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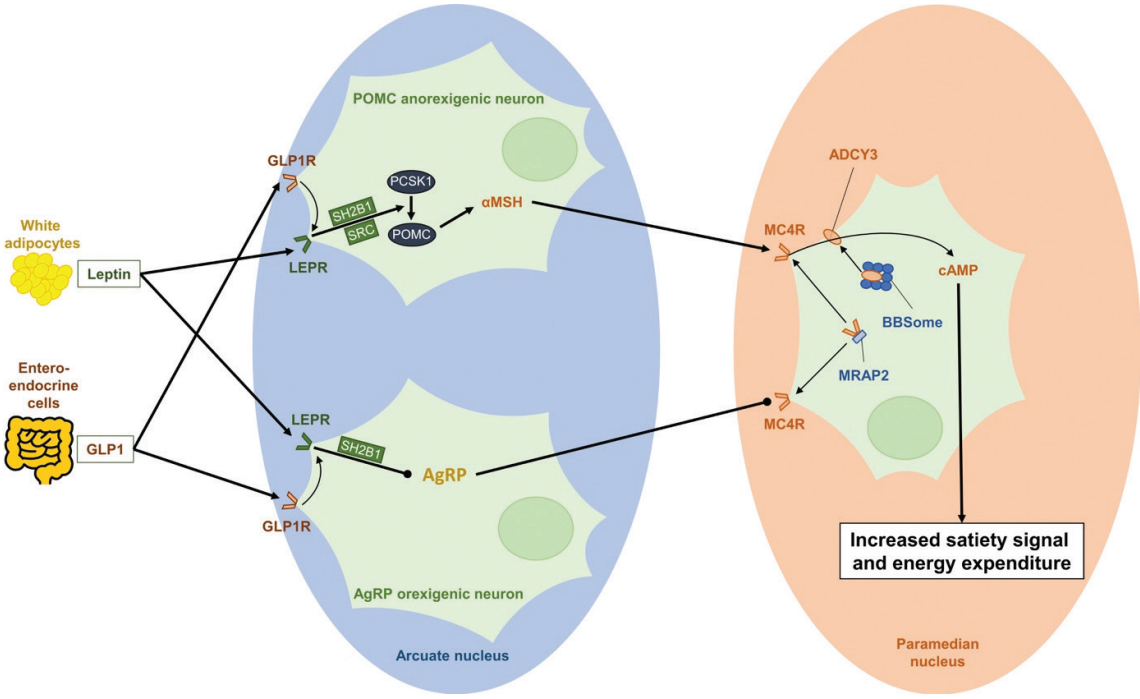

Schematic representation of the leptin-melanocortin pathway in the hypothalamic nuclei.

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REVIEW

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Current Treatments for Patients with Genetic Obesity

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Abstract

Obesity derives from impaired central control of body weight, implying interaction between environment and an individual genetic predisposition. Genetic obesities, including monogenic and syndromic obesities, are rare and complex neuro-endocrine pathologies where the genetic contribution is predominant. Severe and early-onset obesity with eating disorders associated with frequent comorbidities make these diseases challenging. Their current estimated prevalence of 5-10% in severely obese children is probably underestimated due to the limited access to genetic diagnosis. A central alteration of hypothalamic regulation of weight implies that the leptin-melanocortin pathway is responsible for the symptoms. The management of genetic obesity has so far been only based, above all, on lifestyle intervention, especially regarding nutrition and physical activity. New therapeutic options have emerged in the last years for these patients, raising great hope to manage their complex situation and improve quality of life. Implementation of genetic diagnosis in clinical practice is thus of paramount importance to allow individualized care. This review describes the current clinical management of genetic obesity and the evidence on which it is based. Some insights will also be provided into new therapies under evaluation.

Keywords: Genetic obesity, syndromic obesity, personalized medicine, setmelanotide

Introduction

Obesity is a multifactorial and complex disease defined as an excess of body fat resulting from an inadequate energy balance over the long term. It is driven by the interaction between genetic predisposition and environmental factors and can manifest in early childhood with a lifelong burden (1).

Obesity is a major public health issue in our modern society, and its incidence has been increasing significantly among children in recent decades. According to the World Health Organization (WHO) in 2020, 12% of children aged 7-9 years in the 33 participating countries of the European Region can be considered obese (2). Worldwide, WHO has estimated the number of overweight or obese children under the age of 5 to be 39 million (3).

Obesity derives from impaired central control of body weight with a high genetic heritability (up to 80%) in populations developing severe and early obesity, before the age of six years (4,5). Within this genetic susceptibility, frequent polygenic variants with small effects may be distinguished from rare pathogenic variants with large effects causing monogenic and syndromic obesities. Regarding the latter, most of these genes are part of the leptin-melanocortin pathway, which is crucial in central nervous system regulation of body weight. Patients affected by these genetics anomalies show major eating disorder, such as impaired satiety and disruptive food-seeking behavior, from the first years of life resulting in severe early-onset obesity. Some of these patients may also suffer from childhood from neuropsychological and psychiatric disorders, endocrine comorbidities, and complications deriving from obesity.

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Thus, the clinical considerations in these obesities are often complex and challenging. The treatment of genetic obesity has so far been based on environmental control, starting as early as possible, to avoid obesity progression and to help the acquisition of appropriate eating and exercise behavior. The recent development of new therapeutic options for the management of these genetic obesities has made early diagnosis crucial to avoid massive weight gain in childhood and its negative effects on the health of affected children.

In this review, we will briefly describe the principal clinical pictures observed in patients with genetic obesity and then we will outline the distinct aspects of its current management, with a special focus on innovative therapeutics targeting hyperphagia.

Clinical Features of Genetic and Syndromic Obesities

Monogenic and syndromic obesities are part of the same spectrum of hypothalamic pathologies affecting the satiety signal. Both show early-onset obesity, defined for children by body mass index (BMI) higher than the International Obesity Task Force curve corresponding to BMI 30 kg/m^2 in adulthood before six years of age. A very early adiposity rebound before three years of age, or the lack of rebound, is regularly observed. This is related to eating behavior disorders which can be observed from the first months of life. Parents often describe a lack of satiety, intolerance of food restriction, and conflicts about limiting food intake. Later on, patients may have obsessions with food that interfere with other activities, and foraging strategies that may include stealing and night-time feeding (6). An important phenotypic variability is evident between patients with similar genetic disorders. It is partly explained by its interaction with environmental factors such as family and social conditions, ethnicity, and gender. Most common syndromic and monogenic obesities with associated genetic alteration and specific clinical features besides severe early-onset obesity are summarized in Table 1. They are mainly

Table 1. Most prevalent syndromic and monogenic obesities including the specific clinical features, and genetic alterations

	Affected gene	Specific clinical features
Syndromic obesity		
Prader Willi syndrome	Abnormal parental genomic imprinting of paternal 15q11-q13 region.	Neonatal hypotonia, suckling disorders in the first months, hyperphagia and food impulsivity around 4 years, moderate intellectual disability, social interaction and behavioral disorders, endocrine abnormalities (growth hormone deficiency, hypogonadism), dysmorphia, scoliosis.
16p11.2 microdeletion syndrome	Autosomal dominant transmission, small region of chromosome 16.	Developmental delay, intellectual disability.
Fragile X syndrome	X-linked dominant transmission, CGG trinucleotide expansion of *FMR1* promotor leading to a lack of transcription.	Intellectual deficiency and dysmorphic features of varying degree, more severe and frequent in males. 40% of obesity with some PWS-like phenotypes.
Bardet-Biedl syndrome	Autosomal recessive transmission, 22 genes known.	Retinal dystrophy, polydactyly, renal abnormalities, hypogonadism, hepatic fibrosis, learning disabilities.
Alström syndrome	Autosomal recessive transmission, *ALMS1* gene.	Retinal dystrophy, dysmorphic features, short stature, central deafness, endocrine abnormalities (central or peripheral hypogonadism and hypothyroidism, polycystic ovary syndrome) dilated cardiomyopathy, liver and renal fibrosis and no intellectual disability.
Pseudohypoparathyroidism	Autosomal dominant transmission, *GNAS* gene.	Dysmorphia, shot bones, short stature, subcutaneous ossifications, variable developmental delay, hypocalcemia, hypothyroidism, pubertal delay, epilepsy.
MYT1L	Autosomal dominant transmission, *MYT1L* gene.	Developmental and language delay, intellectual disability, behavioral disorders and dysmorphic features.
Monogenic obesity		
LEP	*LEP, LEPR, POMC, PCSK1, MC4R* genes: Autosomal recessive transmission: severe, early-onset obesity and eating disorders with related signs (see beside). Milder phenotype in heterozygous patients without related signs and more metabolic obesity complications.	Endocrine abnormalities (gonadotropic and thyrotropic insufficiency).
LEPR		Endocrine abnormalities (gonadotropic, somatotropic and thyrotropic insufficiency).
POMC		Endocrine abnormalities (corticotropic, gonadotropic, somatotropic and mild thyrotropic insufficiency), red hair.
PCSK1		Severe neonatal diarrhea, endocrine abnormalities (corticotropic, gonadotropic, somatotropic and thyrotropic insufficiency), hypoglycemia.
MC4R		Increased height growth in childhood.

ALMS1: Alström syndrome associated protein 1, FMR1: fragile x messenger ribonucleoprotein 1, LEP: leptin, LEPR: leptin receptor, POMC: proopiomelanocortin, PCSK1: prohormone subtilisin/kexin 1 convertase, MC4R: melanocortin receptor type 4, MYT1L: myelin transcription factor 1 like

related to dysfunction of the leptin-melanocortin pathway, a main contributor to the satiety signal and energy expenditure regulated in the hypothalamic arcuate and paramedian nuclei (Figure 1). This pathway involves the hormone leptin, synthesized by the leptin gene (*LEP*) in adipocytes, that activates its receptor (*LEPR*) inducing, in anorexigenic neurons, prohormone subtilisin/kexin 1 convertase (*PCSK1*) activity which converts proopiomelanocortin (*POMC*) to alpha-melanocyte stimulating hormone (α-MSH). The latter is the natural ligand of the melanocortin receptor type 4 (*MC4R*) which induces a satiety signal by its activation (7). Other genes are also implicated in the regulation of this pathway including *MRAP2* encoding for the melanocortin receptor accessory protein 2 (8,9), *ADCY3* encoding for adenylate cyclase 3 which transmits the MC4R activation signal intracellularly (10). Several genes involved in development of the hypothalamus or MC4R regulation have been reported to influence this signaling, including semaphorin 3A-G (*SEMA3A-G),* plexinA1–4 (*PLXNA1–4*), neuropilin1–2 (*NRP1–2*) (11), kinase suppressor of ras 2 (*KSR2*) (12), and the steroid-receptor co-activator 1 (*SRC-1*) (13). Genetic alterations in these genes lead to the phenotype described in both monogenic obesity and also in syndromic obesity.

Monogenic obesity (ORPHANET 98267) is due to a pathogenic variant on a gene involved in the leptin-melanocortin pathway (14-16). Most variants in the genes described above (*LEP, LEPR, POMC, PCSK1, MC4R* mainly) lead to severe and early obesity with eating disorders when the mutation is homozygous or compound heterozygous, with inconstant association to various endocrine disorders. Cohort studies have shown that heterozygous variant bearers in the same genes display milder phenotypes with a frequency of 10-12% of heterozygous variants in severe early-onset obesity cohorts, especially among children (17,18). Of these, *MC4R* variants are known to be frequent with an incidence of 0.3% in the general population from a cohort of screened newborns in the UK (19), and more than 5% in children with severe obesity (20). Besides these five genes, the other

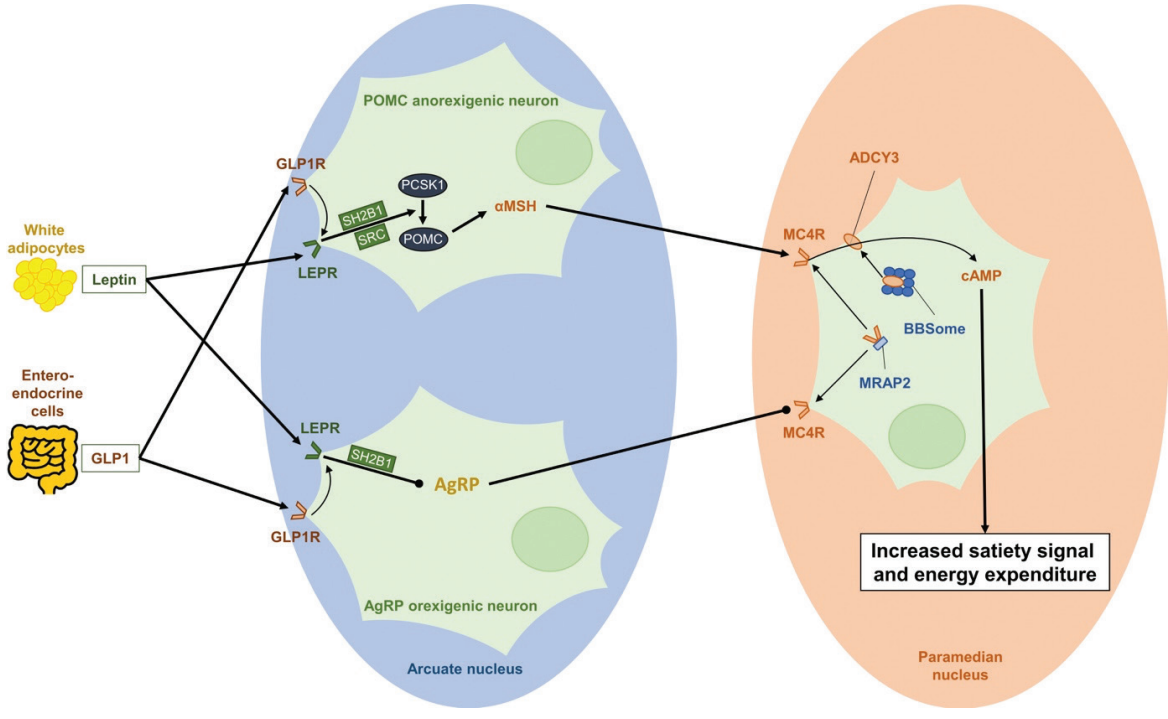

Figure 1. Schematic representation of the leptin-melanocortin pathway in the hypothalamic nuclei.

Peak-ended arrows represent stimulation, circle-ended arrows represent inhibition.

SH2B1 and SRC are activated secondary to leptin liaison on its receptor and potentialize its effect on POMC and PCSK1 in anorexigenic neurons, and SH2B1 potentialize LEPR inhibition effect on Agouti-related protein in orexigenic neuron. GLP1-R activation facilitates LEPR activation in both neuron populations. BBSome is an octameric complex composed of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9 proteins which mediates transmembrane proteins localization in the primary cilium, including ADCY3. MRAP2 addresses MC4R to the cellular membrane.

α-MSH: melanocyte stimulating hormone type α, BBSome: Bardet-Biedl syndrome associated protein complex, BDNF: brain-derived neurotrophic factor, GLP1: glucagon-like peptide 1, GLP1-R: GPL1 receptor, LEPR: leptin receptor, MC4R: melanocortin receptor type 4, MRAP2: melanocortin receptor accessory protein 2, NTRK2: neurotrophic receptor tyrosine kinase 2, PCSK1: prohormone convertase subtilisin-kexin 1, POMC: pro-opiomelanocortin, SH2B1: SRC homology 2 B adapter protein 1, SRC1: steroid receptor coactivator 1

genes cited above have been reported to cause obesity in human in cases series or in rodent models but the frequency of mutations in cohorts of patients with early and severe obesity remains currently unknown.

Syndromic obesities (ORPHANET 240371) are characterized by association with malformations, dysmorphic features and/or neurodevelopmental disorders (psychomotor development delay, intellectual disability, autism spectrum disorders). Around 80 syndromes have been identified to date, some without elucidated genetic cause (21). The leptin melanocortin pathway is involved in several of them.

The Prader-Willi syndrome (PWS) is the most extensively studied form of syndromic obesity with an incidence of about 1/15000 births. It is often diagnosed in the neonatal period in the presence of severe hypotonia with feeding difficulties and dysmorphic traits. The evolution in childhood is marked by the appearance of challenging hyperphagia with an intense impulsivity which lead to early morbid obesity. The combination of obesity with interaction and behavioral disorders makes these symptoms even more problematic for care management (22). This syndrome also has a major impact on the quality of life and is also associated with an important mortality at all ages, with an average life expectancy of 30 years (23,24). The impact of severe obesity is most frequently involved in the cause of death, showing the great importance of its control in this specific population. PCSK1 deficiency and alterations of the orexigenic Agouti-related protein hypothalamic neurons have been described in PWS (25) as the inactivation of *MAGEL2* with decreased density of MSH neurons in rodents (26).

Bardet-Biedl syndrome (BBS), with a prevalence of about 1/125000 births, is also associated with severe early-onset obesity and with retinal dystrophy, polydactyly, renal abnormalities, dysmorphism, and learning disabilities. It is caused by a genetic alteration in the function of the primary cilium, with more than 20 involved genes identified to date. Current evidence suggests that the impairment of the primary cilium induces a hypothalamic dysfunction in the leptin-melanocortin pathway and partly explains the obesity phenotype with severe hyperphagia (27,28,29).

TO HEREThe 16p11.2 microdeletion syndrome has been more recently described and is the most frequent syndromic obesity known to date, with an estimated incidence of 1/2000 births. It is characterized by an altered satiety responsiveness leading to early-onset obesity, with developmental delay, neurodevelopmental disorders and is even more prevalent in patients with autism spectrum disorder. This syndrome is sometimes associated with non-specific malformations or dysmorphism (30,31). The mechanism related to obesity may be the deletion of *SH2B1* contained in this chromosomal region, which is involved in regulation of the melanocortin pathway. The specific deletion of *SH2B1* leads to hyperphagia and early obesity (32).

Another recently described genetic disorder is due to myelin transcription factor 1-like (*MYT1L*) variants. MYT1L is involved in development of the hypothalamus and its heterozygous variants are associated with syndromes showing severe obesity with abnormal feeding behavior, intellectual disability, neurobehavioral disorders and dysmorphic features (33).

All together, these descriptions illustrate the blurred distinction between syndromic and non-syndromic monogenic obesity and the overlap of phenotypes, also with similarity to those of hypothalamic obesity.

Lifestyle Modification Therapies in Genetic Obesities

In common obesity, the cornerstone of clinical management is to provide appropriate nutritional, behavioral and exercise intervention with the help of trained health professionals. The intervention of dieticians, psychologists and teachers of adapted physical activity is recommended for every patients suffering from genetic or syndromic obesity or at-risk of severe obesity later in life, in cases with early diagnosis (6,14). The instruction of caregivers is essential to enable environmental control. These measures should be implemented as early as possible in childhood, as they limit the development and aggravation of obesity and eating behavior disorders and maintained throughout life with increased vigilance during the transition from childhood to adulthood.

Concerning diet, the overall measures focus on avoiding uncontrolled food intake. Restricting food access, establishing a reassuring eating routine, and ritualization of food intake help to limit the impulsivity that leads to hyperphagia and disruptive food-seeking behavior. If dietary autonomy is seldom possible in genetic obesity with eating disorders, this strategy still improves the quality of life of patients and facilitates their social integration by easing their relationship with food. In monogenic obesities, absence of satiety is extremely severe, life-long and responsible for stigma and suffering for the patients (34). On the other hand, the early restriction of food intake through environment control has been shown to benefit PWS patients by slowing the progression of obesity (35).

It is also crucial to begin adapted physical activity. In patients with PWS, a decrease in baseline physical activity is noticed

compared to patients with non-syndromic obesity (36). Two recent systematic reviews about exercise in PWS showed improvement of physical capacities (maximum oxygen uptake, muscle strength, walking distance) but no weight or fat loss without associated dietary intervention (37,38). Children with pathogenic *MC4R* variants who received nutritional, physical, psychological, and family intervention for one year were able to lose as much weight as matched obese children without *MC4R* variation, approximately 0.4 BMI-standard deviation score (SDS) (39). Unfortunately, they were unable to maintain weight loss, unlike their mutation-free counterparts. Multicomponent lifestyle interventions thus have a positive effect on the health outcome of these patients but need to be intensive and sustained to remain effective over time.

Holistic and comprehensive approaches are essential to improve patients' clinical conditions and need expertise in specialized centers. Psychological follow-up is beneficial, both to manage the frequent neuropsychiatric comorbidities and the major psychosocial repercussions of these obesities and the resulting stigma. Neuropsychological evaluation may identify cognitive dysfunction or other specific learning disability to guide and improve psychological and educational support. Screening and treatment of specific comorbidities associated with the genetic defect (Table 1) may also prevent further complications and should thus be given special attention. Genetic obesity is often associated with hormonal deficiency, with better outcomes if treated before becoming symptomatic. Sleep disorders, digestive disorders, and orthopedic deformations, as well as associated congenital malformations, require additional attention and often assistance from other specialized physicians. Complications of obesity may also arise and necessitate additional treatment.

Transition between pediatric and adult care may also be a critical period in such complex patients. In a retrospective cohort study, PWS patients which received transitional care had a lower BMI by 10 kg/m² and less antidepressant treatments (40). All these supports allow patients' quality of life improvement and help them to integrate with social structures and build their own lifestyle.

Pharmacological Treatments

Even though not widely used in practice and often of modest efficacy, some treatments are now approved to treat common obesity (41). In the future, these treatments could be proposed for use in patients with syndromic or monogenic obesity, but only after careful clinical evaluation. GLP1 analogs are, amongst these treatments, probably the most promising molecules being investigated. Human GLP-1, an incretin secreted by entero-endocrine cells in response to food intake, enhances insulin secretion by the pancreatic ß-cell and improves insulin sensitivity. It reduces appetite through a reduction of gastric emptying and central effects on satiety signaling. These mechanisms allow improvements of glucose metabolism and body weight. GLP-1 analogs were first developed for type 2 diabetes before being explored as a treatment for obesity. Reported side effects include frequent nausea, dizziness, pain or local reaction at the injection site, abdominal pain and low blood sugar. Other rare serious side effects have been reported, including anaphylactic reactions, pancreatitis, gallbladder and biliary diseases (42) and acute renal failure. Close attention is needed regarding the tolerance of these treatments given the 2-to-3-fold higher doses used in obesity compared to diabetes. Furthermore, less is known about their long-term safety, and these treatments may need to be prolonged to maintain a significant effect on weight.

Among GLP-1 analogs, liraglutide is supported by the most extensive scientific reports. A double-blind randomized controlled trial (RCT) was conducted for treatment of common obesity in 251 adolescents (12-17 years) with liraglutide combined with lifestyle intervention (43). The assessment after 56 weeks of treatment revealed a significant decrease in BMI-SDS of -0.22 compared to placebo. BMI reduction of more than 5% was completed more frequently with liraglutide than placebo (43.3% vs 18.7%). These results are consistent with data available for adults with a body weight change of about -5% (44). Afterwards, liraglutide (Saxenda®) was approved by the Food and Drug Association (FDA) in 2020 and by the European Medicines Agency at a dose of 3 mg per day subcutaneously for the treatment of obesity in adolescents aged 12–17 years. Given these significant but modest effects on weight loss and the mode of administration (e.g., daily subcutaneous injection), the appropriateness of using this treatment in adolescent obesity remains controversial (45,46,47).

Exenatide, another GLP-1 analog with weekly injections, showed comparable results in a double-blinded RCT against placebo in 44 obese adolescents. Six months of treatment combined with lifestyle intervention permitted a significant but mild reduction in BMI-SDS (-0.09), BMI (-0.8 kg/m^2) and weight (-3 kg) (48). Exenatide has not been approved for the treatment of obesity to date.

More recently, semaglutide showed promising results for common obesity in two RCT investigating adults on the one hand and adolescents on the other (49,50). Concerning adolescents, a double-blind RCT published in 2022 analyzed weekly subcutaneous injection of semaglutide for 68 weeks against placebo in 201 obese adolescents with at least

one weight-related comorbidity. Lifestyle intervention was proposed in both groups. The treatment resulted in a major mean change in BMI of -16.1 % (against + 0.6 % with placebo) at the end of the study period. Moreover, 73 % of patients had lost > 5 % of weight and 62 % had lost > 10 % of weight after 68 weeks on semaglutide, against 18 % and 8 % in the placebo group, respectively. There was a significant improvement of weight-related quality of life and dyslipidemia in the semaglutide group. Semaglutide has been shown to be significantly more effective in weight loss than other GLP-1 analogues. It could pave the way for new therapeutic strategies against obesity in the years to come.

Regarding syndromic obesities, daily liraglutide combined with diet and exercise intervention was administrated to 55 adolescents and children with PWS in a 52 weeks multicenter RCT. There was no significant change in BMI SDS from baseline with an estimated difference around -0.1 SDS. A significant reduction in hyperphagia score was observed at week 52 for liraglutide compared to no treatment in adolescents but not in children (51). No RCT assessing PWS and the other GLP-1 agonists are available to date. The effect of GPL-1 agonists thus appears uncertain in PWS, the only syndromic obesity studied in regard of these treatments so far.

Among patients with monogenic obesity, a trial compared daily 3 mg liraglutide efficacy in 14 carriers of *MC4R* pathogenic variants against 28 non-mutated patients. An equivalent weight loss between the two groups of about 6 % of body weight after 16 weeks of treatment was observed with similar improvement in body fat mass, waist circumference, and glucose tolerance (52). These data suggest a preserved efficacy of GLP-1 agonists for genetic obesity with decreased MC4R signaling. There is no available evidence for other types of monogenic obesity and GLP-1 agonists to date. Further studies are now needed given the substantial expected benefit for these patients, especially considering its promising results in hypothalamic obesity (53).

PWS has benefited from the most intense therapeutic research among syndromic obesity due to its severity and frequency (41,54). PWS leads to a hypothalamic dysfunction involved in satiety deficiency but also results in impaired oxytocin (OXT) signaling and growth hormone (GH) deficiency (55). GH supplementation is recommended for PWS patients from diagnosis and throughout the growth phase. It has been shown to normalize height growth in children, increase lean mass, decrease body fat and improve psychomotor development (56). Continuation of the treatment during adulthood may help patients maintaining a better BMI, body composition and exercise capacity (57,58). Contradictory outcomes emerged from RCT on intranasal OXT supplementation for PWS patients (59,60), but it recently showed promising results, specifically in the youngest ones (61). The ghrelin pathway is indeed impaired in PWS and provides a potential therapeutic target. Livoletide, a non-acylated ghrelin analog, provided promising results concerning food behavior in a RCT of 40 PWS patients undergoing 14 days of treatment (62). Ghrelin O-acyltransferase (GOAT) is the enzyme catalyzing the conversion of ghrelin into its inactive form. A GOAT inhibitor is currently being evaluated in PWS (63).

Targeting the leptin melanocortin pathway has also led to development of successful innovative therapeutics, taking a great step towards personalized medicine in genetic obesity. Montague et al. (64) described the first human cases of congenital leptin deficiency, an exceptionally rare condition secondary to homozygous pathogenic variants in the *LEP* gene. When treated with recombinant leptin (metreleptin), these patients exhibited great weight loss with normalization of metabolic and neuroendocrine alterations (65,66,67). This success raised great hope for the treatment of common obesity. Unfortunately, common obesity is associated with leptin-resistance and its treatment with leptin monotherapy did not lead to sufficient efficacy (68,69,70). In addition, recombinant leptin is not indicated in other types of monogenic obesity with signal interruption downstream in the leptin-melanocortin pathway as in LEPR or POMC deficiency (71,72,73).

Since then, intense research efforts have led to the development of several MC4R agonists. Unfortunately, the first ones were responsible for cardiovascular side-effects. Recently, a better tolerated, highly selective MC4R agonist was discovered, setmelanotide (Imcivree, also initially known as RM-493). Indeed, due to the pivotal role of MC4R in weight, appetite, and energy expenditure regulation, this G protein-coupled receptor is a key target to increase energy expenditure and reduce food intake, causing a negative energy balance when activated. Daily subcutaneous injection of setmelanotide for one year resulted in significant appetite control, consequently resulting in weight loss in a trial assessing POMC and LEPR deficient patients stemming from *POMC* and *PCSK1* or *LEPR* homozygous mutations (74). In the POMC deficient group (10 patients), the mean weight loss was 25.6 % with 80 % losing at least 10 % of initial weight and induced an important decrease in the hunger score of 27 %. Regarding the 11 LEPR deficient patients, the efficiency was also significant with 12.6 % of mean weight loss, 45 % of them losing more than 10 % of weight and a decrease in hunger score of 44 %. The safety profile was characterized by frequent cutaneous hyperpigmentation,

but no other serious adverse events were reported. Transient digestive manifestations and local cutaneous reaction after injection were also frequently reported. These effects seem sustainable in the two first POMC deficient patients treated for more than 7 years with setmelanotide (75). The FDA approved setmelanotide in 2020, followed by the EMA in 2021, in the treatment of obesity in adults and children aged six years and older with confirmed genetic diagnosis of *POMC*, *PCSK1* and *LEPR* deficiency.

The effects of setmelanotide in *MC4R* variant carriers are more controversial. Setmelanotide is a markedly more powerful MC4R agonist than the endogenous ligand (α-MSH). In cellular models, this increased affinity allowed the rescue of intracellular signaling despite defective *MC4R* mutants. The study of rodent models has shown an intermediate response of *MC4R* heterozygous mutant to setmelanotide. These mice had less weight gain under high fat diet than the control *MC4R* heterozygous mice injected with saline. The beneficial effect of setmelanotide was less pronounced than in wild-type mice, while *MC4R* homozygous knock-out mice showed no effect of this molecule. A phase 1 RCT evaluated continuous subcutaneous infusion of setmelanotide during 28 days in eight patients carrying *MC4R* heterozygous pathogenic variants compared to 49 obese patients free of mutation. A significant change in weight loss for setmelanotide compared to placebo were observed for both *MC4R* heterozygous and obese control groups, with a similar effect of -3.48 kg and -3.07 kg, respectively (76). Further studies are required to decipher whether setmelanotide can efficiently induce significant weight loss in subjects with MC4R deficiency.

Concurrently, setmelanotide was studied in BBS patients because of its proven impaired leptin-melanocortin signaling associated to hyperphagia (28) in a 52 weeks multicenter phase 3 RCT that included 32 BBS obese patients more than six years old. The primary endpoint was significant, showing 32.3% of patients with BBS losing more than 10% of bodyweight after 52 weeks of setmelanotide, associated with a reduction in hunger scores (77). As a result of this trial, the EMA approved setmelanotide in 2021 as treatment for BBS patients older than six years, followed by the FDA in 2022.

One phase 3 RCT is in progress to assess setmelanotide treatment in Smith Magenis syndrome and 16p11.2 deletion (NCT03013543). Concerning the younger pediatric populations, a phase 3 open-label clinical trial assessing setmelanotide in PCSK1, POMC and LEPR deficient, and BBS child between 2 and 6 years of age is ongoing (NCT04966741).

Several pharmacologic therapies are now emerging, implying different affected molecular pathways. Some trials targeting hypothalamic obesity may also advance the field for genetic and syndromic obesity, given their similarities. Non-pharmacologic interventions such as deep brain stimulation are also being evaluated.

Bariatric Surgery

Presently, the most common surgical techniques are sleeve gastrectomy (SG) and Roux-en-Y gastric bypass (RYGB). These interventions result in a sustainable weight reduction and remission of comorbidities in most of patients with common obesity (78,79). Bariatric surgery has regularly been undertaken for syndromic and monogenic obesity due to their severity, as the most effective treatment for patients with complicated severe obesity (80). These intervention outcomes remain, however, uncertain over the long term, as the evidence on its use in syndromic obesity are limited and heterogenous.

In syndromic obesity, SG has been studied in one monocentric pediatric study of 24 PWS patients with a mean BMI of 46.2 kg/m² compared to 72 children with common obesity matched for age, gender, and BMI. The PWS group started regaining weight from the fourth year of follow-up, with a BMI loss of 11 kg/m² after 5 years (7 patients' data) significantly lower than the 19 kg/m² loss observed in children without PWS. More than 80% of PWS patients experienced remission of their obesity comorbidities, mainly obstructive sleep apnea, and the safety was good with no major surgical complication (81). A recent systematic review assessed bariatric surgery outcomes for 202 adults and pediatrics patients with obesity associated with hyperphagia (114 patients with PWS, 43 with *MC4R* mutations, 38 with hypothalamic obesity and 7 with BBS). Statistical analysis included 96 PWS patients with a median age of 17 years, median weight of 97 kg and median BMI of 49 kg/m^2 with duration of follow up from 6 months to 14 years. These patients had a median weight loss of 24% within one year of surgery, but showed an important weight regain leading to a non-significant weight change five years after surgery. Surgical morbidity was also problematic with 10 deaths reported out of 104 patients with PWS, including five who died within one year after surgery. Moreover, 13 PWS patients underwent a second bariatric surgery. Long-term outcomes in other hyperphagic obesities were heterogenous but showed a trend towards less weight loss and increased surgical reinterventions (82). These finding suggests that PWS patients may be more likely to regain weight long-term and more prone to surgical complications. In other type of syndromic obesity, isolated

cases of patients undergoing bariatric surgery have been reported, with varying interventions, follow-up and results. It is worth pointing out that no study assessed psychiatric and nutritional complications, more frequent in these particularly vulnerable patients. Caution should be required as patients with syndromic obesity show severe behavioral disorders, developmental disorders and compulsive food behavior which could interfere with lifestyle changes mandatory after bariatric surgery and might lead to worse outcomes. Syndromic obesity therefore appears to be an inadequate indication for bariatric surgery.

Regarding monogenic non-syndromic obesity, the most evidence concerns long-term outcome of bariatric surgery in terms of retrospective genetic analyses. The most important of these published studies assessed the effect of heterozygous variants in the leptin-melanocortin pathway on the long-term outcomes after RYGB in a retrospective case-control study with 50 heterozygous variant carriers and seven genes were analyzed: *LEPR*, *PCSK1*, *POMC*, *SH2B1*, *SRC1*, *MC4R*, and *SIM1*, while 100 matched (sex, age, BMI, and time since surgery) controls free of mutation were also assessed. Mean age was 51 ± 11 years and BMI 46 ± 7 kg/m^2 at the time of surgery. The percentage weight loss 15 years after surgery was -16.6 ± 10.7% for variant carriers against -28.7 ± 12.9% in matched controls. The weight regain after maximum weight loss was also greater in heterozygous patients with 52.7 ± 29.7 kg compared to 29.8 ± 20.7 kg for non-carriers. These data show a lower long-term efficiency of RYGB in heterozygous variant carriers secondary to more weight regain, possibly due to eating behavior disorders (83). These results were consistent with a former retrospective genetic analysis in 131 obese adults who underwent SG surgery, showing that the 10 patients carrying heterozygous variants in the leptin-melanocortin pathway had less weight loss over both the short-term and long-term (84). However, another study of 1014 patients who underwent bariatric surgery which included 30 patients with a heterozygous variant in the leptin-melanocortin pathway (12 in *POMC*, 11 in *MC4R*, 5 in *PCSK1*) showed similar weight loss among mutation carriers and controls after a short follow-up of two years (85). A recent multicenter case-control study also compared outcomes of 35 patients with heterozygous likely-pathogenic *MC4R* variants compared with 70 mutation-free controls matched for age, sex, BMI and surgical procedure. Five years after bariatric surgery, a trend towards greater weight regain after nadir was observed for the *MC4R* variant carriers, which was greater after SG than after RYGB (86).

Concerning homozygous variant carriers, the largest case series available to date reported eight patients with *POMC*, *LEPR*, and *MC4R* mutations. Long-term outcomes were unsatisfactory and experienced by every patient with a median weight regain of 24.1 kg after an initial median weight loss of 21.5 kg (87).

Thus, melanocortin pathway heterozygous variants, in the absence of major eating or neurodevelopmental/psychiatric disorders, are not an absolute contraindication to bariatric

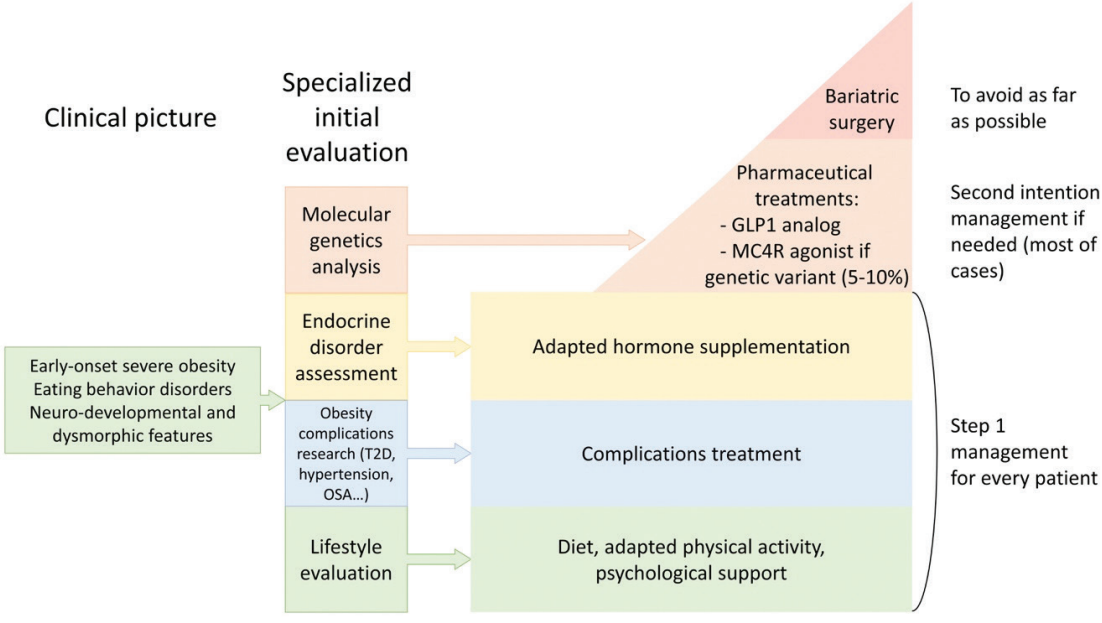

Figure 2. Emerging strategy for management of genetic and syndromic obesity.

OSA: obstructive sleep apnea, T2D: type 2 diabetes

surgery. However, with the emergence of new effective treatments, caution and multidisciplinary discussion to accurately judge the benefit-risk balance are warranted before opting for surgery (Figure 2).

Conclusion and Perspectives

Until recently, only multicomponent lifestyle interventions were proposed for patients with syndromic or monogenic obesity. While it remains the basis of their clinical management, the emergence of innovative, targeted treatments in recent years has changed this reality and paved the way for personalized medicine for these diseases in the future. Bariatric surgery now has pharmaceutical challengers for weight loss, which should probably be preferred in these situations to avoid irreversible anatomical changes and uncertain outcomes. However, further efforts are still needed to clarify the position of each treatment in each of these rare and complex clinical conditions. Early genetic diagnosis remains a major concern for these patients while it permits access to specialized multidisciplinary care, new molecules, and ongoing clinical trials to optimize their management. Genetic analyses should be offered to every child with rapid weight gain and additional clinical suggestive features. This population, confronting a lifelong struggle with obesity and its complications, certainly require special attention, which may prevent the development of obesity related complications, avoid the failure of conservative treatment approaches, and reduces the stigmatization of patients and their families. Intensive lifestyle intervention may help to improve these features, particularly when held on an outpatient basis as close to home as possible. Specific healthcare pathways are currently available in France to explore this hypothesis. This management will hopefully lead to a better prognosis for these patients in adulthood.

Research is thankfully still producing new solutions. Patients with monogenic forms of obesity may benefit in the future from CriSPr-mediated gene editing via induced pluripotent stem cell technologies (88) or direct defective gene repairing (89). Given the clinical severity of these patients, involvement and cooperation from both physicians and scientists is still required to improve their conditions and outcomes.

Ethics

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Nathan Faccioli, Christine Poitou, Karine Clément, Béatrice Dubern, Design: Nathan Faccioli, Christine Poitou, Karine Clément, Béatrice Dubern, Literature Search: Nathan Faccioli, Christine Poitou, Karine Clément, Béatrice Dubern, Writing: Nathan Faccioli, Christine Poitou, Karine Clément, Béatrice Dubern.

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References

1. Bouchard C. Genetics of Obesity: What We Have Learned Over Decades of Research. Obesity (Silver Spring) 2021;29:802-820.
2. Childhood obesity in European Region remains high: new WHO report presents latest country data [Internet]. 2020 [cited 2022 Dec 23]. Available from: https://www.who.int/europe/news/item/08-11-2022-childhood-obesity-in-european-region-remains-high--new-who-report-presents-latest-country-data
3. WHO. Obesity and overweight [Internet]. 2019 [cited 2022 Dec 21]. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
4. Silventoinen K, Jelenkovic A, Sund R, Hur YM, Yokoyama Y, Honda C, Hjelmborg Jv, Möller S, Ooki S, Aaltonen S, Ji F, Ning F, Pang Z, Rebato E, Busjahn A, Kandler C, Saudino KJ, Jang KL, Cozen W, Hwang AE, Mack TM, Gao W, Yu C, Li L, Corley RP, Huibregtse BM, Christensen K, Skytthe A, Kyvik KO, Derom CA, Vlietinck RF, Loos RJ, Heikkilä K, Wardle J, Llewellyn CH, Fisher A, McAdams TA, Eley TC, Gregory AM, He M, Ding X, Bjerregaard-Andersen M, Beck-Nielsen H, Sodemann M, Tarnoki AD, Tarnoki DL, Stazi MA, Fagnani C, D'Ippolito C, Knafo-Noam A, Mankuta D, Abramson L, Burt SA, Klump KL, Silberg JL, Eaves LJ, Maes HH, Krueger RF, McGue M, Pahlen S, Gatz M, Butler DA, Bartels M, van Beijsterveldt TC, Craig JM, Saffery R, Freitas DL, Maia JA, Dubois L, Boivin M, Brendgen M, Dionne G, Vitaro F, Martin NG, Medland SE, Montgomery GW, Chong Y, Swan GE, Krasnow R, Magnusson PK, Pedersen NL, Tynelius P, Lichtenstein P, Haworth CM, Plomin R, Bayasgalan G, Narandalai D, Harden KP, Tucker-Drob EM, Öncel SY, Aliev F, Spector T, Mangino M, Lachance G, Baker LA, Tuvblad C, Duncan GE, Buchwald D, Willemsen G, Rasmussen F, Goldberg JH, Sørensen TIa, Boomsma DI, Kaprio J. Genetic and environmental effects on body mass index from infancy to the onset of adulthood: an individual-based pooled analysis of 45 twin cohorts participating in the COllaborative project of Development of Anthropometrical measures in Twins (CODATwins) study. Am J Clin Nutr 2016;104:371-379. Epub 2016 Jul 13
5. Wardle J, Carnell S, Haworth CM, Plomin R. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. Am J Clin Nutr 2008;87:398-404.
6. Poitou C, Mosbah H, Clément K. Mechanisms in Endocrinology: Update on treatments for patients with genetic obesity. Eur J Endocrinol 2020;183:149-166.
7. Brouwers B, de Oliveira EM, Marti-Solano M, Monteiro FBF, Laurin SA, Keogh JM, Henning E, Bounds R, Daly CA, Houston S, Ayinampudi V, Wasiluk N, Clarke D, Plouffe B, Bouvier M, Babu MM, Farooqi IS, Mokrosiński J. Human MC4R variants affect endocytosis, trafficking and dimerization revealing multiple cellular mechanisms involved in weight regulation. Cell Rep 2021;34:108862.
8. Baron M, Maillet J, Huyvaert M, Dechaume A, Boutry R, Loiselle H, Durand E, Toussaint B, Vaillant E, Philippe J, Thomas J, Ghulam A, Franc S, Charpentier G, Borys JM, Lévy-Marchal C, Tauber M, Scharfmann R, Weill J, Aubert C, Kerr-Conte J, Pattou F, Roussel R, Balkau B, Marre M, Boissel M, Derhourhi M, Gaget S, Canouil M, Froguel P, Bonnefond A. Loss-of-function mutations in MRAP2 are pathogenic in hyperphagic obesity with hyperglycemia and hypertension. Nat Med 2019;25:1733-1738. Epub 2019 Nov 7
9. Bernard A, Ojeda Naharros I, Yue X, Mifsud F, Blake A, Bourgain-Guglielmetti F, Ciprin J, Zhang S, McDaid E, Kim K, Nachury MV,

Reiter JF, Vaisse C. MRAP2 regulates energy homeostasis by promoting primary cilia localization of MC4R. JCI Insight 2023;8:e155900.

10. Saeed S, Bonnefond A, Tamanini F, Mirza MU, Manzoor J, Janjua QM, Din SM, Gaitan J, Milochau A, Durand E, Vaillant E, Haseeb A, De Graeve F, Rabearivelo I, Sand O, Queniat G, Boutry R, Schott DA, Ayesha H, Ali M, Khan WI, Butt TA, Rinne T, Stumpel C, Abderrahmani A, Lang J, Arslan M, Froguel P. Loss-of-function mutations in ADCY3 cause monogenic severe obesity. Nat Genet 2018;50:175-179. Epub 2018 Jan 8
11. van der Klaauw AA, Croizier S, Mendes de Oliveira E, Stadler LKJ, Park S, Kong Y, Banton MC, Tandon P, Hendricks AE, Keogh JM, Riley SE, Papadia S, Henning E, Bounds R, Bochukova EG, Mistry V, O'Rahilly S, Simerly RB; INTERVAL; UK10K Consortium; Minchin JEN, Barroso I, Jones EY, Bouret SG, Farooqi IS. Human Semaphorin 3 Variants Link Melanocortin Circuit Development and Energy Balance. Cell 2019;176:729-742. Epub 2019 Jan 17
12. Pearce LR, Atanassova N, Banton MC, Bottomley B, van der Klaauw AA, Revelli JP, Hendricks A, Keogh JM, Henning E, Doree D, Jeter-Jones S, Garg S, Bochukova EG, Bounds R, Ashford S, Gayton E, Hindmarsh PC, Shield JP, Crowne E, Barford D, Wareham NJ; UK10K consortium; O'Rahilly S, Murphy MP, Powell DR, Barroso I, Farooqi IS. KSR2 mutations are associated with obesity, insulin resistance, and impaired cellular fuel oxidation. Cell 2013;155:765-777.
13. Cacciottolo TM, Henning E, Keogh JM, Bel Lassen P, Lawler K, Bounds R, Ahmed R, Perdikari A, Mendes de Oliveira E, Smith M, Godfrey EM, Johnson E, Hodson L, Clément K, van der Klaauw AA, Farooqi IS. Obesity Due to Steroid Receptor Coactivator-1 Deficiency Is Associated With Endocrine and Metabolic Abnormalities. J Clin Endocrinol Metab 2022;107:2532-2544.
14. Huvenne H, Dubern B, Clément K, Poitou C. Rare Genetic Forms of Obesity: Clinical Approach and Current Treatments in 2016. Obes Facts 2016;9:158-173. Epub 2016 Jun 1
15. van der Klaauw AA, Farooqi IS. The hunger genes: pathways to obesity. Cell 2015;161:119-132.
16. Loos RJF, Yeo GSH. The genetics of obesity: from discovery to biology. Nat Rev Genet 2022;23:120-133. Epub 2021 Sep 23
17. Courbage S, Poitou C, Le Beyec-Le Bihan J, Karsenty A, Lemale J, Pelloux V, Lacorte JM, Carel JC, Lecomte N, Storey C, De Filippo G, Coupaye M, Oppert JM, Tounian P, Clément K, Dubern B. Implication of Heterozygous Variants in Genes of the Leptin-Melanocortin Pathway in Severe Obesity. J Clin Endocrinol Metab 2021;106:2991-3006.
18. Nalbantoğlu Ö, Hazan F, Acar S, Gürsoy S, Özkan B. Screening of non-syndromic early-onset child and adolescent obese patients in terms of LEP, LEPR, MC4R and POMC gene variants by next-generation sequencing. J Pediatr Endocrinol Metab 2022;35:1041-1050.
19. Wade KH, Lam BYH, Melvin A, Pan W, Corbin LJ, Hughes DA, Rainbow K, Chen JH, Duckett K, Liu X, Mokrosiński J, Mörseburg A, Neaves S, Williamson A, Zhang C, Farooqi IS, Yeo GSH, Timpson NJ, O'Rahilly S. Loss-of-function mutations in the melanocortin 4 receptor in a UK birth cohort. Nat Med 2021;27:1088-1096. Epub 2021 May 27
20. Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. N Engl J Med 2003;348:1085-1095.
21. Kaur Y, de Souza RJ, Gibson WT, Meyre D. A systematic review of genetic syndromes with obesity. Obes Rev 2017;18:603-634. Epub 2017 Mar 27
22. Butler MG, Miller JL, Forster JL. Prader-Willi Syndrome - Clinical Genetics, Diagnosis and Treatment Approaches: An Update. Curr Pediatr Rev 2019;15:207-244.
23. Butler MG, Manzardo AM, Heinemann J, Loker C, Loker J. Causes of death in Prader-Willi syndrome: Prader-Willi Syndrome Association (USA) 40-year mortality survey. Genet Med 2017;19:635-642. Epub 2016 Nov 17
24. Pacoricona Alfaro DL, Lemoine P, Ehlinger V, Molinas C, Diene G, Valette M, Pinto G, Coupaye M, Poitou-Bernert C, Thuilleaux D, Arnaud C, Tauber M. Causes of death in Prader-Willi syndrome: lessons from 11 years' experience of a national reference center. Orphanet J Rare Dis 2019;14:238.
25. Burnett LC, LeDuc CA, Sulsona CR, Paull D, Rausch R, Eddiry S, Carli JF, Morabito MV, Skowronski AA, Hubner G, Zimmer M, Wang L, Day R, Levy B, Fennoy I, Dubern B, Poitou C, Clement K, Butler MG, Rosenbaum M, Salles JP, Tauber M, Driscoll DJ, Egli D, Leibel RL. Deficiency in prohormone convertase PC1 impairs prohormone processing in Prader-Willi syndrome. J Clin Invest 2017;127:293-305. Epub 2016 Dec 12
26. Maillard J, Park S, Croizier S, Vanacker C, Cook JH, Prevot V, Tauber M, Bouret SG. Loss of Magel2 impairs the development of hypothalamic Anorexigenic circuits. Hum Mol Genet 2016;25:3208-3215. Epub 2016 Jun 10
27. Tsang SH, Aycinena ARP, Sharma T. Ciliopathy: Bardet-Biedl Syndrome. Adv Exp Med Biol 2018;1085:171-174.
28. Vaisse C, Reiter JF, Berbari NF. Cilia and Obesity. Cold Spring Harb Perspect Biol 2017;9:a028217.
29. Chennen K, Scerbo MJ, Dollfus H, Poch O, Marion V. Syndrome de Bardet-Biedl: cils et obésité - de la génétique aux approches intégratives [Bardet-Biedl syndrome: cilia and obesity - from genes to integrative approaches]. Med Sci (Paris) 201430:1034-1039. Epub 2014 Nov 10
30. Maillard AM, Hippolyte L, Rodriguez-Herreros B, Chawner SJ, Dremmel D, Agüera Z, Fagundo AB, Pain A, Martin-Brevet S, Hilbert A, Kurz S, Etienne R, Draganski B, Jimenez-Murcia S, Männik K, Metspalu A, Reigo A, Isidor B, Le Caignec C, David A, Mignot C, Keren B; 16p11.2 European Consortium; van den Bree MB, Munsch S, Fernandez-Aranda F, Beckmann JS, Reymond A, Jacquemont S. 16p11.2 Locus modulates response to satiety before the onset of obesity. Int J Obes (Lond) 2016;40:870-876. Epub 2015 Dec 1
31. Chung WK, Roberts TP, Sherr EH, Snyder LG, Spiro JE. 16p11.2 deletion syndrome. Curr Opin Genet Dev 2021;68:49-56. Epub 2021 Mar 2
32. Doche ME, Bochukova EG, Su HW, Pearce LR, Keogh JM, Henning E, Cline JM, Saeed S, Dale A, Cheetham T, Barroso I, Argetsinger LS, O'Rahilly S, Rui L, Carter-Su C, Farooqi IS. Human SH2B1 mutations are associated with maladaptive behaviors and obesity. J Clin Invest 2012;122:4732-4736. Epub 2012 Nov 19
33. Coursimault J, Guerrot AM, Morrow MM, Schramm C, Zamora FM, Shanmugham A, Liu S, Zou F, Bilan F, Le Guyader G, Bruel AL, Denommé-Pichon AS, Faivre L, Tran Mau-Them F, Tessarech M, Colin E, El Chehadeh S, Gérard B, Schaefer E, Cogne B, Isidor B, Nizon M, Doummar D, Valence S, Héron D, Keren B, Mignot C, Coutton C, Devillard F, Alaix AS, Amiel J, Colleaux L, Munnich A, Poirier K, Rio M, Rondeau S, Barcia G, Callewaert B, Dheedene A, Kumps C, Vergult S, Menten B, Chung WK, Hernan R, Larson A, Nori K, Stewart S, Wheless J, Kresge C, Pletcher BA, Caumes R, Smol T, Sigaudy S, Coubes C, Helm M, Smith R, Morrison J, Wheeler PG, Kritzer A, Jouret G, Afenjar A, Deleuze JF, Olaso R, Boland A, Poitou C, Frebourg T, Houdayer C, Saugier-Veber P, Nicolas G, Lecoquierre F. MYT1L-associated neurodevelopmental disorder: description of 40 new cases and literature review of clinical and molecular aspects. Hum Genet 2022;141:65-80. Epub 2021 Nov 8
34. Wabitsch M, Fehnel S, Mallya UG, Sluga-O'Callaghan M, Richardson D, Price M, Kühnen P. Understanding the Patient Experience of Hunger and Improved Quality of Life with Setmelanotide Treatment in POMC and LEPR Deficiencies. Adv Ther 2022;39:1772-1783. Epub 2022 Feb 22
35. Kimonis VE, Tamura R, Gold JA, Patel N, Surampalli A, Manazir J, Miller JL, Roof E, Dykens E, Butler MG, Driscoll DJ. Early Diagnosis in Prader-

Willi Syndrome Reduces Obesity and Associated Co-Morbidities. Genes (Basel) 2019;10:898.

36. Bellicha A, Coupaye M, Hocquaux L, Speter F, Oppert JM, Poitou C. Increasing physical activity in adult women with Prader-Willi syndrome: A transferability study. J Appl Res Intellect Disabil 2020;33:258-267. Epub 2019 Oct 2
37. Morales JS, Valenzuela PL, Pareja-Galeano H, Rincón-Castanedo C, Rubin DA, Lucia A. Physical exercise and Prader-Willi syndrome: A systematic review. Clin Endocrinol (Oxf) 2019;90:649-661. Epub 2019 Mar 18
38. Bellicha A, Coupaye M, Mosbah H, Tauber M, Oppert JM, Poitou C. Physical Activity in Patients with Prader-Willi Syndrome-A Systematic Review of Observational and Interventional Studies. J Clin Med 2021;10:2528.
39. Reinehr T, Hebebrand J, Friedel S, Toschke AM, Brumm H, Biebermann H, Hinney A. Lifestyle intervention in obese children with variations in the melanocortin 4 receptor gene. Obesity (Silver Spring) 2009;17:382-389. Epub 2008 Nov 6
40. Paepegaey AC, Coupaye M, Jaziri A, Ménesguen F, Dubern B, Polak M, Oppert JM, Tauber M, Pinto G, Poitou C. Impact of transitional care on endocrine and anthropometric parameters in Prader-Willi syndrome. Endocr Connect 2018;7:663-672. Epub 2018 Apr 17
41. Kühnen P, Biebermann H, Wiegand S. Pharmacotherapy in Childhood Obesity. Horm Res Paediatr 2022;95:177-192.
42. He L, Wang J, Ping F, Yang N, Huang J, Li Y, Xu L, Li W, Zhang H. Association of Glucagon-Like Peptide-1 Receptor Agonist Use With Risk of Gallbladder and Biliary Diseases: A Systematic Review and Meta-analysis of Randomized Clinical Trials. JAMA Intern Med 2022;182:513-519.
43. Kelly AS, Auerbach P, Barrientos-Perez M, Gies I, Hale PM, Marcus C, Mastrandrea LD, Prabhu N, Arslanian S; NN8022-4180 Trial Investigators. A Randomized, Controlled Trial of Liraglutide for Adolescents with Obesity. N Engl J Med 2020;382:2117-2128. Epub 2020 Mar 31
44. Pi-Sunyer X, Astrup A, Fujioka K, Greenway F, Halpern A, Krempf M, Lau DC, le Roux CW, Violante Ortiz R, Jensen CB, Wilding JP; SCALE Obesity and Prediabetes NN8022-1839 Study Group. A Randomized, Controlled Trial of 3.0 mg of Liraglutide in Weight Management. N Engl J Med 2015;373:11-22.
45. Anker MS, Butler J, Anker SD. Liraglutide for Adolescents with Obesity. N Engl J Med 2020;383:1192.
46. Page LC, Freemark M. Role of GLP-1 Receptor Agonists in Pediatric Obesity: Benefits, Risks, and Approaches to Patient Selection. Curr Obes Rep 2020;9:391-401. Epub 2020 Oct 21
47. Kelishadi R. Liraglutide for management of adolescent obesity. Nat Rev Endocrinol 2020;16:405-406.
48. Weghuber D, Forslund A, Ahlström H, Alderborn A, Bergström K, Brunner S, Cadamuro J, Ciba I, Dahlbom M, Heu V, Hofmann J, Kristinsson H, Kullberg J, Ladinger A, Lagler FB, Lidström M, Manell H, Meirik M, Mörwald K, Roomp K, Schneider R, Vilén H, Widhalm K, Zsoldos F, Bergsten P. A 6-month randomized, double-blind, placebo-controlled trial of weekly exenatide in adolescents with obesity. Pediatr Obes 2020;15:e12624. Epub 2020 Feb 16
49. Wilding JPH, Batterham RL, Calanna S, Davies M, Van Gaal LF, Lingvay I, McGowan BM, Rosenstock J, Tran MTD, Wadden TA, Wharton S, Yokote K, Zeuthen N, Kushner RF; STEP 1 Study Group. Once-Weekly Semaglutide in Adults with Overweight or Obesity. N Engl J Med 2021;384:989-1002. Epub 2021 Feb 10
50. Weghuber D, Barrett T, Barrientos-Pérez M, Gies I, Hesse D, Jeppesen OK, Kelly AS, Mastrandrea LD, Sørrig R, Arslanian S; STEP TEENS Investigators. Once-Weekly Semaglutide in Adolescents with Obesity. N Engl J Med 2022;387:2245-2257. Epub 2022 Nov 2
51. Diene G, Angulo M, Hale PM, Jepsen CH, Hofman PL, Hokken-Koelega A, Ramesh C, Turan S, Tauber M. Liraglutide for Weight Management in Children and Adolescents With Prader-Willi Syndrome and Obesity. J Clin Endocrinol Metab 2022;108:4-12.
52. Iepsen EW, Zhang J, Thomsen HS, Hansen EL, Hollensted M, Madsbad S, Hansen T, Holst JJ, Holm JC, Torekov SS. Patients with Obesity Caused by Melanocortin-4 Receptor Mutations Can Be Treated with a Glucagon-like Peptide-1 Receptor Agonist. Cell Metab 2018;28:23-32. Epub 2018 May 31
53. Roth CL, Perez FA, Whitlock KB, Elfers C, Yanovski JA, Shoemaker AH, Abuzzahab MJ. A phase 3 randomized clinical trial using a once-weekly glucagon-like peptide-1 receptor agonist in adolescents and young adults with hypothalamic obesity. Diabetes Obes Metab 2021;23:363-373. Epub 2020 Oct 25
54. Tan Q, Orsso CE, Deehan EC, Triador L, Field CJ, Tun HM, Han JC, Müller TD, Haqq AM. Current and emerging therapies for managing hyperphagia and obesity in Prader-Willi syndrome: A narrative review. Obes Rev 2020;21:e12992. Epub 2019 Dec 30
55. Grugni G, Sartorio A, Crinò A. Growth hormone therapy for Prader-willi syndrome: challenges and solutions. Ther Clin Risk Manag 2016;12:873-881.
56. Wolfgram PM, Carrel AL, Allen DB. Long-term effects of recombinant human growth hormone therapy in children with Prader-Willi syndrome. Curr Opin Pediatr 2013;25:509-514.
57. Vogt KS, Emerick JE. Growth Hormone Therapy in Adults with Prader-Willi Syndrome. Diseases 2015;3:56-67.
58. Oto Y, Tanaka Y, Abe Y, Obata K, Tsuchiya T, Yoshino A, Murakami N, Nagai T. Exacerbation of BMI after cessation of growth hormone therapy in patients with Prader-Willi syndrome. Am J Med Genet A 2014;164A:671-675. Epub 2014 Jan 17
59. Kuppens RJ, Donze SH, Hokken-Koelega AC. Promising effects of oxytocin on social and food-related behaviour in young children with Prader-Willi syndrome: a randomized, double-blind, controlled crossover trial. Clin Endocrinol (Oxf) 2016;85:979-987. Epub 2016 Aug 26
60. Einfeld SL, Smith E, McGregor IS, Steinbeck K, Taffe J, Rice LJ, Horstead SK, Rogers N, Hodge MA, Guastella AJ. A double-blind randomized controlled trial of oxytocin nasal spray in Prader Willi syndrome. Am J Med Genet A 2014;164A:2232-2239.
61. Tauber M, Boulanouar K, Diene G, Çabal-Berthoumieu S, Ehlinger V, Fichaux-Bourin P, Molinas C, Faye S, Valette M, Pourrinet J, Cessans C, Viaux-Sauvelon S, Bascoul C, Guedeney A, Delhanty P, Geenen V, Martens H, Muscatelli F, Cohen D, Consoli A, Payoux P, Arnaud C, Salles JP. The Use of Oxytocin to Improve Feeding and Social Skills in Infants With Prader-Willi Syndrome. Pediatrics 2017;139:e20162976.
62. Allas S, Caixàs A, Poitou C, Coupaye M, Thuilleaux D, Lorenzini F, Diene G, Crinò A, Illouz F, Grugni G, Potvin D, Bocchini S, Delale T, Abribat T, Tauber M. AZP-531, an unacylated ghrelin analog, improves food-related behavior in patients with Prader-Willi syndrome: A randomized placebo-controlled trial. PLoS One 2018;13:e0190849.
63. Miller JL, Lacroix A, Bird LM, Shoemaker AH, Haqq A, Deal CL, Clark KA, Ames MH, Suico JG, de la Peña A, Fortier C. The Efficacy, Safety, and Pharmacology of a Ghrelin O-Acyltransferase Inhibitor for the Treatment of Prader-Willi Syndrome. J Clin Endocrinol Metab 2022;107:2373-2380.
64. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency

is associated with severe early-onset obesity in humans. Nature 1997;387:903-908.

65. Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, Hughes IA, McCamish MA, O'Rahilly S. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. N Engl J Med 1999;341:879-884.

66. Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest 2002;110:1093-1103.

67. Licinio J, Caglayan S, Ozata M, Yildiz BO, de Miranda PB, O'Kirwan F, Whitby R, Liang L, Cohen P, Bhasin S, Krauss RM, Veldhuis JD, Wagner AJ, DePaoli AM, McCann SM, Wong ML. Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. Proc Natl Acad Sci U S A 2004;101:4531-4536. Epub 2004 Mar 9

68. Hukshorn CJ, Saris WH, Westerterp-Plantenga MS, Farid AR, Smith FJ, Campfield LA. Weekly subcutaneous pegylated recombinant native human leptin (PEG-OB) administration in obese men. J Clin Endocrinol Metab 2000;85:4003-4009.

69. Zelissen PM, Stenlof K, Lean ME, Fogteloo J, Keulen ET, Wilding J, Finer N, Rössner S, Lawrence E, Fletcher C, McCamish M; Author Group. Effect of three treatment schedules of recombinant methionyl human leptin on body weight in obese adults: a randomized, placebo-controlled trial. Diabetes Obes Metab 2005;7:755-761.

70. Scarpace PJ, Zhang Y. Leptin resistance: a prediposing factor for diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 2009;296:493-500. Epub 2008 Dec 17

71. Clément K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougnères P, Lebouc Y, Froguel P, Guy-Grand B. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 1998;392:398-401.

72. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Grüters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet 1998;19:155-157.

73. Vaisse C, Clement K, Guy-Grand B, Froguel P. A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet 1998;20:113-114.

74. Clément K, van den Akker E, Argente J, Bahm A, Chung WK, Connors H, De Waele K, Farooqi IS, Gonneau-Lejeune J, Gordon G, Kohlsdorf K, Poitou C, Puder L, Swain J, Stewart M, Yuan G, Wabitsch M, Kühnen P; Setmelanotide POMC and LEPR Phase 3 Trial Investigators. Efficacy and safety of setmelanotide, an MC4R agonist, in individuals with severe obesity due to LEPR or POMC deficiency: single-arm, open-label, multicentre, phase 3 trials. Lancet Diabetes Endocrinol 2020;8:960-970. Epub 2020 Oct 30

75. Kühnen P, Clément K. Long-Term MC4R Agonist Treatment in POMC-Deficient Patients. N Engl J Med 2022;387:852-854.

76. Collet TH, Dubern B, Mokrosinski J, Connors H, Keogh JM, Mendes de Oliveira E, Henning E, Poitou-Bernert C, Oppert JM, Tounian P, Marchelli F, Alili R, Le Beyec J, Pépin D, Lacorte JM, Gottesdiener A, Bounds R, Sharma S, Folster C, Henderson B, O'Rahilly S, Stoner E, Gottesdiener K, Panaro BL, Cone RD, Clément K, Farooqi IS, Van der Ploeg LHT. Evaluation of a melanocortin-4 receptor (MC4R) agonist (Setmelanotide) in MC4R deficiency. Mol Metab 2017;6:1321-1329. Epub 2017 Jul 8

77. Haqq AM, Chung WK, Dollfus H, Haws RM, Martos-Moreno GÁ, Poitou C, Yanovski JA, Mittleman RS, Yuan G, Forsythe E, Clément K, Argente J. Efficacy and safety of setmelanotide, a melanocortin-4 receptor agonist, in patients with Bardet-Biedl syndrome and Alström syndrome: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial with an open-label period. Lancet Diabetes Endocrinol 2022;10:859-868. Epub 2022 Nov 7

78. Poirier P, Cornier MA, Mazzone T, Stiles S, Cummings S, Klein S, McCullough PA, Ren Fielding C, Franklin BA; American Heart Association Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Bariatric surgery and cardiovascular risk factors: a scientific statement from the American Heart Association. Circulation 2011;123:1683-1701. Epub 2011 Mar 14

79. Sjöström L. Review of the key results from the Swedish Obese Subjects (SOS) trial - a prospective controlled intervention study of bariatric surgery. J Intern Med 2013;273:219-234. Epub 2013 Feb 8

80. Vos N, Oussaada SM, Cooiman MI, Kleinendorst L, Ter Horst KW, Hazebroek EJ, Romijn JA, Serlie MJ, Mannens MMAM, van Haelst MM. Bariatric Surgery for Monogenic Non-syndromic and Syndromic Obesity Disorders. Curr Diab Rep 2020;20:44.

81. Alqahtani AR, Elahmedi MO, Al Qahtani AR, Lee J, Butler MG. Laparoscopic sleeve gastrectomy in children and adolescents with Prader-Willi syndrome: a matched-control study. Surg Obes Relat Dis 2016;12:100-110. Epub 2015 Jul 22

82. Gantz MG, Driscoll DJ, Miller JL, Duis JB, Butler MG, Gourash L, Forster J, Scheimann AO. Critical review of bariatric surgical outcomes in patients with Prader-Willi syndrome and other hyperphagic disorders. Obesity (Silver Spring) 2022;30:973-981. Epub 2022 Apr 13

83. Campos A, Cifuentes L, Hashem A, Busebee B, Hurtado-Andrade MD, Ricardo-Silgado ML, McRae A, De la Rosa A, Feris F, Bublitz JT, Hensrud D, Camilleri M, Kellogg TA, Eckel-Passow JE, Olson J, Acosta A. Effects of Heterozygous Variants in the Leptin-Melanocortin Pathway on Roux-en-Y Gastric Bypass Outcomes: a 15-Year Case-Control Study. Obes Surg 2022;32:2632-2640. Epub 2022 Jun 3

84. Li Y, Zhang H, Tu Y, Wang C, Di J, Yu H, Zhang P, Bao Y, Jia W, Yang J, Hu C. Monogenic Obesity Mutations Lead to Less Weight Loss After Bariatric Surgery: a 6-Year Follow-Up Study. Obes Surg 2019;29:1169-1173.

