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Sample References

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

Papers Only Published with DOI Numbers: Knops NB, Sneeuw KC, Brand R, Hile ET, de Ouden AL, Wit JM, Verloove-Vanhorick SP. Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. BMC Pediatrics 2005 doi: 10.1186/1471-2431-5-26.

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

Books: Practical Endocrinology and Diabetes in Children. Raine JE, Donaldson MDC, Gregory JW, Savage MO. London, Blackwell Science, 2001;37-60.

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Review

Original Articles

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20-23 May 2017, Lisbon, Portugal

ECO 2017 (24th European Congress on Obesity)
17-20 May 2017, Porto, Portugal

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Review

Current Status of Childhood Hyperinsulinemic Hypoglycemia in Turkey

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Ankara University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey

ABSTRACT

Congenital hyperinsulinism (CHI) is a rare disease characterized by dysregulated insulin secretion from pancreatic β-cells. Recurrent hypoglycemia can lead to neurological insult and permanent brain injury. Recently, there are important advances in understanding the genetic mechanisms, histological characteristics, imaging, and surgical techniques of congenital hyperinsulinemic hypoglycemia that could reflect to improvement in the clinical care of infants with this disorder. In Turkey, there is a high rate of consanguinity, thus, the incidence of CHI is expected to be high. Until now, there are no nationwide data regarding the disorder, and some individual case reports or case series had been published. Determining the characteristics of Turkish patients with CHI can help develop a different perspective on this rare disease. In this review, we evaluated the clinical and molecular characteristics of Turkish patients with CHI based on reports published in the literature. The most frequently seen mutations were *ABCC8* gene mutations (n=37), followed by *HADH* (n=11) and *KCNJ11* gene (n=7) mutations. A total of 141 Turkish patients with CHI were reported until now. Among them, 115 patients had been genetically analyzed, and 56 of them had one of the mutation leading to hyperinsulinism.

Keywords: Hyperinsulinism, hypoglycemia in infancy, congenital hyperinsulinism, hyperinsulinemic hypoglycemia

Conflict of interest: None declared
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Introduction

Hyperinsulinemic hypoglycemia (HH) consist of a group of heterogeneous disorders characterized by unregulated insulin secretion from pancreatic β-cells (1). It is the commonest cause of both persistent and transient hypoglycemia in neonates and infants (2,3,4). Because there can be severe brain damage related to hypoglycemia, it is vital to diagnose and treat patients with HH correctly (1,2,3,4).

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The clinical presentation of HH can be very heterogeneous ranging from very subtle presentation to severe disease needing pancreatectomy (2,3,4,5). It can be either permanent or transient; the permanent form is usually known as congenital. Transient forms of congenital hyperinsulinism (CHI) usually occur in newborns with certain risk factors like maternal diabetes mellitus, intrauterine growth retardation, perinatal asphyxia. Some case with transient HH might have *HNF4A* gene, *HNF1A* gene, or ATP-sensitive potassium (K_{ATP}) channel mutations.

CHI is the most severe and persistent form of hereditary HH (1,2,3,4). Persistent CHI can be caused by mutations in nine genes regulating the insulin secretion from the β-cells (*ABCC8, KCNJ11, GLUD1, HADH, GCK, HNF4A, HNF1A, SLC16A1*, and *UCP2* genes) (6,7). The most common causes are mutations in the *ABCC8* and *KCNJ11* which encode the SUR1 and Kir6.2 subunits of the pancreatic β-cell K_{ATP} channel (2,3,4). K_{ATP} channels in pancreatic β-cells regulate the flux of K ions across cell membranes. Glucose phosphorylation by glucokinase controls glucose-regulated insulin secretion. Glucose 6 phosphatase produces ATP by getting metabolized. This leads to an increase in intracytosolic ATP:ADP ratio. Increased ATP:ADP ratio inhibits the activity of the K_{ATP} channel. Following from this, ATP channel gets closed and membrane depolarization occurs. From this point on, voltage-dependent calcium channels open resulting in more calcium going into the beta cells. Higher concentrations of calcium trigger secretory granules to release insulin (8,9).

Mutations in ABCC8 and KCNJ11 can be either autosomal-recessive or autosomal-dominant (10).

Histologically, CHI is classified in three subgroups: diffuse, focal, and atypical forms. Most of the times, diffuse form is inherited autosomal-recessively. Autosomal dominant hereditation is also seen but not as often. Focal form, which occurs sporadically, can be seen with the combination of paternal heterozygous germline mutation in one of the *ABCC8* and *KCNJ11* genes and uniparental disomy of the maternal chromosome 11p15. In patients with atypical disease, the histological abnormalities may be diffuse with coexistence of normal and abnormal islets (9).

The incidence of CHI was reported as 1 in 40 000 in the general population to 1 in 2500 in certain communities (11). In Turkey, there is a high rate of consanguinity, thus the incidence of CHI is expected to be high (12,13). Until now, there are no nationwide data regarding this disorder, and only some individual case reports and studies including a total of 141 patients had been published (6,13,14,15,16,17,18, 19,20,21,22,23,24,25,26,27,28,29,30,31,32). Determining the characteristics of Turkish patients with CHI may gain a different perspective to this rare disorder.

In this review, we aimed to evaluate the all published papers reporting Turkish patients with CHI and to explore both diagnostic and follow-up characteristics of these patients.

Methods

The PubMed, SCOPUS, and Web of Science electronic databases were systematically searched from inception to February 10, 2016. The search terms were "hyperinsulinism" or "CHI" or "nesidioblastosis" or "hypoglycemia" or "persistent hyperinsulinemic hypoglycemia of infancy" or "hyperinsulinemic hypoglycemia of infancy" or "HI" or "hyperinsulinaemic" or "hyperinsulinemic" and "Turkey" or "Turkish". No search filters or language restrictions were imposed. All case reports, case series, and studies on Turkish patients with HH were evaluated. Papers reporting other than congenital or persistent HH were excluded from the analysis. The data of cases in authors' center were also given.

General Characteristics of Patients

A total of 18 manuscripts reporting Turkish patients with CHI had been published. A total number of 12 case reports including 1 to 3 patients were published between 1997 and 2016. Because some patients were presented in different papers, we counted them once. Only six papers reporting 4 to 35 patients' data had been published, including some patients individually reported before. The number of all reported Turkish patients was 141. Among them, 115 patients had undergone molecular genetic analysis, and 56 of them had one of the mutations leading to hyperinsulinism. A total of 26 patients had not undergone molecular studies (6,13,14,15,16,17,18,19,20, 21,22,23,24,25,26,27,28,29,30,31,32).

Admission Characteristics of Turkish Patients

It was reported that the most common presentation of HH is during the neonatal period (1,2,3,4). These finding is similar to the characteristics of Turkish patients. The majority of reported Turkish patients were diagnosed during the neonatal period.

Delivery of macrosomic fetus is a sign of intrauterine hyperinsulinemic state. Among the reported Turkish cases, the rate of macrosomic babies was 34%.

Etiology of Turkish Hyperinsulinemic Hypoglycemia of Patients

HH can be transient. Among the causes for transient form of HH, maternal diabetes mellitus, intra-uterine growth retardation, perinatal asphyxia, erythroblastosis fetalis, maternal administration of drugs, intravenous glucose infusions during delivery, as well as Sotos syndrome can be counted (10,33). There were only two papers reporting prolonged transient HH in Turkish children (15,32). Although transient HH usually resolves spontaneously in a few weeks, in some patients, the duration of hyperinsulinism can be protracted, requiring diazoxide treatment (10). In those patients, the mechanism for hyperinsulinism is not clear. Güven et al (32) reported a case series of CHI patients, and in 7 of 13 patients which responded to diazoxide therapy, treatment had been stopped between 15

days to 12 years. Ağladıoğlu et al (15) presented 17 HH cases in their series, eight of which had transient HH; diazoxide therapy had been ceased between 3 to 154 months. No etiological or molecular genetic studies were done in these patients.

Recently, it has appeared that some transient HH patients have *HNF4A* gene, *HNF1A* gene, or K_{ATP} channel mutations (16,34). HNF4-MODY and *HNF1A-MODY* were well-known subtypes of autosomal-dominant diabetes. Early postnatal HH could be accompanied in individuals with those mutations (34).

Huopio et al (35) described the first dominantly inherited *ABCC8* mutation (E1507K, previously reported as E1506K) that caused HH in early life and predisposed to early-onset insulin deficiency. It was suggested that some adults with a dominant K_{ATP} channel mutation were asymptomatic, and these mutations could have been missed in infancy (36).

We followed up two siblings with transient HH who were heterozygous for *ABCC8* missense mutation, A1367D (c.4100C>A; p.Ala1367Asp). The clinical presentation of these patients with a dominant *ABCC8* mutation was milder than that of patients with the recessive form of the disease; they responded well to medical therapy. Both siblings have been diagnosed with autoantibody-negative diabetes mellitus during the prepubertal period following a remission of the HH in childhood. Their mother, maternal aunt, and maternal grandfather were also heterozygous for the same mutation. The mother was diagnosed with type 2 diabetes mellitus at the age of 28 years, and the maternal aunt and grandfather had a medical history of postprandial hypoglycemic attacks; there was no family history of hypoglycemia in neonatal period.

Another interesting patient that we diagnosed as transient HH was born after caesarian delivery with birth weight of 4250 g. He had been diagnosed with nonketotic HH at 2 days of life (when blood sugar was 38 mg/dL and serum insulin level was found as 7.3 mIU/mL). The patient responded well to diazoxide treatment in a dose of 10 mg/kg/day. During follow-up, diazoxide dose was tapered gradually and ceased at 3 months of life. Now, he is 2.5 years of age and good in condition. He was heterozygous for a novel *KCNJ11* gene missense mutations and a *KCNJ11* frameshift mutation [Exon1B/Exon1C;c.130G>A/c.405dup; p.Val144Met/p.Arg136fs (p.V44M/p.R136fs)]. The patient had inherited the p.V44M mutation from his clinically unaffected mother.

In the light of these findings, it should be kept in mind that transient HH in infancy can be related to K_{ATP} channel mutations, and diabetes mellitus may develop later in life.

Genetic Mutations of Patients

Molecular studies showed that the congenital form of HH is due to mutations in eight different genes (*ABCC8, KCNJ11, GLUD1, CGK, HADH, SLC16A1, HNF4A*, and *UCP2*). Most of the CHI cases are caused by K_{ATP} channel mutations. K_{ATP} channels in pancreatic β-cells are composed of four inward-rectifying potassium channel (Kir6.2) subunits and four high-affinity sulfonylurea receptor 1 (SUR 1) subunits (37). After excluding the recurrent reported cases, a total of 115 cases had been analyzed for genetic mutations; in 26 cases, no genetic analysis had been carried out. 44 of patients with mutation analysis done had K_{ATP} channel genes mutations (Figure 1). The most frequently seen mutations were *ABCC8* gene mutations (n=37), followed by *HADH* (n=11), *KCNJ11* (n=7) and *GLUD1* (n=1) gene mutations (6,13,14,15,16,17,18,19,20, 21,22,23,24, 25,26,27,28,29,30,31,32).

ABCC8 gene mutations have been reported as the commonest cause of CHI by Demirbilek et al (24) in the largest cohort consisting of Turkish CHI patients. They estimated the frequency of *ABCC8* mutation in the CHI patients as 40% (14/35). In this cohort, eight different *ABCC8* mutations had been identified. One of the commonest mutations in their cohort (5/14) was p.L1171fs (c.3512del), a frameshift mutation on exon 28 of *ABCC8* gene. Güven et al (32) also reported that 9 of 12 mutation-positive patients had *ABCC8* gene mutation.

KCNJ11 gene mutations had been detected in seven patients (7/56). Three of them were reported in a cohort (24). The authors of this review detected three patients with *KCNJ11* mutation. All except one patient with this mutation were diazoxide-unresponsive and required pancreatectomy. One diazoxide-unresponsive patient with *KCNJ11* gene mutation responded well to octreotide therapy (32).

A female patient's follow-up in the authors' center revealed two novel mutations in the *KCNJ11* gene in exon 1 which were

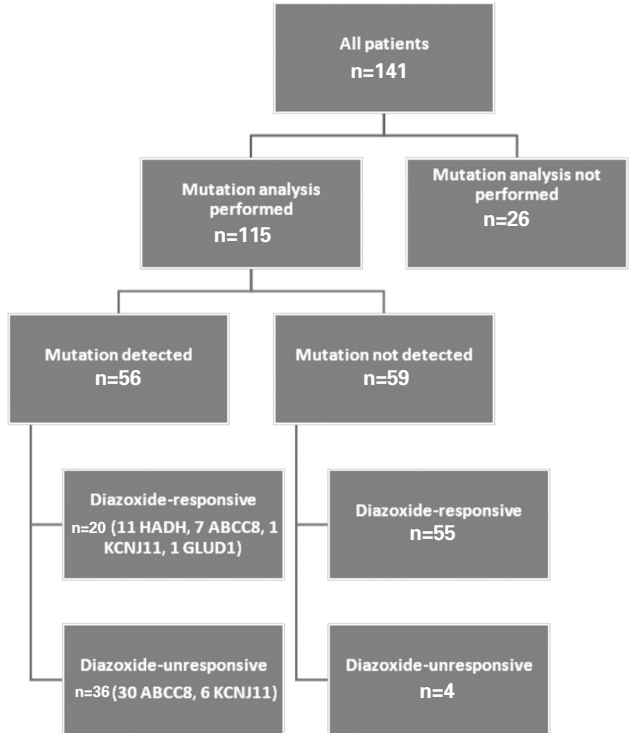

Figure 1. Distribution of patients according to mutation analysis results and diazoxide responsiveness

paternally inherited (p.R221H and p.Q299H). Her father was not clinically affected and mother had no K_{ATP} channel mutation. Interestingly, in this case, maternal loss of heterozygosity between chromosomes 11p15.5 and 11p15.1 covering the region (mosaic uniparental disomy) that may lead to Beckwith-Wiedemann syndrome was found. In histopathological evaluation, diffuse lesions were detected.

The distinction between focal and diffuse forms of hyperinsulinism cannot be made by clinical or biochemical means in cases with CHI. Positron emission tomography/computed tomography by 18 fluoro-L-DOPA (^{18}F-DOPA PET/CT) is a noninvasive scanning tool with the capability to distinct between focal and diffuse forms (38). Unfortunately, ^{18}F-DOPA PET/CT scanning is not available in Turkey.

