from a standard normal distribution table.

The tool is under protection of our national society, will be kept updated, and will incorporate new features.

Ethics

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Korcan Demir, Samim Özen, Ergün Konakçı, Murat Aydın, Feyza Darendeliler, Design: Korcan Demir, Samim Özen, Ergün Konakçı, Data Collection and Processing: Korcan Demir, Samim Özen, Feyza Darendeliler, Analysis and Interpretation: Korcan Demir, Samim Özen, Murat Aydın, Feyza Darendeliler, Literature Research: Korcan Demir, Samim Özen, Feyza Darendeliler, Writing: Korcan Demir, Samim Özen, Ergün Konakçı, Murat Aydın, Feyza Darendeliler.

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