85. Cooiman MI, Kleinendorst L, Aarts EO, Janssen IMC, van Amstel HKP, Blakemore AI, Hazebroek EJ, Meijers-Heijboer HJ, van der Zwaag B, Berends FJ, van Haelst MM. Genetic Obesity and Bariatric Surgery Outcome in 1014 Patients with Morbid Obesity. Obes Surg 2020;30:470-477.

86. Cooiman MI, Alsters SIM, Duquesnoy M, Hazebroek EJ, Meijers-Heijboer HJ, Chahal H, Le Beyec-Le Bihan J, Clément K, Soula H, Blakemore AI, Poitou C, van Haelst MM. Long-Term Weight Outcome After Bariatric Surgery in Patients with Melanocortin-4 Receptor Gene Variants: a Case-Control Study of 105 Patients. Obes Surg 2022;32:837-844. Epub 2022 Jan 4

87. Poitou C, Puder L, Dubern B, Krabusch P, Genser L, Wiegand S, Verkindt H, Köhn A, von Schwartzenberg RJ, Flück C, Pattou F, Laville M, Kühnen P, Clément K. Long-term outcomes of bariatric surgery in patients with bi-allelic mutations in the POMC, LEPR, and MC4R genes. Surg Obes Relat Dis 2021;17:1449-1456. Epub 2021 May 8

88. Rajamani U, Gross AR, Hjelm BE, Sequeira A, Vawter MP, Tang J, Gangalapudi V, Wang Y, Andres AM, Gottlieb RA, Sareen D. Super-Obese Patient-Derived iPSC Hypothalamic Neurons Exhibit Obesogenic Signatures and Hormone Responses. Cell Stem Cell 2018;22:698-712. Epub 2018 Apr 19

89. Zhu L, Yang X, Li J, Jia X, Bai X, Zhao Y, Cheng W, Shu M, Zhu Y, Jin S. Leptin gene-targeted editing in ob/ob mouse adipose tissue based on the CRISPR/Cas9 system. J Genet Genomics 2021;48:134-146. Epub 2021 Mar 30

REVIEW

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Frequently Asked Questions and Evidence-Based Answers on Medical Nutritional Therapy in Children with Type 1 Diabetes for Health Care Professionals

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Abstract

Medical nutrition therapy is a cornerstone in type 1 diabetes management and is based on the principles of healthy eating. The recommendations presented are valid for all children and their families. A number of frequently asked questions will be addressed in this article. Although carbohydrates are the main nutrient that affects postprandial blood glucose in individuals with type 1 diabetes, intake of carbohydrates (type and amount), protein and fat content of the meal, and glycemic index affect the postprandial glycemic response. In recent years, the relative increase in studies about Ramadan fasting for individuals with type 1 diabetes has indicated that health professionals should be informed about this issue. The difficulties in nutritional management of preschool children should be solved with a professional approach. The increasing frequency of celiac disease in people with type 1 diabetes and an increasing interest in a gluten-free diet for non-celiac reasons (popular diet trends for weight loss or healthy eating) further complicate diabetes management. This review provides evidence-based approaches to frequently encountered problems on medical nutrition therapy in children and adolescents with type 1 diabetes.

Keywords: Type 1 diabetes, medical nutrition therapy, nutritional management

Introduction

Type 1 diabetes is caused by autoimmune damage to the insulin-producing β-cells of the pancreatic islets, leading to endogenous insulin deficiency (1). The aim of diabetes care and management is to support individuals with type 1 diabetes to live a long and healthy life. In addition to complex insulin regimens, sufficient knowledge and skills are required to prevent hypoglycemia and hyperglycemia and to maintain euglycemia (2).

The main goal in diabetes management is to maintain normoglycemia for as long as possible. National and international guidelines accepted that proper nutrition therapy is an important part of diabetes management (3,4). The purpose of nutrition therapy of type 1 diabetes is multiple: to improve general health and to encourage people with diabetes to gain healthy eating habits; to achieve/maintain a healthy body weight; to provide metabolic control; to prevent/delay diabetes-related acute/chronic complications; and to determine nutritional needs based on individual and cultural preferences, health literacy level and access to healthy food options. An individualized meal plan with prandial insulin dose adjustments is important for improving glycemic control (4). In the meal plan, it is important to provide practical information to children and adolescents with diabetes and their family, to match the insulin doses with the composition of the meal, and to apply the advanced carbohydrate counting method.

However, implementation of appropriate nutritional intervention and eventual adherence to the plan remains a challenge for several reasons. One of the most important problems is the availability of nutritional information

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from many sources for individuals with diabetes, their parents, and health professionals (5). Individuals with type 1 diabetes should be referred to a dietitian who has knowledge and experience in providing diabetes-specific, individualized nutritional recommendations in line with diabetes technology (1). Medical nutrition therapy given by an experienced dietitian has been found to provide a 1.0-1.9% (11-21 mmol/mol) reduction in hemoglobin A1c when integrated into general diabetes management (4). However, there is no consensus on the best nutrition therapy for people with diabetes, and an ongoing debate in the popular press may confuse people with diabetes, diabetes care providers, and healthcare professionals (5). The aim of this review is to answer frequently asked questions concerning medical nutrition therapy in children with type 1 diabetes, based on the latest evidence.

How much Carbohydrates should Children and Adolescents with Type 1 Diabetes Intake?

Energy requirement varies greatly in children and adolescents with type 1 diabetes, depending on age, growth rate, gender, physical activity and, unsurprisingly, is similar to their healthy peers. Energy intake should be sufficient to maintain optimal growth and maintain ideal body weight (3,6). About half of daily energy requirement should come from carbohydrates as they are the main energy source for the body. Foods containing carbohydrates are also important sources of dietary fiber and some vitamins and minerals (7,8). The American Diabetes Association (ADA) recommends nutrient-dense carbohydrate sources that are high in fiber and minimally processed, regardless of the amount of carbohydrates in the diet. Both children and adults with diabetes should be encouraged to minimize intake of added sugars and instead focus on carbohydrates from vegetables, legumes, fruits, dairy products (milk and yogurt), and grains (4).

While the ADA makes no recommendation about the ideal distribution of carbohydrates, proteins, and fats for all people with diabetes (4), the International Society of Pediatric and Adolescents Diabetes (ISPAD) suggests that, although the optimal macronutrient distribution varies depending on individualized targets of children and adolescents with type 1 diabetes, carbohydrate should supply approximately 45-55% of energy (3).

Since the energy needs of children and adolescents with type 1 diabetes will increase with increased growth or physical activity, the amount of carbohydrates that should be taken will need adjustment. For example, if the daily energy requirement of a 17-year-old boy who exercises regularly is estimated to be 2,800 kcal/d, the recommended carbohydrate intake is 315-385 gram/d (45-55% of energy), while the recommended amount of carbohydrate for a girl with a sedentary lifestyle at the same age is 180-220 g/d (45-55% of energy) for 1600 kcal/d energy needs. Therefore, when evaluating the daily carbohydrate intake of children and adolescents with diabetes, not only the amount but also the proportion of the energy they provide should be considered.

The effectiveness of carbohydrate restriction in nutrition therapy of type 1 diabetes has been an active and controversial issue in recent years. With increasing media attention about low-carb/carbohydrate-restricted diets, healthcare professionals and people with diabetes and their families can implement this approach as part of diabetes management (9). If carbohydrate intake causes postprandial glycemic excursions, "lower carbohydrate intake produces a lower glycemic response or less insulin is better" beliefs are accepted by some families and health care professionals.

The definition of a "low carbohydrate diet" varies. The ADA defines a carbohydrate intake of <130 g/d or $<26\%$ of energy from carbohydrates as a "low-carb diet". Feinman et al. (10) defined three categories of low-carbohydrate diets: (a) 25-50 grams carbohydrate/day or $<10\%$ of the 2000 kcal/d diet as a "very-low-carbohydrate ketogenic diet"; (b) <130 g/day or $<26\%$ total energy as "low-carbohydrate diet"; and (c) "moderate carbohydrate diets" in which carbohydrates are limited to 130-225 g per day or, 26-45% of total energy intake. De Bock et al. (11) published a case series showing that carbohydrate restriction in children with diabetes may cause growth and developmental retardation and increase the cardiovascular disease risk profile due to increased fat intake. Lennerz et al. (12) reported that the height z-score of 34 children who were on a low-carbohydrate diet for an average of 2.3 years, which was 0.41 at diagnosis, decreased to 0.2 after a low-carbohydrate diet. Franceschi et al. (13) reported growth and developmental retardation in two children who continued to be fed with a low-carbohydrate diet (12% and 17% of total energy) after the honeymoon period. If the energy deficit caused by the low carbohydrate intake is not compensated by increased fat and protein intake, the reduction in total energy intake together with the loss of body weight will result in a potentially negative effect on growth in children and adolescents (14). Although low-carbohydrate diets seem like a rational approach to lower postprandial glucose levels, carbohydrate restriction is not recommended in children and adolescents with diabetes because of the evidence that this negative effect on growth (3). In addition, low carbohydrate diets have the potential to increase the risk of hypoglycemia and/or reduce the effect of glucagon in the treatment of hypoglycemia (15).

Excessive dietary restriction can contribute to impaired glycemic control, causing binge eating disorders, and make accurate insulin dose adjustment difficult. In addition, low carbohydrate diets can cause social isolation during mealtimes with peers (9,11). Given the increasing popularity of low-carb diets for improving glycemic control among children and adolescents with type 1 diabetes, diabetes team members should inform them and their families on medical and psychosocial risks of these diets and investigate the reasons to follow a low-carbohydrate diet (3,9).

Can a Low Glycemic Index Food be Freely Consumed in the Diet?

The glycemic index (GI) is defined as the ratio of the glycemic response of the test food containing 50 g carbohydrate within 2 hours to the reference food (glucose or white bread) containing the same amount of carbohydrate. Foods are classified as low (0-55), medium (56-69), and high (≥70) GIs (16). There are various factors that affect the GI of a food. Physical characteristics of the food (grated, pureed, squeezed juice), degree of ripeness, degree of food processing, type of starch (amylose/amylopectin ratio, resistant starch), method of preparation (cooking method, time), presence of other nutrients (fat, protein) or non-nutrient components (phytate, lectin, tannin, phenolic compounds, α-amylase inhibitors, some organic acids, saponin) affect the GI of foods. Contrary to popular belief, the GI value decreases as fruits ripen. Unripe fruits have a higher starch and lower sugar content, while ripe or over-ripe fruit typically have a lower starch and higher sugar content (17). Another method used to predict the postprandial glucose response is the concept of Glycemic Load (GL). The GL takes into account both the GI and serving size of a carbohydrate-containing food. The GL of the meal can be classified low (0-10), medium (11-19), and high (≥20) depending on the portion of consumed foods. GI and GL should be evaluated together in achieving good metabolic control. A low GI value of a food does not mean that it is a healthy food, similarly, a high GI value of a food is not proof that it is unhealthy food. Although chocolate (GI = 40) is a low GI food, it should not be consumed freely in the diet. One carbohydrate exchange of watermelon (15 g), which is a high GI fruit (GI = 72), is 220 grams. If half, one, and two exchanges are consumed, it causes low, medium, and high GL in the diet, respectively. Therefore, carbohydrate-rich foods should be evaluated according to the type of carbohydrate and the amount consumed. In a study conducted on children with diabetes in which the effects of diet quality and macronutrient distribution on glycemic control was found that both general diet quality (natural sugar, fiber, low GI, low saturared fat) and macronutrient distribution were associated with optimal glycemic control (18). The use of GI provides additional benefit to glycemic control, when total carbohydrate is considered alone. In type 1 diabetes, the GI concept should not be used in isolation but should be used with a carbohydrate assay method. In a controlled study in children using low-GI foods instead of high-GI foods, a lower GI diet was found to improve glycemic control after 12 months compared to a higher GI diet. In clinical practice, GI is used as a tool to minimize postprandial glucose excursions and to enhance the quality of the diet (3). In practice the mismatch between the rapid glucose absorption due to consumption of a high-GI meal and the relatively delayed action of subcutaneous insulin may be difficult to overcome. Therefore, there are some recommendations for adjusting prandial insulin doses according to the GI of foods. Increasing the prandial insulin dose is not a solution to reduce the rapid rise in blood glucose after high GI food consumption and may increase the risk of hypoglycemia. In addition, it may lead to excessive insulinization in the postprandial period. In this case, bolus insulin 15-20 minutes before a meal or the use of the "Super bolus" option in those receiving insulin pump therapy is recommended to provide a better match between insulin action and glucose absorption following consumption of foods with a high GI (19).

How should the Nutrition Program be Arranged for Adolescents with Type 1 Diabetes Who want to Fast during Ramadan?

As per Islamic rules, all healthy adolescents and adults can fast during Ramadan, but those who think that fasting will adversely affect their health and have a chronic illness are exempt from fasting. However, despite being aware of the potential complications, many adolescents with type 1 diabetes fast during Ramadan to match their peers and avoid social stigma. This poses a challenge for pediatric diabetes teams to ensure blood glucose regulation of adolescents with type 1 diabetes who wish to fast during Ramadan (20,21).

There are limited studies focusing on Ramadan fasting of adolescents with type 1 diabetes (22,23). The lack of pre-fasting assessment and appropriate/adequate diabetes education in adolescents with type 1 diabetes are considered to be major barriers to "safe Ramadan fasting" (24,25). In the consensus report published in 2020, ISPAD stated that adolescents with type 1 diabetes can fast on the condition that they receive education related to fasting before the month of Ramadan with their families (20).

Pre-Ramadan fasting focused diabetes education should include: i) emergency management of hypoglycemia, hyperglycemia and diabetic ketoacidosis and adjustment of nutrition, physical activity, and insulin adjustment; ii) medical assessment, including assessment of hypoglycemia awareness; iii) optimization of glycemic control to reduce

potential risks associated with fasting and minimize glucose fluctuations; and iv) frequent blood glucose monitoring or continuous glucose monitoring systems and interpretation of results. However, adolescents with type 1 diabetes wishing to fast should be counseled on the permissibility and necessity of interventions that disrupt the integrity of the skin for blood glucose level monitoring and insulin injection during fasting (20,21).

To ensure the safety of young people who are planning to fast, it is essential to evaluate nutrition therapy and provide advanced nutrition education before Ramadan. In addition, an individualized medical nutrition therapy should be created according to the energy needs of the adolescent, the foods commonly consumed in Ramadan, the timing of sahur and iftar meals, insulin, and exercise regimen. To help prevent hypoglycemia and hyperglycemia, food consumption should be constantly monitored by adjusting the appropriate insulin dose during Ramadan. It should be recommended to consume liquids, such as water or unsweetened beverages, at regular intervals during non-fasting hours to prevent dehydration. Meals should include low-GI carbohydrate sources, vegetables, fruit, yogurt, and protein sources such as lean meat, chicken, and fish. The quality and quantity of foods consumed during Ramadan should be carefully monitored to prevent acute complications, excessive body weight gain, and adverse changes in lipid profile. Therefore, sweets and fried foods should be limited, sugary foods and beverages should be avoided, and mono-unsaturated and polyunsaturated fats should be used instead of saturated fats in cooking. Sahur (the pre-dawn meal) should be eaten as late as possible to reduce the duration of fasting during the day. Hypoglycemia/hyperglycemia can be prevented by accurate carbohydrate counting at sahur and iftar meals. Preprandial bolus insulin should be preferred to insulin administered during or after meals, and consistency in carbohydrate intake should be ensured for those who inject insulin twice daily. Consistent snacking throughout the night between iftar and sahur should be avoided (20,21).

When should the Fat and Protein Content of the Meal be Considered? How should the Insulin Dose be Adjusted in High-fat and High-protein Meals?

Postprandial hyperglycemia, plays a significant role in the emergence of late macrovascular complications in individuals with diabetes. Recent studies with type 1 diabetics receiving intensive insulin therapy show that high-protein or high-fat foods affect blood glucose levels and the peak time of blood glucose in the long term, especially in the postprandial 6-hour period (26,27). While protein affects the blood glucose at a minimum level in the presence of sufficient insulin, it increases the glucose level rapidly through the gluconeogenesis pathway in insulin deficiency (28). In a study investigating postprandial glycemia in children using intensive insulin therapy and consuming low-protein (5 g) and high-protein (40 g) meals with a fixed carbohydrate content, it was reported that after a high-protein meal high glycemic excursions occurred for the first postprandial 3-5 hours and increased insulin requirement. In the same study, it was found that a high protein meal reduced the risk of hypoglycemia (29). A high-fat meal, on the other hand, decreases the postprandial glucose response in the early period (2-3 hours), delays stomach emptying and results in a later timing of peak postprandial glucose (postprandial > 3 hours) (28,30,31). A high-fat meal is usually defined as a meal containing more than 40 grams of fat, while a high-fat and high-protein meal is often defined as a meal containing more than 40 grams of fat and 25 grams of protein (19,32). In a systematic review, it was stated that when 35 g fat was added to the meal, there was an increase in blood glucose of 2.3 mmol/L, while the insulin requirement doubled when 50 g fat was added (19). Thus, there is increasing evidence that the effect of the fat and protein content of a meal should be taken into account in determining the bolus insulin dose and mode of administration (29,33,34,35,36). Pańkowska et al. (33) developed an algorithm for protein and fat counting in 2003 (37) and this algorithm has been tested in many studies. However, in some clinical studies conducted using the Pańkowska algorithm, hypoglycemia (~70 mg/dL) was observed, especially in the postprandial 6-8 hour period and therefore this method may be insufficient to manage meals containing high-fat and high-protein (32,38). To provide postprandial normoglycemia after consumption of high-fat and high-protein meals, the preprandial insulin dose should be adjusted according to the amount of fat and protein as well as carbohydrates. However, there is still no simple and easy-to-use insulin dose calculation algorithm for fats and proteins (4). ISPAD guidelines recommend a 15-20% increase in the prandial bolus dose adjusted for the carbohydrate amount of the meal for a controlled starting point (3). However, the glycemic response to high-fat and high-protein meals shows individual variation. Therefore, in clinical practice, individualized modifications should be made by evaluating each diabetic individual's blood glucose diaries and food consumption records together (39).

What are the Possible Solutions for the Nutritional Problems Encountered in Preschool Children with Type 1 Diabetes?

Lifestyle choices and food preferences in the pre-school period provide an opportunity for children to acquire healthy habits that will be maintained throughout their life.

Variable or inconsistent appetites, unpredictable food preferences, and food refusal in preschoolers with type 1 diabetes often make mealtimes difficult for parents/caregivers. In addition, the lack of ability of daytime caregivers (nursery staff, grandparents, etc.) to determine the amount of carbohydrates intake and fear of hypoglycemia can result in force-feeding, grazing continually through the day, and postprandial insulin administration, causing prolonged periods of hyperglycemia (40). Poor glycemic control is notable in children who have irregular eating behavior and frequent meals (41).

Family-centered meal times are important in establishing healthy eating behaviors, preventing frequent feeding of the child throughout the day, and supporting the consumption of new foods. In addition, it reduces the risk of cardiovascular disease by improving glycemic control (40,42).

It cannot be overemphasized that nutritional behavior and food choices acquired in the pre-school period will be carried over to adulthood. Therefore, family members should be encouraged to increase the consumption of vegetables and fruits and reduce the intake of saturated fatty acids in the child with diabetes in the early stages, and necessary initiatives should be taken in this regard (43).

Preschool children should be offered regular meals that include healthy food choices, constant snacking should be prevented and they should start the meal hungry. The prandial insulin administration time is also important. Preprandial bolus insulin should be preferred to insulin administered during or after meals and should be routinely recommended for all preschool children with diabetes. However, in children consuming inconsistent amounts of food or when new foods are introduced to the child, the bolus insulin dose may be split between preprandial and meal times (19,40). During the pre-school period, age-specific, family-centered nutrition education should be given to parents/caregivers by a pediatric diabetes dietitian to achieve metabolic goals. In addition, appropriate glucose monitoring should be provided with flexible insulin therapy accompanied by carbohydrate counting.

Should Children and Adolescents with Type 1 Diabetes without a Diagnosis of Celiac Disease Follow a Gluten-free Diet?

Due to the increased incidence of celiac disease in children diagnosed with type 1 diabetes, some parents may prefer a prophylactic gluten-free diet to reduce the risk of celiac disease. However, there is currently no scientific evidence that a gluten-free diet can prevent type 1 diabetes or reduce the risk of developing celiac disease in children with type 1 diabetes. On the contrary, this approach can cause some difficulties the diabetes management of children with type 1 diabetes (44). Although the macronutrient content of gluten-free foods is different compared to their gluten-containing counterparts, they often have low fiber and protein content, and high carbohydrate, fat, and GI values (45). In children diagnosed with type 1 diabetes and celiac disease, glucose peaks may be higher in a shorter time (46). For this reason, insulin dose and time should be determined by the macronutrient content of gluten-free foods (19,47). In addition, it is important to give detailed nutritional counseling to individuals with diabetes who have been diagnosed with celiac disease. Some gluten-free products may contain very low carbohydrates, so administering standard insulin doses can lead to severe hypoglycemia. Label information of packaged products must be accurate and must be read and evaluated correctly (47,48).

There is no evidence to support the benefits of a gluten-free diet in individuals without celiac disease or gluten intolerance. Furthermore, gluten consumption is necessary to avoid false negative results on celiac disease testing and thus enables an appropriate diagnosis in those children with diabetes who may develop celiac disease in the future. Therefore, the gluten-free diet should not be a medical recommendation for children and adolescents with type 1 diabetes (44,49,50).

Conclusion

Individuals with type 1 diabetes merit better, higher-quality research evidence about what their optimal nutrition therapy should be. Current evidence suggests that the meal plan should be individualized to meet the needs of each person with diabetes, taking into account their lifestyle, habits, socio-economic factors, cultural backgrounds, and motivations. The guidance about lifestyle change and support needed requires teamwork involving an endocrinologist, dietitian, nurse, and psychologist. Diabetes team members should use a common language for treatment and management strategies should adapt to the metabolic goals, wishes, and use of diabetes technologies of the diabetic individual.

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Authorship Contributions

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References

1. Holt RIG, DeVries JH, Hess-Fischl A, Hirsch IB, Kirkman MS, Klupa T, Ludwig B, Nørgaard K, Pettus J, Renard E, Skyler JS, Snoek FJ, Weinstock RS, Peters AL. Correction to: The management of type 1 diabetes in adults. A consensus report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetologia 2022;65:255.
2. Mobasseri M, Shirmohammadi M, Amiri T, Vahed N, Hosseini Fard H, Ghojazadeh M. Prevalence and incidence of type 1 diabetes in the world: a systematic review and meta-analysis. Health Promot Perspect 2020;10:98-115.
3. Smart CE, Annan F, Higgins LA, Jelleryd E, Lopez M, Acerini CL. ISPAD Clinical Practice Consensus Guidelines 2018: Nutritional management in children and adolescents with diabetes. Pediatr Diabetes 2018;19(Suppl 27):136-154.
4. American Diabetes Association Professional Practice Committee; 5. Facilitating Behavior Change and Well-being to Improve Health Outcomes: Standards of Medical Care in Diabetes-2022. Diabetes Care 2022; 45(Suppl 1):60-82.
5. Gray A, Threlkeld RJ. Nutritional Recommendations for Individuals with Diabetes. 2019 Oct 13. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, Dungan K, Hershman JM, Hofland J, Kalra S, Kaltsas G, Koch C, Kopp P, Korbonits M, Kovacs CS, Kuohung W, Laferrère B, Levy M, McGee EA, McLachlan R, Morley JE, New M, Purnell J, Sahay R, Singer F, Sperling MA, Stratakis CA, Trence DL, Wilson DP, (eds). Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000.
6. Rodríguez G, Moreno LA, Sarría A, Fleta J, Bueno M. Resting energy expenditure in children and adolescents: agreement between calorimetry and prediction equations. Clin Nutr 2002;21:255-260.
7. American Academy of Pediatrics Committee on Nutrition. Micronutrients and Macronutrients. In: Kleinman RE, Greer FR, eds. Pediatric Nutrition. 8th ed. Itasca, IL: American Academy of Pediatrics, 2019;481-508.
8. Trumbo P, Schlicker S, Yates AA, Poos M; Food and Nutrition Board of the Institute of Medicine, The National Academies. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. J Am Diet Assoc 2002;102:1621-1630.
9. Gallagher KAS, DeSalvo D, Gregory J, Hilliard ME. Medical and Psychological Considerations for Carbohydrate-Restricted Diets in Youth With Type 1 Diabetes. Curr Diab Rep 2019;27;19:27.
10. Feinman RD, Pogozelski WK, Astrup A, Bernstein RK, Fine EJ, Westman EC, Accurso A, Frassetto L, Gower BA, McFarlane SI, Nielsen JV, Krarup T, Saslow L, Roth KS, Vernon MC, Volek JS, Wilshire GB, Dahlqvist A, Sundberg R, Childers A, Morrison K, Manninen AH, Dashti HM, Wood RJ, Wortman J, Worm N. Dietary carbohydrate restriction as the first approach in diabetes management: critical review and evidence base. Nutrition 2015;31:1-13. Epub 2014 Jul 16
11. de Bock M, Lobley K, Anderson D, Davis E, Donaghue K, Pappas M, Siafarikas A, Cho YH, Jones T, Smart C. Endocrine and metabolic consequences due to restrictive carbohydrate diets in children with type 1 diabetes: An illustrative case series. Pediatr Diabetes 2018;19:129-137. Epub 2017 Apr 11
12. Lennerz BS, Barton A, Bernstein RK, Dikeman RD, Diulus C, Hallberg S, Rhodes ET, Ebbeling CB, Westman EC, Yancy WS Jr, Ludwig DS. Management of Type 1 Diabetes With a Very Low-Carbohydrate Diet. Pediatrics 2018;141:e20173349. Epub 2018 May 7
13. Franceschi R, Rizzardi C, Cauvin V, Berchielli F, Liguori A, Soffiati M. Carbohydrate Restriction and Growth Failure in Two Children with Type 1 Diabetes: A Case Report. Dubai Diabetes Endocrinol J 2020;26:134-138.
14. Seckold R, Fisher E, de Bock M, King BR, Smart CE. The ups and downs of low-carbohydrate diets in the management of Type 1 diabetes: a review of clinical outcomes. Diabet Med 2019;36:326-334.
15. Ranjan A, Schmidt S, Damm-Frydenberg C, Steineck I, Clausen TR, Holst JJ, Madsbad S, Nørgaard K. Low-Carbohydrate Diet Impairs the Effect of Glucagon in the Treatment of Insulin-Induced Mild Hypoglycemia: A Randomized Crossover Study. Diabetes Care 2017;40:132-135. Epub 2016 Oct 21
16. Atkinson FS, Foster-Powell K, Brand-Miller JC. International tables of glycemic index and glycemic load values: 2008. Diabetes Care 2008;31:2281-2283. Epub 2008 Oct 3
17. American Academy of Pediatrics Committee on Nutrition. Nutrition in Acute and Chronic Illness. In: Kleinman RE, Greer FR, eds. Pediatric Nutrition. 8th ed. Itasca, IL: American Academy of Pediatrics, 2019;851-886.
18. Nansel TR, Lipsky LM, Liu A. Greater diet quality is associated with more optimal glycemic control in a longitudinal study of youth with type 1 diabetes. Am J Clin Nutr 2016;104:81-87. Epub 2016 May 18
19. Bell KJ, Smart CE, Steil GM, Brand-Miller JC, King B, Wolpert HA. Impact of fat, protein, and glycemic index on postprandial glucose control in type 1 diabetes: implications for intensive diabetes management in the continuous glucose monitoring era. Diabetes Care 2015;38:1008-1015.
20. Deeb A, Elbarbary N, Smart CE, Beshyah SA, Habeb A, Kalra S, Al Alwan I, Babiker A, Al Amoudi R, Pulungan AB, Humayun K, Issa U, Jalaludin MY, Sanhay R, Akanov Z, Krogvold L, de Beaufort C. ISPAD Clinical Practice Consensus Guidelines: Fasting during Ramadan by young people with diabetes. Pediatr Diabetes 2020;21:5-17. Epub 2019 Oct 28
21. Hassanein M, Afandi B, Yakoob Ahmedani M, Mohammad Alamoudi R, Alawadi F, Bajaj HS, Basit A, Bennakhi A, El Sayed AA, Hamdy O, Hanif W, Jabbar A, Kleinebreil L, Lessan N, Shaltout I, Mohamad Wan Bebakar W, Abdelgadir E, Abdo S, Al Ozairi E, Al Saleh Y, Alarouj M, Ali T, Ali Almadani A, Helmy Assaad-Khalil S, Bashier AMK, Arifi Beshyah S, Buyukbese MA, Ahmad Chowdhury T, Norou Diop S, Samir Elbarbary N, Elhadd TA, Eliana F, Ezzat Faris M, Hafidh K, Hussein Z, Iraqi H, Kaplan W, Khan TS, Khunti K, Maher S, Malek R, Malik RA, Mohamed M, Sayed Kamel Mohamed M, Ahmed Mohamed N, Pathan S, Rashid F, Sahay RK, Taha Salih B, Sandid MA, Shaikh S, Slim I, Tayeb K, Mohd Yusof BN, Binte Zainudin S. Diabetes and Ramadan: Practical guidelines 2021. Diabetes Res Clin Pract 2022;185:109185. Epub 2022 Jan 8
22. Kaplan W, Afandi B. Blood glucose fluctuation during Ramadan fasting in adolescents with type 1 diabetes: findings of continuous glucose monitoring. Diabetes Care 2015;38:162-163. Epub 2015 Aug 20
23. Hassanein M, Alamoudi RM, Kallash MA, Aljohani NJ, Alfadhli EM, Tony LE, Khogeer GS, Alfadhly AF, Khater AE, Ahmedani MY, Buyukbese

MA, Shaltout I, Belkhadir J, Hafidh K, Chowdhury TA, Hussein Z, Elbarbary NS. Ramadan fasting in people with type 1 diabetes during COVID-19 pandemic: The DaR Global survey. Diabetes Res Clin Pract 2021;172:108626. Epub 2020 Dec 13

24. Elbarbary N, Deeb A, Habeb A, Beshyah SA. Management of diabetes during Ramadan fasting in children and adolescents: A survey of physicians' perceptions and practices in the Arab Society for Paediatric Endocrinology and Diabetes (ASPED) countries. Diabetes Res Clin Pract 2019;150:274-281.
25. Sahay RK, Nagesh VS. Type 1 diabetes and fasting during Ramzan. J Soc Health Diabetes 2016;4:11-16.
26. Carstensen S, Huber J, Schönauer M, Thomas A. Effects of evening meals with complex nutrient content on the nocturnal blood glucose levels of type 1 diabetes patients. Diabetologia 2010;52(Suppl 1):403-404.
27. Neu A, Behret F, Braun R, Herrlich S, Liebrich F, Loesch-Binder M, Schneider A, Schweizer R. Higher glucose concentrations following protein- and fat-rich meals - the Tuebingen Grill Study: a pilot study in adolescents with type 1 diabetes. Pediatr Diabetes. Pediatr Diabetes 2015;16:587-591. Epub 2014 Oct 20
28. Paterson M, Bell KJ, O'Connell SM, Smart CE, Shafat A, King B. The Role of Dietary Protein and Fat in Glycaemic Control in Type 1 Diabetes: Implications for Intensive Diabetes Management. Curr Diab Rep 2015;15:61.
29. Smart CE, Evans M, O'Connell SM, McElduff P, Lopez PE, Jones TW, Davis EA, King BR. Both dietary protein and fat increase postprandial glucose excursions in children with type 1 diabetes, and the effect is additive. Diabetes Care 2013;36:3897-3902. Epub 2013 Oct 29
30. Freckmann G, Hagenlocher S, Baumstark A, Jendrike N, Gillen RC, Rössner K, Haug C. Continuous glucose profiles in healthy subjects under everyday life conditions and after different meals. J Diabetes Sci Technol 2007;1:695-703.
31. Clegg ME, Pratt M, Markey O, Shafat A, Henry CYK. Addition of different fats to a carbohydrate food: Impact on gastric emptying, glycaemic and satiety responses and comparison with in vitro digestion. Food Res Int 2012;48:91-97.
32. Bell KJ, Toschi E, Steil GM, Wolpert HA. Optimized Mealtime Insulin Dosing for Fat and Protein in Type 1 Diabetes: Application of a Model-Based Approach to Derive Insulin Doses for Open-Loop Diabetes Management. Diabetes Care 2016;39:1631-1634. Epub 2016 Jul 7
33. Pańkowska E, Błazik M, Groele L. Does the fat-protein meal increase postprandial glucose level in type 1 diabetes patients on insulin pump: the conclusion of a randomized study. Diabetes Technol Ther 2012;14:16-22. Epub 2011 Oct 20
34. Wolpert HA, Atakov-Castillo A, Smith SA, Steil GM. Dietary fat acutely increases glucose concentrations and insulin requirements in patients with type 1 diabetes: implications for carbohydrate-based bolus dose calculation and intensive diabetes management. Diabetes Care 2013;36:810-816. Epub 2012 Nov 27
35. Kaya N, Kurtoğlu S, Gökmen Özel H. Does meal-time insulin dosing based on fat-protein counting give positive results in postprandial glycaemic profile after a high protein-fat meal in adolescents with type 1 diabetes: a randomised controlled trial. J Hum Nutr Diet 2020;33:396-403. Epub 2019 Oct 24
36. Abdou M, Hafez MH, Anwar GM, Fahmy WA, Abd Alfattah NM, Salem RI, Arafa N. Effect of high protein and fat diet on postprandial blood glucose levels in children and adolescents with type 1 diabetes in Cairo, Egypt. Diabetes Metab Syndr 2021;15:7-12. Epub 2020 Nov 26
37. Pańkowska E, Błazik M. Bolus Calculator with Nutrition Database Software, a New Concept of Prandial Insulin Programming for Pump Users. J Diabetes Sci Technol 2010;4:571-576.
38. Kordonouri O, Hartmann R, Remus K, Bläsig S, Sadeghian E, Danne T. Benefit of supplementary fat plus protein counting as compared with conventional carbohydrate counting for insulin bolus calculation in children with pump therapy. Pediatr Diabetes. Pediatr Diabetes 2012;13:540-544. Epub 2012 Jul 6
39. García-López JM, González-Rodriguez M, Pazos-Couselo M, Gude F, Prieto-Tenreiro A, Casanueva F. Should the amounts of fat and protein be taken into consideration to calculate the lunch prandial insulin bolus? Results from a randomized crossover trial. Diabetes Technol Ther 2013;15:166-171. Epub 2012 Dec 21
40. Sundberg F, Barnard K, Cato A, de Beaufort C, DiMeglio LA, Dooley G, Hershey T, Hitchcock J, Jain V, Weissberg-Benchell J, Rami-Merhar B, Smart CE, Hanas R. ISPAD Guidelines. Managing diabetes in preschool children. Pediatr Diabetes. Pediatr Diabetes 2017;18:499-517. Epub 2017 Jul 20
41. Monaghan M, Herbert LJ, Wang J, Holmes C, Cogen FR, Streisand R. Mealtime behavior and diabetes-specific parent functioning in young children with type 1 diabetes. Health Psychol 2015;34:794-801. Epub 2015 Feb 9
42. Edelson LR, Mokdad C, Martin N. Prompts to eat novel and familiar fruits and vegetables in families with 1-3 year-old children: Relationships with food acceptance and intake. Appetite 2016;99:138-148. Epub 2016 Jan 11
43. Kaikkonen JE, Mikkilä V, Magnussen CG, Juonala M, Viikari JS, Raitakari OT. Does childhood nutrition influence adult cardiovascular disease risk?--insights from the Young Finns Study. Ann Med 2013;45:120-128. Epub 2012 Apr 12
44. Hatun Ş, Dalgıç B, Gökşen D, Aydoğdu S, Savaş Erdeve Ş, Kuloğu Z, Doğan Y, Aycan Z, Yeşiltepe Mutlu G, Uslu Kızılkan N, Keser A, Beşer ÖF, Özbek MN, Bideci A, Ertem D, Evliyaoğlu O, Eliuz Tipici B, Gökçe T, Muradoğlu S, Taşkın OÇ, Koca T, Tütüncüler F, Baş F, Darendeliler F, Selimoğlu MA. Recommendations for Clinical Decision-making in Children with Type 1 Diabetes and Celiac Disease: Type 1 Diabetes and Celiac Disease Joint Working Group Report. J Clin Res Pediatr Endocrinol 2022;14:1-9. Epub 2021 Aug 18
45. Kaur N, Bhadada SK, Minz RW, Dayal D, Kochhar R. Interplay between Type 1 Diabetes Mellitus and Celiac Disease: Implications in Treatment. Dig Dis 2018;36:399-408. Epub 2018 Jul 25
46. Pham-Short A, Donaghue KC, Ambler G, Garnett S, Craig ME. Greater postprandial glucose excursions and inadequate nutrient intake in youth with type 1 diabetes and celiac disease. Sci Rep 2017;7:45286.
47. Scaramuzza AE, Mantegazza C, Bosetti A, Zuccotti GV. Type 1 diabetes and celiac disease: The effects of gluten free diet on metabolic control. World J Diabetes 2013;4:130-134.
48. Camarca ME, Mozzillo E, Nugnes R, Zito E, Falco M, Fattorusso V, Mobilia S, Buono P, Valerio G, Troncone R, Franzese A. Celiac disease in type 1 diabetes mellitus. Ital J Pediatr 2012;38:10.
49. Jones AL. The Gluten-Free Diet: Fad or Necessity? Diabetes Spectr 2017;30:118-123.
50. Serena G, Camhi S, Sturgeon C, Yan S, Fasano A. The role of gluten in celiac disease and type 1 diabetes. Nutrients 2015;7:7143-7162.

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The Importance of Extended High Frequencies in Hearing Evaluation of Pediatric Patients with Type 1 Diabetes

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What is already known on this topic?

Diabetes-induced hearing loss is considered a progressive sensorineural hearing loss with a gradual onset typically occurring at high frequencies (HFs). However, studies investigating extended HFs (EHFs) in pediatric patients with type 1 diabetes (T1D) are limited.

What this study adds?

There was a higher prevalence of hearing loss at EHFs in children with T1D, although the patients did not complain of hearing loss. This finding highlights the need for auditory evaluation of children with T1D to be performed both at the frequency range used in conventional audiometry and at EHFs.

Abstract

Objective: Type 1 diabetes (T1D), one of the most common childhood diseases worldwide, can cause hearing loss through systemic effects. Diabetes-induced hearing loss is considered a progressive sensorineural hearing loss with a gradual onset, typically occurring at high frequencies (HFs). Extended HF (EHF) hearing sensitivity in children with T1D who did not complain of hearing loss was investigated as an early marker for hearing loss at the standard/conventional frequency range of hearing.

Methods: Forty-two children (21 with T1D and 21 healthy controls) were evaluated in a case-control design. Conventional and EHF (14,000, 16,000, and 18,000 Hz) audiometry were performed. The diabetes group underwent routine blood biochemistry and glycated hemoglobin A1c measurements. The data were analyzed by the Student's t-test, Mann-Whitney U test, chi-square test, and logistic regression analysis.

Results: The mean hearing thresholds were significantly higher ($p < 0.05$) in the diabetes group than in controls at 500, 2,000, 4,000, and 8,000 Hz [all < 15 decibel hearing level (dB HL)]. The number of ears with thresholds > 15 dB HL at 14,000-18,000 Hz but ≤ 15 dB HL at 500-4,000 Hz was significantly higher in the diabetes group than in the control group ($p = 0.049$).

Conclusion: Children with diabetes showed normal hearing thresholds within the conventional audiometric frequency range but they had higher hearing thresholds during EHF audiometry when compared with controls. Audiometry in these children should be performed using frequencies above 8,000 Hz combined with the conventional frequency range. EHF audiometry may be an effective method for identifying subclinical hearing loss in children with diabetes. Thus, diabetic children with an EHF mean hearing threshold above 15 dB HL should be monitored more closely in terms of blood glucose regulation to prevent diabetes-related hearing loss at the conventional frequency range.

Keywords: Type 1 diabetes, children, hearing, extended high-frequency audiometry, hearing impairment

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Introduction

Type 1 diabetes (T1D) is one of the most prevalent long-term diseases of childhood worldwide. The young population with documented T1D in Turkey represents ~3% of the approximately 500,000 T1D cases worldwide (1). Diabetes is a chronic disorder of carbohydrate metabolism induced by absolute or relative insulin deficiency that impedes several organ systems. The multiorgan effects of diabetes include microangiopathy and/or neuropathy throughout the disease duration (2). Hearing impairment and loss of balance due to neuropathy and angiopathy are well-known clinical manifestations in both type 1 and type 2 diabetes (3,4,5,6). Neuropathy, vascular thrombosis, and arteriolar spasm, gradually developing in patients with diabetes, can cause loss of hearing (3,5,6,7). Diabetes-induced hearing impairment is characterized by a moderate loss of sensorineural hearing involving a lack of perception of high-frequency sounds; it affects both ears and leads to progressive hearing loss (5,6,8).

Although the underlying mechanism remains controversial, it is suggested that microangiopathy could lead to hearing loss in diabetic patients (9,10). Another potential mechanism involves changes in glucose metabolism (11). It is assumed that excess free oxygen radicals, formed because of nonenzymatic glycation in individuals with diabetes, may cause toxicity in the outer hair cells of the ears, eventually leading to hearing loss.

Previous studies on the presence, pattern, and severity of hearing loss and its relationship with metabolic control in patients with diabetes were inconclusive (12) and mostly involved adults with type 2 diabetes. To date, only a limited number of studies have documented marked hearing loss in young patients with T1D, particularly children with a relatively short disease duration (13). Hearing loss in adult patients with diabetes could be related to aging rather than solely to diabetes-induced neurovascular degeneration, whereas hearing loss in children with T1D most likely reflects the primary effects of diabetes (14). In their 2017 meta-analysis, Teng et al. (15) revealed a relationship between T1D and auditory dysfunction and reported that although hearing loss is mild and subclinical in T1D, the probability of hearing loss is higher compared with that in controls.

A young, healthy individual can often hear pure tones up to approximately 20 kHz. However, clinical audiometry, the gold standard for detecting hearing loss, typically measures tonal sensitivity up to 8 kHz (16). This suggests that achieving a normal pure-tone hearing threshold on an audiogram does not mean that there is no pathology in the cochlea or the central auditory nervous system (17). Therefore, conventional pure-tone audiometry should be complemented by extended high-frequency (EHF) (> 8 kHz) audiometry (17,18,19) to achieve an accurate diagnosis for people with a normal conventional audiogram who have listening difficulties or people with history of noise exposure and/or disorders that affect basal regions of the cochlea, such as T1D. This type of audiometry may be useful in the early diagnosis of hearing loss in certain situations, such as the ototoxic effect of cisplatin-based treatment, noise exposure, or oral misunderstanding, especially in noisy environments (20) and with T1D. EHF hearing is important for our understanding of speech in noise (17), potentially affecting academic success in school-age children. Therefore, EHF audiometry could also be a useful tool for the early diagnosis of hearing impairment in childhood (18,19). To date, only one study has investigated EHFs in children with T1D (21). The main difference between this and the present study is identification of diabetic children with subclinical hearing loss by using different frequency ranges, especially EHFs. Thus, we tested the diagnostic value of EHF audiometry in diabetic children.

Methods

This study was approved by the Ethics Committee of Trakya University and was conducted as per the tenets of the Declaration of Helsinki (decision no: 07/08, date: 13.04.2020). Informed consent was obtained from all subjects and their parents.

Participants

Forty-two children (84 ears from 21 patients with T1D and 21 healthy controls) were included. All subjects were aged 5-18 years and the study had a case-control design. Diabetic children were recruited from the pediatric endocrinology department, and the control group comprised healthy children who were referred to our center for an auditory evaluation for a school/course application. Our primary inclusion criterion for children in both groups was the absence of complaints of hearing loss. The exclusion criteria were any middle ear pathology, such as acute/chronic otitis media and otosclerosis, or history of middle ear surgery, ototoxic medication use, known family history of hearing loss, severe febrile illness, or previous head trauma.

A detailed pediatric and ear-nose-throat (ENT) physical examination was performed in both groups. Weight and height were measured and used to calculate body mass index (BMI) using the standard formula. Systolic/diastolic blood pressure (BP) was measured, and individuals with BP in the 95th percentile or higher were considered hypertensive

(22). For the diabetes group, we collected baseline venous blood samples after a 12-hour fasting period to determine the levels of fasting blood glucose, urea nitrogen, creatinine, total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and glycated hemoglobin (HbA1c). HbA1c values under 7.5% were considered to indicate good metabolic control, values of 7.5-9.0% indicated moderate metabolic control, and those above 9% indicated poor metabolic control (23). Dyslipidemia was diagnosed if one or more of the following parameters were met: LDL-C >2.6 mmol/L, HDL-C <1.1 mmol/L or TG >1.5 mmol/L (24). Microalbuminuria was defined as an albumin excretion rate of 30-300 mg/24 hours in 2 out of 3 early morning urine samples within 3-6 months of the first positive urine test (25). Tests were performed for vibration, pressure sensation, and proprioception to screen for diabetic polyneuropathy. A retinal examination was performed by an ophthalmologist in the diabetes group.

Audiological Evaluations

Otoscopic examination of all children was performed by the same ENT physician. Children with bilaterally normal otoscopic examination were included in the study, and audiological evaluations were made. The same audiologist performed the audiological tests in all children, which included pure-tone audiometry and tympanometry. Tympanometry and acoustic reflex testing were performed using an Interacoustics AZ26 (Interacoustics, Assens, Denmark) impedance audiometer. In immittance measurements, middle ear pressures and acoustic reflexes were measured with a probe tone of 226 Hz and an intensity of 85 dB sound pressure level. In the automatic evaluation, pressure between +200 daPa and -400 daPa was applied, and tympanogram types of all children were obtained. Both ipsilateral acoustic reflex and contralateral stapes reflex thresholds were evaluated as present/absent. Pure-tone audiometry was performed in a sound-treated booth using an Interacoustics AC40 (Interacoustics, Assens, Denmark) audiometer and Telephonics TDH-39P (Telephonics, USA) earphones. Children with normal tympanic membranes, type A tympanogram [Jerger et al.'s (26) classification], ipsilateral and contralateral stapes reflexes (at 1 kHz) within normal limits, and without conductive hearing loss on audiogram were included in the study, as long as they did not have middle ear pathology. Air and bone conduction were tested at frequencies of 250-8,000 Hz and 250-4,000 Hz, respectively. An air-bone gap over 10 dB HL is defined as conductive hearing loss. Children with an air-bone gap at any frequency above 10 dB HL were excluded. Children with an airway threshold average above 20 dB HL at speech frequencies (SF) of 500-4,000 Hz in the audiogram were also excluded from the study. In addition to using the 250-8,000 Hz frequency range in conventional audiometry, we performed auditory evaluations at EHFs of 14,000, 16,000, and 18,000 Hz in all children included in the study. Koss R/80 (Koss Co., USA) earphones were used for high-frequency audiometry. Pure-tone audiometry measurements were first performed at the conventional frequency range, followed by the EHF range. The ascending method was used to obtain the pure-tone audiometry thresholds (27). The step size used to measure the threshold was 5 dB. This step size provides the opportunity to obtain the hearing threshold more quickly and to control the accuracy of the threshold in most children with normal hearing (28). Special care was taken with each of the young participants as they could potentially become bored, and they were given breaks if necessary. Patients who recorded thresholds above 15 dB HL in the conventional pure-tone audiogram were considered to have hearing loss (18,19). Based on previous studies, we also performed statistical evaluations using a threshold value of 15 dB HL for EHFs (18,19). While analyzing the hearing measurements, the threshold values for each measured frequency and the pure-tone average threshold values of some frequency ranges (500-4,000 Hz as the human SF range; 4,000-8,000 Hz as the high frequency (HF) range; 14,000-18,000 Hz as the EHF range) were computed and used to reveal the exact frequency range that could be more predictive as a diagnostic approach.

Whether there was sensorineural hearing loss at the EHF in the diabetes group compared with the control group was investigated. The age at the time of diagnosis, duration of disease, HbA1c values, and data on microalbuminuria, dyslipidemia, retinopathy, and neuropathy were evaluated to investigate whether the potential sensorineural hearing loss in the diabetes group was related to metabolic control of T1D.

Statistical Analysis

The Shapiro-Wilk test was used to evaluate the normality of distribution of quantitative variables. Mean ± standard deviation was used as descriptive statistics for normally distributed quantitative data, median (minimum-maximum) values were used for non-normally distributed quantitative data, and numbers (%) were used for categorical variables.

The sample size was calculated as 36 ears for each group based on an effect size of 0.873 at 16,000 Hz frequency (21) with an alpha level of 5% and power of 95%. Considering the possibility of missing data, we included 42 ears in each group.

The Student's t-test was used to compare normally distributed quantitative data (age and BMI) between the diabetes and control groups and the Mann-Whitney U test to compare non-normally distributed quantitative hearing thresholds and HbA1c (%) between groups (healthy controls vs diabetes, disease duration <5 vs. ≥5 years). Comparison of mean hearing thresholds among frequency ranges was done using the Freidman test. The Pearson chi-square, Yates correction, and Fisher's exact tests were used as appropriate to compare categorical data (hearing thresholds >15 vs. ≤15 dB HL) between the groups. Potential confounders were analyzed by logistic regression analysis with the enter method. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was done with Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM SPSS Statistics for Windows, version 20.0. Armonk, NY: IBM Corp.).

Results

The general characteristics of the study population are given in Table 1. Both groups were similar in terms of age, gender distribution, and BMI.

Table 2 summarizes the audiometric test results. When the mean differences in frequency-specific hearing thresholds were analyzed after performing the auditory evaluations using both conventional and EHF methods, it was found that

Table 1. General characteristics of study groups

	Diabetes (n = 42 ears)	Control (n = 42 ears)	p
Age, years	11.9 ± 2.6	11.3 ± 2.6	0.448
Sex (male/female)	22/20	20/22	1.000
BMI, kg/m^2	19.1 ± 3.1	18.4 ± 2.0	0.302
BMI SDS	-0.030 ± 1.105	-0.162 ± 0.721	0.529
HbA1c	9.22 ± 1.56	-	-
Microalbuminuria, yes	4 (9.5%)	-	-
Dyslipidemia, yes	16 (38.1%)	-	-
Disease duration, years	5.19 ± 2.78	-	-
Mean age at the time of diagnosis, years	6.76 ± 3.17	-	-

BMI: body mass index; BMI SDS: BMI standard deviation score, HbA1c: glycated hemoglobin

Table 2. Hearing thresholds (dB HL) of ears and the number of ears with a threshold value above 15 dB HL at each frequency in the diabetes and control groups

Frequency (Hz)	dB HL	Diabetes (n = 42 ears)	Control (n = 42 ears)	p
250	Mean ± SD	11.6 ± 4.2	11.6 ± 5.0	0.924
	>15	5 (11.9%)	7 (16.7%)	0.755
500	Mean ± SD	12.0 ± 4.8	9.0 ± 3.1	**0.002**
	>15	4 (9.5%)	0 (0.0%)	0.116
1,000	Mean ± SD	7.3 ± 4.8	6.0 ± 2.3	0.327
	>15	4 (9.5%)	0 (0.0%)	0.116
2,000	Mean ± SD	8.0 ± 4.5	5.4 ± 2.1	**<0.001**
	>15	2 (4.8%)	0 (0.0%)	0.494
4,000	Mean ± SD	8.9 ± 5.2	5.5 ± 1.6	**<0.001**
	>15	3 (7.1%)	0 (0.0%)	0.241
8,000	Mean ± SD	11.9 ± 5.6	6.6 ± 3.0	**<0.001**
	>15	7 (16.7%)	0 (0.0%)	**0.012**
14,000	Mean ± SD	8.5 ± 12.9	7.2 ± 5.4	0.081
	>15	6 (14.3%)	3 (7.1%)	0.483
16,000	Mean ± SD	11.0 ± 15.0	8.5 ± 7.3	0.103
	>15	11 (26.2%)	6 (14.3%)	0.277
18,000	Mean ± SD	10.3 ± 11.0	7.2 ± 5.0	0.993
	>15	12 (28.6%)	2 (4.8%)	**0.008**

dB HL: decibel hearing level, SD: standard deviation.
n (%)

the diabetes group had higher mean hearing thresholds than the control group at all frequencies except 250 Hz; however, all the children's mean hearing thresholds were under 15 dB HL. The mean thresholds of both groups were equal at a frequency of 250 Hz. The higher mean hearing thresholds in the diabetes group were statistically significant only at 500, 2,000, 4,000, and 8,000 Hz (Table 2). Table 2 also shows the number of ears with hearing thresholds above 15 dB HL at each frequency in the diabetes and control groups. The number of ears with a threshold value above 15 dB HL was significantly higher in the diabetes group than in the control group at frequency ranges of 8,000 Hz ($p = 0.012$), 18,000 Hz ($p = 0.008$), and 14,000-18,000 Hz ($p = 0.023$, Tables 2 and 3). There was no significant between-group difference in the number of ears with mean hearing threshold values above 15 dB HL at the 500-4,000 Hz frequency range ($p = 0.241$, Table 3). Although there was no ear with a mean hearing threshold value above 15 dB HL at this frequency range in the control group, there were three ears with threshold values above 15 dB HL in the diabetes group, all of which were ≤20 dB HL (Table 3).

Table 3 shows hearing thresholds and the number of ears with a threshold value above 15 dB HL at different frequency ranges. It was found that the mean hearing thresholds at the SF (500-4,000 Hz), HF (4,000-8,000 Hz), and EHF (14,000-18,000 Hz) ranges were higher in the diabetes group than in the control group, but they were statistically significant only in the SF and HF ranges.

Table 4 shows the number of ears with a normal hearing threshold (≤15 dB HL) at the SF range but above 15 dB HL at the HF and EHF ranges in both the diabetes and control groups. According to conventional SF test results, 39 ears in the diabetes group and 42 ears in the control group were within normal limits. However, extending the audiometry to EHF in these healthy ears revealed that 10 (25%) ears in the diabetes group and 3 (7%) ears in the control group had subclinical hearing loss. Additionally, mean hearing thresholds at the EHF range (25.7 ± 9.3 dB HL) were significantly higher in the diabetes group (n = 10) compared with the mean hearing threshold at the SF range (8.8 ± 2.8 dB HL; $p = 0.005$). In the control group (n = 3), there was no significant difference between mean hearing thresholds at the EHF (22.2 ± 6.9 dB HL) and those at the SF range (10.8 ± 4.0 dB HL; $p = 0.109$).

To evaluate the role of age in the hearing thresholds of children with T1D, the correlation of hearing thresholds at each frequency with age was investigated. A significant positive correlation was found between age and threshold values at 8,000 Hz in the diabetes group ($r = 0.460$; $p = 0.036$). There was no significant correlation between mean hearing threshold and age at other frequencies. In the control group, there was no significant correlation between age and hearing thresholds at any frequency (data not shown).

Table 5 gives a comparison of the median hearing thresholds of patients with T1D at each frequency based on disease duration. In the diabetes group, the mean duration of disease for T1D was 5.19 ± 2.78 years. Mean hearing thresholds at 250, 500, 2,000, 4,000, 500-4,000, and 4,000-8,000 Hz were significantly higher in patients with a disease duration

Table 3. Hearing thresholds (dB HL) and the number of ears with a threshold value above 15 dB HL of ears at different frequency ranges in diabetes and control groups

Pure-tone audiometry	dB HL	Diabetes (n = 42 ears)	Control (n = 42 ears)	p
Speech frequency (500-4,000 Hz)	Mean ± SD	9.1 ± 3.7	6.5 ± 1.8	**< 0.001**
	> 15, n (%)	3 (7.1%)	0 (0%)	0.241
High frequency (4,000-8,000 Hz)	Mean ± SD	10.4 ± 4.9	6.1 ± 2.0	**< 0.001**
	> 15, n (%)	5 (11.9%)	0 (0%)	0.055
Extended high frequency (14,000-18,000 Hz)	Mean ± SD	10.0 ± 12.1	7.6 ± 5.2	0.385
	> 15, n (%)	12 (28.6%)	3 (7.1%)	**0.023**

dB HL: decibel hearing level, SD: standard deviation

Table 4. The number of ears with normal hearing threshold (≤15 dB HL) at speech frequency range but above 15 dB HL at high and EHF ranges

Diabetes (n = 39)		Hearing loss at high frequency (4,000-8,000 Hz) (> 15 dB HL)			Hearing loss at EHF (14,000-18,000 Hz) (> 15 dB HL)		
		Control (n = 42)	p	Diabetes (n = 39)	Control (n = 42)	p	
Speech frequency (500-4,000 Hz)	≤15 dB HL	2 (5.1%)	0 (0%)	0.229	10 (25.6%)	3 (7.1%)	**0.049**

dB HL: decibel hearing level, EHF: extended high frequency.
n (%)

of ≥5 years compared with those with a disease duration of <5 years (Table 5).

The mean HbA1c value of all diabetic children included in the study group was 9.22 ± 1.56%. A subgroup analysis was performed based on HbA1c and compared the HbA1c values between patients with or without hearing loss at all tested frequencies (Table 6). It was found that those with thresholds above 15 dB HL at 2,000 and 4,000 Hz also had significantly higher HbA1c ($p < 0.05$). Among the patients with a normal hearing result in the conventional SF range, the mean HbA1c value was higher in patients with a hearing threshold >15 dB HL at the EHF range compared with patients with a hearing threshold ≤15 dB HL at the EHF range (9.47% vs. 8.94%, respectively). However, this difference failed to reach significance ($p = 0.421$). In addition, a correlation analysis was performed between HbA1c and mean hearing threshold at different frequency ranges. No significant correlation was identified (Supplementary Table 1).

When patients were grouped according to HbA1c values (see Methods section), the frequency of hearing thresholds above 15 dB HL at 500 Hz was significantly higher in the group with moderate metabolic control than in the good and poor control groups ($p = 0.005$). There was no significant difference at other frequencies. The percentage of diabetic patients with good, moderate, or poor metabolic control was compared among the hearing threshold groups at different frequency ranges (Figure 1; $p > 0.05$).

Table 5. Comparison of the median hearing thresholds (dB HL) of patients with type 1 diabetes at each frequency based on disease duration

Frequency	Disease duration		p[a]
	<5 years (n = 11)	≥5 years (n = 10)	
250 Hz	10 (5-15)	15 (10-20)	**0.045**
500 Hz	10 (5-15)	15 (10-25)	**0.005**
1000 Hz	5 (5-10)	5 (5-20)	0.690
2000 Hz	5 (5-5)	7.5 (5-20)	**0.009**
4000 Hz	5 (5-10)	10 (5-25)	**0.014**
8000 Hz	10 (5-20)	12.5 (10-25)	0.081
14000 Hz	5 (0-50)	0 (0-45)	0.681
16000 Hz	5 (0-55)	2.5 (0-40)	0.556
18000 Hz	10 (0-30)	10 (0-30)	0.856
500-4000 Hz	6.25 (5.25-7.5)	10 (6.25-18.75)	**0.002**
4000-8000 Hz	7.5 (5-15)	10 (7.5-25.5)	**0.017**
14000-18000 Hz	6.67 (0-45)	6.67 (0-38.3)	0.774

dB HL: decibel hearing level.
[a]Mann-Whitney U test, median (minimum-maximum).

Table 6. Comparison of the HbA1c values of patients with type 1 diabetes based on their hearing thresholds at each frequency

Frequency	Hearing ≤15 dB HL	Hearing >15 dB HL	p[a]
250 Hz	9.2 (6.9-12.6)	8.4 (8.0-12.6)	0.938
500 Hz	9.2 (6.9-12.6)	8.4 (8.0-8.5)	0.230
1000 Hz	9.2 (6.9-12.6)	8.4 (8.0-12.6)	0.864
2000 Hz	9.05 (6.9-11.8)	12.6 (12.6-12.6)	**0.018**
4000 Hz	8.9 (6.9-11.8)	12.6 (9.6-12.6)	**0.024**
8000 Hz	9.2 (6.9-11.8)	9.3 (8.0-12.6)	0.457
14000 Hz	9.05 (6.9-12.6)	10.8 (8.0-12.6)	0.098
16000 Hz	8.9 (6.9-11.8)	11.0 (7.3-12.6)	0.063
18000 Hz	9.2 (6.9-11.8)	9.45 (7.3-12.6)	0.419
500-4000 Hz	9.2 (6.9-11.8)	12.6 (8.4-12.6)	0.096
4000-8000 Hz	9.2 (6.9-11.8)	9.6 (8.1-12.6)	0.243
14000-18000 Hz	9.05 (6.9-11.8)	10.4 (7.3-12.6)	0.112

HbA1c: glycated hemoglobin, dB HL: decibel hearing level.
[a]Mann-Whitney U test, median (minimum-maximum).

In terms of mean hearing threshold ≤15 dB HL or >15 dB HL at each frequency measured, patients with and without microalbuminuria and dyslipidemia were comparable. Presence or absence of microalbuminuria and/or dyslipidemia was not a distinguishing factor between the patients who had hearing loss at different frequency ranges (Figures 2 and 3; p>0.05). There were no patients with retinopathy or neuropathy in the diabetes group.

The effect of potential confounders (age, BMI, disease duration, and HbA1c) on hearing loss at different frequency ranges (SF, HF, and EHF) of pure-tone audiometry was analyzed by logistic regression, and no significant effect on hearing loss was found (Table 7).

Discussion

The findings of this study imply that using EHFs during audiometric evaluation in diabetic children may reveal hearing impairment, which in turn may be evidence of

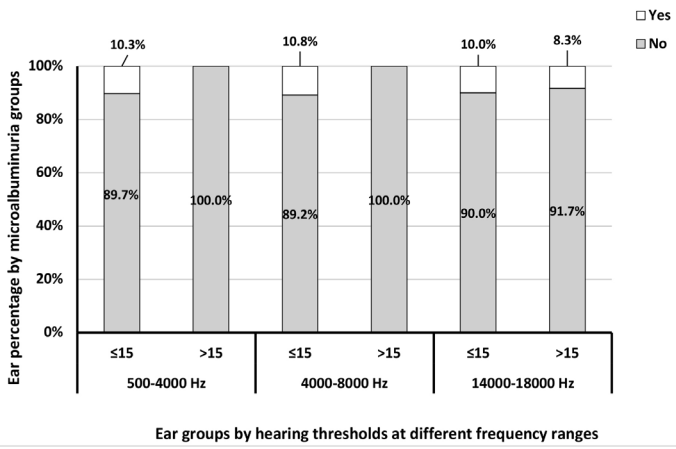

Figure 2. Distribution of microalbuminuria groups based on diagnostic criteria among ears with a hearing threshold >15 or ≤15 dB HL at different frequency ranges

dB HL: decibel hearing level, Yes: microalbuminuria present, No: microalbuminuria absent

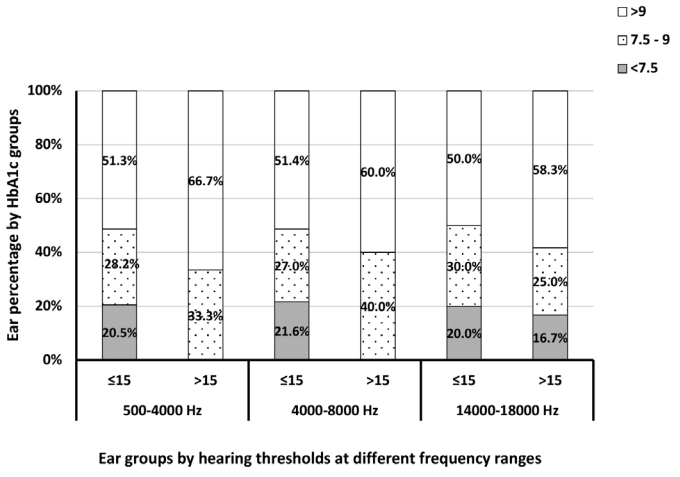

Figure 1. Distribution of metabolic control groups based on HbA1c levels (1: <7.5%; 2: 7.5-9%; 3: >9%) among ears with a hearing threshold >15 or ≤15 dB HL at different frequency ranges

HbA1c: glycated hemoglobin, dB HL: decibel hearing level

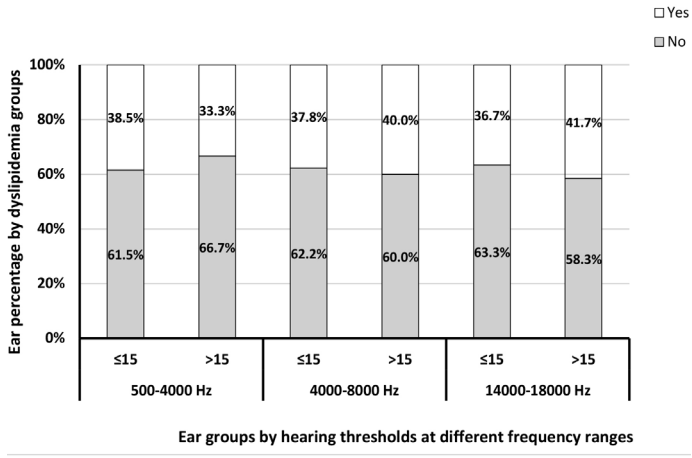

Figure 3. Distribution of dyslipidemia groups based on diagnostic criteria among ears with a hearing threshold >15 or ≤15 dB HL at different frequency ranges

dB HL: decibel hearing level, Yes: dyslipidemia present, No: dyslipidemia absent

Table 7. The effect of potential confounders on hearing loss (>15 dB HL) at different frequency ranges (speech frequency, high frequency, and EHF) of pure-tone audiometry by logistic regression

	Hearing loss at speech frequency			Hearing loss at high frequency			Hearing loss at EHF		
	Wald statistics	p	OR (95% CI)	Wald statistics	p	OR (95% CI)	Wald statistics	p	OR (95% CI)
Age, years	1.07	0.302	0.57 (0.20-1.66)	0.93	0.334	0.78 (0.47-1.29)	2.53	0.112	0.75 (0.52-1.07)
BMI (kg/m^2)	1.24	0.266	0.10 (0-5.92)	0.73	0.392	1.16 (0.82-1.65)	1.84	0.175	0.77 (0.53-1.12)
Disease duration, years	1.21	0.271	0.48 (0.13-1.78)	0.01	0.934	1.02 (0.64-1.63)	1.19	0.275	1.24 (0.85-1.81)
HbA1c (%)	0.64	0.424	1.64 (0.49-5.49)	0.63	0.427	1.42 (0.60-3.40)	0.89	0.345	1.31 (0.75-2.28)

OR: odds ratio, CI: confidence interval, BMI: body mass index, HbA1c: glycated hemoglobin, dB HL: decibel hearing level, EHF: extended high frequency

early changes related to hearing loss. Hearing impairment in patients with diabetes has been investigated for several years but previous studies on the presence, pattern, and severity of hearing loss and its relationship with metabolic control have been inconclusive (12). Diabetes-induced hearing loss is considered a progressive sensorineural type of hearing loss with a gradual onset typically occurring at HFs (5,6,8). A higher prevalence of hearing loss in EHF was found in the present study, although there was no complaint of hearing loss in the children with T1D. Diabetic children with an EHF mean hearing threshold above 15 dB HL should be monitored more closely in terms of regulation of blood glucose levels to prevent diabetes-related hearing loss.

In conventional audiometry, the air conduction pathway is examined at 250-8,000 Hz and the bone conduction pathway at 500-4,000 Hz. In our study, the mean hearing thresholds of patients with T1D were higher than those of the healthy controls. At frequencies of 500, 2,000, 4,000, and 8,000 Hz, this difference was statistically significant but the mean hearing threshold was not higher than 15 dB HL at any frequency. Among diabetic patients, the number of ears with a normal mean hearing threshold (≤15 dB HL) at the SF range but a mean hearing threshold above 15 dB HL at the EHF range was significantly higher compared with that in the healthy controls. Although the increase in mean hearing thresholds at the EHF range was not significant in the diabetes group compared with that in the control group, the fact that the number of ears with a mean hearing threshold ≤15 dB HL at the SF range but >15 dB HL at the EHF range was significantly higher in patients with T1D suggests that these frequencies should be further investigated. These ears might be overlooked when only conventional audiometry frequencies are considered while analyzing EHFs could contribute to early recognition of the pathogenetic process already initiated in the inner ear in children with T1D.