We observed that *HADH* gene mutations were relatively frequent in Turkish CHI patients, accounting for 20% (11/56) of all mutation-studied cases (6,13,24,32), despite the fact that the mutations in that gene are reported as a rare cause of recessively inherited HH (7,10). *HADH* gene encodes the mitochondrial enzyme L-3-hydroxyacyl-coenzyme A dehydrogenase (*HADH*). Loss-of-function mutations in the *HADH* gene cause short-chain L-3-hydroxyacyl-CoA (SCHAD) deficiency. The mechanism behind unregulated insulin secretion in SCHAD deficiency is not fully understood but may involve changes in protein-protein interactions with glutamate dehydrogenase (GDH) (6).

These patients exhibit severe protein (especially leucine) sensitivity, with some subjects having raised plasma levels of hydroxybutyrylcarnitine as well as elevated urinary levels of medium-chain dicarboxylic and 3-hydroxydicarboxylic metabolites and 3-hydroxyglutarate (6). The clinical presentation is mainly neonatal- or early infancy-onset CHI, but mild late-onset phenotype can be seen (6,10). So far, approximately 40 patients with CHI resulting from a mutation in the *HADH* gene have been reported, and all of them have responded well to diazoxide therapy (6).

Because the consanguinity is high in the Turkish population, occurrence of recessively inherited *HADH* gene mutation in increased rate seems to be inevitable. It is convenient that sequencing of *HADH* gene be recommended in all patients with diazoxide-responsive HH.

The method for detection of *HADH* gene mutation is also important which could be different in the centers. Most of the Turkish patients (n=8) with *HADH* gene mutation were reported by Flanagan et al (13). They detected deep intronic mutations of that gene by using next-generation sequencing analysis which have not been demonstrated before by Sanger sequencing.

Among the reported Turkish patients, there was no case with exercise-induced hyperinsulinism (*SLC16A1*), glucokinase-induced hyperinsulinism, or mutations in the *UCP2, HNF4A,* and *HNF1A* genes, while one patient had *GLUD1* gene mutation (32).

Treatment Modalities of Turkish Patients

For preventing hypoglycemia-related irreversible brain damage, aggressive and early intervention remains the mainstay of treatment in HH. The first-line drug management of persistent CHI is diazoxide therapy. Diazoxide binds to SUR1 component of K_{ATP} channels resulting in their opening. It is effective in most of the CHI forms except in those due to recessive (and some dominant) inactivating mutations in *ABCC8* and *KCNJ11* and in patients with focal CHI (10). The second-line medical treatment is octreotide therapy. If octreotide fails to control the hypoglycemia, surgical intervention is usually required.

Most of the Turkish patients with CHI were responsive to diazoxide treatment (100/141, 71%), while 18 of them had the transient form. Among patients with reported genetic mutation analysis (n=115), 75 were diazoxide-responsive.

All Turkish patients with *HADH* gene mutation were diazoxide-responsive, thus no surgical treatment was applied, as expected. Pancreatectomy was implemented in 28 of diazoxide-unresponsive CHI patients which accounts for 19.8% of all CHI patients. All of them, except one, had K_{ATP} channel mutations.

Pathological examination of pancreatic tissues revealed the diffuse form of hyperinsulinism in 27 of 28 cases. Moreover, the focal form of hyperinsulinism has been reported in the literature in almost half of all patients treated surgically (7); only one patient with focal form has been reported from our country (32).

Because ^{18}F-DOPA PET/CT is not available in our country, genetic analysis would be important to discriminate the focal disease before the decision of pancreatectomy. Even in patients with mutation analysis indicating focal disease, this form might not be pathologically proven in every case. It is suspected that: Focal disease might not really have been absent among the patients. The pathological examination might have been insufficient in some patients.

It has been reported that pancreatectomy is associated with a high incidence of diabetes mellitus and pancreatic exocrine insufficiency. For that reason, surgical treatment should be reserved for the cases with unsuccessful medical treatment (37,39). Among the reported Turkish patients with CHI, there is very scarce long-term information after pancreatectomy with aspect to development of diabetes and other possible complications. In a case series, follow-up time was relatively low as near 6 years (32). In addition, there is no knowledge about disease-free survival. It would be important to demonstrate the follow-up of characteristics revealing the improvement in the management of these patients.

Very recently, a new medical treatment option has emerged for diffuse CHI unresponsive to diazoxide and/or octreotide treatment. The mammalian target of rapamycin inhibitor sirolimus has been successfully used in some diazoxide- and octreotide-unresponsive CHI patients. It reduces the pancreatic B-cell proliferation and inhibits insulin production (40).

Neurological outcome: One of the most important long-term complications of CHI is severe brain damage such as cerebral palsy, epilepsy, developmental delay (1,2,3). Among the reported cases from Turkey, there are no extensive data about long-term neurological consequences. From the two largest series, it was seen that neurological sequelae were

encountered in almost one third of patients (34% and 29%) (15,24). Especially diazoxide-unresponsive patients were under high risk for development of neurological sequelae.

The most favorable neurological outcomes were seen in patients with *HADH* gene mutation. The longest follow-up was reported in a female patient with *HADH* gene mutation who had no neurological findings (6).

Early and aggressive treatment of patients with severe CHI is necessary to prevent brain damage, and diazoxide responsiveness gives an important clue for good prognosis and further treatment.

In conclusion, CHI is a heterogeneous disorder. K_{ATP} channel mutation is the most frequent etiological factor among mutation-studied Turkish patients with CHI. Patients with *HADH* gene mutation are relatively frequent among them. Transient form of HH in infancy could be caused by K_{ATP} channel mutations, and diabetes mellitus may develop later in life. Because long-term neurological damage is high in diazoxide-unresponsive patients, early and prompt intervention is needed in such patients. There is no clear information and follow-up data, including development of diabetes, about patients who have undergone pancreatectomy. Multicenter studies are needed to obtain long-term follow-up characteristics of such patients at national base.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Zeynep Şıklar and Merih Berberoğlu, Design: Zeynep Şıklar and Merih Berberoğlu, Data Collection and Processing: Zeynep Şıklar, Analysis and Interpretation: Zeynep Şıklar and Merih Berberoğlu, Literature Research: Zeynep Şıklar, Writing: Zeynep Şıklar and Merih Berberoğlu.

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Original Article

Breast-Milk Iodine Concentrations, Iodine Status, and Thyroid Function of Breastfed Infants Aged 2-4 Months and Their Mothers Residing in a South African Township

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ABSTRACT

Objective: Lactating women and their infants are susceptible to iodine deficiency and iodine excess. In South Africa, no data exist on the iodine status and thyroid function of these vulnerable groups.

Methods: In a cross-sectional study, urinary iodine concentrations (UIC), thyroid function, and breast-milk iodine concentrations (BMIC) were assessed in 100 lactating women from a South African township and their 2-4-month-old breastfed infants. Potential predictors of UIC, thyroid function, and BMIC, including household salt iodine concentrations (SIC) and maternal sodium excretion, were also investigated.

Results: The median (25^{th}-75^{th} percentile) UIC was 373 (202-627) µg/L in infants and 118 (67-179) µg/L in mothers. Median household SIC was 44 (27-63) ppm. Household SIC and maternal urinary sodium excretion predicted UIC of lactating mothers. Median BMIC was 179 (126-269) µg/L. Age of infants, SIC, and maternal UIC predicted BMIC. In turn, infant age and BMIC predicted UIC of infants. Forty-two percent of SIC values were within the South African recommended salt iodine fortification level at production of 35-65 ppm, whilst 21% of SIC were >65 ppm. Thyroid-stimulating hormone, total thyroxine, and thyroglobulin concentrations in the dried whole blood spot specimens from the infants were 1.3 (0.8-1.9) mU/L, 128±33 mmol/L, and 77.1 (56.3-105.7) µg/L, respectively, and did not correlate with infant UIC or BMIC.

Conclusion: Our results suggest that the salt fortification program in South Africa provides adequate iodine to lactating women and indirectly to their infants via breast milk. However, monitoring of salt iodine content of the mandatory salt iodization program in South Africa is important to avoid over-iodization of salt.

Keywords: Breast-milk iodine concentration, urinary iodine concentration, salt iodine concentration, lactating women, infants, thyroid hormones

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

South African school children and women of reproductive age have adequate iodine intake. However, more recent data point gaps in iodine nutrition of South Africans, as more than a third of the population still lacks access to adequately iodized salt. Furthermore, no data exist on iodine status in lactating women and infants. Breast-milk iodine concentrations are dependent on maternal iodine intake.

WHAT THIS STUDY ADDS?

This is the first study to report iodine status, breast-milk iodine concentrations (BMIC), and thyroid function of breastfed infants and lactating mothers in South Africa. Iodine in household salt (SIC) and maternal urinary iodine concentration (UIC) were predictors of BMIC, which in turn predicted the UIC of infants. Our results indicate a successful universal salt iodization program in South Africa providing adequate iodine for infants via breast milk. However, fortification of salt needs to be monitored, to avoid over-iodization of salt.

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Introduction

Dietary iodine is an essential substrate for thyroid hormone [thyroxine (T_4) and triiodothyronine (T_3)] synthesis and is as such required for normal brain development, growth, and metabolism (1). Both low and high iodine intake can lead to thyroid dysfunction (2). Infants may be particularly vulnerable to iodine deficiency and iodine excess as the fetal and newborn thyroid has limited iodine stores and adapts poorly to high intakes (3,4,5). Acute iodine excess from for example maternal iodine supplements and iodine containing skin disinfectants may cause hypothyroidism in newborns (6,7). Recent data indicate that older infants may be able to adapt to high iodine intakes and maintain their euthyroid state (8). However, little is known about the effects of habitual high iodine intake on thyroid function in breastfed infants.

Programs of universal salt iodization have made remarkable progress in improving iodine status worldwide, but in a handful of countries, salt iodine fortification is poorly monitored and the iodine intake is excessive (1,5).

In South Africa, in order to achieve a level of 30 ppm at retail and 15 ppm in households, iodization of table salt at a concentration of 35-65 ppm at the point of production was revised in 2006/2007 (9,10). The legislation does not involve fortification of agricultural salt or salt for processed foods. The introduction of universal salt iodization remarkably improved the iodine status of school children and of women of reproductive age. The 2005 South African Food-Based National Food Consumption Survey (NFCS-FB-I) reported a median urinary iodine concentration (UIC) in South African school children and women of reproductive age of 215 µg/L and 177 µg/L, respectively, indicating overall adequate iodine intake (11). However, more recent data point gaps in iodine nutrition of South Africans, as more than a third of the population still lacks access to adequately iodized salt (9,12). Furthermore, no data exist on iodine status in lactating women and infants.

The iodine requirements as recommended by World Health Organization (WHO) increase to 250 µg during lactation: additional to the recommended daily intake of 150 µg iodine for women of reproductive age, lactating women should consume 100 µg/day extra in order to cover the additional iodine need of their breastfed infants (10). Breastfed infants depend on iodine from breast milk for the synthesis of thyroid hormones and to build up intra-thyroidal iodine stores (13,14). Breast-milk iodine concentrations (BMIC) are determined by the maternal iodine intake; population medians have been shown to range from 9-32 µg/L in iodine deficient goitrous areas to 146 µg/L in iodine sufficient Chinese women (15,16). The WHO recommends a dietary iodine intake of 90 µg/day for infants aged 0-6 months (10).

Population iodine status is assessed by UIC, as 90% of ingested iodine is excreted through the renal system and median spot UIC directly reflects recent dietary iodine intake (1,10). In lactating women and in children <2 years, a median UIC <100 µg/L indicates insufficient iodine intake (10).

Measurement of serum or dried blood spot thyroglobulin (Tg) can be an additional useful biomarker of iodine status to accompany UIC measurements. Zimmermann et al, (17) showed that Tg is a sensitive marker for both low and high iodine intakes in school-aged children. Tg is also a sensitive indicator for iodine deficiency in adults (18,19).

Despite the importance of adequate iodine status and thyroid health in lactating women and their breastfed infants, to date, no data exist on BMIC or iodine status of infants and lactating South African women. This study therefore assessed iodine status, BMIC, and thyroid function of breastfed infants and their lactating mothers living in a township located in the North-West Province of South Africa and further explored potential predictors of UIC, thyroid function, and BMIC.

Methods

Participants

This study included a convenient sample of 100 apparently healthy infants aged 2-4 months and their lactating mothers residing in two peri-urban settlements (Ikageng and Promosa) on the fringes of Potchefstroom in the Kenneth Kaunda District municipal area, in the North West Province of South Africa. The majority of residents in these townships are of Black African descent, the socio-economic status is low, and unemployment is high. Recruitment of mother-infant pairs was done at local health clinics in Ikageng and Promosa. Infants included in the study were: 1) generally healthy; 2) singletons; 3) had no history of thyroid disease; 4) currently being breastfed; and 5) not using any iodine containing supplements. Mothers included in the study were: 1) generally healthy; 2) had no history of thyroid disease; 3) currently breastfeeding; and 4) not using any iodine-containing supplements.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Health Research Ethics Committee of the North-West University (NWU-00016-13-A1). Permission was also granted from the Provincial and District Health Departments in the North West Province to recruit mother-infant pairs for this study at local health clinics. The study protocol was fully explained by a trained study assistant fluent in the local language (Setswana or Afrikaans) and written informed consent was obtained from participating women.

Data Collection

The study design was cross-sectional. Lactating mothers and their infants were invited to the metabolic clinic at the North-West University, South Africa, where the study procedures were conducted between 08:00 am and 12:00 am. Mothers were asked to bring samples of salt (10 g)

and water (10 mL) from their homes. Upon arrival, the study protocol was fully explained to the mothers in their home language (Setswana or Afrikaans) and they signed informed consent. A detailed questionnaire was used to collect information on socio-economic characteristics, use of iodized salt, consumption of iodine-containing foods, use of iodine-containing supplements (currently and during pregnancy), and breastfeeding practices. Breastfeeding practices were divided into three categories, namely: 1) Exclusive breastfeeding; 2) Predominantly breastfeeding; 3) Partial breastfeeding (20).