Most previous studies on T1D and hearing used ≤8,000 Hz pure-tone audiometry. Four studies used >8,000 Hz pure-tone audiometry. Of these, only one was performed in the pediatric age group (21), and the mean age of cases in the other three studies was over 20 years (29,30,31). Abd El Dayem et al. (21) performed pure-tone audiometry (250-18,000 Hz) and transient-evoked otoacoustic emission (TEOAE). Similar to our study, the thresholds in patients with diabetes were higher than those in the controls at all frequencies. However, significantly higher hearing thresholds were recorded at 8,000, 16,000, 17,000, and 18,000 Hz in the right ear and at 4,000, 8,000, 16,000, 17,000, and 18,000 Hz in the left ear. They found no significant difference between patients with diabetes and controls at low and medium frequencies ≤4,000 Hz. Their findings show significant decreases in the signal/noise ratio in TEOAE at 4,000 Hz in the right ear and at 1,000, 1,500, and 4,000 Hz in the left ear in patients compared with controls, suggesting cochlear pathology. The authors concluded that audiometric evaluation at HF and EHF could help detect underlying hearing impairments in children with diabetes more effectively than conventional audiometry and our results support this finding. In our study, in frequency-based comparisons, we found a significant increase in thresholds of diabetic children at 500, 2,000, 4,000, and 8,000 Hz, but we did not detect a significant increase in EHF. However, the mean hearing values at each frequency in all of our cases were less than 15 dB HL. We found a higher prevalence of hearing loss in EHF in type 1 diabetic children with clinically normal hearing. We paid particular attention to the fact that all patients included in the study did not have hearing loss complaints. In the study of Abd El Dayem et al. (21), the average hearing thresholds were higher than in ours. The lower mean age and mean HbA1c value of our diabetic patients may have contributed to this difference.

Two other studies (30,31) investigated auditory involvement in adults with T1D. Dabrowski et al. (30) reported that the mean hearing thresholds at frequencies of 3,000, 4,000, 6,000, 8,000, and 12,000 Hz were significantly higher in patients with T1D. Malucelli et al. (31) also found that the mean hearing values of both ears at 250, 500, 9,000, 10,000, 11,200, 12,500, 14,000, and 16,000 Hz in the patient group were significantly higher than in the control group. They also detected thresholds of under 20 dB at frequencies ≤10,000 Hz and above 20 dB at frequencies ≥10,000 Hz. Similar to our findings, Dabrowski et al. (30) found that all mean thresholds were under 20 dB. Their report of higher mean hearing thresholds could be attributed to the fact that the mean age of patients in our study was 11.3 ± 2.6 years, whereas both abovementioned studies enrolled adults aged over 25 years. In 1980, Osterhammel and Christau (29) evaluated high-frequency hearing and stapedius reflex thresholds at 250-20,000 Hz in 61 patients with insulin-dependent diabetes, aged 20-50 years, and compared their results with normative data of nondiabetic matched controls. They reported that, unlike in our study, there was no significant difference between the two groups in the hearing and stapedius reflex thresholds.

Two meta-analyses revealed the relationship between T1D and auditory dysfunction (14,15). In one study, the prevalence of hearing loss was higher in patients with diabetes compared with controls, even if the hearing impairment was mild and subclinical (15). The other study reported that hearing loss indicated subclinical microvascular damage and should be recognized as equivalent to subclinical neuropathy,

retinopathy, and nephropathy, which can require a stringent treatment/management regimen to prevent late disease complications (14).

Aiming to evaluate functional hearing and general communication skills in school-age children with T1D, Rance et al. (32) investigated both cochlear and auditory neural function using auditory brainstem response (ABR), pure-tone audiometry at 250-8,000 Hz, otoacoustic emissions (OAEs), and behavioral testing techniques. Although the hearing value was ≤15 dB in both groups, the hearing thresholds of the patients were significantly higher than those of the healthy controls. Furthermore, these authors reported lower mean response amplitudes in distortion product OAE (DPOAE) and decreased V-wave amplitudes and prolonged I-V waves in the ABR of the patients. Additionally, their patients had impaired bilateral speech perception in noisy environments, and their perceptual ability and degree of neural deterioration in the auditory brainstem were correlated. The authors concluded that a functional hearing impairment that is severe enough to limit communication and threaten academic progress is common in school-age children with T1D and that standard audiometry was not an effective screening method in their population. We conclude that EHFs should be included in the standard follow-up regimens of these children and auditory screening tests in schools.

The relationship between hearing, duration of disease, and other metabolic changes in patients with T1D remains inconclusive. Comparison of diabetic patients with a disease duration of greater than versus less than 5 years revealed that hearing thresholds were higher in the group with longer disease duration. Grouping patients based on HbA1c levels enabled additional comparisons among patients with various metabolic control. As indirect evidence from Figure 1, we suggest that the presence of patients with good metabolic control in EHF tests and absence of patients with good metabolic control in conventional frequencies could indicate the sensitivity of using EHF to detect hearing loss at early stages. Abd El Dayem et al. (21) found that the rate of failed OAE in patients with a disease duration of more than 10 years was significantly higher than those with a disease duration of less than 10 years. However, they did not find a significant relationship between hearing, disease duration, and HbA1c at EHFs (16,000, 17,000, and 18,000 Hz). Dąbrowski et al. (30) reported that diabetes duration and metabolic control were not related to hearing thresholds and ABR results. Rance et al. (32) found no relationship between the mean hearing level, age at the time of disease onset, duration of disease, and HbA1c levels. However, Mujica-Mota et al. (14) found that the duration of diabetes contributed markedly to the development of hearing loss and concluded that the relative risk increases over time. The fact that the number, mean age, and duration of disease of diabetic cases were higher in the studies included in Mujica-Mota et al.'s (14) meta-analysis than in other studies may explain this difference.

Study Limitations

The most important limitation of our study was our inability to perform other electrophysiological tests (OAE, ABR) to evaluate hearing. Another limitation could be lack of other extended frequencies, such as 10 and 12.5 kHz, which would provide additional information. The relationship between the metabolic control of T1D and hearing loss remains controversial, and future studies using electrophysiological tests in addition to pure-tone audiometry on a greater number of pediatric patients could contribute significantly to the current literature. Although the number of patients was sufficient in the power analysis for the study, the number was insufficient for the subgroup analysis. If more patients had been included, the result might be more robust.

Conclusion

In conclusion, our findings suggest that the auditory evaluation of children with T1D should be performed both at the frequency range used in conventional audiometry and at EHFs, although larger-scale studies will be required in the future to support and confirm these results. Pure-tone audiometers are more widely used, more accessible, and cheaper. Therefore, we believe that EHF audiometric evaluation has potential for early detection of subclinical hearing impairment in children with T1D. Early detection of impaired hearing at higher frequencies could be an early and useful warning sign that will enable intervention which may aid in preservation of hearing in these children. Diabetic children with an EHF mean hearing threshold above 15 dB HL should be monitored more closely in terms of regulation of blood glucose levels to prevent diabetes-related hearing loss. Therefore, this approach combined with increased metabolic control could allow for an improved disease process and a more stable academic life for these children.

Acknowledgments

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Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Trakya University and was conducted as per the tenets of the Declaration of Helsinki (decision no: 07/08, date: 13.04.2020).

Informed Consent: Informed consent was obtained from all subjects and their parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Selis Gülseven Güven, Çiğdem Binay, Concept: Selis Gülseven Güven, Design: Selis Gülseven Güven, Data Collection or Processing: Selis Gülseven Güven, Çiğdem Binay, Analysis or Interpretation: Selis Gülseven Güven, Çiğdem Binay, Literature Search: Selis Gülseven Güven, Çiğdem Binay, Writing: Selis Gülseven Güven, Çiğdem Binay.

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References

1. Yeşilkaya E, Cinaz P, Andıran N, Bideci A, Hatun Ş, Sarı E, Türker T, Akgül Ö, Saldır M, Kılıçaslan H, Açıkel C, Craig ME. First report on the nationwide incidence and prevalence of Type 1 diabetes among children in Turkey. Diabet Med Diabet Med 2017;34:405-410. Epub 2016 Feb 12
2. Aladağ I, Kurt S, Eyibilen A, Güven M, Erkorkmaz U. Early evaluation of auditory dysfunction in patients with type 2 diabetes mellitus. KBB Ihtis Derg 2008;18:203-210.
3. Chávez-Delgado ME, Vázquez-Granados I, Rosales-Cortés M, Velasco-Rodríguez V. Disfuncion cócleo-vestibular en pacientes con diabetes mellitus, hipertensión arterial sistémica y dislipidemia [Cochleovestibular dysfunction in patients with diabetes mellitus, hypertension and dyslipidemia]. Acta Otorrinolaringol Esp 2012;63:93-101. Epub 2011 Dec 6
4. Horikawa C, Kodama S, Tanaka S, Fujihara K, Hirasawa R, Yachi Y, Shimano H, Yamada N, Saito K, Sone H. Diabetes and risk of hearing impairment in adults: a meta-analysis. J Clin Endocrinol Metab 2013;98:51-58. Epub 2012 Nov 12
5. Klagenberg KF, Zeigelboim BS, Jurkiewicz AL, Martins-Bassetto J. Vestibulocochlear manifestations in patients with type I diabetes mellitus. Braz J Otorhinolaryngol 2007;73:353-358.
6. ukushima H, Cureoglu S, Schachern PA, Paparella MM, Harada T, Oktay MF. Effects of type 2 diabetes mellitus on cochlear structure in humans. Arch Otolaryngol Head Neck Surg 2006;132:934-938.
7. Lin SW, Lin YS, Weng SF, Chou CW. Risk of developing sudden sensorineural hearing loss in diabetic patients: A population-based cohort study. Otol Neurotol 2012;33:1482-1488.
8. Kulak Burun Boğaz Hastalıkları ve Baş Boyun Cerrahisi. Birinci Baskı. Çelik O. Güneş Tıp Kitapevi., 2004;71-87. Available from: https://www.kitantik.com/product/Kulak-Burun-Bogaz-Hastaliklari-ve-Bas-Boyun-Cerrahisi_1br9qfwkvog5tx714ql
9. Jorgensen MB. The inner ear in diabetes mellitus: Histological studies. Arch Otolaryngol 1961;74:373-381.
10. Jorgensen MB, Buch NH. Studies on inner-ear function and cranial nerves in diabetics. Acta Otolaryngol 1961;53:350-364.
11. Lisowska G, Namysłowski G, Morawski K, Strojek K. Early identification of hearing impairment in patients with type 1 diabetes mellitus. Otol Neurotol 2001;22:316-320.
12. ALDajani N, ALkurdi A, ALMutair A, ALdraiwesh A, ALMazrou KA. Is type 1 diabetes mellitus a cause for subtle hearing loss in pediatric patients? Eur Arch Otorhinolaryngol 2015;272:1867-1871. Epub 2014 Mar 14
13. Okhovat SA, Moaddab MH, Okhovat SH, Al-Azab AA, Saleh FA, Oshaghi S, Abdeyazdan Z. Evaluation of hearing loss in juvenile insulin dependent patients with diabetes mellitus. J Res Med Sci 2011;16:179-183.
14. Mujica-Mota MA, Patel N, Saliba I. Hearing loss in type 1 diabetes: Are we facing another microvascular disease? A meta-analysis. Int J Pediatr Otorhinolaryngol l 2018;113:38-45. Epub 2018 Jul 10
15. Teng ZP, Tian R, Xing FL, Tang H, Xu JJ, Zhang BW, Qi JW. An association of type 1 diabetes mellitus with auditory dysfunction: A systematic review and meta-analysis. Laryngoscope 2017;127:1689-1697. Epub 2016 Oct 7
16. Hunter LL, Monson BB, Moore DR, Dhar S, Wright BA, Munro KJ, Zadeh LM, Blankenship CM, Stiepan SM, Siegel JH. Extended high frequency hearing and speech perception implications in adults and children. Hearing Res 2020;397:107922. Epub 2020 Feb 18
17. Motlagh Zadeh L, Silbert NH, Sternasty K, Swanepoel W, Hunter LL, Moore DR. Extended high-frequency hearing enhances speech perception in noise. Proc Natl Acad Sci U S A 2019;116:23753-23759. Epub 2019 Nov 4
18. Peñaranda D, Pérez-Herrera LC, Hernández D, Moreno-López S, Perea I, Jacome M, Suetta-Lugo N, García JM, Peñaranda A. Prevalence of extended high-frequency hearing loss among adolescents from two rural areas in Colombia. Int J Audiol 2021;60:365-373. Epub 2020 Oct 12
19. Anastasio AR, Radael RD, Cavalcante JM, Hatzopoulos S. A report of extended high frequency audiometry thresholds in school-age children with no hearing complaints. Audiol Res 2012;2:8.
20. Rodríguez Valiente A, Roldán Fidalgo A, Villarreal IM, García Berrocal JR. Extended high-frequency audiometry (9,000-20,000 Hz). Usefulness in audiological diagnosis. Acta Otorrinolaringol Esp 2016;67:40-44. Epub 2015 May 27
21. Abd El Dayem SM, Abd El Ghany SM, Beshr AE, Hassan AG, Attaya MS. Assessment of hearing in children with type 1 diabetes mellitus. J Pediatr Endocrinol Metab 2014;27:393-402.
22. National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents. The fourth report on the diagnosis, evaluation, and treatment of high blood pressure in children and adolescents. Pediatrics 2004;114(2 Suppl 4th Report):555-576.
23. Rewers M, Pihoker C, Donaghue K, Hanas R, Swift P, Klingensmith GJ. Assessment and monitoring of glycemic control in children and adolescents with diabetes. Pediatr Diabetes 2009;10(Suppl 12):71-81.
24. American Diabetes Association. Management of dyslipidemia in children and adolescents with diabetes. Diabetes Care 2003;26:2194-2197.
25. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH, Steffes MW; American Diabetes Association. Nephropathy in diabetes. Diabetes Care 2004;27(Suppl 1):79-83.
26. Jerger J, Jerger S, Mauldin L. Studies in impedance audiometry. I. Normal and sensorineural ears. Arch Otolaryngol 1972;96:513-523.

27. ISO/TC 43, ISO 8253-1:2010. Pure-tone air and bone conduction audiometry. Retrieved: December 12, 2021. Available from: https://www.iso.org/standard/43601.html

28. Arlinger SD. Comparison of ascending and bracketing methods in pure tone audiometry. A multi-laboratory study. Scand Audiol 1979;8:247-251.

29. Osterhammel D, Christau B. High frequency audiometry and stapedius muscle reflex thresholds in juvenile diabetics. Scand Audiol 1980;9:13-18.

30. Dąbrowski M, Mielnik-Niedzielska G, Nowakowski A. Involvement of the auditory organ in type 1 diabetes mellitus. Endokrynol Pol 2011;62:138-144.

31. Malucelli DA, Malucelli FJ, Fonseca VR, Zeigeboim B, Ribas A, Trotta Fd, Silva TP. Hearing loss prevalence in patients with diabetes mellitus type 1. Braz J Otorhinolaryngol 2012;78:105-115.

32. Rance G, Chisari D, Edvall N, Cameron F. Functional hearing deficits in children with type 1 diabetes. Diabet Med 2016;33:1268-1274. Epub 2016 Feb 12

Click for Supplementary Table 1 access link: http://glns.co/meoy0

ORIGINAL ARTICLE

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Novel Modified Algorithm for High Fat/High Energy Density Meal in Type 1 Diabetes: Less Hypoglycemia

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What is already known on this topic?

Optimal postprandial glycemia depends on matching insulin to the carbohydrate, protein, and fat contents of meal, after a high fat/high energy density meal in individuals with type 1 diabetes.

What this study adds?

Additional insulin dose (64%) for high fat/high energy density meal increased time in normoglycemia without hypoglycemia.

Abstract

Objective: This aim of this study was to investigate the effect of additional insulin dosing for high fat/high energy density mixed meal over 12 hours.

Methods: In this single-center, non-blinded, randomized, cross-over study, a high fat/high energy density test meal was used to study the impact on glycemic response of either carbohydrate counting (CC) on the first day and the Pańkowska algorithm (PA) on the second test day. The two methods were compared in 20 adolescents with type 1 diabetes (T1D), aged 9-18 years, using insulin pump therapy and continuous glucose monitoring on postprandial early (0-120 min), late (120-720 min), and total (0-720 min) glycemic response.

Results: There was no difference between groups in the duration of normoglycemia in the early period. Postprandially, 50% of patients developed hypoglycemia using the PA at a median of 6.3 (5.6-7.9) hours and the PA was subsequently modified for the remaining ten patients. Area under the curve (AUC) for the early period decreased non-significantly in the CC group, indicating less normoglycemia. No significant difference was found in the AUC of the PA (no hypoglycemia n = 4) and modified PA groups (no hypoglycemia n = 6) over the whole period (0-12 hours). AUC for level 2 hyperglycemia was statistically greater in the PA-no hypoglycemia patients compared to modified PA-no hypoglycemia patients.

Conclusion: There were inter-individual differences in glycemic response to high fat/high energy density meals. An individualized approach to insulin dosing by evaluating food diary and postprandial glucose monitoring appears to be optimal for children and adolescents with T1D.

Keywords: High fat, glycemic variability, insulin pump therapy, type 1 diabetes mellitus

Introduction

The primary goal of diabetes management is to achieve normal or near-normal blood glucose levels. Food and nutrition interventions that reduce postprandial blood glucose excursions are important in this regard since dietary carbohydrate is the major determinant of postprandial glucose levels (1). Thus, carbohydrate counting (CC) is conventionally recommended for preprandial insulin dose calculation for individuals with type 1 diabetes mellitus (T1D) on intensive insulin therapy and insulin infusion pump therapy. Although carbohydrate is the predominant macronutrient affecting postprandial blood glucose excursions, recent research has shown that dietary fat

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and protein can also significantly impact the postprandial glycemic profile (2,3,4,5,6,7,8,9).

When consumed separately, both protein and fat may cause an increase in postprandial glycemia, depending on the quantity (6,10,11). However, most meals contain both fat and protein and when a meal containing high levels of both fat and protein is consumed, the combined impact is additive and causes significantly higher glucose excursions. Closed-loop studies have suggested that for high-fat meals the insulin dose needs to be increased by 42% and for high fat/high protein mixed meals by 39% (6,12). However, it should be noted that the increased insulin requirement after high-fat meal consumption can show great differences between individuals. These findings suggest that a change in insulin dose is warranted and, in most patients, additional insulin may be required but there is no international consensus about the preprandial insulin dose estimation for high fat/high protein mixed meals. The American Diabetes Association acknowledges that for people with diabetes who are prescribed a flexible insulin therapy program, education on how to use CC and on dosing for fat and protein content should be used to determine mealtime insulin dosing (13). The International Society for Paediatric and Adolescent Diabetes (ISPAD) has noted that the optimal insulin bolus dose and delivery for meals high in fat and protein are undefined with randomized controlled trials required (14).

A novel insulin dosing algorithm has been proposed which takes account of the glycemic impact of fat/protein when calculating mealtime insulin dose. Pańkowska et al. (15) developed an algorithm for calculating the preprandial insulin dose based on all macronutrients (carbohydrate, fat, and protein) of the meal and described a "fat/protein unit (FPU)" as 100 kcal from fat and/or protein.

The aim of the present study was to compare the impact of additional dosing with extended insulin bolus, as described by the Pańkowska algorithm (PA) versus CC on postprandial glucose excursions for high fat/high energy density mixed meal on postprandial glucose excursions for the first 12 hours after the meal in adolescents with T1D using insulin pump therapy (IPT) and a continuous glucose monitoring system (CGMS).

Methods

A single-center, non-blinded, randomized, cross-over study was performed between July 2017-April 2018.

Participants

Twenty adolescents with T1D were recruited. The inclusion criteria were: T1D for at least one year and treatment with IPT for at least six months; body mass index (BMI) z-score of >-1 <2; and total daily insulin use of ≥0.5 U/kg to avoid inclusion of participants in the remission phase of diabetes. Exclusion criteria were: concomitant dietary restrictions (eg, Celiac disease or food allergy); cystic fibrosis; concurrent conditions that can be associated with delayed gastric emptying or altered digestion; and the use of any medication that is known to modify glycemia, such as glucocorticoids or oral antidiabetic drugs.

Study Design

Participants attended the clinic a week before for the insertion of Guardian™ Connect (Medtronic MiniMed, Inc., Northridge, California) CGMS. In the seven days leading up to the study, participants or their caregivers were contacted daily by the pediatric endocrinologist to review the CGMS blood glucose levels of participants, and the food and activity diary. CGMS readings were used to adjust basal rates, insulin carbohydrate ratio (ICR), and sensitivity factors so that normoglycemia was achieved within the week prior to the study.

On study days, participants were admitted to the hospital, and meals were served at 6.30 pm. The meal was a high fat/high energy density test meal containing 80 g carbohydrate (34%), 70 g fat (66%), and 35 g protein (14%). The total energy of the meal was 4563 kJ (1090 kcal).

The detailed composition and ingredients of the test meal are given in Supplementary Table 1. Participants should have no glucose fluctuations in the two hours before study entry as measured by CGMS, no correction boluses for at least four hours before test meal consumption, and fasting glycemia in the range 70-180 mg/dL on both study days.

On the first study day participants calculated the insulin dose for the test meal by CC. On the second study day, the insulin dose was calculated using the PA algorithm or modified PA algorithm. The cross-over design allowed the

Table 1. Characteristics of the study subjects

	Participants (n = 20) Median (min-max)
Female/male (n)	11/9
Age (years)	14.42 (9-21)
BMI z-score	0.13 (-1.17-1.9)
Diabetes duration (years)	7 (2.08-17.83)
HbA1c (%)	7.3 (5.7-10.4)
HbA1c (mmol/mol)	56 (39-90)
Insulin (IU/kg/day)	0.8 (0.55-0.97)
Basal insulin to total daily insulin (%)	42 (33-64)

BMI: body mass index, HbA1c: hemoglobin A1c, min-max: minimum-maximum

comparison of each patient eating the same meal twice but using the two different counting methods to calculate an appropriate insulin dose. Consumption of the test meal was completed in 20 minutes under supervision by a caregiver and a research team dietician. The flow diagram of the study is presented in Figure 1.

Algorithm for Calculating and Delivering Preprandial Bolus Insulin

Initially, two insulin algorithms were used to calculate preprandial insulin dose: CC and PA. However, during the study half of the patients using the published PA experienced hypoglycemic events and so the PA was modified for the remaining patients.

For CC each participant's individualized ICR was expressed as insulin per one carbohydrate unit (CU = 10 g carbohydrate) and this was used to calculate individual preprandial insulin doses, which were delivered in a standard bolus.

For PA, the insulin dose was calculated according to the carbohydrate content (1 CU = 10 g carbohydrate) but also took into account the fat and protein content (1 FPU = 100 kcal from fat and protein) of the test meal. The participant's individualized ICR was calculated, expressed as insulin per 1 CU and 1 FPU with the same insulin ratio used for 1 CU or 1 FPU. The total insulin dose was delivered for CU in a standard bolus and FPU in an extended bolus. According to Pańkowska et al. (15), the extended bolus should be given for eight hours for a meal containing ≥4 FPU. As the test meal had 7.7 FPUs, the extended bolus was delivered over eight hours.

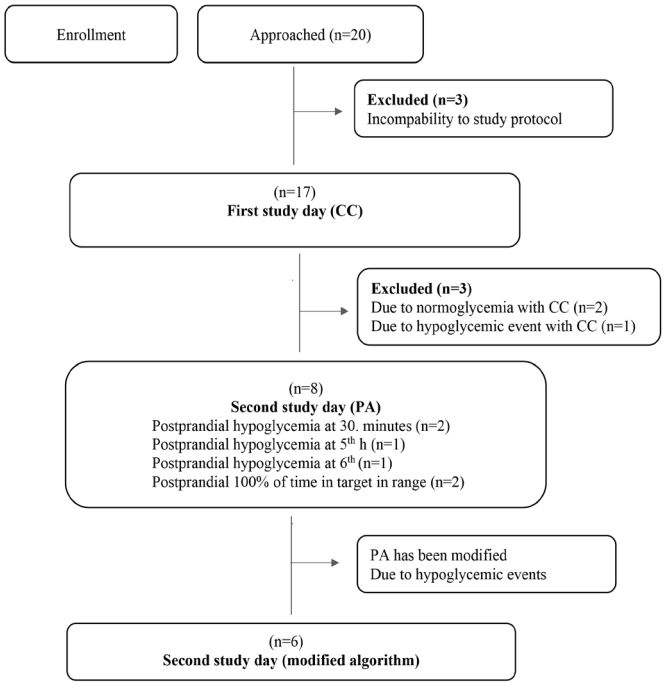

Figure 1. Flow diagram

CC: carbohydrate counting, PA: Pańkowska algorithm

The PA was subsequently modified as follows. The PA participant's individualized ICR was calculated, expressed as insulin per 1 CU and 1 FPU. However, 1 FPU was now equated to 150 kcal from fat and protein (the same insulin ratio was used for 1 CU or 1 FPU). The test meal was now calculated to contain five FPUs, and so still required the extended bolus to be delivered over eight hours (15).

During the study period, no additional meals, snacks, or other food and no physical activity were allowed and no correction boluses were administered.

Measurement of Glycemia

Postprandial glycemia was measured by CGMS during the subsequent twelve hours. Postprandial glucose excursions were evaluated by reference to the International Consensus on Use of Continuous Glucose Monitoring, as described by Danne et al. (16) level 1 hypoglycemia glucose value of 70-54 mg/dL (3.9-3.0 mmol/L) with or without symptoms, level 2 hypoglycemia glucose level of < 54 mg/dL (< 3.0 mmol/L) with our without symptoms; level 1 hyperglycemia glucose value of > 180 mg/dL (10 mmol/L) and glucose ≤250 mg/dL (13.9 mmol/L) and level 2 hyperglycemia glucose level of > 250 mg/dL (13.9 mmol/L). If hypoglycemia occurred during the study period, participants consumed 0.3 g/kg carbohydrate (white sugar). The data of the participants experiencing hypoglycemic events with the PA were not included in the twelve-hour data analysis of the study.

Primary and Secondary Outcomes

The primary outcomes were glucose area under the curve (AUC) and % of time in range (TIR) according to CC, PA, and modified PA algorithms. The secondary outcomes were the number of hypoglycemic events over the study period which were verified by capillary blood glucose measurements.

Statement of Ethics

The study protocol was reviewed and approved by the Ege University Medical Faculty Ethics Committee, approval number: 16-12.1/44 and the Ministry of Health of Turkey (date: 22.07.2016). This trial was registered at www.clinicaltrials.gov as NCT05152121.

Statistical Analysis

SAS® software (SAS system for Windows, version 9.3; SAS Institute, Cary, NC, USA) was used for statistical analysis. Significance was assumed with a p value of < 0.05. AUC calculation was performed according to the Trapezoidal

rule. The Wilcoxon Rank Sums test and Wilcoxon Signed Rank test were used to compare between and within-group differences in terms of AUC. The minimum sample size for p = 0.01 and 1-p = 0.99 was calculated as 18, with an error of 4% (d = 0.04) at the 95% ($\alpha = 0.05$) confidence interval limits for 0.80 power.

Results

Characteristics of the Participants

Twenty adolescents participated in the study. Their ages ranged from 9-18 years and the sex mix was 11 male (55%) and nine female (45%). One was excluded due to incompatibility during the preparation of the study, and two refused the consumption of the test meal on the second study day. Seventeen of the participants completed the CC protocol, while eight of them used the standard PA, and six of them used the modified algorithm on the second study day (Figure 1). The study session was not completed due to one hypoglycemic event and two episodes of normoglycemia after the test meal on CC as there may be a risk of hypoglycemia when additional doses are given for fat and protein on the second study day.

Demographic data of study participants are given in Table 1. Participants median (range) age was 14.4 (9-18) years, BMI z-score was 0.13 (-1.17-1.9), duration of diabetes was 7 (2-17.8) years, HbA1c was 56 (39-90) mmol/L [equivalent to 7.3% (5.7-10.4%)], total insulin requirement was 0.8 (0.55-0.97) IU/kg and basal insulin ratio was 42% (33-64). The level of HbA1c was not a criterion for inclusion in the study, because the doses of insulin were adjusted for seven days before the study. The median basal insulin dose was 0.34 IU/kg (0.24-0.47).

First Study Day

After consumption of test meal calculating insulin dose by CC (n = 17), one patient was hypoglycemic postprandially at the second hour and two patients remained normoglycemic for 12 hours. This resulted in these three patients being excluded from the second part of the study since the additional dose of insulin calculated for fat and protein can cause hypoglycemia. For the first postprandial 12 hours CC patients were in TIR in 26.4% and experienced level 1 and level 2 hyperglycemia for 28.5% and 17.4%, respectively.

Second Study Day

On the second day of the study, 4/8 participants using the standard PA algorithm developed hypoglycemia at a median (range) time of 6.25 (5.58-7.91) hours. The PA algorithm was then modified as previously described. In the six participants using the modified PA, hypoglycemia did not develop in the ensuing 12 hours.

There were no significant differences between HbA1c, BMI z-score, insulin dose/kg, and diabetes duration between participants who used PA and the modified algorithm. There was no difference in the time spent in normoglycemia in the first two hours after meal consumption in the three groups; median AUC for PA = 119.99; modified PA = 91.64, and CC = 69.75). The AUC for the initial two hours was decreased non-significantly in the CC group indicating less normoglycemia (data not shown).

There was no significant difference between the different methods of insulin dose calculation during the first 5.58 hours postprandial when the first hypoglycemic event developed with unmodified PA. Although it was not statistically significant, the AUC decreased in level 1 and level 2 hyperglycemia with the modified algorithm (Table 2).

There was no significant difference in the AUC of the participants who completed the PA algorithm with no hypoglycemic event (n = 4) when compared to participants who used the modified algorithm (n = 6) over the postprandial 12 hours. Although not statistically significant, the median AUC in level 2 hyperglycemia decreased with the modified algorithm (Table 3).

Although median time spent in normoglycemia was decreased with the modified PA compared to unmodified PA, level 2 hyperglycemia was decreased in the modified algorithm for the postprandial period 2-12 hours (Table 4).

Table 2. Postprandial AUC for 5.58th hour (time first hypoglycemic event detected)

AUC	PA hypoglycemia (-) Median (min-max) n = 4	PA hypoglycemia (+) Median (min-max) n = 4	Modified algorithm Median (min-max) n = 6
< 3 mmol/L (< 54 mg/dL)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
3-3.8 mmol/L (54-69 mg/dL)	0.0 (0.0-0.0)	0.0 (0.0-11.1)	0.0 (0.0-5.6)
3.9-10 mmol/L (70-180 mg/dL)	201.9 (23.5-293.5)	290.9 (237.7-340.7)	186.8 (79.1-285.5)
10.1-13.9 mmol/L (181-250 mg/dL)	39.6 (0.0-96.3)	7.5 (0.0-11.9)	24.9 (0.0-89.5)
> 13.9 mmol/L (> 250 mg/dL)	22.5 (0.0-69.7)	0.0 (0.0-0.0)	0.0 (0.0-200.2)

AUC: area under the curve (mg/dL x min), PA: Pańkowska algorithm, min-max: minimum-maximum

Table 3. Postprandial glucose AUC with PA and modified algorithm for 0-12 hours

AUC	PA with no hypoglycemia Median (min-max) n = 4	Modified algorithm Median (min-max) n = 6	p*
< 3 mmol/L (< 54 mg/dL)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	1.000
3-3.8 mmol/L (54-69 mg/dL)	0.0 (0.0-7.471)	0.0 (0.0-24.468)	0.667
3.9-10 mmol/L (70-180 mg/dL)	277.950 (202.579-589.268)	218.856 (15.735-461.048)	0.476
10.1-13.9 mmol/L (181-250 mg/dL)	27.446 (0.0-94.126)	84.090 (0.0-295.821)	0.609
> 13.9 mmol/L (> 250 mg/dL)	23.037(0.0-66.296)	0.0 (0.0-259.687)	0.005

*Wilcoxon signed-rank test.
AUC: area under the curve (mg/dL x min), PA: Pańkowska algorithm, min-max: minimum-maximum

Table 4. AUC for PA and modified algorithm total time for 2-12 hours

Total time	PA with no hypoglycemia Median (min-max) n = 4	Modified algorithm Median (min-max) n = 6	p*
< 3 mmol/L (< 54 mg/dL)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	1.000
3-3.8 mmol/L (54-69 mg/dL)	0.0 (0.0-11.0)	0.0 (0.0-24.0)	0.666
3.9-10 mmol/L (70-180 mg/dL)	90.0 (69.0-115.0)	80.0 (2.0-104.0)	0.476
10.1-13.9 mmol/L (181-250 mg/dL)	20.5 (0.0-28.0)	41.5 (0.0-116.0)	0.347
> 13.9 mmol/L (> 250 mg/dL)	12.5 (0.0-30.0)	0.0 (0.0-76.0)	0.500

*Wilcoxon signed-rank test.
AUC: area under the curve (mg/dL x min), PA: Pańkowska algorithm, min-max: minimum-maximum

Discussion

This is the first study to compare traditional mealtime insulin dose estimation (CC) with one novel (PA) and one new insulin-dosing algorithm (the modified PA) for high fat/high energy density meals for the postprandial 12-hour period. Our results showed an increased time in normoglycemia without hypoglycemia with the new algorithm but an increased incidence of hypoglycemia using the PA when compared with the traditional CC. The insulin dose calculated for PA and the modified algorithm was 94% and 64% higher, respectively, than the insulin dose for the CC method. This novel modified algorithm achieves better glycemic control with less hypoglycemia in children and adolescents with T1D with a longer duration of follow-up than the PA.

Using PA, 50% of the patients were hypoglycemic at a median postprandial time of 6.25 hours. This finding was similar to previous studies which have compared CC and the unmodified PA for high-fat meals and high protein meals (4,17,18,19,20). In contrast, Pańkowska et al. (4) reported no difference in hypoglycemia between PA and CC but postprandial glucose monitoring was only performed for two hours. In our study, when the insulin dose was calculated with PA, hypoglycemia occurred around six hours postprandially. However, no hypoglycemic event occurred in patients using the modified algorithm for the whole 12 hours monitoring period and AUC was lower than for CC. Similar to our modified algorithm experience, Smith et al. (19) found that an additional 60% of the meal insulin dose significantly reduces the glycemic excursion up to postprandial five hours without increasing the incidence of hypoglycemia. AUC for normoglycemia, and level 1 and level 2 hyperglycemia was similar during the latter ten hours of monitoring (2-12 hours postprandial) with both the modified algorithm and PA, with the caveat that only patients without hypoglycemia were included. Compared to the PA, the median time spent in level 2 hyperglycemia decreased and the time spent in normoglycemia decreased with the modified algorithm (Table 4). Thus the PA resulted in more time spent in normoglycemia, but at the cost of an increased risk of hypoglycemia.

There is no consensus about the insulin dose required for high fat/high energy density meals. ISPAD guidelines recommend an increase of 15% to 20% of the bolus for high fat/high protein meals (14). A systematic review for high-fat meals (≥40 g of fat), recommended bolus dose increase up to 30-35% accompanied by using combo bolus with 50/50 split over 2-2.5 hours and review late postprandial glucose and adjust total insulin dose as indicated (21). Wolpert et al. (6) suggested a mean insulin dose increase of 42% for a high-fat meal (60 g fat) compared to a low-fat meal (10 g fat), with marked significant individual differences, with some

participants requiring more than twice as much insulin while others required no extra insulin. We showed non-significant lower AUC for normoglycemia than CC with the modified algorithm with an insulin dose increase of 64% for a high-fat meal (70 g fat), with no hypoglycemia for 12 hours. In the current study, a postprandial observation period of 12 hours was chosen. Wolpert et al. (6) demonstrated in their closed-loop study that after a 60 g high-fat meal, the impact of added fat continues for at least five hours. We, therefore, designed a longer observation period to assess the effect of insulin on meals with high fat/high energy density meals. Since participants consumed the test meal in the evening, we were able to follow for a full twelve hours.

Study Limitations

Our study has strengths and limitations. One of the strengths was that glycemic stability was evaluated daily for one week before the study and this also allowed the optimization of individual carbohydrate/insulin ratio and sensitivity factors for each participant. Another strength of the study was the extended postprandial monitoring using CGMS. The main limitation of this study was the small sample size, partly due to poor adherence to the study protocol in adolescent participants. However, the findings are of interest and may provide an improvement on the original PA so we believe that this warrants confirmation of the findings using larger group sizes, which should be adequately powered.

This study was also limited to participants using IPT to take advantage of more sensitive insulin dosing and the use of the dual-wave bolus option. Therefore, the study should also be performed in patients using multiple daily injections.

Conclusion

Although carbohydrates are the primary determinant of postprandial glucose levels, recent research has shown that insulin dosing based on carbohydrate quantity alone is inadequate for optimal glycemic control after a high fat/high energy density meal in individuals with T1D. The dose and delivery type of preprandial insulin may need adjustment, not only to carbohydrate quantity but also to the fat content of the meal to achieve stable postprandial normoglycemia. However, our study has shown marked inter-individual differences in response to the test meal. We, therefore, suggest that, due to these differences and the lack of large-scale prospective data, an individualized approach to insulin dosing for high fat/high energy density meals should be adopted currently. This can be done by evaluating food diaries and the use of postprandial glucose monitoring. This may represent the present best practice for children and adolescents with T1D.

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Ethics

Ethics Committee Approval: The study was approved by the Ege University of Local Ethics Committee (protocol number: 16-12.1/44, date: 20.12.2016).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Günay Demir, Hafize Çetin, Samim Özen, Concept: Yasemin Atik Altınok, Damla Gökşen, Design: Yasemin Atik Altınok, Damla Gökşen, Data Collection or Processing: Günay Demir, Hafize Çetin, Analysis or Interpretation: Yasemin Atik Altınok, Günay Demir, Literature Search: Yasemin Atik Altınok, Günay Demir, Writing: Yasemin Atik Altınok, Samim Özen, Şükran Darcan, Damla Gökşen.

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References

1. American Diabetes Association; Bantle JP, Wylie-Rosett J, Albright AL, Apovian CM, Clark NG, Franz MJ, Hoogwerf BJ, Lichtenstein AH, Mayer-Davis E, Mooradian AD, Wheeler ML. Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association. Diabetes Care 2008;31(Suppl 1):61-78.
2. Jones SM, Quarry JL, Caldwell-McMillan M, Mauger DT, Gabbay RA. Optimal insulin pump dosing and postprandial glycemia following a pizza meal using the continuous glucose monitoring system. Diabetes Technol Ther 2005;7:233-240.
3. Wolever TMS, Mullan YM. Sugars and fat have different effects on postprandial glucose responses in normal and type 1 diabetic subjects. Nutr Metab Cardiovasc Dis 2011;21:719-725.
4. Pańkowska E, Błazik M, Groele L. Does the Fat-Protein Meal Increase Postprandial Glucose Level in Type 1 Diabetes Patients on Insulin Pump: The Conclusion of a Randomized Study. Diabetes Technol Ther 2012;14:16-22. Epub 2011 Oct 20
5. Smart CE, Evans M, O'Connell SM, McElduff P, Lopez PE, Jones TW, Davis EA, King BR. Both dietary protein and fat increase postprandial glucose excursions in children with type 1 diabetes, and the effect is additive. Diabetes Care. 2013;36:3897-3902. Epub 2013 Oct 29
6. Wolpert HA, Atakov-Castillo A, Smith SA, Steil GM. Dietary fat acutely increases glucose concentrations and insulin requirements in patients with type 1 diabetes: implications for carbohydrate-based bolus dose calculation and intensive diabetes management. Diabetes Care 2013;36:810-816. Epub 2012 Nov 27

7. Paterson MA, Smart CEM, Lopez PE, Howley P, McElduff P, Attia J, Morbey C, King BR. Increasing the protein quantity in a meal results in dose-dependent effects on postprandial glucose levels in individuals with Type 1 diabetes mellitus. Diabet Med 2017;34:851-854. Epub 2017 Mar 19
8. 8 van der Hoogt M, van Dyk JC, Dolman RC, Pieters M. Protein and fat meal content increase insulin requirement in children with type 1 diabetes - Role of duration of diabetes. J Clin Transl Endocrinol 2017;10:15-21.
9. Neu A, Behret F, Braun R, Herrlich S, Liebrich F, Loesch-Binder M, Schneider A, Schweizer R. Higher glucose concentrations following protein- and fat-rich meals - the Tuebingen Grill Study: a pilot study in adolescents with type 1 diabetes. Pediatr Diabetes. Pediatr Diabetes 2015;16:587-591. Epub 2014 Oct 20
10. Paterson MA, Smart CE, Lopez PE, McElduff P, Attia J, Morbey C, King BR. Influence of dietary protein on postprandial blood glucose levels in individuals with Type 1 diabetes mellitus using intensive insulin therapy. Diabet Med 2016;33:592-598. Epub 2015 Dec 6
11. Evans M, Smart CEM, Paramalingam N, Smith GJ, Jones TW, King BR, Davis EA. Dietary protein affects both the dose and pattern of insulin delivery required to achieve postprandial euglycaemia in Type 1 diabetes: a randomized trial. Diabet Med 2019;36:499-504. Epub 2019 Feb 20
12. Gingras V, Bonato L, Messier V, Roy-Fleming A, Smaoui MR, Ladouceur M, Rabasa-Lhoret R. Impact of macronutrient content of meals on postprandial glucose control in the context of closed-loop insulin delivery: A randomized cross-over study. Diabetes Obes Metab 2018;20:2695-2699. Epub 2018 Jul 18
13. American Diabetes Association Professional Practice Committee. 5. Facilitating Behavior Change and Well-being to Improve Health Outcomes: Standards of Medical Care in Diabetes-2022. Diabetes Care. 2022;45(Suppl 1):60-82.
14. Smart CE, Annan F, Higgins LA, Jelleryd E, Lopez M, Acerini CL. ISPAD Clinical Practice Consensus Guidelines 2018: Nutritional management in children and adolescents with diabetes. Pediatr Diabetes 2018;19(Suppl 27):136-154.
15. Pańkowska E, Szypowska A, Lipka M, Szpotańska M, Błazik M, Groele L. Application of novel dual wave meal bolus and its impact on glycated hemoglobin A1c level in children with type 1 diabetes. Pediatr Diabetes 2009;10:298-303. Epub 2008 Oct 20
16. Danne T, Nimri R, Battelino T, Bergenstal RM, Close KL, DeVries JH, Garg S, Heinemann L, Hirsch I, Amiel SA, Beck R, Bosi E, Buckingham B, Cobelli C, Dassau E, Doyle FJ, Heller S, Hovorka R, Jia W, Jones T, Kordonouri O, Kovatchev B, Kowalski A, Laffel L, Maahs D, Murphy HR, Nørgaard K, Parkin CG, Renard E, Saboo B, Scharf M, Tamborlane WV, Weinzimer SA, Phillip M. International Consensus on Use of Continuous Glucose Monitoring. Diabetes Care 2017;40:1631-1640.
17. Lopez PE, Evans M, King BR, Jones TW, Bell K, McElduff P, Davis EA, Smart CE. A randomized comparison of three prandial insulin dosing algorithms for children and adolescents with Type 1 diabetes. Diabet Med 2018;35:1440-1447. Epub 2018 Jun 19
18. Kordonouri O, Hartmann R, Remus K, Bläsig S, Sadeghian E, Danne T. Benefit of supplementary fat plus protein counting as compared with conventional carbohydrate counting for insulin bolus calculation in children with pump therapy. Pediatr Diabetes 2012;13:540-544. Epub 2012 Jul 6
19. Smith T, Fuery M, Knight B, Harris M, Howley P, King B, Smart C. In young people with Type 1 Diabetes using insulin pump therapy giving an additional sixty percent of the mealtime insulin dose improves postprandial glycemia following a high fat, high protein meal. 44th Annual Conference of ISPAD, P234. Available from: http://medialibrary.ispad.cyim.com/mediatheque/media.aspx?mediaId = 49532&channel = 9857
20. Piechowiak K, Dżygało K, Szypowska A. The additional dose of insulin for high-protein mixed meal provides better glycemic control in children with type 1 diabetes on insulin pumps: randomized cross-over study. Pediatr Diabetes 2017;18:861-868. Epub 2017 Jan 24
21. Bell KJ, Smart CE, Steil GM, Brand-Miller JC, King B, Wolpert HA. Impact of fat, protein, and glycemic index on postprandial glucose control in type 1 diabetes: implications for intensive diabetes management in the continuous glucose monitoring era. Diabetes Care 2015;38:1008-1015.

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ORIGINAL ARTICLE

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Evaluation of The Effects of Carob (*Ceratonia siliqua* L.) Fruits on the Puberty of Rats

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What is already known on this topic?

Natural and organic nutrition, which parents prefer to support their children's immunity and development, can sometimes act as endocrine disruptors due to the constituents of the food and the frequency of consumption.

What this study adds?

This is the first study showing that the use of carob in the prepubertal period causes early puberty and tissue damage by increasing doses. *C. siliqua,* preferred by parents for organic nutrition, induces early puberty and increases spermiogenesis and folliculogenesis. Furthermore, antioxidant mechanisms can come into effect and cause tissue damage at high doses.

Abstract

Objective: This study was planned to determine the effects of carob use on puberty because of the observation of early puberty or pubertal variants due to the long-term use of carob in our clinic.

Methods: Forty-eight Wistar albino rats, on postnatal day 21, were assigned into two groups female (n = 24) and male (n = 24). Groups were divided into four groups Control, and Carob-150, Carob-300, and Carob-600. *Ceratonia siliqua* L. extract was given to rats in a 0.5% carboxymethylcellulose (CMC) solution. CMC (0.5%) was given to the control, *Ceratonia siliqua* L. extract was given 150 mg/kg/day to the Carob-150, 300 mg/kg/day to the Carob-300, 600 mg/kg/day to the Carob-600 by oral gavage. The treatments were performed once daily until the first sign of puberty. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, total testosterone, leptin, glutathione, glutathione peroxidase (GPx), and malondialdehyde were measured by commercial rat-specific ELISA kits. Testis, uterus and ovarian tissue were examined histologically.

Results: The median time of preputial separation in male rats was 38th, 31st, 31st, and 31st days in the Control, Carob-150, Carob-300, and Carob-600 groups, respectively (p = 0.004). The median day of vaginal opening day was the 39th, 31st, 34th, and 31st days in the Control, Carob-150, Carob-300, and Carob-600 groups, respectively (p = 0.059). FSH, LH, testosterone (male), estradiol (female) and leptin levels of the groups were similar. However, GPx levels were higher in male and female animals given *C. siliqua* extract compared to the Control (male p = 0.001 and female p = 0.008). Testicular and ovarian tissues were concordant with the pubertal period in all groups. As the dose

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of *Ceratonia siliqua* extract increased, it induced spermatogenesis and spermiogenesis, causing abnormal changes, such as ondulation in the basement membrane, capillary dilatation, and increased congestion in males. In females, edema in the medulla gradually increased with increased dosage, and granulosa cell connections were separated in Carob-300 and Carob-600 groups.
Conclusion: This study demonstrated that *C. siliqua* caused early puberty and increased spermiogenesis and folliculogenesis. Antioxidant mechanisms were impaired with increasing dose, possibly leading to tissue damage at high doses.
Keywords: *Ceratonia siliqua* L., carob, puberty, antioxidant

Introduction

In recent years, chemical substances such as sweeteners, flavoring, and preservatives have been increasingly used in foods. These foods are the primary source of chemicals in our daily lives. Moreover, natural and organic nutrition can sometimes act as endocrine disruptors due to the content of the food and the frequency of consumption (1,2,3,4).

After weaning, parents tend to prefer organic foods, such as carob, for their children. Carob (Harnup-*Ceratonia siliqua* L.) is a plant species belonging to the legumes (*Fabaceae*) family and grows naturally in a Mediterranean climate (5). It produces a pod-like fruit, consisting of two parts, fruit (pod), and seeds. Carob is a sweet fruit considered a healthy food by families and may be consumed as raw fruit, flour or syrup. These forms are obtained from the fruity pod of the carob (6). Carob is also a natural sweetener and a source of vegetable carbohydrates. *C. siliqua* is rich in polyphenols and flavonoids in addition to its carbohydrate, protein, and fat content (7,8). Due to its rich polyphenol and mineral content, it is used especially for enhancing immune function. Several studies have demonstrated the antioxidant, anti-inflammatory, analgesic, and lipid-lowering effects of *C. siliqua* and there is also evidence of blood sugar regulation (9,10,11,12).

When the medical histories of children attending our clinic because of early puberty and puberty variants, anecdotal evidence emerged of long-term and regular use of *C. siliqua* in some cases. This animal study was planned to experimentally investigate the effects of long-term use of *C. siliqua* on puberty. To the best of our knowledge, this is the first study to examine the effect of *C. siliqua* on puberty.

Methods

Ceratonia siliqua L. Extract Preparation

Ceratonia siliqua L. fruits were provided from Doğal Kurucu Gıda Sanayi ve Ticaret Limited Şirketi, Malatya, Turkey as collected fruit material from Tarsus district of Mersin province, Turkey, in 2021. After the fruits (500 g) were dried and separated from their seeds and crushed, a 50% aqueous-alcoholic extract was prepared. The extract was concentrated in a Rotavapor® R-100 (Buchi, Switzerland) under reduced pressure and at a temperature not exceeding 40 °C. The resulting dry extract was prepared to be given to rats in a 0.5% carboxymethylcellulose (CMC) aqueous solution.

When previous *in vivo* studies on carob was reviewed, aqueous-alcoholic fruit extracts were administered to animals by oral gavage at doses of 50 mg/kg to 2000 mg/kg. In studies on the reproductive system, it has been reported that the extracts have been studied at dose ranges of 150 mg/kg and 600 mg/kg (13,14). In light of this, the dosing groups for experimanetal animals were planned to be 0 mg/kg, 150 mg/kg, 300 mg/kg, and 600 mg/kg and designated control, Carob-150, Carob-300, and Carob-600, respectively. According to the guideline, the tested extract doses in rats (150, 300, 600 mg/kg) can be converted to a human dose based on body surface area as 0.72 mg, 1.44 mg, 2.88 mg per day for a child weighing 30 kg (15). The extracts were prepared and administered to the animals in a 0.5% CMC aqueous solution. The same volume of the vehicle without extract (0.5% CMC) was administered orally to the control.

Animals and Study Design

Forty-eight Wistar albino rats weaned on postnatal day 21, were assigned into two groups female (n = 24) and male (n = 24). Animals were kept in a 12-hour light and 12-hour dark cycle and fed standard rodent chow (Korkuteli Food Industry, Turkey). Female and male groups were divided into control, Carob-150, Carob-300, and Carob-600 with six animals in each group for the male and female sub-groups. CMC (0.5%) was given to the control, and *Ceratonia siliqua* L. extract was given at 150 mg/kg/day to the Carob-150, 300 mg/kg/day to the Carob-300, and 600 mg/kg/day to the Carob-600 by oral gavage. The treatments were performed once daily (6 days/week), at the same time (between 8:00 and 10:00 AM), until the first sign of puberty. The first sign of puberty in male rats is preputial separation and for female rats is the first oestrus stage following vaginal opening. Body weights were recorded, and weight gain was calculated by the formula weight gain (%) = (Last day-First day)/First day. Vaginal cytology was performed to determine the estrus stage (cornified epithelial cells) after vaginal opening. Vaginal secretion was collected with a plastic pipette filled

with 10 IU of normal saline (NaCl 0.9%) by inserting the tip into the vagina. The vaginal fluid was dripped onto glass slides and was evaluated under the light microscope (Leica CME Microscope, 1349522X, NY, USA, 40x objective lenses) according to Cora et al. (16). Female rats in the first estrus stage and preputial separated male rats were euthanized by taking intracardiac blood under ketamine 45 mg/kg and xylazine 5 mg/kg anesthesia.

This study was performed in Gazi University Laboratory Animal Breeding and Experimental Research Center and approved by the Ethical Animal Research Committee of Gazi University (protocol no: G.Ü.ET-21.053, date: 09.07.2021). The experimental procedures and animal care are conducted per the EU Directive 2010/63/EU.

Biochemical Methods

Collected blood was centrifuged at 3000 rpm for 10 minutes at 4 °C and stored at -80 °C.

EA0015Ra rat follicle-stimulating hormone (FSH), EA0013Ra rat luteinizing hormone (LH), E0259Ra rat testosterone, E0174Ra rat estradiol, E0561Ra rat leptin, E1101Ra rat glutathione, E1759Ra rat glutathione peroxidase (GPx) (antioxidative markers), and E0156Ra rat malondialchehyche (MDA) as an oxidative marker were measured (Bioassay Technology Laboratory, Shangai, China).

Histopathological Methods

In the female rat groups, after euthanasia, ovaries and uterus were dissected, and ovarian (right and left) and uterus weights were measured. After euthanasia, testes (right and left separately) were dissected in male rat groups, and their lengths were measured. Uterus, ovarian (single), and testis (single) were fixed in Bouin's fixative, paraffin blocks were obtained, and 4-5 micron-thick sections were taken from the paraffin blocks. Sections taken were stained with hematoxylin-eosin. Ten sections from each rat tissue were evaluated, and data were obtained by examining ten independent fields in each section. The histomorphological changes in the obtained samples were examined with light microscopy using the Leica DM4000 (Leica, Wetzlar, Germany) computer-assisted imaging system. Captured images were evaluated using the Leica-Qwin program.

Statistical Analysis

Statistical Package for the Social Sciences, version 26 was used for statistical analysis (IBM Inc., Armonk, NY, USA). The Kruskal-Wallis test was used when comparing the medians of four independent groups in the data that did not fit the normal distribution, and the Mann-Whitney U test and the Spearman's correlation test were used when comparing the medians of two independent groups. Bonferroni correction was used in *post-hoc* tests. Statistically, $p < 0.05$ was considered significant. A power analysis was performed using GPower version 3.1.9.7 to determine the minimum sample size required of male and female rat groups to test the study hypothesis. Results indicated that a sample size of $n = 18$ is required to achieve 80% power for detecting a large effect at a significance of $\alpha = 0.05$.

Results

Male Results

At the beginning of the study, the mean weight of the male rat groups control, Carob-150, Carob-300, and Carob-600 were 45±2.9, 47±11, 49±10, 48±8.8 g, respectively. The median time of preputial separation in male rats was 38th (37-39th), 31st, (30-35th) 31st (30-34th), and 31st (30-34th) days in control, Carob-150, Carob-300, and Carob-600. The day of the beginning of puberty was statistically significantly earlier in all groups given *C. siliqua* extract than in the control group ($p = 0.04$). The median (minimum-maximum) of % weight gain was 126% (83-133), 72.5% (53-121), 60% (52-82), and 60.1% (45-89) in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. Percentage weight gain (%) was higher in the control group than in all groups given *C. siliqua* extract ($p = 0.006$). A positive correlation was found between weight gain and the day of the beginning of puberty ($p = 0.001$ $r = 0.636$). There was no statistical difference between the groups in terms of FSH, LH, testosterone and leptin (Table 1).

Table 1. The mean ± SD hormone levels of the male groups

	FSH (mIU/mL)	LH (mIU/mL)	Testosteron (ng/L)	Leptin (ng/mL)
Control	14.5 ± 7.3	70 ± 45	263 ± 52	3.3 ± 0.3
Carob-150	11.6 ± 5	92.2 ± 29.5	232.6 ± 25.5	2.8 ± 0.3
Carob-300	6.1 ± 2.8	50.4 ± 38.3	227.2 ± 73.3	3.2 ± 0.6
Carob-600	7.6 ± 2.5	68.6 ± 24.5	285.2 ± 21.5	3.2 ± 0.7
p value	0.051	0.273	0.136	0.319

FSH: follicle stimulating hormone, LH: luteinizing hormone, SD: standard deviation

The mean ± standard deviation (SD) glutathione level was 166.8 ± 28.8 mg/L, 164.5 ± 27.4 mg/L, 158.9 ± 26.8 mg/L and 178.2 ± 15.8 mg/L in the control, Carob-150, Carob-300, and Carob-600 groups, respectively, while the MDA levels were 1 ± 0.3 nmol/mL, 0.9 ± 0.2 nmol/mL, 1 ± 0.2 nmol/mL and 1.2 ± 0.1 nmol/mL in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. There was no difference between the groups regarding glutathione and MDA levels (p = 0.612, p = 0.144). The mean ± SD GPx level was 85.9 ± 11.4 U/mL, 123.4 ± 12.4 U/mL, 128 ± 20.9 U/mL and 144.0 ± 21.7 U/mL in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. There was a significantly higher level of GPx in Carob-300 (p = 0.042) and Carob-600 (p = 0.001) compared to the control group (p = 0.002) (Figure 1).

Testicular lengths in animals from the control group were longer than the groups given *C. siliqua* extract. Although % weight gain was higher in control animals, a positive correlation was found between % weight gain and both right and left testicular length (right testis p = 0.038, r = 0.425 and left testis p = 0.019, r = 0.474). Left testis length was shorter in Carob-150 and Carob-300 than in the control group and this was statistically significant (p = 0.011) (Table 2).

On histological evaluation, the seminiferous tubules of the animals from the control group were observed to have the usual histomorphology with regular contoured basement membranes. Secondary spermatocytes and spermatids were observed in some of the tubules, while spermatogonium and primary spermatocytes were observed in most of the tubules. No sperm were found in the lumen of any tubule. These findings indicated that the spermatogenesis process had just started in the tubules, and cells belonging to the later stages of the series did not differentiate. Leydig cells and capillaries in the interstitial area were in normal formation (Figure 2A).

In the Carob-150 group, the basement membranes of seminiferous tubule contours were regular. This group's secondary spermatocytes and spermatids distribution were similar to the control group. Leydig cells and capillaries in the interstitial area were observed to have normal structure and distribution (Figure 2B).

In the Carob-300 group, the most striking finding was ondulation in the basement membranes of the all seminiferous tubules. The length of the seminiferous epithelium was elongated in most tubules, and secondary spermatocytes, spermatids and spermatazoa were present in all tubules. Congestion and dilatation were detected in the capillaries. Leydig cells were observed to have normal structure (Figure 2C).

The seminiferous tubule basement membrane ondulation, seen in the Carob-300 group, was much more common and prominent in the Carob-600 group. In all tubules, thickened seminiferous epithelium containing every cell type of

Table 2. Mean ± SD testis lengths of the groups

	Right testicular length (mm)	Left testicular length (mm)
Control	14.6 ± 0.9	15 ± 1.1
Carob-150	12.2 ± 2.2	10 ± 4.8
Carob-300	12.1 ± 1.9	11.8 ± 1.4*
Carob-600	12.3 ± 1.6	12.2 ± 2*
p value	0.059	**0.011**

Values represent mean ± SD. *p < 0.05 vs. control.
SD: standard deviation

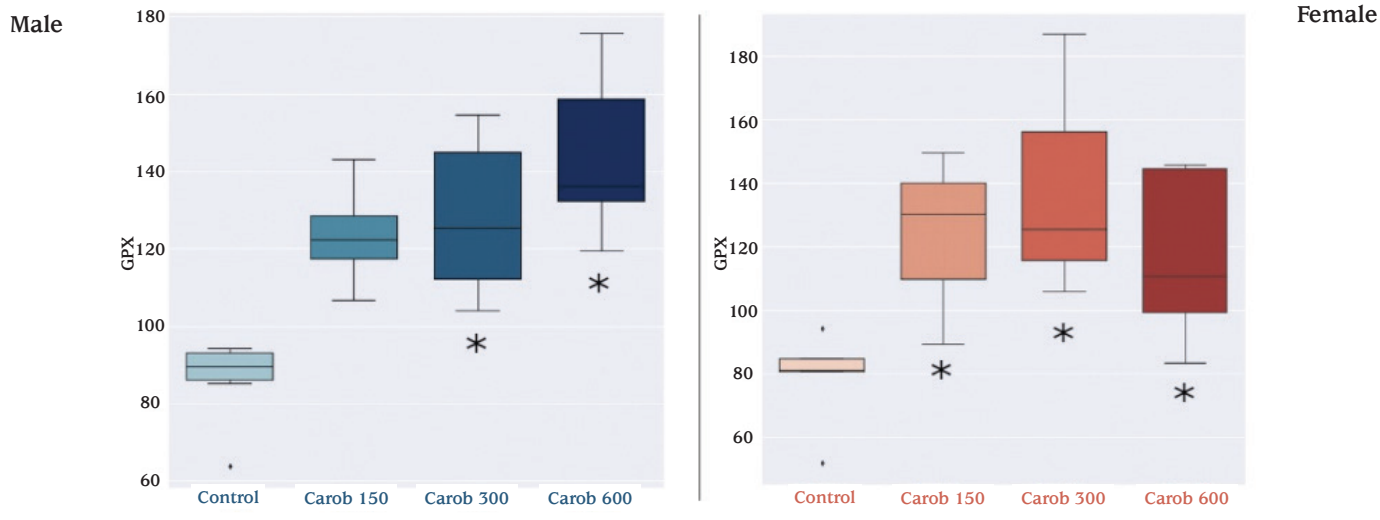

Figure 1. Glutathione peroxidase of male and female rat groups (*p≤0.05)

the spermatogenic series and sperm were evident. The seminiferous tubule walls were significantly thicker. In this group, capillary congestion and dilatation in the interstitial area were more common and prominent. Leydig cells were detected with typical histological structure near these capillaries (Figure 2D).

Testicular tissue was concordant with the pubertal period in all groups. As the dose of carob extract increased, it was observed that spermatogenesis and spermiogenesis became more evident, and although this did not cause structural and numerical changes in Leydig and Sertoli cells there were abnormal changes, such as ondulation in the basement membrane, capillary dilatation, and increased congestion.

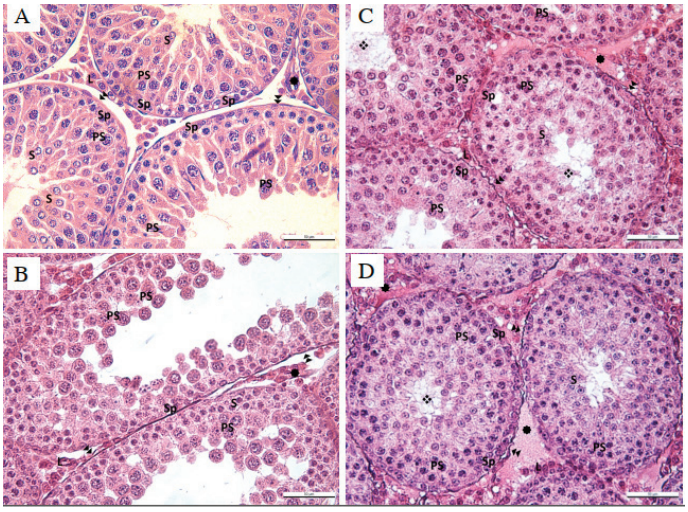

Figure 2. Histological findings of testis. **A)** Control group: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ◂◂: basement membrane of the seminiferous tubule in normal configuration, L: Leydig cell, ✹: normal blood vessel (H&E x200). B) Group 1: Sp: spermatogonia, PS: primary spermatocyte, ◂◂: basement membrane of seminiferous tubule in normal configuration, L: Leydig cell, ✹: normal blood vessel (H&E x200). **C)** Group 2: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ❖: tails of sperm in the stage of spermiogenesis, ◂◂: the corrugated basement membrane of the seminiferous tubule, L: Leydig cell, ✹: congested and dilated blood vessel (H&E x200). **D)** Group 3: Sp: spermatogonia, PS: primary spermatocyte, S: secondary spermatocytes and spermatids, ❖: tails of sperm in the stage of spermiogenesis, ◂◂: densely corrugated seminiferous tubule basement membrane, L: Leydig cell, ✹: extensively congested and dilated blood vessel (H&E x200)

The mean seminiferous tubule thickness for the groups (control, Carob-150, Carob-300, and Carob-600) was 234.3 ± 34.5 µm, 258.9 ± 46.4 µm, 301 ± 36.2 µm, 383.8 ± 76.4 µm, respectively. The seminiferous tubule thickness was significantly higher in Carob-300 and Carob-600 than in control and Carob 150 (p = 0.001).

Female Results

At the beginning of the study, the mean weight of the female rats was 42.5±4.7, 50±9.5, 49±6.7, 51.1±4.5 g in the control, Carob-150, Carob-300 and Carob-600 groups, respectively. The median (minimum-maximum) day of vaginal opening in female rats was 39th (37-39th), 31st (30-35th) 31st (30-34th), and 31st (30-34th) in the control, Carob-150, Carob-300, and Carob-600 groups, respectively. The day of the beginning of puberty was earlier in all groups given *C. siliqua* extract compared to the control group, but this was not significant. The median (minimum-maximum) % weight gain was 165 (127-186) mg/L, 151 (127-197) mg/L, 143 (128-184) mg/L, and 141 (131-156) mg/L in the control group, control, Carob-150, Carob-300, and Carob-600 groups with % weight gain being higher in the control group than in all groups given *C. siliqua* extract, but again this was not significant. A positive correlation was found between weight gain and the time of the beginning of puberty (p = 0.001, r = 0.682).

There were no statistical differences between the groups in terms of FSH, LH, estradiol, and leptin levels (Table 3).

The mean ± SD glutathione levels were 161.7 ± 19.7 mg/L, 162.3 ± 31.1 mg/L, 149.3 ± 18.8 mg/L and 141.5 ± 9.2 mg/L in the control, Carob-150, Carob-300, and Carob-600 groups respectively while the MDA levels were 0.9 ± 0.5 nmol/mL, 1.0 ± 0.0 nmol/mL, 1.0 ± 0.3 nmol/mL and 0.9 ± 0.2 nmol/mL in the same groups. There was no difference between the groups in terms of mean glutathione and MDA levels (p = 0.277 and p = 0.976). The mean ± SD GPx levels were 79.1 ± 14.3 U/mL, 121.4 ± 25.5 U/mL, 123.9 ± 32.1 U/mL and 113.7 ± 25.8 U/mL in the Control, Carob-150, Carob-300, and Carob-600. GPx levels were higher in all groups given

Table 3. The mean ± SD hormone levels of the female groups

	FSH (mIU/mL)	LH (mIU/mL)	Estradiol (ng/L)	Leptin (ng/mL)
Control	15.8 ± 4	65.7 ± 23	99.9 ± 37.4	3.2 ± 0.6
Carob-150	12.3 ± 4.3	101.3 ± 45.3	105.9 ± 30	3.3 ± 0.4
Carob-300	6.1 ± 6	56.6 ± 41.8	89.7 ± 40.4	3.2 ± 0.3
Carob-600	11.4 ± 5.9	95.6 ± 33.6	88.6 ± 28	3.1 ± 0.5
p value	0.051	0.167	0.81	0.963

FSH: follicle stimulating hormone, LH: luteinizing hormone, SD: standard deviation

C. siliqua extract compared to the control group (p = 0.008) (Figure 1).

There was no difference between the groups in terms of ovarian length, ovarian weight or uterus weight (p > 0.05) (Table 4).

On histological evaluation, the ovarian tissue of the control group was observed to exhibit normal histomorphology, compatible with puberty. While all follicles belonging to the developmental stage were seen in the sections, no corpus luteum formation was observed in any section. (Figure 3A).

In the Carob-150 group, vasodilatation and edema were evident in the medulla. Typical follicle structures at the developmental stage were observed in the cortex. However, there was no evidence of corpus luteum in this group (Figure 3B).

In the Carob-300 group, vasodilatation and findings of edema of the medulla edema were increased compared to the Carob-150 group (Figure 3C). In the multilaminar primary follicle structure, separation and pericellular edema were observed in the junctional units between the granulosa cells (Figure 3D in 3C). It is possible that these type of follicles may lead to atresia.

In the Carob-600 group, corpus luteum-like structures was detected in many areas. Degenerative changes were detected in the granulosa cell layer in the multilaminar primary follicle structure. Interstitial edema was observed in regions containing granulosa cells. Edema and congestion in the medulla were found most frequently and markedly in this group. Similarly, corpus luteum was only observed in this group (Figure 3E).

Ovarian tissue was concordant with the pubertal period in all groups and primary, antral and tertiary follicles were observed in all groups but corpus luteum was only seen in the Carob-600 group. Edema and congestion in the medulla gradually increased in all groups starting from the group that received the lowest dose of carob extract. Separation of granulosa cell connections was detected in the two highest dose groups (Carob-300 and Carob-600).

Uterine tissue was observed to exhibit normal structure through all layers in the control and Carob-150 groups (Figure 4A). However, relatively minor edema was observed in the lamina propria in Carob-150 animals (Figure 4B). In Carob-300 vasodilatation and edema in the lamina propria and congestion in the muscle layer were observed, in contrast to the control and Carob-150 groups (Figure 4C). In

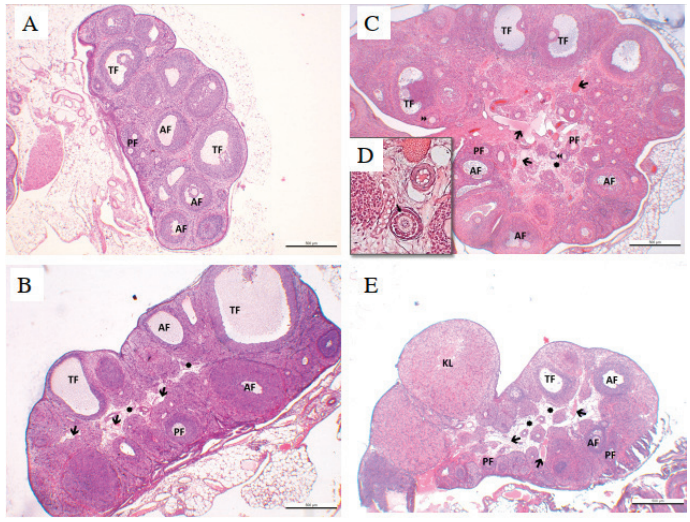

Figure 3. Histological findings of over. **A)** Control: Normal histomorphological ovarian tissue. At various developmental stages, with their normal structures PF: primary follicle, AF: antral follicle, TF: tertiary follicle (H&E x40). **B)** Carob-150: Vasodilatation in all areas, especially in the medulla (➔), edema in the medulla (✹). At various developmental stages, with their normal structures PF: primary follicle, AF: antral follicle, TF: tertiary follicle (H&E x40). **C)** Carob-300: Vasodilatation increased in all areas, especially in the medulla (➔). Common edema in the medulla (✹). At various developmental stages PF: primary follicle, AF: antral follicle, TF: tertiary follicle, particularly in primary follicles, separations between granulosa cells (⏪) (H&E x40). D) Separation (➔) and pericellular edema in the junctional units between granulosa cells, in the multilaminar primary follicle structure. **E)** Carob-600: corpus luteum (CL), significant vasodilatation and congestion in all areas (➔) at the highest level in this group. Progressive medullary edema (✹). At various developmental stages PF: primary follicle, AF: antral follicle, TF: tertiary follicle, particularly in primary follicles, separations between granulosa cells (⏪) (H&E x40)

Table 4. Mean ± SD ovarian lengths and ovarian and uterus weights of the groups

	Right ovarian (mm)	Left ovarian (mm)	Ovarian weight (mg)	Uterus weight (mg)
Control	4.7 ± 0.7	4.9 ± 0.7	75 ± 33.9	441.7 ± 240.9
Carob-150	5.1 ± 0.7	5.2 ± 0.5	110.8 ± 17.5	451.3 ± 114
Carob-300	4.4 ± 0.9	4.8 ± 1.4	95.1 ± 32.4	353.7 ± 154.4
Carob-600	4.6 ± 0.9	4.4 ± 0.4	97.7 ± 27.7	384 ± 163.8
p value	0.565	0.18	0.22	0.734

p < 0.05 vs. control.
SD: standard deviation

Carob-600, mitosis activation, characteristic of proliferation, and edema in the lamina propria, were more prominent in the juxta epithelial region. Due to this proliferation, the mucosa corrugated towards the lumen. Vasodilatation was very prominent in this group (Figure 4D). These findings suggested that carob extract affected the uterine cycle in a dose-dependent manner, and histological changes mimicked the proliferation phase in the control, Carob-150 and Carob-300 groups and the secretory phase in the Carob-600 group. It appeared that high dose carob extract was especially potent at accelerating the cycle in rat uterus.

Discussion

In this study, the association between ingestion of *C. siliqua* and precocious puberty was investigated in an animal model. *C. siliqua* extract accelerated the time to puberty in male and female rats. Previous studies have investigated the relationship between *C. siliqua* and the fertility of male and female adult rats (13,14,17,18). In these studies, *C. siliqua* extract was given after exposure to gonadotoxic agents such as doxorubicin, cyclophosphamide, lead and monosodium glutamate. Results reported included an increase in gonadotropins, testosterone, and estradiol after different doses of *C. siliqua*. In the present study, gonadotropin and sex steroid levels (total testosterone in males, estradiol in females) of the control group and the groups given varying doses of *C. siliqua* extract were similar. The gonadotropin levels of the control group and *C. siliqua* extract-exposed groups suggest that the onset of puberty was associated with central activity.

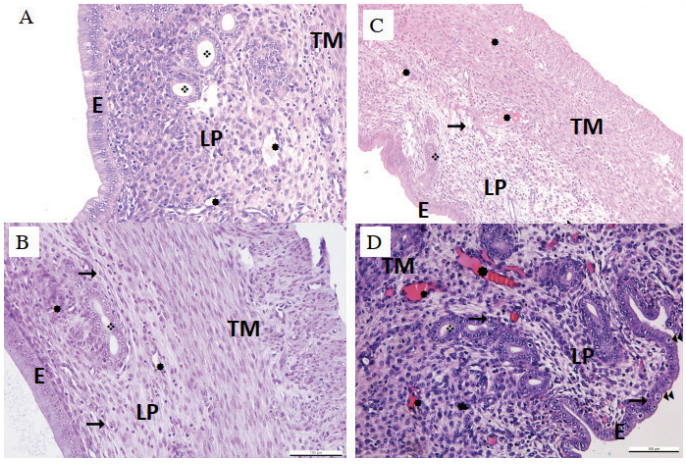

Figure 4. Histological findings of uterus. **A)** Control: Epithelium (E) lamina propria (LP), uterine glands (❖), vascular structures (✹) and muscle layer (Tunica muscularis) (TM) observed with normal histological structures (H&E x100). **B)** Carob-150: Epithelium E, lamina propria (LP), uterine glands (❖),vascular structures (✹) and muscle layer (Tunica muscularis) (TM) were observed with normal histological structures, minimal edema in the lamina propria (→) (H&E x100). **C)** Carob-300: Vasodilatation and congestion in the vessels in the lamina propria and muscle layer (✹). More prominent edema in the lamina propria (→) (❖): normal uterine glands (H&E x100). **D)** Carob-600: Relatively increased mitosis activation in epithelial cells (E) (◂◂). Increased edema in the lamina propria, more prominent in the juxta epithelial region (→). Corrugated mucosa towards the lumen. Vasodilatation is very prominent in this group (✹). (❖): normal uterine glands (H&E x100).

In the male animals exposed to *C. siliqua* extract, the onset of puberty was earlier and the day of start of puberty were the same in all three groups. The histological appearance of the testicular tissue was concordant with the pubertal period in all the male animals given *C. siliqua* extract; increasing doses of carob extract induced spermatogenesis and spermiogenesis and increased seminiferous tubule thickness. Notably, as the dose increased, tissues were more likely to be damaged.

The onset of puberty was also earlier in all female animals exposed to *C. siliqua* extract. Ovarian tissue was consistent with the pubertal period in all female groups given *C. siliqua* extract, and primary, antral and tertiary follicles were observed in all groups. As the dose increased, the follicle structures of oogenesis tended to be at a more advanced stage. It was observed that increasing the dose of *C. siliqua* extract accelerated the cycle in the rat uterus. It is hypothesized that this may have occurred in response to the increased hormone level due to ovarian-induced folliculogenesis. In the present study *C. siliqua* caused early puberty and increased spermiogenesis and folliculogenesis on histological examination of reporoductive tissues. Similarly, there are studies reporting that *C. siliqua* increases spermiogenesis and folliculogenesis after different doses in adult rats after exposure to gonadotoxic agents (13,14,18). Some studies also report an increase in the number of Sertoli and Leydig cells in the tissue, but this was not observed in the present study (13,14).

One of the main factors leading to puberty in rats is excess weight gain. Increasing leptin levels with the increase of adipose tissue is a trigger to initiate puberty (19,20). Weight gain percentage was calculated to evaluate the effect of weight gain on puberty since increasing doses of extract increased the calories taken. Percentage weight gain in the control groups was higher in both female and male rats compared to groups given the extract. However, the beginning of puberty in the control group was later than the groups exposed to *C. siliqua* extract (21,22). Leptin is known to affect the onset of puberty. In humans, weight gain and an increase in leptin caused delayed puberty in boys and early puberty in girls (23,24). In the present study, leptin levels were similar between the groups.

Fertility markers that were negatively affected at both the hormonal and tissue levels after exposure to gonadotoxic agents showed improvement after *C. siliqua* extract was administered (25). It was suggested that this occured because of the rich polyphenol, vitamin, and mineral content of *C. siliqua* and up-regualtion of antioxidant mechanisms. Arachidonic acid and aspartic acid, present in *C. siliqua*, increase the synthesis of annular adenosine monophosphate and cAMP, stimulating testosterone production (25). In addition, *C. siliqua* may exert an antioxidant effect through polyphenols (gallic acid-tannin) and through iron, manganese, zinc, copper, selenium, and vitamin E, which are cofactors of antioxidant pathways. Antioxidants are critical for protection against oxidative stress created by free radicals. Antioxidants can scavenge free radicals and prevent cell damage (26). GPX, one of the enzymatic antioxidants, breaks down hydrogen peroxide into water in the mitochondria and cytosol. GPX activity is selenium-dependent, and there are eight identified GPXs (27). GPX plays a role in cell differentiation and proliferation in gametes. GPX4 is located primarily in the testis, and its expression pattern in the testis suggests that it may be related to the onset of puberty (28). In the rat study of Roveri et al. (29), it was reported that under stimulation by gonadotropins, GPX increased in the testicular tissue of rats and stimulated spermatogenesis.

In the present study, GPX levels were significantly higher in male and female animals given the extract. Thus, antioxidant processes may also play a role in the progression of puberty, as previously suggested (30). In addition to inducing puberty at high doses, *C. siliqua* also caused histopathological changes, including ondulation in the basement membrane in males and degeneration of intercellular junctions in granulosa cells in females. These findings suggest that high doses may cause damage to gametes. When antioxidants are taken in high doses, they may become pro-oxidants in the tissue and cause tissue damage and death (31,32).

Study Limitations

Limitations of the present study include not analyzing kisspeptin and neurokinin B levels, both of which are mediators involved in the onset of puberty. If these markers had been measured, we hypothesize that there would be results more supportive of the central onset of puberty. Also, we were unable to demonstrate hyperplasia of gonadotroph cells in the rat pituitary histopathologically. This was not studied because the rats were in a small age group, and the tissue was difficult to dissect. Another limitation of our study was *C. siliqua* fruits are rich in flavonoids, phenolic acids, carbohydrates, proteins, vitamins and minerals. It is not known which of these ingredients induces puberty. We did not conduct a content analysis in the extract. But in future studies it will be possible to show the effect of more specific active ingredients.

Conclusion

Extracts of *C. siliqua* appeared to cause early puberty and increased spermiogenesis and folliculogenesis in a rat model. It is suggested that antioxidant mechanisms may also be involved but may cause tissue damage at high doses. We caution that foods consumed for their organic nutrition may become endocrine disruptors when the amount and duration of use increase.

Ethics

Ethics Committee Approval: This study was performed in Gazi University Laboratory Animal Breeding and Experimental Research Center and approved by the Ethical Animal Research Committee of Gazi University (protocol no: G.Ü.ET-21.053, date: 09.07.2021).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Aylin Kılınç Uğurlu, Aysun Bideci, Design: Aylin Kılınç Uğurlu, Aysun Bideci, Elvan Anadol, İpek Süntar, Gülnur Take Kaplanoğlu, Özlem Gülbahar, Zeynep Şafak Teksin, Duygu Dayanır, Tuba Saadet Deveci Bulut, Canan Uluoğlu, M. Orhun Çamurdan, Data Collection or Processing: Aylin Kılınç Uğurlu, Elvan Anadol, İpek Süntar, Gülnur Take Kaplanoğlu, Özlem Gülbahar, Zeynep Şafak Teksin, Duygu Dayanır, Tuba Saadet Deveci Bulut, Canan Uluoğlu, Analysis or Interpretation: Aylin Kılınç Uğurlu, Aysun Bideci, Elvan Anadol, Gülnur Take Kaplanoğlu, Özlem Gülbahar, Duygu Dayanır, M. Orhun Çamurdan, Literature Search: Aylin Kılınç Uğurlu, Aysun Bideci, Elvan Anadol, Gülnur Take Kaplanoğlu, Duygu Dayanır, Writing: Aylin Kılınç Uğurlu, Aysun Bideci, Elvan Anadol, Gülnur Take Kaplanoğlu, Duygu Dayanır.

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References

1. Sürekli Karakuş Ö, Arabacı Tamer S, Levent HN, Kaygusuz SB, Turan S, Akakın D, Guran T, Yeğen BC, Bereket A. Is quail egg a potential endocrine disruptor? Horm Res Paediatr 2021;94:371.
2. Elmaoğulları S, Kadan E, Anadol E, Gökçeoğlu A, Çetinkaya S, Yarım GF, Uçaktürk SA, Aycan Z. Effects of 5-Hydroxymethylfurfural on Pubertal Development of Female Wistar Rats. J Clin Res Pediatr Endocrinol 2020;12:79-85. Epub 2019 Sep 2

3. Rashid H, Alqahtani SS, Alshahrani S. Diet: A Source of Endocrine Disruptors. Endocr Metab Immune Disord Drug Targets 2020;20:633-645.
4. Patisaul HB. Endocrine disruption by dietary phyto-oestrogens: impact on dimorphic sexual systems and behaviours. Proc Nutr Soc 2017;76:130-144. Epub 2016 Jul 8
5. Lachkar N, Al-Sobarry M, El-Hajaji H, Lamkinsi T, Lachkar M, Cherrah Y, Alaoui K. Anti-inflammatory and antioxidant effect of Ceratonia siliqua L. Methanol barks extract. J Chem Pharm Res 2016;8:202-210.
6. Papaefstathiou E, Agapiou A, Giannopoulos S, Kokkinofta R. Nutritional characterization of carobs and traditional carob products. Food Sci Nutr 2018;6:2151-2161.
7. El-Haskoury R, Kriaa W, Lyoussi B, Makni M. Ceratonia siliqua honeys from Morocco: Physicochemical properties, mineral contents, and antioxidant activities. J Food Drug Anal 2018;26:67-73.
8. Lakkab I, Hajaji HE, Lachkar N, Bali BE, Lachkar M, Ciobica A. Phytochemistry, bioactivity: suggestion of Ceratonia siliqua L. as neurodegenerative disease therapy. J Complement Integr Med 2018:15.
9. Custódio L, Fernandes E, Escapa AL, López-Avilés S, Fajardo A, Aligué R, Albericio F, Romano A. Antioxidant activity and in vitro inhibition of tumor cell growth by leaf extracts from the carob tree (Ceratonia siliqua). Pharm Biol 2009;47:721-728.
10. Agrawal A, Mohan M, Kasture S, Foddis C, Frau MA, Loi MC, Maxia A. Antidepressant activity of Ceratonia siliqua L. fruit extract, a source of polyphenols. Nat Prod Res 2011;25:450-456.
11. Valero-Muñoz M, Ballesteros S, Ruiz-Roso B, Pérez-Olleros L, Martín-Fernández B, Lahera V, de Las Heras N. Supplementation with an insoluble fiber obtained from carob pod (Ceratonia siliqua L.) rich in polyphenols prevents dyslipidemia in rabbits through SIRT1/PGC-1α pathway. Eur J Nutr 2019;58:357-366. Epub 2017 Dec 22
12. Navarro JA, Decara J, Medina-Vera D, Tovar R, Suarez J, Pavón J, Serrano A, Vida M, Gutierrez-Adan A, Sanjuan C, Baixeras E, Fonseca FR. D-Pinitol from Ceratonia siliqua Is an Orally Active Natural Inositol That Reduces Pancreas Insulin Secretion and Increases Circulating Ghrelin Levels in Wistar Rats. Nutrients 2020;12:2030.
13. Khani HM, Shariati M, Forouzanfar M, Hosseini SE. Protective effects of Ceratonia siliqua extract on protamine gene expression, testicular function, and testicular histology in doxorubicin-treated adult rats: An experimental study. Int J Reprod Biomed 2020;18:667-682.
14. Sadeghzadeh F, Sadeghzadeh A, Changizi-Ashtiyani S, Bakhshi S, Mashayekhi FJ, Mashayekhi M, Poorcheraghi H, Zarei A, Jafari M. The effect of hydro-alcoholic extract of Ceratonia Silique L. on spermatogenesis index in rats treated with cyclophosphamide: An experimental study. Int J Reprod Biomed 2020;18:295-306.
15. Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). Pharmacology and Toxicology 2005. Available from: https://www.fda.gov/media/72309/download
16. Cora MC, Kooistra L, Travlos G. Vaginal Cytology of the Laboratory Rat and Mouse:Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol 2015;43:776-793. Epub 2015 Mar 3
17. Soleimanzadeh A, Kian M, Moradi S, Mahmoudi S. Carob (Ceratonia siliqua L.) fruit hydro-alcoholic extract alleviates reproductive toxicity of lead in male mice: Evidence on sperm parameters, sex hormones, oxidative stress biomarkers and expression of Nrf2 and iNOS. Avicenna J Phytomed 2020;10:35-49.
18. El-Beltagy AE-FBM, Elghaweet HA. Adverse effects of monosodium glutamate on the reproductive organs of adult Female albino rats and the possible ameliorated role of carob (Ceratonia Siliqua). Journal of Bioscience and Applied Research 2016;2:170-184.
19. Engelbregt MJ, van Weissenbruch MM, Popp-Snijders C, Lips P, Delemarre-van de Waal HA. Body mass index, body composition, and leptin at onset of puberty in male and female rats after intrauterine growth retardation and after early postnatal food restriction. Pediatr Res 2001;50:474-478.
20. Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS. Leptin accelerates the onset of puberty in normal female mice. J Clin Invest 1997;99:391-395.
21. Yamasaki K, Sawaki M, Noda S, Muroi T, Takatsuki M. Preputial separation and glans penis changes in normal growing Crj: CD (SD) IGS rats. Reprod Toxicol 2001;15:533-536.
22. Beckman DA, Feuston M. Landmarks in the development of the female reproductive system. Birth Defects Res B Dev Reprod Toxicol 2003;68:137-143.
23. Farooqi IS. Leptin and the onset of puberty: Insights from rodent and human genetics. Semin Reprod Med 2002;20:139-144.
24. Zhang J, Gong M. Review of the role of leptin in the regulation of male reproductive function. Andrologia 2018.
25. Mokhtari M, Sharifi E, Azadian SH. The effects of hydro alcoholic extract of Ceratonia siliqua L. seeds on pituitary--testis hormones and spermatogenesis in rat. Advances in Environmental Biology 2012;6:2778-2784.
26. Sen S, Chakraborty R. The role of antioxidants in human health. ACS Symposium Series 2011;1083:1-37.
27. Buday K, Conrad M. Emerging roles for non-selenium containing ER-resident glutathione peroxidases in cell signaling and disease. Biol Chem 2021;402:271-287.
28. Giannattasio A, Girotti M, Williams K, Hall L, Bellastella A. Puberty influences expression of phospholipid hydroperoxide glutathione peroxidase (GPX4) in rat testis: Probable hypophysis regulation of the enzyme in male reproductive tract. J Endocrinol Invest 1997;20:439-444.
29. Roveri A, Casasco A, Maiorino M, Dalan P, Calligaro A, Ursini F. Phospholipid hydroperoxide glutathione peroxidase of rat testis. Gonadotropin dependence and immunocytochemical identification. J Biol Chem 1992;267:6142-6146.
30. Paltoglou G, Avloniti A, Chatzinikolaou A, Stefanaki C, Papagianni M, Papassotiriou I, Fatouros IG, Chrousos GP, Kanaka-Gantenbein C, Mastorakos G. In early pubertal boys, testosterone and LH are associated with improved anti-oxidation during an aerobic exercise bout. Endocrine 2019;66:370-380. Epub 2019 Aug 4
31. Lu LY, Ou N, Lu QB. Antioxidant induces DNA damage, cell death and mutagenicity in human lung and skin normal cells. Sci Rep 2013;3:3169.
32. Bender D. The antioxidant paradox: Damage and defence. Biochemist 2006;28:9-12.

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Decline in the Age of Menarche in Istanbul Schoolgirls Over the Last 12 Years

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What is already known on this topic?

In the Western world, the mean age at menarche (AAM) decreased from the 1800s until the 1950s which was explained by improved living conditions and nutritional status. Some studies suggest that the AAM has continued to decrease after the 1950s while others suggest that the downward trend has halted.

What this study adds?

The median menarcheal age was 12.04 years and has declined by 0.7 years during the past 12 years in İstanbul. Sequential studies in Turkey indicate a decline in the AAM of 0.91 years in the last half-century -a speed of -0.56 years per generation of 30 years. Besides the strong influence of the maternal menarcheal age, the secular trend towards a younger AAM during the last decade can be mainly explained by increased rates of obesity in Turkey.

Abstract

Objective: Menarche is the endpoint of a sequence of maturational events of female puberty. The timing of menarche is a strongly heritable trait. However, secular trends suggest that lifestyle and environmental factors are important. To assess the trend in age at menarche (AAM), and its associated factors in İstanbul over the last 12 years.

Methods: A cross-sectional study was carried out between March and April 2022 on schoolgirls aged 9-18 years. A predesigned and self-administered questionnaire was filled out anonymously by the students. The data of AAM was included in the statistical analysis if the time of AAM is remembered in both months and years. A probit model was used to calculate the median AAM. The findings were compared with those from a study performed 12 years ago in the same region of İstanbul.

Results: Among 9000 girls to whom the questionnaire was distributed, 1749 (19.5%) responded. The median AAM of 1374 girls whose AAM information was considered valid was 12.04 years (95% confidence interval: 12.01-12.13), 0.7 years lower than was reported 12 years ago ($p < 0.0001$). AAM was correlated positively with maternal AAM, and negatively with body mass index (BMI) standard deviation score and maternal educational status ($p < 0.0001$, $p < 0.0001$ and $p = 0.002$), respectively. There was no correlation between the AAM and birth weight. Girls with BMI percentile ≥85% (n = 251) had earlier menarche than the ones with BMI percentile <85% (n = 1072) (11.5 vs. 12.1 years, $p < 0.0001$). Among the mother-daughter pairs (n = 1162), AAM of girls was 0.91 years (median 0.94 years) earlier than their mothers.

Conclusion: The present study demonstrates a significant downward trend in the menarcheal age in İstanbul over the last twelve years. These findings support a strong contribution from genetic factors and BMI on AAM.

Keywords: Puberty, age at menarche, secular trend, pubertal timing, Turkey

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Introduction

The age at menarche (AAM) displays considerable variation among girls and has undergone changes over time (1,2). Secular trend in the timing and tempo of puberty and AAM is determined by several intrinsic and environmental variables, such as genetics, lifestyle, nutrition, ethnicity, geographical and socioeconomic background, and endocrine-disrupting chemicals (3,4). In Europe, mean AAM has declined over the past two centuries - from 15-16 years in the early 19th century to 12-13 years in the late 20th century (1,3). Furthermore, early menarche has increased globally by 25-33% in recent generations (5,6,7). Longitudinal studies demonstrated a remarkable increase of early pubertal development and an increased rate of sexual precocity in children (4,8). Our observations suggested similar increase in the number of premature thelarche and precocious puberty cases over the last decade in our clinic. Since the age of menarche is highly correlated with the age of pubertal onset (9), the aim was to revisit the AAM data that we reported 12 years ago in İstanbul (10).

Methods

In the first part of the study, hospital records and clinical files were investigated to compare the number of patients presenting with premature thelarche, premature menarche, precocious puberty, and other conditions in our pediatric endocrinology clinic between January 1, 2008 - December 31, 2009, and January 1, 2020 - December 31, 2021 (Table 1).

In the second part, a cross-sectional study was conducted between March 2022 and April 2022 on schoolgirls aged 9-18 years living in the Asian part of İstanbul. A questionnaire and consent forms were distributed three days before the study. The predesigned, pretested, structured and self-administered questionnaire was filled out anonymously by the girls and their parents. A total of 9,000 female students from 22 schools located in the same area in İstanbul studied 12 years ago (10) were invited to participate in the study. Consented girls with no chronic medical condition were included.

The questionnaire was designed to obtain the information about menarcheal age of the participants and their mothers, birth weight, actual weight and height measurements of schoolgirls, the education status of the parents and the financial income of the family. The data about AAM was included only if the time of AAM could be recalled in both months and years. Body mass index (BMI) percentiles and BMI- standard deviation (SD) score (SDS) values of the girls were calculated according to national data (11). Educational status of the parents were classified as high (high school and beyond) or low (below high school). Household income was classified as low (costs > income), middle (costs≈income) or high (costs < income).

The study was performed with the approval of the Ethics Committee of Marmara University Faculty of Medicine (date: 05.11.2021, protocol no: 09.2021.1251), and the Turkish Ministry of Education. Participants and parents provided written informed consent.

In addition, the AAM trends were compared with the previous cohort studies performed in Turkey.

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism® V5.0 software (GraphPad Software Inc., San Diego, California, USA). Results were reported as frequencies and percentages, mean, and median with minimum-maximum, interquartile ranges or 95% confidence intervals (CI) as appropriate. To calculate the median AAM, a probit model was used. Parametric t-test was used for comparison of variables. Pearson's correlation coefficients were used to investigate the relationship between various data, as required. The distribution of categorical variables was compared using chi-square test. Statistical significance was set at $p < 0.05$.

Results

According to our clinical records, the number of patients presenting with precocious puberty relative to the patients with other endocrine disorders was higher in the years 2020-2021 than in 2008-2009 ($p < 0.0001$) (Table 1).

Table 1. The prevalence of early puberty in girls among the total number of patients presenting to our pediatric endocrinology clinic

Time interval	Total number of patients (n)	Premature menarche, n (%)	Premature telarche n (%)	Precocious puberty n (%)	Total early pubertal patients n (%)
1 January 2008-31 December 2009	33328	5 (0.015)	88 (0.26)	44 (0.13)	137 (0.41)
1 January 2020-31 December 2021	35818	13 (0.036)	95 (0.26)	135 (0.37)	243 (0.67)
Chi-square test p value		0.08	0.97	**< 0.0001**	**< 0.0001**

The questionnaire was distributed to 9000 girls who were attending schools located in the area where we performed the study to determine AAM 12 years ago (10). Among those, 1749 (19.5%) consented and filled out the questionnaire. Of them, 1374 recalled the year and month of the AAM which was a median of 12.04 years (95% CI: 12.01-12.13) and a mean ± SD of 12.07 ± 1.11 years. The AAM was approximately 0.7 years lower than reported using the same method 12 years ago in the same region of İstanbul ($p < 0.0001$) (Table 2, Figure 1a). Maternal AAM was reported by 1528 mothers and was a median of 12.96 years (95% CI: 12.92-13.08). There were 1162 mother-daughter pairs providing AAM data which showed that the daughters had a mean of 0.91 years (median 0.94) earlier menarche than their mothers ($p < 0.0001$). However, the AAM of the girls was positively and significantly correlated with their mothers' ($R^2 = 0.048$, $p < 0.0001$).

The prevalence of overweight (BMI ≥85% and <95%) and obesity (BMI ≥95%) was 9.1% (n = 121) and 9.8% (n = 130), respectively. The overweight/obese girls (n = 251) had earlier menarche than the rest of the cohort (n = 1072) [median (95% CI); 11.54 (11.39-11.77) vs. 12.12 (12.04-12.23) years, $p < 0.0001$] (Figure 1b). There was a significant negative correlation between BMI-SDS and AAM ($R^2 = 0.066$, $p < 0.0001$).

Maternal educational status was negatively correlated with AAM. The AAM was lower in girls with mothers of higher educational status (HES) than the ones with mothers of lower educational status (LES) [median (95% CI); 11.96 (11.87-12.04) vs. 12.17 (12.03-12.28) years, $p = 0.002$] (Figure 1c).

The correlation between the AAM and birth weight, paternal educational status or household income were not significant ($p = 0.18$, 0.17, and 0.07, respectively).

Table 2. The number of schoolgirls and the percentage of those having menarche at the respective ages in two studies with 12 years intervals in İstanbul

Age (years)	Atay et al. (10) (2010)	Guran et al. (2022) (current study)
	Menarche, n (%)	Menarche, n (%)
10	520 (1.5)	31 (12.9)
11	501 (12.6)	213 (19.2)
12	463 (42.5)	232 (49.5)
13	535 (78.3)	230 (84.3)
14	355 (92.7)	283 (94.6)
15	311 (98.1)	377 (98.9)
16	203 (100)	311 (99)
17	124 (100)	51 (100)
18	71 (100)	21 (100)

The total prevalence of preterm and small for gestational age (SGA) births was 14.5% in the cohort (n = 200). There was no correlation between AAM and birthweight ($r = 0.03$, $p = 0.18$). There was no significant difference for AAM between preterm +/- SGA and term +/- appropriate for gestational age (AGA) births (mean 12.01 vs. 12.07 years, $p = 0.50$).

Previous studies reporting AAM in the various regions of Turkey also showed an ongoing decline in the menarcheal age since 1970s (Figure 2) (10,12,13,14,15,16,17).

Discussion

This study on a large cohort of schoolgirls demonstrated that the median AAM was 12.04 years and has declined by 0.7 years during the past 12 years in İstanbul. Current data presents evidence of a significant shift in menarche to earlier ages in recent years in Turkey.

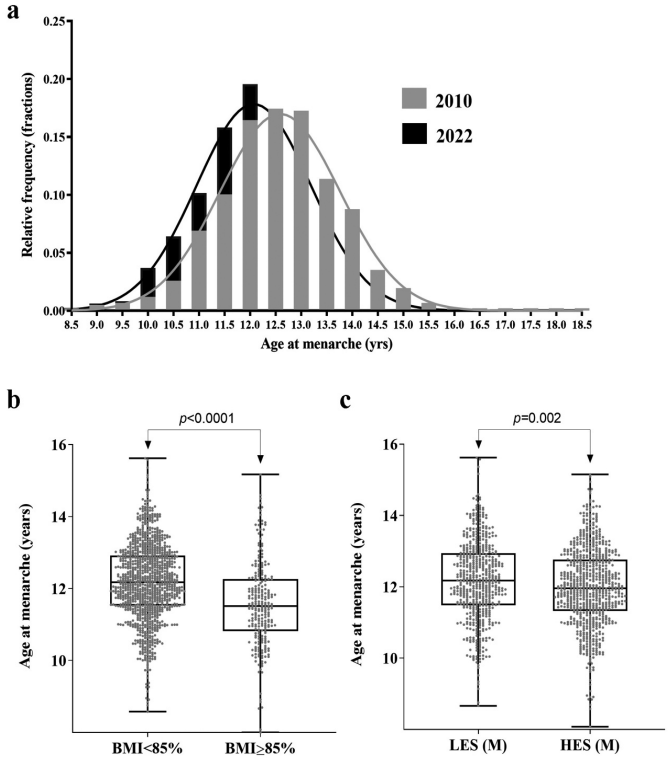

Figure 1. Characteristics of age at menarche (AAM) in İstanbul. a) Change in the AAM in İstanbul in the last 12 years. The results of the study by Atay et al. (10) in 2010 are compared with the current study. Bars indicate the fractions (percentage) of the girls having menarche at the respective ages. b) Comparison of AAM between girls with BMI <85% and ≥85%, c) Comparison of AAM among the girls whose mothers have HES or LES

LES: lower educational status, HES: higher educational status, BMI: body mass index, yrs: years

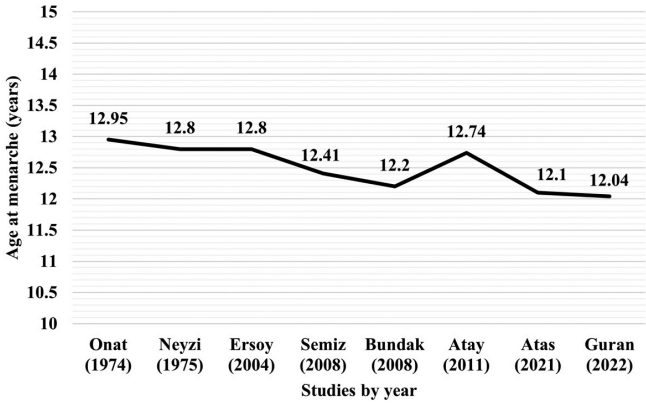

Figure 2. Schematic representation of previous studies determining AAM in Turkey by years (10,11,12,13,14,15,16,17)

AAM: age at menarche

The starting point of our attempt to update our data on AAM was an observation of increased clinical presentation of early puberty in recent years in our department. Although menarche is a relatively late marker of puberty, it is significantly correlated with age at the onset of thelarche and is therefore considered to be an indicator of the onset of puberty (1). Indeed, a higher number of admissions to our outpatient clinic for premature thelarche, premature menarche and precocious puberty by 50% in last 12 years is in line with the finding of a decline in AAM in İstanbul schoolgirls. The AAM was found to be 12.74 years in 2011 by Atay et al. (10) in 1732 girls attending schools at the same region of İstanbul. Not only in İstanbul, in which resides almost 18% of Turkey's population (18), but sequential studies in Turkey indicate a decline in AAM of 0.91 years in the last half-century -a speed of -0.56 years per generation of 30 years (12,13,14,15,16,17).

Data published during 1990-2000 on the AAM in different European countries showed a north-to-south gradient that ranged from 13.4 years in the north to 12 years in the south of Europe. There was also an east-to-west gradient that was reported at 12.6 years in France to lower ages in the Eastern European/Mediterranean countries such as 12.3 years in Greece and 12 years in Italy (1). Geographical, genetic/ethnic, and environmental similarities between Turkey and other Mediterranean countries may partly explain earlier menarche in Turkey compared to Northern/Eastern Europe. Nevertheless, the rate of decline in AAM is sharper in Turkey relative to other European and Mediterranean countries. A study from The Netherlands in 2009 showed a greater decrease in median AAM in Turkish girls between 1997 and 2009 compared to Dutch girls (from 13.15 to 13.05 in Dutch vs. from 12.80 to 12.50 years in Turkish girls). These authors reported that 33% of Turkish girls younger than 12 years start menstruating in primary school (6). Compared to Dutch girls they found a faster decrease in AAM in girls of Turkish descent, even after adjustment for BMI-SDS.

An increased rate of obesity may account for the downward trend of AAM in Turkey, as childhood obesity figures are also on the rise in the comaprative period. Between, 2000 and 2010 different regions of Turkey have demonstrated varying prevalence rates of 10.3-17.6% and 1.9-7.8% for overweight and obesity, respectively, in children aged 6-16 years (19). In the last decade, the prevalence of overweight (including obesity) and obesity among children and adolescents aged 10-19 years raised significantly to 27-28% and 9-10%, respectively (20). Lower AAM in obese/overweight girls and remarkable negative correlation of BMI and AAM in our study supports the major influence of obesity on the tempo of puberty, as shown by several others (21,22,23,24).

Socioeconomic status (SES) may account for variations in the timing of puberty. However, the results of studies into the effects of SES on AAM are inconsistent and differentiate not only between countries but within countries as well (23,25,26,27). According to a general conception, a low socioeconomic living environment may involve nutritional problems, high energy expenditure, insufficient access to health services, large family size, and social and emotional injuries and ultimately delayed puberty and menarche. On the other hand, declining trends in AAM have been reported from high SES populations (23,25,28,29). Previous studies indicated that girls of Turkish descent with high SES had an earlier AAM than girls with low SES (10,14). In the present study we did not observe a correlation between SES and AAM. However, income per capita in Turkey declined by 1156 $/year-nearly 11% in 2021 compared to that in 2010 (10,743 $/year in 2010 while it is 9,587 $/year in 2021) (https://www.macrotrends.net/countries/TUR/turkey/gdp-per-capita), which may have an effect in the decline in AAM.

Similar to SES, parental education has been found to be associated with AAM in some studies (30,31), but this has not been replicated in others (29,32). A similar discrepancy in the effect of parental education on AAM was also observed in studies from Turkey. Ataş Aslan and Ünüvar et al. (17) found that LES was associated with earlier menarcheal age but this was not supported by Ersoy et al. (14). We have found that higher maternal education was associated with earlier onset of menarche, as shown in some previous studies (23,26). Overall, our results and previous studies suggest that obesity is the major and consistent non-genetic variable responsible for declining menarcheal age. Besides the non-genetic factors, we also demonstrated that AAM

of the mothers was significantly positively correlated with the AAM of their daughters, which is the most consistent finding of the majority of the studies reporting AAM (1,3).

SGA children are more prone to have precocious pubarche and an earlier onset of pubertal development and menarche, and faster progression of puberty than children born AGA (33). However, we could not demonstrate a relationship between birth weight and AAM in our cohort and AAM was similar between girls born SGA or AGA.

This study also provides the first AAM data after the Coronavirus disease-2019 (COVID-19) pandemic. There is some evidence of increased frequency of idiopathic central precocious puberty in girls during the COVID-19 pandemic in Turkey and in other countries (34,35,36,37,38,39,40). Some studies found an association between earlier pubertal onset and increased body mass, disturbed sleep patterns or increased screen exposure during COVID-19 lockdown. The possible increased rate of earlier pubertal onset during the pandemic may have contributed to the observed decline in menarcheal age in the present study, and this should be investigated in the future.

Study Limitations

The main limitation of the study, as in the most of the studies reporting AAM, is the collection of AAM based on recall by girls and their mothers, which is susceptible to various forms of error (1). However, the same method was used 12 years ago and the AAM data was included only if both month and year of menarcheal age was remembered precisely. Utilization of the same method for the collection of the data, the same statistical analysis for AAM, and doing the survey in the same area are the strengths of the study.

Conclusion

In conclusion, our data suggests that a downward trend in the AAM continues and a plateau for AAM has not yet been reached in Turkey. Besides the strong influence of the maternal menarcheal age, the secular trend towards a younger AAM during the last decade can be explained mainly by increased rates of obesity among children in Turkey.

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Ethics

Ethics Committee Approval: The study was performed with the approval of the Ethics Committee of Marmara University Faculty of Medicine (date: 05.11.2021, protocol no: 09.2021.1251), and the Turkish Ministry of Education.

Informed Consent: Participants and parents provided written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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References

1. Parent AS, Teilmann G, Juul A, Skakkebaek NE, Toppari J, Bourguignon JP. The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. Endocr Rev 2003;24:668-693.
2. Dratva J, Gómez Real F, Schindler C, Ackermann-Liebrich U, Gerbase MW, Probst-Hensch NM, Svanes C, Omenaas ER, Neukirch F, Wjst M, Morabia A, Jarvis D, Leynaert B, Zemp E. Is age at menopause increasing across Europe? Results on age at menopause and determinants from two population-based studies. Menopause 2009;16:385-394.
3. Brito VN, Canton APM, Seraphim CE, Abreu AP, Macedo DB, Mendonca BB, Kaiser UB, Argente J, Latronico AC. The Congenital and Acquired Mechanisms Implicated in the Etiology of Central Precocious Puberty. Endocr Rev 2023;44:193-221.
4. Soriano-Guillén L, Tena-Sempere M, Seraphim CE, Latronico AC, Argente J. Precocious sexual maturation: Unravelling the mechanisms of pubertal onset through clinical observations. J Neuroendocrinol 2022;34:12979. Epub 2021 Apr 26
5. Henrichs KL, McCauley HL, Miller E, Styne DM, Saito N, Breslau J. Early menarche and childhood adversities in a nationally representative sample. Int J Pediatr Endocrinol 2014;2014:14. Epub 2014 Jul 15

6. Talma H, Schönbeck Y, van Dommelen P, Bakker B, van Buuren S, Hirasing RA. Trends in menarcheal age between 1955 and 2009 in the Netherlands. PLoS One 2013;8:e60056.
7. Rigon F, Bianchin L, Bernasconi S, Bona G, Bozzola M, Buzi F, Cicognani A, De Sanctis C, De Sanctis V, Radetti G, Tatò L, Tonini G, Perissinotto E. Update on age at menarche in Italy: toward the leveling off of the secular trend. J Adolesc Health 2010;46:238-244. Epub 2009 Oct 13
8. Bräuner EV, Busch AS, Eckert-Lind C, Koch T, Hickey M, Juul A. Trends in the Incidence of Central Precocious Puberty and Normal Variant Puberty Among Children in Denmark, 1998 to 2017. JAMA Netw Open 2020;3:e2015665.
9. Tanner JM. Growth at adolescence. 2nd ed, Blackwell Scientific Publications, Oxford, 1962.
10. Atay Z, Turan S, Guran T, Furman A, Bereket A. Puberty and influencing factors in schoolgirls living in Istanbul: end of the secular trend? Pediatrics 2011;128:40-45. Epub 2011 Jun 13
11. Neyzi O, Bundak R, Gökçay G, Günöz H, Furman A, Darendeliler F, Baş F. Reference Values for Weight, Height, Head Circumference, and Body Mass Index in Turkish Children. J Clin Res Pediatr Endocrinol 2015;7:280-293.
12. Onat T, Ertem B. İstanbul kız çocuklarında menarş, seksüel, kemik gelişmesi ve menarş yaşının fiziksel, seksüel ve kemik gelişimi tempoları ve sosyoekonomik seviye ile ilişkileri. Cerrahpaşa Tıp Fak Derg 1973;4:392-415.
13. Neyzi O, Alp H, Orhon A. Sexual maturation in Turkish girls. Ann Hum Biol 1975;2:49-59.
14. Ersoy B, Balkan C, Gunay T, Onag A, Egemen A. Effects of different socioeconomic conditions on menarche in Turkish female students. Early Hum Dev 2004;76:115-125.
15. Semiz S, Kurt F, Kurt DT, Zencir M, Sevinç O. Pubertal development of Turkish children. J Pediatr Endocrinol Metab 2008;21:951-961.
16. Bundak R, Darendeliler F, Günöz H, Baş F, Saka N, Neyzi O. Puberty and pubertal growth in healthy Turkish girls: no evidence for secular trend. J Clin Res Pediatr Endocrinol 2008;1:8-14. Epub 2008 Aug 2
17. Ataş Aslan E, Ünüvar T. Age at Onset of Menarche and Puberty of Girls in Aydin Region and the Factors Affecting Them. Trends in Pediatrics 2021;2:35-42.
18. https://data.tuik.gov.tr/Kategori/GetKategori?p = Nufus-ve-Demografi-109.
19. Bereket A, Atay Z. Current status of childhood obesity and its associated morbidities in Turkey. J Clin Res Pediatr Endocrinol 2012;4:1-7.
20. WHO European Regional Obesity Report 2022: Available from: https://apps.who.int/iris/bitstream/handle/10665/353747/9789289057738eng.pdf?sequence = 1&isAllowed = y
21. Atay Z, Turan S, Guran T, Furman A, Bereket A. The prevalence and risk factors of premature thelarche and pubarche in 4- to 8-year-old girls. Acta Paediatr 2012;101:71-75. Epub 2011 Sep 23
22. Ferrari V, Stefanucci S, Ferrari M, Ciofi D, Stagi S; on the behalf of the Tuscany Menarche Study Group. Retrospective longitudinal analysis of the effects of postnatal weight gain on the timing and tempo of puberty and menarche in a cohort of Italian girls. Ital J Pediatr 2022;48:20.
23. Garenne M. Age at menarche in Nigerian demographic surveys. J Biosoc Sci 2021;53:745-757. Epub 2020 Sep 11
24. Rafique N, AlSheikh MH. Identifying menarcheal age and its association with body mass index in young Saudi females. Saudi Med J 2019;40:958-961.
25. Liu W, Yan X, Li C, Shu Q, Chen M, Cai L, You D. A secular trend in age at menarche in Yunnan Province, China: a multiethnic population study of 1,275,000 women. BMC Public Health 2021;21:1890.
26. Cheng M, Yao Y, Zhao Y, Lin Y, Gao S, Xie J, Zhang X, Zhu H. The influence of socioeconomic status on menarcheal age among Chinese school-age girls in Tianjin, China. Eur J Pediatr 2021;180:825-832. Epub 2020 Sep 12
27. Kim T, Yun JW, Son M, Kim CB, Choe SA. Age at menarche of adolescent girls and the neighbourhood socioeconomic status of their school area. Eur J Contracept Reprod Health Care 2022;28:65-71. Epub 2022 Sep 2
28. Rao S, Joshi S, Kanade A. Height velocity, body fat and menarcheal age of Indian girls. Indian Pediatr 1998;35:619-628.
29. Braithwaite D, Moore DH, Lustig RH, Epel ES, Ong KK, Rehkopf DH, Wang MC, Miller SM, Hiatt RA. Socioeconomic status in relation to early menarche among black and white girls. Cancer Causes Control 2009;20:713-720. Epub 2008 Dec 25
30. Wronka I, Pawlińska-Chmara R. Menarcheal age and socio-economic factors in Poland. Ann Hum Biol 2005;32:630-638.
31. Junqueira Do Lago M, Faerstein E, De Souza Lopes C, Werneck GL; Pró-Saúde Study (Rio de Janeiro, Brazil). Family socio-economic background modified secular trends in age at menarche: evidence from the Pró-Saúde Study (Rio de Janeiro, Brazil). Ann Hum Biol 2003;30:347-352.
32. Lee MH, Kim SH, Oh M, Lee KW, Park MJ. Age at menarche in Korean adolescents: trends and influencing factors. Reprod Health 2016;13:121.
33. Verkauskiene R, Petraitiene I, Albertsson Wikland K. Puberty in children born small for gestational age. Horm Res Paediatr 2013;80:69-77. Epub 2013 Jul 26
34. Acinikli KY, Erbaş İM, Besci Ö, Demir K, Abacı A, Böber E. Has the Frequency of Precocious Puberty and Rapidly Progressive Early Puberty Increased in Girls During the COVID-19 Pandemic? J Clin Res Pediatr Endocrinol 2022;14:302-307.
35. Acar S, Özkan B. Increased frequency of idiopathic central precocious puberty in girls during the COVID-19 pandemic: preliminary results of a tertiary center study. J Pediatr Endocrinol Metab 2021;35:249-251.
36. Yesiltepe Mutlu G, Eviz E, Haliloglu B, Kirmizibekmez H, Dursun F, Ozalkak S, Cayir A, Sacli BY, Ozbek MN, Demirbilek H, Hatun S. The effects of the covid-19 pandemic on puberty: a cross-sectional, multicenter study from Turkey. Ital J Pediatr 2022;48:144.
37. Mondkar SA, Oza C, Khadilkar V, Shah N, Gondhalekar K, Kajale N, Khadilkar A. Impact of COVID-19 lockdown on idiopathic central precocious puberty - experience from an Indian centre. J Pediatr Endocrinol Metab 2022;35:895-900.
38. Chen Y, Chen J, Tang Y, Zhang Q, Wang Y, Li Q, Li X, Weng Z, Huang J, Wang X, Liu S. Difference of Precocious Puberty Between Before and During the COVID-19 Pandemic: A Cross-Sectional Study Among Shanghai School-Aged Girls. Front Endocrinol (Lausanne) 2022;13:839895.
39. Turriziani Colonna A, Curatola A, Sodero G, Lazzareschi I, Cammisa I, Cipolla C. Central precocious puberty in children after COVID-19 outbreak: a single-center retrospective study. Minerva Pediatr (Torino) 2022.
40. Oliveira Neto CP, Azulay RSS, Almeida AGFP, Tavares MDGR, Vaz LHG, Leal IRL, Gama MEA, Ribeiro MRC, Nascimento GC, Magalhães M, Santos WCD, Facundo AN, Faria MDS, Lago DCF. Differences in Puberty of Girls before and during the COVID-19 Pandemic. Int J Environ Res Public Health 2022;19:4733.

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Clinical Characteristics and Genetic Analyses of Patients with Idiopathic Hypogonadotropic Hypogonadism

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What is already known on this topic?

Approximately 50% of all normosmic idiopathic hypogonadotropic hypogonadism (nIHH)/Kalman syndrome cases can be explained by genetic variations reported in more than 50 genes. It has been suggested that gonadotropin releasing hormone receptor variations account for approximately 40-50% of familial, autosomal recessive nIHH and approximately 17% of sporadic nIHH.

What this study adds?

Many variants of uncertain significance (VUS) were obtained in children with idiopathic hypogonadotropic hypogonadism. In this study protein models showed that variants interpreted as VUS according to American College of Medical Genetics and Genomics guidelines could account for the clinical IHH.

Abstract

Objective: Idiopathic hypogonadotropic hypogonadism (IHH) is classified into two groups-Kalman syndrome and normosmic IHH (nIHH). Half of all cases can be explained by mutations in >50 genes. Targeted gene panel testing with nexrt generation sequencing (NGS) is required for patients without typical phenotypic findings. The aim was to determine the genetic etiologies of patients with IHH using NGS, including 54 IHH-associated genes, and to present protein homology modeling and protein stability analyzes of the detected variations.

Methods: Clinical and demographic data of 16 patients (eight female), aged between 11.6-17.8 years, from different families were assessed. All patients were followed up for a diagnosis of nIHH, had normal cranial imaging, were without anterior pituitary hormone deficiency other than gonadotropins, had no sex chromosome anomaly, had no additional disease, and underwent genetic analysis with NGS between the years 2008-2021. Rare variants were classified according to the variant interpretation framework of the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology. Changes in protein structure caused by variations were modeled using RoseTTAFold and changes in protein stability resulting from variation were analyzed.

Results: Half of the 16 had no detectable variation. Three (18.75%) had a homozygous (pathogenic) variant in the *GNRHR* gene, one (6.25%) had a compound heterozygous [likely pathogenic-variants of uncertain significance (VUS)] variant in *PROK2* and four (25%) each had a heterozygous (VUS) variant in *HESX1, FGF8, FLRT3* and *DMXL2*. Protein models showed that variants interpreted as VUS according to ACMG could account for the clinical IHH.

Conclusion: The frequency of variation detection was similar to the literature. Modelling showed that the variant in five different genes, interpreted as VUS according to ACMG, could explain the clinical IHH.

Keywords: Protein modelling, hypogonadotropic hypogonadism, genetic analyses

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Introduction

Normal pubertal development depends on the production and appropriate activity of gonadotropin-releasing hormone (GnRH) produced by neurons in the ventromedial hypothalamus (1,2). Isolated GnRH deficiency, also called idiopathic hypogonadotropic hypogonadism (IHH), is a group of genetic disorders associated with defects in the production and/or action of this hypothalamic peptide that controls human reproduction (3). IHH is divided into two main groups: Kalman syndrome (KS) and normosmic IHH (nIHH). IHH can be congenital (congenital heart disease) or acquired. The majority of hereditary causes of IHH are congenital (4).

Recently, with advances in genetic techniques, such as next generation sequencing (NGS), approximately 50 % of all nIHH/KS cases can be explained by genetic variations reported in more than 50 genes (4,5,6). Since the identification of the role of *ANOS1* (formerly *KAL1*) in the pathogenesis of X-linked KS, variants in *ANOS1, FGFR1, GNRH/GNRHR* and *PROK2/PROKR2* associated with IHH have been reported in several studies in the Human Gene Mutation Database as "disease-causing" (7). *GNRHR* is the first gene found to be responsible for isolated nIHH with deficiencies in follicle stimulating hormone (FSH) and luteinizing hormone (LH) (8,9,10). It has been suggested that *GNRHR* variations account for approximately 40-50 % of familial, autosomal recessive nIHH and approximately 17 % of sporadic nIHH (11). As a result of genetic studies performed in the last two decades, it has been found that many genes are associated with IHH (4). Genetic heterogeneity, variable expression and incomplete penetrance make it difficult to correlate the genotype-phenotype of IHH (12,13,14). Genetic tests are recommended for diagnosis of IHH and are necessary to determine the prognosis of IHH and to provide relevant genetic counseling (15).

Despite these recent advances in our understanding of the pathogenesis of IHH, it is likely that many more pathogenic genes remain to be discovered. While Sanger sequencing analyzes may be indicative for patients with specific findings or a family history, multi-gene panel testing NGS is required for patients who do not have typical phenotypic findings and/or no family history (16). Perhaps the most current challenge in the molecular genetic diagnosis of nIHH is the evaluation of variants of unknown clinical significance (VUS). Segregation analysis of family members is very important to reveal the genetic etiology. In addition, comprehensive *in silico* analyzes to assess the structural and functional impact of each genetic change on the protein product may be useful.

Mutations can cause changes in protein functional properties and protein-protein interactions by triggering changes in protein structure and stability. These changes are the basis of the development mechanism of many diseases (17,18,19). It should be kept in mind that the changes caused by mutations will trigger changes not only in the mutant protein but also in other proteins and structures with which it interacts. Therefore, elucidating the molecular mechanism of diseases is a complex and heterogeneous process. In recent years, *in silico* tools have significantly contributed to making many data and findings meaningful in this complex problem. In particular, computational studies that reduce the experimental processes that can take years to brief periods in the development of drugs and vaccines that can be the solution to global health problems come to the fore with their high reliability. It has been confirmed by numerous scientific studies that artificial intelligence-supported applications that use technical scientific data in the analysis of protein structure and stability provide high-reliability data. It has also been shown that computational tools used in protein homology modeling and stability analysis produce results that are equal to the data obtained by experimental methods, and some applications even produce better results than experimental data (20,21).

This study was conducted to determine the genetic etiology of patients with IHH by targeted gene panel including 54 genes known to cause IHH and to present protein homology modeling and protein stability analyzes of any detected variations.

Methods

Clinical and demographic data of patients followed up with the diagnosis of nIHH in the Pediatric Endocrine Departments of İnönü University Faculty of Medicine and Malatya Training and Research Hospital between the years of 2008 and 2021 were analyzed.

The diagnosis of nIHH was made according to the following criteria:

1) Absence or insufficient development of secondary sexual characteristics after the age of 13 in girls and after the age of 14 in boys;

2) Clinical signs or symptoms of hypogonadism;

3) Insufficient (low) sex steroid concentrations [testosterone or estradiol (E2)], and LH and FSH concentrations during the GnRH test;

4) Normal levels of free thyroxine, thyroid stimulating hormone, prolactin, insulin-like growth factor-1, adrencorticotropic hormone, and cortisol;

5) No evidence of structural lesions on imaging of the hypothalamic-pituitary region;

6) No evidence of chronic systemic diseases (such as uremia, thalassemia, poorly controlled diabetes mellitus), eating disorders (such as anorexia nervosa, bulimia), or protein energy malnutrition;

7) No patients reported olfactory problems;

8) None had features typical of Bardet-Biedl, Biemond, or Prader-Willi syndrome;

9) Absence of sex chromosome abnormalities (6,22,23,24).

GnRH test was done at 08:00 in the morning. Blood samples for FSH, LH, E2 or testosterone were taken. Then 100 mcg of GnRH was administered intravenously. Blood samples were taken for FSH and LH levels at 20, 40, 60 and 90 minutes after drug administration.

The study was approved by the Ethics Committee of İnönü University Faculty of Medicine (approval number: 2022/2650, date: 11.01.2022). Written consent was obtained from all patients or their legal guardians, if under eighteen years.

Clinical and Endocrinological Evaluation

Medical records including, clinical features, sense of smell, family history, associated anomalies, micropenis-cryptorchidism history, and laboratory-radiological findings were retrospectively reviewed. Pubertal development was graded according to the guidelines recommended by Marshall and Tanner (11). Testicular volume was measured with a Prader orchidometer. Olfactory function of the patients was evaluated by anamnesis, olfactory function test could not be used to diagnose olfactory abnormalities.

Statistical Analysis

Descriptive statistical method was used in this study. Data were summarized as count (percentage).

Next Generation Sequencing and Bioinformatics Analysis

Genetic Analyses

Genomic DNA was extracted from peripheral blood and NGS was performed by capture of the coding regions and splice sites of the following target genes: *ANOS1, CHD7, CYP19A1, DUSP6, DMXL2, DUSP6, ESR1, FEZF1, FGF8, FGFR1, FSHB, FGF17, FLRT3, GH1, GLCE, GLI2, GNRH1, GNRHR, HESX1, HS6ST1, IHX3, IL17RD, KISS1, KISS1R, LEP, LEPR, LHX3, LHB, LHX4, LHCGR, NROB1, NR5A1, NSMF, OTX2, OTUD4, PNPLA6, POLR3A, POLR3B, POU1F1, PROK2, PROKR2, PROP1, RNF216, SEMA3A, SEMA3E, SOX2, SOX3, SOX10, SPRY4, STUB1, TACR3, TUBB3, TAC3, WDR11*. An Illumina custom enrichment panel was used for this (Illumina, San Diego, CA, USA).

After library enrichment and quality control, the samples were sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 100-bp paired-end reads at an average sequencing depth of 100×.

The sequencing reads were aligned to the human reference genome assembly (GRCh37: Genome Reference Consortium Human Build 37) using BWA. Then, BAM files were sorted, indexed and de-duplicated using SAMtools and Picard. For the filtering process, exonic and splicing variants, including missense/nonsense variants, and indels were selected. Annotation of detected variants was performed using Illumina BaseSpace Variant Interpreter, InterVar, Franklin, VarSome, ClinVar, OMIM, and Pubmed. Variants with a frequency higher than 0.1% were filtered out. dbNSFP, which contains SIFT, PolyPhen-2, LRT, and Mutation Taster, was used to predict the pathogenicity of variants. Rare variants were classified according to the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology variant interpretation framework (25).

All variants identified by NGS were confirmed by Sanger sequencing. Sanger sequencing was performed using the Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA). Detected variants were classified as "pathogenic", "likely pathogenic (LP)", or "variant of uncertain significance (VUS)" according to the international guidelines of the ACMG. To assess the association between any identified genetic variants and IHH, hypothetical protein structures were constructed and analyzed *in silico* (see below).

Protein Homology Modeling

Modeling of changes in protein structure caused by variations was performed with RoseTTAFold, which uses deep learning-based, three-track neural network algorithms. Rosetta provides both *ab initio* and comparative models of protein domains. Comparative models are built from structures detected and aligned by HHSEARCH, SPARKS, and Raptor. Loop regions are assembled from fragments and optimized to fit the aligned template structures. *De novo* models are built using the Rosetta *de novo* protocol (26). Since the protein structures of some of the IHH-related genes (*PROK2, DMXL2* and *PROP1*) examined in this study were not previously defined, wild-type protein structures were also modeled in this study for the first time. Identification of the reference sequence data for the variants is given in Table 1. The *GNRHR*, *FLRT3* and *FGF8* homology models were created using templates from the Protein Data Bank: 7BR3, 5CMP and 2FDB. Protein model quality analyzes were performed with ProSA and QMEANDisco (27,28).

Topological differences between wild-type and mutant protein were analyzed by TM-score (29). Superimpositional and conformational analysis of proteins were performed with DDS and PyMOL (ver2.4.1).

Protein Stability Analyzes

Changes in protein stability after variation was analyzed with mCSMstability (30), DUET (31), SDM (32), and DynaMut2 (33) bioinformatics tools. All interatomic contacts calculated with Arpeggio were displayed using NGL viewer (34,35).

Results

Of 39 patients with IHH whose file data were available, 18 (46%) were male and 21 (54%) were female. Of these, 16 unrelated patients (eight female) with the diagnosis of IHH and whose genetic panel had been performed were included in the study. Mean age of the patients at presentation was 14.8 years. All of the patients presented with delayed puberty. None of the patients reported problems with sense of smell. There was a history of delayed puberty in the family of six (37.5%) patients.

Six (75%) male patients had micropenis. A patient with a normal penis size (patient number F15P15) had received six doses of intramuscular testosterone therapy in an external center before attending our clinic. Three patients (F1P1, F2P2 and F3P3) had a history of unilateral cryptorchidism. No patient had a history of bilateral cryptorchidism. The pubertal stage of 14 patients (87.5%; seven girls and seven boys), was evaluated as Tanner stage 1. One male patient (6.25%) was Tanner stage 2, and one female patient (6.25%) was at Tanner stage 4 of puberty. Both patients (patients F15P15 and F12P12) who had started puberty had received sex steroid replacement therapy in an external center before attending our clinic. GnRH stimulation test was performed in all patients. The clinical and laboratory findings of the patients at presentation are summarized in Table 2.

Molecular Findings

Eight (50%) had a variation in one of the genes included in the panel while eight had no detectable variant in the gene panel used. Three (18.75%) had a pathogenic, homozygous variant in the *GNRHR* gene, one (6.25%) had LP, compound heterozygous variant in *PROK2*, and four (25%) had a VUS in one each of four different genes, *HESX1*, *FGF8*, *FLRT3* and *DMXL2* (Table 1). The variants detected in the study and the assessment of pathogenicity are shown in Table 1 (25).

The previously reported hot spot pathogenic variant c.415C>T in the *GNRHR* gene, was detected homozygously in our three index cases. Parents were shown to be carriers by segregation analyzes, and parents had a history of delayed puberty. The same variation was present in a homozygous fashion in two siblings of P1 and the twin of P2. The three siblings were being followed in our clinic due to delayed

Table 1. The pathogenicity assessment of the detected variants

Patient number	Gene	Transcript number	Nucleotide change	AA change	MAF by gnomAD	Zyg	Variant location	Variant type	ClinVar	ACMG class	ACMG pat crit
F1P1	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F2P2	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F3P3	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F4P4	*PROK2*	NM_001126128.2	c.217C>T	p.Arg73Cys	0.0000716	Het	Exon 2	Mis	Pat	LP	PM2, PP3, PP5
	PROK2	NM_001126128.2	c.1A>C	p.Met1Leu	-	Het	Exon 1	Mis	NP	VUS	PVS1, PM2
F5P5	*HESX1*	NM_003865.3	c.18G>C	p.Gln6His	-	Het	Exon 1	Mis	VUS	VUS	PM2, PM6, PP2
F6P6	*FGF8*	NM_033163.5	c.476C>T	p.Thr159Met	-	Het	Exon 6	Mis	NP	VUS	PP2, PP3, BS2, PM6
F7P7	*FLRT3*	NM_013281.3	c.1541A>G	p.Asn514Ser	0.00000798	Het	Exon 2	Mis	NP	VUS	PM1, PM2
F8P8	*DMXL2*	NM_001174116.3	c.5915A>T	p.Glu1972Val	-	Het	Exon 24	Mis	NP	VUS	PM2

AA: amino acid, MAF: minor allele frequency, Zyg: zygosity, ACMG Class: The American College of Medical Genetics and Genomics Classification, ACMG Pat Crit: ACMG Pathogenicity Criteria, Het: heterozygous, Hom: homozygous, Del: deletion, Frms: frameshift, Mis: missense, Splc: splicing, NP: not provided, Pat: pathogenic, LP: likely pathogenic, VUS: variant of uncertain significance

Table 2. Age, clinical presentation, family history, Tanner stage and GnRH test findings of all patients

	Sex	Age at diagnosis (years)	Clinical presentation	Family history	Stretched penile length (cm)	Tanner stage of gonads at diagnosis	GnRH test peak	
							FSH (mIU/mL)	LH (mIU/mL)
F1P1	M	11y 9/12	Cryptorchidism, micropenis	Pubertal delay in brother, dad and grandfather. Sister has no menstruation	4.5	1	1.88	0.2
F2P2	M	11y 7/12	Cryptorchidism micropenis	Pubertal delay in twins. Parents had a child with *in vitro* fertilization.	3.7	1	1.74	0.66
F3P3	M	13y 7/12	Cryptorchidism, micropenis	Micropenis in brother	3	1	0.45	0.24
F4P4	F	14y 9/12	No menstruation	-		1	3.56	1.28
F5P5*	M	16y 10/12	Pubertal delay	Pubertal delay in father and brother.	8	1	5.3	7.1
F6P6	F	14y 3/12	No menstruation	-		1	9.54	2.03
F7P7	M	14y 7/12	Micropenis	-	4	1	5.98	0.93
F8P8	F	14y 11/12	No menstruation	Late menstruation in mother		1	1.66	1.89
F9P9	F	15y 7/12	No menstruation	-		1	3.64	1.02
F10P10	F	15y 5/12	No menstruation	-		1	3.28	1
F11P11	F	15y 2/12	No menstruation	-		1	2.52	0.77
F12P12	F	16y 6/12	No menstruation	-		4	4.1	3.2
F13P13	M	10y 4/12	Micropenis	-	4	1	5.89	0.8
F14P14	F	16y 10/12	No menstruation	-		1	5.2	1.75
F15P15	M	17y 10/12	Pubertal delay	Pubertal delay in uncle		2	1.14	0
F16P16*	M	16y 8/12	Pubertal delay micropenis	-	6	1	3.9	8.4

NB brain magnetic resonance imaging of all patients was evaluated as normal.
*The 5th and 16th patients had late puberty. Pubertal induction therapy was given to them. They were followed up for one year at pediatric endocrinology department and their testicular volumes remained < 4 mL.
y: year, F: family, P: patient, F: female, M: male, N: not detected, GnRH: gonadotropin-releasing hormone, FSH: follicle stimulating hormone, LH: luteinizing hormone

puberty and were receiving pubertal induction therapy.

In one patient, c.1A>C and c.217C>T variants in the *PROK2* gene were detected in a compound heterozygous fashion. According to the ACMG classification, these variants are interpreted as VUS/LP. As a result of segregation analysis, the heterozygous c.271C>T variant was found in the mother of the patient, and the heterozygous c.1A>C variant was found in the father. There was no history suggestive of hypogonadism in the parents.

In one patient, a heterozygous variation, c.18G>C, was detected in *HESX1*, and this was found to be a *de novo* mutation.

A c.476C>T, heterozygous variant was detected in *FGF8* in one patient. The segregation analysis showed no such variant in the mother, while heterozygous variation was found in the same gene in the father. It was learned that the father had puberty tarda and had children without any therapy.

A c.1541A>G heterozygous variation was detected in *FLRT3* in one patient. Genetic analysis could not be performed in the parents of this patient.

A heterozygous variation, c.5915A>T was detected in *DMXL2* in one patient. While heterozygous variation was detected in the same gene in the mother, no variation was found in the father. It was learned that the mother had late menstruation but had children spontaneously.

Protein Structural Analysis

In this study, the relationship between the changes in protein structure caused by seven variations in six different genes (*GNRHR, PROK2, HESX1, FGF8*, *FLRT3* and *DMXL2*)

and IHH was investigated. Tertiary models of proteins containing mutant residues were created using deep learning algorithms. The protein tertiary models created were within the quality limits of X-ray and NMR. QMEAN scores ranged from -2.06 to 0.66. The *GNRHR*.p.Arg139Cys variation is associated with HH disease. *GNRHR* is a G-protein-coupled GnRH receptor, regulates LH and FSH secretion and has seven transmembrane segments and an extracellular amino terminus (36). *GNRHR*.p.Arg139Cys variations were noted for their highly destabilizing effects (-2.35 and -2.086 kcal. mol^{-1}, respectively) and increased solvent accessibility. The *GNRHR*.p.Arg139Cys variation changed the protein topology (rmsd 0.157 Å). The Arg139Cys variation in the cytoplasmic region may affect the coupling of the G protein with the receptor. The Arg139 residue in wild-type *GNRHR* contributes to cytoplasmic region stability with twenty-one weak bond interactions (Figure 1a). It was observed that the number of these interactions decreased to thirteen due to the changed conformation in the mutant protein, and the two hydrophobic and one polar interaction with Met76 was abolished (Figure 1b). Solvent accessibility of residue 139 increased approximately 3-fold after variation.

The *PROK2*.p.Met1Leu variation resulted in a possible 43 amino acid shortening of the mature protein length and changed topology (Figure 2a, 2b). The rmsd was 0.930 Å in superimpose. The *PROK2*.p.Met1Leu variation may have shifted the start signal to the methionine codon at the 44th codon. Therefore, stability assessment of the *PROK2*.p.Met1Leu variation was performed at the conformational level, since the mutant protein did not contain mutant residue. The rmsd was 0.582 Å for the *PROK2*.p.Arg73Cys variation at superimpose. The variation caused a change in conformation (Figure 1c, 1d) and topology (Figure 2c, 2d) of the protein product.

In this paper a three-dimensional model of *HESX1* is presented for the first time. The model developed was within the NMR quality limitations (Z score -4.1). The Gln6His variation caused limited change in protein structure. The -NE2 group 5.2 Å moved away from the main backbone (Figure 3a) as a result of the variation, increasing exposure to solvent accessibility (Table 3). After the variation, the two hydrophobic and one polar contact created between residue-1 and residue-6 were abolished (Figure 3b, 3c). The interaction between residue-3 and -6 with two polar and three hydrogen bonds was reduced to three polar interactions after the variation. The conformational change induced by the variation revealed one van der Waals (vdw) and one polar interaction between residue-6 and residue-10 that was not present in the wild type. *HESX1*.p.Gln6His variation caused a decrease in protein stability (-0.732 kcal. mol^{-1}).