Height and weight of mothers and weight, length, and head circumference of infants were measured using standard anthropometric techniques (21). For the measurements, mothers removed their shoes, emptied their pockets, and wore minimal clothing. Height measurements were done using a rigid stadiometer and recorded to the nearest 0.5 cm. Weights of the women were measured on a high capacity electronic flat scale (seca 813; Germany) and recorded to the nearest 0.1 kg. Measurements of infants were done using an infant scale (seca 334; Germany) to the nearest 2 g with no clothing or nappy. To measure length, infants wore only their nappy, and measurements were taken to the nearest centimeter on a ShorrBoard portable height-length measuring board with auto-lock sliding foot piece (Weigh and measure, LLC; USA). Head circumference was also measured using a head circumference measuring tape for infants (seca 212; Germany) to the nearest centimeter. Body mass index-for-age z-scores (BAZ) were calculated using the WHO (2006) growth standards. Wasting was defined as BAZ <-2, normal weight as BAZ ≥-2 and ≤2, risk for overweight as BAZ >1, and overweight as BAZ >2 (22).

A standard breakfast was served to the mothers at arrival at the metabolic clinic and before collection of biological samples. Spot urine samples (5 mL) were obtained from the mothers (within a maximum interval of 30 minutes after breakfast consumption), aliquoted, and stored at -80 ^{0}C until analysis. Breast milk samples (5 mL of foremilk) were obtained by manual expression. To obtain foremilk, mothers were asked to express milk from the breast that was not used at the last feed. The baby was then allowed to suckle the breast until fully satisfied. Breast milk samples were aliquoted and stored at -20 ^{0}C until analysis. Spot urine samples were collected from infants using a urine collection pad (SteriSets Uricol Set), aliquoted, and stored at -80 ^{0}C until analysis. Whole blood obtained via venipuncture or foot prick was spotted onto filter paper (Whatman 903; GE Healthcare) and allowed to dry at room temperature for 24 hours. They were then stored at -20 ^{0}C in sealed low-density polyethylene bags containing desiccant packets until analysis of thyroid hormones.

Laboratory Analysis

UIC in spot urine samples from infants and mothers was measured in duplicate at the North-West University in Potchefstroom using the Pino modification of the Sandell-Kolthoff reaction with spectrophotometric detection (10,23). The laboratory successfully participates in the Program to Ensure the Quality of Urinary Iodine Procedures (EQUIP, U.S. Centers for Disease Control and Prevention, Atlanta GA, USA) (24). Iodine in spot urine samples from infants and lactating mothers were expressed as median concentrations (µg/L). A median UIC greater than 100 µg/L was considered to indicate adequate iodine intake in lactating women and infants (10).

In lactating mothers, we additionally determined the iodine:creatinine ratio (µg iodine/g creatinine) to reduce the intra-individual variation in daily urine volume and also to adjust for fluid intake (25,26). Urinary creatinine and sodium concentrations in spot urine from mothers were analyzed using the UniCel® DxC800 System (Beckman Coulter) at a commercial pathological laboratory (Ampath Johannesburg).

BMIC was analyzed at the Laboratory of Human Nutrition of ETH Zurich, Switzerland (27). Iodine was extracted from the samples using a modified tetramethylammonium hydroxide (TMAH) extraction procedure (28,29). The iodine content in filtered TMAH extracts was measured using a multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS [Finnigan NEPTUNE, Thermo Scientific™ Waltham, MA, USA]). Quantification was done using isotope dilution analysis with ^{129}I (SRM 4949C, National Institute of Standards and Technology [NIST], Gaithersburg, MD, USA). Tellurium (AppliChem, Darmstadt, Germany) was used for mass bias correction of the measured ^{127}I/^{129}I intensity ratio according to Russell's law. The iodine concentrations of the milk samples were calculated using the dilution factors applied to each milk sample. Standard reference material (SRM 1549a, Whole Milk Powder, NIST, Gaithersburg, MD, USA) was analyzed as external control with each ICP-MS run (30). The method was recently validated at the Human Nutrition Laboratory of ETH Zurich, Switzerland. The mean [± standard deviation (SD)] iodine content for the NIST SRM1549a reference material was 3502 (±89) ng/g (n=16), well within the certified acceptable range (3040-3640 ng/g). The total-assay variability of the method is 2.6%. The within-assay variability is 1.1% and the between-assay variability is 1.3%. The limit of detection of the method is 0.26 ng/g.

Dried whole blood spots were analyzed for thyroid-stimulating hormone (TSH) (DELFIA NeoTSH kit, PerkinElmer Life Sciences, Turku, Finland) and total thyroxine (TT_4) (Delfia Neonatal T_4 kit, PerkinElmer Life Sciences, Turku, Finland) using automated fluoroimmunoassay (31). Analysis of dried blood spots-Tg (DBS-Tg) was done by a new sandwich enzyme-linked immunosorbent assay that was recently developed and validated at the Human Nutrition Laboratory of ETH Zurich, Switzerland (32). Serum control samples (Liquicheck Tumor Marker Control, Bio-Rad, Hercules, CA, USA) were used as standards for the DBS-Tg assay. Normal reference ranges for TSH and T_4 as supplied by the manufacturer were as follows: TSH of 0.1-4.5 mU/L and 0.1-3.7 mU/L for 60-155-day-old

infants and for subjects 1-99 years of age, respectively; TT_4 of 80-165 nmol/L and 65-165 nmol/L for 60-155-day-old infants and for subjects 1-99 years of age, respectively. Normal range reference values for DBS-Tg are only available for school-age children (4-40 µg/L) but not for young children and infants (33,34).

Salt iodine concentrations (SIC) and water iodine concentrations (WIC) were determined by using the Pino modification of the Sandell-Kolthoff reaction with spectrophotometric detection (23). Household SIC was expressed as median and classified into three categories, according to ranges in ppm based on the 2006/2007 South African mandatory fortification level for table salt at the point of production (9). Salt samples were considered inadequately, adequately, and over-iodized when SIC was <35 ppm, 35-65 ppm, and >65 ppm, respectively. Household SIC were also classified into the three fortification categories indicating inadequately (SIC <15 ppm), adequately (SIC 15-40 ppm), or excessively (SIC >40 ppm) iodized salt at household level as recommended by WHO (35).

Data on the intake of potentially iodine-rich foods in lactating mothers were collected using an unquantified food frequency questionnaire and presented as the number (%) of mothers who consumed specific iodine-rich foods.

Statistical Analysis

All data processing and analysis were done using IBM SPSS statistics version 20. Data were checked for normality using Q-Q plots and the Shapiro-Wilk test. Normally distributed data were presented as mean ± SD. Non-normally distributed data were presented as median (25^{th}-75^{th} percentiles) values. For between-group comparisons, the Mann-Whitney U or Kruskal-Wallis tests were used for non-parametric data. Spearman correlations were performed to determine associations between variables. Multiple linear regression analyses were used to explore whether household SIC, salt intake of mothers (urinary sodium excretion), UIC of mothers (only for BMIC and UIC of infants as dependent variable), age of mothers and infants, and BMIC (only for UIC of mothers and infants as dependent variables) were predictors of BMIC and UIC in lactating mothers and breastfed infants. Other dietary and maternal factors [e.g. mode of breastfeeding and delivery, smoking habits, human immunodeficiency virus (HIV) status] were also tested using a stepwise procedure, but none of those were significant predictors of BMIC or UIC of mothers and infants and were therefore not included in the final regression models. Non-parametric dependent variables were transformed prior to analysis. Furthermore, we examined the odds ratios (OR) for infants to have abnormal thyroid hormone concentrations with excessive or inadequate iodine intake using binary logistic regression analyses, adjusting for age of mothers and infants, as well as HIV status of mothers. A p-value <0.05 was considered significant.

Results

One hundred mother-infant pairs participated in the study. Characteristics of the infants and mothers are shown in Table 1. Infants were aged 3-4 months (mean ± SD: 3.0±1.1); 54 were females and 46 were males. Of all the infants, 67% were exclusively breastfed, whilst 9% and 24% were predominantly and partially breastfed, respectively.

The median (25^{th}-75^{th} percentiles) UIC of infants (n=92) was 373 (202-627) µg/L (Table 2). The median UIC of mothers was 118 (67-179) µg/L and the iodine:creatinine ratio was 126 (86-207) µg/g. Figure 1 illustrates the frequency distribution of infant and maternal spot UIC, and BMIC. Thirty-nine percent of mothers had UIC <100 µg/L, whereas, only 4% of infants had UIC <100 µg/L. Fifty-three per cent of infants had a UIC >300 µg/L and 26.1% >600 µg/L. UIC of mothers were positively correlated with the UIC of infants (r_s=0.425, p<0.001) (Figure 2A). The UIC of both infants and mothers were positively correlated with BMIC (infants: r_s=0.552, p<0.001; mothers: r_s=0.593, p<0.001) (Figure 2B, 2C). Infants of obese mothers had higher UIC [495.3 (141.8-1060.9) µg/L; p=0.04] than infants of mothers that were normal weight (n=39). We found no other association of UIC in infants and mothers with participant characteristics and frequency of iodine-containing foods consumed by mothers. Generally, cow's milk was consumed either every day or sometimes by 82% of mothers. Whilst 60% consumed fish sometimes, 69% consumed meat always (Table 3).

Median BMIC was 179 (126-269) µg/L. Median SIC (n=85) was 44 (27-63) ppm. The majority of women (90%) used adequately iodized salt in the household (≥15 ppm) as defined by WHO, 42% consumed salt that was within the South African recommended salt iodine fortification level at production (35-65 ppm), whilst 21% of households consumed salt that was iodized above 65 ppm. Iodine in water collected from the different households was below detection limit (<10 µg/L).

The UICs of infants and their mothers were positively correlated with household SIC (infants: r_s=0.341, p<0.001; mother: r_s=0.252, p<0.001). The median UIC of mothers from households with SIC above 65 ppm [185 (117-411) µg/L] was higher than that of mothers from households with iodized salt containing 35-65 ppm [105 (68-163) µg/L; p=0.024] and that of mothers from households with SIC below 35 ppm [117 (62-136) µg/L; p=0.004]. Likewise, the median UIC of infants from households with SIC above 65 ppm [719 (478-911) µg/L] was higher than of infants from households with iodized salt at 35-65 ppm [346 (194-530) µg/L; p=0.006] and with SIC below 35 ppm [250 (177-411) µg/L; p<0.001].

Using multiple linear regression analysis, household SIC and maternal urinary sodium excretion significantly predicted UIC of lactating mothers (Table 4). Household SIC, maternal UIC, and age of infants significantly predicted BMIC. In turn, BMIC as well as infant age significantly predicted UIC in infants.

Table 1. Characteristics of breastfed infants and their lactating mothers

	%	Mean	Standard deviation
Infants (n=100)			
Males (%)	46		
Females (%)	54		
Age (months)		3.0	1.1
Length (cm)		58.3	3.4
Weight (kg)		5.7	1.0
BAZ (%)			
Wasted (BAZ <-2)	3		
Normal (-2≥ BAZ ≤1)	80		
Risk for overweight (BAZ >1)	11		
Overweight (BAZ >2)	6		
Mothers (n=100)			
Age (years)		27.7	6.8
Number of children		2.2	1.2
HIV-positive (%)	22		
Smoking now (%)	10		
Smoking before pregnancy (%)	8		
Education (%)			
Primary	5		
Secondary	90		
Tertiary	4		
Other	1		
Employed (%)	11		
Mode of delivery (%)			
Vaginal delivery	79		
Caesarean	21		
Height (m)		1.57	0.05
Weight (kg)		66.5	14.7
BMI (%)			
Underweight (BMI <18.5)	3		
Normal weight (BMI 18.5-24.9)	39		
Overweight (BMI 25.0-29.9)	31		
Obese (BMI ≥30)	27		
Breastfeeding practice (%)			
Exclusive	67		
Predominant	9		
Partial	24		

BMI: body mass index, BAZ: body mass index-for-age z-scores, HIV: human immunodeficiency virus

Thyroid hormone concentrations in lactating mothers and their infants are shown in Table 2. Infant TSH, TT_4, and Tg concentrations were 1.3 (0.8-1.9) mU/L, 128±33 mmol/L, and 77.1 (56.3-105.7) µg/L, respectively. Mother TSH, TT_4, and Tg concentrations were 0.8 (0.6-1.0) mU/L, 69.6±15.9 mmol/L, and 22.2 (14.4-30.7) µg/L, respectively. We found that 99% of infants had TSH concentrations within the normal range. No associations of UIC were found with Tg, TSH, and thyroid hormone concentrations in infants or mothers. However, median TSH concentrations were significantly higher in HIV-positive [0.95 (0.0-1.7) mU/L] than HIV-negative [0.7 (0.0-2.2) mU/L] mothers (p=0.021). Further, maternal TT_4 concentrations were associated with TT_4 concentrations of their infants (r=0.236, p=0.020). TT_4 concentrations were significantly lower in HIV-positive (61.0±15.3 nmol/L) than HIV-negative (72.1±15.3 nmol/L) mothers (p=0.004). In turn, the odds for having low TT_4 concentrations were significantly higher in HIV-positive (TT_4 <65 nmol/L=63%) than HIV-negative (TT_4 <65 nmol/L=37%) mothers (OR=2.95, 95% confidence interval: 1.11-7.90). We did not observe any significant differences in Tg concentrations by HIV status. Furthermore, the thyroid hormone status of the infants did not differ with regard to maternal HIV status.