FGF8.p.Thr159Met variation increased protein instability (-0.444 kcal.mol^{-1}). An increase in the solvent accessibility of the 159th residue after the variation was identified (Table 3). The variation detected in our patient in protein modeling caused a putative change in the conformational structure of *FGF8* (rmsd 0.184 Å) (Figure 4a). Changes in the conformation and topology of two consecutive heterodimer helix-turn-helix motifs located in the N-terminal domain of the FGF8 protein may result in changes in protein functional

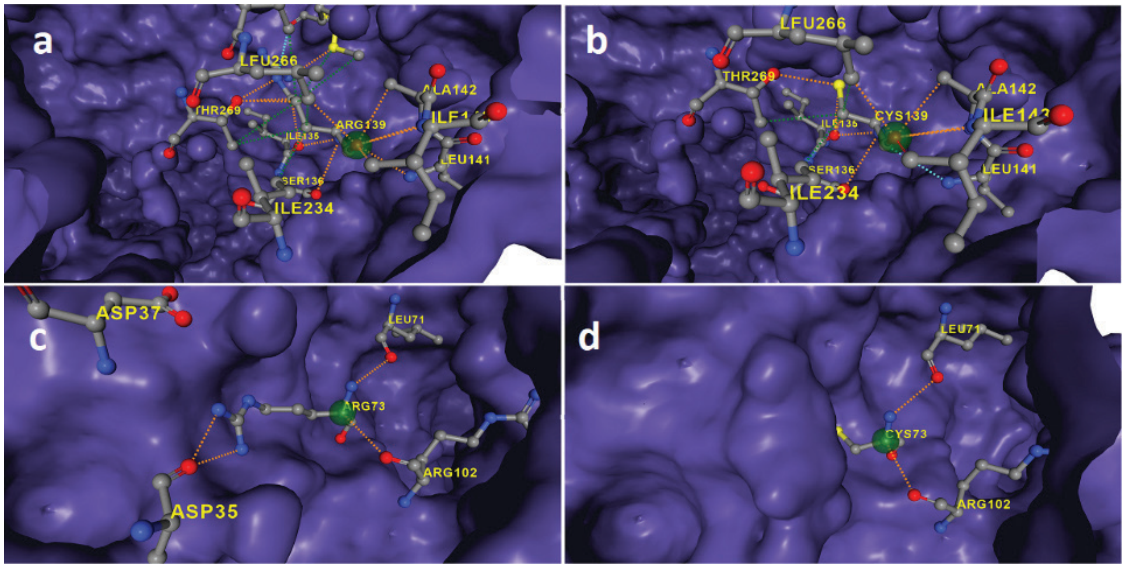

Figure 1. Surface/stick representation of changes in protein stability and bond formation caused by variations. Green transparent sphere indicates mutant position. Colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar. a) *GNRHR* wild-type, b) *GNRHR* mutant, c) *PROK2* wild-type, d) *PROK2* mutant

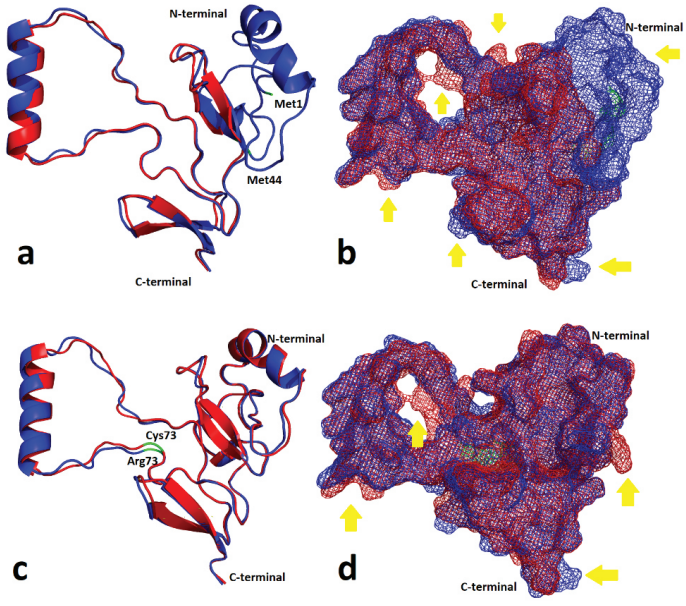

Figure 2. Superimpose representation of the changes in protein conformation and topology caused by *PROK2* variations

Blue: wild-type *PROK2*, red: mutant *PROK2*, yellow arrow: indicates change, green: mutant residue. a) Cartoon representation of the *PROK2*.p.Met1Leu variation, b) Mesh topological representation of the *PROK2*.p.Met1Leu variation, c) Cartoon representation of the *PROK2*.p.Arg73Cys variation, d) Mesh topological representation of the *PROK2*.p.Arg73Cys variation

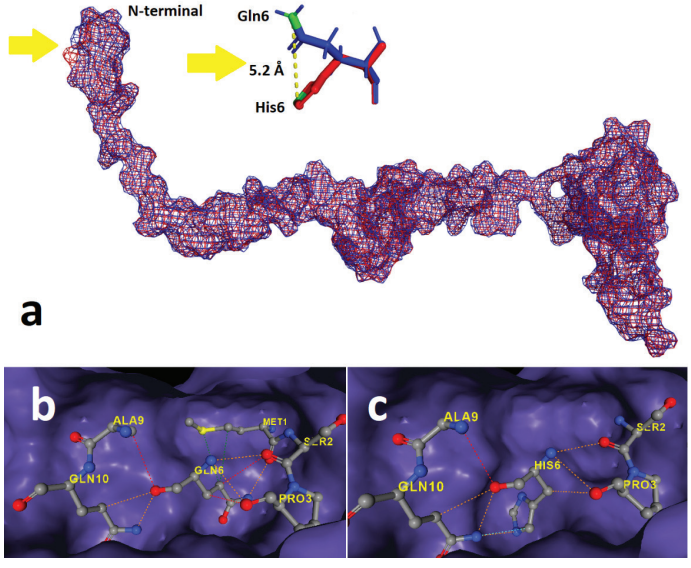

Figure 3. Representation of the changes in protein congormation and topology caused by HESX1 variation

a) Superimpose (blue: wild-type, red: mutant, yellow arrow: indicates change), b) Surface/stick representation of wild-type *HESX1*, c) Surface/stick representation of mutant *HESX1* (colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

properties, protein-protein/DNA, and receptor interaction (Figure 4b). The interaction between Thr159 and Arg125 in wild-type FGF8 was abolished in the mutant protein (Figure 4c, 4d).

The *FLRT3*.p.Asn514Ser variation changed the topology of the tunnel formation located near the transmembrane domain (residue 529-549) (Figure 5a, 5b). The two polar interactions between Asn514 and Glu516 in the wild-type FLRT3 protein were abolished in the mutant FLRT3 (Figure 5c, 5d). It was observed that the interaction between wild-type Asn514 and Gln517 with a hydrophobic force of one hydrogen was provided by two hydrogen bonds and a polar interaction between Ser514 and Gln517 in the mutant protein. The interaction between residue 514 and residue 517 was achieved with one hydrogen bond and one hydrophobic force in wild-type FLRT3, while in mutant FLRT3 this was changed to two hydrogen bonds and one polar interaction. The *FLRT3*.p.Asn514Ser variation decreased protein stability and solvent accessibility (-0.188 kcal.mol^{-1}) (Table 3).

The *DMXL2*.p.Glu1972Val variation increased protein stability (0.139 kcal.mol^{-1}). The p.Glu1972Val variation abolished the two polar interactions between residue 1973 and Lys2013 (Table 3).

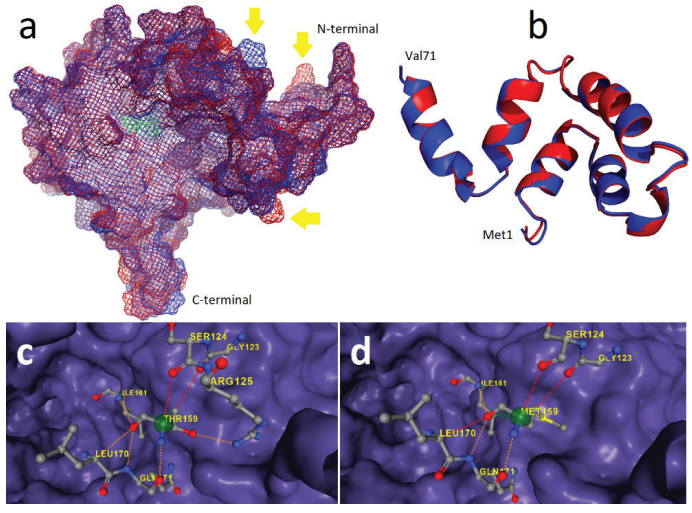

Figure 4. Illustration of variation-induced change in *FGF8*

a) Mesh topological representation of topological changes in *FGF8*, b) Cartoon representation of N-terminal domain of *FGF8* (blue: wild-type *FGF8*, red: mutant *FGF8*, yellow arrow: indicates change, green: mutant residue), c) Surface/stick presentation of residue interactions of wild-type *FGF8*, d) Surface/stick presentation of residue interactions of mutant *FGF8* (colors in dashed lines represent- green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

Table 3. Effects of mutant residues on protein stability

Protein . Mutation	Protein stability (ΔΔG kcal.mol^{-1})				Output	%RSA exchange
	mCSMstability	DUET	SDM	DynaMut2		
GNRHR.p.Arg139Cys	-2.086	-2.267	-1.57	-1.61	Highly destabilizing	0.9 → 2.4
PROK2.p.Arg73Cys	0.13	0.104	-0.18	0.56	Stabilizing	45.4 → 68.1
HESX1.pGln6His	-0.732	-0.466	-0.33	-0.26	Destabilizing	60.2 → 74.3
FGF8.p.Thr159Met	-0.444	-0.283	0.08	-0.3	Destabilizing	26.2 → 35.4
FLRT3.p.Asn514Ser	-0.188	-0.002	-0.33	0.29	Destabilizing	99.6 → 81.7
DMXL2.p.Glu1972Val	0.139	0.539	0.78	0.3	Stabilizing	70.7 → 72.3

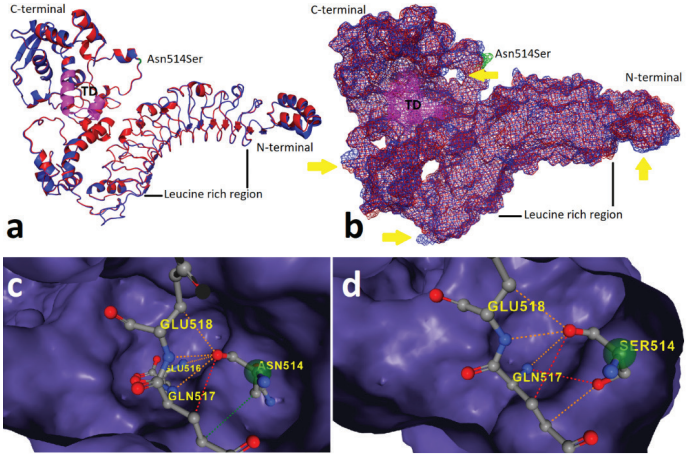

Figure 5. Representation of the changes caused by the *FLRT3* variation

a) Superimpose cartoon representation of the FLRT3.p.Asn514Ser variation, b) Superimpose mesh topological representation of the FLRT3.p.Asn514Ser variation (blue: wild-type FLRT3, red: mutant FLRT3, yellow arrow: indicates topological change, green: mutant residue, magenta: transmembrane domain (TD), c) Surface/stick presentation of residue interactions of wild-type FLRT3, d) Surface/stick presentation of residue interactions of mutant *FLRT3* (colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

Discussion

In this study targeted NGS analysis was used in patients with nIHH of unknown genetic etiology. We found a genetic etiology in 50% (8/16) of cases. The most common variation was the C.415C>T homozygous variation in the *GNRHR* gene, which was interpreted as pathogenic according to the ACMG Classification. The c.415C>T (p.Arg139Cys) variant, which was present homozygously in our patients, is a known hot spot variation in the *GNRHR* gene. This variant was first reported by Topaloglu et al. (37) in 2009 and it was found in two Turkish sisters (aged 16 and 23), whose parents were first-degree cousins, who presented with delayed puberty. *GNRHR* variations are known to account for approximately 40-50% of familial nIHH (11) and our results were compatible with this. Protein models showed that the *GNRHR*.p.Arg139Cys variation was highly destabilizing and increased solvent accessibility. The *GNRHR*.p.Arg139Cys variation also changed the protein topology on *in silico* modeling. It is possible that these variational changes decrease intracellular signaling mechanism effectiveness and lead to reduced activation of phospholipase-C, rather than receptor binding affinity. De Roux et al. (8) revealed that variations in the cytoplasmic loop did not change the binding of GnRH to the receptor, but decreased activation of the effector macromolecule phospholipase-C.

The *PROK2* gene encodes prokinetecin 2, an 81 amino acid peptide that signals through the G protein-linked product of the *PROKR2* gene (38). Variations in *PROKR2* and *PROK2* are generally seen in combination with other variations with oligogenic inheritance in IHH (4). In our study, c.217C>T (p.Arg73Cys), interpreted as LP according to ACMG classification, and c.1A>C (p.Met1Leu), interpreted as VUS, were found to be combined in a heterozygous fashion in one patient in *PROK2*. Protein models showed that the *PROK2*.p.Met1Leu variation resulted in a possible 43 amino acid shortening of the mature protein length and changed topology. The absence of the -AVITGA- sequence, which is highly conserved across species and thought to be important for the functional properties of *PROK2*, may result in impaired protein function (39,40,41). We hypothesize that this variation, which is currently interpreted as VUS according to the ACMG classification, may be associated with HH. The *PROK2*.p.Arg73Cys variation caused a putative change in conformation and topology of the protein product. The cysteine residue introduced by the p.Arg73Cys variation is likely to affect the formation of disulfide bonds in the protein (42). The decrease in receptor affinity caused by the changed protein structure with these identified *PROK2* variations may be the reason for the decrease in receptor signaling, intracellular calcium mobilization, and MAPK signaling that will result in the HH phenotype and lack of GnRH (43,44). The patient's mother was carrying the c.271C>T variant, and her father was heterozygous

for the c.1A > C variant. There was no history of delayed puberty in the parents. It was thought that the compound heterozygous variation in our patient may have caused thier clinical findings.

The *HESX1* gene is part of a family of homeobox genes that act during early embryonic development to control the formation of many body structures. HESX1 protein is a transcription factor that plays an important role in early-stage brain development. The HESX1 protein is required for the structural development of the forebrain and pituitary. HESX1 exerts its effects in combination with PROP1 and many other proteins during embryonic development to coordinate the formation of different parts of the brain through the control of gene expression (45,46,47). It is not clear whether *HESX1* variations cause mild forms of IHH, or partial or complete absence of puberty due to GnRH deficiency/impaired gonadotropins (48). Newbern et al. (48) investigated the presence of *HESX1* variation in 217 patients, followed up with the diagnosis of KS or IHH and in whom other anterior pituitary deficiencies were excluded and a control group of 192 patients. They detected a *HESX1* heterozygous variant in three patients, two of whom were Turkish. In the control group, no variation was detected and no variation was found in the 1,000 genomes database. In our study, one patient was heterozygous for *HESX1*, which was interpreted as VUS according to ACMG classification. Segregation analysis confirmed that the variant was *de novo*. We evaluated this change, which we believe may explain the patient's clinical picture. Protein models showed that *HESX1*.p.Gln6His variation caused a decrease in protein stability. We suggest that heterozygous variations of the *HESX1* gene, whose homozygous variations lead to severe phenotypes, such as septo-optic dysplasia, may cause IHH. However, further studies are needed to confirm this hypothesis.

Studies have shown that there is a 30-50 % decrease in total GnRH neurons in mice harboring heterozygous *FGF8* gene variations, while a greater reduction in GnRH neurons is seen in mice with co-variation in *FGFR1* and *FGF8* genes (32). Olsen et al. (49) showed that variation of Phe32Ala in the N-terminal region of *FGF8*b, the isomer of *FGF8*, resulted in decreased receptor affinity and changes in protein functional properties. In the presence of other gene variations accompanying p.Glu176Lys and p.Arg184Cys variations in *FGF8*, in addition to HH, some clinical problems reflected in the phenotype, such as dental agenesis, hearing loss and hand malformation, have been reported (32). In our study, a heterozygous variation of c.476C > T, interpreted as VUS according to the ACMG classification, was detected in *FGF8* in one patient. While no variation was detected in the mother of the patient, the same heterozygous variation was found in her father. It was learned that her father had delayed puberty but had children spontaneously. It was thought that this variant may explain the patient's clinical picture, but more studies are needed.

The *FGF8*.p.Thr159Met variation, detected in our patient, caused a change in the conformational structure of *FGF8* (rmsd 0.184 Å) on protein modelling. The fact that the patient and her father had a history of delayed puberty together with the predicted decreased protein stability of the detected variant suggest that this variant may explain the HH in the patient. In this study, we report the association of the *FGF8*.p.Thr159Met variation with HH for the first time.

A heterozygous variation of c.1541A > G, interpreted as VUS according to ACMG classification, was detected in *FLRT3* in one patient. Genetic analysis could not be performed in the parents of this patient. The variation changed the putative protein conformation and decreased protein stability in protein modelling. We suggest that this variation, which is currently interpreted as VUS according to the ACMG classification, may be associated with HH.

A heterozygous variation of c.5915A > T, was detected in *DMXL2*, which was interpreted as VUS according to the ACMG Classification, in one patient. While heterozygous variation was detected in the same gene in the mother of the patient, no variation was found in the father. The mother had a history of late menstruation but had children spontaneously. The *DMXL2*.p.Glu1972Val variation increased protein stability (0.139 kcal.mol^{-1}) in protein modeling. We hypothesize that this variation, which caused delayed puberty in both mother and the patient, and abolished two polar interactions on protein modeling, could be the etiology of our patient's HH.

Amato et al. (50) performed genetic analyzes of 130 CHH patients using NGS (including 29 known and seven candidate genes) and detected pathogenic/LP variations in 43 (33 %). In this study, as in our study, the most common variation detected in nIHH patients was in the *GNRHR* gene.

Study Limitations

The number of our patients was small as we were working with a rare genetic disease group. Genetic analysis could not be performed on the parents of a patient whose genetic variation was determined as VUS according to ACGM. Olfactory function test were not performed because they are not available at our hospital. Olfactory function of the patients was evaluated by anamnesis and this may be unreliable. Finally, functional analysis was not performed in variations classified as VUS by the ACGM.

Conclusion

In this study, pathogenic/LP variation was detected in 25% of 16 patients and VUS in a further 25%, while no variation was detected in 50% using a panel containing 54 genes associated with IHH. The frequency of detection of variants is similar to the literature. The most frequently detected variation was in the *GNRHR* gene, a finding consistent with several previous reports. Protein models showed that variants interpreted as VUS (*PROK2*, *HESX1*, *FGF8*, *FLRT3* and *DMXL2*) according to ACMG could account for the clinical IHH. Association of the *FGF8*.p.Thr159Met variation with HH was reported for the first time in this study. Large-scale genetic studies are needed to understand the genetic aspects of nIHH in Turkey and in other populations. Overall, the practical yield of this study is considerable because it reflects professional experience gained in a single center and represents one of the first studies in Turkish children including molecular analysis of 54 causal IHH-related genes. Confirmatory genetic testing in patients with suspected nIHH allows for definitive diagnoses, which may guide management and provide rationales for screening other family members presymptomatically. In studies conducted with NGS, as in our study, through advancing molecular testing and identification of new genes, the number of patients with nIHH may be expected to rise rapidly. It is reasonable and appropriate to conclude here that verification of these candidate genes would not only help treatment plans for these patients, but would also facilitate further research into GnRH neuronal migration.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of İnönü University Faculty of Medicine (approval number: 2022/2650, date: 11.01.2022).

Informed Consent: Written consent was obtained from all patients or their legal guardians, if under eighteen years.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Mustafa Doğan, Leman Kayaş, Concept: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Design: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Data Collection or Processing: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Mustafa Doğan, Leman Kayaş, Analysis or Interpretation: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Literature Search: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Writing: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Leman Kayaş.

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References

1. Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. Nature 1989;338:161-N164.
2. Wray S. From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. J Neuroendocrinol 2010;22:743-753.
3. Pitteloud N, Crowley WF, Ravikumar B. Isolated gonadotropin-releasing hormone deficiency (idiopathic hypogonadotropic hypogonadism). UpTo Date. March, 2023. Available from: https://www.uptodate.com/contents/isolated-gonadotropin-releasing-hormone-deficiency-idiopathic-hypogonadotropic-hypogonadism
4. Topaloğlu AK. Update on the Genetics of Idiopathic Hypogonadotropic Hypogonadism. J Clin Res Pediatr Endocrinol 2017;9(Suppl 2):113-122. Epub 2017 Dec 27
5. Quaynor SD, Bosley ME, Duckworth CG, Porter KR, Kim SH, Kim HG, Chorich LP, Sullivan ME, Choi JH, Cameron RS, Layman LC. Targeted next generation sequencing approach identifies eighteen new candidate genes in normosmic hypogonadotropic hypogonadism and Kallmann syndrome. Mol Cell Endocrinol 2016;437:86-96. Epub 2016 Aug 5
6. Young J, Xu C, Papadakis GE, Acierno JS, Maione L, Hietamäki J, Raivio T, Pitteloud N. Clinical Management of Congenital Hypogonadotropic Hypogonadism. Endocr Rev 2019;40:669-710.
7. Stenson PD, Mort M, Ball EV, Shaw K, Phillips A, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Hum Genet 2014;133:1-9.
8. De Roux N, Young J, Misrahi M, Genet R, Chanson P, Schaison G, Milgrom E. A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor. N Engl J Med 1997;337:1597-1602.
9. Layman LC, Cohen DP, Jin M, Xie J, Li Z, Reindollar RH, Bolbolan S, Bick DP, Sherins RR, Duck LW, Musgrove LC, Sellers JC, Neill JD. Mutations in gonadotropin-releasing hormone receptor gene cause hypogonadotropic hypogonadism. Nat Genet 1998;18:14-15.
10. de Roux N, Young J, Brailly-Tabard S, Misrahi M, Milgrom E, Schaison G. The same molecular defects of the gonadotropin-releasing hormone receptor determine a variable degree of hypogonadism in affected kindred. J Clin Endocrinol Metab 1999;84:567-572.
11. Beranova M, Oliveira LM, Bédécarrats GY, Schipani E, Vallejo M, Ammini AC, Quintos JB, Hall JE, Martin KA, Hayes FJ, Pitteloud N, Kaiser UB, Crowley WF Jr, Seminara SB. Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab 2001;86:1580-1588.
12. Beate K, Joseph N, Nicolas de R, Wolfram K. Genetics of isolated hypogonadotropic hypogonadism: role of GnRH receptor and other genes. Int J Endocrinol 2012;2012:147893.
13. Fathi AK, Luo X. Normosmic idiopathic hypogonadotropic hypogonadism: update on the genetic background and future challenges. J Pediatr Endocrinol Metab 2013;26:405-415.

14. Liu Q, Yin X, Li P. Clinical, hormonal, and genetic characteristics of 25 Chinese patients with idiopathic hypogonadotropic hypogonadism. BMC Endocr Disord 2022;22:30.

15. Sykiotis GP, Hoang XH, Avbelj M, Hayes FJ, Thambundit A, Dwyer A, Au M, Plummer L, Crowley WF Jr, Pitteloud N. Congenital idiopathic hypogonadotropic hypogonadism: evidence of defects in the hypothalamus, pituitary, and testes. J Clin Endocrinol Metab 2010;95:3019-3027. Epub 2010 Apr 9

16. Behjati S, Tarpey PS. What is next generation sequencing? Arch Dis Child Educ Pract Ed 2013;98:236-238. Epub 2013 Aug 28

17. Meyer K, Kirchner M, Uyar B, Cheng JY, Russo G, Hernandez-Miranda LR, Szymborska A, Zauber H, Rudolph IM, Willnow TE, Akalin A, Haucke V, Gerhardt H, Birchmeier C, Kühn R, Krauss M, Diecke S, Pascual JM, Selbach M. Mutations in Disordered Regions Can Cause Disease by Creating Dileucine Motifs. Cell 2018;175:239-253. Epub 2018 Sep 6

18. Ancien F, Pucci F, Godfroid M, Rooman M. Prediction and interpretation of deleterious coding variants in terms of protein structural stability. Sci Rep 2018;8:4480.

19. Akbulut E. Investigation of changes in protein stability and substrate affinity of 3CL-protease of SARS-CoV-2 caused by mutations. Genet Mol Biol 2022;45:e20210404.

20. Pak MA, Ivankov DN. Best templates outperform homology models in predicting the impact of mutations on protein stability. Bioinformatics 2022;38:4312-4320.

21. Akdel M, Pires DEV, Pardo EP, Jänes J, Zalevsky AO, Mészáros B, Bryant P, Good LL, Laskowski RA, Pozzati G, Shenoy A, Zhu W, Kundrotas P, Serra VR, Rodrigues CHM, Dunham AS, Burke D, Borkakoti N, Velankar S, Frost A, Basquin J, Lindorff-Larsen K, Bateman A, Kajava AV, Valencia A, Ovchinnikov S, Durairaj J, Ascher DB, Thornton JM, Davey NE, Stein A, Elofsson A, Croll TI, Beltrao P. A structural biology community assessment of AlphaFold2 applications. Nat Struct Mol Biol 2022;29:1056-1067.

22. Boehm U, Bouloux PM, Dattani MT, de Roux N, Dodé C, Dunkel L, Dwyer AA, Giacobini P, Hardelin JP, Juul A, Maghnie M, Pitteloud N, Prevot V, Raivio T, Tena-Sempere M, Quinton R, Young J. Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism--pathogenesis, diagnosis and treatment. Nat Rev Endocrinol 2015;11:547-564.

23. Binder G, Schweizer R, Blumenstock G, Braun R. Inhibin B plus LH vs GnRH agonist test for distinguishing constitutional delay of growth and puberty from isolated hypogonadotropic hypogonadism in boys. Clin Endocrinol (Oxf) 2015;82:100-105. Epub 2014 Oct 23

24. Binder G, Schweizer R, Haber P, Blumenstock G, Braun R. Accuracy of Endocrine Tests for Detecting Hypogonadotropic Hypogonadism in Girls. J Pediatr 2015;167:674-678. Epub 2015 Jun 18

25. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-424. Epub 2015 Mar 5

26. Baek M, DiMaio F, Anishchenko I, Dauparas J, Ovchinnikov S, Lee GR, Wang J, Cong Q, Kinch LN, Schaeffer RD, Millán C, Park H, Adams C, Glassman CR, DeGiovanni A, Pereira JH, Rodrigues AV, van Dijk AA, Ebrecht AC, Opperman DJ, Sagmeister T, Buhlheller C, Pavkov-Keller T, Rathinaswamy MK, Dalwadi U, Yip CK, Burke JE, Garcia KC, Grishin NV, Adams PD, Read RJ, Baker D. Accurate prediction of protein structures and interactions using a three-track neural network. Science 2021;373:871-876. Epub 2021 Jul 15

27. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 2007;35:407-410. Epub 2007 May 21

28. Studer G, Rempfer C, Waterhouse AM, Gumienny R, Haas J, Schwede T. QMEANDisCo-distance constraints applied on model quality estimation. Bioinformatics 2020;36:1765-1771.

29. Xu J, Zhang Y. How significant is a protein structure similarity with TM-score = 0.5? Bioinformatics 2010;26:889-895. Epub 2010 Feb 17

30. Pires DE, Ascher DB, Blundell TL. mCSM: predicting the effects of mutations in proteins using graph-based signatures. Bioinformatics 2014;30:335-342. Epub 2013 Nov 26

31. Pires DE, Ascher DB, Blundell TL. DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. Nucleic Acids Res 2014;42:314-319. Epub 2014 May 14

32. Pandurangan AP, Ochoa-Montaño B, Ascher DB, Blundell TL. SDM: a server for predicting effects of mutations on protein stability. Nucleic Acids Res 2017;45:229-235.

33. Rodrigues CHM, Pires DEV, Ascher DB. DynaMut2: Assessing changes in stability and flexibility upon single and multiple point missense mutations. Protein Sci 2021;30:60-69. Epub 2020 Sep 11

34. Jubb HC, Higueruelo AP, Ochoa-Montaño B, Pitt WR, Ascher DB, Blundell TL. Arpeggio: A Web Server for Calculating and Visualising Interatomic Interactions in Protein Structures. J Mol Biol 2017;429:365-371. Epub 2016 Dec 10.

35. Rose AS, Bradley AR, Valasatava Y, Duarte JM, Prlic A, Rose PW. NGL viewer: web-based molecular graphics for large complexes. Bioinformatics 2018;34:3755-3758.

36. Amato LGL, Montenegro LR, Lerario AM, Jorge AAL, Guerra Junior G, Schnoll C, Renck AC, Trarbach EB, Costa EMF, Mendonca BB, Latronico AC, Silveira LFG. New genetic findings in a large cohort of congenital hypogonadotropic hypogonadism. Eur J Endocrinol 2019;181:103-119.

37. Topaloglu AK, Lu ZL, Farooqi IS, Mungan NO, Yuksel B, O'Rahilly S, Millar RP. Molecular genetic analysis of normosmic hypogonadotropic hypogonadism in a Turkish population: identification and detailed functional characterization of a novel mutation in the gonadotropin-releasing hormone receptor gene. Neuroendocrinology 2006;84:301-308. Epub 2006 Dec 19

38. Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY. Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. Science 2005;308:1923-1927.

39. Kaser A, Winklmayr M, Lepperdinger G, Kreil G. The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. EMBO Rep 2003;4:469-473.

40. Bullock CM, Li JD, Zhou QY. Structural determinants required for the bioactivities of prokineticins and identification of prokineticin receptor antagonists. Mol Pharmacol 2004;65:582-588.

41. Magnan C, Migrenne-Li S. Pleiotropic effects of prokineticin 2 in the control of energy metabolism. Biochimie 2021;186:73-81. Epub 2021 Apr 29

42. Chen J, Kuei C, Sutton S, Wilson S, Yu J, Kamme F, Mazur C, Lovenberg T, Liu C. Identification and pharmacological characterization of prokineticin 2 beta as a selective ligand for prokineticin receptor 1. Mol Pharmacol 2005;67:2070-2076.

43. Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toublanc JE, Wolczynski S, Delpech M, Petit C, Young J, Hardelin JP. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. PLoS Genet 2006;2:175. Epub 2006 Sep 1

44. Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, Hughes VA, Dwyer AA, Raivio T, Hayes FJ, Seminara SB, Huot C, Alos N, Speiser P, Takeshita A, Van Vliet G, Pearce S, Crowley WF Jr, Zhou QY, Pitteloud N. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. J Clin Endocrinol Metab 2008;93:3551-3559. Epub 2008 Jun 17

45. Takagi M, Takahashi M, Ohtsu Y, Sato T, Narumi S, Arakawa H, Hasegawa T. A novel mutation in HESX1 causes combined pituitary hormone deficiency without septo optic dysplasia phenotypes. Endocr J 2016;63:405-410. Epub 2016 Jan 15

46. Brickman JM, Clements M, Tyrell R, McNay D, Woods K, Warner J, Stewart A, Beddington RS, Dattani M. Molecular effects of novel mutations in Hesx1/HESX1 associated with human pituitary disorders. Development 2001;128:5189-5199.

47. Carvalho LR, Woods KS, Mendonca BB, Marcal N, Zamparini AL, Stifani S, Brickman JM, Arnhold IJ, Dattani MT. A homozygous mutation in HESX1 is associated with evolving hypopituitarism due to impaired repressor-corepressor interaction. J Clin Invest 2003;112:1192-1201.

48. Newbern K, Natrajan N, Kim HG, Chorich LP, Halvorson LM, Cameron RS, Layman LC. Identification of HESX1 mutations in Kallmann syndrome. Fertil Steril 2013;99:1831-1837. Epub 2013 Mar 1

49. Olsen SK, Li JY, Bromleigh C, Eliseenkova AV, Ibrahimi OA, Lao Z, Zhang F, Linhardt RJ, Joyner AL, Mohammadi M. Structural basis by which alternative splicing modulates the organizer activity of FGF8 in the brain. Genes Dev 2006;20:185-198. Epub 2005 Dec 29

50. Amato LGL, Montenegro LR, Lerario AM, Jorge AAL, Guerra Junior G, Schnoll C, Renck AC, Trarbach EB, Costa EMF, Mendonca BB, Latronico AC, Silveira LFG. New genetic findings in a large cohort of congenital hypogonadotropic hypogonadism. Eur J Endocrinol 2019;181:103-119.

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Chronic Disease Management of Children Followed with Type 1 Diabetes Mellitus

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What is already known on this topic?

The daily life of the child with type 1 diabetes mellitus (T1DM) and their family usually functions normally, to the extent that the family is able to manage the chronic illness and cope with the difficulties experienced. Improving the management of the disease may be possible by evaluating the possible future effects of the disease.

What this study adds?

High education level, increase in family income, use of an insulin pump and longer duration since diagnosis positively affected the management of T1DM and the daily life of the child with T1DM. However, the presence of chronic diseases other than T1DM negatively affects diabetes management.

Abstract

Objective: With the diagnosis of chronic illness in children, a stressful period is likely to begin for both the affected child and their families. The aim of this study was to investigate the factors affecting chronic disease management by the parents of children diagnosed with type 1 diabetes mellitus (T1DM).

Methods: The sample consisted of 110 children, aged between 4-17 years and their mothers. The patients had been diagnosed with T1DM for at least one year, and had attended pediatric endocrinology outpatients or were hospitalized in a single center. First, sociodemographic information about the child with T1DM were obtained. Then, the "Family Management Measure" (FaMM) was applied. The FaMM is constructed to measure family functioning and management in families who have a child with a chronic illness.

Results: Paternal years of education ($p = 0.036$), family income ($p = 0.008$), insulin pump use ($p = 0.011$), and time elapsed after diagnosis ($p = 0.048$) positively affected both the management of T1DM and the child's daily life. However, presence of chronic diseases in addition to T1DM ($p = 0.004$) negatively affected diabetes management. Higher maternal education year ($p = 0.013$) and family income level ($p = 0.001$) increased parental mutuality scores. However, as the time after diagnosis increased, parental mutuality scores decreased.

Conclusion: It is important to evaluate the child with chronic disease with a biopsychosocial approach. This approach aims to evaluate the problems of the child and his/her family who experience the disease with a holistic approach.

Keywords: Type 1 diabetes mellitus, chronic disease, children, family management measure

Introduction

Mokkink et al. (1) provided a consensus definition of childhood chronic disease, consisting of four criteria as follows: "a disease or condition is considered to be a chronic condition in childhood if: (1) it occurs in children aged 0 up to 18 years; (2) the diagnosis is based on medical scientific knowledge and can be established using reproducible and valid methods or instruments according to professional standards; (3) it is not (yet) curable or, for mental health

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conditions, it is highly resistant to treatment; and (4) it has been present for longer than three months or it will, very probably, last longer than three months, or it has occurred three times or more during the past year and will probably reoccur". As stated in the Turkey Chronic Diseases and Risk Factors Survey, the prevalence of chronic diseases is increasing rapidly in our country, as well as globally (2). In recent years, the prevalence of chronic diseases in children has increased and now affects around 12-16% of children (3). It is estimated that this frequency is between 10-15% in the population under the age of thirteen years. If children with mental, emotional, learning and behavioral problems are included, the incidence of chronic disease can increase up to 30-40% (4). In the thesis study conducted by Mustafayev (5) in 2019, investigating the risks impairing child development in our country, it was reported that having a chronic disease had the highest risk rate among the independent risk factors identified.

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease characterized by insulin deficiency and hyperglycemia, which occurs when beta cells of the pancreas are affected by autoimmune or non-autoimmune pathologies (6). The estimated prevalence of diabetes among children and adolescents has also been increasing in recent years globally (7). It is estimated that there are approximately 20,000 children under the age of 18 years living with T1DM in Turkey, at least 15,000 of these are of school age and around 1500-1700 children are diagnosed with T1DM each year (8). The overall mean incidence of T1DM was 16.7/100000 persons per year. The regional incidence rates of T1DM are reported to vary from 10.2 to 24.1/100000 persons per year, between 2009 and 2019 (9).

The diagnosis of chronic illness in children is likely to be accompanied by increased stress, both for the affected child and their families. Thus, the entire family system is affected. The daily life of both the child and the family usually functions normally, to the extent that the family is able to manage the illness and cope with the difficulties (10). However, they often need the help of healthcare professionals in their efforts to manage the disease. Incorporating the management of a chronic disease into the mechanisms of family life and making it a natural part of daily life is possible when the child's disease care needs have been fully identified, strategies for the management of the disease have been developed, routines have been established and the possible future effects of the disease have been evaluated.

To date, there is no published study in Turkey concerning the factors affecting chronic disease management of families of children with T1DM. The aim of this study, which was undertaken in İnönü University Faculty of Medicine, Departments of Developmental Pediatrics and Pediatric Endocrinology, was to investigate the factors affecting chronic disease management of the parents of children with T1DM.

Methods

The study center serves as a referral hospital in the East and Southeast regions of Turkey. Children who were admitted to the pediatric endocrinology outpatient clinic or who were hospitalized and diagnosed with T1DM at least one year previously together with their families were eligible for the study. A one-year duration since diagnosis ensured that the child and family had time to understand the reality of the diagnosis and develop an approach to condition management. Exclusion criteria were maternal caregivers who could not read or speak Turkish. Ethics committee approval for the study was obtained from İnönü University Health Sciences Research and Publication Ethics Committee (approval number: E-129717, date: 06/01/2022).

At the beginning of the interview, the principal researcher explained the purpose, content, duration, and how the descriptive study would be conducted to the mothers of the children who met the sampling conditions. The consent form was read aloud or read to the families, and their informed written consent was obtained by asking whether they would like to participate. Subsequently, a face-to-face interview was conducted by the researcher with the mothers who consented to participate. It was emphasized throughout the process that participation was voluntary. First, sociodemographic information and information about the child with T1DM were obtained. Then, the "Family Management Measure" (FaMM) was completed with the mothers. The duration of the interview was between 22-47 minutes. The author conducted the data collection between January 2022 and April 2022. In addition, an invitation to participate in the study was sent to a social media group for T1DM patients followed in the pediatric endocrinology outpatient clinic. The invitation to participate included the contact details of the first and second authors. Parents were given the option to complete an online or print version of the questionnaire. If they completed the survey online, the consent form was issued as part of the online survey. Parents were encouraged to contact the correspondent author if they had any questions or concerns. All data were checked by the researcher and transferred to a database.

Data Collection Tool

Family Management Measure

The FaMM is constructed to measure family functioning and management in families who have a child with a chronic

illness. The FaMM has good internal consistency, measured by Cronbach's alpha, ranging from 0.72 to 0.90 for mothers (10). The Turkish validity and reliability studies of the scale, which was originally in English, were carried out by Ergun et al. (11). Data were collected from a total of 395 parents with a child diagnosed with chronic disease. The general content validity index was 95% and the results were found to be valid, reliable, appropriate and satisfactory for Turkish culture and psychometric characteristics. The Turkish version of the scale, which originally consisted of 53 items and six sub-dimensions, consists of 42 items and three sub-dimensions (Table 1). Each item in the scale is scored using a five-point Likert scale. There are reverse scored items in each sub-dimension.

High scores in the sub-dimension of disease management and the child's daily life (19 questions) indicate a more normal life and that families find themselves more capable with disease management. High scores in the sub-dimension (16 questions) related to life difficulties and the occurrence of disease effects indicate that the situation is more serious and more difficulties are experienced. The last sub-dimension is related to parental mutuality (7 questions). High scores indicate that parents are working together for the child's disease management. The Cronbach's alpha of the first dimension was 0.68, while for the second dimension this was 0.76 and for the third dimension it was 0.80.

The sub-dimensions focus specifically on how chronic disease management is incorporated into daily life, how families define family life in the context of a child's chronic illness, and key aspects of management. The aim is to explain the perspectives of families about the management activities of their children's disease and how they make sense of them. The sub-dimensions also contribute to the development and testing of interventions to change problematic aspects of family management and strengthen aspects that support optimal child and family outcomes.

Statistical Analysis

Categorical (qualitative) variables were expressed as numbers (percentage). Quantitative variables are summarized as mean ± standard deviation and median and interquartile range (25^{th} to the 75^{th} quartile). Mann-Whitney U, independent groups t, One-Way ANOVA and Kruskal-Wallis tests were used where appropriate. Spearman's rank correlation coefficient was calculated for the variables thought to be related to the scale scores. Statistical tests with a $p < 0.05$ were considered significant. All statistical analyzes were performed using IBM Statistical Package for the Social Sciences for Windows, version 26.0 (IBM Inc., Armonk, NY, USA) (12).

Results

The sample consisted of 110 children, aged between 4-17 years, and their mothers. Of the children, 63 (57.3%) were girls and 47 (42.7%) were boys. The mean age of the mothers of the children was 38.5 ± 6.0 years, and the mean age of the fathers was 42.9 ± 6.5 years. Other sociodemographic data is given in Table 2.

The median (interquartile range) time after diagnosis of T1DM was 29.92 (16.3-54.7) months. Ninety-eight (89.1%) of the children were going to school and 87 (79.1%) of the families reported that they regularly visited the outpatient clinic. Most (n = 93, 84.5%) of the families had received diabetes training. Eighteen of the children (16.4%) were using an insulin pump. Chronic disease other than T1DM had been diagnosed in 14 of the children (12.7%). Pubertal staging at the last hospital visit showed that 47 (42.7%) were in the prepubertal stage and 63 (57.3%) were in the pubertal stage. Hemoglobin A1c (HbA1c) levels in the previous one year were analyzed from file records and assessed according to The International Society for Pediatric and Adolescent Diabetes 2018 criteria which are: target HbA1c < 7, therefore below 7% is considered good control, between 7% and 9% is considered moderate control and above 9% is considered poor control. Based on these criteria 27 (24.5%) patients were in the poor control group, 59 (53.6%) were in the moderate control group and 24 (21.8%) were in the good control group.

When the families were asked what challenges they faced with T1DM, they reported regulating the diet of their children (n = 76, 69%), monitoring blood sugar (n = 60, 54%), regulating meals (n = 55, 50%), adjusting insulin doses (n = 45, 41%), exercising (n = 45, 41%) and difficulties in obtaining drugs and materials (n = 31, 28%).

FaMM scale scores are given in Table 3, 4 and 5. There was a significant difference in condition management and child's daily life scores when comparing the groups stratified by presence or absence of other chronic disease (p = 0.004), the years of education of the father (p = 0.036), the income level of the family (p = 0.008), insulin pump use (p = 0.011), and time since diagnosis (p = 0.048) (Table 3). There was no significant difference between the groups of the variables in terms of family life difficulty and view of condition impact score (Table 4). Significant variables affecting parental mutuality scores were limited to years of education of the mother (p = 0.013) and the income level of the family (p = 0.001) (Table 5).

Table 1. Psychometric properties of Turkish version of the FaMM (42 items)

Factors and items
Condition management and child's daily life
1. Our child's everyday life is similar to that of other children his/her age.
2. In the future we expect our child to take care of the condition.
3. Taking care of our child's condition is often overwhelming.
4. We have some definite ideas about how to help our child live with the condition.
5. Our child is different from other children his/her age because of the condition.
6. It is difficult to know when our child's condition must come first in the family.
7. We are looking forward to a happy future with our child.
8. When something unexpected happens with our child's condition, we usually know how to handle it.
9. Our child's friendships are different because of the condition.
10. We feel we are doing a good job taking care of our child's condition.
11. People with our child's condition have a normal length of life.
12. We often feel unsure about what to do to take care of our child's condition.
13. We have not been able to develop a routine for taking care of our child's condition.
14. Even though our child has the condition, we have a normal family life.
15. We have goals in mind to help us manage our child's condition.
16. It is difficult to fit care of our child's condition into our usual family routine.
17. Dealing with our child's condition makes family life more difficult.
18. We know when our child needs to be a child.
19. I am unhappy about the way my partner and I share the management of our child's condition.
Family life difficulty and view of condition impact
20. Our child's condition is like a roller coaster with lots of ups and downs.
21. Our child's condition is the most important thing in our family.
22. It is very hard for us to take care of our child's condition.
23. Because of the condition, we worry about our child's future.
24. We have enough money to manage our child's condition.
25. A condition like the one our child has makes family life very difficult.
26. Our child's condition rarely interferes with other family activities.
27. Our child's condition will be harder to take care of in the future.
28. We think about our child's condition all the time.
29. It seems as if our child's condition controls our family life.
30. It is hard to get anyone else to help us with our child's condition.
31. It takes a lot of organization to manage our child's condition.
32. We are sometimes undecided about how to balance the condition and family life.
33. It is hard to know what to expect of our child's condition in the future.
34. Our child would do better in school if he/she didn't have the condition.
35. A condition like the one our child has makes it hard to live a normal life.
Parental mutuality
36. We are confident that we can take care of our child's condition.
37. We are a closer family because of how we deal with our child's condition.
38. I am pleased with how my partner and I work together to manage our child's condition.
39. My partner and I argue about how to manage our child's condition.
40. My partner and I consult with each other before we make a decision about our child's care.
41. My partner and I have similar ideas about how we should be raising our child.
42. My partner and I support each other in taking care of our child's condition.

FaMM: Family Management Measure

Table 2. Descriptive statistics on children and family		
Age of child, (mean ± SD)	10.83 ± 3.81	
Order of child Median (25-75% percentiles)	2 (1-2)	
Number of children Median (25-75% percentiles)	3 (2-3)	
Mother education years, (mean ± SD)	10.09 ± 4.66	
Father education years, (mean ± SD)	11.30 ± 4.31	
Mother working status, n (%)	Working	19 (17.27)
	Not working	91 (82.73)
Father working status, n (%)	Working	88 (80)
	Not working	22 (20)
Family income level, n (%)	Less than minimum wage	25 (22.73)
	Minimum wage*	47 (42.73)
	More than minimum wage	38 (34.55)
Family structure, n (%)	Nuclear family	87 (79.09)
	Extended family	16 (14.55)
	Broken family	7 (6.36)
Place of residence, n (%)	City	67 (60.91)
	Suburbs	32 (29.09)
	Village	11 (10)
Type of accommodation, n (%)	Apartment	82 (74.55)
	Other	28 (25.45)
Contact with endocrinologist, n (%)	Yes	61 (55.45)
	No	49 (44.55)
Health insurance status, n (%)	Yes	87 (79.09)
	No	23 (20.91)
Access to diabetes nurse, n (%)	Yes	94 (85.45)
	No	16 (14.55)

*Minimum wage: The lowest wage level that can legally be paid to workers. In January 2022 this was 4,253 TL per month.
SD: standard deviation, FaMM: Family Management Measure

On correlation analysis, two significant relationships were identified. The first was a negative correlation between duration since diagnosis (months) and parental mutuality score [Spearman rank (r) = -0.204, p = 0.033]. Thus, as duration from diagnosis increases there appears to be a decrease in parental co-operation. Secondly, a positive correlation was found between HbA1c level and time after diagnosis (months) (r = 0.275, p = 0.004).

Discussion

When diagnosed with a chronic illness, sick children and their families face a variety of challenges (13). The daily life of both the child and the family functions normally, as long as the family is able to manage the illness and cope with the difficulties experienced. It has been shown that variables, including family demographics, are closely related to the child's and family's adaptation to the disease and management outcomes (14).

In addition to the medical problems related to treatment and care in the period starting with the diagnosis of a chronic disease, limited economic resources are one of the problems encountered (15). In the review of Didsbury et al. (16), which included 6957 children and young patients with T1DM, it was reported that there was a significant relationship between at least one socio-economic determinant and quality of life. These authors showed that low parental education and low income were associated with low quality of life in children with chronic diseases (16). In the present study, low disease management scores were associated with lower family income levels and when father had fewer years of education. If the income level is low, it will be difficult for the parents to adjust the family budget for their child's illness and to cope with the difficult treatment process (17). Having a high level of education will not only make it easier for fathers to manage the process, but it will also make it easier to adapt to the life-style changes. Interestingly, no relationship was found between maternal education level

Table 3. Comparison of Turkish FaMM subscale scores for condition management and child's daily life

		Condition management and child's daily life		
		Mean ± SD	Median (25^{th}-75^{th} quantile)	p value
Gender	Girl		65 (57-70)	0.53*
	Boy		67 (55-72)	
School status	Pre-school	62.1 ± 8.2		0.37**
	School group	65.2 ± 11.5		
Presence of other chronic disease	Yes	56.8 ± 11.6		**0.004****
	No	66.0 ± 10.7		
Mother education years	Eight years and below	63.6 ± 11.4		0.25**
	More than 8 years	66.0 ± 10.9		
Father education years	Eight years and below	61.8 ± 10.5		0.036**
	More than 8 years	66.5 ± 11.3		
Family income level	Lower than minimum wage		59.5 (52-67.5)	**0.008*****
	Minimum wage		65 (54-71)	
	More than minimum wage		67 (63-75)	
Use of insulin pump	Yes	71 ± 9.5		**0.011****
	No	63.6 ± 11.1		
Regular outpatient visits	Yes	64.6 ± 10.9		0.63**
	No	65.9 ± 12.4		
Received diabetes education	Yes	65.3 ± 11.6		0.28**
	No	62.2 ± 8.4		
Disease control****	HbA1c > 9.0% (poor control)		62 (56-74)	0.90***
	HbA1c 7.0 to ≤9.0% (moderate control)		67 (55-73)	
	HbA1c < 7.0% (good control)		66.50 (61.5-69)	
Pubertal stage	Prepubertal period	63.7 ± 9.8		0.34**
	Pubertal period	65.7 ± 12.1		
Post diagnosis period	Less than three years	63.4 ± 9.5		**0.048****
	Over three years	67.4 ± 13.3		

*Mann-Whitney U test, **Independent sample t-test, ***Kruskal-Wallis test.
****According to ISPAD 2018.
SD: standard deviation, FaMM: Family Management Measure, ISPAD: The International Society for Pediatric and Adolescent Diabetes

and disease management score, although there was an association with parental mutuality scores.

Chronic illness of a family member can also affect the relationship between all family members. When parents support each other, parents' trust in each other increases, but conflict between spouses causes stress and decreases parental motivation (18). In the present study, there was a significant difference between the groups when divided by maternal years of education and the income level of the family in the parental mutuality score. Parental mutuality appeared to increase as both the education level of the mother increased and the income level of the family increased.

It has been shown that conflicts between spouses, divorce, financial problems, lack of social support, and problems that may occur in family functionality may make it difficult for the child to adapt to their disease (19). Case et al. (20) conducted a prospective, longitudinal study of 127 children, aged 5-9 years and their parents, within 12 months of diagnosis of T1DM at two pediatric diabetes clinics in the USA and followed participants for 27 months. They found that as the time after diagnosis increased, parental mutuality decreased and parental conflict increased. The results of our study are consistent with these findings, because as the time after diagnosis increased, the parental mutuality score decreased. We believe that this is the result of the financial problems that the family may face as the duration of chronic illness increases, the increasing anxiety caused by having a child who requires constant monitoring, and the decrease in the motivation to cope with the stress of the disease over time.

When a school-age child is diagnosed with T1DM, one of the first problems parents face is the difficulties in adapting

Table 4. Comparison of Turkish FaMM subscale scores for family life difficulty and view of condition impact

		Family life difficulty and view of condition impact		
		Mean ± SD	Median	p value
Gender	Girl	56.7 ± 13.2		0.38**
	Boy	54.5 ± 12.7		
School status	Pre-school	59.7 ± 12.8		0.26**
	School group	55.3 ± 13.0		
Presence of other chronic disease	Yes	59.5 ± 13.0		0.25**
	No	55.2 ± 13.0		
Mother education year	Eight years and below		57 (45-67)	0.943*
	More than eight years		55 (48-65)	
Father education year	Eight years and below	57.5 ± 12.3		0.287*
	More than eight years	54.8 ± 13.3		
Family income level	Lower than minimum wage		58 (46-69)	0.59***
	Minimum wage		58 (47-66)	
	More than minimum wage		53.5 (45-65)	
Use of insulin pump	Yes		56 (41-61)	0.29*
	No		57 (47.5-67.5)	
Regular outpatient visits	Yes	55.66 ± 13.28		0.81*
	No	56.30 ± 12.24		
Received diabetes education	Yes		57 (45-66)	0.392*
	No		54 (52-68)	
Disease control*****	HbA1c > 9.0% (poor control)	56.67 ± 14.90		0.27****
	HbA1c 7.0 to ≤9.0% (moderate control)	53.98 ± 12.81		
	HbA1c < 7.0% (good control)	58.96 ± 10.60		
Pubertal stage	Prepubertal period	57.1 ± 12.3		0.36**
	Pubertal period	54.8 ± 13.5		
Post diagnosis period	Less than three years		57 (48-68)	0.22*
	Over three years		57 (45-63)	

*Mann-Whitney U test, **Independent sample t-test, ***Kruskal-Wallis test, ****One-Way ANOVA test.
*****According to ISPAD 2018.
SD: standard deviation, FaMM: Family Management Measure, ISPAD: The International Society for Pediatric and Adolescent Diabetes, HbA1c: hemoglobin A1c

school life to their child's illness (21). In the qualitative study of Beacham and Deatrick (22), using FaMM with thirty-two school-going children aged 8-13 years with chronic illness, the children mentioned the effort they needed to deal with their illness, the difficulties in managing the illness during school days, and the illness disrupting school. Patients stated that disease management was much easier at weekends or on non-school days. However, in the present study, no significant association was observed between school attendance and disease management. In our country, the School Diabetes Program was started in 2010 as a part of the national diabetes program, and the program is continuing successfully (23,24). It is likely that disease management did not fail in school in our patient group because of the positive effect of this program, but the low number of individuals in the preschool group (n = 12, 10.9%) may have affected the statistical comparison.

Parents are faced with multiple stressors during and after the diagnosis process. Life changes can affect family routines, relationships, and parenting styles, due to the long-term burden of the disease, dietary restrictions, medications, and frequent visits to outpatient clinics (25). In the present study, more than half of the families stated that they had difficulties in regulating their children's diet, blood sugar monitoring and regulating meals during T1DM follow-up. Given the complexity of prioritizing diabetes treatment goals in themselves, prioritizing goals in multiple chronic conditions can be a challenge for families. When co-management of concurrent chronic diseases is required, the remaining time and energy to care for diabetes can be significantly reduced. Even if the combined management of concurrent chronic diseases is not attempted, the control of diabetes-specific risk factors may be poorer and this may negatively affect patients and cause them to miss

Table 5. Comparison of Turkish FaMM subscale scores for parental mutuality

		Parental mutuality	
		Median (25th-75th quantile)	**p value**
Gender	Girl	27 (23-31)	0.65*
	Boy	28 (23-31)	
School status	Pre-school	29.5 (20.5-31)	0.85*
	School group	27 (23-31)	
Presence of other chronic disease	Yes	24.5 (20-31)	0.28*
	No	28 (23-31)	
Mother education year	Eight years and below	26 (20.5-30)	**0.013***
	More than eight years	29 (26-32)	
Father education year	Eight years and below	26 (23-31)	0.294*
	More than eight years	28 (23-31)	
Family income level	Lower than minimum wage	24 (19.5-27.5)	**0.001*****
	Minimum wage	28 (23-31)	
	More than minimum wage	30.5 (27-33)	
Use of insulin pump	Yes	27.5 (26-29)	0.87*
	No	28 (22-31)	
Regular outpatient visits	Yes	28 (23-31)	0.51*
	No	27 (22-31)	
Received diabetes education	Yes	28 (23-31)	0.38*
	No	27 (22-30)	
Disease control******	HbA1c >9.0% (poor control)	28 (24-31)	0.85***
	HbA1c 7.0 to ≤9.0% (moderate control)	27 (21-31)	
	HbA1c <7.0% (good control)	27.50 (24.5-30.5)	
Pubertal stage	Prepubertal period	28 (23-31)	0.84*
	Pubertal period	28 (23-31)	
Post diagnosis period	Less than three years	28 (24-31)	0.25*
	Over three years	27 (21.5-31)	

*Mann-Whitney U test, **Independent sample t-test, ***Kruskal-Wallis test.
*****According to ISPAD 2018.
FaMM: Family Management Measure, ISPAD: The International Society for Pediatric and Adolescent Diabetes, HbA1c: hemoglobin A1c

opportunities to improve their quality of life. In a qualitative study by Beacham and Deatrick (22), an example was given of a child athlete with both diabetes and asthma who had to stop frequently before, during, and after training or matches to control blood sugar levels or to take inhalation treatments, and that it was difficult to manage these two diseases. However, in the study of Al-Hadhrami et al. (26), in which 210 Omani adults diagnosed with T1DM and the factors affecting the self-management of diabetes were evaluated, it was reported that those with additional chronic diseases had better disease self-management than those without diabetes. They interpreted the reason for this as individuals with T1DM affected by other chronic diseases fear that their condition will progress or worsen and thus gave higher priority to necessary lifestyle changes. This contrasts with the findings in the present study, and others (22,27). The presence of a chronic disease in addition to diabetes adversely affected disease management of the families. Considering the difficulty of prioritizing the treatment goals of diabetes, it seems reasonable to accept that having more than one chronic disease may pose an increased challenge for families, which may further complicate diabetes management.

The insulin treatment option to be used also has an effect on disease management. Insulin pump therapy can provide a more comfortable life style for the patient by eliminating continuous insulin injections during the day. In a cohort study by Karges et al. (28), among patients younger than 20 years of age with T1DM and a duration of diabetes greater than one year, insulin pump therapy was associated with better glycemic control and lower risks of severe hypoglycemia and diabetic ketoacidosis in the last year of therapy compared to insulin injection therapy. When the data obtained from The International Pediatric Registry SWEET for 25,654 participants with T1DM between the

ages of 1-18 years were examined, lower HbA1c level, fewer diabetic ketoacidosis episodes and a lower rate of severe hypoglycemia were detected in the participants using pumps (29). Kardaş and Gürol (30) found that children using insulin pumps achieved better metabolic control and their quality of life increased as HbA1c levels decreased. These findings provide evidence for improved clinical outcomes associated with insulin pump therapy compared to injection therapy in children, adolescents, and young adults with T1DM. These benefits to young patients are likely to facilitate disease management by the parents (28). The findings from the present study, that the use of an insulin pump positively affected disease management, are consistent with these earlier studies.

The first period after diagnosis is a period that requires rapid knowledge and skill acquisition for disease management by parents and children, including blood glucose monitoring, insulin administration and carbohydrate counting. This may complicate the establishment of effective parent-child cooperation and disease management for diabetes care. The study of Case et al. (20) showed that children with a diagnosis of T1DM had significantly higher Diabetes Self-Management Questionnaire-Summary scores at 27 months, mostly reported by their mothers. In our study, a significant increase was found in the disease management scores of the parents at three years after diagnosis compared to earlier. This finding suggests that families experience difficulties in accepting and understanding T1DM in the first years after diagnosis.

HbA1c measurements are made to evaluate longer-term glycemic control in the follow-up of diabetes patients. Nirantharakumar et al. (31) investigated HbA1c levels and the time elapsed since diagnosis in a study of 4.525 patients diagnosed with T1DM from The Health Improvement Network database, between 1995 and 2015. HbA1c levels increased after diagnosis and started to stabilize after an average of five years after diagnosis. In our study, a positive correlation was found between HbA1c level and the duration (months-years) since diagnosis. This may be due to less stringent disease management over time, or it may be due to the result of falsely low assessment of HbA1c levels due to increased hypoglycemia rates in the early stages of the disease. Studies evaluating the time spent in target blood glucose range with devices that measure blood glucose continuously will give more accurate results in this regard. Further studies are needed in this area.

Study Limitations

Firstly, all participants were treated in the same large children's hospital which may have led to homogeneity of participating families experience of disease follow-up and treatment, which in turn may have affected the FaMM scores. Secondly, we specifically requested the participation of mothers in our study, as we hypothesized that mothers would play an important role in the management of T1DM in their children. However, the views of other family members, in particular the patients themselves, but also their fathers, siblings and other relatives involved in disease management may have provided additional insights into disease management in this cohort. Future research to address the limitations of the current study is needed.

Conclusion

It is important to evaluate the child with chronic disease using a biopsychosocial approach. Such an approach aims to evaluate the problems of the child and his/her family who experience this disease through a holistic approach because the chronic disease experienced by the child is a complex and trying process that affects not only the child but also their families for many years. The aim should be to strengthen the patients, ensure the functionality of their families, and provide additional psychological, practical and emotional support to ameliorate the physical challenges of chronic illness. The use of FaMM provided a better understanding of the family unit by identifying the strengths that families and children develop, as well as their weaknesses, that will help improve the results of interventions.

Ethics

Ethics Committe Approval: Ethics committee approval for the study was obtained from İnönü University Health Sciences Research and Publication Ethics Committee (approval number: E-129717, date: 06/01/2022).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: Şenay Güven Baysal, Nurdan Çiftci, Mehmet Akif Büyükavcı, İsmail Dündar, Emine Çamtosun, Derya Doğan, Ayşehan Akıncı, Concept: Şenay Güven Baysal, Nurdan Çiftci, Mehmet Akif Büyükavcı, İsmail Dündar, Derya Doğan, Ayşehan Akıncı, Design: Şenay Güven Baysal, Nurdan Çiftci, Mehmet Akif Büyükavcı, İsmail Dündar, Literature Search: Şenay Güven Baysal, Nurdan Çiftci, Data Collection or Processing: Şenay Güven Baysal, Nurdan Çiftci, Analysis or Interpretation: Fatma Hilal Yagın, Şenay Güven Baysal, Nurdan Çiftci, Writing: Şenay Güven Baysal, Nurdan Çiftci, Mehmet Akif Büyükavcı, İsmail Dündar.

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References

1. Mokkink LB, van der Lee JH, Grootenhuis MA, Offringa M, Heymans HS; Dutch National Consensus Committee Chronic Diseases and Health Conditions in Childhood. Defining chronic diseases and health conditions in childhood (0-18 years of age): national consensus in the Netherlands. Eur J Pediatr 2008;167:1441-1447. Epub 2008 Mar 14
2. Türkiye Cumhuriyeti Sağlık Bakanlığı Türkiye Halk Sağlığı Kurumu. Türkiye Kronik Hastalıklar ve Risk Faktörleri Sıklığı Çalışması, 2013.
3. Raphael JL, Rueda A, Lion KC, Giordano TP. The role of lay health workers in pediatric chronic disease: a systematic review. Acad Pediatr 2013;13:408-420.
4. Çavuşoğlu H. Çocuk sağlığı hemşireliği Cilt 1. Ankara, Sistem Ofset Basımevi, 2011.
5. Mustafayev R. Gelişimi İzleme ve Destekleme Rehberi Uluslararası Standardizasyon Çalışması'nın Türkiye örnekleminde gelişimsel risklerin belirlenmesi. T.C. Ankara Üniversitesi Tıpta UzmanlıkTezi, Ankara, 2019.
6. Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, Cavan D, Shaw JE, Makaroff LE. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract 2017;128:40-50. Epub 2017 Mar 31
7. Lawrence JM, Divers J, Isom S, Saydah S, Imperatore G, Pihoker C, Marcovina SM, Mayer-Davis EJ, Hamman RF, Dolan L, Dabelea D, Pettitt DJ, Liese AD; SEARCH for Diabetes in Youth Study Group. Trends in Prevalence of Type 1 and Type 2 Diabetes in Children and Adolescents in the US, 2001-2017. JAMA 2021;326:717-727.
8. Yeşilkaya E, Cinaz P, Andıran N, Bideci A, Hatun Ş, Sarı E, Türker T, Akgül Ö, Saldır M, Kılıçaslan H, Açıkel C, Craig ME. First report on the nationwide incidence and prevalence of Type 1 diabetes among children in Turkey. Diabet Med 2017;34:405-410. Epub 2016 Feb 12
9. Esen I, Okdemir D. Trend of type 1 diabetes incidence in children between 2009 and 2019 in Elazig, Turkey. Pediatr Diabetes 2020;21:460-465. Epub 2020 Jan 24
10. Knafl KA, Deatrick JA, Havill NL. Continued development of the family management style framework. J Fam Nurs 2012;18:11-34. Epub 2012 Jan 5
11. Ergun A, Sisman FN, Erol S, Gur K, Kolac N, Kadioglu H. The Family Management of Childhood Chronic Conditions: Measurement in a Turkish Sample. J Pediatr Nurs 2019;47:16-23. Epub 2019 Apr 23
12. IBM SPSS Statistics for Macintosh, Version 26.0. Available from: https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-26
13. Knafl K, Deatrick JA, Gallo A, Dixon J, Grey M, Knafl G, O'Malley J. Assessment of the psychometric properties of the Family Management Measure. J Pediatr Psychol 2011;36:494-505. Epub 2009 May 18
14. August GJ, Realmuto GM, Joyce T, Hektner JM. Persistence and desistance of oppositional defiant disorder in a community sample of children with ADHD. J Am Acad Child Adolesc Psychiatry 1999;38:1262-1270.
15. Erdem E, Korkmaz Z, Tosun Ö, Avcı Ö, Uslu N, Bayat M. The burden of care in the mothers of the children with chronic disease. J Health Sci 2013;22:150-157.
16. Didsbury MS, Kim S, Medway MM, Tong A, McTaggart SJ, Walker AM, White S, Mackie FE, Kara T, Craig JC, Wong G. Socio-economic status and quality of life in children with chronic disease: A systematic review. J Paediatr Child Health 2016;52:1062-1069.
17. Hood A, Grange DK, Christ SE, Steiner R, White DA. Variability in phenylalanine control predicts IQ and executive abilities in children with phenylketonuria. Mol Genet Metab 2014;111:445-451.
18. Han JW, Lee H. Actor and partner effects of parenting stress and co-parenting on marital conflict among parents of children with atopic dermatitis. BMC Pediatr 2020;20:141.
19. Soliday E, Kool E, Lande MB. Psychosocial adjustment in children with kidney disease. J Pediatr Psychol 2000;25:93-103.
20. Case H, Williams DD, Majidi S, Ferro D, Clements MA, Patton SR. Longitudinal associations between family conflict, parent engagement, and metabolic control in children with recent-onset type 1 diabetes. BMJ Open Diabetes Res Care 2021;9:e002461.
21. Glaab LA, Brown R, Daneman D. School attendance in children with Type 1 diabetes. Diabet Med 2005;22:421-426.
22. Beacham BL, Deatrick JA. Children with chronic conditions: perspectives on condition management. J Pediatr Nurs 2015;30:25-35. Epub 2014 Oct 22
23. Hatun Ş. National childhood diabetes program activities in Turkey. J Clin Res Pediatr Endocrinol 2015;7:1-6.
24. Hatun Ş, Yeşiltepe Mutlu G, Gökçe T, Avcı Ö, Yardım N, Aycan Z, Darendeliler F. Care and Support of Children with Type 1 Diabetes at School: The Turkish Experience. J Clin Res Pediatr Endocrinol 2021;13:370-374. Epub 2021 May 20
25. Hatzmann J, Valstar MJ, Bosch AM, Wijburg FA, Heymans HS, Grootenhuis MA. Predicting health-related quality of life of parents of children with inherited metabolic diseases. Acta Paediatr 2009;98:1205-1210. Epub 2009 Apr 21
26. Al-Hadhrami R, Al-Rawajfah O, Muliira J. Diabetes Self-Management and the Associated Factors Among Adult Omanis with Type 1 Diabetes. Sultan Qaboos Univ Med J 2020;20:339-345. Epub 2020 Dec 21
27. Piette JD, Kerr EA. The impact of comorbid chronic conditions on diabetes care. Diabetes Care 2006;29:725-731.
28. Karges B, Schwandt A, Heidtmann B, Kordonouri O, Binder E, Schierloh U, Boettcher C, Kapellen T, Rosenbauer J, Holl RW. Association of Insulin Pump Therapy vs Insulin Injection Therapy With Severe Hypoglycemia, Ketoacidosis, and Glycemic Control Among Children, Adolescents, and Young Adults With Type 1 Diabetes. JAMA 2017;318:1358-1366.
29. Cardona-Hernandez R, Schwandt A, Alkandari H, Bratke H, Chobot A, Coles N, Corathers S, Goksen D, Goss P, Imane Z, Nagl K, O'Riordan SMP, Jefferies C; SWEET Study Group. Glycemic Outcome Associated With Insulin Pump and Glucose Sensor Use in Children and Adolescents With Type 1 Diabetes. Data From the International Pediatric Registry SWEET. Diabetes Care 2021;44:1176-1184. Epub 2021 Mar 2
30. Kardaş GN, Gürol A. The Metabolic Control And Quality Of Life Levels Of Children With Type 1 Diabetes Using Insulin Pen And Insulin Pumps. KOU Sag Bil Derg 2022;8:65-71.
31. Nirantharakumar K, Mohammed N, Toulis KA, Thomas GN, Narendran P. Clinically meaningful and lasting HbA1c improvement rarely occurs after 5 years of type 1 diabetes: an argument for early, targeted and aggressive intervention following diagnosis. Diabetologia 2018;61:1064-1070. Epub 2018 Feb 24

ORIGINAL ARTICLE

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Comparison of Makorin Ring Finger Protein 3 Levels Between Obese and Normal Weight Patients with Central Precocious Puberty

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What is already known on this topic?

Puberty is initiated by the complex interaction of stimulatory and suppressive factors. Obesity in girls can cause early puberty by affecting the hypothalamic-pituitary-gonadal axis. Makorin ring finger protein 3 (MKRN3) is the primary inhibitor of gonadotropin-releasing hormone secretion.

What this study adds?

Serum MKRN3 levels were found to be negatively correlated with levels of follicle stimulating hormone and estradiol, and also body mass index (BMI), uterine length and ovarian volumes. Serum MKRN3 level was lowest in the central precocious puberty (CPP)-obese group. The negative correlation between BMI and MKRN3, and lower MKRN3 levels in CPP-obese patients, suggest that adipose tissue has a role in the onset of puberty.

Abstract

Objective: Genetic studies of familial central precocious puberty (CPP) have suggested that makorin ring finger protein 3 (MKRN3) is the primary inhibitor of gonadotropin-releasing hormone secretion. Obesity in girls can cause early puberty by affecting the hypothalamic-pituitary-gonadal axis. This study evaluated serum MKRN3 levels of patients with CPP and its relationship with body mass index (BMI).

Methods: The study included 92 CPP and 86 prepubertal healthy controls (HC) aged 6-10 years. The CPP and HC groups were divided into obese and non-obese subgroups to evaluate whether BMI affects MKRN3. Patients' presenting complaints, chronological age, height age, bone age, Tanner stage, standard deviation scores for weight, height, and BMI, levels of follicle-stimulating hormone, luteinizing hormone, estradiol, and MKRN3, and pelvic ultrasonography findings were recorded.

Results: Serum MKRN3 levels were lower in the CPP group and lowest in the CPP-obese subgroup. There were significant differences in MKRN3 levels between the CPP-obese and CPP-normal weight ($p = 0.02$), CPP-obese and HC-obese ($p < 0.001$), and CPP-obese and HC-normal weight ($p = 0.03$) groups. MKRN3 and BMI were negatively correlated in all cases ($r = -0.326$, $p < 0.001$).

Conclusion: The negative correlation between BMI and MKRN3, and lower MKRN3 levels in CPP-obese patients, suggests that adipose tissue has a role in the onset of puberty. More comprehensive studies are needed to determine the relationship between MKRN3 and adipose tissue.

Keywords: Makorin ring finger protein 3, central precocious puberty, obesity, children

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Introduction

Puberty is a period of rapid growth, marking the transition from sexual immaturity to sexual maturity. It is characterized by the appearance of secondary sexual characteristics, the achievement of reproductive capacity, and psychological changes. Puberty results from complex, co-ordinated, neuroendocrine mechanisms involving the maturation and activation of the hypothalamic-pituitary-gonadal (HPG) axis and pulsatile release of gonadotropin-releasing hormone (GnRH) (1).

Obesity affects all organ systems, especially the neuroendocrine system. The interaction of numerous genes controlling puberty activates the HPG axis and initiates puberty. The epigenetic effects of adipokines secreted from adipose tissue affect the functions of these genes. The ability of adipose tissue to accumulate sex hormones and inter-convert them enzymatically also affects pubertal development (2,3). The relationship between obesity and pubertal timing is thought to be controlled by adipokines, hyperandrogenism, the aromatase effect of adipose tissue, insulin resistance, and hyperinsulinemia (4).

Makorin ring finger protein 3 (MKRN3) is an intronless gene located on chromosome 15q11.2 in the Prader-Willi syndrome critical region that was first identified by Jong et al. (5) in 1999. A 2013 study of families with central precocious puberty (CPP) by Abreu et al. (6) found that the *MKRN3* gene has effects on children entering puberty. MKRN3 is also a major inhibitor of GnRH secretion in childhood (6,7). An indirect way to determine the function of MKRN3 in humans is to investigate serum levels in different conditions. Studies have shown that serum MKRN3 levels decrease before puberty (8,9,10,11) and are negatively correlated with gonadotropin levels (9,12,13). Grandone et al. (13) and Li et al. (14) observed a negative correlation between MKRN3 and body mass index (BMI). The present study examined the relationship between obesity and MKRN3 in CPP by comparing serum MKRN3 levels between obese and normal-weight CPP patients.

Methods

Patients and Controls

The study recruited 92 girls with CPP, and 86 age-matched prepubertal girls as healthy controls (HC), from the Pediatric Endocrinology Department from June 2019 to July 2021. To evaluate whether BMI affects MKRN3, the CPP and HC groups were divided into obese and non-obese subgroups. The presence of breast development before the age of 8 years, menarche before the age of 10 years, advanced bone age [a standard deviation (SD) score (SDS) of +2 relative to the chronological age], basal luteinizing hormone (LH) ≥1 mIU/mL or peak LH ≥5 mIU/mL in the GnRH stimulation test, uterine length ≥35 mm, and ovarian volume ≥2 mL on obstetric ultrasonography were used to diagnose CPP in the girls. Obesity was defined as a BMI above the 95th percentile or +2 SDS (15). In our pediatric endocrinology outpatient clinic, we obtain a history, perform a physical examination, examine routine laboratory tests (fasting blood glucose, insulin, thyroid function tests, triglyceride, cholesterol, and ALT) and perform abdominal ultrasonography to differentiate exogenous and endogenous obesity. All examinations of the patients included in this study were normal. In addition, obesity due to Cushing's syndrome, chronic drug use (for example, corticosteroids), and monogenic obesity syndromes were excluded. The prepubertal stage was defined clinically as the absence of breast budding and pubic hair (Tanner stage 1). Children with tumors, organic or endocrine disease, premature thelarche, or syndromic disease, and those taking medications, were excluded.

The study protocol was in line with the Declaration of Helsinki and was approved by the Eskişehir Osmangazi University Faculty of Medicine, Non-Interventional Clinical Research Ethics Committee (protocol no: 10, date: 25.06.2019). Informed consent was obtained from all individuals included in this study and their parents. This study was supported by the Eskisehir Osmangazi University Scientific Research Projects Coordination Unit (project no. TTU-2021-1630).

Evaluation of Growth and Development

All girls underwent physical examination, including weight and height, BMI, and Tanner breast development stage. BMI was calculated as the weight in kilograms divided by the height in meters squared. Height, weight, and BMI were expressed as SDS using growth reference percentiles for Turkish children and adolescents (16,17).

The left wrist was X-rayed to determine bone age according to the Greulich-Pyle method (18). Gynecological ultrasound was performed to observe the ovarian volume, uterine length, and fundus/cervix ratio, as well as for secondary follicle determination. Pituitary and cranial magnetic resonance imaging were performed in patients diagnosed with CPP younger than six years of age.

Biochemical Analysis

All blood samples were drawn between 8.00 a.m. and 10.00 a.m. from an antecubital vein, clotted, and centrifuged; serum was stored at -80 °C until hormone analyses were performed. For CPP girls, blood samples were withdrawn

before GnRH analog treatment was started. The serum LH, follicle-stimulating hormone (FSH), and estradiol (E_2) levels were measured by immunochemiluminometric assays using a COBAS 8000 autoanalyzer (Roche Diagnostics, Mannheim, Germany). The lowest LH and FSH level determined by this method is 0.1 mIU/mL, and the lowest E_2 level is 5 pg/mL. Gonadorelin acetate (Ferring, Germany) was used for the GnRH stimulation test, with an injected dose of 2.5 µg/kg (maximum dose = 100 µg). LH and FSH were measured before the injection, and 20, 40, 60, and 90 min thereafter (19,20). The serum MKRN3 levels were measured using human MKRN3 ELISA kits (BT Lab, China), with a 0.019 ng/mL detection limit. The intra- and inter-assay coefficients of variation were less than 8% and 10%, respectively.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences software (version 21.0; IBM Corp., Armonk, NY, USA). Data were tested for normality using the Shapiro-Wilk test and values >0.05 were considered normal. For normally distributed continuous variables, the data are expressed as the mean ± SD, and for non-normally distributed variables, they are expressed as the median and interquartile range. Independent samples t-tests and analysis of variance (ANOVA) were utilized to compare normally distributed continuous variables. The Mann-Whitney and Kruskal-Wallis non-parametric tests were used to compare non-normally distributed variables. The chi-square test was used for categorical variables. One-way ANOVA and the Kruskal-Wallis test were used to determine whether the study data differed between the normal weight and obese groups. To determine which groups were responsible for differences, the least significant difference post hoc test was used when variance was homogeneous; Tamhane's T2 test was used when the variance was not homogeneous. The relationships of MKRN3 with other biochemical indicators were evaluated using Spearman's correlation. P values <0.05 were considered statistically significant.

Results

No secondary sex characteristics were detected in the HC girls. All CPP girls had bilateral breast development. Of these girls, 30 (33%), 43 (47%), 18 (29%), and 1 (1%) were Tanner stages II, III, IV and V, respectively. Nineteen CPP patients had progressed to menarche. Table 1 summarizes the girls' clinical and biochemical characteristics. Bone age was increased in the CPP patients compared with the controls. As expected, CPP girls had higher serum LH, FSH, and E_2 levels than the HC group (all $p<0.001$). The serum MKRN3 levels were lower in the CPP than HC group ($p<0.001$) (Figure 1).

Table 1. Clinical and biochemical characteristics of the CPP and HC girls

	CPP n = 92	HC n = 86	p value
Weight SDS	1.23 ± 1.09	1.06 ± 1.70	0.42
Height SDS	0.63 ± 0.97	0.48 ± 1.33	0.37
BMI (kg/m_2)	19.72 ± 3.53	19.15 ± 3.97	0.31
BMI SDS	1.17 ± 1.16	1.05 ± 1.59	0.55
CA (years)	8.84 ± 0.74	8.63 ± 0.72	0.58
BA (years)	10.49 ± 1.07	8.96 ± 1.35	< 0.001
BA-CA	1.57 ± 0.80	0.71 ± 0.84	< 0.001
FSH (mIU/mL)	4.03 (2.58-5.57)	1.54 (1.18-2.49)	< 0.001
LH (mIU/mL)	0.80 (0.30-4.16)	0.10 (0.10-0.10)	< 0.001
E_2 (pg/mL)	18.15 (7.70-32.72)	5.00 (5.00-7.19)	< 0.001
p-FSH (mIU/mL)	12.45 (9.42-16.00)		
p-LH (mIU/mL)	13.95 (9.12-23.60)		
p-LH/p-FSH	1.18 (0.80-2.10)		
MKRN3 (ng/mL)	1.40 (0.76-2.66)	1.68 (1.26-4.60)	< 0.001
Length of the uterus (mm)	45.5 (39.3-49.8)	27.0 (10.0-34.0)	< 0.001
Right ovarian volume (mL)	4.0 (3.0-5.9)	1.7 (0.8-1.8)	< 0.001
Left ovarian volume (mL)	4.0 (3.0-5.4)	1.2 (1.0-1.7)	< 0.001

The median and interquartile ranges were shown except for weight, height, BMI, age which were expressed as means ± SDS.
CPP: central precocious puberty, HC: healthy controls, BMI: body mass index, SDS: standard deviation score, CA: chronological age, BA: bone age, FSH: follicle stimulating hormone, LH: luteinizing hormone, E_2: estradiol, p: peak, MKRN3: makorin ring finger protein 3

Table 2 shows the clinical and biochemical characteristics of the obese and non-obese subgroups. Although their chronological ages were similar, bone age was increased most in the CPP-obese group. Serum MKRN3 levels were lower in the CPP-obese group compared to the other groups ($p < 0.001$).

Serum MKRN3 levels were inversely correlated with BMI (Figure 1), Tanner stage, FSH, E_2, uterus length, and right and left ovarian volumes, as shown in Table 3.

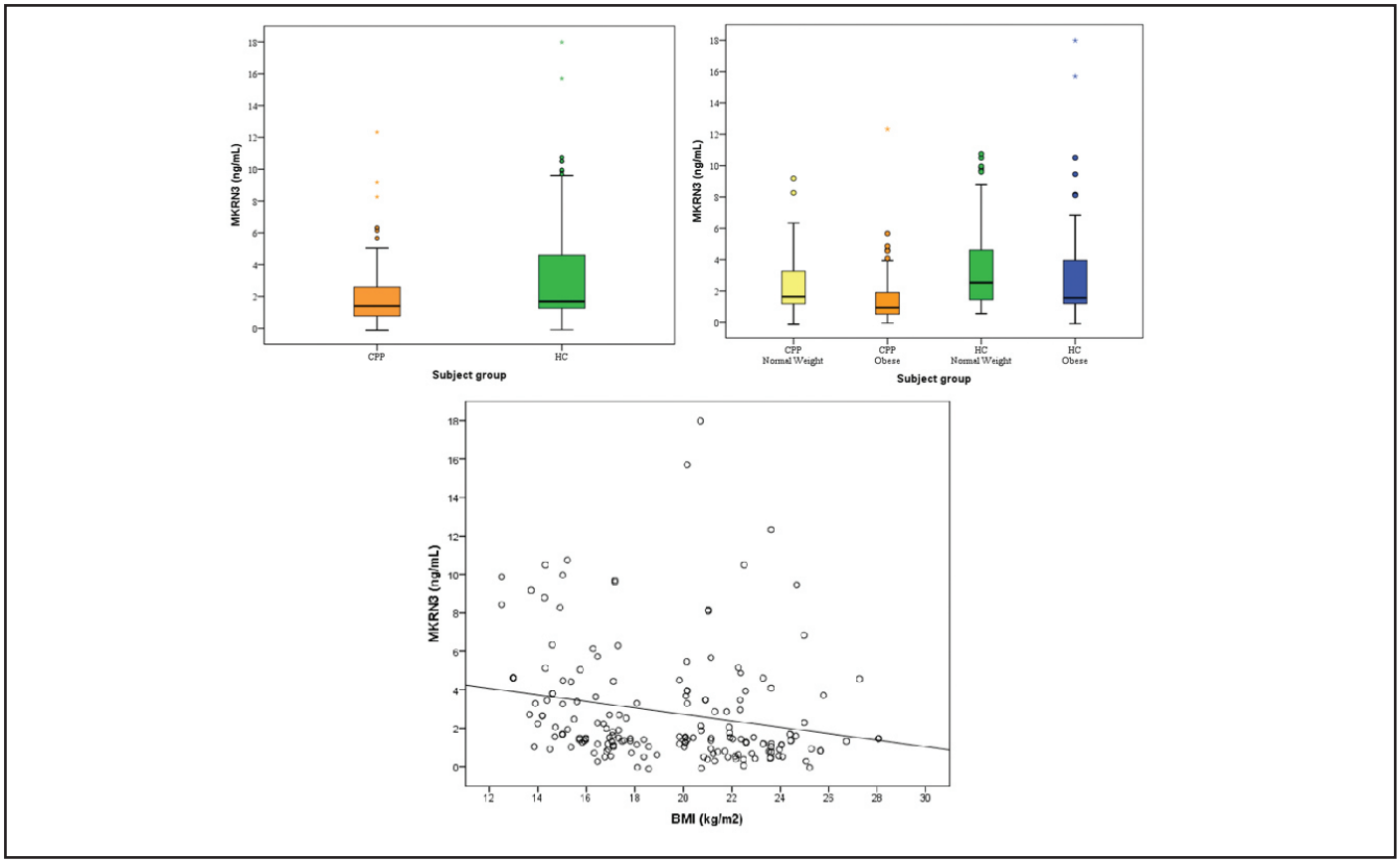

Figure 1. Serum MKRN3 concentrations, and negative correlations between serum MKRN3 levels and BMI in all cases

CPP: central precocious puberty, HC: healthy controls, BMI: body mass index, MKRN3: makorin ring finger protein 3

Table 2. Clinical and biochemical characteristics of the obese and non-obese groups

n	CPP	CPP	HC	HC	p	Post-hoc p < 0.05
	Normal weight	Obese	Normal weight	Obese		
	49	43	43	43		
BMI (kg/m^2)	16.83 ± 1.71	23.02 ± 1.67	15.78 ± 2.04	22.52 ± 2.10	< 0.001	2 > 1 > 3, 4 > 1 > 3
BMI SDS	0.24 ± 0.76	2.24 ± 0.27	-0.32 ± 1.08	2.42 ± 0.40	< 0.001	2 > 1 > 3, 4 > 1 > 3
CA (years)	8.85 ± 0.80	8.83 ± 0.61	8.68 ± 0.82	8.60 ± 0.60	0.281	
BA (years)	10.33 ± 1.08	10.67 ± 1.03	9.13 ± 1.31	8.79 ± 1.39	< 0.001	2 > 1 > 3 > 4
FSH (mIU/mL)	2.81 (2.21-4.62)	5.00 (2.84-6.32)	1.60 (1.18-2.74)	1.39 (1.06-2.46)	< 0.001	1 > 3, 1 > 4, 2 > 3, 2 > 4
LH (mIU/mL)	0.4 (0.3-1.6)	3.1 (0.4-5.9)	0.3 (0.3-0.3)	0.3 (0.3-0.3)	< 0.001	2 > 1 > 3, 2 > 1 > 4
E_2 (pg/mL)	18.0 (8.3-30.6)	20.6 (5.3-38.0)	5.0 (5.0-7.0)	5.0 (5.0-7.9)	< 0.001	1 > 3, 1 > 4, 2 > 3, 2 > 4
MKRN3 (ng/mL)	1.63 (1.14-3.28)	0.93 (0.50-2.05)	2.52 (1.40-4.61)	1.55 (1.19-3.95)	< 0.001	1 > 2, 3 > 2, 4 > 2

The median and interquartile ranges were shown except for BMI, age which were expressed as means ± SDS.
CPP: central precocious puberty, HC: healthy controls, BMI: body mass index, SDS: standard deviation score, CA: chronological age, BA: bone age, FSH: follicle stimulating hormone, LH: luteinizing hormone, E_2: estradiol, p: peak, MKRN3: makorin ring finger protein 3

Table 3. Correlations between MKRN3 and other biochemical indicators

	All cases			CPP group			HC group		
	n	r	p	n	r	p	n	r	p
BMI	178	-0.326	< 0.001	92	-0.349	0.001	86	-0.284	0.008
BMI SDS	178	-0.261	< 0.001	92	-0.315	0.002	86	-0.237	0.028
Tanner stage	178	-0.272	< 0.001	92	-0.143	0.173			
FSH	178	-0.218	0.003	92	-0.133	0.206	86	-0.036	0.739
LH	178	-0.128	0.09	92	-0.099	0.346	86	0.239	0.027
E_2	178	-0.175	0.02	92	-0.049	0.644	86	-0.014	0.899
p-FSH				60	0.178	0.174			
p-LH				60	0.164	0.211			
Length of the uterus	178	-0.206	0.006	92	-0.009	0.930	86	0.057	0.604
Right ovarian volumes	178	-0.194	0.009	92	-0.003	0.976	86	-0.026	0.811
Left ovarian volumes	178	-0.189	0.12	92	-0.004	0.972	86	0.053	0.629

CPP: central precocious puberty, HC: healthy controls, BMI: body mass index, SDS: standard deviation score, FSH: follicle stimulating hormone, LH: luteinizing hormone, E_2: estradiol, p: peak, MKRN3: makorin ring finger protein 3

Discussion

Puberty is a complex developmental process that leads to sexual maturation and reproductive capacity, resulting in spermatogenesis in boys and ovulation in girls. This arises from a coordinated sequence of events controlled by genetic, neurochemical, metabolic, and environmental factors (21).

In 1970, Frisch and Revelle (22,23) suggested there is a critical body weight controlling pubertal timing and menarche in girls. Many studies have shown that girls with more body fat undergo puberty earlier (4,24,25,26). Several cross-sectional studies have reported significant correlations between obesity and earlier menarche (27,28,29). Wang (30) investigated the relationship between obesity and early sexual development in 1,501 girls and 1,520 boys aged 8-14 years. In girls, they found positive correlations between early sexual development and BMI, obesity, and subcutaneous adipose tissue thickness. The increased risk of early puberty in girls may be related to the recent increase in childhood obesity (31).

The *MKRN3* gene, located on chromosome 15 in the Prader-Willi syndrome-associated region (15q11-q13), was found to be mutated in five families with familial precocious puberty (6). These included frameshift, nonsense, and missense mutations (32). *MKRN3* is expressed in the hypothalamus and other tissues (33). *MKRN3* expression is also high in the hypothalamus of prepubertal mice, rats, and primates; it decreases rapidly before puberty and remains low thereafter (6,34). Thus, MKRN3 is thought to inhibit the pathways leading to the onset of puberty. Abreu et al. (34) reported that MKRN3 is expressed in KISS1 neurons of the mouse hypothalamic arcuate nucleus and that MKRN3 repressed the promoter activity of human KISS1 and TAC3. MKRN3 also has ubiquitinase activity, which is reduced by *MKRN3* mutations affecting the RING finger domain; these mutations compromise the ability of *MKRN3* to suppress KISS1 and TAC3 promoter activity. Thus, MKRN3 is thought to act at the level of kisspeptin or GnRH neurons.

This study investigated how serum MKRN3 levels change with obesity in girls with CPP. Previous studies revealed that the serum MKRN3 level was significantly lower in girls with CPP than prepubertal girls (11,13,14). We found that median serum MKRN3 levels were 1.40 (0.76-2.66) ng/mL in the CPP group and 1.68 (1.26-4.60) ng/mL in the HC group. The decreased MKRN3 levels in girls with CPP support the association of MKRN3 with the inhibition of GnRH secretion and pubertal initiation, and concur with previous reports of peripubertal changes in serum MKRN3 levels (9,11,12,13,35).

Hagen et al. (9) reported that MKRN3 was negatively correlated with gonadotropin levels in prepubertal girls. Grandone et al. (13) reported that MKRN3 was negatively correlated with gonadotropins and E_2 in CPP, normal age prepubertal, and pubertal girls. Ge et al. (12) reported that MKRN3 was negatively correlated with gonadotropin levels in girls with premature thelarche and CPP. The prepubertal decline in MKRN3, and its negative correlation with gonadotropins, support the notion that MKRN3 is a major inhibitor of hypothalamic GnRH secretion during childhood. Inter-individual variation in circulating MKRN3 indicates that there is no standard threshold with respect to when MKRN3 initiates puberty. We found that serum MKRN3 levels were negatively correlated with FSH and E_2, and non-significantly correlated with LH. We also found negative

correlations between MKRN3 and the uterine length and ovarian volume, also supporting a relationship between MKRN3 decline and the onset of puberty. FSH and LH are hormones produced by the anterior pituitary in response to GnRH from the hypothalamus (36). In men, Leydig cells produce testosterone under the control of LH. However, in women, FSH stimulates granulosa cells in the ovarian follicles to synthesize aromatase, which converts androgens produced by the thecal cells to E_2 (37). Our study revealed that, the effect of the peripubertal decline in MKRN3 on FSH is more prominent than its effect on LH.

Grandone et al. (13) and Li et al. (14) found negative correlations between MKRN3 and BMI, while Jeong et al. (11) did not (35). The BMI and BMI SDS of the patient and control groups in these studies were within the normal range. In our study, the MKRN3 level was lowest in the CPP-obese group, and there was a significant difference between the CPP-obese and other subgroups (CPP-normal weight, HC-normal weight, and HC-obese). There was no difference in MKRN3 level among the CPP-normal weight, HC-normal weight, and HC-obese groups. Although the MKRN3 level in the CPP group was lower than in the HC group, the MKRN3 levels of the CPP-normal weight group did not differ from the HC-normal weight and HC-obese groups, which indicates there is a relationship between obesity and MKRN3. In addition, there was no significant difference between the HC-normal weight and HC-obese groups; the median value was higher in the HC-normal weight group. We hypothesize that MKRN3 levels may decrease due to the effects of obesity. Finally, MKRN3 levels do not appear to represent a marker for discriminating precocious puberty between CPP-normal weight and HC-obese groups. This suggests that puberty is not only affected by MKRN3 or obesity and that the mechanisms are complex. There was also a negative correlation between MKRN3 and BMI. The relationship between adiposity and the onset of puberty, as well as between obesity and early menarche, is known (38). The negative correlation with BMI suggests that MKRN3 in girls is modulated by nutritional factors and adipokines, such as leptin.

Leptin is a peptide hormone released from adipose tissue in proportion to its mass. Leptin levels are associated with the energy reserve required for pubertal development, and levels convey this status to the hypothalamus. Leptin acts in the sensitization of hypothalamic GnRH neurons and stimulates GnRH by binding to the leptin receptor and activating kisspeptin (39,40). Leptin permits puberty to progress only if adequate body energy reserves are available (41), although a recent study showed that the peripubertal decrease in MKRN3 expression was independent of the effect of leptin in a leptin-deficient mouse model (42). Therefore, the interactions and relationships between neuroendocrine factors and adipokines at the onset of puberty have not yet been fully elucidated. The negative correlation between BMI and MKRN3, and lower MKRN3 levels in obese patients in early puberty, suggests that another factor modulates the effect of adipose tissue on the onset of puberty. Unfortunately, leptin was not measured in our patients.

Study Limitations

MKRN3 gene analysis was not performed in our study but selective genetic testing should be performed in patients with very low or very high MKRN3 values. Since our study group was divided into obese and non-obese cases, overweight cases were not evaluated separately. Finally, the relationship between leptin and MKRN3 has not been evaluated. In future studies, the limitations of our study can be eliminated by evaluating a larger sample group and investigating MKRN3 levels in patients with overweight and/or morbid obesity. Furthermore, this design would enable the relationship between leptin and MKRN3 to be evaluated.

Conclusion

In conclusion, serum MKRN3 levels were lower in girls with CPP than controls, supporting the finding that MKRN3 levels decrease at the onset of puberty and have a role therein. The negative correlation between BMI and MKRN3, and the lower MKRN3 levels in CPP-obese cases, suggest that another factor modulates the effect of adipose tissue on the onset of puberty. More comprehensive studies are needed to determine the relationship between MKRN3 and adipose tissue.

Ethics

Ethics Committee Approval: The study protocol was in line with the Declaration of Helsinki and was approved by the Eskişehir Osmangazi University Faculty of Medicine, Non-Interventional Clinical Research Ethics Committee (protocol no: 10, date: 25.06.2019).

Informed Consent: Informed consent was obtained from all individuals included in this study and their parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: Sümeyye Emel Eren, Enver Şimşek.

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References

1. Choi JH, Yoo HW. Control of puberty: genetics, endocrinology, and environment. Curr Opin Endocrinol Diabetes Obes 2013;20:62-68.
2. Soliman AT, Yasin M, Kassem A. Leptin in pediatrics: a hormone from adipocyte that wheels several functions in children. Indian J Endocrinol Metab 2012;16(Suppl 3):577-587.
3. Shalitin S, Gat-Yablonski G. Associations of obesity with linear growth and puberty. Horm Res Paediatr 2022;95:120-136. Epub 2021 Jun 15
4. Li W, Liu Q, Deng X, Chen Y, Liu S, Story M. Association between Obesity and Puberty Timing: A Systematic Review and Meta-Analysis. Int J Environ Res Public Health 2017;14:1266.
5. Jong MT, Gray TA, Ji Y, Glenn CC, Saitoh S, Driscoll DJ, Nicholls RD. A novel imprinted gene, encoding a ring zinc-finger protein, and overlapping antisense transcript in the Prader-Willi syndrome critical region. Hum Mol Genet 1999;8:783-793.
6. Abreu AP, Dauber A, Macedo DB, Noel SD, Brito VN, Gill JC, Cukier P, Thompson IR, Navarro VM, Gagliardi PC, Rodrigues T, Kochi C, Longui CA, Beckers D, de Zegher F, Montenegro LR, Mendonca BB, Carroll RS, Hirschhorn JN, Latronico AC, Kaiser UB. Central precocious puberty caused by mutations in the imprinted gene MKRN3. N Engl J Med 2013;368:2467-2475. Epub 2013 Jun 5
7. Macedo DB, Abreu AP, Reis AC, Montenegro LR, Dauber A, Beneduzzi D, Cukier P, Silveira LF, Teles MG, Carroll RS, Junior GG, Filho GG, Gucev Z, Arnhold IJ, de Castro M, Moreira AC, Martinelli CE Jr, Hirschhorn JN, Mendonca BB, Brito VN, Antonini SR, Kaiser UB, Latronico AC. Central precocious puberty that appears to be sporadic caused by paternally inherited mutations in the imprinted gene makorin ring finger 3. J Clin Endocrinol Metab 2014;99:1097-1103. Epub 2014 Mar 14
8. Busch AS, Hagen CP, Almstrup K, Juul A. Circulating MKRN3 levels decline during puberty in healthy boys. J Clin Endocrinol Metab 2016;101:2588-2593. Epub 2016 Apr 8
9. Hagen CP, Sørensen K, Mieritz MG, Johannsen TH, Almstrup K, Juul A. Circulating MKRN3 levels decline prior to pubertal onset and through puberty: a longitudinal study of healthy girls. J Clin Endocrinol Metab 2015;100:1920-1926. Epub 2015 Feb 19
10. Varimo T, Dunkel L, Vaaralahti K, Miettinen PJ, Hero M, Raivio T. Circulating makorin ring finger protein 3 levels decline in boys before the clinical onset of puberty. Eur J Endocrinol 2016;174:785-790. Epub 2016 Mar 29
11. Jeong HR, Lee HJ, Shim YS, Kang MJ, Yang S, Hwang IT. Serum Makorin ring finger protein 3 values for predicting Central precocious puberty in girls. Gynecol Endocrinol 2019;35:732-736. Epub 2019 Feb 26
12. Ge W, Wang HL, Shao HJ, Liu HW, Xu RY. Evaluation of serum makorin ring finger protein 3 (MKRN3) levels in girls with idiopathic central precocious puberty and premature thelarche. Physiol Res 2020;69:127-133. Epub 2019 Dec 19
13. Grandone A, Cirillo G, Sasso M, Capristo C, Tornese G, Marzuillo P, Luongo C, Rosaria Umano G, Festa A, Coppola R, Miraglia Del Giudice E, Perrone L. MKRN3 levels in girls with central precocious puberty and correlation with sexual hormone levels: a pilot study. Endocrine 2018;59:203-208. Epub 2017 Mar 15
14. Li M, Chen Y, Liao B, Tang J, Zhong J, Lan D. The role of kisspeptin and MKRN3 in the diagnosis of central precocious puberty in girls. Endocr Connect 2021;10:1147-1154.
15. Lakshman R, Elks CE, Ong KK. Childhood obesity. Circulation 2012;126:1770-1779.
16. Neyzi O, Furman A, Bundak R, Gunoz H, Darendeliler F, Bas F. Growth references for Turkish children aged 6 to 18 years. Acta Paediatr 2006;95:1635-1641.
17. Bundak R, Furman A, Gunoz H, Darendeliler F, Bas F, Neyzi O. Body mass index references for Turkish children. Acta Paediatr 2006;95:194-198.
18. Greulich WW, Pyle SI. Radiographic atlas of skeletal development of the hand and wrist. 2nd ed ed. Stanford, CA, Stanford University Press, 1971.
19. Ab Rahim SN, Omar J, Tuan Ismail TS. Gonadotropin-releasing hormone stimulation test and diagnostic cutoff in precocious puberty: a mini review. Ann Pediatr Endocrinol Metab 2020;25:152-155. Epub 2020 Jul 30
20. Kandemir N, Demirbilek H, Özön ZA, Gönç N, Alikaşifoğlu A. GnRH stimulation test in precocious puberty: single sample is adequate for diagnosis and dose adjustment. J Clin Res Pediatr Endocrinol 2011;3:12-17. Epub 2011 Feb 23
21. Maione L, Naulé L, Kaiser UB. Makorin ring finger protein 3 and central precocious puberty. Curr Opin Endocr Metab Res 2020;14:152-159. Epub 2020 Aug 26
22. Frisch RE, Revelle R. Height and weight at menarche and a hypothesis of critical body weights and adolescent events. Science 1970;169:397-399.
23. Frisch RE, Revelle R. The height and weight of girls and boys at the time of initiation of the adolescent growth spurt in height and weight and the relationship to menarche. Hum Biol 1971;43:140-159.
24. Wagner IV, Sabin MA, Pfäffle RW, Hiemisch A, Sergeyev E, Körner A, Kiess W. Effects of obesity on human sexual development. Nat Rev Endocrinol 2012;8:246-254.
25. Rosenfield RL, Lipton RB, Drum ML. Thelarche, pubarche, and menarche attainment in children with normal and elevated body mass index. Pediatrics 2009;123:84-88.
26. Biro FM, Kiess W. Contemporary trends in onset and completion of puberty, gain in height and adiposity. Endocr Dev 2016;29:122-133. Epub 2015 Dec 17
27. Barcellos Gemelli IF, Farias EDS, Souza OF. Age at Menarche and Its Association with Excess Weight and Body Fat Percentage in Girls in the Southwestern Region of the Brazilian Amazon. J Pediatr Adolesc Gynecol 2016;29:482-488. Epub 2016 Mar 8
28. Bau AM, Ernert A, Schenk L, Wiegand S, Martus P, Grüters A, Krude H. Is there a further acceleration in the age at onset of menarche? A cross-sectional study in 1840 school children focusing on age and bodyweight at the onset of menarche. Eur J Endocrinol 2009;160:107-113. Epub 2008 Oct 30
29. Wronka I. Association between BMI and age at menarche in girls from different socio-economic groups. Anthropol Anz 2010;68:43-52.
30. Wang Y. Is obesity associated with early sexual maturation? A comparison of the association in American boys versus girls. Pediatrics 2002;110:903-910.
31. Obesity and overweight. World Health Organization; 2021. who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed December 26, 2021.
32. Valadares LP, Meireles CG, De Toledo IP, Santarem de Oliveira R, Gonçalves de Castro LC, Abreu AP, Carroll RS, Latronico AC, Kaiser UB, Guerra ENS, Lofrano-Porto A. MKRN3 Mutations in Central Precocious Puberty: A Systematic Review and Meta-Analysis. J Endocr So 2019;3:979-995.

33. Känsäkoski J, Raivio T, Juul A, Tommiska J. A missense mutation in MKRN3 in a Danish girl with central precocious puberty and her brother with early puberty. Pediatr Res 2015;78:709-711. Epub 2015 Sep 2

34. Abreu AP, Toro CA, Song YB, Navarro VM, Bosch MA, Eren A, Liang JN, Carroll RS, Latronico AC, Rønnekleiv OK, Aylwin CF, Lomniczi A, Ojeda S, Kaiser UB. MKRN3 inhibits the reproductive axis through actions in kisspeptin-expressing neurons. J Clin Invest 2020;130:4486-4500.

35. Jeong HR, Yoon JS, Lee HJ, Shim YS, Kang MJ, Hwang IT. Serum level of NPTX1 is independent of serum MKRN3 in central precocious puberty. J Pediatr Endocrinol Metab 2021;34:59-63.

36. Stamatiades GA, Kaiser UB. Gonadotropin regulation by pulsatile GnRH: Signaling and gene expression. Mol Cell Endocrinol 2018;463:131-141. Epub 2017 Nov 2

37. Biro FM, Pinney SM, Huang B, Baker ER, Walt Chandler D, Dorn LD. Hormone changes in peripubertal girls. J Clin Endocrinol Metab 2014;99:3829-3835. Epub 2014 Jul 16

38. German A, Shmoish M, Hochberg ZE. Predicting pubertal development by infantile and childhood height, BMI, and adiposity rebound. Pediatr Res 2015;78:445-450. Epub 2015 Jul 7

39. Yu WH, Kimura M, Walczewska A, Karanth S, McCann SM. Role of leptin in hypothalamic-pituitary function. Proc Natl Acad Sci U S A 1997;94:1023-1028.

40. Reinehr T, Roth CL. Is there a causal relationship between obesity and puberty? Lancet Child Adolesc Health 2019;3:44-54. Epub 2018 Nov 14

41. Tena-Sempere M. Keeping puberty on time: novel signals and mechanisms involved. Curr Top Dev Biol 2013;105:299-329.

42. Roberts SA, Abreu AP, Navarro VM, Liang JN, Maguire CA, Kim HK, Carroll RS, Kaiser UB. The Peripubertal Decline in Makorin Ring Finger Protein 3 Expression is Independent of Leptin Action. J Endocr Soc 2020;4:bvaa059.

ORIGINAL ARTICLE

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Can Serum 25-Hydroxy Vitamin D Levels Predict the Severity of Multisystem Inflammatory Syndrome in Children and COVID-19?

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What is already known on this topic?

Serum vitamin D levels are lower in patients with Coronavirus disease-2019 (COVID-19) and multisystem inflammatory syndrome in children (MIS-C).

What this study adds?

The severity of COVID-19 was associated with low serum vitamin D levels. In MIS-C there was a moderate correlation between the number of affected organ systems and serum 25-hydroxy vitamin D levels. MIS-C patients who required intensive care had considerably lower vitamin D levels than those who did not.

Abstract

Objective: To determine the clinical significance of serum 25-hydroxy (OH) vitamin D levels in pediatric patients with multisystem inflammatory syndrome in children (MIS-C) and compare the vitamin D levels of these patients with those patients with Coronavirus disease-2019 (COVID-19) and healthy controls.

Methods: This study was designed for pediatric patients aged 1 month to 18 years and conducted between July 14 and December 25, 2021. Fifty-one patients with MIS-C, 57 who were hospitalized with COVID-19, and 60 controls were enrolled in the study. Vitamin D insufficiency was defined as a serum 25 (OH) vitamin D level of less than 20 ng/mL. Severe MIS-C was classified as necessitating intensive care due to cardiovascular instability, the necessity for non-invasive or invasive mechanical ventilation, and/or a diminishing Glasgow coma scale. World Health Organization definition criteria were used to describe the clinical stages of COVID-19 in children and patients were divided into four groups according to the clinical severity of COVID-19: asymptomatic, mild, moderate, and severe/critical.

Results: The median serum 25 (OH) vitamin D was 14.6 ng/mL in patients with MIS-C, 16 ng/mL in patients with COVID-19, and 21.1 ng/mL in the control group ($p < 0.001$). Vitamin D insufficiency was present in 74.5% (n = 38) of patients with MIS-C, 66.7% (n = 38) of patients with COVID-19, and 41.7% (n = 25) of the controls ($p = 0.001$). The percentage of four or more affected organ systems was 39.2% in patients with MIS-C. The correlation between the number of affected organ systems and serum 25 (OH) vitamin D levels was evaluated in patients with MIS-C and there was a moderate negative correlation ($r = -0.310$; $p = 0.027$). A weak negative correlation was found between the severity of COVID-19 and serum 25 (OH) vitamin D ($r = -0.320$, $p = 0.015$).

Conclusion: Vitamin D levels were insufficient in both the MIS-C and COVID groups. Furthermore, vitamin D levels correlated with the number of affected organ systems in MIS-C and the severity of COVID-19.

Keywords: Vitamin D, COVID-19, MIS-C, children

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Introduction

The Coronavirus disease-2019 (COVID-19) pandemic, caused by Severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) infection, has spread rapidly worldwide. While the nature of this disease is gradually being discovered, it has been observed that the clinical course is milder in children compared with adults (1). Nevertheless, recent evidence has shown that children may develop signs of multiorgan failure several weeks after primary infection, manifesting in cardiovascular dysfunction leading to life-threatening shock and even requiring a stay in the intensive care unit (ICU) due to the systemic inflammatory response (2). This novel syndrome was later termed multisystem inflammatory syndrome in children (MIS-C) (3,4). This postinfectious process is thought to be caused by non-neutralizing antibodies through antibody-dependent amplification, causing immune system dysregulation by SARS-CoV-2 with a racial genetic predisposition (5,6).

Vitamin D is well-known for its role in regulating calcium and phosphorus metabolism. More recently, the role of vitamin D in non-skeletal functions, including inflammation and immune regulation, has also been investigated (7). One of the mechanistic effects of vitamin D on immune function is via the vitamin D receptor, which is expressed in most cell types and can influence genomic and non-genomic pathways related to the immune system (8). Vitamin D can induce monocyte differentiation into macrophages, increase the activity of lysosomal enzymes in macrophages, and facilitate cytotoxic activity by increasing the rate of phagocytosis (9). Many studies have provided evidence that vitamin D reduces the risk of viral infection by suppressing the release of inflammatory cytokines derived from the adaptive immune system, particularly interleukin-2 and interferon-gamma (10,11). Vitamin D has been reported to inhibit inflammatory processes by stimulating T-regulatory cells and increasing cellular immunity (10,11). Vitamin D is also known to exert direct antibacterial and antiviral effects via cathelicidin. Cathelicidin is an antimicrobial peptide that promotes the induction of reactive oxygen radical synthesis, which has direct microbicidal effects and elicits immunomodulatory responses to pathogen-associated stimuli by recruiting neutrophils, monocytes, and T cells to microbial invasion sites (12,13). The effect of vitamin D in MIS-C is thought to be due to its well-established role in modulating adaptive and innate immunity, including regulation of inflammatory cytokine release (5,6).

There are many studies on vitamin D deficiency in children with various infectious diseases (14,15). However, there are insufficient studies on vitamin D status in children with MIS-C. This study aimed to investigate the clinical significance of serum 25-hydroxy (OH) vitamin D levels in pediatric patients with MIS-C and to compare 25 (OH) vitamin D levels in patients hospitalized for COVID-19 and healthy controls.

Methods

Study Design

This prospective, observational study was designed for pediatric patients who were aged 1 month to 18 years and was conducted between July 14th and December 25th, 2021. Hospitalized patients who met the diagnostic criteria for MIS-C were enrolled in the study. During the study period, hospitalized pediatric patients with a diagnosis of COVID-19 confirmed by a positive reverse transcriptase-polymerase chain reaction (RT-PCR) were included in the study. Healthy volunteers who were admitted to general pediatric polyclinics were defined as the control group and serum samples were during similar months to the patient group to negate the well-known seasonal effect on vitamin D levels. The control group was randomly selected, starting with the 50th patient out of roughly 3000 attendants to pediatric outpatient clinics, as well as patients who were multiples of that patient.

Patient demographics, underlying disease, medication history, symptoms, laboratory results, system involvement, and outcomes were extracted from medical records. Clinical and laboratory parameters (lymphocyte count, neutrophil count, blood pressure, respiratory rate, and heart rate) were recorded as age-specific normal ranges. The need for ICU care due to inotropic support or fluid resuscitation, the need for invasive/non-invasive mechanical ventilation, or extracorporeal membrane oxygenation were assessed. Treatment modalities were recorded. The case definition of MIS-C was used, as defined by the Centers for Disease Control and Prevention and the World Health Organization (3,4). Severe MIS-C was classified as necessitating intensive care due to cardiovascular instability, the necessity for non-invasive or invasive mechanical ventilation, and/or a diminishing Glasgow coma scale. World Health Organization definition criteria were used to describe the clinical stages of COVID-19 in children (16). Patients were divided into four groups according to the clinical severity of COVID-19: asymptomatic, mild, moderate, and severe/critical.

Cut-off values for serum 25 (OH) vitamin D have been previously published with global consensus recommendations from pediatric endocrinologists: Vitamin

D sufficiency is defined as a serum 25 (OH) vitamin D level of at least 20 ng/mL (50 nmol/L), whereas insufficiency is defined as 12 to 20 ng/mL (range, 30-50 nmol/L) and deficiency is less than 12 ng/mL (< 30 nmol/L) (17). Serum 25 (OH) vitamin D levels were measured during the first three days after hospitalization.

Patients who had taken vitamin supplements, who had bone metabolism disorders, and who did not want to participate in the study were excluded. Written informed consent was obtained from the patients and their parents. Ethical committee approval was obtained from University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/07-14, date: 14.07.2021).

RT-PCR Assay

Combined nasopharyngeal and oropharyngeal swab specimens were collected from children with suspected COVID-19 and sent to the medical microbiology laboratory. SARS-CoV-2 was detected using RT-PCR (Bio-Speedy SARS-CoV-2 double Gene RT-qPCR Kit). Specifically, two target genes, including open reading frame 1ab (ORF1ab) and nucleocapsid protein (N), were tested during the RT-PCR assay.

Vitamin D Assay

Blood samples were placed in gel-containing tubes with a clot activator (BD Vacutainer SST II Advance, USA) and centrifuged at 1500 g for 10 minutes to separate serum from clot. Serum 25 (OH) vitamin D was measured by chemiluminescence immunoassay on an Advia Centaur XP analyzer (Siemens Healthineers, Erlangen, Germany). The intra-assay and inter-assay coefficients of variation for the 25 (OH) vitamin D assay were less than 8% and 12%, respectively.

Statistical Analysis

The median, first quartile, and third quartile [interquartile range (IQR)] were used to represent continuous variables that were not normally distributed. Differences between two or three groups were analyzed using the Mann-Whitney U test and the Kruskal-Wallis test, respectively. An independent t-test was used to compare normally distributed data. Categorical variables were compared using the chi-square test or Fisher's exact test. A $p < 0.05$ was considered significant. Spearman's rank correlation test was performed to determine the association between serum 25 (OH) vitamin D and the severity of MIS-C or COVID-19 pneumonia. Spearman's correlation analysis was used to determine the correlation between laboratory results and serum 25 (OH) vitamin D levels. Statistical analyses were performed using Statistical Package for the Social Sciences for Windows, version 25 (IBM, Armonk, NY, USA).

Results

This prospective observational study was performed with 51 patients with MIS-C, 57 patients with COVID-19, and 60 controls. When the sex and median age distribution of the groups were evaluated, there were no statistical differences between the three groups ($p = 0.446$ and $p = 0.089$, respectively) (Table 1). The median serum 25 (OH) vitamin D level was 14.6 ng/mL in patients with MIS-C, 16 ng/mL in patients with COVID-19, and 21.1 ng/mL in the controls ($p < 0.001$). In subgroup comparison, serum 25 (OH) vitamin D levels were significantly lower in patients with MIS-C compared with controls (MIS-C vs. controls $p < 0.001$; MIS-C vs. COVID-19 $p = 0.240$; COVID-19 vs. controls $p = 0.058$). Vitamin D insufficiency was present in 74.5% (n = 38/51) of patients with MIS-C, 66.7% (n = 38/57) of patients with COVID-19, and 41.7% (n = 25/60) of the controls (Figure 1).

Table 1. Characteristics and serum vitamin D levels between patients with MIS-C, hospitalized COVID-19 and the control group

	MIS-C	COVID-19	Control group	p value	p value		
					MIS-C vs. COVID-19	MIS-C vs. control	COVID-19 vs. control
Patient number, n (%)	51	57	60	-	-	-	-
Age, years (IQR)	8.8 (5.6-12.3)	11.8 (3.8-15.7)	10 (6.2-16.4)	0.089	-	-	-
Sex, n (%)							
Boy	33 (64.7)	30 (52.6)	35 (58.3)	0.446	-	-	-
Girl	18 (35.3)	27 (47.4)	25 (41.7)				
25 (OH) vitamin D levels (IQR)	14 (9.3-20)	16 (9.1-23.4)	21.1 (13.7-27.5)	< 0.001*	0.240	< 0.001	0.058
Vitamin D status, n (%)				0.001	0.373	< 0.001	0.007
Vitamin D sufficiency	13 (25.5)	19 (33.3)	35 (58.3)				
Vitamin D insufficiency	38 (74.5)	38 (66.7)	25 (41.7)				

*Fisher's exact probability test was used for cross-classification tables.
IQR: interquartile range, 25 (OH): 25-hydroxy, MIS-C: multisystem inflammatory syndrome in children, COVID-19: Coronavirus disease-2019

The characteristics of patients with MIS-C according to adequate/inadequate serum 25 (OH) vitamin D levels are shown in Table 2. Thirty-eight (74.5%) patients had vitamin D insufficiency and 13 (25.5%) had vitamin D sufficiency. The median (IQR) age of patients with MIS-C was 8.8 (5.6-12.3) years. Patients with adequate serum 25 (OH) vitamin D levels were younger compared with patients with inadequate serum 25 (OH) vitamin D (6 vs. 10.3 years; p = 0.034) (Table 2). Thirty-three (64.7%) patients with MIS-C were male and 28.9% (n = 15) were overweight-obese. The median length of hospital stay was eight days in the inadequate vitamin D group and five days in the adequate vitamin D group (p = 0.085). In the evaluation of admission symptoms (fever, fatigue, muscle ache, any gastrointestinal symptoms, conjunctival inflammation mucous membrane changes, rash, arthralgia, any respiratory symptoms), there were no statistically significant differences between the adequate and inadequate vitamin D groups with MIS-C (p > 0.05 for all).

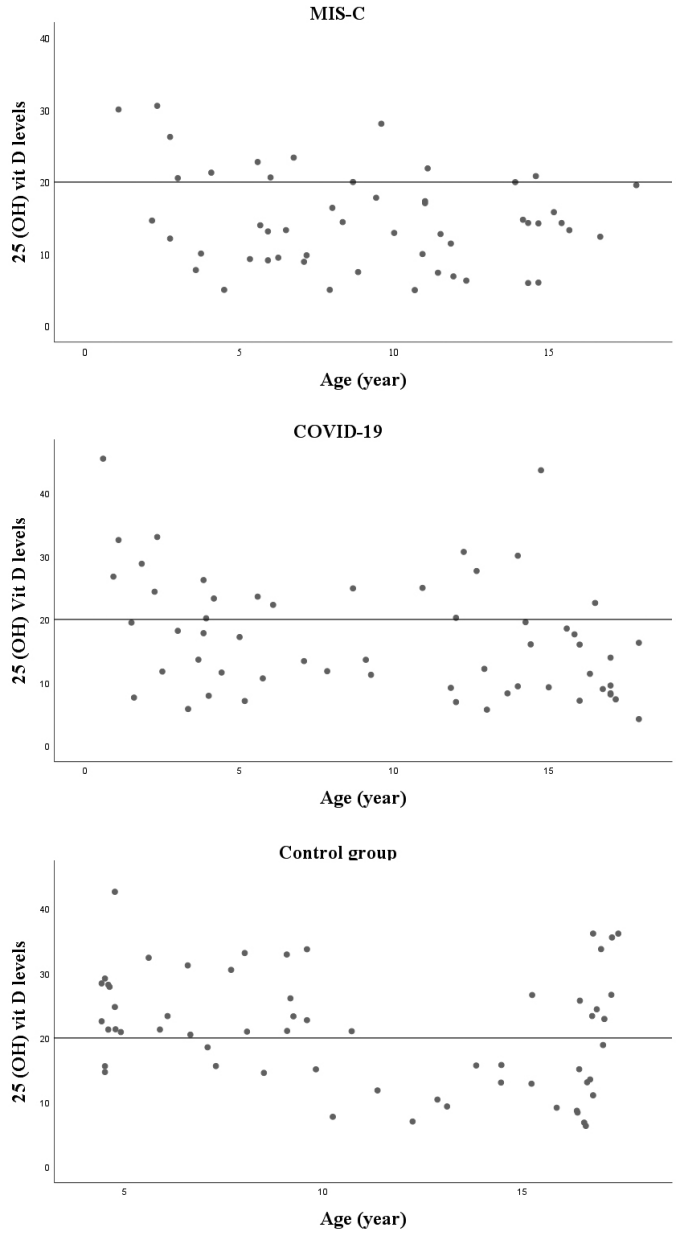

Figure 1. Serum 25-hydroxy vitamin D values in patients with MIS-C, COVID-19, and healthy controls

COVID-19: Coronavirus disease-2019, MIS-C: multisystem inflammatory syndrome in children, 25 (OH): 25-hydroxy

The affected organ systems (cardiac, renal, respiratory, hematologic, gastrointestinal, dermatologic, or neurologic) were assessed in patients with MIS-C. The percentage of four or more affected organ systems was 39.2% among patients with MIS-C. It was found that the prevalence of patients with ≥4 involved organ systems was significantly higher in the group with inadequate vitamin D (47.4%, n = 18) compared with the group with adequate vitamin D (15.4%, n = 2) (p = 0.041). When the correlation between the number of affected organ systems and serum 25 (OH) vitamin D levels was evaluated, there was a moderate negative correlation (r = -0.310; p = 0.027). ICU stay was required in 15.7% (n = 8) of patients with MIS-C, and all of these patients were in the inadequate vitamin D group (p = 0.096). The pediatric ICU (PICU) group had significantly lower serum 25 (OH) vitamin D levels compared with the non-PICU group (11.8 vs. 15.1; p = 0.039) (Figure 2). Similarly, hypotension was noted in 34.2% (n = 13) of patients, and shock developed in 26.3% (n = 10) of patients; all of these patients were in the inadequate vitamin D group (p = 0.023 and p = 0.048, respectively). There were no deaths in the study population.

The characteristics of patients with COVID-19 are shown in Table 3. Thirty-eight (66.7%) of 57 hospitalized COVID-19 patients had vitamin D insufficiency. The median (IQR) age was 11.8 years (3.8-15.7) and 52.6% (n = 30) of patients were male. When evaluating the clinical characteristics of the patients, dry cough was significantly more frequent in the group with inadequate serum vitamin D levels (73.7% vs. 47.4%, respectively; p = 0.049). When evaluating the laboratory results, the lymphocyte count was significantly lower in the group with inadequate serum vitamin D levels (1300 vs. 2200 cells/uL, p = 0.049). When evaluating the correlation between the severity of COVID-19 and serum 25 (OH) vitamin D, a weak negative correlation was found (r = -0.320, p = 0.015) and this was also found for the length of hospital stay (r = -0.304, p = 0.022). The correlation between serum 25 (OH) vitamin D levels and laboratory results was evaluated. There was a moderate positive correlation between serum 25 (OH) vitamin D levels and aspartate aminotransferase levels (r = 0.530; p < 0.001),

and a weak positive correlation with lactate dehydrogenase levels (r = 0.269, p = 0.043).

Discussion

To the best of our knowledge, this is one of the first studies to analyze vitamin D levels in pediatric patients with MIS-C and a hospitalized pediatric COVID-19 group. In the present study, the median serum 25 (OH) vitamin D level was inadequate in both patients with MIS-C and COVID-19 compared with the control group. It was lowest in the MIS-C group followed by COVID-19 and then healthy controls.

There are few published studies on vitamin D status in patients with MIS-C. In a study by Darren et al. (18), 16 of 18 (89%) patients with MIS-C had vitamin D insufficiency, and the mean 25 (OH) vitamin D level was 6.8 ng/mL. They also reported that the PICU group (n = 12) tended to have lower mean 25 (OH) vitamin D levels compared with the non-PICU group (8.9 vs. 5.6 ng/mL, respectively; p = 0.110), but these results were not significant. Zengin et al. (19) compared the serum vitamin D levels of 34 MIS-C patients requiring ICU with those of 34 control patients in a retrospective study. They reported that patients with MIS-C had considerably lower serum 25 (OH) vitamin D levels than those without MIS-C (9 vs. 19 ng/mL). Consistent with previous reports, 75% of patients in the present study with MIS-C had either vitamin D deficiency or vitamin D insufficiency and all patients who required ICU stay (n = 8/51, 21%) were in the vitamin D insufficiency group. The PICU group had significantly lower 25 (OH) vitamin D levels than the non-

Table 2. Characteristics of the patients with MIS-C according to serum 25-hydroxy vitamin D levels

	All patients n = 51	Vitamin D insufficiency n = 38	Vitamin D sufficiency n = 13	p value
Age, years, median (IQR)	8.8 (5.6-12.3)	10.3 (6.1-13)	6 (2.6-10.3)	0.034
Overweight/obese n/total (%)	13/45 (28.9)	10/35 (28.6)	3/10 (30)	0.608
Sex, n (%)				
Girl	18 (35.3)	15 (39.5)	3 (21.1)	0.336
Boy	33 (64.7)	23 (60.5)	10 (76.9)	
25 (OH) vitamin D levels, median (IQR)				
Girl	11.6 (6.7-18.2)	9.8 (6.3-12.7)	20.6 (20.5--)	-
Boy	14.4 (10.7-20.4)	13.3 (9.3-14.6)	23.1 (20.6-28.6)	-
Underlying medical condition, n (%)	17 (33.3)	14 (36.8)	3 (23.1)	0.502
Duration of hospitalization, median (IQR)	7 (4-11)	8 (5-13.2)	5 (3-8.5)	0.085
Number of organ systems involvements				
2-3	31 (60.8)	20 (52.6)	11 (84.6)	**0.041**
≥4	20 (39.2)	18 (47.4)	2 (15.4)	
Treatment				
Intravenous immunoglobulin n (%)	36 (70.6)	28 (73.7)	8 (61.5)	0.487*
Corticosteroids n (%)	31 (60.8)	26 (68.4)	5 (38.5)	0.098*
Anticoagulants n (%)	39 (76.5)	32 (84.2)	7 (53.8)	0.053*
Acetyl salicylic acid n (%)	5 (9.8)	3 (7.9)	2 (15.4)	0.591*
Inotropes n (%)	9 (17.6)	8 (21.1)	1 (7.7)	0.417*
Immunomodulatory therapy n (%)	4 (7.8)	4 (10.5)	0	0.561*
Need for oxygen n (%)	10 (19.6)	10 (26.3)	0	0.048*
Outcomes				
Hypotension n (%)	13 (25.5)	13 (34.2)	0	**0.023***
Extracorporeal membrane oxygenation n (%)	3 (5.9)	3 (7.9)	0	0.561*
Prone position n (%)	4 (7.8)	4 (10.5)	0	0.342*
Plasma exchange n (%)	4 (7.8)	4 (10.5)	0	0.295*
NIMV/MV n (%)	4 (7.8)	4 (10.5)	0	0.561*
Shock n (%)	10 (19.6)	10 (26.3)	0	**0.048***
Need for ICU n (%)	8 (15.7)	8 (21.1)	0	0.096*

*Fisher's exact probability test was used for cross-classification tables.
IQR: interquartile range, ICU: intensive care unit, NIMV/MV: non-invasive mechanical ventilation/mechanical ventilation, 25 (OH): 25-hydroxy, MIS-C: multisystem inflammatory syndrome in children

ICU group (11.8 vs. 15.1 ng/mL, respectively). This finding warrants further investigation in larger MIS-C cohorts.

Although studies on vitamin D status in patients with MIS-C are limited, some studies focused on its relation to disease severity. In the study by Torpoco Rivera et al. (20), the authors found that the seriousness of MIS-C, especially cardiac involvement, was associated with severe vitamin D deficiency [25 (OH) vitamin D level < 10 ng/mL]. In the study conducted by Mamishi et al. (21), 122 patients with MIS-C were divided into two groups (mild-moderate and severe). Mild-to-moderate MIS-C was present in 97, while severe MIS-C was present in 25. Serum 25 (OH) vitamin D levels were considerably lower in patients with severe MIS-C (8.5 vs. 20.5 ng/mL). In a review by Feketea et al. (22), the authors concluded that serum vitamin D levels might help predict severe forms of MIS-C and that correction of abnormal levels in severe MIS-C could influence the progression of the syndrome. Consistent with these speculations, we found a moderate negative correlation between serum 25 (OH) vitamin D and the number of affected organ systems in patients with MIS-C. These results suggest that patients with inadequate vitamin D status had a more severe disease course. However, vitamin D is an acute-phase reactant, and its blood level might decrease during the inflammatory process. MIS-C disease is known to occur as a result of cytokine storms. It is thought that an excess of cytokines could lead to more severe inflammation and cause a further decrease in serum vitamin D levels. Similarly, in a study by Peterson and Heffernan (23), the authors found serum concentrations of tumor necrosis factor-alpha or C-reactive protein were inversely correlated with serum vitamin D concentrations. As another mechanism, it is worth noting that the need for active vitamin D, which has an anti-inflammatory effect, increases when a severe inflammatory process occurs. Therefore, the turnover of vitamin D from serum and cells involved in immunomodulation increases, resulting in a decrease of inactive vitamin D from serum. From this point of view, the low serum vitamin D level in severe disease could be a consequence of severity and not a predisposing factor (24).

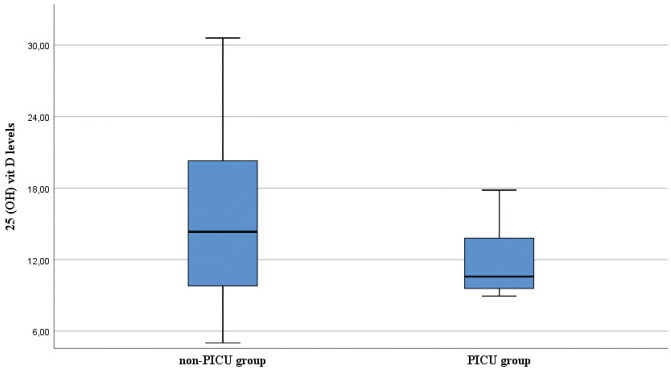

Figure 2. The comparison of serum 25-hydroxy vitamin D levels of patients with MIS-C according to a need for a stay in an intensive care unit

PICU: pediatric intensive care unit, 25 (OH): 25-hydroxy, MIS-C: multisystem inflammatory syndrome in children

Apart from the well-known effect of vitamin D on calcium metabolism in humans, it regulates immune responses by increasing the production of anti-inflammatory cytokines,

Table 3. Characteristics of hospitalized patients with COVID-19 according to serum 25-hydroxy vitamin D levels

	All patients n = 57	Vitamin D insufficiency n = 38	Vitamin D sufficiency n = 19	p value
Age, years, median (IOR)	11.8 (3.8-15.7)	13.3 (4.8-16.2)	5.5 (2.2-12.2)	**0.007**
Sex, n (%)				0.091
Girl	27 (47.4)	21 (55.3)	6 (31.6)	-
Boy	30 (52.6)	17 (44.7)	13 (68.4)	-
25 (OH) vitamin D levels, median (IQR)				-
Girl	11.2 (8.1-19.5)	9.2 (7.6-13.7)	25.8 (23.5-32.9)	-
Boy	17.7 (12-25.3)	13.3 (9.8-16.7)	26.2 (22.4-31.6)	-
Underlying medical condition, n (%)	20 (35.1)	15 (39.5)	5 (26.3)	0.326
Duration of hospitalization, median (IQR)	5 (3-7)	5 (2.7-7)	4 (3-6)	0.274
Severity of COVID-19 pneumonia n (%)				0.292*
Mild	17 (29.8)	9 (23.7)	8 (42.1)	-
Moderate	22 (38.6)	15 (39.5)	7 (36.8)	-
Severe/critical	18 (31.6)	14 (36.9)	4 (21.1)	-
Need for oxygen treatment n (%)	18 (31.6)	14 (36.8)	4 (21.1)	0.227
Need for ICU n (%)	5 (8.8)	3 (7.9)	2 (10.5)	1.000*

*Fisher's exact probability test was used for cross-classification tables.
IQR: interquartile range, ICU: intensive care unit, COVID-19: Coronavirus disease-2019, 25 (OH): 25-hydroxy

reducing plasma cells and the release of immunoglobulins, decreasing the production of proinflammatory cytokines, and thus stimulating the production of antimicrobial peptides in the respiratory system (7,25). One such study by Katz (26) examined 987,849 patients, 887 individuals tested positive for COVID-19, while 31,950 were diagnosed with vitamin D deficiency. Additionally, 87 patients had both vitamin D deficiency and COVID-19. They found that patients with vitamin D deficiency were 4.6 times more likely to have positive COVID-19 status than patients without deficiency [95% confidence interval (CI), 3.713-5.783]. Many studies conducted on adult patients showed a significant association between vitamin D deficiency and the severity of COVID-19 (26,27,28). In contrast, there are few studies in children because of the milder clinical course of COVID-19. A study by Alpcan et al. (29) retrospectively analyzed serum 25 (OH) vitamin D levels in 75 pediatric patients with COVID-19 and 80 healthy controls. The mean serum vitamin D level was significantly lower in the COVID-19 group than in the control group (21.5 vs. 28.0 ng/mL). They also showed that 84% of patients with COVID-19 had vitamin D insufficiency, as in the study by Karakaya Molla et al. (30), which reported a rate of 82% (29). Similar to previous reports, 66.7% of hospitalized patients with COVID-19 had vitamin D insufficiency in our population. Although the median serum vitamin D level was lower in the hospitalized COVID-19 group than in the control group, this was not significant, although there was a weak negative correlation between the severity of COVID-19 and serum vitamin D levels.

In a recent study comparing clinical features associated with COVID-19, according to vitamin D status, dyspnea, weakness, anosmia, headache, myalgia, and loss of taste were significantly more common in the insufficient vitamin D group (29). Regression analysis showed that low vitamin D level was a risk factor for the occurrence of dyspnea (Odds ratio = -0.268, 95% CI: -15.920 to -1.406) (29). The present study showed that only dry cough was significantly more frequent in the group with insufficient vitamin D in patients with COVID-19 (73.7% vs. 47.4%). In a study evaluating laboratory results and serum vitamin D levels, vitamin D was positively correlated with leukocyte count, lymphocyte count, and platelet count. In contrast, it was negatively correlated with age and length of hospital stay (30). Our results showed that there was a moderate positive correlation between serum 25 (OH) vitamin D and aspartate aminotransferase and a weak positive correlation with lactate dehydrogenase levels. Importantly, we found a weak negative correlation between serum 25 (OH) vitamin D levels and length of hospital stay.

Study Limitations

First, serum vitamin D levels were taken during the active inflammation phase. Serum vitamin D levels decrease during active inflammation in the human body. A more valid comparison would be possible if these patients' serum vitamin D levels before infection and inflammation were known. However, it is practically impossible to know in advance which patient will have MIS-C or COVID-19, unless widespread population studies are performed.

Conclusion

This study sheds light on the relationship between vitamin D status in patients with MIS-C and COVID-19. Serum 25 (OH) vitamin D levels were correlated with the severity of MIS-C, as represented by patients with >4 involved organ systems and severity of COVID-19. However, it is unclear whether low vitamin D status is more common in patients with MIS-C than in the general population because there are no clinical trial data. Our study is the first to compare vitamin D levels in patients with MIS-C and healthy controls. Evaluation of serum vitamin D status of patients with MIS-C and COVID-19 before and during the disease will provide a better understanding of the pathophysiologic mechanism of this issue.

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Ethics

Ethics Committee Approval: Ethical committee approval was obtained from University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/07-14, date: 14.07.2021).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Yıldız Ekemen Keleş, Dilek Yılmaz, Gülnihan Üstündağ, Ayşegül Elvan Tuz, Ahu Kara Aksay, Ayfer Çolak, Concept: Yıldız Ekemen Keleş, Dilek Yılmaz, Selin Taşar, Aslıhan Şahin, Ayşegül Elvan Tuz, Aslıhan Arslan Maden, Eda Karadağ Öncel, Ayfer Çolak, Design: Yıldız Ekemen Keleş, Dilek Yılmaz, Ahu Kara Aksay, Eda Karadağ Öncel, Ayfer Çolak, Data Collection or Processing: Yıldız Ekemen Keleş, Dilek Yılmaz, Selin Taşar, Gülnihan Üstündağ, Ayşegül Elvan Tuz, Aslıhan Şahin, Aslıhan Arslan Maden, Ahu Kara Aksay, Analysis or Interpretation: Eda

Karadağ Öncel, Ayfer Çolak, Yıldız Ekemen Keleş, Dilek Yılmaz, Gülnihan Üstündağ, Literature Search: Yıldız Ekemen Keleş, Dilek Yılmaz, Selin Taşar, Aslıhan Şahin, Ayşegül Elvan Tuz, Aslıhan Arslan Maden, Ahu Kara Aksay, Writing: Yıldız Ekemen Keleş, Dilek Yılmaz, Eda Karadağ Öncel, Gülnihan Üstündağ, Ayfer Çolak, Aslıhan Şahin, Ahu Kara Aksay.