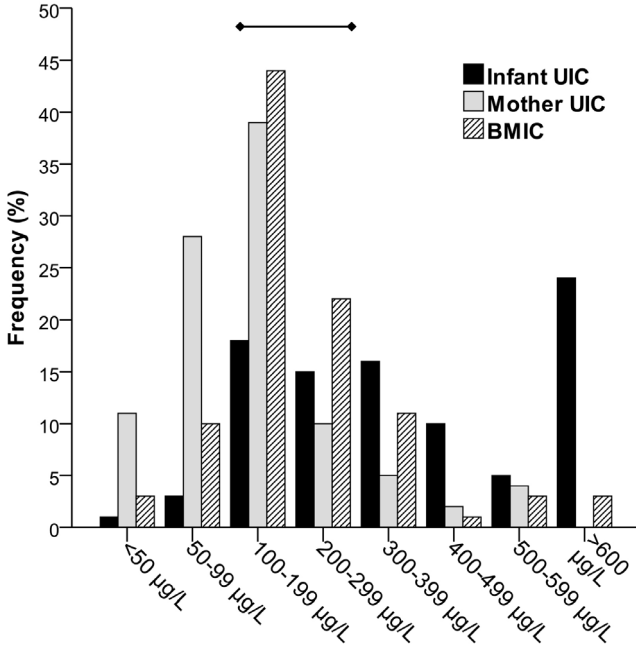

Figure 1. Frequency distribution of spot urinary iodine concentrations of lactating mothers (n=100) and their breastfed infants (n=92), and breast-milk iodine concentrations in µg/L. The urinary iodine concentrations range indicating sufficient iodine intake in lactating women and infants is highlighted
UIC: urinary iodine concentration, BMIC: breast-milk iodine concentration

Discussion

To our knowledge, this is the first study to report iodine status, BMIC, and thyroid function of breastfed infants and lactating mothers in South Africa. Our findings suggest adequate iodine status in both lactating women and their breastfed infants in this convenience sample. Based on a BMIC of 179 µg/L and a breast-milk consumption of 0.78 L at 3 months (36), infants consumed 140 µg iodine/day, well above the recommended daily iodine intake of 90 µg and 110 µg for infants below 6 months of age by WHO and the Institute of Medicine (IOM), respectively (10,36). WHO applies the threshold of ≥100 µg/L for the median UIC to determine iodine sufficiency in children less than 2 years of age. This cut-off sharply disagrees with the intake recommendations from both WHO and IOM. By assuming a urine volume in infants of approximately 500 mL/day and 90% bioavailability and excretion (10,36), the UIC corresponding to the recommended dietary iodine intake of 90-110 µg/day would be in the range of 160-240 µg/L. Although the median UIC in the infants in our study is more than 3 times higher than the WHO UIC threshold, it should be noted that no range for median UIC reflecting optimal iodine nutrition during infancy has been defined. Considering the small urine volume in infants, a wide UIC range is expected. Previous studies in

Table 2. Urinary iodine and thyroid hormone concentrations in South African lactating mothers and breastfed infants

Infants		**n**	**Median**	**25th percentile**	**75th percentile**
Urinary iodine concentration (µg/L)		92	373	202.0	627.0
Estimated 24 h iodine intake from breast milk (µ/day)[2]		97	140	97.9	209.4
TSH (mU/L)		96	1	0.8	1.9
	TSH >4.5 mU/L (n [%])		1 [1]		
T4 (mmol/L)		97	128.0 (32.8)*		
	T_4 <80 nmol/L (n [%])		5 [5.2]		
	80≥ T_4 ≤165 nmol/L (n [%])		81 [83.5]		
	T_4 >165 nmol/L (n [%])		11 [11.3]		
Tg (µg/L) (n=66)[1]			77	56.3	105.7
Subclinical hypothyroidism (n [%])			0 [0]		
Overt hypothyroidism (n [%])			0 [0]		
Hypothyroxinemia (n [%])			5 [5.2]		
Mothers					
Urinary iodine concentration (µg/L)		100	118	67	179
Iodine-creatinine ratio (µg/g)		100	126	86	207
Breast-milk iodine concentration (µg/L)		100	179	126	269
Urinary sodium excretion in spot samples (mmol/g creatinine)		88	154	99	220
TSH (mU/L)		100	0.8	0.6	1.0
	TSH >3.7 mU/L (n [%])		0 [0]		
T_4 (mmol/L)		100	69.6 (15.9)*		
	T_4 <65 nmol/L (n [%])		43 [57]		
Tg (µg/L)		96	22.2 (14.4-30.7)	14.4	30.7
	Tg >40 µg/L (n [%])		16 [17]		
Subclinical hypothyroidism (%)		100	0		
Overt hypothyroidism (%)		100	0		
Hypothyroxinemia (%)		100	43		

T_4: thyroxine, TSH: thyroid-stimulating hormone, Tg: thyroglobulin.
Subclinical hypothyroidism defined as elevated TSH (relative to age-specific cutoffs) and normal T_4; overt hypothyroidism defined as elevated TSH (relative to age-specific cutoff) and low T_4 (relative to age-specific cutoffs); and hypothyroxinemia defined as T_4 less than age-specific cutoff and normal TSH.
[1]Eight values were above measuring range of 150 µg/L.
[2]24 h iodine intake from breast milk was estimated on the basis of the measured breast-milk iodine concentrations and assuming that the mean breast milk intake in infants 0-6 months of age is 0.78 L/day (36).
*Normally distributed data presented as mean (standard deviation)

Niger and Algeria also reported high median UICs (220 µg/L and 728 µg/L, respectively) in breastfed infants (33,37,38). Lower median UICs were observed in exclusively breastfed infants from the Boston area in the U.S. (204 µg/L) and Switzerland (82 µg/L) which are both considered iodine-sufficient populations (39,40). The scientific basis for the dietary iodine requirements during infancy is weak. The IOM intake recommendation is an Adequate Intake (AI) because there were insufficient data to establish an Estimated Average Requirement (EAR) for this age group and no upper intake level has been defined (10,36). Work is on-going to add data to this knowledge gap (ClinicalTrials.gov: Project NCT02045784).

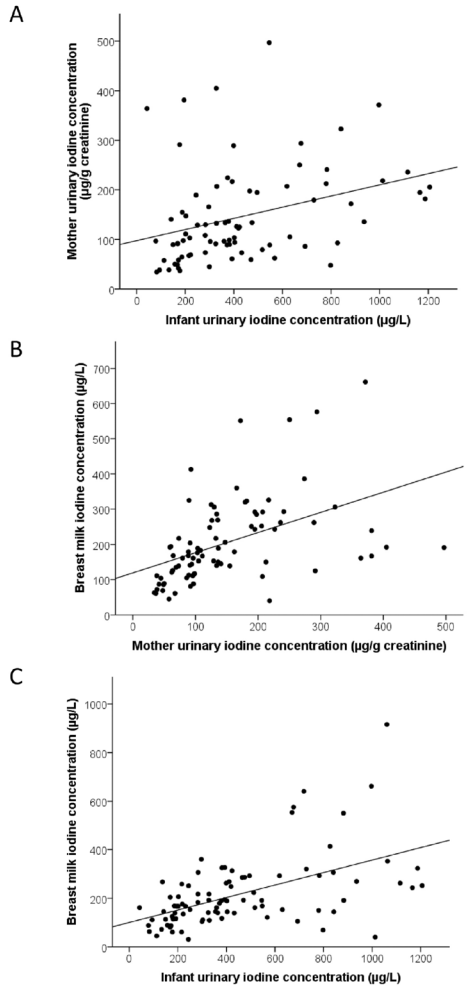

Figure 2. Scatter plots indicating the Spearman correlations between (A) urinary iodine concentrations of South African lactating mothers and their breastfed infants (Spearman correlations: r_S=0.425 and p<0.001); (B) Urinary iodine concentrations of lactating mothers and breast-milk iodine concentrations (r_S=0.593 and p<0.001); (C) Urinary iodine concentrations of infants and breast-milk iodine concentrations (r_S=0.552 and p<0.001)

The median Tg concentrations of 77.1 µg/L is six times higher than reported in iodine-sufficient school-aged children (17). Pediatric reference ranges for serum-Tg assays indicate physiologically elevated Tg concentrations during the first two years of life (41,42). The Tg concentrations gradually decline with age. Infant reference values are lacking for DBS-Tg as well as data on DBS-Tg in iodine-sufficient breastfed infant populations. However, the magnitude of the elevated DBS-Tg concentration in infants in our study compared to median DBS-Tg concentration observed in iodine-sufficient school children suggests that Tg production may increase in response to a marginally high iodine intake in infants. However, none of the infants had subclinical hypothyroidism, and we found no associations of infant UIC with Tg, TSH, and T_4 concentrations, indicating a possible adaptation of the thyroid gland.

Based on median UIC, lactating women in the present study have adequate iodine status. The iodine intake in the mothers is estimated to 320 µg/day; 140 µg iodine is excreted in the breast milk and 180 µg in the urine (assuming a urine volume of 1.5 liters/day). All women have normal TSH concentrations. The high prevalence of hypothyroxinemia should be interpreted with caution; we applied the TT_4 thresholds defined for women of reproductive age in the absence of normal reference ranges for maternal TT_4 in breastfeeding women. Cross-sectional data suggest lower TT_4 concentrations during early lactation (43,44). In addition, we observed higher TSH and lower TT_4 concentration in HIV-positive women than in HIV-negative women. Abnormalities in thyroid function of HIV patients have been previously described (45,46). Most HIV-positive mothers in our study reported to be on the highly active antiretroviral therapy (HAART). Increased prevalence of sub-clinical hypothyroidism is known to occur in HIV treated patients, especially those on HAART (47,48). Madeddu et al (49) emphasized the need to sequentially check thyroid function in HIV patients on HAART after they

Table 3. Consumption patterns of iodine-containing foods

General frequency of consumption (%)	Foods				
	Cow's milk (n=100)	Fish (n=99)	Seafood (n=100)	Eggs (n=100)	Meat (n=99)
Never	4.0	16.2	89.0	7.0	1.0
Rarely	9.0	20.2	0	32.0	1.0
Sometimes	52.0	60.6	11.0	46.0	10.1
Often	5.0	3.0	0	6.0	18.2
Always	30.0	0	0	9.0	69.7

Table 4. Predictors of breast-milk iodine concentration and urinary iodine concentration in South African lactating mothers and breastfed infants

	UIC (μg/g creatinine) in mothers		BMIC (μg/L)		UIC (μg/L) in infants	
Multiple linear regression	β β	**p**	β	**p**	β	**p**
Salt iodine concentrations (μg/g)	0.340	0.003	0.329	0.005	0.120	0.349
Sodium excretion (mmol Na/g creatinine)	0.461	<0.001	0.174	0.115	0.215	0.061
Age infant (months)	-0.084	0.455	-0.386	<0.001	0.281	0.019
Age mother (years)	0.123	0.241	0.001	0.989	0.126	0.216
BMIC (μg/L)	-	-	-	-	0.522	<0.001
Maternal UIC (μg/g creatinine)	-	-	0.325	0.005	-0.117	0.338
Adjusted R^2		0.243		0.273		0.347

Dependent variables were log-transformed to perform multiple linear regression analysis. UIC: urinary iodine concentration, BMIC: breast-milk iodine concentration, Na: sodium

observed elevated TSH levels in these patients as compared to naive patients and controls. This might be particularly crucial in lactating women, considering the positive associations that we observed between TT_4 concentrations in mothers and their infants.

Confirming the influential role of maternal iodine intake on the iodine status of breastfed infants (15), we found maternal UIC to be a predictor for BMIC, which in turn was a predictor for UIC in infants. Most infants included in the present study were exclusively breastfed (67%), but some mothers reported to occasionally feed their infants with commercial infant formula or other foods (for example, maize meal porridge and commercial infant cereals). We did not observe any differences in infant UIC based on feeding practices. Our findings, however, show that infant age is a strong negative predictor for BMIC, confirming that BMIC may decline within the first six months of lactation (50). Furthermore, discrepancies in literature exist regarding the relationship between maternal and infant UIC. In agreement with our study, various authors have found positive correlations between infant and mother UIC (38,51,52), while others did not (53,54).

Our results indicate that household salt was a major source of iodine for mothers; SIC predicted both BMIC and maternal UIC. The majority (90%) of women consumed adequately iodized salt (>15 ppm) and our data indicate that the iodized salt coverage in the Potchefstroom area is high and meets the WHO criteria for a successful salt iodization program (10). In 2005, a national study reported that 77% of households in South Africa had access to and consumed adequately-iodized salt (9). The median iodine concentration in the household salt was 44 ppm, ranging from 0-153 ppm, slightly above the upper level of 40 ppm recommended by WHO (10). Iodine is a volatile micronutrient (55) and the iodine fortification level in the South African program (35-65 ppm) has been set to account for possible losses in salt iodine concentrations before salt reaches households. However, our data also show that 21% of households consumed salt iodized above the upper level of 65 ppm. It has been previously documented that poor implementation and insufficient monitoring of universal salt iodization programs worldwide have resulted in inadequate and even excess intakes of iodine in several countries (56). In South Africa, the median iodine concentration in household salt has previously been reported to range from 6 ppm to 42 ppm across all provinces and 30 ppm nationwide (57).

The daily quantity of salt consumed by mothers was beyond the scope of this study and sodium excretion was measured only in spot urine samples, which is not recommended for estimating individual sodium intake. However, 90% of mothers indicated that they used salt every day for food preparation. The South African population is known to have high salt intakes. The mean salt consumption in the country is 6-12 g per day per person, which is higher than the WHO recommendation of ≤5 g of salt (<2000 mg sodium) per day per person (58). Currently, policies are being implemented to reduce the salt intake of the general population. Salt intakes as low as 5 g per day are known to have adequate amounts of iodine, provided the salt is sufficiently iodized (12). Our results indicate that the amount of iodine added to salt by some producers may be to too high and that the compliance with the current legislation (35-65 ppm) is not properly monitored and may therefore lead to over-iodized salt in the market.

In South Africa, fortification of salt is only mandatory for table salt and not for salt used in processed foods. In this study, sodium excretion and household SIC were independent predictors of maternal UIC, indicating that iodized household salt may not have been the sole source for iodine. Thus, the possibility of obtaining iodine from other food sources cannot be over-ruled. For example, there was a reported frequent consumption of cow's milk, and the assessment of various brands of milk available in the local market showed that the iodine content in milk ranged from 116-366 μg/L (unpublished results). Only few studies have determined whether processed foods in South Africa contain iodized salt. Bread is the major source of dietary salt intake for adults, especially urban black

dwellers, who are said to obtain 49-54% of their salt intake from bread and cereal food groups (59). Several manufacturing companies have previously reported to use salt containing substantial amounts of iodine (39-69 ppm), especially for food items that were frequently distributed countrywide (60). These reports are, however, old and it is likely that more food manufacturing companies now use iodized salt.

A limitation of this study is the small sample size. However, the vast amount of data collected in the study provides a complete picture of the iodine status in the studied infants and their mothers. We did not collect data on the use of any iodine containing disinfectants applied for maternal wound disinfection or continuous umbilical care of the infants and acknowledge this limitation (61). Based on information received from clinics and the local hospital, the most commonly used disinfectant in theatre during caesarean delivery is HibiTane® (containing chlorhexidine) in either alcohol or iodine, and water in chlorhexidine is also being used for perineal laceration. Iodine is preferably used over alcohol as it is believed to cause less irritation. However, we did not observe any significant difference in UIC between infants born via vaginal delivery or caesarean, therefore ruling out possible contamination from maternal wound disinfectants. Furthermore, in clinics, mothers are mainly advised to use alcohol (surgical spirit) for umbilical care and hence iodine contamination is unlikely.