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References

1. Karbuz A, Akkoc G, Bedir Demirdag T, Yilmaz Ciftdogan D, Ozer A, Cakir D, Hancerli Torun S, Kepenekli E, Erat T, Dalgic N, Ilbay S, Karaaslan A, Erdeniz EH, Aygun FD, Bozdemir SE, Hatipoglu N, Emiroglu M, Sahbudak Bal Z, Ciftci E, Bayhan GI, Gayretli Aydin ZG, Ocal Demir S, Kilic O, Hacimustafaoglu M, Sener Okur D, Sen S, Yahsi A, Akturk H, Cetin B, Sutcu M, Kara M, Uygun H, Tural Kara T, Korukluoglu G, Akgun O, Üstündağ G, Demir Mis M, Sali E, Kaba O, Yakut N, Kılıc O, Kanik MK, Cetin C, Dursun A, Cicek M, Kockuzu E, Sevketoglu E, Alkan G, Guner Ozenen G, İnce E, Baydar Z, Ozkaya AK, Ovali HF, Tekeli S, Celebi S, Cubukcu B, Bal A, Khalilova F, Kose M, Hatipoglu HU, Dalkiran T, Turgut M, Basak Altas A, Selcuk Duru HN, Aksay A, Saglam S, Sari Yanartas M, Ergenc Z, Akin Y, Duzenli Kar Y, Sahin S, Tuteroz SK, Bilen NM, Ozdemir H, Senoglu MC, Pariltan Kucukalioglu B, Besli GE, Kara Y, Turan C, Selbest Demirtas B, Celikyurt A, Cosgun Y, Elevli M, Sahin A, Bahtiyar Oguz S, Somer A, Karadag B, Demirhan R, Turk Dagi H, Kurugol Z, Taskin EC, Sahiner A, Yesil E, Ekemen Keles Y, Sarikaya R, Erdem Eralp E, Ozkinay F, Konca HK, Yilmaz S, Gokdemir Y, Arga G, Ozen S, Coksuer F, Vatansever G, Tezer H, Kara A. Epidemiological, Clinical, and Laboratory Features of Children With COVID-19 in Turkey. Front Pediatr 2021;9:631547.
2. Riphagen S, Gomez X, Gonzalez-Martinez C, Wilkinson N, Theocharis P. Hyperinflammatory shock in children during COVID-19 pandemic. Lancet 2020;395:1607-1608. Epub 2020 May 7
3. Centers for Disease Control and Prevention (2020) Multisystem Inflammatory Syndrome in Children (MIS-C) Associated with Coronavirus Disease 2019 (COVID-19). https://www.cdc.gov/mmwr/volumes /69 /wr /mm6932e2.htm. Accessed August 2020
4. World Health Organization (2020) Multisystem inflammatory syndrome in children and adolescents with COVID-19. https://www.who.int/news-room/commentaries/detail/multisystem-inflammatory-syndrome-in-children-and-adolescents-with-covid-19. Accessed May 2020
5. Rowley AH. Understanding SARS-CoV-2-related multisystem inflammatory syndrome in children. Nat Rev Immunol 2020;20:453-454.
6. Sacco K, Castagnoli R, Vakkilainen S, Liu C, Delmonte OM, Oguz C, Kaplan IM, Alehashemi S, Burbelo PD, Bhuyan F, de Jesus AA, Dobbs K, Rosen LB, Cheng A, Shaw E, Vakkilainen MS, Pala F, Lack J, Zhang Y, Fink DL, Oikonomou V, Snow AL, Dalgard CL, Chen J, Sellers BA, Montealegre Sanchez GA, Barron K, Rey-Jurado E, Vial C, Poli MC, Licari A, Montagna D, Marseglia GL, Licciardi F, Ramenghi U, Discepolo V, Lo Vecchio A, Guarino A, Eisenstein EM, Imberti L, Sottini A, Biondi A, Mató S, Gerstbacher D, Truong M, Stack MA, Magliocco M, Bosticardo M, Kawai T, Danielson JJ, Hulett T, Askenazi M, Hu S; NIAID Immune Response to COVID Group; Chile MIS-C Group; Pavia Pediatric COVID-19 Group; Cohen JI, Su HC, Kuhns DB, Lionakis MS, Snyder TM, Holland SM, Goldbach-Mansky R, Tsang JS, Notarangelo LD. Immunopathological signatures in multisystem inflammatory syndrome in children and pediatric COVID-19. Nat Med 2022;28:1050-1062. Epub 2022 Feb 17
7. Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. Inflamm Res 2014;63:803-819. Epub 2014 Jul 22
8. Hewison M. Antibacterial effects of vitamin D. Nat Rev Endocrinol 2011;7:337-345.
9. Boltz-Nitulescu G, Willheim M, Spittler A, Leutmezer F, Tempfer C, Winkler S. Modulation of IgA, IgE, and IgG Fc receptor expression on human mononuclear phagocytes by 1 alpha,25-dihydroxyvitamin D3 and cytokines. J Leukoc Biol 1995;58:256-262.
10. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zügel U, Gallo RL, Eisenberg D, Hewison M, Hollis BW, Adams JS, Bloom BR, Modlin RL. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 2006;311:1770-1173. Epub 2006 Feb 23
11. Lemire JM, Adams JS, Kermani-Arab V, Bakke AC, Sakai R, Jordan SC. 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. J Immunol 1985;134:3032-3035.
12. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. Immunol 2007;179:2060-2063.
13. De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 2000;192:1069-1074.
14. Dumache R, Enache A, Cut T, Paul C, Mihailescu A, Ionescu A, Novacescu D, Marinescu A, Ciocan V, Muresan C, Voicu A. Deficiency of Vitamin D, a Major Risk Factor for SARS-CoV-2 Severity. Clin Lab 2022:68.
15. Aglipay M, Birken CS, Parkin PC, Loeb MB, Thorpe K, Chen Y, Laupacis A, Mamdani M, Macarthur C, Hoch JS, Mazzulli T, Maguire JL; TARGet Kids! Collaboration. Effect of High-Dose vs Standard-Dose Wintertime Vitamin D Supplementation on Viral Upper Respiratory Tract Infections in Young Healthy Children. JAMA 2017;318:245-254.
16. World Health Organization (WHO). Clinical management of COVID-19. Last Accessed Date: 27 May 2020. Available from: https://apps.who.int/iris/rest/bitstreams/1278777/retrieve
17. Munns CF, Shaw N, Kiely M, Specker BL, Thacher TD, Ozono K, Michigami T, Tiosano D, Mughal MZ, Mäkitie O, Ramos-Abad L, Ward L, DiMeglio LA, Atapattu N, Cassinelli H, Braegger C, Pettifor JM, Seth A, Idris HW, Bhatia V, Fu J, Goldberg G, Sävendahl L, Khadgawat R, Pludowski P, Maddock J, Hyppönen E, Oduwole A, Frew E, Aguiar M, Tulchinsky T, Butler G, Högler W. Global Consensus Recommendations on Prevention and Management of Nutritional Rickets. J Clin Endocrinol Metab 2016;101:394-415. Epub 2016 Jan 8
18. Darren A, Osman M, Masilamani K, Habib Ali S, Kanthimathinathan HK, Chikermane A, Al-Abadi E, Welch SB, Hackett S, Scholefield BR, Uday S, Jyothish D. Vitamin D status of children with paediatric inflammatory multisystem syndrome temporally associated with severe acute respiratory syndrome coronavirus 2 (PIMS-TS). Br J Nutr 2022;127:896-903. Epub 2021 May 12
19. Zengin N, Bal A, Goren TA, Bayturan SS, Alkan F, Akcali S. Serum Vitamin D Levels in Relation to Development of Multisystem Inflammatory Syndrome in Pediatric COVID-19. J Pediatr Infect Dis 2022:308-316.
20. Torpoco Rivera D, Misra A, Sanil Y, Sabzghabaei N, Safa R, Garcia RU. Vitamin D and morbidity in children with Multisystem inflammatory syndrome related to Covid-19. Prog Pediatr Cardiol. Prog Pediatr Cardiol 2022;66:101507. Epub 2022 Mar 1

21. Mamishi S, Olfat M, Pourakbari B, Eshaghi H, Abdolsalehi MR, Shahbabaie MA, Jalali F, Safari F, Mahmoudi S. Multisystem inflammatory syndrome associated with SARS-CoV-2 infection in children: update and new insights from the second report of an Iranian referral hospital. Epidemiol Infect 2022;150:179.
22. Feketea G, Vlacha V, Bocsan IC, Vassilopoulou E, Stanciu LA, Zdrenghea M. Vitamin D in Corona Virus Disease 2019 (COVID-19) Related Multisystem Inflammatory Syndrome in Children (MIS-C). Front Immunol 2021;12:648546.
23. Peterson CA, Heffernan ME. Serum tumor necrosis factor-alpha concentrations are negatively correlated with serum 25(OH)D concentrations in healthy women. J Inflamm (Lond) 2008;5:10.
24. Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. Lancet Diabetes Endocrinol 2014;2:76-89. Epub 2013 Dec 6
25. Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, Goleva E. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J Immunol 2012;188:2127-2135. Epub 2012 Feb 1
26. Katz J. Increased risk for COVID-19 in patients with vitamin D deficiency. Nutrition 2021;90:111361. Epub 2021 May 30
27. De Smet D, De Smet K, Herroelen P, Gryspeerdt S, Martens GA. Serum 25(OH)D Level on Hospital Admission Associated With COVID-19 Stage and Mortality. Am J Clin Pathol 2021;155:381-388.
28. Ye K, Tang F, Liao X, Shaw BA, Deng M, Huang G, Qin Z, Peng X, Xiao H, Chen C, Liu X, Ning L, Wang B, Tang N, Li M, Xu F, Lin S, Yang J. Does Serum Vitamin D Level Affect COVID-19 Infection and Its Severity?-A Case-Control Study. J Am Coll Nutr 2021;40:724-731. Epub 2020 Oct 13
29. Alpcan A, Tursun S, Kandur Y. Vitamin D levels in children with COVID-19: a report from Turkey. Epidemiol Infect 2021;149:180.
30. Karakaya Molla G, Ünal Uzun Ö, Koç N, Özen Yeşil B, Bayhan Gİ. Evaluation of nutritional status in pediatric patients diagnosed with Covid-19 infection. Clin Nutr ESPEN 2021;44:424-428. Epub 2021 May 11

CASE REPORT

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Primary Thyroid Diffuse Large B-cell Lymphoma in a Child with Hashimoto's Thyroiditis: A Case Report

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What is already known on this topic?

Primary thyroid diffuse large B-cell lymphoma (DLBCL) is extremely rare in children and an uncommon malignancy in adults. Hashimoto thyroiditis (HT) is a risk factor for DLBCL. Core needle biopsy is usually required for diagnosis.

What this study adds?

DLBCL should be considered in the differential diagnosis of a thyroid mass in adolescents with a history of HT. Diagnosis is difficult. Chemotherapy and/or radiology seems to be the most effective treatment, even in children. Surgical removal of the thyroid gland is limited to cases where chemotherapy fails.

Abstract

Primary thyroid lymphoma (PTL) is a rare thyroid gland cancer, with diffuse large B-cell lymphomas (DLBCL) being extremely rare in children and adolescents. Thus, optimal therapy is debatable. We describe a rare case of thyroid DLBCL in an adolescent girl with a history of Hashimoto thyroiditis (HT), the difficulty in diagnosis and the outcome of treatment. A 12-year-old girl with a nine-year history of HT was admitted with a right-sided painless progressive swelling of the neck. Physical examination and imaging including ultrasound (US), computed tomography (CT) and positron emission tomography/CT revealed an enlarged thyroid gland with right side lymphadenopathy and no metastasis. Two fine needle aspirations were done showing suspected lymphoblastic lesions for non-Hodgkin lymphoma without precise diagnosis. US guided core needle biopsy was finally performed confirming the diagnosis of DLBCL. She was treated according to LMB 96-group B protocol with no surgical removal of thyroid. The patient responded very well to treatment and 14 months later there is no evidence of relapse or metastases. PTL is an extremely rare cause of thyroid malignancy in children. However, it should be considered in the differential diagnosis of a thyroid mass in adolescents presenting with a rapidly enlarging neck mass and a history of HT. It is a treatable condition with a good prognosis, even in aggressive histological subtypes, with no need for thyroidectomy.
Keywords: Primary thyroid lymphoma, diffuse large B-cell lymphoma, children

Introduction

Studies in adults have shown that primary thyroid lymphoma (PTL) accounts for <5% of thyroid malignancies and <2% of extranodal lymphomas, with an annual estimated incidence of 2 cases per 1 million. PTL is extremely rare in children, with only a few cases published (1). We describe a 12-year-old girl with Hashimoto thyroiditis (HT) and diffuse large B-cell lymphoma (DLBCL).

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Case Report

A 12-year-old girl was referred to our department with a painless right sided enlargement of the neck, which was evident three weeks prior to presentation. There was a progressive deterioration, with no other symptoms. An ultrasound (US) elastography of the thyroid gland performed at that time showed a 3 cm hypoechoic solid nodule, with mild lobulated borders. That mass was highly suspicious of non-Hodgkin lymphoma (NHL), based on the fine needle aspiration biopsy (FNA) result, which was performed a few days before her presentation to us. However, no definite diagnosis could be made.

The patient's past medical history was remarkable for HT and she had been under treatment with levothyroxine, since the age of two years (Table 1). There was a family history of thyroidopathy in her two older sisters. Her middle sister had HT since the age of 13 years and the eldest sister had thyroidectomy at the age of 23 years because of a thyroid nodule [classified according to the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC)- category II-benign].

Physical examination revealed an enlarged thyroid gland with a notable soft mass (3 cm x 3 cm) on the right side of the thyroid and ipsilateral cervical lymphadenopathy. After admission, laboratory tests were performed. Full blood count, lactate dehydrogenase, and renal and liver function tests were all normal (Table 1). Her thyroid function tests are also shown in Table 1.

Table 1. Demographic, clinical and biochemical data of the patient

Age (years)	$12^{9/12}$	
Sex	Female	
Race	White Caucasian	
Past medical history	Hashimoto thyroiditis since 2 years of age	
Medical treatment	Thyroxine (1.2 μg/kg/d)	
Height	155 cm (50^{th} percentile)	
Weight	49 kg (50^{th} percentile)	
Body mass index	19.38 kg/m^2 (25^{th}-50^{th} percentile)	
Tanner stage	5	
Blood tests		**Reference range**
Full blood count		
WBC	6200/μL (NE: 50.2%, LY: 41.1 %, EO: 1.3%)	4.5-13.0x10^3
Hb	**11.1 gr/dL**	11.5-15.5
HBC (MCV)	**34% (78.3 fl)**	35-45
Platelets	308000/μL	130-400x10^3
ESR	5 mm/h	< 10
Biochemistry		
Urea	19 mg/dL	5-45
Creatinine	0.6 mg/dL	0.5-1
CRP	0 mg/dL	< 5
SGOT	11 U/L	5-45
SGPT	7 U/L	5-45
γGT	6 U/L	< 26
Lactate dehydrogenase	188 U/L	< 300
Hormones		
TSH	0.603 μIU/mL	0.4-5
fT4	1.37 ng/dL	0.9-1.9
T3	1.270 ng/dL	0.83-2.13
T4	8.15 μg/dL	5.6-13.9
Tg	**69.94 ng/dL**	3.5-31.1
Anti-TPO	**278.7 IU/mL**	< 16
Anti-TG	**1287 IU/mL**	< 100
Calcitonin	2.2 pg/mL	< 10

CRP: C-reactive protein, WBC: white blood cell, Hb: hemoglobin, HBC: hemoglobin C, MCV: mean corpuscular volume, ESR: erythrocyte sedimentation rate, EO: eosinophil, NE: neutrophil, LY: lymphocyte, TSH: thyroid stimulating hormone

A more detailed US examination of the thyroid gland was performed and showed an increase in the size, with a notable solid hypoechoic nodule (3.40 x 2.93 x 4.62 cm), with two jagged edges, lobulated borders, calcifications and intranodular vascularization on the right side of the gland. Furthermore, two hypoechoic nodules, smaller in size and with well-defined borders, and without internal vascularization were present on the left side of the gland. Multiple cervical lymph nodes were also found bilaterally. However, US was still non-diagnostic. A second FNA was performed, which showed suspicious lymphoblastic lesions. Due to the difficulty of making the diagnosis, a subsequent US-guided core needle biopsy (CNB) was carried out and confirmed the diagnosis (Figure 1) by histological examination.

Histopathological examination showed destruction of thyroid follicles and diffuse growth of lymphocytes (Figure 2a, 2b). Immunohistochemistry was positive for CD20 (Figure 3) with co-expression of PAX-5 transcription factor, and was positive for CD5, CD23, CD30, cyclinD and moderately positive for CD3. These markers are key immunohistochemical features for distinguishing between DLBCL and mucosa-associated lymphoid tissue (MALT)-derived subtypes. Antibody testing against antibody/proteins showed CD10 $<30\%$, bcl-6 $<30\%$ and MUM-1/IRF4 $>30\%$. The Ki-67 index was 60%. Bcl-2 was positive in $>90\%$ of the cell population examined. Fluorescence *in-situ* hybridization analysis revealed no translocation of the *MYC*, *BCL-2*, *DUSP22* and *IRF4* genes, indicating good prognosis (2).

Staging of the lymphoma included computed tomography (CT) scan of the neck, chest and abdomen, positron emission tomography/CT (PET/CT), bone marrow aspiration and cerebral spinal fluid (CSF) analysis. The CT scan of the

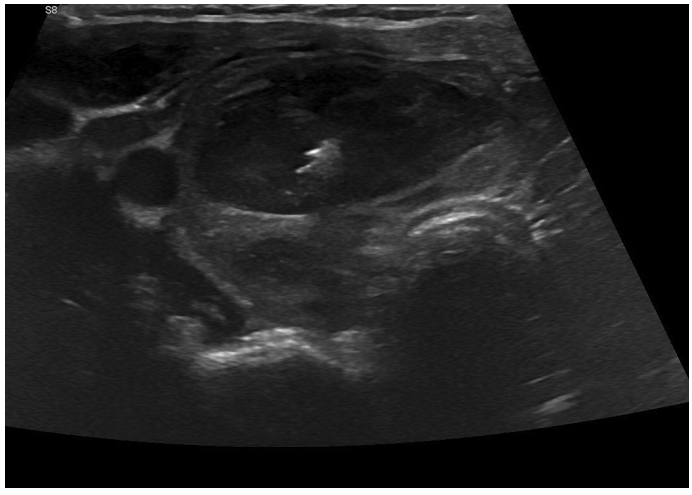

Figure 1. Ultrasound guided core needle biopsy

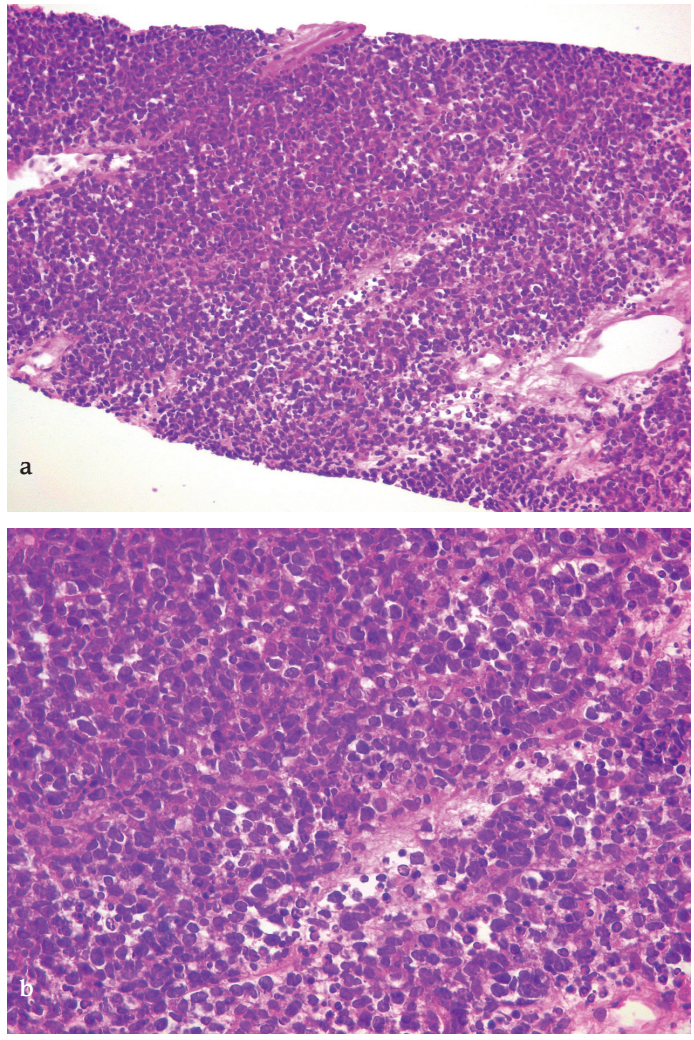

Figure 2. a, b) Histological examination with hematoxylin and eosin staining of thyroid follicles destruction and diffuse growth of lymphocytes

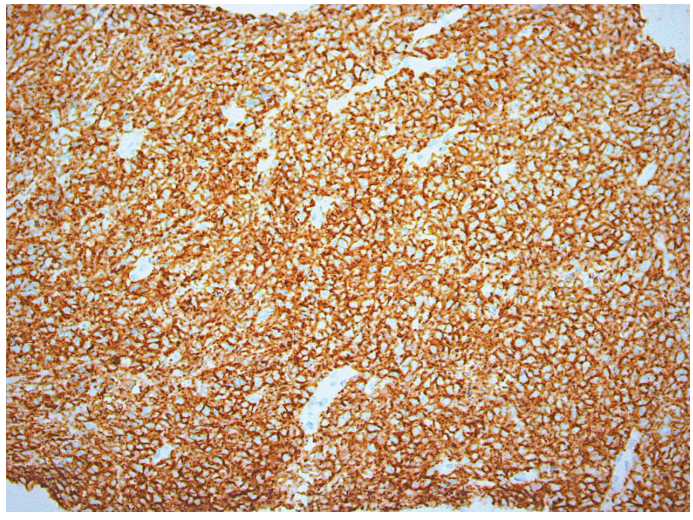

Figure 3. Positive immunohistochemistry stain for CD20

neck revealed a nodular alteration of 2.95 x 3.9 x 5.2 cm in the right thyroid gland and multiple lymph nodes in the neck bilaterally. PET/CT scan confirmed these findings with SUV_{max} = 16.5 and 2.5 in the right thyroid gland and lymph nodes, respectively. Chest and abdomen CT scans were normal. Flow cytometry, morphology and cytogenetic analysis did not show any evidence of bone marrow involvement and the CSF was negative for infiltration. The above findings indicated a categorization in the intermediate risk group.

Due to the rarity of the disease in children, the optimal therapy (thyroidectomy or chemotherapy) was debatable. It was finally decided to start chemotherapy only, according to the lymphomes malins B (LMB) 96-group B protocol. This protocol consists of initial induction chemotherapy with intravenous (IV) COP [cyclophosphamide (300 mg/m^2), vincristine (1 mg/m^2), and prednisolone (60 mg/m^2-7 days)] and intrathecal (IT) methotrexate (15 mg) and hydrocortisone (15 mg), followed by two courses of IV COPADM [doxorubicin 60 mg/m^2, methotrexate 3 g/m^2, cyclophosphamide 500 mg/m^2/day-5 days, vincristine (2 mg/m^2), and prednisolone (60 mg/m^2-5 days)], IT methotrexate (15 mg) with hydrocortisone (15 mg) and two courses of IV CYM (cytarabine 100 mg/m^2/day-5 days and methotrexate 3 g/m^2) and IT methotrexate (15 mg), hydrocortisone (15 mg) and cytrabine (30 mg).

The patient responded very well to treatment with a rapid decrease in the size of the thyroid mass after COP. An US of the thyroid gland performed after completion of COP revealed an 80% decrease in the size of the mass. A follow-up PET/CT scan after the first course of CYM showed that the tumor had totally disappeared. At the time of writing, she was disease-free, 14 months after end of treatment.

Discussion

PTL is an extremely rare malignancy in children (3), and represents only 1-5% of all malignancies of thyroid gland in adults (3). To the best of our knowledge, this is only the second case of a young adolescent with PTL reported in the literature (1).

A comparison of findings between adults and adolescents is depicted in Table 2.

Previous studies have shown that most patients were females aged 50-80 years (4,5,6). PTL presents with progressive swelling of the neck. Compressive symptoms (dyspnea, dysphagia, cough, hoarseness) may develop, as well as general symptoms, such as weight loss, night sweats and fever in 10% (7). The presented patient, however, had no such symptoms (Table 2). HT is a well-known risk factor for PTL with patients having a relative risk of 67-80 times compared to those without thyroiditis (8). The presented patient had a nine-year history of HT before the diagnosis of PTL. Various theories have been proposed to explain the association between HT and PTL. It has been suggested that chronic antigen stimulation of lymphocytes may lead to malignant differentiation (8). In a recent large scale-report of PTL, 154 out of 171 adult patients (90%) had HT diagnosed 1-362 months prior to the diagnosis of lymphoma (9).

Table 2. Comparison between adults and children (two cases reported in literature)

	Adults	Children
Incidence	2/10^6 per year	2 cases
F / M	Five times more common in F	1 F/1 M
Rapid painless enlargement	Yes	Both
Compressive symptoms	Common	None
Cervical lymphadenopathy	Common	None
Hashimoto thyroiditis	Common (67-80-fold risk)	One
Ultrasound findings:		
- Diffuse heterogenous hypoechoic parenchyma	Yes (25%)	Both
- Hypoechoic mass	Yes (67%)	Both
Final diagnosis:		
- FNA	Yes (50-60%)	One
- Core biopsy	In doubtful cases	One
Treatment		
- Chemotherapy and radiotherapy	Yes	Both only chemotherapy
- Surgery	No	None
Prognosis		
- Survival at 5 years	74%	Unknown (both are disease-free 2 years
- Survival at 10 years	71%	after diagnosis)

FNA: fine needle aspiration, M: male, F: female

Many previous studies agree that the most common subtype of PTL is DLBCL followed by MALT lymphoma mixed type. Histopathologically, it is very important to distinguish between the above-mentioned subtypes, as therapeutic management and prognosis are different. On immunohistochemical staining, CD5, CD10 and CD23 are negative in MALT cases and CD19, CD20 and CD45 are usually positive in DLBCL (10). Most DLBCL are Bcl-6 positive and almost half are Bcl-2 positive (11). The presented case was positive for Bc-2 and negative for *MYC*, *DUSP22* and *IRF4* translocations.

US is often the first line investigation in patients with thyroid enlargement and nodules but it is sometimes non diagnostic for PTL. In DLBCL, US usually shows homogenous and hypoechoic internal echoes, with indistinct borders between the lymphomatous and non-lymphomatous tissues. These findings, however, are also typical of severe chronic thyroiditis (12). In a retrospective study of 165 patients with US-suspected malignant thyroid lymphoma based on the US findings, 79 (47.9%) were confirmed as having lymphoma (12). The positive predictive value for diagnosis of diffuse type was reported to be lower, compared to nodular or mixed type (12).

US-guided FNA and CNB are the next steps for the diagnostic strategy. FNA is widely accepted as a diagnostic tool due to its simplicity, safety and its high sensitivity of 83-98% and specificity of 70-92% (13). However, FNA results may be non-diagnostic in 2-24% (14). CNB has been suggested as a complimentary method to FNA. CNB is safe, well-tolerated and reduces the possibility of inconclusive results, as a larger tissue sample is taken when performed by an expert. However, a recent meta-analysis by Li et al. (15) found that FNA and CNB don't differ significantly in sensitivity and specificity for the diagnosis of thyroid malignancy. In the presented case, two FNAs and a CNB were necessary to confirm the diagnosis.

Previously, open surgical biopsy was used to differentiate lymphoma from thyroiditis and carcinoma (16). However, recent advances in immunochemistry have improved the accuracy of FNA. In 119 patients with thyroid lymphoma, Matsuzuka et al. (16) showed that only 78.3% who underwent FNA without immunotyping were diagnosed correctly, while another 12% had borderline cytologic results. In another study, FNA results were highly suggestive of thyroid lymphoma in only five out of 17 (29.4%) (17). Based on such studies, many specialists recommended surgical intervention and open biopsy in all patients due to the perceived limited role of FNA in diagnosing thyroid lymphoma. More recent studies, however, have shown that FNA together with immunophenotyping improves the accuracy of the results. Therefore, CNB or surgical biopsies are now less often needed (18,19). The expertise of the physician performing the FNA, the amount of tissue taken and the pathologist's experience in interpreting FNA results are important for accurate diagnosis. Therefore, CNB or open biopsies (to obtain enough cells) are the most preferable techniques. CNB and surgical biopsy are comparable regarding the accuracy, but the latter is usually accompanied by trauma and possible complications (18,19).

Regarding the treatment, experience in children and adolescents is limited, since DLBCL is rare in this age group with sparce data on incidence and treatment. Therefore, the optimal approach remains controversial (20). For these reasons the treatment for pediatric/adolescent DLBCL is generally based on established treatment regimen for other extra-nodal NHLs (21). According to histology findings and cancer staging, chemotherapy, loco-regional radiotherapy and surgery may be combined for successful treatment. Surgery seems to play a limited role and is only really necessary in large tumors with compressive symptoms (21). Surgical biopsy and resection have been used for the diagnosis and therapeutic management with significant survival benefit (21). In the presented case, the patient commenced on chemotherapy based on staging.

Our patient responded very well to the chemotherapy protocol with rapid decrease in the thyroid mass. The role of surgical removal of thyroid remains questionable (16). It is not a first line treatment and is limited only either to cases that have failed to respond successfully to chemotherapy or to cases where CNB has failed to establish the precise diagnosis (20).

The intermediate risk disease group, B-cell-NHL (B-NHL), is the largest and most heterogeneous. In FAB/LMB studies 70% of patients can be classified as intermediate risk (group B) and have a 4-year event free survival of 90% (20).

Conclusion

A case of NHL which belonged to the DLBCLs is presented. This was a primary tumor in the thyroid gland, which is extremely rare in children and adolescents. The case responded very well to chemotherapy. NHLs should be considered in the differential diagnosis in children and adolescents presenting with rapidly increasing, hard, and painless mass in the neck, especially on a background of HT.

Ethics

Informed Consent: All authors comply with the guidelines for human studies and also comply with Ethics Guidelines.

The patient and her parents have given their written informed consent to publish this case.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Maria Xatzipsalti, Evangelos Bourousis, Maria Nikita, Dimitra Rontogianni, Myrsini. G. Gkeli, Dionisios Chrysis, Aristeidis Giannakopoulos, Dimitrios Delis, Margarita Baka, Andriani Vazeou, Concept: Maria Xatzipsalti, Evangelos Bourousis, Dionisios Chrysis, Aristeidis Giannakopoulos, Dimitrios Delis, Margarita Baka, Andriani Vazeou, Design: Maria Xatzipsalti, Evangelos Bourousis, Andriani Vazeou, Data Collection or Processing: Maria Xatzipsalti, Evangelos Bourousis, Dionisios Chrysis, Analysis or Interpretation: Maria Xatzipsalti, Evangelos Bourousis, Dimitra Rontogianni, Myrsini. G. Gkeli, Dionisios Chrysis, Aristeidis Giannakopoulos, Dimitrios Delis, Margarita Baka, Andriani Vazeou, Literature Search: Maria Xatzipsalti, Evangelos Bourousis, Maria Nikita, Andriani Vazeou, Writing: Maria Xatzipsalti, Evangelos Bourousis, Maria Nikita, Dimitra Rontogianni, Myrsini. G. Gkeli, Dionisios Chrysis, Aristeidis Giannakopoulos, Andriani Vazeou.

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References

1. Marwaha RK, Pritchard J. Primary thyroid lymphoma in childhood: treatment with chemotherapy alone. Pediatr Hematol Oncol 1990;7:383-388.
2. Aukema SM, Siebert R, Schuuring E, van Imhoff GW, Kluin-Nelemans HC, Boerma EJ, Kluin PM. Double-hit B-cell lymphomas. Blood 2011;117:2319-2331. Epub 2010 Nov 30
3. Graff-Baker A, Roman SA, Thomas DC, Udelsman R, Sosa JA. Prognosis of primary thyroid lymphoma: demographic, clinical, and pathologic predictors of survival in 1,408 cases. Surgery 2009;146:1105-1115.
4. Chai YJ, Hong JH, Koo do H, Yu HW, Lee JH, Kwon H, Kim SJ, Choi JY, Lee KE. Clinicopathological characteristics and treatment outcomes of 38 cases of primary thyroid lymphoma: a multicenter study. Ann Surg Treat Res 2015;89:295-299. Epub 2015 Nov 27
5. Derringer GA, Thompson LD, Frommelt RA, Bijwaard KE, Heffess CS, Abbondanzo SL. Malignant lymphoma of the thyroid gland: a clinicopathologic study of 108 cases. Am J Surg Pathol 2000;24:623-639.
6. Trovato M, Giuffrida G, Seminara A, Fogliani S, Cavallari V, Ruggeri RM, Campennì A. Coexistence of diffuse large B-cell lymphoma and papillary thyroid carcinoma in a patient affected by Hashimoto's thyroiditis. Arch Endocrinol Metab 2017;61:643-646.
7. Holm LE, Blomgren H, Löwhagen T. Cancer risks in patients with chronic lymphocytic thyroiditis. N Engl J Med 1985;312:601-604.
8. Kossev P, Livolsi V. Lymphoid lesions of the thyroid: review in light of the revised European-American lymphoma classification and upcoming World Health Organization classification. Thyroid 1999;9:1273-1280.
9. Watanabe N, Noh JY, Narimatsu H, Takeuchi K, Yamaguchi T, Kameyama K, Kobayashi K, Kami M, Kubo A, Kunii Y, Shimizu T, Mukasa K, Otsuka F, Miyara A, Minagawa A, Ito K, Ito K. Clinicopathological features of 171 cases of primary thyroid lymphoma: a long-term study involving 24553 patients with Hashimoto's disease. Br J Haematol 2011;153:236-243. Epub 2011 Mar 4
10. Dralle H, Musholt TJ, Schabram J, Steinmüller T, Frilling A, Simon D, Goretzki PE, Niederle B, Scheuba C, Clerici T, Hermann M, Kußmann J, Lorenz K, Nies C, Schabram P, Trupka A, Zielke A, Karges W, Luster M, Schmid KW, Vordermark D, Schmoll HJ, Mühlenberg R, Schober O, Rimmele H, Machens A; German Societies of General and Visceral Surgery; Endocrinology; Nuclear Medicine; Pathology; Radiooncology; Oncological Hematology; and the German Thyroid Cancer Patient Support Organization Ohne Schilddrüse leben e.V. German Association of Endocrine Surgeons practice guideline for the surgical management of malignant thyroid tumors. Langenbecks Arch Surg 2013;398:347-375. Epub 2013 Mar 3
11. Niitsu N, Okamoto M, Nakamura N, Nakamine H, Bessho M, Hirano M. Clinicopathologic correlations of stage IE/IIE primary thyroid diffuse large B-cell lymphoma. Ann Oncol 2007;18:1203-1208. Epub 2007 Apr 11
12. Ota H, Ito Y, Matsuzuka F, Kuma S, Fukata S, Morita S, Kobayashi K, Nakamura Y, Kakudo K, Amino N, Miyauchi A. Usefulness of ultrasonography for diagnosis of malignant lymphoma of the thyroid. Thyroid 2006;16:983-987.
13. American Thyroid Association (ATA) Guidelines Taskforce on Thyroid Nodules and Differentiated Thyroid Cancer; Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, Mazzaferri EL, McIver B, Pacini F, Schlumberger M, Sherman SI, Steward DL, Tuttle RM. Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid. 2009;19:1167-1214.
14. Yoon JH, Moon HJ, Kim EK, Kwak JY. Inadequate cytology in thyroid nodules: should we repeat aspiration or follow-up? Ann Surg Oncol 2011;18:1282-1289. Epub 2011 Feb 19
15. Li L, Chen BD, Zhu HF, Wu S, Wei D, Zhang JQ, Yu L. Comparison of pre-operation diagnosis of thyroid cancer with fine needle aspiration and core-needle biopsy: a meta-analysis. Asian Pac J Cancer Prev 2014;15:7187-7193.
16. Matsuzuka F, Miyauchi A, Katayama S, Narabayashi I, Ikeda H, Kuma K, Sugawara M. Clinical aspects of primary thyroid lymphoma: diagnosis and treatment based on our experience of 119 cases. Thyroid 1993;3:93-99.
17. Sarinah B, Hisham AN. Primary lymphoma of the thyroid: diagnostic and therapeutic considerations. Asian J Surg 2010;33:20-24.
18. Cha C, Chen H, Westra WH, Udelsman R. Primary thyroid lymphoma: can the diagnosis be made solely by fine-needle aspiration? Ann Surg Oncol 2002;9:298-302.
19. Sangalli G, Serio G, Zampatti C, Lomuscio G, Colombo L. Fine needle aspiration cytology of primary lymphoma of the thyroid: a report of 17 cases. Cytopathology 2001;12:257-263.
20. Egan G, Goldman S, Alexander S. Mature B-NHL in children, adolescents and young adults: current therapeutic approach and emerging treatment strategies. Br J Haematol 2019;185:1071-1085.
21. Stein SA, Wartofsky L. Primary thyroid lymphoma: a clinical review. J Clin Endocrinol Metab 2013;98:3131-3138. Epub 2013 May 28

CASE REPORT

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Prolyl Endopeptidase-like Deficiency Associated with Growth Hormone Deficiency

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What is already known on this topic?

Prolyl endopeptidase-like (PREPL) deficiency (MIM#616224) is a rare congenital disorder characterized by neonatal hypotonia and feeding difficulties, growth hormone (GH) deficiency and hypergonadotropic hypogonadism. This syndrome is an autosomal recessive disease resulting from mutations in the *PREPL* gene (MIM#609557).

What this study adds?

This report describes a novel previously undescribed mutation in *PREPL*. We also describe a typical presentation of the syndrome, with early growth impairment in infancy due to GH deficiency and a good response to GH treatment. The description of new patients with PREPL deficiency syndrome is essential to better delineate the phenotypic and genotypic spectrum of the disease.

Abstract

Prolyl endopeptidase-like (PREPL) deficiency (MIM#616224) is a rare congenital disorder characterised by neonatal hypotonia and feeding difficulties, growth hormone (GH) deficiency and hypergonadotropic hypogonadism. This syndrome is an autosomal recessive disease resulting from mutations in the *PREPL* gene (MIM#609557). Herein we report a 7-year-old female patient with biallelic mutations in *PREPL* (c.1528C > T in one allele and whole gene deletion in the other) with early growth impairment in infancy. GH deficiency was confirmed at 20 months of life. Recombinant GH treatment was introduced with a good response. Her clinical features were similar to those of previously reported cases. The description of new patients with PREPL deficiency syndrome is essential to better delineate the phenotypic and genotypic spectrum of the disease.

Keywords: Prolyl endopeptidase-like, growth hormone deficiency, genetics

Introduction

The prolyl endopeptidase-like gene (*PREPL*) is ~43 kb long, located in 2p21 and encodes the PREPL protein which is a cytoplasmatic serine hydrolase belonging structurally to an oligopeptidase family (1). Historically PREPL deficiency was described as part of a recessive contiguous gene deletion syndrome involving *PREPL* and *SLC3A1*, known as hypotonia cystinuria syndrome (HCS). While cystinuria in HCS is caused by SLC3A1 deficiency, the other symptoms (neonatal hypotonia, growth impairment and cognitive problems) arise from PREPL deficiency (2). This second isolated PREPL deficiency is also known as congenital myasthenic syndrome 22 (MIM#616224).

To date, only fourteen mutations have been described in the *PREPL* gene that are associated with HCS or congenital myasthenic syndrome (3). Here we report a female child with isolated PREPL deficiency, with a single nucleotide

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variant (c.1528 C > T) in *PREPL* and a 0.031Mb deletion in 2p21 (including *PREPL* only).

Most of the available literature about PREPL deficiency focuses on neurological symptoms. In this report, we outline the hormonal disorders associated with this syndrome.

Case Report

The proband is a 7-year-old female, born to nonconsanguineous caucasian healthy parents, with a healthy younger brother. Pregnancy was appropriately monitored and without major medical problems, teratogenic exposures or hospitalizations. The patient was born by C-section for breech presentation at 39 weeks of gestation. At birth, her weight was 2.855 g [-0.9 standard deviation (SD)], length 47 cm (-1.5 SD) and cranial perimeter 34.5 cm (+0.2 SD). Dysmorphic features were noted at birth, including broad nasal root, microretrognatia, mild thenar hypoplasia and bilateral 5th finger clinodactyly. She presented with neonatal hypotonia and was poorly reactive to stimulus. She suffered from neonatal hypoglycaemia due to feeding problems, so required a nasogastric tube for nutrition for the first month after birth. Among the diagnostic possibilities for the neonatal hypotonia, infection, cardiopathy and toxics were excluded. Additionally, she had a normal metabolic workup, no cystinuria and a normal electrocardiogram and brain magnetic resonance imaging (MRI) at birth. Electromyography at four months of age was also normal. Muscle biopsy was not performed, but levels of creatine-kinase were normal. Thyroid hormones were also normal. Thus, having ruled out other causes, array-CGH and also MLPA for Prader-Willi syndrome were performed with neither revealing any alterations.

Neurological evaluation at five months revealed persistence of global hypotonia with axial dominance, with apparent improvement over time. She acquired autonomous standing at one year of life and began autonomous ambulation at 18 months. She needed motor rehabilitation and stimulation in a specialized center.

Stunted growth became evident with a height of 72 cm (-3.8 SD) at 20 months of life. The serum insulin-like growth factor (IGF-1) level was low (27 ng/mL), as was the binding protein, IGFBP-3 level (1.78 mg/L). Pharmacological testing with glucagon showed no response, with the highest peak of growth hormone (GH) at the start (3.22 ng/mL) and only 0.48 ng/mL at 60 minutes. Additionally, she had a delayed bone age (9 months at a chronological age of 14 months). Celiac disease and hypothyroidism were excluded as part of the work-up for GH deficiency and a hypothalamic-hypophyseal MRI did not reveal any alteration. Being diagnosed with GH deficiency, substitutive treatment was started at an age of 2 years and 8 months. She rapidly responded to GH treatment, significantly increasing growth velocity from 7 cm/year to 11 cm/year.

Currently, at 7 years and 5 months old, she is still under GH treatment with a good response (Figure 1), with a weight of 18 kg (-1.75 SD), a height of 116.5 cm (-1.8 SD) and a prepuberal Tanner staging (P1S1). Her bone-age is still younger than her chronological age. She eats all kinds of food in small quantities without dysphagia. On physical examination, she only presents left ptosis associated with fatigue, no hypomimia, and a normal axial tone. Dysmorphologic evaluation shows epicanthus, mandibular retrognathia, ogival palate, a notch in the earlobe and mild clinodactyly of the 5th finger with small but proportionate feet and hands. Ligament laxity is also evident. She has a nasal voice. Social development and educational attainment

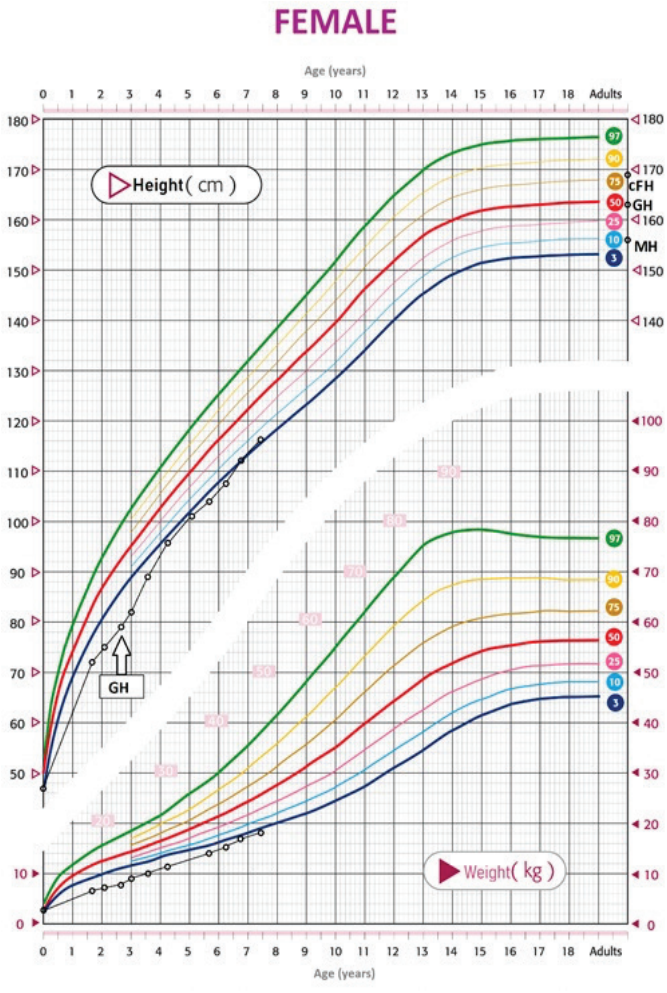

Figure 1. Growth chart of our patient. The start of somatotropin treatment is indicated (GH), with a subsequent good response

GH: growth hormone

are normal. Her motor exam revealed that the patient has a normal muscular axial tone and correctly aligned rachis, with normal osteotendinous reflexes and tendency to toe walking.

To identify the genetic condition of this patient, we first performed a next-generation sequencing study including genes *IGF2, IGF1R, IGF1, NPR2, GH1, GHR, GHRHR, IGFALS, STAT5B, CCDC8* and *GHSR* without revealing any pathogenic variant. Furthermore, methylation analysis of the Silver-Russell syndrome region was also normal. Therefore, whole-exome sequencing was carried out after obtaining informed consent from the patient's family. We identified an apparently homozygous variant in *PREPL* c.1528C > T, recognized as pathogenic in VarSome (4). The progenitor direct genetic study revealed that this was from paternal inheritance. Although the explanation for this apparently homozygous state could be an isodisomy, given that deletion of *PREPL* has been frequently described, an array-CGH (with exonic coverage of *PREPL*) was performed and it showed a 0.031Mb deletion in the 2p21 chromosome region (including the *PREPL* gene), classified as pathogenic with a recessive inheritance. The deletion was inherited from the mother. Therefore, the PREPL deficiency in the patient was due to a point mutation in one allele and a whole gene deletion in the other.

Discussion

Hypotonia in early infancy may be a sign of a central nervous disorder, a primary neuromuscular disorder or a genetic syndrome associated with hypotonia. However these signs most frequently occur as a consequence of common neonatal conditions, such as congenital infections, hypothyroidism or drug toxicity. In the presented case, these more common conditions were excluded, so genetic syndromes were considered.

HCS has been described as a disorder with cystinuria and congenital myasthenia resulting from the recessive deletions in *SLC3A1* and *PREPL* (2,5,6,7,8). To date, only 11 patients (2,3,5,6,8,9) with isolated PREPL deficiency have been reported. Isolated PREPL deficiency causes an autosomal recessive inherited congenital myasthenic syndrome characterized by severe neonatal hypotonia that improves spontaneously with age, and endocrinology problems, such as GH hormone deficiency and hypergonadotropic hypogonadism. In late childhood (6-11 years) obesity can appear due to hyperphagia. Patients also show mild facial dysmorphism (9).

In this case, we found a novel heterozygous variant in c.1528C > T p.(Arg510Ter) in one allele associated with a whole gene deletion of 0.031Mb in 2p21 in the other. Further analysis showed that the novel mutation c.1528C > T p.(Arg510Ter), inherited from the father, results in a change of an arginine to a premature-stop-codon, resulting in a truncated protein or the absence of it, thus leading to a loss of function. This variant has not been identified previously in the public databases consulted (1000 genomes, exome variant server, exome aggregation consortium). The other variant, which was maternally inherited, was a 0.031Mb deletion in 2p21, implying a *PREPL* gene abnormality (Figure 2).

Patients with PREPL deficiency often present with growth deficiency and GH therapy has a positive effect in the cohort of cases that exhibit GH deficiency (2,5,6). In the presented case, the patient has received treatment with GH with a good response. However, the mechanism of GH deficiency associated with PREPL deficiency is unknown.

The *PREPL* gene is located in 2p21 and encodes the cytoplasmic PREPL protein which is ubiquitously expressed, with highest levels in brain, kidney, and skeletal muscle, in descending order (10). *PREPL* encodes a putative serine peptidase from the prolyl peptidase family (11). Prolyl peptidases cleave peptides on the C-terminal side of proline residues, modulating the levels of different peptides and hormones. Nonetheless, substrates for PREPL have not yet been identified and its exact cellular function remains unknown (1). Some clues might be found in the literature based on the function of its homologues PREP (prolyl oligopeptidase) and OpdB (oligopeptidase B) which suggest a proteolytic activity can be expected of PREPL. However, PREPL seems to have important cellular and physiological effects besides peptide cleavage, such as a role in growth cone development, acting as a binding partner of tubulin and influencing protein secretion, which are primarily due to protein-protein interactions (12).

Prolyl peptidases have the potential to participate in a wide range of cellular regulatory processes, as their substrates are involved in regulating different signalling pathways (13). Based on the clinical observation that patients with isolated PREPL deficiency exhibit GH deficiency, it has been hypothesized that PREPL might be involved in the secretion and/or processing of peptide hormones. It is possible that PREPL plays a role in signalling pathways, leading to, for instance, GH secretion. In addition, normal downstream signalling of the GH receptor is apparent from the reported good response of these patients to GH administration.

Patients with PREPL deficiency often develop obesity due to hyperphagia in late childhood but at the time of writing at an age of 7 years and 5 months, our patient has low intake and

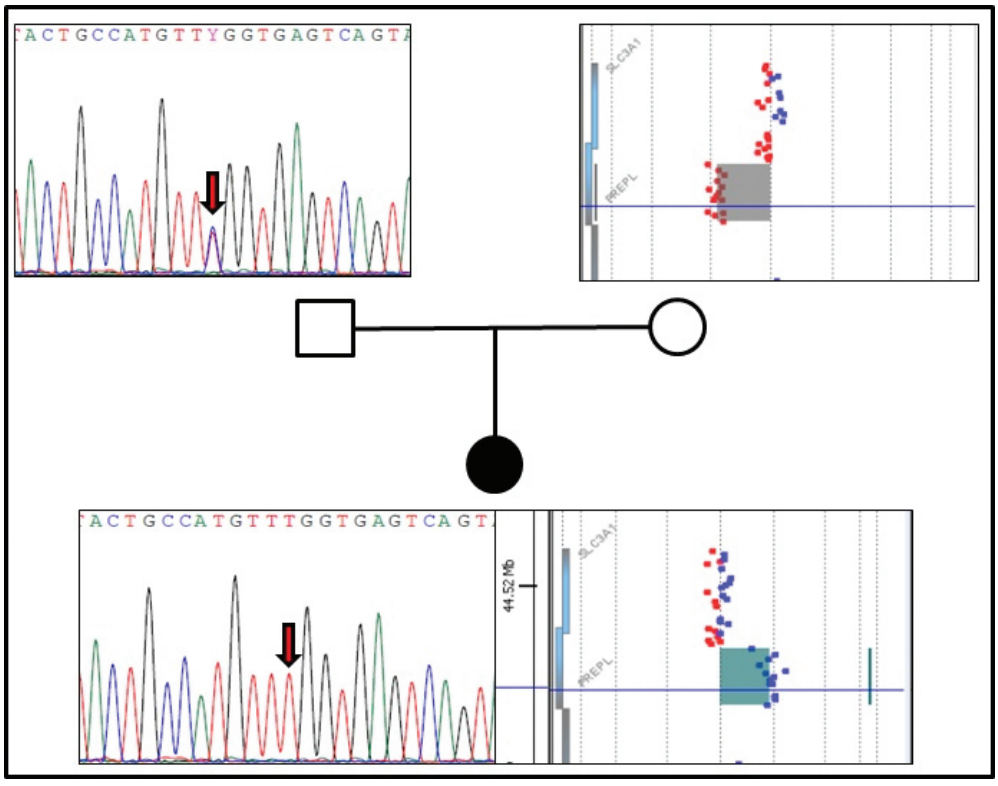

Figure 2. Study of the genetic condition. Whole-exome sequencing identified an apparent homozygous variant in *PREPL* c.1528C > T from paternal inheritance. The array-CGH (with exonic coverage of *PREPL*) showed a 0.031Mb deletion in 2p21 chromosome (including *PREPL* gene) inherited from the mother. In the array-CGH, the DNA from the patient is signalled with CY3 red, whereas the DNA from the mother is signalled with CY5 blue

PREPL: prolyl endopeptidase-like gene

a normal body mass index (13.2 kg/m^2; eighth percentile, -1.5 SD). Although hypergonadotropic hypogonadism has been observed in some patients with isolated PREPL deficiency (2), sexual hormones have not yet been tested in the proband because she has not reached a puberal age.

Previous studies (3,14) described moderate intellectual disability (ID) in PREPL deficient patients. Silva et al. (10) observed that biallelic *PREPL* mutations alone (without involvement of other genes) can cause ID. Besides the motor delay present in early infancy, the presented patient does not have developmental delay and has only needed some logopaedic therapy for diction problems. She also had the common phenotype associated with PREPL deficiency, including neonatal hypotonia and feeding problems during the first months after birth.

Conclusion

Further follow-up of this patient is needed to report longer term outcomes and evaluate the response to GH treatment including the final height attained in adulthood.

Ethics

Informed Consent: Consent form was filled out by the patient's family.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Laura Sayol-Torres, Maria Irene Valenzuela, Maria Clemente, Diego Yeste, Concept: Laura Sayol-Torres, Maria Irene Valenzuela, Rosangela Tomasini, Paula Fernández-Alvarez, Maria Clemente, Diego Yeste, Design: Maria Irene Valenzuela, Maria Clemente, Diego Yeste, Data Collection or Processing: Maria Clemente, Diego Yeste, Analysis or Interpretation: Maria Clemente, Diego Yeste, Literature Search: Maria Clemente, Diego Yeste, Writing: Laura Sayol-Torres, Maria Irene Valenzuela, Maria Clemente, Diego Yeste.

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References

1. Szeltner Z, Alshafee I, Juhász T, Parvari R, Polgár L. The PREPL A protein, a new member of the prolyl oligopeptidase family, lacking catalytic activity. Cell Mol Life Sci 2005;62:2376-2381.
2. Régal L, Mårtensson E, Maystadt I, Voermans N, Lederer D, Burlina A, Juan Fita MJ, Hoogeboom AJM, Olsson Engman M, Hollemans T, Schouten M, Meulemans S, Jonson T, François I, Gil Ortega D, Kamsteeg EJ, Creemers JWM. PREPL deficiency: delineation of the phenotype and development of a functional blood assay. Genet Med 2018;20:109-118. Epub 2017 Jul 20
3. Yang Q, Hua R, Qian J, Yi S, Shen F, Zhang Q, Li M, Yi S, Luo J, Fan X. PREPL Deficiency: A Homozygous Splice Site PREPL Mutation in a Patient With Congenital Myasthenic Syndrome and Absence of Ovaries and Hypoplasia of Uterus. Front Genet 2020;11:198.
4. Kopanos C, Tsiolkas V, Kouris A, Chapple CE, Albarca Aguilera M, Meyer R, Massouras A. VarSome: the human genomic variant search engine. Bioinformatics 2019;35:1978-1980.
5. Jaeken J, Martens K, Francois I, Eyskens F, Lecointre C, Derua R, Meulemans S, Slootstra JW, Waelkens E, de Zegher F, Creemers JW, Matthijs G. Deletion of PREPL, a gene encoding a putative serine oligopeptidase, in patients with hypotonia-cystinuria syndrome. Am J Hum Genet 2006;78:38-51. Epub 2005 Nov 23
6. Régal L, Shen XM, Selcen D, Verhille C, Meulemans S, Creemers JW, Engel AG. PREPL deficiency with or without cystinuria causes a novel myasthenic syndrome. Neurology 2014;82:1254-1260. Epub 2014 Mar 7
7. Legati A, Reyes A, Nasca A, Invernizzi F, Lamantea E, Tiranti V, Garavaglia B, Lamperti C, Ardissone A, Moroni I, Robinson A, Ghezzi D, Zeviani M. New genes and pathomechanisms in mitochondrial disorders unraveled by NGS technologies. Biochim Biophys Acta 2016;1857:1326-1335. Epub 2016 Mar 8
8. Tucker EJ, Jaillard S, Grover SR, van den Bergen J, Robevska G, Bell KM, Sadedin S, Hanna C, Dulon J, Touraine P, Sinclair AH. TP63-truncating variants cause isolated premature ovarian insufficiency. Hum Mutat 2019;40:886-892. Epub 2019 Mar 29
9. Laugwitz L, Redler S, Buchert R, Sturm M, Zeile I, Schara U, Wieczorek D, Haack T, Distelmaier F. Isolated PREPL deficiency associated with congenital myasthenic syndrome-22. Klin Padiatr 2018;230:281-283. Epub 2018 Jun 18
10. Silva S, Miyake N, Tapia C, Matsumoto N. The second point mutation in PREPL: a case report and literature review. J Hum Genet 2018;63:677-681. Epub 2018 Feb 26
11. Martens K, Derua R, Meulemans S, Waelkens E, Jaeken J, Matthijs G, Creemers JW. PREPL: a putative novel oligopeptidase propelled into the limelight. Biol Chem 2006;387:879-883.
12. Bartholdi D, Asadollahi R, Oneda B, Schmitt-Mechelke T, Tonella P, Baumer A, Rauch A. Further delineation of genotype-phenotype correlation in homozygous 2p21 deletion syndromes: first description of patients without cystinuria. Am J Med Genet A 2013;161:1853-1859. Epub 2013 Jun 21
13. Boonen K, Régal L, Jaeken J, Creemers JW. PREPL, a prolyl endopeptidase-like enzyme by name only?--Lessons from patients. CNS Neurol Disord Drug Targets 2011;10:355-360.
14. García-Horsman JA, Männistö PT, Venäläinen JI. On the role of prolyl oligopeptidase in health and disease. Neuropeptides 2007;41:1-24. Epub 2006 Dec 29

CASE REPORT

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A Potentially Fatal Outcome of Oral Contraceptive Therapy: Estrogen-Triggered Hereditary Angioedema in an Adolescent

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What is already known on this topic?

Factor 12-related hereditary angioedema is an autosomal dominant disease with incomplete penetrance. Angioedema attacks in this syndrome are known to occur more frequently with higher estrogen levels. Polycystic ovary syndrome (PCOS) is a relatively common disorder and combined oral contraceptives (OCs) which contain estrogen and progesterone are considered first-line drugs in adolescents with PCOS, for control of symptoms due to hyperandrogenism.

What this study adds?

To the best of our knowledge, this case is the first pediatric case of hereditary angioedema due to factor 12 mutation that is induced by estradiol-containing OC in the literature.

Abstract

Hereditary angioedema (HAE) is characterized by recurrent angioedema attacks with no urticaria. This disease has a high mortality due to asphyxia. Level of complement component 4 (C4), C1 esterase inhibitor (C1-INH) level and function, and genetic mutations determine different endotypes of HAE. Clinical presentation and the triggers of vasogenic edema may change according to the endotypes. An adolescent girl with oligomenorrhea, obesity, hirsutism, and acanthosis nigricans was diagnosed with polycystic ovary syndrome and prescribed ethinyl estradiol and cyproterone acetate containing oral contraceptive (OC). On the sixteenth day of treatment, she developed angioedema of the face, neck, and chest leading to dyspnea. Adrenaline, antihistamine, and corticosteroid treatments were ineffective. In the family history, the patient's mother and two cousins had a history of angioedema. C1-INH concentrate was administered with a diagnosis of HAE. C4 and C1-INH level and activity were normal. Genetic analysis identified a mutation in the factor 12 (*F12*) gene, and the diagnosis of F12-related HAE was made. OC treatment was discontinued. She has had no additional angioedema attacks in the follow-up period of two years. OC containing estrogen may induce the life-threatening first attack of F12-related HAE even in children. Recurring angioedema attacks in the family should be asked before prescribing estrogen-containing OC pills.

Keywords: Hereditary angioedema type 3, hereditary angioedema, angioedema, factor 12, polycystic ovary syndrome

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Introduction

Hereditary angioedema (HAE) is a genetic disorder that presents with an abrupt swelling caused by bradykinin-related vasogenic edema. The mechanism of HAE is non-inflammatory and non-allergic but involves increased production of bradykinin. Until now, five different genes have been identified to cause HAE which are serine protease inhibitor G1 (*SERPING1*), factor 12 (*F12*), plasminogen (*PLG*), angiopoietin (*ANGPT1*), and kininogen (*KNG1*) (1,2,3). Different types of HAE consist of low C1 inhibitor (C1-INH) activity caused by low production or loss of function of C1-INH due to *SERPING1* gene mutations, or normal level and activity of C1-INH (HAEnCI) due to mutations of *F12, PLG, ANGPT1*, and *KNG1* genes. HAEnCI presentation is similar to the other forms of HAE and characterized by recurring attacks of angioedema without urticaria that can be fatal due to laryngeal swellings. In this form of the disease, the most common defective plasma protein is FXII (3,4). Factor 12 is a critical molecule where pathways of coagulation, complement activation, contact reaction, and fibrinolysis meet. The promoter region of the *F12* gene carries an estrogen-responsive element and there are multiple case reports that associate high levels of estrogen and HAE attacks (5,6,7). This manuscript describes a patient presenting with her first angioedema attack after initiation of oral contraceptive (OC) treatment for polycystic ovary syndrome (PCOS) who was subsequently diagnosed with HAEnCI with *F12* mutation.

Case Report

A thirteen-year-old girl presented to the emergency department with dyspnea and swelling in her upper body. Her medical history was notable for PCOS which was diagnosed two weeks earlier in another pediatric endocrinology clinic. She was reported to have irregular menstruation and hirsutism and diagnosed with PCOS with high androgen levels (Table 1) and polycystic ovary morphology on pelvic ultrasound. Ethinyl estradiol and cyproterone acetate-containing OC pill was initiated. On the sixteenth day of treatment, she developed periorbital swelling which spread over the face, the neck, and the upper body in a matter of hours (Figure 1). With the initial diagnosis of anaphylaxis, epinephrine, antihistamines, and steroids were administered but her swellings were unresponsive. Her family history was positive for recurring angioedema attacks in her mother and two cousins. Therefore, she was given 500 IU of C1 esterase inhibitor concentrate with a pre-diagnosis of HAE. She was hospitalized in the Koç University, Pediatric Intensive Care Unit due to the possible risk of laryngeal edema. During her stay in pediatric intensive care, two more doses of 500 IU of C1 esterase inhibitor concentrate were given. The swellings began to recede after 12 hours and they had completely waned within 48 hours. The physical examination was also remarkable for obesity with a body mass index of 29.7 kg/m^2 (> 99th percentile, +2.51 standard deviation score), acanthosis nigricans on the neck, and severe hirsutism with a Ferriman-Gallwey score of 25.

Her laboratory workup showed complement 4 levels of 31 mg/dL (normal range: 10-40 mg/dL) and normal plasma levels (0.31 g/L, normal range: 0.21-0.39 g/L) and activity of C1 esterase inhibitor (107.7%, normal range: 70-130%) which indicated a diagnosis of HAEnCI. Genetic analysis of *F12* gene revealed a heterozygous mutation in the ninth exon, C>A variant which resulted in p.Thr328Lys variation.

Table 1. Laboratory tests showing serum hormone levels

	Value	Normal range
1.4-androstenedion (ng/mL)	2.38	0.24-1.73
Testosterone (ng/mL)	0.24	0.24-1.67
Sex hormone binding globulin (nmol/L)	10.6	11-120
17-alpha hydroxyprogesterone (ng/dL)	107.0	13-185
Fasting blood glucose (mg/dL)	91.0	60-100
Fasting insulin (µU/mL)	66.8	2.6-25

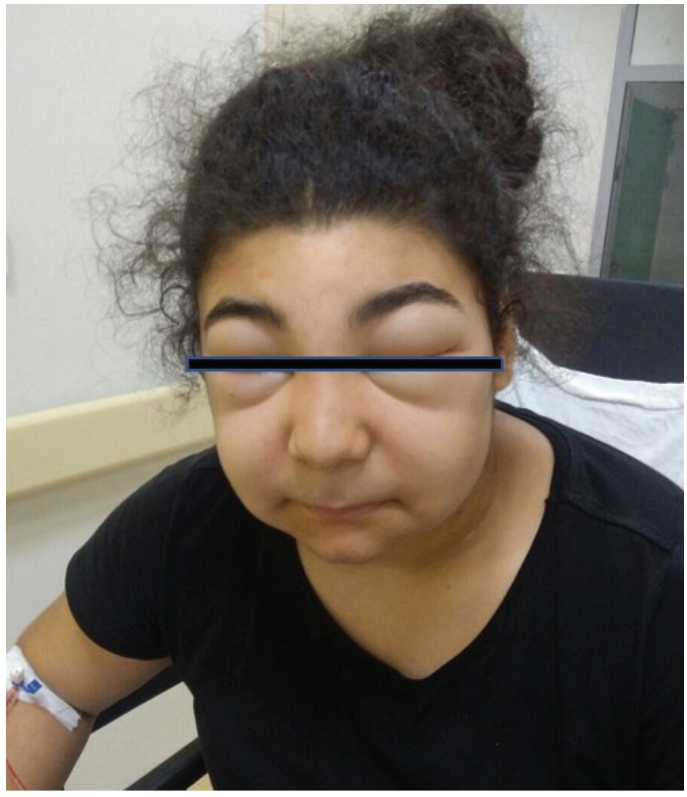

Figure 1. The face of the patient at admission

The OC pill was discontinued and life-style interventions were recommended. No attack occurred during her follow-up over 20 months.

Discussion

We report a female adolescent with a diagnosis of PCOS in another clinic who presented with an angioedema attack after OC medication was initiated. The final diagnosis of HAEnCI was made after genetic analysis of the *F12* gene showed a heterozygous mutation. *F12*-related HAEnCI is an autosomal dominant condition that shows incomplete penetrance. In most cases, the first symptoms appear before the third decade. Published series of HAE patients report a clear female predominance (8). in addition, women are more likely to be symptomatic than men. Hormonal factors play an important role in the worsening of the condition in women. There are differences in the overall frequency of angioedema symptoms depending on different female life stages (childhood, adolescence, menstruation, pregnancy, and menopause). It has also been reported that administration of estrogen, not progestin, in women with HAE may lead to the emergence or worsening of angioedema symptoms (7). A case series conducted in 61 women with *F12*-related HAEnCI showed that 95% of the women presented with at least one angioedema attack during periods of high estrogen exposure (OC pill, hormone replacement therapy, or pregnancy) (4). Estrogen as a trigger for HAE attacks could not be explained by a single mechanism. However, the limited literature indicates that the main culprit could be the estrogen-responsive element on the 5' flank of the *F12* gene (5,6). Another possible effect of estrogens in the pathogenesis of HAE is that estrogen-containing medications can decrease angiotensin-converting enzyme, which is also a protein responsible for degradation of bradykinin. Once angiotensin-converting enzyme activity decreased, accumulation of bradykinin could occur (7). Although some authors proposed that PCOS might have a protective role regarding HAE attacks due to increased levels of androgen and more stable levels of estradiol in PCOS patients (7), compelling evidence showing the protective effect of hyperandrogenism is lacking. The frequent co-occurrence of PCOS and HAE may suggest a link between the neuroendocrine and immune system, consisting of the presence of a pathology related with hypothalamic-pituitary dysregulation and an immunological disorder (6). However, the relationship between these two disorders needs to be clarified.

The present case also reminds us of the challenges associated with the diagnosis of PCOS in adolescence. Diagnostic criteria for PCOS in adolescence remain controversial because the diagnostic pathological features used in adults, including irregular menses up to two years beyond menarche, cystic acne, and polycystic ovarian morphology, may be normal pubertal physiological events (9). The present case had a history of oligomenorrhea and hirsutismus. She had presented to another pediatric endocrinology clinic with these chief complaints. The pelvic ultrasound which was performed in that center showed PCO morphology and laboratory tests revealed a high level of 1.4 androstenedione but a normal testosterone level. Although serum free testosterone level was unavailable, the low level of sex hormone binding globulin suggested it might be elevated. After being diagnosed with PCOS she was started on OC therapy. No pharmacological treatment has yet been approved by the Food and Drug Administration/European Medicines Agency for use in adolescents with PCOS but some pharmacological interventions, including OC, have been frequently used to manage PCOS symptoms (9). The treatment approach in this patient was discontinuing the estrogen-containing OC pill together with life-style interventions (calorie restricted diet, exercise and behavioral treatment) to provide weight loss. There are some reports showing the efficacy of progestin-only OC in decreasing the attack incidence in HAE (10,11), however the use of these in adolescence is debatable. Another pillar of treatment in HAE is patient education. Patients should be advised to avoid estrogen-containing products and cooperate with their doctors when planning to become pregnant in the future.