In conclusion, our results suggest that iodized salt is a major contributor to iodine status in lactating mothers and their infants. The results also show that the salt iodization program in South Africa not only supplies sufficient iodine for children and women of reproductive age, but also for lactating mothers and breastfed infants. However, salt iodine levels appear to be poorly monitored. There is a dire need for on-going monitoring and surveillance of salt fortification at production, to avoid over-iodized salt and ensure sustenance of optimal iodine status in vulnerable population groups.

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Ethics

Ethics Committee Approval: This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Health Research Ethics Committee (HREC) of the North-West University (NWU-00016-13-A1), Informed Consent: Permission was also granted from the Provincial and District Health Departments in the North West Province to recruit mother-infant pairs for this study at local health clinics.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Jennifer Osei, Maria Andersson, Cornelius M. Smuts, Jeannine Baumgartner, Design: Jennifer Osei, Maria Andersson, Cornelius M. Smuts, Jeannine Baumgartner, Data Collection or Processing: Jennifer Osei, Olivia van der Reijden, Susanne Dold, Jeannine Baumgartner, Analysis or Interpretation: Jennifer Osei, Jeannine Baumgartner, Literature Search: Jennifer Osei, Writing: Jennifer Osei.

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Original Article

Luteinizing Hormone Secretion during Gonadotropin-Releasing Hormone Stimulation Tests in Obese Girls with Central Precocious Puberty

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ABSTRACT

Objective: Girls with precocious puberty have high luteinizing hormone (LH) levels and advanced bone age. Obese children enter puberty at earlier ages than do non-obese children. We analyzed the effects of obesity on LH secretion during gonadotropin-releasing hormone (GnRH) tests in girls with precocious puberty.

Methods: A total of 981 subjects with idiopathic precocious puberty who had undergone a GnRH stimulation testing between 2008 and 2014 were included in the study. Subjects were divided into three groups based on body mass index (BMI). Auxological data and gonadotropin levels after the GnRH stimulation test were compared.

Results: In Tanner stage 2 girls, peak stimulated LH levels on GnRH test were 11.9±7.5, 10.4±6.4, and 9.1±6.1 IU/L among normal-weight, overweight, and obese subjects, respectively (p=0.035 for all comparisons). In Tanner stage 3 girls, peak stimulated LH levels were 14.9±10.9, 12.8±7.9, and 9.6±6.0 IU/L, respectively (p=0.022 for all comparisons). However, in Tanner stage 4 girls, peak stimulated LH levels were not significantly different among normal, overweight, and obese children. On multivariate analysis, BMI standard deviation score was significantly and negatively associated with peak LH (β=-1.178, p=0.001).

Conclusion: In girls with central precocious puberty, increased BMI was associated with slightly lower peak stimulated LH levels at early pubertal stages (Tanner stages 2 and 3). This association was not valid in Tanner stage 4 girls.

Keywords: Luteinizing hormone, body mass index, precocious puberty

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Weight gain has an effect on pubertal development, such as the timing of pubertal initiation and age at menarche.

WHAT THIS STUDY ADDS?

In girls with central precocious puberty, increased body mass index affects peak stimulated luteinizing hormone levels during the early pubertal stages (Tanner stages 2 and 3). Excess adiposity may suppress gonadotropin secretion during early puberty through complex hormonal interactions.

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Introduction

Over the past decades, many studies have reported, in various ethnic groups, an earlier age of onset of puberty and menarche in girls (1,2,3). The timing of puberty is primarily driven by genetic factors. Several genetic mutations have been identified in patients with idiopathic hypothalamic hypogonadism and central precocious puberty (CPP) (4,5). Although genetic factors play a critical role in the timing of puberty, nutrition and environmental factors also influence pubertal development (6). Excess adiposity may be one of the most important causes of alterations in pubertal development, such as the timing of onset of puberty and age of menarche. There are many studies which report the correlation between increasing body mass index (BMI) and early maturation and which also take into account relevant racial and genetic factors (7,8,9,10).

Precocious puberty is characterized by early activation of the pituitary-gonadal axis which leads to increased growth rate and development of secondary sexual characteristics (11). The secretion of gonadotropin-releasing hormone (GnRH) is low during the juvenile period in mammals and increases in amount and in frequency at the onset of puberty (12). So, girls with precocious puberty have high luteinizing hormone (LH) levels and a high LH/follicle-stimulating hormone (FSH) ratio for their age. However, recent studies have shown that the LH increase with the onset of sleep, the earliest hormonal change in puberty, was blunted in otherwise healthy girls with very high BMIs (13,14). In our previous study, high BMI was also associated with lower LH response to the GnRH stimulation test in boys with CPP (15). These findings indicate that obesity affects GnRH secretion and pubertal maturation. Therefore, the purpose of this study was to evaluate LH secretion during GnRH stimulation tests in a subset of normal-weight and obese girls with idiopathic CPP.

Methods

The study sample included 981 girls who were diagnosed with idiopathic CPP at Ajou University Hospital between 2008 and 2014. All subjects underwent GnRH stimulation tests as part of their clinical evaluation. Precocious puberty was defined as the appearance of breast development before the age of 8 years, advanced bone age (BA) [one year above chronological age (CA)], and increased LH response to the GnRH stimulation test (peak LH >5 IU/L) on immunoradiometric assay (IRMA) (16). Tanner stage was evaluated by palpation of glandular breast tissue while the subjects raised their arms and evaluations were done by one pediatric endocrinologist. Patients with organic intracranial lesions such as brain tumors, were excluded after neuroradiological examinations using magnetic resonance imaging of the hypothalamic-pituitary region. Subjects with previously identified endocrine disorders, previous use of hormonal medications, those with chromosomal abnormalities, as well as subjects with abnormal androgen secretion and congenital adrenal hyperplasia were excluded from the study. Plasma thyroxin and thyroid-stimulating hormone concentrations were measured in order to exclude hypothyroidism. Ovarian disorders were ruled out on the basis of a pelvic ultrasound. None of the subjects had experienced menarche. The interval between the onset of puberty and the age at diagnosis was 0.60±0.1 year.

The GnRH stimulation test was performed in the daytime. Serum LH and FSH levels were determined at baseline and 30, 45, 60, and 90 min after injection of GnRH (100 μg Relefact; Sanofi-Aventis, Frankfurt, Germany). Basal estradiol (E2) was measured before injection of GnRH. Height was assessed with a Harpenden stadiometer. Weight was measured with a calibrated digital scale. BMI was calculated as weight/height^2. Pubertal status (Tanner stage for breast development) was assessed by inspection and palpation and documented by two pediatric endocrinologists. Patients were categorized by pubertal stage (Tanner 2-5) (17). BA was determined using an X-ray of the left hand using the Greulich and Pyle method (18). The standard deviation scores (SDS) for height, weight, and BMI were calculated based on the 2007 Korean National Growth Charts (19).

Serum LH and FSH levels were measured by IRMA (BioSource, Nivelles, Belgium). The detection limits for LH and FSH were 0.1 IU/L and 0.2 IU/L, with an intra-assay coefficient of variation (CV) ranging from 1.4-3.9% to 1.1-2.0% and an inter-assay CV ranging from 3.4-8.0% to 2.4-4.4%, respectively. E2 levels were measured by radioimmunoassay with a detection limit of 5 pg/mL, with an intra-assay CV ranging from 4.0-7.0% and an inter-assay CV ranging from 4.2-8.1% (RIA; Coat-A-Coung, Diagnostic Products, Los Angeles, CA, USA).

Statistical Analysis

Statistical analysis was performed using SPSS version 21.0 (SAS Institute, Chicago, USA). BMI status was stratified as normal (BMI between the 5th and 85th percentiles), overweight (BMI between the 85th and 95th percentiles), and obese (BMI ≥95th percentile). For comparison of clinical parameters according to BMI, ANOVAs with Tukey's post-hoc tests were performed for each Tanner stage. Spearman's correlation was used to examine the relationship between peak LH and clinical parameters according to the Tanner stage because LH levels were not normally distributed. After finding a significant association with peak LH, linear regression was performed in multivariate analysis with stepwise variable selection, including age at diagnosis, BMI SDS, difference between BA and CA, basal LH, basal FSH, and basal E2 levels. Statistical significance was defined as $p<0.05$. Results are given as mean ± standard deviation unless otherwise stated.

Results

Mean age at diagnosis of the study group was 8.2±0.86 years. The majority of the children were in Tanner stage 2 (n=610, 62.2%), with 270 (27.5%) children in Tanner stage 3 and 101 (10.3%) in Tanner stage 4 of puberty. Mean BMI SDS was 0.43±0.88 and ranged from -2.39 to 3.16. The numbers of normal-weight, overweight, and obese girls were 733 (74.7%), 169 (17.2%), and 79 (8.1%), respectively. In all Tanner stages, obese girls were significantly taller than normal-weight girls, and BA was more advanced in obese children. As expected, obese children were heavier and had greater BMI values at all Tanner stages (Table 1).

As shown in Table 2, in Tanner stage 2 and 3 girls, the LH response to GnRH stimulation was clearly influenced by BMI status. Peak stimulated LH levels were significantly lower in overweight subjects and lower still in obese subjects. However, peak stimulated LH levels were not significantly different between the groups in Tanner stage 4 girls. There were no differences in basal LH, FSH, or E2 levels in any Tanner stage groups.

On Spearman's correlation analysis, BMI SDS was significantly and negatively associated with peak LH levels in Tanner stage 2 and 3 girls (r=-0.137, and -0.157; p=0.001 and p=0.010, respectively), while BMI SDS was not significantly associated with peak stimulated LH levels in Tanner stage 4 girls (r=-0.080; p=0.427). Basal LH and BA-CA were significantly associated with peak LH levels (Table 3).

To identify the determinants of peak LH response to the GnRH stimulation test, stepwise multivariate regression analysis was performed. BMI SDS, Tanner stage, basal LH, and BA-CA were significant predictors of peak LH levels (Table 4). BMI SDS was the only negative predictor of peak LH levels.

Table 1. Baseline characteristics of study subjects stratified by body mass index and Tanner stage

	Normal	Overweight	Obese	p-value
Tanner 2 subjects	**(n=494)**	**(n=86)**	**(n=30)**	
Age at diagnosis (years)	8.1±0.8	8.2±0.6	7.7±0.9	0.009
Height SDS	0.6±0.8	1.0±0.7	1.1±0.8	<0.001
Weight SDS	0.3±0.7	1.4±0.4	1.9±0.4	<0.001
BMI (kg/m^2)	16.5±1.4	19.5±0.9	21.4±1.8	<0.001
BMI SDS	-0.04±0.7	1.29±0.2	1.93±0.3	<0.001
BA (years)	9.8±1.0	10.2±0.8	9.7±1.1	0.015
BA SDS	3.3±1.1	3.6±1.0	3.6±1.0	0.030
BA-CA	1.6±0.7	1.9±0.7	2.0±0.7	0.001
Tanner 3 subjects	**(n=183)**	**(n=58)**	**(n=29)**	
Age at diagnosis (years)	8.2±1.0	8.3±0.5	8.2±0.6	0.091
Height SDS	0.9±0.8	1.1±0.8	1.4±0.8	0.003
Weight SDS	0.6±0.5	1.4±0.3	2.1±0.3	<0.001
BMI (kg/m^2)	17.2±1.4	19.7±0.8	22.2±1.8	<0.001
BMI SDS	0.26±0.6	1.31±0.2	2.02±0.3	<0.001
BA (years)	10.3±1.3	10.6±0.7	10.4±1.1	0.253
BA SDS	3.8±1.1	4.2±0.9	4.2±1.0	0.021
BA-CA (years)	2.0±0.8	2.3±0.6	2.2±0.7	0.041
Tanner 4 subjects	**(n=56)**	**(n=25)**	**(n=20)**	
Age at diagnosis (years)	8.5± 0.4	8.5±0.3	8.0±1.5	0.033
Height SDS	1.2±0.7	1.6±0.8	2.0±0.6	<0.001
Weight SDS	0.8±0.5	1.6±0.7	2.0±0.6	<0.001
BMI (kg/m^2)	17.6±1.3	19.9±0.8	22.2±1.3	<0.001
BMI SDS	0.42±0.5	1.28±0.2	1.99±0.2	<0.001
BA (years)	10.9±0.6	11.0±0.6	10.9±1.3	0.842
BA SDS	4.3±0.9	4.5±0.8	5.3±1.4	0.002
BA-CA (years)	2.4±0.6	2.5±0.6	2.9±0.5	0.016

BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age

Table 2. Hormone levels of study subjects stratified by body mass index and Tanner stage

	Normal	Overweight	Obese	p-value
Tanner 2 subjects	**(n=494)**	**(n=86)**	**(n=30)**	
LH at baseline (mIU/mL)	1.3±0.8	1.4±0.8	1.4±0.8	0.754
Peak LH (mIU/mL)	11.9±7.5	10.4±6.4	9.1±6.1	0.035
FSH at baseline (pg/mL)	2.5±1.2	2.6±1.6	2.6±1.6	0.782
Peak FSH (mIU/mL)	14.3±5.2	13.0±4.2	15.1±5.1	0.053
E2 at baseline (pg/mL)	8.1±4.3	8.2±4.0	6.7±2.3	0.197
Peak E2 (pg/mL)	8.7±4.7	8.8±4.2	7.4±2.8	0.287
Peak LH/FSH ratio	0.9±0.6	0.8±0.4	0.6±0.3	0.006
Tanner 3 subjects	**(n=183)**	**(n=58)**	**(n=29)**	
LH at baseline (mIU/mL)	1.5±1.1	1.4±0.8	1.6±1.1	0.673
Peak LH (mIU/mL)	14.9±10.9	12.8±7.9	9.6±6.0	0.022
FSH at baseline (pg/mL)	2.7±1.5	2.6±1.7	2.7±1.4	0.782
Peak FSH (mIU/mL)	14.2±7.9	12.6±4.6	11.9±3.5	0.137
E2 at baseline (pg/mL)	9.5±6.3	8.1±3.5	9.9±4.7	0.205
Peak E2 (pg/mL)	10.1±6.5	8.7±3.9	10.7±5.1	0.233
Peak LH/FSH ratio	1.2±0.8	1.1± 0.6	0.9±0.5	0.066
Tanner 4 subjects	**(n=56)**	**(n=25)**	**(n=20)**	
LH at baseline (mIU/mL)	1.9±1.1	1.8±0.9	2.4±1.8	0.203
Peak LH (mIU/mL)	22.6±17.6	17.4±13.1	29.4±30.1	0.141
FSH at baseline (pg/mL)	3.6±1.5	3.3±1.5	3.2±1.2	0.405
Peak FSH (mIU/mL)	13.3±4.6	11.1±3.9	12.0±4.5	0.106
E2 at baseline (pg/mL)	11.3±8.1	13.0±9.3	14.0±14.3	0.523
Peak E2 (pg/mL)	12.0±8.2	13.6±9.5	15.3±14.6	0.456
Peak LH/FSH ratio	1.7±1.1	1.5±0.8	2.2±1.7	0.106