Conclusion

To the best of our knowledge this case is the first pediatric case of HAE due to *F12* mutation induced by estradiol containing OC to be reported. Individuals with HAE-related mutations may not have any attack until encountering a trigger, such as estrogen-containing drugs. Thus, we highlight the importance of obtaining a thorough family history regarding any HAE attack before initiation of OC, as this care may be lifesaving.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

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References

1. Banday AZ, Kaur A, Jindal AK, Rawat A, Singh S. An update on the genetics and pathogenesis of hereditary angioedema. Genes Dis 2020;7:75-83.
2. Bork K, Wulff K, Rossmann H, Steinmüller-Magin L, Braenne I, Witzke G, Hardt J. Hereditary angioedema cosegregating with a novel kininogen 1 gene mutation changing the N-terminal cleavage site of bradykinin. Allergy 2019;74:2479-2481. Epub 2019 Jun 7
3. Zuraw BL. Hereditary angioedema with normal C1 inhibitor: Four types and counting. J Allergy Clin Immunol 2018;141:884-885. Epub 2018 Feb 2
4. Bork K, Wulff K, Witzke G, Hardt J. Hereditary angioedema with normal C1-INH with versus without specific F12 gene mutations. Allergy 2015;70:1004-1012. Epub 2015 May 22
5. Farsetti A, Misiti S, Citarella F, Felici A, Andreoli M, Fantoni A, Sacchi A, Pontecorvi A. Molecular basis of estrogen regulation of Hageman factor XII gene expression. Endocrinology 1995;136:5076-5083.
6. Perricone R, Pasetto N, De Carolis C, Vaquero E, Noccioli G, Panerai AE, Fontana L. Cystic ovaries in women affected with hereditary angioedema. Clin Exp Immunol 1992;90:401-404.
7. Iahn-Aun M, Aun MV, Motta AA, Kalil J, Giavina-Bianchi P, Hayashida SA, Baracat EC, Maciel GA. The Complex Interaction Between Polycystic Ovary Syndrome and Hereditary Angioedema: Case Reports and Review of the Literature. Obstet Gynecol Surv 2017;72:417-424.
8. Bork K, Meng G, Staubach P, Hardt J. Hereditary angioedema: new findings concerning symptoms, affected organs, and course. Am J Med 2006;119:267-274.
9. Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, Dabadghao P, Darendeliler F, Elbarbary NS, Gambineri A, Garcia Rudaz C, Hoeger KM, López-Bermejo A, Ong K, Peña AS, Reinehr T, Santoro N, Tena-Sempere M, Tao R, Yildiz BO, Alkhayyat H, Deeb A, Joel D, Horikawa R, de Zegher F, Lee PA. An International Consortium Update: Pathophysiology, Diagnosis, and Treatment of Polycystic Ovarian Syndrome in Adolescence. Horm Res Paediatr 2017;88:371-395. Epub 2017 Nov 13
10. Bork K, Wulff K, Witzke G, Hardt J. Treatment for hereditary angioedema with normal C1-INH and specific mutations in the F12 gene (HAE-FXII). Allergy 2017;72:320-324. Epub 2016 Dec 1
11. Saule C, Boccon-Gibod I, Fain O, Kanny G, Plu-Bureau G, Martin L, Launay D, Bouillet L, Gompel A. Benefits of progestin contraception in non-allergic angioedema 2013;43:475-482.

CASE REPORT

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Nephrogenic Syndrome of Inappropriate Antidiuresis Mimicking Hyporeninemic Hypoaldosteronism: Case Report of Two Infants

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What is already known on this topic?

Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is a very rare disorder caused by activating mutations in the arginine vasopressin (AVP) receptor-2 gene (*AVPR2*). Patients with NSIAD can manifest at any age from birth with euvolemic hyponatremia and concentrated urine excretion. Undetectable AVP levels distinguish this syndrome from inappropriate antidiuretic hormone secretion.

What this study adds?

In NSIAD, plasma renin activity is suppressed and aldosterone level is relatively low. This profile can be confused with hyporeninemic hypoaldosteronism, especially in infants with no apparent gross cranial, pulmonary and renal pathology. Diagnosing NSIAD correctly may prevent complications, such as hyponatremia, and unnecessary treatment with fludrocortisone, which would most likely result in hypertension.

Abstract

Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is an X-linked disease caused by activating mutations in the arginine vasopressin (AVP) receptor-2 (*AVPR2*) gene. Affected patients excrete concentrated urine despite very low levels of AVP, and consequently develop euvolemic hyponatremia. Due to its low frequency, patients may be misdiagnosed and treated incorrectly. We report two related male infants with NSIAD that was initially confused with hyporeninemic hypoaldosteronism (HH). First, a 2-month-old male presented with hyponatremia, low plasma osmolality, relatively high urine osmolality, and low plasma renin-aldosterone levels. These clinical and laboratory findings were compatible with syndrome of inappropriate antidiuretic hormone (ADH) secretion without apparent cause. Consequently, fludrocortisone was initiated with a presumptive diagnosis of HH. While correcting hyponatremia, fludrocortisone treatment led to hypertension and was discontinued promptly. The second patient, aged one year, was admitted with a history of oligohydramnios, had been hospitalized four times due to hyponatremia since birth, and had a diagnosis of epilepsy. Similarly, the second infant had clinical and laboratory findings compatible with syndrome of inappropriate ADH secretion with no apparent cause. Fluid restriction normalized his serum sodium despite the plasma AVP level being undetectable. In both infants, *AVPR2* gene analysis revealed a known mutation (c.409C > T; p.R137C) and confirmed the diagnosis of NSIAD. In conclusion, NSIAD should be considered in all patients with unexplained euvolemic hyponatremia despite high urine osmolality. If NSAID is not considered, the plasma renin-aldosterone profile can be confused with HH, especially in infants.

Keywords: *AVPR2* gene, hyponatremia, inappropriate antidiuretic hormone secretion

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Introduction

The vasopressin type 2 receptor (V2R) plays a central role in the control of water homeostasis by the kidney (1). While inactivating mutations in the V2R gene (AVPR2) causes X-linked nephrogenic diabetes insipidus (1,2), activating mutations result in nephrogenic syndrome of inappropriate antidiuresis (NSIAD) (3). NSIAD, first described in 2005, is a rare disorder with about 30 cases reported so far. It shares the same clinical features with syndrome of inappropriate antidiuretic hormone secretion (SIADH). Both syndromes are associated with euvolemic hyponatremia, decreased serum osmolality, and inappropriate increases in urine osmolality, but differ in arginine-vasopressin (AVP) levels, which are high in SIADH and not detectable in NSIAD (3,4). Increased extracellular volume due to the excessive effect of AVP induces atrial natriuretic peptide (ANP) secretion. ANP directly reduces plasma renin activity (PRA), and thereby decreased aldosterone production which leads to increased output of urinary sodium and water (5,6). This compensatory event, which occurs due to inappropriate antidiuresis, can give a false impression that the primary pathological process underlying hyponatremia is hyporeninemic hypoaldosteronism (HH). Therefore, if NSIAD is not considered in the differential diagnosis of hyponatremia, this rare disorder can be mistaken for HH. Presence of chronic hyperkalemia distinguishes HH from NSIAD (7,8), thus hyponatremic but normokalemic infants have been reported with a diagnosis of HH (9,10). Here, we present two related male infants with NSIAD that was initially confused with HH. The aim of this report is to raise awareness of NSIAD, which is very rare cause of chronic or recurrent hyponatremia.

Case Reports

Case 1

A 2-month-old male who was scheduled for inguinal hernia surgery was seen because of hyponatremia. He was the second child of non-consanguineous parents. His parents and older sister were healthy, but his cousins suffered from hyponatremia (Figure 1). His mother was not taking any medications. Personal medical history was unremarkable with a normal pregnancy, birth by cesarean section at 39 weeks of gestation, and a birth weight of 3290 g. He was exclusively breastfed. On admission, the infant was an apparently healthy male with normal physical exam findings. On examination, his weight was 5.83 kg [-0.27 standard deviation score (SDS)], length 59 cm (-0.37 SDS), temperature 36.5 °C, blood pressure 80/40 mm Hg (49th percentile/77th percentile). Initial laboratory testing was as follows: serum sodium 126 mEq/L [reference range (RR): 135-145], potassium 5.7 mEq/L (RR: 3.5-5.5), serum osmolality 257 mOsm/kg (RR: 275-295), uric acid 1.5 mg/dL (RR: 1.8-5.0), PRA 0.02 ng/mL/h (RR: 0.4-15), aldosterone 26 ng/dL (RR: 5-90), urine osmolality 132 mOsm/kg (RR: 50-1400) and urine sodium 24 mEq/L (RR: 54-190). Other laboratory values of the patient are given in Table 1. When serum sodium was normalized subsequently by treatment, urinary sodium concentration increased to 87 mEq/L.

The findings of an inappropriately concentrated urine (>100 mOsm/kg), low serum osmolality (<280 mOsm/kg) and serum sodium (<135 mEq/L) were compatible with SIADH, but there was no apparent cause to explain it, including cranial or pulmonary pathologies and drugs. Kidney ultrasound was also normal. Other hyponatremic states were also ruled out on the basis of his history, physical examination and laboratory studies (Table 1). Due to suppressed PRA and relatively low levels of aldosterone despite hyponatremia and mild hyperkalemia, a presumptive diagnosis of HH was made. To correct hyponatremia, fludrocortisone treatment (0.1 mg/day) was started together with oral sodium chloride supplement (6 mEq/kg/day). He did not have marked variations in weight. Subsequently serum sodium increased to 140 mEq/L within two weeks, sodium chloride was discontinued and the dose of fludrocortisone was reduced to 0.05 mg/day. At the end of two-month follow-up, blood pressure was found to be elevated [100/60 mmHg (96th percentilep/99th percentile)], and therefore fludrocortisone was discontinued. During the subsequent observation period of four months, serum

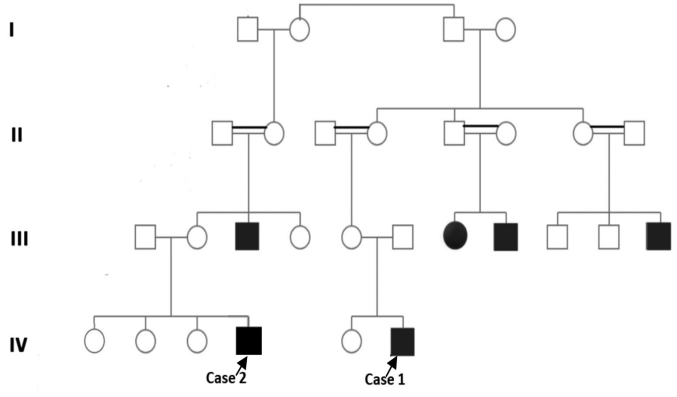

Figure 1. Patients pedigree

Black squares with arrows indicate genetically confirmed NSIAD. Black squares and circles indicate the cases that are followed up with hyponatremia

NSIAD: nephrogenic syndrome of inappropriate antidiuresis

sodium level remained in the range of 130-136 mEq/L, and blood pressure was normalized without intervention.

Case 2

Six months after the first patient's admission, the second boy, at the age of one year, was referred to our clinic for recurrent hyponatremia. He was born weighing 3000 g at 37 weeks of gestation by emergency C-section due to oligohydramnios, which developed within the last trimester. He was treated for diagnoses of hyponatremia and sepsis for ten days after birth. Subsequently, he was hospitalized for hyponatremia three more times up to the age of six months. At the age of 10 months, he had a seizure and oxcarbazepine treatment was started. When inquiring about the family history, his non-consanguineous parents and three sisters were healthy but his maternal uncle had a history of hyponatremia and epilepsy (Figure 1). On physical examination, his temperature was 36.8 °C, blood pressure 85/45 mmHg (60^{th} percentile/85^{th} percentile), pulse of 75 beats per minute, weight was 9.2 kg (-0.7 SDS) and length was 72.8 cm (-1.27 SDS). He appeared well and euvolemic. Initial serum sodium level was 120 mEq/L, PRA was 0.02 ng/mL/h and all laboratory findings were compatible with SIADH, as shown in Table 1. After fluid restriction to 1000 mL/m^2/day, serum sodium concentration increased up to 141 mEq/L and PRA 19.2 ng/mL/h. Since oxcarbazepine was known to cause SIADH and the patient's electroencephalography was normal, this treatment was discontinued. Further studies were conducted to determine the cause of SIADH. Chest X-ray and magnetic resonance imaging of the brain were normal. When daily fluid intake became unrestricted, hyponatremia recurred. After exclusion of usual causes of SIADH, a nephrogenic origin of inappropriate antidiuresis was considered and the AVP level was checked. Plasma AVP level, measured by double-antibody radioimmunoassay, was undetectable (< 0.5 pg/mL) in the presence of euvolemic hyponatremia (128 mEq/L). Therefore, we switched our clinical diagnosis of SIADH to NSIAD. The diagnosis of NSIAD was confirmed by genetic testing, which showed a known mutation in *AVPR2* c.409C > T, corresponding to arginine to cysteine mutation at amino acid 137 (p.R137C). Detailed pedigree analysis showed that the second case was a relative of the first case (Figure 1). Plasma AVP level was also measured in the first case and was also undetectable (< 0.5 pg/mL) in the presence of euvolemic hyponatremia (126 mEq/L). Clinical and laboratory data of both cases were also similar (Table 1). Therefore, genetic analysis was also performed in the first case, and confirmed the diagnosis of NSIAD by detecting the same mutation in the *AVPR2* gene. Both patients are continuing to be followed up with normal serum sodium levels on limited fluid intake for three years. Their motor and mental development is normal.

Table 1. Laboratory values of cases

Laboratory studies	Case 1	Case 2	Reference ranges
Serum/plasma			
Sodium (mEq/L)	126	120	135-145
Potassium (mEq/L)	5.7	4.9	3.5-5.5
Chloride (mEq/L)	95.1	93.2	98-106
Glucose (mg/dL)	77	79	70-111
Bicarbonate (mmol/L)	22	21	22-29
Creatinine (mg/dL)	0.22	0.21	0.2-0.4
Urea (mg/dL)	2	2,8	5-18
Uric acid (mg/dL)	1.5	2,1	1.8-5.0
Albumin (g/dL)	3.72	3,6	3.5-5.0
Osmolality (mOsm/kg H_2O)	254	236	275-295
Renin activity (ng/mL/h)	0.02	0.02	0.4-15
Aldosterone (ng/dL)	26	84	5-90
Arginine vasopressin (pg/mL)	< 0.05	< 0.05	1.5-12[a]
fT4 (ng/dL)	1.69	1.2	0.96-1.77
TSH (µIU/mL)	1.97	2.55	0.7-5.97
ACTH (pg/mL)	22.9	24	25-100
Cortisol (µg/dL)	16.4	22.4	8.5-23
Urine			
Osmolality (mOsm/kg H_2O)	132	571	50-1400[b]
Sodium (mEq/L)	24	58	54-190[c]
Potassium (mEq/L)	13	14	20-80
Sweat chloride (mEq/L)	19	ND	< 60

[a]Serum osmolality dependent.
[b]Fluid intake dependent.
[c]Diet dependent.
ACTH: adrenocorticotropic hormone, TSH: thyroid stimulating hormone

Discussion

We report two consanguineous male infants with a diagnosis of NSIAD, which was confirmed by genetic analysis which revealed a known mutation in *AVPR2*. NSIAD is a very rare disorder reported in about 30 cases since 2005 when it was first described (3,11-21). The prevalence of activating mutation of *AVPR2* is unknown. Due to as many as 10% of patients with SIADH having undetectable levels of AVP, activating mutations of *AVPR2* are likely to account at least for some of these cases (11). Due to its low frequency, it is not usually considered in the differential diagnosis of euvolemic hyponatremia. Therefore, lack of awareness of this rare disease may cause delay in determining the etiology of hyponatremia and even misdiagnosis. Indeed, in our first case with hyponatremia, the laboratory data (low

renin-aldosterone and borderline-high potassium levels) were erroneously interpreted as HH. This confusion led to fludrocortisone treatment. A therapeutic approach to correct the serum sodium level by increasing renal sodium and water reabsorption in a patient with elevated plasma volume naturally resulted in hypertension. This erroneous management was immediately corrected, but the patient remained undiagnosed for a while. Luckily, the second case presented with similar clinical and laboratory findings in a short time. Even though unable to identify an etiology, we established a clinical diagnosis of SIADH by observing that the hyponatremia improved with fluid restriction. Then, it was shown that the syndrome was of renal origin by way of undetectable AVP levels. Thus, molecular analysis of the renal AVP receptor gene confirmed the diagnosis of NSIAD.

HH, the initial diagnosis of our first case, in fact pointed to the renal origin of underlying defect. Hyporeninemia occurs in many kidney diseases, including diabetic nephropathy, lupus nephritis, sickle cell anemia, amyloidosis, urinary tract obstructions, and due to abuse of drugs impairing renin production. The typical patient with HH usually presents at elderly ages with mild renal insufficiency and metabolic acidosis, and asymptomatic chronic hyperkalemia, without hyponatremia (7,8). Therefore, a diagnosis of HH does not seem to be appropriate for a hyponatremic infant without any apparent renal pathology. On the other hand, HH has been rarely described in infants who have hyponatremia, but no hyperkalemia and hyperchloremic acidosis (9,10). Unlike the clinical picture in adults, this electrolyte profile in the infants has been related to renal characteristics of the age period, and the absence of gross renal pathology (10) but the etiology of HH in these infants has remained undetermined. Interestingly, as seen in our first case, fludrocortisone treatment led to hypertension in one of male siblings described by Landier et al. (10). HH has been occasionally defined in children with acquired, chronic or acute kidney diseases (22,23), whereas its congenital form has been reported only in a few infants (9), and an underlying genetic defect has not been identified to date. However, in a retrospective analysis by Storey et al. (24), the prevalence of genetic defects of the mineralocorticoid pathway including hypoaldosteronism and pseudohypoaldosteronism was considerably higher than expected in the hyponatremic neonates and infants. However, no infant in this large patient group had HH. As a result, in our infant cases, after initial confusion with HH, we correctly described NSIAD as a genetic cause of hyponatremia originating from the kidney. We also consider that the unusual hyponatremic infant cases of HH reported before recognition of NSIAD might be the earliest examples of undiagnosed NSIAD.

NSIAD is a disorder characterized by hyponatremia, normal or slightly elevated plasma volume, an inappropriately concentrated urine and normal-to-high urine sodium (3). SIADH and NSIAD have the same clinical features of impaired free water excretion (4). In affected patients, plasma volume increases due to reduced free water excretion. The volume increment results in high secretion of natriuretic peptides, leading to suppression of renin-aldosterone levels. Secondary mineralocorticoid deficiency causes renal salt wasting and hyponatremia (5,6). When SIADH or NSIAD are not correctly identified as a main source, the patients can be mistakenly diagnosed as HH. Thus, in a patient with suspected SIADH, if its classical causes of cranial and pulmonary origin or the use of drugs inducing AVP secretion are not found, NSIAD should be considered first in the differential diagnosis. Despite the findings compatible with SIADH, the demonstration of undetectable plasma AVP levels makes a clinical diagnosis of NSIAD (3,13,14).

Feldman et al. (3) first reported hemizygous gain of function point mutations (p.Arg137Cys and p.Arg137Leu) in *AVPR2* in two male infants with NSIAD. Almost all the patients with NSIAD presented in the literature have had one of these two *AVPR2* mutations (3,11,12,13,14,20,21). Our NSIAD patients also had p.Arg137Cys mutation. Functional analysis reported by previous studies have already shown that this variant is responsible for a constitutive activation of the AVP type 2 receptor, leading to inadequate water reabsorption in spite of low AVP levels.

In our case study, detailed family inquiry revealed that these two infants who independently presented were related and also had the five adult relatives with history of hyponatremia and/or epilepsy. We learned that these adults were not diagnosed with NSIAD, but consumed limited fluid of their own free will. Since the cousins of the first infant's mother and the second infant's uncle lived abroad, we could not perform genetic testing for these family members. While symptoms in our infant cases began in the neonatal and even antenatal period, manifested by oligohydramnios due to low urination, the other family members' complaints, including tiredness, headache and seizures, had started at different ages ranging from childhood to later life. So, the age range in the seven patients (one female) in our large family varied from infancy to adulthood. Decaux et al. (11) demonstrated that NSIAD shows a wide variation of expressivity. It is not limited to infants, and the diagnosis should also be considered in adults. Albeit NSIAD is an X-linked genetic disease, it has been reported in heterozygous females, and this is explained as random X-inactivation (25).

Early detection and treatment of NSIAD are essential to prevent severe hyponatremia, which can have dangerous effects on neonates and infants, and can potentially lead to death or, if survived, neurological sequelae. The goal of therapy is to limit free-water intake (3,11-21). Since AVP stimulates thirst, low to undetectable levels of AVP encountered in NSIAD could induce a diminished thirst sensation and thus explain the good compliance to water restriction (26). Fludrocortisone treatment rescues otherwise potentially life-threatening hyponatremia due to renal salt wasting and the secondary mineralocorticoid deficiency driven by elevated ANP and/or brain natriuretic peptide (27). However, long-term use of mineralocorticoids can lead to hypertension, as seen in our first case. The vaptans, AVP antagonists that interfere with the hormone's antidiuretic effect by competitively binding to AVPR2, are effective in SIADH but ineffective in NSIAD due to the receptor's constitutive activation (11,17). Therefore, fluid restriction remains the mainstay of therapy, as applied in the two cases presented herein.

Conclusion

In conclusion, NSIAD should be considered as a diagnosis in patients presenting at any ages with unexplained hyponatremia and low plasma osmolality, despite relatively high urine osmolality. As a first step in the investigation, plasma AVP levels should be measured. In patients with undetectable AVP levels, genetic testing of AVPR2 can simply confirm diagnosis. It should be noted that if NSIAD is not considered, the plasma renin-aldosterone profile can be confused with HH, especially in infants.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Jamala Mammadova, Cengiz Kara, Eda Çelebi Bitkin, Elif İzci Güllü, Murat Aydın, Concept: Jamala Mammadova, Cengiz Kara, Murat Aydın, Design: Jamala Mammadova, Cengiz Kara, Murat Aydın, Data Collection or Processing: Jamala Mammadova, Cengiz Kara, Analysis or Interpretation: Jamala Mammadova, Cengiz Kara, Literature Search: Jamala Mammadova, Cengiz Kara, Writing: Jamala Mammadova, Cengiz Kara.

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References

1. van den Ouweland AM, Dreesen JC, Verdijk M, Knoers NV, Monnens LA, Rocchi M, van Oost BA. Mutations in the vasopressin type 2 receptor gene (AVPR2) associated with nephrogenic diabetes insipidus. Nat Genet 1992;2:99-102.
2. Bockenhauer D, Carpentier E, Rochdi D, van't Hoff W, Breton B, Bernier V, Bouvier M, Bichet DG. Vasopressin type 2 receptor V88M mutation: molecular basis of partial and complete nephrogenic diabetes insipidus. Nephron Physiol 2009;114:1-10. Epub 2009 Oct 8
3. Feldman BJ, Rosenthal SM, Vargas GA, Fenwick RG, Huang EA, Matsuda-Abedini M, Lustig RH, Mathias RS, Portale AA, Miller WL, Gitelman SE. Nephrogenic syndrome of inappropriate antidiuresis. N Engl J Med 2005;352:1884-1890.
4. Ellison DH, Berl T. Clinical practice. The syndrome of inappropriate antidiuresis. N Engl J Med 2007;356:2064-2072.
5. Cuneo RC, Espiner EA, Nicholls MG, Yandle TG, Loyce SL, Gilchrist NL. Renal, hemodynamic, and hormonal responses to atrial natriuretic peptide infusions in normal man, and effect of sodium intake. J Clin Endocrinol Metab 1986;63:946-953.
6. Cuneo RC, Espiner EA, Nicholls MG, Yandle TG, Livesey JH. Effect of physiological levels of atrial natriuretic peptide on hormone secretion: inhibition of angiotensin-induced aldosterone secretion and renin release in normal man. J Clin Endocrinol Metab 1987;65:765-772.
7. Sousa AG, Cabral JV, El-Feghaly WB, de Sousa LS, Nunes AB. Hyporeninemic hypoaldosteronism and diabetes mellitus: Pathophysiology assumptions, clinical aspects and implications for management. World J Diabetes 2016;7:101-111.
8. Arai K, Papadopoulou-Marketou N, Chrousos GP. Aldosterone Deficiency and Resistance. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, et al. (eds). Endotext South Dartmouth (MA): MDText.com, Inc.; 2000.
9. Monnens L, Fiselier T, Bos B, van Munster P. Hyporeninemic hypoaldosteronism in infancy. Nephron 1983;35:140-142.
10. Landier F, Guyene TT, Boutignon H, Nahoul K, Corvol P, Job JC. Hyporeninemic hypoaldosteronism in infancy: a familial disease. J Clin Endocrinol Metab 1984;58:143-148.
11. Decaux G, Vandergheynst F, Bouko Y, Parma J, Vassart G, Vilain C. Nephrogenic syndrome of inappropriate antidiuresis in adults: high phenotypic variability in men and women from a large pedigree. J Am Soc Nephrol 2007;18:606-612. Epub 2007 Jan 17
12. Gupta S, Cheetham TD, Lambert HJ, Roberts C, Bourn D, Coulthard MG, Ball SG. Thirst perception and arginine vasopressin production in a kindred with an activating mutation of the type 2 vasopressin receptor: the pathophysiology of nephrogenic syndrome of inappropriate antidiuresis. Eur J Endocrinol 2009;161:503-508. Epub 2009 Jun 19
13. Bes DF, Mendilaharzu H, Fenwick RG, Arrizurieta E. Hyponatremia resulting from arginine vasopressin receptor 2 gene mutation. Pediatr Nephrol 2007;22:463-466. Epub 2006 Nov 18
14. Marcialis MA, Faà V, Fanos V, Puddu M, Pintus MC, Cao A, Rosatelli MC. Neonatal onset of nephrogenic syndrome of inappropriate antidiuresis. Pediatr Nephrol 2008;23:2267-2271. Epub 2008 Jul 12
15. Soule S, Florkowski C, Potter H, Pattison D, Swan M, Hunt P, George P. Intermittent severe, symptomatic hyponatraemia due to the nephrogenic syndrome of inappropriate antidiuresis. Ann Clin Biochem 2008;45:520-523.
16. Bockenhauer D, Penney MD, Hampton D, van't Hoff W, Gullett A, Sailesh S, Bichet DG. A family with hyponatremia and the nephrogenic syndrome of inappropriate antidiuresis. Am J Kidney Dis 2012;59:566-568. Epub 2011 Dec 9

17. Vandergheynst F, Brachet C, Heinrichs C, Decaux G. Long term treatment of hyponatremic patients with nephrogenic syndrome of inappropriate antidiuresis: personal experience and review of published case reports. Nephron Clin Pract 2012;120:168-172. Epub 2012 Jun 19
18. Brachet C, Vandergheynst F, Heinrichs C. Nephrogenic syndrome of inappropriate antidiuresis in a female neonate: review of the clinical presentation in females. Horm Res Paediatr 2015;84:65-67. Epub 2015 Apr 25
19. Erdélyi LS, Mann WA, Morris-Rosendahl DJ, Groß U, Nagel M, Várnai P, Balla A, Hunyady L. Mutation in the V2 vasopressin receptor gene, AVPR2, causes nephrogenic syndrome of inappropriate diuresis. Kidney Int 2015;88:1070-1078. Epub 2015 Jul 1
20. Ranchin B, Boury-Jamot M, Blanchard G, Dubourg L, Hadj-Aïssa A, Morin D, Durroux T, Cochat P, Bricca G, Verbavatz JM, Geelen G. Familial nephrogenic syndrome of inappropriate antidiuresis: dissociation between aquaporin-2 and vasopressin excretion. J Clin Endocrinol Metab 2010;95:37-43. Epub 2010 Jul 14
21. Cho YH, Gitelman S, Rosenthal S, Ambler G. Long-term outcomes in a family with nephrogenic syndrome of inappropriate antidiuresis. Int J Pediatr Endocrinol 2009;2009:431527. Epub 2010 Jan 28
22. Rodríguez-Soriano J, Vallo A, Sanjurjo P, Castillo G, Oliveros R. Hyporeninemic hypoaldosteronism in children with chronic renal failure. J Pediatr 1986;109:476-482.
23. Watanabe T, Nitta K. Transient hyporeninemic hypoaldosteronism in acute glomerulonephritis. Pediatr Nephrol 2002;17:959-963. Epub 2002 Oct 11
24. Storey C, Dauger S, Deschenes G, Heneau A, Baud O, Carel JC, Martinerie L. Hyponatremia in children under 100 days old: incidence and etiologies. Eur J Pediatr 2019;178:1353-1361. Epub 2019 Jul 13
25. Hague J, Casey R, Bruty J, Legerton T, Abbs S, Oddy S, Powlson AS, Majeed M, Gurnell M, Park SM, Simpson H. Adult female with symptomatic AVPR2-related nephrogenic syndrome of inappropriate antidiuresis (NSIAD). Endocrinol Diabetes Metab Case Rep 2018;2018:17-0139.
26. de Arruda Camargo LA, Saad WA, Cerri PS. Effects of V1 and angiotensin receptor subtypes of the paraventricular nucleus on the water intake induced by vasopressin injected into the lateral septal area. Brain Res Bull 2003;61:481-487.
27. Kleanthous K, Maratou E, Spyropoulou D, Dermitzaki E, Papadimitriou A, Zoupanos G, Moutsatsou P, Mastorakos G, Urano F, Papadimitriou DT. Lessons from Wolfram Syndrome: Initiation of DDAVP Therapy Causes Renal Salt Wasting Due to Elevated ANP/BNP Levels, Rescued by Fludrocortisone Treatment. Indian J Pediatr 2021;88:582-585. Epub 2020 Nov 18

CASE REPORT

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An Alternative Route of Treatment in Transient Hypothyroxinemia of Prematurity: Rectal Administration of Levothyroxine

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What is already known on this topic?

When levothyroxine treatment is indicated in newborns enteral administration is the preferred route. Rectal administration of the drug has not previously been reported in preterm infants although it has been used successfully in adult patients with poor oral absorbtion.

What this study adds?

In preterm babies with serious gastrointestinal problems rectal administration of levothyroxine tablet may be effective in the treatment of transient hypothyroxinaemia of prematurity.

Abstract

Transient hypothyroxinaemia of prematurity (THOP) is a disorder encountered particularly in extremely low birth weight and preterm newborns. In recent years, the survival rates of these babies have increased, owing to the advances in neonatal care, thereby increasing the incidence of THOP. Controversies about the management of this disorder still continues while accompanying morbidites may create difficulties in the treatment of these patients. A preterm baby boy, born at $25^{6/7}$ gestational weeks with a birthweight of 665 g who developed short bowel syndrome after necrotizing enterocolitis surgery and who was treated with rectal levothyroxine, is presented.
Keywords: Levothyroxine, prematurity, short bowel, rectal

Introduction

Transient hypothyroxinemia of prematurity (THOP) is defined as thyroid dysfunction with low circulating free and total throxine (T4) without an expected increase in thyroid stimulating hormone (TSH) (1). It has been reported that THOP occurs in almost half of the babies born at or less than 30 weeks of gestation (2,3).

In preterm babies, the TSH surge is delayed and free T4 levels (fT4) remain low due to several factors, including discontinuation of maternal and placental thyroid hormone support, immaturity of the hypothalamo-pituitary-thyroid axis, limitation of iodine intake and retention, and insufficient volume and capacity of the thyroid gland (1,4). It has been reported that THOP may increase the risk of perinatal mortality and morbidity but the management of this thyroid dysfunction in premature infants is still controversial (2).

Parallel to the improved survival of more immature preterm babies, studies concerning THOP have also increased. Conflicting results have been reported considering neurodevelopmental, auditory and cognitive outcomes of very low birth weight babies with or without THOP, some showing no significant difference between the two groups (5). Reports on the effect of treatment of THOP on neurodevelopmental outcome in preterm babies are also

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controversial. Some studies have shown no significant effect of treatment, while others have found better language skills, motor and cognitive functions in the group given thyroxine treatment (6). Hence, studies comparing the long-term effects of treatment in preterm babies with a diagnosis of THOP are still needed (7,8,9). When a treatment decision is made, serious gastrointestinal problems in some very low birth weight babies may create difficulties in the administration of levothyroxine when oral formulation is the only option.

In this paper, a case of THOP in a preterm baby who was born at $25^{6/7}$ gestational week and treated with rectal levothyroxine is presented. The baby developed short bowel syndrome after necrotizing enterocolitis (NEC) surgery and did not respond to oral administration of the drug.

Case Report

A baby boy was born by emergency cesarean section, due to severe preeclampsia in the mother, at $25^{6/7}$ gestational week with a birthweight of 665 g. He was intubated in the delivery room and transferred to the neonatal intensive care unit. He was on mechanical ventilation. Total parenteral nutrition (TPN) and minimal enteral nutrition with breast milk were started on the postnatal first day. The baby had delayed meconium passage and developed abdominal distension with increased gastric residuals. Laboratory and radiological findings were compatible with NEC. Minimal enteral feeding was discontinued, gastric free drainage and broad-spectrum antibiotic therapy were initiated. He was operated on the postnatal sixth day due to perforated NEC (Figure 1). As there were multiple areas of perforation and circulatory disturbances in the intestinal wall, a long segment including the jejunum and ileum was resected. A stoma was formed with the proximal end whereas the distal end was left closed in the abdomen. The baby was on TPN until the postoperative seventh day when minimal enteral feeding was started and gradually increased. However TPN support could not be discontinued as enteral nutrition alone was insufficient due to short bowel syndrome.

On the fourteenth postnatal day, thyroid screening tests revealed serum levels of fT4: 0.87 ng/dL and TSH: 0.061 mIU/L, while cortisol was 5.75 µg/dL. Serum total bilirubin level was 12.12 mg/dL, predominant component being direct reacting bilirubin (DB: 11.48 mg/dL). One week later, as the fT4 level was decreasing and close to the lower limit of normal, enteral levothyroxine 5 µg/kg/day was started. There was no response to treatment during follow-up and the enteral dose of levothyroxine was increased to 10 µg/kg/day (Table 1, Figure 2). However there was still no increase in fT4 levels which was thought to be the result of poor absorption of the drug because the main site of oral thyroxine absorbtion is the duodenum, jejenum and ileum which were incomplete in this case. Since parenteral and suppository levothyroxine preparations were not available, the tablet form of the drug was ground and one tablet (25 µg) was diluted with 10 mL of saline to be administered rectally at a dose of 10 µg/kg/day (4 mL/kg) by a 6 Fr feeding tube. After nine days of rectal levothyroxine treatment fT4 levels increased and bilirubin levels decreased (Table 1, Figure 2).

Unfortunately the baby died on postnatal 77^{th} day, while still on rectal levothyroxine treatment; cause of death was a combination of severe bronchopulmonary dysplasia, surgical NEC, short bowel syndrome and sepsis. A written informed consent was obtained from the patient's family for publication.

Discussion

Transient hypothyroxinemia is the most common thyroid dysfunction in preterm infants. Although it is controversial, it has been reported that some preterm babies can benefit from treatment of THOP, but issues such as the timing and duration of therapy are not yet clear (1,2,4). In the presented case, the gradual decrease in fT4 levels together

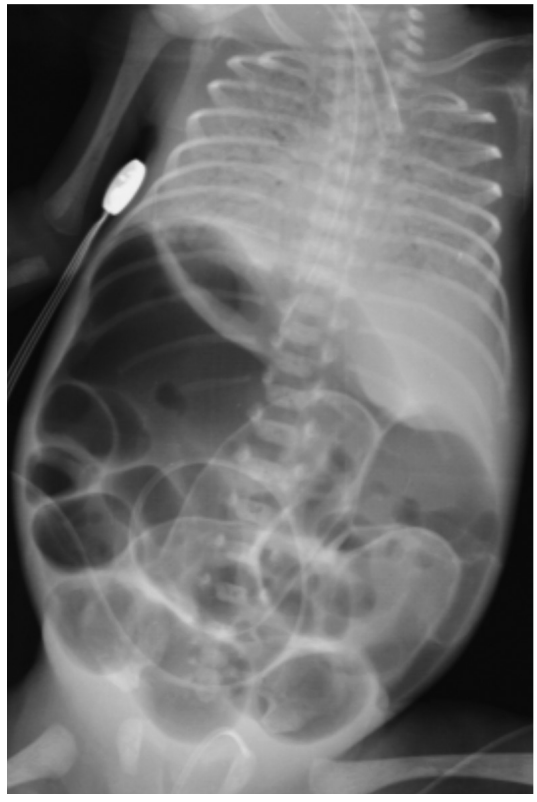

Figure 1. The abdomen X-ray of the baby with diffuse distention in necrotizing enterocolitis

with increasing TSH levels prompted us to initiate treatment. However the baby had short bowel syndrome after NEC surgery, when the main sites of absorbtion of oral thyroxine had been largely removed and fT4 levels did not respond to incremental doses of oral levothyroxine.

In a recently published article, alternative routes of levothyroxine administration were discussed (10). If refractory hypothyroidism persists despite oral therapy, it has been suggested to try different formulae. Among these, it was proposed that since the gastrointestinal transit time is longer, gel and capsules or in cases where absorption is not possible, intravenous and rectal forms could be tried. Since other forms that would prolong the stay of the drug in the gastrointestinal tract were not available, it was decided to give a diluted tablet form by the rectal route in this case.

There are a few publications reporting on the use of levothyroxine rectally. The efficacy of rectal levothyroxine treatment in suppository form was investigated in a study which reported both animal and human data. The authors examined the levels of fT4 after the administration of the

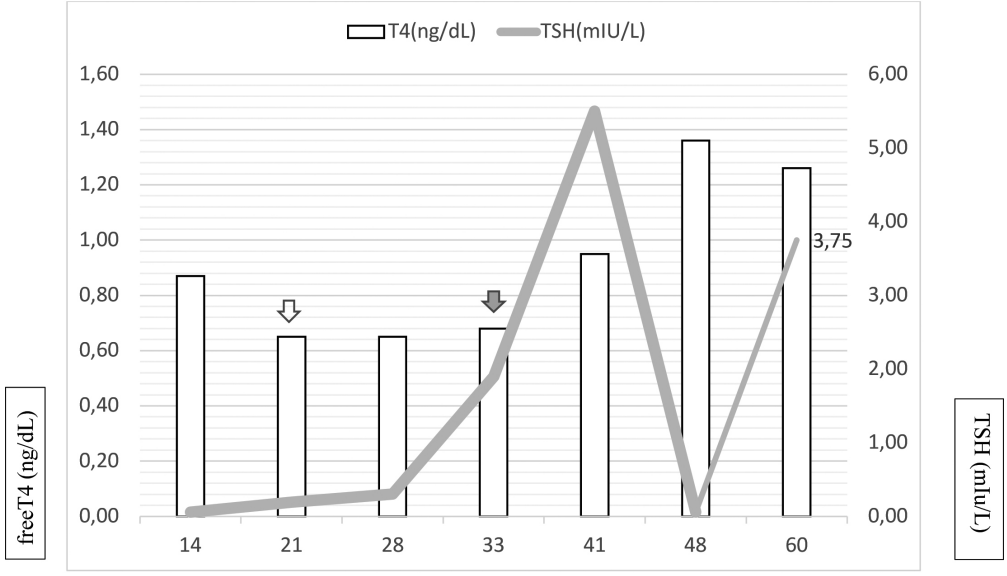

Figure 2. Thyroid hormone levels and treatment

TSH: thyroid stimulating hormone

Enteral levothyroxine

Rectal levothyroxine

Table 1. Thyroid functions, bilirubin values and treatment

Postnatal age, day	Postmenstrual age, week	fT4, ng/dL (N)*	TSH, mIU/L (N)*	Cortisol µg/dL	TSB/DB/IB**, mg/dL	Treatment
14	$27^{6/7}$	0.87 **(0.6-2.2)**	0.061 **(0.2-30.3)**	5.75	12.12/11.48/0.64	No treatment
21	$28^{6/7}$	0.65 **(0.6-3.4)**	0.191 **(0.2-20.6)**		18.9/4.4/4.7	5 µg/kg/day levothyroxine, enteral
28	$29^{6/7}$	0.65 **(0.6-3.4)**	0.301 (0.2-20.6)		9.05/8.24/0.81	10 µg/kg/day levothyroxine, enteral
33	$30^{4/7}$	0.68 **(0.6-3.4)**	1.9 (0.2-20.6)		6.46/6.17/0.29	10 µg/kg/day levothyroxine, rectal
41	$31^{6/7}$	0.95 **(1.0-3.8)**	5.5 (0.7-27.9)		8.7/7.89/0.81	10 µg/kg/day levothyroxine, rectal
48	$33^{0/7}$	1.36 **(1.0-3.8)**	0.06 (0.7-27.9)	0.51	-	10 µg/kg/day levothyroxine, rectal
60	$34^{3/7}$	1.26 **(1.2-4.4)**	3.73 (1.2-21.6)	0.96	8.8/7.4/1.4	10 µg/kg/day levothyroxine, rectal

*Normal values for postmenstrual age (9).
TSB: total serum bilirubin, DB: direct bilirubin, IB: indirect bilirubin

drug in suppository form to thyroidectomized rats and subsequently to six adult patients with hypothyroidism. The results showed that the bioavailability of levothyroxine was lower after rectal administration than after receiving oral medication. However it was suggested that T4 levels can be maintained if the suppository formulation was used at a dose 1.8 times higher than that of the oral dose and can be an alternative route in clinical practice (11).

In another study a 4-month-old baby who developed short bowel syndrome after multiple surgical operations due to gastroschisis was diagnosed with hypothyroidism while being investigated for direct hyperbilirubinemia and reduced intestinal motility. Since oral absorption was insufficient in this baby, the levothyroxine tablet was administered rectally. The initial dose was 12.5 µg/day (5 µg/kg/day) and increased to 25 µg/day (10 µg/kg/day) after one week. The tablet was diluted in 3 mL of saline and administered in bolus, with a size 8 rectal probe, which was flushed with 5 mL of water. Before each administration the drug was prepared freshly. Clinical and laboratory recovery was achieved at the end of four weeks of rectal treatment (12).

In another case report, a 58-year-old adult who had poor oral intake due to gastrointestinal system malignancy and who had impaired thyroid function was unresponsive to oral treatment. Due to the lack of parenteral preparations and rectal suppositories of levothyroxine, high doses of tablet formulation were ground and dissolved in 500 mL of normal saline and administered as a rectal enema for 21 days, after which thyroid function tests returned to normal (13).

The presented case who had short bowel syndrome, was unresponsive to oral tablet formulation of levothyroxine very probably because of poor intestinal absorbtion. Due to the lack of intravenous and suppository forms of the drug as alternative formulations, the oral tablet form of levothyroxine was administered by the rectal route after being ground and diluted with saline. Laboratory recovery was determined after nine days of rectal treatment with increasing fT4 levels and decreasing direct bilirubin levels.

However, the fact that our patient did not survive for a long time limits our long-term follow-up and interpretation of THOP and treatment. Nevertheless, to the best of our knowledge, this case is the first premature infant, or even newborn infant, who was treated with rectal levothyroxine to be published.

Conclusion

In conclusion, rectal administration of the diluted oral form of levothyroxine may be used as an alternative route of drug administration in the absence of availability of other forms of the drug in preterm neonates with impaired oral intake or absorption.

Ethics

Informed Consent: A written informed consent was obtained from the patient's family for publication.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Zeynep İnce, Erhan Aygün, Asuman Çoban, Concept: Duygu Tunçel, Asuman Çoban, Design: Duygu Tunçel, Asuman Çoban, Data Collection or Processing: Duygu Tunçel, Zeynep İnce, Erhan Aygün, Asuman Çoban, Analysis or Interpretation: Duygu Tunçel, Asuman Çoban, Literature Search: Duygu Tunçel, Zeynep İnce, Erhan Aygün, Asuman Çoban, Writing: Duygu Tunçel, Zeynep İnce, Erhan Aygün, Asuman Çoban.

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References

1. Eerdekens A, Langouche L, Van den Berghe G, Verhaeghe J, Naulaers G, Vanhole C. Review shows that thyroid hormone substitution could benefit transient hypothyroxinaemia of prematurity but treatment strategies need to be clarified. Acta Paediatr 2019;108:792-805. Epub 2019 Jan 4
2. Yoon SA, Chang YS, Ahn SY, Sung SI, Park WS. Incidence and severity of transient hypothyroxinaemia of prematurity associated with survival without composite morbidities in extremely low birth weight infants. Sci Rep 2019;9:9628.
3. Williams FL, Simpson J, Delahunty C, Ogston SA, Bongers-Schokking JJ, Murphy N, van Toor H, Wu SY, Visser TJ, Hume R; Collaboration from the Scottish Preterm Thyroid Group. Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. J Clin Endocrinol Metab 2004;89:5314-5320.
4. Iijima S. Current knowledge of transient hypothyroxinemia of prematurity: to treat or not to treat? J Matern Fetal Neonatal Med 2019;32:2591-2597. Epub 2018 Feb 22
5. Tan LO, Tan MG, Poon WB. Lack of association between hypothyroxinemia of prematurity and transient thyroid abnormalities with adverse long term neurodevelopmental outcome in very low birth weight infants. PLoS One 2019;14:e0222018.
6. van Wassenaer AG, Kok JH, de Vijlder JJ, Briët JM, Smit BJ, Tamminga P, van Baar A, Dekker FW, Vulsma T. Effects of thyroxine supplementation on neurologic development in infants born at less than 30 weeks' gestation. N Engl J Med 1997;336:21-26.
7. Ng SM, Turner MA, Weindling AM. Neurodevelopmental Outcomes at 42 Months After Thyroxine Supplementation in Infants Below 28 Weeks' Gestation: A Randomized Controlled Trial. Thyroid 2020;30:948-954. Epub 2020 Mar 17
8. van Wassenaer AG, Kok JH. Trials with thyroid hormone in preterm infants: clinical and neurodevelopmental effects. Semin Perinatol 2008;32:423-430.

9. Adams LM, Emery JR, Clark SJ, Carlton EI, Nelson JC. Reference ranges for newer thyroid function tests in premature infants. J Pediatr 1995;126:122-127.
10. Ritter MJ, Gupta S, Hennessey JV. Alternative routes of levothyroxine administration for hypothyroidism. Curr Opin Endocrinol Diabetes Obes 2020;27:318-322.
11. Kashiwagura Y, Uchida S, Tanaka S, Watanabe H, Masuzawa M, Sasaki T, Namiki N. Clinical efficacy and pharmacokinetics of levothyroxine suppository in patients with hypothyroidism. Biol Pharm Bull 2014;37:666-670.
12. Ybarra M, Dos Santos TJ, Pinheiro CTC, Dichtchekenian V, Damiani D. Rectal Levothyroxine for the Treatment of Hypothyroidism: A Case Study. Pediatrics 2018;142:20173317. Epub 2018 Jul 12
13. Obeidat KA, Saadeh NA, As'ad A, Bakkar S. Successful management of hypothyroidism in gastric outlet obstruction using levothyroxine rectal enemas: a case report. Am J Case Rep 2018;19:903-905.

CASE REPORT

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Liraglutide Treatment in a Morbidly Obese Adolescent with a *MC4R* Gene Variant: Side Effects Reduce Success

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What is already known on this topic?

Melanocortin-4 receptor gene (*MC4R*) defects cause monogenic obesity. In this situation, management of obesity is challenging because of excessive appetite and standard methods are unlikely to achieve weight loss over the long term. Recently, liraglutide treatment has been reported to provide weight loss in these patients with only gastrointestinal side effects.

What this study adds?

We present a long (43 weeks) experience of liraglutide treatment in an adolescent patient carrying a *MC4R* variant. Unfortunately, the drug could not be tolerated for a longer period due to gastrointestinal side effects, and discontinuation of treatment led to rapid weight gain.

Abstract

Variants of the melanocortin-4 receptor (*MC4R*) gene are the most common cause of monogenic obesity. It has been shown that, while obesity cannot be controlled with diet and exercise, glucagon-like-peptide-1 receptor agonists (GLP-1 RA) provide weight loss in the short term. In this paper, our experience with liraglutide treatment in an adolescent patient carrying a *MC4R* gene variant is presented. A female patient was admitted first at the age of 12.5 years with a complaint of progressive weight gain. She had marked excess of appetite since infancy. On physical examination of the pubertal female patient with a body mass index (BMI) of 36.1 kg/m^2 (3.48 standard deviation score), there was no pathological finding except diffuse acanthosis nigricans. Laboratory examinations revealed only insulin resistance. Weight loss was not achieved with lifestyle changes, metformin and orlistat treatments. On genetic examination, a sporadic heterozygous c.206T > G(p.I69R) variant that had been reported previously, was found in *MC4R* gene. Treatment with the GLP-1 RA, liraglutide, was initiated and a 19.2% reduction was achieved in the body weight and BMI at the end of 32 weeks. However, the patient, whose treatment compliance was disrupted due to significant gastrointestinal complaints, returned to her former weight within a few months (13 weeks) after treatment was stopped. In this case with a known pathogenic variant in *MC4R* gene, decrease of appetite and weight loss were achieved with liraglutide treatment, but side-effects of this treatment led to discontinuation of therapy. In such cases, there is need for effective and tolerable treatment options.

Keywords: Melanocortin-4 receptor defect, obesity, treatment, liraglutide, side effect

Introduction

Variants of the melanocortin-4 receptor (*MC4R*) gene are the most common cause of non-syndromic monogenic obesity (1). The interaction of MC4R with alpha-melanin stimulating hormone causes a decrease in appetite and food intake. Pathogenic variants of the *MC4R* gene located on chromosome 18q21.32 cause early onset, severe obesity. Dominant inherited obesity due to variants of the *MC4R* gene in humans was first described in 1998 (2). Today, more than 300 variants in the gene are known (3). The frequency of variants in the *MC4R* gene in individuals

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with early onset and severe obesity has been reported to be 5.7-8.6% (1,4,5). Early-onset severe obesity, tall stature, hyperphagia, increased lean body mass, normal pubertal age, and normal fertility have been reported in these individuals (1,4). Hyperinsulinemia is a common finding of the disease, and no pathology has been found in other hormones. Farooqi et al. (1) reported that 23 of 29 cases, in whom they detected variants in the *MC4R* gene, carried heterozygous variants, while six carried homozygous variants, and the phenotype was more severe in cases carrying homozygous variants. Currently, there are no specific treatment method recommended for the management of obesity due to *MC4R* variants. Long-term success is unlikely with lifestyle changes (diet, exercise, behavioral therapy) alone (6,7). A study has been published showing that subcutaneous use of the glucagon-like-peptide-1 receptor agonist (GLP-1 RA) liraglutide 3 mg/day for 16 weeks reduced appetite in these cases and provided associated weight loss compared to obese controls (8). This study also reported gastrointestinal side effects (nausea, vomiting, abdominal pain, diarrhea, constipation, reflux) which were generally mild and transient. Subcutaneous liraglutide was generally well tolerated in clinical trials among obese and overweight adults. Most of the side effects reported during treatment were gastrointestinal complaints. The most common causes of discontinuation of treatment were nausea, vomiting and diarrhea (9). In an another study, conducted on a small number of patients with heterozygous *MC4R* variants, weight loss, which was not different from placebo, was found after four weeks of treatment with the MC4R agonist Setmelanotide (Phase 1b study) (3). However, these two drugs have not yet been used in larger patient groups with *MC4R* variants, especially in the long-term. In studies evaluating the efficacy of bariatric surgery in this patient group, the most frequently used method was gastric bypass and in most of these studies, similar weight loss was obtained compared to obese patients without *MC4R* variants (10). However, long-term results are contradictory and there was lower success in some variants compared to others. In this report, we present our experience with liraglutide treatment in a morbidly obese, adolescent girl with a variant in the *MC4R* gene.

Case Report

The female patient was first admitted at the age of 12.5-years with a complaint of excess weight. She was born at 3000 g at term, and fed with breast milk until the age of three, supplementary food was added after six months of age, her motor-mental development was normal and she was obese since infancy. She was followed up for cyclical neutropenia and recurrent urinary tract infection, and had tonsillectomy and appendectomy operations. There was no history of serious obesity in the family. On admission, her body weight (BW) was 89 kg (> 97th percentile), height was 157 cm (50-75 percentile), and BMI was 36.1 kg/m^2 [+3.48 standard deviation score (SDS)]. On physical examination, there was no goiter and the pubertal stage was Tanner 3. Cervical and axillary acanthosis nigricans was present. She looked obese with a diffuse body fat distribution. Her mental status was normal. There was no dysmorphism. Laboratory examinations revealed leukopenia and high fasting insulin level (Table 1). Hepatosteatosis was not detected on abdominal ultrasonography. The patient, who was recommended dietary, exercise, and metformin 2x500 mg (oral) treatments, could not adapt to diet and exercise, and did not use metformin regularly. In the oral glucose tolerance test, performed at the age of 13.9 years, serum glucose and insulin levels at the 120th minute were 163 mg/dL and 244 mcIU/mL, respectively. Glycohemoglobin was 5.5% (normal range 4.5-6.5). When she was 17.2 years old, her BMI was 48 kg/m^2 (+4.5 SDS). She had excessive appetite, could not stay on diet and did not use metformin treatment regularly because of nausea and dizziness. She was recommended orlistat 2x120 mg orally in addition to diet, exercise, and metformin treatment. Since the patient had early onset severe obesity and hyperphagia, a gene panel targeted for genetic obesity was performed. The patient was heterozygous for the c.206T>G(p.I69R) (NM_005912.3) variant in the *MC4R* gene, which had been previously described (10,11). Since this variant was not detected in the parents, it was considered *de novo*. When the patient was 17.9 years old, her BMI was 52.87 kg/m^2 (+4.89 SDS), and upon obtaining the consent of the family, liraglutide treatment was initiated (8). In the first week of treatment, a dose of 0.6 mg/day was administered subcutaneously, then the dose was increased by 0.6 mg once a week, and increased to the full dose (3 mg/day) in the fifth week. Her appetite reduced with treatment. Weight loss achieved after five weeks of treatment was 4.8%. However, nausea, bloating, belching, intermittent abdominal pain, and gas-related pain became more pronounced with the increase to the full dose. Since gastrointestinal side effects associated with liraglutide therapy have been described (8,9), the dose was reduced to 1.8 mg/day, which the patient reported she could tolerate. During the treatment process, the menstrual cycle delayed once. When the dose of liraglutide was increased to 2.4 mg/day, due to slowing of the weight reduction rate, the gastrointestinal complaints recurred and the dose was reduced again to 1.8 mg/day. At the end of 32 weeks of regular use of the drug, a 19.2% reduction was achieved in her BW and BMI (Table 2). Since gastrointestinal

complaints started with the initiation of the drug and became more pronounced with increasing the dose, and were relieved with dose reduction, these gastrointestinal symptoms were thought very likely to be drug side effects. The patient, who could not tolerate the gastrointestinal side effects, was observed to gain weight when she stopped taking the drug for two weeks. After starting the drug at a dose of 1.8 mg/day, the weight gain stopped, but the patient decided to discontinue the drug and did not come to the follow up visits after 43 weeks of treatment initiation. When the patient was contacted by phone, it was learned that she returned to her pre-treatment weight (145 kg) a few months after she discontinued the drug.

Table 1. Results of laboratory analysis of the patient on first admission

Test	Results	Reference range
White blood cell count (10^3/mL)	2.5	4.3-10.3
Hemoglobin (g/dL)	12.6	13.6-17.2
Mean corpuscular volume (fL)	80.7	80.7-95.5
Platelet count (10^3/mL)	242.000	150-400
Alanine aminotransferase (IU/L)	15	0-55
Aspartate aminotransferase (IU/L)	19	5-34
Uric acid (mg/dL)	5.4	2-5.5
Total cholesterol (mg/dL)	151	< 170
Low density lipoprotein (mg/dL)	98	< 130
High density lipoprotein (mg/dL)	44	40-60
Triglyceride (mg/dL)	45	< 150
Free thyroxine (ng/dL)	0.89	0.65-2.3
Thyroid stimulating hormone (µIU/mL)	3.5	0.33-6.0
Cortisol µg/dL (nmol/L)	9.67 (266.8)	5-23 (138-635)
ACTH pg/mL (pmol/L)	23.6 (5.20)	7.2-63.3 (1.6-13.9)
Cortisol after 1 mg dexamethasone µg/dL (nmol/L)	1.0 (27.6)	< 1.8 (49.7)
Luteinising hormone (IU/L)	0.77	0.1-12 Tanner 3
Follicle stimulating hormone (IU/L)	5.7	1.5-12.8 Tanner 3
Estradiol (pg/mL)	80.2	7-60 Tanner 3
Fasting glucose mg/dL (mmol/L)	97 (5.4)	60-100 (3.3-5.6)
Fasting insulin µIU/mL (pmol/L)	50.8 (352.8)	6-27 (41.7-187.5)
Glucohemoglobin (%)	6	4-6
Oral glucose tolerance test 120' glucose mg/dL (mmol/L) 120' insulin mg/dL (mmol/L)	 112 (6.2) 41.5 (288.2)	 < 140 (< 7.8) < 75 (< 520.8)

ACTH: adrenocorticotropic hormone

Table 2. Changes in body weight and body mass index during liraglutide treatment in the follow-up period

Week	Dosage mg/day s.c	Weight kg	Loss of weight kg (%)	BMI kg/m²	Loss of BMI kg/m² (%)
0	0.6	144.8	-	52.87	-
5	3*	137.9	6.9 (4.8)	50.32	2.55 (4.8)
8	1.8	134	10.8 (7.5)	48.90	3.97 (7.5)
19	2.4	130.8	14.0 (9.7)	47.75	5.12 (9.7)
26	2.4*	124	20.8 (14.4)	45.27	7.6 (14.4)
32	1.8*	117	27.8 (19.2)	42.72	10.15 (19.2)
36	After two weeks of treatment cessation	124	20.8 (14.4)	45.27	7.6 (14.4)
38	1.8	124	20.8 (14.4)	45.27	7.6 (14.4)
43	1.8	126	18.8 (13.0)	46.00	6.87 (13.0)
56	0 (13 weeks after treatment cessation)	145**			

*Significant gastrointestinal side effects. s.c: subcutaneus. **Weight measurement at home was learned over the phone.
BMI: body mass index

Genetic Methods

DNA obtained from the patient's peripheral blood sample for genetic analysis was subjected to fragmentation, barcoding, library creation, target enrichment and amplification, and loaded into the next generation sequencing device according to the protocol suggested by the manufacturer (MiSeq, Illumina, San Diego, California). A custom panel containing 41 obesity-related genes (*DYRK1B, LEP, LEPR, MC4R, NR0B2, POMC, UCP3, ADRB2, ADRB3, AGRP, MC3R, NTRK2, PCSK1, SIM1, CARTPT, ENPP1, PPARB, PPARGC SDC3, UCP1, ADIPOQ, NAMPT, CFD, RETN, PPARGC1A, CCK, NPY, SLC2A4, ADD1, SREBF1, PTPN1, IRS-1, GHRL, BDNF, NEGR1, SH2B1, GIPR, TMEM18, FTO, SLC22*) was used for sequencing. Bioinformatics analyzes were performed using Qiagen Bioinformatics solutions (Quiagen, Hilden, Germany) software (QCI Analyze Universal 1.5.0 and Qiagen Clinical Insight Interpret) (4). The c.206T>G, p.I69R variant in *MC4R* gene detected and was also analyzed and confirmed by Sanger sequencing. The amplicon was analyzed by direct sequencing with ABI 3500 (Life Technologies, Waltham, MA, USA). Analysis of the sequence result was performed by Variant Surveyor Programme (SoftGenetics, USA).

Discussion

In this study, an adolescent obese girl with a heterozygous variant in *MC4R* gene who was treated with liraglutide was presented. She achieved weight loss with liraglutide treatment, but could not continue therapy due to gastrointestinal side effects and regained weight after discontinuing the drug. MCR4 defects are characterized by early onset severe obesity, hyperphagia (more prominent especially in younger ages), increased linear growth, and insulin resistance (1). Our patient had excessive appetite and hyperphagia from infancy, and her obesity was worsening. After considering that her obesity may have a genetic cause, gene panel testing was performed and a heterozygous, sporadic variant, c.206T>G, p.I69R, was detected in the *MC4R* gene This variant was previously reported in two morbidly obese children of Iraqi origin (11,12). In keeping with this previous report, the pubertal development of the presented case case was normal and menarche age was 13.5 years. In addition to clinical and laboratory findings of insulin resistance, she also had cyclical neutropenia and recurrent urinary tract infection. It was thought that these findings, which were not described in the previous report, may be incidental.

A standard method for obesity management has not been defined in obese patients with *MC4R* variants. In some of the studies investigating the effect of lifestyle change on weight loss in these patients, it was reported that patients with variants achieved weight loss similar to controls, but this could not be sustained in the long term (6,13). Trier et al. (7) reported that BMI SDS could be reduced in the control group after an average of 1 year of lifestyle change but not in cases with *MC4R* variants. Initially, lifestyle changes and oral metformin were recommended to our patient for obesity management. However, she could not limit food intake, continued to binge, and BMI, and BMI SDS increased at each visit. The addition of orlistat, which is an Food and Drug Administration (FDA) approved drug in the treatment of obesity in children, was also not effective. It has been reported that patients with heterozygous variants in the *MC4R* gene experienced a similar weight loss (6%) compared to the control group after 16 weeks of treatment with GLP-1A liraglutide (3 mg/d, subcutaneous) (8). There were no studies reporting long term data and evaluation after the discontinuation of the treatment. Bariatric surgery, especially with gastric bypass, has been reported to have similar results in terms of weight loss for 1-3 years, in patients with *MC4R* defect compared to control obese patients. In some studies it has been shown that the long-term effects continued for 5-7 years and in others it was reported that the patients regained weight at the end of 5 years; notably, some studies have not elucidated the mechanisms of pathogenicity of the variants (10).

Liraglutide was approved by the FDA in 2014 for the treatment of obesity in adults. Later, in April 2020, the FDA approved the use of the drug in adolescents who met the criteria of age ≥12 years, BMI ≥30 kg/m^2 and BW >60 kg). In the presented patient, liraglutide treatment was started on 20th June 2019, only 1-2 months before she was 18 years of age. In our patient, after 32 weeks of treatment with liraglutide, a 19.2% reduction was achieved in BW and BMI. However, the treatment could not be continued due to intolerable gastrointestinal complaints, especially at doses above 1.8 mg/day. It was learned that the patient returned to her initial weight within months after stopping the treatment. Gastrointestinal complaints were the most commonly reported side-effects during liraglutide therapy. In clinical studies of adult obese patients, it was reported that these side effects were usually well tolerated but also that they led to discontinuation of treatment in a small proportion of patients (1.4-2.9%) (9).

Since appetite control is poor due to genetic pathology in this group of patients, it seems that it is more difficult to maintain long-term effectiveness of the treatment. Therefore, in order to increase success in obesity management and to maintain it for a longer period, the side effects of existing treatment options should be decreased or surgical and medical

treatments should be combined or new treatment options should be investigated.

Conclusion

Monogenic obesity should be considered in patients with early onset obesity and in whom appetite control cannot be achieved. In this case with a known *MC4R* variant, liraglutide treatment provided a decrease in appetite and 19.2% reduction in BW and BMI after 32 weeks of treatment. However, the treatment could not be continued due to side effects and she returned to her previous weight after a period of a few months after the discontinuation of the drug. In such cases, there is a need for effective treatment options with tolerable side effects for effective long-term management.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Emine Çamtosun, Ayşehan Akıncı, Leman Kayaş, Nurdan Çiftci, Concept: Emine Çamtosun, Ayşehan Akıncı, Design: Emine Çamtosun, Ayşehan Akıncı, Data Collection or Processing: Emine Çamtosun, Analysis or Interpretation: Emine Çamtosun, Ayşehan Akıncı, İbrahim Tekedereli, Literature Search: Emine Çamtosun, Writing: Emine Çamtosun, Ayşehan Akıncı, İbrahim Tekedereli.

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References

1. Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S. Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. N Engl J Med 2003;348:1085-1095.
2. OMIM. Melanocortin 4 Receptor; MC4R. Last Accessed Date: 09.05.2023. Available from: https://www.omim.org/entry/155541
3. Collet TH, Dubern B, Mokrosinski J, Connors H, Keogh JM, Mendes de Oliveira E, Henning E, Poitou-Bernert C, Oppert JM, Tounian P, Marchelli F, Alili R, Le Beyec J, Pépin D, Lacorte JM, Gottesdiener A, Bounds R, Sharma S, Folster C, Henderson B, O'Rahilly S, Stoner E, Gottesdiener K, Panaro BL, Cone RD, Clément K, Farooqi IS, Van der Ploeg LHT. Evaluation of a melanocortin-4 receptor (MC4R) agonist (Setmelanotide) in MC4R deficiency. Mol Metab 2017;6:1321-1329. Epub 2017 Jul 8
4. Akıncı A, Türkkahraman D, Tekedereli İ, Özer L, Evren B, Şahin İ, Kalkan T, Çürek Y, Çamtosun E, Döğer E, Bideci A, Güven A, Eren E, Sangün Ö, Çayır A, Bilir P, Törel Ergür A, Ercan O. Novel Mutations in Obesity-related Genes in Turkish Children with Non-syndromic Early Onset Severe Obesity: A Multicentre Study. J Clin Res Pediatr Endocrinol 2019;11:341-349. Epub 2019 Apr 17
5. Aykut A, Özen S, Gökşen D, Ata A, Onay H, Atik T, Darcan Ş, Özkinay F. Melanocortin 4 receptor (MC4R) gene variants in children and adolescents having familial early-onset obesity: genetic and clinical characteristics. Eur J Pediatr 2020;179:1445-1452. Epub 2020 Mar 18
6. Reinehr T, Hebebrand J, Friedel S, Toschke AM, Brumm H, Biebermann H, Hinney A. Lifestyle intervention in obese children with variations in the melanocortin 4 receptor gene. Obesity (Silver Spring) 2009;17:382-389. Epub 2008 Nov 6
7. Trier C, Hollensted M, Schnurr TM, Lund MAV, Nielsen TRH, Rui G, Andersson EA, Svendstrup M, Bille DS, Gjesing AP, Fonvig CE, Frithioff-Bøjsøe C, Balslev-Harder M, Quan S, Gamborg M, Pedersen O, Ängquist L, Holm JC, Hansen T. Obesity treatment effect in Danish children and adolescents carrying Melanocortin-4 Receptor mutations. Int J Obes (Lond) 2021;45:66-76. Epub 2020 Sep 13
8. Iepsen EW, Zhang J, Thomsen HS, Hansen EL, Hollensted M, Madsbad S, Hansen T, Holst JJ, Holm JC, Torekov SS. Patients with Obesity Caused by Melanocortin-4 Receptor Mutations Can Be Treated with a Glucagon-like Peptide-1 Receptor Agonist. Cell Metab 2018;28:23-32. Epub 2018 May 31
9. Scott LJ. Liraglutide: a review of its use in the management of obesity. Drugs 2015;75:899-910.
10. Vos N, Oussaada SM, Cooiman MI, Kleinendorst L, Ter Horst KW, Hazebroek EJ, Romijn JA, Serlie MJ, Mannens MMAM, van Haelst MM. Bariatric Surgery for Monogenic Non-syndromic and Syndromic Obesity Disorders. Curr Diab Rep 2020;20:44.
11. Wangensteen T, Kolsgaard ML, Mattingsdal M, Joner G, Tonstad S, Undlien D, Retterstol L. Mutations in the melanocortin 4 receptor (MC4R) gene in obese patients in Norway. Exp Clin Endocrinol Diabetes 2009;117:266-273. Epub 2009 Mar 19
12. Khandelwal D, Birla S, Sharma A, Khadgawat R. MC4R Variant in Early-onset Severe Childhood Obesity-Genotype-phenotype Correlation, US Endocrinology 2017;13:69.
13. Hainerová I, Larsen LH, Holst B, Finková M, Hainer V, Lebl J, Hansen T, Pedersen O. Melanocortin 4 receptor mutations in obese Czech children: studies of prevalence, phenotype development, weight reduction response, and functional analysis. J Clin Endocrinol Metab 2007;92:3689-3696. Epub 2007 Jun 19