LH: luteinizing hormone, FSH: follicle-stimulating hormone

Table 3. Spearman's correlation of peak stimulated luteinizing hormone levels with various parameters in all subjects (n=981)

Parameter	Tanner 2		Tanner 3		Tanner 4	
	r	p	r	p	r	p
BMI SDS	-0.137	0.001	-0.157	0.010	-0.080	0.427
Height SDS	0.029	0.478	-0.007	0.906	0.153	0.126
Weight SDS	-0.077	0.056	-0.124	0.042	0.047	0.639
BA-CA	0.141	<0.001	0.170	0.005	0.392	<0.001
Basal LH	0.090	0.026	0.177	0.004	0.576	<0.001
Basal FSH	0.177	<0.001	0.233	<0.001	0.248	0.013
Basal E2	0.094	0.021	0.097	0.114	-0.005	0.959

BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age LH: luteinizing hormone, FSH: follicle-stimulating hormone

Discussion

The present study investigated how obesity affects the LH response to GnRH stimulation test in girls with idiopathic CPP and at different stages of puberty. During the GnRH stimulation test, while peak LH levels were significantly lower in obese and overweight subjects than in normal-weight subjects, in Tanner stage 2 and 3 girls, in girls at later pubertal stages (Tanner stage 4), obesity was not associated with LH secretion. These findings indicate that excess adiposity or fat accumulation may

Table 4. Multivariate analysis of factors associated with peak luteinizing hormone values (n=981, r^2=0.220, p<0.001)

Variables	Estimate	SE	p-value
BMI SDS	-1.178	0.364	0.001
BA-CA	1.530	0.408	<0.001
Tanner stage	3.443	0.489	<0.001
Basal LH	3.592	0.312	<0.001

Stepwise multivariate regression analysis included the following independent variables: age at diagnosis, body mass index standard deviation score, difference between bone age and chronological age, Tanner stage, basal luteinizing hormone, basal follicle-stimulating hormone, and basal E2 levels.
BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age LH: luteinizing hormone, SE: standard error

affect gonadotropin secretion in girls at early pubertal stages but not at later pubertal stages.

The beginning of puberty is characterized by marked increases in GnRH and gonadotropin secretion (12). The increased prevalence of overweight and obesity may be triggering early pubertal development (20,21,22). The mechanisms whereby obese children grow faster beginning in early childhood remain unclear. Adipocytes secrete leptin in direct proportion to adipose tissue mass as well as to nutritional status. Leptin and its regulation may be important in the initiation and/or progression of puberty and may play a role in the earlier onset of puberty in obese children compared to normal-weight children (23,24,25). Leptin concentrations are directly correlated with fat mass, and leptin serves as a signal to the hypothalamus regarding energy stores in the adipose tissue compartment (26). Animals lacking functional leptin or its receptor show marked suppression of pulsatile LH secretion and are infertile (27,28). Leptin may also have more direct stimulatory effects on GnRH and gonadotropin secretion (7). So, we hypothesized that obese girls with precocious puberty would have higher levels of LH than normal-weight girls with precocious puberty. However, our results show that excessive adiposity, as assessed by elevated BMI, was correlated with decreased LH secretion in girls with CPP.

There are a small number of reported studies on LH secretion and obesity in children. McCartney et al (14) reported that obesity in prepubertal and early pubertal girls was associated with reduced LH secretion and reduced nocturnal changes in LH compared to their normal-weight counterparts but with increased frequency of LH secretion during later puberty. In another study by Bordini et al (29), spontaneous sleep-related gonadotropin rise was blunted in healthy excessive weight girls undergoing puberty at a normal age. They also reported that peak LH response to a GnRH agonist was not associated with BMI percentile, in contrast to the results of our study. This may be related to the use of a different test agent (GnRH agonist) and the small number of subjects undergoing normal puberty. Recently, Fu et al (30) evaluated peak LH levels in 865 girls with idiopathic CPP. They reported that LH secretion after a GnRH provocation test was lower in overweight and obese girls than in normal-weight girls with CPP, but they did not investigate the differences in LH secretion based on Tanner stage.

The mechanisms by which increased BMI is associated with decreased LH levels are unclear. Sex steroids may directly affect sexual maturation. Estrogen has an essential role in the initiation and progression of puberty, and increased estrogen levels are linked to excess adipose tissue (31). Estrone (E1) and E2 are synthesized from androstenedione and testosterone. Decreased hepatic inactivation by estrogen-2 hydroxylation in the context of obesity leads to reduced estrogen clearance (32). Girls with obesity exhibit increased total testosterone production and reduced hepatic sex hormone binding globulin (SHBG) production, and a decrease in the levels of SHBG could result in increased sex steroid bioavailability (33). Also, increased adiposity could lead to increased aromatase activity, resulting in increased and accelerated conversion of androgens to estrogens (34). Relatively inactive androgens may induce advanced breast development, while the hypothalamic-pituitary axis remains relatively dormant. Another potentiating/mediating factor influencing the effect of BMI on LH is insulin resistance or hyperinsulinemia associated with obesity. Increased fat accumulation leads to increased insulin resistance, which may affect sex hormone production (6). Increased insulin levels in obese girls stimulate androgen production by acting on the adrenal glands, liver, and ovaries. Furthermore, increased androgen levels may affect central neurosecretory function (35).

GnRH secretion is extremely sensitive to negative feedback from sex steroids during early puberty (36). Although our study showed that E2 levels were not significantly different in normal-weight children and obese children in all Tanner stages, lower LH response to GnRH stimulation tests may partly reflect a negative feedback effect by estrogen. Tanner stage 2 and 3 girls may experience relative immaturity of hypothalamic-pituitary function (37). The amount of estrogen required to suppress gonadotropins in peripubertal girls is lower than the amount required in adults (38). Also, the prepubertal hypothalamic-pituitary axis is estimated to be six- to 15-fold more sensitive than that of adults (39). Our study showed that BMI was not significantly associated with peak LH levels in Tanner stage 4 girls. These results suggest that sensitivity to negative feedback decreases as the reproductive axis matures, permitting increasing GnRH and gonadotropin secretion (33).

In our study subjects, obese girls in Tanner stage 2 were significantly younger than those in the other groups. LH levels tend to increase in the later stages of puberty, although FSH levels rise during the early stages of puberty (40). So, early detection of precocious puberty in Tanner stage 2 obese girls may also be the cause of low peak LH levels in the GnRH stimulation test.

This study has a few limitations stemming from its retrospective design. We did not evaluate a variety of other hormones known to link obesity to gonadotropin secretion, such as insulin, SHBG, and testosterone; as a result, we cannot prove causality. Also, our sample size of Tanner stage 4 girls was smaller than that of Tanner stage 2 and 3 subjects. Furthermore, although BMI and peak LH levels were significantly correlated in early pubertal stages, there was a lack of statistical power. Finally, any recruitment bias was especially unlikely to have influenced the results in Tanner stage 2 and 3 girls. Obese girls may present earlier than normal-weight girls because of greater concerns over psychological and health-related body issues. Furthermore, it can be difficult to distinguish lipomastia from true breast tissue in overweight and obese girls. In order to reduce bias, Tanner stage was evaluated by palpation of glandular breast tissue while the subjects raised their arms and evaluations were done by one pediatric endocrinologist. Regardless of these limitations, our findings indicate a potential association between gonadotropin secretion and excess adiposity.

In conclusion, our results suggest that higher BMI during early puberty is associated with slightly lower LH levels evoked by GnRH stimulation in girls with precocious puberty, but increased BMI is not associated with LH secretion in girls with CPP in later pubertal stages. Therefore, BMI should be considered when interpreting the results of GnRH stimulation tests. Further studies are needed to explore the mechanisms by which BMI affects gonadotropin secretion.

Ethics

Ethics Committee Approval: Retrospective study, Informed Consent: Retrospective study.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Hae Sang Lee, Jin Soon Hwang, Design: Hae Sang Lee, Jin Soon Hwang, Data Collection or Processing: Hae Sang Lee, Jong Seo Yoon, Analysis or Interpretation: Hae Sang Lee, Jin Soon Hwang, Literature Search: Hae Sang Lee, Jong Seo Yoon, Writing: Hae Sang Lee.

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Original Article

The Role of Osteopontin in the Pathogenesis and Complications of Type 1 Diabetes Mellitus in Children

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ABSTRACT

Objective: Type 1 diabetes mellitus (T1DM) is the most common chronic metabolic disorder of childhood and adolescence. Osteopontin plays a significant role in the development and progression of several autoimmune diseases. Moreover, osteopontin promotes adipose tissue inflammation, dysfunction, and insulin resistance. To investigate the levels of serum osteopontin in pediatric patients with T1DM and to explore if these levels have a role in the prediction of diabetes complications.

Methods: This was a case–control study conducted at the Endocrinology unit of the Children's Hospital of Zagazig University in Egypt, from October 2014 to December 2015. Sixty patients with T1DM and 60 healthy subjects were enrolled. A detailed medical history was taken from all patients/parents. A full clinical examination including ophthalmoscopy was performed on all patients. Fasting blood glucose, hemoglobin A1c (HbA1c), urine albumin/creatinine ratio, and serum osteopontin levels were also determined in all subjects.

Results: Patients with T1DM had significantly higher serum osteopontin levels compared with controls (mean ± standard deviation: 13.7±3.4 µg/L vs. 8.9±2.9 µg/L, $p<0.001$). Also, serum osteopontin concentrations were higher in patients with microalbuminuria than in patients with normal albumin excretion rate and in the control group. Similarly, those who had retinal disease had higher osteopontin concentrations than those without (16.8±2 vs. 12.4±3 mg/L; $p=0.005$). Serum osteopontin levels correlated with a diagnosis of T1DM, and in diabetic patients, correlated with higher systolic and diastolic blood pressure, body mass index values and with lower high density lipoprotein values, diagnosis of retinopathy, and microalbuminuria. No correlation was found between osteopontin levels and HbA1c, insulin dose, co-medications, and diabetes duration in T1DM patients. The association between high osteopontin levels and T1DM was independent from all confounders.

Conclusion: This study shows that increased osteopontin levels are independently associated with T1DM in pediatric patients and supports the hypothesis that osteopontin may have a role in the prediction of microvascular diabetes complications.

Keywords: Type 1 diabetes mellitus, osteopontin, cytokines, microalbuminuria, retinopathy

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Type 1 diabetes mellitus (T1DM) is the most common chronic metabolic disorder of childhood and adolescence. Many patients with diabetes eventually develop physical and emotional complications, including neuropathy, nephropathy, retinopathy, and cardiovascular disease.

WHAT THIS STUDY ADDS?

Increased osteopontin levels are independently associated with T1DM in pediatric patients.

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Introduction

Type 1 diabetes mellitus (T1DM) is the most common chronic metabolic disorder of childhood and adolescence. It is characterized as a disorder in the metabolism of carbohydrates, lipids and amino acids as a result of decreased insulin. Many patients with diabetes eventually develop physical and psychological complications, including neuropathy, nephropathy, retinopathy, and cardiovascular disease (1).

T1DM develops as a result of an autoimmune process, leading to beta-cell destruction (2). In the early stages of insulitis, activated natural killer cells, dendritic cells, macrophages, and T-cells are attracted to the islets. This early phase is followed by production of cytokines and free radicals, which contribute to beta-cell dysfunction and death (3).

Osteopontin (OPN) is a phosphoprotein with adhesive and cell signaling functions; it can act either as an extracellular matrix component in mineralized tissue or as a soluble cytokine in inflamed tissue and serum (4). It plays a vital role in the regulation of immune cell response as it modulates T cell function by affecting the differentiation of T lymphocytes into Th1 and Th2 cells, regulating the balance between Th1 and Th2, and participating in the cell-induced immunological response (5).

OPN was demonstrated to induce adipose tissue inflammation and to increase pro-inflammatory cytokines release in the bloodstream (6). Also, itself acts as a pro-inflammatory cytokine by chemoattracting monocytes, macrophages, and lymphocytes (7). It also stimulates B lymphocytes to express multi-clone antibodies (8). Consequently, OPN promotes the destruction of pancreatic beta-cell and development of T1DM. Several authors reported that increased OPN levels were found to be a predictor of coronary calcification, nephropathy, and coronary artery disease in patients with type 2 diabetes mellitus, independent of traditional risk factors (9,10). However, there are limited data regarding the role of OPN in TIDM in children.

The aim of this study was to investigate the levels of serum OPN in pediatric patients with T1DM compared to non-diabetic participants and to explore if it has a role in the prediction of microvascular and macrovascular complications of diabetes.

Methods

This case-control study was conducted on 60 children with T1DM recruited from those attending the Pediatric Endocrinology Outpatient Clinic at the Children's Hospital, Zagazig University, Egypt, from October 2014 to December 2015, fulfilling the following inclusion and exclusion criteria:

Inclusion criteria: 1) Age: less than 18 years old. 2) Gender: both males and females. 3) Insulin dependency for controlling blood sugar within normal ranges.

Exclusion criteria: 1) Conditions which may lead to insulin resistance such as obesity, acanthosis nigricans. 2) Acute inflammatory illness (including a common cold, infections) as it can affect the serum OPN level. 3) Children with end-stage renal diseases.

Sixty apparently healthy children were included as a control group.

Written informed consent was obtained from the parents of the patients involved in the study as recommended by the Institutional Ethics Committee of Zagazig University and in accordance with the Helsinki declaration after a full explanation of the purpose and nature of all procedures used.

All studied patients were subjected to the following: Detailed history taken laying stress on age, gender, symptoms of diabetes, duration of the disease, frequency of self-monitoring blood glucose (SMBG) by asking patients to estimate approximately how many times a week they usually monitored their blood glucose, recent history of infections or serious illness, daily insulin dose (IU/kg/day), response to insulin therapy, complications of diabetes or insulin therapy, and concomitant medications at the time of study enrollment [angiotensin-converting enzyme inhibitors (ACE-I)].

All subjects underwent a careful physical examination. Weight, height, systolic and diastolic blood pressure (SBP, DBP, mmHg) were measured. Body mass index (BMI) (kg/m^2) was calculated and manifestation of insulin side effects were recorded in each subject.

Ophthalmoscopy was performed at the Zagazig University Hospital ophthalmology outpatient clinic by an ophthalmologist with expertise in diabetes. Ophthalmoscopy was followed by retinal fluoroangiography, when indicated. Retinal examination was used to identify and quantify diabetic retinopathy (DR) according to the International Clinical DR Disease Severity Scale (11).

All study participants underwent blood sampling for biochemistry after an overnight fasting.

Fasting blood glucose (FBG) and hemoglobin A1c (HbA1c) were measured by high-performance liquid chromatography (Tosoh 2.2; Tosoh Bioscience, South San Francisco, CA).

Blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), aspartate aminotransferase (AST, IU/L), and alanine aminotransferase (ALT, IU/L) values were estimated (Cobas Integra 400 plus, Roch Germany). Total cholesterol (mg/dL), low-density lipoprotein-cholesterol (mg/dL), high-density lipoprotein-cholesterol (mg/dL), and triglyceride (mg/dL) levels were also determined.

Microalbuminuria was estimated as the albumin/creatinine ratio in a random spot urine specimen. To this end, the first morning midstream urine samples (10 mL) were collected in sterile containers and centrifuged at 2000-3000 rpm for 20 minutes. The supernatant was removed. The urinary microalbumin and urinary creatinine were measured immediately after centrifugation. Microalbuminuria was assayed by the immunoturbidimetric method (Biosystems SA, Costa Brava, Barcelona, Spain). Creatinine was assayed by Modified Jaffes method using a fully automated Chemistry Analyzer of Cobas Integra 400 plus. Urinary microalbumin/creatinine ratio was calculated as urine microalbumin (mg)/urine creatinine (g).

Serum OPN level was measured using sandwich enzyme immunoassay technique by Enzyme-Linked ImmunoSorbent Assay kit provided by Glory Science Co., Ltd, USA.

Statistical Analysis

Data were checked, entered, and statistically analyzed by SPSS (Statistical Package for Social Sciences version 19, Chicago, IL, USA) and were expressed as mean ± standard deviation for quantitative variables and number and percentage for categorical variables.

Student's t-test for continuous variables and χ^2 test for categorical variables were used to compare two independent groups. Means were compared using ANOVA test for more than 2 groups. For nonparametric data, Mann-Whitney U-test was used to compare quantitative variables between two groups. Correlations between continuous variables and ordinal parameters were calculated by Pearson's coefficient and Spearman's coefficient, respectively. For all of the above, a p-value <0.05 was considered statistically significant and a p-value <0.001 was considered highly statistically significant.

Bivariate logistic regression analysis was used to detect the association between serum OPN levels, considered as a continuous variable, and all possible ordinal variables. The presence of diabetes was categorized as: 0=absence and 1=presence. The presence of DR was categorized as: 0=absence of DR and 1=non-proliferative DR. Diabetic nephropathy (DN) was categorized as 0=absence of DN and 1=presence of persistent microalbuminuria in at least two of three urine samples collected over 24 h.

Results

Our study included 60 patients with T1DM (Male/Female: 42/18) of a mean age of 11.8±2.2 years, with a mean diabetes duration of 6.1±1.6 years (range 4-10 years) and 60 healthy subjects (Male/Female: 39/21, age 12±2.2 years) as a control group. Clinical and biochemical characteristics of the study population are shown in Table 1. Patients with T1DM had significantly lower BMI, higher FBG, and higher HbA1c values than the control group. The clinical and biochemical findings of

Table 1. Clinical and biochemical characteristics in type 1 diabetes mellitus patients and in the controls

	Patients (n=60)	Controls (n=60)	Test statistics	p
Age (years)	11.8±2.2 (9-16)	12±2.1 (9-16)	t=0.432	0.61
Sex (Male/Female)	42/18	39/21	χ^2=0.12	0.744
BMI (kg/m^2)	21.8±4.2	24.6±3.18	t=2.4	0.02*
SBP (mmHg)	114±10.2	113±9.7	t=0.397	0.72
DBP (mmHg)	76±9.4	75±8.8	t=0.412	0.69
FBG (mg/dL)	170 (90-250)	94 (78-112)	Z=5	<0.001**
HbA1c (%)	8.2±1.5	4.1±0.46	t=11.5	<0.001**
Creatinine (mg/dL)	0.7±0.2	0.75±0.18	t=0.382	0.75
BUN (mg/dL)	26.4±6.3	24.9±4.1	t=1.77	0.16
AST (IU/L)	20 (15-40)	18.7 (13-44)	t=1.25	0.28
ALT (IU/L)	21.3 (10-45)	19.8 (12-43)	t=1.34	0.25
Total cholesterol (mg/dL)	169.4±32.7	167.4±30.5	t=1.22	0.3
HDL cholesterol (mg/dL)	56.5±14.9	56.9±12.6.	t=0.09	0.9
LDL cholesterol (mg/dL)	111.7±15.4	110.8±10.5	t=0.21	0.83
Triglycerides (mg/dL)	120±32.6	118±26.5	t=1.9	0.06
Urine albumin/creatinine ratio (mg/g)	20 (10–70)	15 (8-22)	Z=-5.4	<0.001**
OPN (µgLl)	13.7±3.4 (10-20)	8.9±2.9 (4-14)	7	<0.001**

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure FBG: fasting blood glucose, HbA1c: hemoglobin A1c, BUN: blood urea nitrogen, HDL: high-density lipoprotein, LDL: low-density lipoprotein, OPN: osteopontin

Values are expressed by mean ± standard deviation, median (minimum-maximum), or rate of subjects, as appropriate.

t: independent Student's t-test, χ2: Chi-square test, Z: Mann-Whitney U test

*p-value <0.05, **p-value <0.001

patients with T1DM revealed no significant gender difference in diabetes duration, daily insulin dose, BMI, SBP, DBP, HbA1c, and lipid profile.

The overall mean value for HbA1c in patients with T1DM was 8.2±1.5 (8.1±1.2 in males and 8.6±2.2 in females). Acceptable HbA1c (≤8%) values for glycemic control were found in 40% (24 out of 60) of the subjects.

In our subjects, the prevalence of DR in the form of non-proliferative DR was 25% (n=15/60). In this group, diabetes duration was 7±1.5 years and HbA1c (mean ± standard deviation): 8.6±1.4%. Prevalence of DN in the form of microalbuminuria was 21.6% (n=13/60) in patients with a diabetes duration of 7.7±1.9 years and the mean HbA1c value in this group was 9.6±1.6%. We found that 15% (9/60) of T1DM patients had combined DR and DN. There was no significant gender difference regarding DR and DN in patients with T1DM. The prevalence of antihypertensive agents (ACE-I) use was 20% (12/60) in a group with a with diabetes duration of 7.3±1.6 years and with a SBP value of 118±2 mmHg.

Patients with T1DM had significantly higher serum OPN levels compared with controls (13.7±3.4 µg/L vs. 8.9±2.9 µg/L, p<0.001). However, no significant gender difference regarding OPN levels were found in patients with T1DM. Serum OPN concentrations were higher in patients with microalbuminuria than patients with normal albumin excretion rate (AER) and controls. OPN concentrations were also higher in patients with normal AER than the control group (Table 2).

Similarly, patients with retinopathy had higher OPN concentrations than those without (16.8±2 vs. 12.4±3 mg/L; p=0.005). Patients with retinopathy without signs of nephropathy (normal AER) had higher serum OPN concentrations than those who did not have retinal pathology (16.3±2 mg/L vs. 12.4±3 mg/L; p=0.02).

In T1DM patients (n=60), serum OPN levels correlated with higher SBP, DBP, and BMI, lower HDL, and microalbuminuria. However, no correlation was found of OPN levels with HbA1c, insulin dose, and diabetes duration in T1DM patients (Table 3).

To rule out an influence of ACE-I treatment on OPN levels, serum OPN levels were also evaluated separately in T1DM patients according to the use of ACE-Is and no difference was detected between T1DM patients with (n=12) and without ACE-I therapy (n=48). Mean OPN value in T1DM patients treated with ACE-I was 16.4±2.3 mg/L, while mean OPN value in T1DM patients not treated with ACE-I was 15.2±18.5 mg/L (p=0.74).

Finally, the bivariate logistic regression analysis demonstrated that higher serum OPN levels were associated with the diagnosis of T1DM independent of all possible confounders. Also, OPN was independently associated with the development of retinopathy and microalbuminuria in patients with T1DM (Table 4).

Discussion

T1DM is a heterogeneous disorder characterized by autoimmune-mediated destruction of pancreatic beta cells culminating in absolute insulin deficiency (12).

Our study demonstrated that serum OPN levels are significantly higher in pediatric patients with T1DM compared to healthy participants. This finding is in agreement with the findings of Karamizadeh et al (13) who found increased serum OPN levels in pediatric patients with T1DM compared with healthy children in an Iranian study. Also, our findings are in accordance with previous studies on adult T1DM patients (14). Aspord et al (15) studied the pattern of expression of 524 immune-related genes in the Langerhans cells of diabetic mice and found OPN as one of the early genes that was expressed in the primary phases of diabetes and concluded that OPN may play a vital role in T1DM. Also, OPN has been found as one of the auto-antigens which are expressed by human islet somatostatin cells (16). Moreover, previous studies described OPN as a key regulator of adipose tissue inflammation as both serum levels and adipose tissue expression of some pro-inflammatory cytokines such as tumor necrosis factor alpha, interleukin-6, and inducible nitric oxide synthase were significantly reduced in mice lacking the OPN gene (17). Other studies concluded that OPN deficiency led to reduced adipose tissue inflammation and increased insulin sensitivity (17,18).

Frequency of cardiovascular diseases increases in diabetic patients by two- to fourfold. They are the most

Table 2. Comparison of osteopontin levels between controls and patients subgroups

Variable	Controls (n=60)	T1DM with microalbuminuria (n=13)	T1DM with normal albumin excretion rate (n=47)	F	p	LSD
OPN (µg/L)	8.9±2.9	18.2±2.4	12.3±2.3	37	<0.001	<0.001[1] 0.001[2] <0.001[3]

OPN: Osteopontin, T1DM: type 1 diabetes mellitus, LSD: least significant difference

[1]Control group vs. type 1 diabetes mellitus with microalbuminuria

[2]Control group vs. type 1 diabetes mellitus with normal albumin excretion

[3]Type 1 diabetes mellitus with microalbuminuria vs. type 1 diabetes mellitus with normal albumin excretion

F: one-way ANNOVA test

p-value <0.05 is significant

Table 3. Correlations of osteopontin with clinical and biochemical characteristics of the type 1 diabetes mellitus group

	Osteopontin	
	r	p
BMI	0.50	0.022
SBP	0.644	0.001
DBP	0.486	0.025
Creatinine	0.263	0.352
BUN	0.187	0.434
Total cholesterol	0.171	0.475
HDL cholesterol	0.451-	0.046
LDL cholesterol	0.163	0.493
Triglycerides	0.134	0.521
HbA1c	0.21	0.371
Insulin dose	0.427	0.06
Microalbuminuria	0.511	0.021
Diabetes duration	0.371	0.1

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure FBG: fasting blood glucose, HbA1c: hemoglobin A1c,
BUN: blood urea nitrogen, HDL: high-density lipoprotein, LDL: low-density lipoprotein, r: Pearson's correlation coefficient

Table 4. Logistic regression analysis for osteopontin as an independent predictor of type 1 diabetes mellitus, diabetic retinopathy, and nephropathy

Dependent variable	Odd ratio	p	95% confidence interval
T1DM	1.73	0.004*	1.19-2.5
Diabetic retinopathy	2.07	0.045*	0.98-4.34
Diabetic nephropathy	2.2	0.026*	1.09-4.4

*p-value <0.05
T1DM: type 1 diabetes mellitus

common cause of death in patients with T1DM. However, the risk is low before age 30 years (19,20). Risk factors that independently increase cardiovascular risk in patients with diabetes include hypertension, dyslipidemia, renal dysfunction, and hyperglycemia (21).

The current study revealed that serum OPN levels directly correlate with several cardio-metabolic risk factors, such as higher BMI, SBP, DBP, lower HDL, diagnosis of T1DM, but not with insulin dose, diabetes duration, and HbA1c. The only previous study done for assessment of OPN in pediatric patients by Karamizadeh et al (13) did not investigate the presence of clinical and biochemical determinants of high OPN levels. However, our findings are in agreement with a previous study conducted on adult patients with T1DM which demonstrated that circulating OPN levels directly correlate with higher BMI, SBP, and DBP, and lower HDL, but not with diabetes complications, insulin dose, C-peptide level, or disease duration (14). Moreover, the serum OPN levels directly correlated with microalbuminuria. Similar results were reported by Gordin et al (22) who also found an association between higher OPN levels and microalbuminuria at baseline in a cohort study conducted on a large population of adult patients with T1DM. On the other hand, Barchetta et al (14) did not find a significant correlation between serum OPN and microalbuminuria.

In the present work, multivariate logistic regression analyses revealed an association of increased OPN levels with diabetes, DR, and DN, independent from clinical and biochemical confounders. This finding is in agreement with a previous study conducted in adult T1DM patients (14). Also, Gordin et al (22), in a 10-year follow-up study on a cohort of adult patients with T1DM, demonstrated that serum OPN level was an independent predictor of DN, of cardiovascular events, and of all-cause mortality. This finding can be explained by the ability of OPN to increase the pro-inflammatory cytokines in these tissues.

In the present study, only 40% (24 out of 60) of the subjects were found to have achieved acceptable HbA1c levels indicating good glycemic control. This is a serious situation and needs to be underlined since in T1DM patients, poor metabolic control is usually due to inadequacies in insulin regimens and these patients are at highest risk for complications and cardiovascular disorders even at an early age (23).

The main limitation of the present study was the relatively small number of patients participating in the study. Also, the single-center approach limits making generalizations from the results of the study. However, we consider this present study as a baseline for a future cohort study which we plan to conduct on a large population of pediatric patients with T1DM presenting to our endocrinology unit.

In conclusion, this study shows that increased OPN levels are independently associated with T1DM in pediatric patients and identify patients with an unfavorable metabolic profile. Therefore, our data provide a support to the hypothesis that OPN may have a role in the prediction of microvascular diabetes complications. Future studies are needed to demonstrate the clinical benefit of OPN as a possible novel marker of vascular dysfunction and a useful tool for risk stratification in pediatric patients with T1DM.

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Ethics

Ethics Committee Approval: Institutional Ethical Committee of Zagazig University; September 2014, Informed Consent: Written informed consent was obtained from the parents of the patients involved in the study as recommended by the Institutional Ethics Committee of Zagazig University and in

accordance with the Helsinki declaration after a full explanation of the purpose and nature of all procedures used.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Mohamed A. Talat, Design: Mohamed A. Talat, Data Collection or Processing: Mohamed A. Talat, Anwar Ahmed Rass, Maha Mahmoud Hamed Sakr, Analysis or Interpretation: Mohamed A. Talat, Hosam Fathy El-Saadany, Anwar Ahmed Rass, Literature Search: Mohamed A. Talat, Rabab M. Saleh, Writing: Mohamed A. Talat, Laila Metwaly Sherief.

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Original Article

Association between Common Genetic Variants and Polycystic Ovary Syndrome Risk in a Chinese Han Population

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ABSTRACT

Objective: Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies affecting 5-7% of reproductive age women worldwide. The aim of our study was to explore the PCOS-related single nucleotide polymorphism (SNP) associations between common genetic variants and PCOS risk in a Han Chinese women population.

Methods: In this case-control study, 285 Chinese Han women aged 28.50±6.858 years with PCOS and 299 controls of a mean age of 32.66±7.018 years were compared. We selected recently published genome-wide association studies (GWAS) which identified several genetic loci in PCOS. All the SNPs were genotyped by Sequenom Mass-ARRAY technology. Associations between the gene and the risk of PCOS were tested using various genetic models by Statistical Package for the Social Sciences and Plink.

Results: We found that rs705702 in the *RAB5B/SUOX* was associated with PCOS (odds ratio=1.42; 95% confidence interval=1.08-1.87, p=0.011) and increased the PCOS risk. The genotypic model analysis also showed that rs705702 was associated with PCOS risk.

Conclusion: Our results suggest that SNPs rs705702 in gene *RAB5B/SUOX* was associated with PCOS in Han Chinese women.

Keywords: Polycystic ovary syndrome, single nucleotide polymorphism, Chinese Han women

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The first genome-wide association studies and subsequent follow-up performed on Han Chinese populations identified the following Polycystic ovary syndrome (PCOS) candidate loci: *DENND1A, INSR, YAP1, C9orf3, RAB5B, HMGA2, TOX3, SUMO1P1/ZNF217, THADA*, follicle-stimulating hormone receptor, luteinizing hormone/choriogonadotropin receptor.

WHAT THIS STUDY ADDS?

Single nucleotide polymorphisms rs705702 in gene *RAB5B/SUOX* was associated with PCOS in Han Chinese women.

Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive disorder in women. It is a complex, heterogeneous disorder characterized by chronic anovulation, clinical and/or

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biochemical hyperandrogenism and polycystic ovaries. It is the most common endocrine disorder in premenopausal women and affects up to 10% of them (1). The pathogenesis of PCOS is not fully understood, but it is accepted as a multifactorial disorder that arises from interactions between genetic, environmental, and intrauterine factors (2,3). Several family and twin studies indicate a strong genetic basis and twin studies of heredity provide the most rigorous demonstration that the disorder has a genetic component. Twin studies have provided heritability estimates for PCOS of 70% (4). Several groups have undertaken genetic studies to identify the etiology of PCOS. Genome-wide association studies (GWAS) offer a potentially powerful approach to identify the associations between millions of single-nucleotide polymorphisms (SNPs) and specific traits or disorders (5). The first GWAS and subsequent follow-up performed on Han Chinese populations identified the following PCOS candidate loci: *DENND1A, INSR, YAP1, C9orf3, RAB5B, HMGA2, TOX3, SUMO1P1/ ZNF217, THADA*, follicle-stimulating hormone receptor (FSHR), luteinizing hormone/choriogonadotropin receptor (LHCGR) (6,7). At 19p13.3, rs2059807 is located in the intron of the INSR (insulin receptor) gene and in previous studies, common SNPs in the *INSR* gene have been reported to be associated with PCOS in both Han Chinese individuals and those of European ancestry. *INSR* has an important role in insulin metabolism, consistent with a very common explanation for the pathogenesis of PCOS, namely, insulin resistance (8). Mutations affecting the tyrosine kinase domain of the insulin receptor are known to cause severe hyperinsulinemia and insulin resistance (9). LHCGR is expressed on the surface of target cells of reproductive organs such as the ovary, uterus, fallopian tube, and in a variety of other tissues including vascular endothelium (10,11). Studies conducted over the past decade have built a convincing argument that genetic factors contribute to development of PCOS. Despite advances in genetic technologies, very few PCOS susceptibility genes have been validated. To date, there are only a few published genome-wide associated variants for PCOS in Han Chinese women. Indeed, these studies will be important because the susceptibility variants may differ in individual ethnic groups as do the phenotypic features of PCOS. It has been well established that ethnic background adds to the phenotypic diversities in PCOS patients. Although one group recently replicated two of these loci (12), another did not (13). We examined the same susceptibility variants in additional PCOS case-control sets in Han Chinese women. We also investigated variants that correlate strongly with the risk variants in the Chinese population for association with PCOS in the case-control study.

Methods

In this case-control study, 584 women were recruited from the Second Affiliated Hospital, Xi'an Jiatong University, during 2009 to 2013. Among these subjects who were all Han Chinese women, 285 were diagnosed as PCOS according to the National Institutes of Health criteria (14). The mean age of this study group was 28.50±6.858 years. None of the subjects had any other diseases and none of them had undergone chemotherapy or radiotherapy. All were unrelated individuals of reproductive age who had received no hormonal therapy for at least three months prior to the study. The control group consisted of 299 healthy women of a mean age of 32.66±7.018 years.

The study was approved by the ethics committee of the Second Affiliated Hospital, Xi'an Jiatong University, and written informed consent was obtained from all participants.

Weight and height were measured in all subjects by standard protocol and calibrated instruments. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Demographic and personal data were collected through a face to face interview using a standardized epidemiological questionnaire, including age, ethnicity, residential region, and family history of PCOS. In addition, relevant information for PCOS patients was collected through consultation with treating physicians or through a review of the medical chart.

A peripheral blood sample of 5 mL was taken from all participants.

Single-Nucleotide Polymorphism Selection and Genotyping

From the previously published work, we selected 10 SNPs which could be associated with PCOS. Additionally, minor allele frequency (MAF) of these SNPs in the HapMap CHB (Chinese Han Beijing) population was >5%. We used the GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag Co. Ltd. Xi'an City, China) for the extraction of genomic DNA from whole blood, and DNA concentration was measured by NanoDrop 2000 (Gene Company Limited). We used Sequenom MassARRAY Assay Design 3.0 Software to design Multiplexed SNP MassEXTEND assay (15). We performed Sequenom MassARRAY RS1000 to genotype the SNPs using the standard protocol recommended by the manufacturer (15). Finally, data management and analysis were performed by Sequenom Typer 4.0 Software (15,16). Laboratory personnel were blinded to the genotyping results of all samples.

Statistical Analysis

Microsoft Excel and Statistical Package for the Social Sciences 19.0 statistical package (SPSS, Chicago, IL, USA) were used to perform the statistical analyses. A p-value <0.05 was considered statistically significant. The validation of each SNP frequency in control subjects was tested for departure from Hardy-Weinberg Equilibrium (HWE) using an exact test. The χ^2 test was used to compare the distribution of genotypes and allele frequencies between patients and control subjects. The most common genotype in the controls was used as reference group. The associations between the genes and the risk of PCOS were tested using genetic models (co-dominant, dominant, recessive, over-dominant, and log-additive) analysis

by SNP stats, website software from http://bioinfo.iconcologia.net/snpstats/start.htm. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression analysis adjusted for age and gender (17). We used the Haploview software package (version 4.2) and SHEsis software platform (http://www.nhgg.org/analysis/) for analyses of linkage disequilibrium (LD), haplotype construction, and genetic association at polymorphism loci, and D' >0.8 indicated that the related tSNPs formed one block (18,19).

Results

The characteristics of PCOS and control subjects are presented in Table 1. The PCOS group was older than the control group (p<0.001). There were no significant differences in BMI (p=0.585). As listed in Table 2, a multiplexed SNP MassEXTEND assay was designed with the Sequenom MassARRAY Assay Design 3.0 Software. No significant deviation of allele frequencies from HWE was found in PCOS and control groups. We investigated the possibility of the minor allele of each SNP being a risk factor compared with the wild-type allele. As shown in Table 3, the allele and genotype frequencies of the ten SNP were not significantly different between PCOS patients and healthy controls. As shown in Table 3, we found that rs705702 in the *SUOX* was associated with PCOS (OR=1.42; 95% CI=1.08-1.87, p=0.011) and increased the PCOS risk. The genotypic model analysis showed that rs705702 was associated with PCOS risk (Table 4) and logistic regression analysis adjusted for age and BMI also showed an association with PCOS risk. Using the AA genotypes combined as a reference, the AG/GG genotype of rs705702 was significantly associated with an increased risk of PCOS (adjusted p=0.018, OR=1.52; 95% CI=1.07-2.16, Dominant model). In the log additive model, we found that rs705702 was significantly associated with an increased risk of PCOS (adjusted p=0.014, OR=1.42; 95% CI=1.07-1.98). Since the pattern of LD is highly structured into conserved blocks of sequence separated by hotspots of recombination, the final function of a conserved haplotype may be the result of interaction among polymorphisms within the block. We analyzed linkage LDs among the SNPs and these SNPs showed no tight links.

Table 1. Clinical characteristics of the Polycystic ovary syndrome cases and of the control group

	Age (years)		BMI (kg/m^2)	
	Cases	Controls	Cases	Controls
n	285	299	285	299
Mean ± SD	28.5±6.858	32.66±7.018	20.49±2.713	20.61±2.801
p-value	< 0.001		0.585	

SD: standard deviation, BMI: body mass index
p-value ≤0.05 indicates statistical significance

Discussion

In this case-control study, we aimed to determine whether variants recently identified in a GWAS for PCOS in Chinese Han subjects would be associated with PCOS in Xi'an Chinese Han people. This is the first study to report the frequencies of genotypes and alleles in ten different SNPs (rs13405728, rs3802457, rs1894116, rs705702, rs2272046, rs1961177, rs1048943, rs4784165, rs2059807, rs6022786), which were identified by GWAS, between PCOS and healthy Xi'an Han

Table 2. Polymerase chain reaction primers

SNP ID	1st-PCR primer sequences	2nd-PCR primer sequences	UEP sequences
rs13405728	ACGTTGGATGCTTCAATATCCTGGGCTTAC	ACGTTGGATGGATTTAGAAACCTGCTCTGG	gCACCATAATGCAGCCATTTGT
rs3802457	ACGTTGGATGGTTGGGAAAGCCAGTTTCGG	ACGTTGGATGTTCACTACTCTCCAGGAAGC	GGAGGATAAGGCATATTCAT
rs1894116	ACGTTGGATGAAATTTAGTTGCATTGAGG	ACGTTGGATGAAGGATTGACCACTGTCAAG	CTACATAATATTGATTCTAGACAATT
rs705702	ACGTTGGATGACGAGAACTAAGCGATTGAC	ACGTTGGATGCCACTTTAAACCCAGGGTAG	GTAGTTGTAGTTGCAACAG
rs2272046	ACGTTGGATGGGATTCAGTAATTGGCCTTG	ACGTTGGATGACATTCTGCATGCATTGTCC	cTGGCCTTGGGACATTTG
rs6022786	ACGTTGGATGTTTAACCCCCTCAGTTTCTC	ACGTTGGATGCCTAGAGAAATTGCTTAGAC	GACTATTTTAGCTGGTGAC
rs2059807	ACGTTGGATGATGTGAATCAGACCTCTTGC	ACGTTGGATGAGCCAATAACCATATCAAGG	TCAGACCTCTTGCTTTTAA
rs4784165	ACGTTGGATGACTGATCCTCTGCCTACTTC	ACGTTGGATGCCAGCCGTACATTAATCCAC	TTTCCCTATTAAAGAACATCC
rs1048943	ACGTTGGATGTGGGCAAGCGGAAGTGTATC	ACGTTGGATGCTGAATTCCACCCGTTGCAG	gagctGAAGTGTATCGGTGAGACC
rs1961177	ACGTTGGATGGAAATTCCAGAAGTCTAAGG	ACGTTGGATGCTTCCCCTATAAGTCACTTG	aagAAGGATGAAAAGAAAATCTTACA

PCR: polymerase chain reaction, SNP: single-nucleotide polymorphism, UEP: unique-event polymorphism

Table 3. Basic information of candidate single-nucleotide polymorphisms in this study

SNP ID	Chromosome	Alleles A/B	Gene	MAF		p-HWE	OR (95% CI)	p-value
				Cases	Controls			
rs13405728	2p16.3	G/A	*LHCGR*	0.221	0.232	0.002	0.94 (0.71-1.23)	0.642
rs3802457	9q22.32	A/G	*C9orf3*	0.133	0.167	0.409	0.77 (0.55-1.06)	0.106
rs1894116	11q22.1	G/A	*YAP1*	0.256	0.219	0.612	1.23 (0.94-1.61)	0.136
rs705702	12q13.2	G/A	*SUOX*	0.261	0.199	0.467	1.42 (1.08-1.87)	0.011
rs2272046	12q14.3	C/A	*HMGA2*	0.075	0.084	0.706	0.89 (0.58-1.37)	0.606
rs1961177	15q21.2	T/C	*GLDN*	0.190	0.159	0.665	1.23 (0.91-1.67)	0.174
rs1048943	15q24.1	C/T	*CYP1A1*	0.311	0.315	0.894	0.98 (0.76-1.26)	0.869
rs4784165	16q12.1	G/T	*TOX3*	0.388	0.403	1.000	0.94 (0.74-1.19)	0.593
rs2059807	19p13.2	G/A	*INSR*	0.460	0.426	1.000	1.14 (0.91-1.44)	0.253
rs6022786	20q13.2	A/G	*SUMO1P1*	0.472	0.465	0.642	1.03 (0.82-1.29)	0.809

* p-value ≤0.05 indicates statistical significance; A/B stands for minor/major alleles on the control sample frequencies. HWE: Hardy-Weinberg equilibrium; MAF: minor allele frequency, OR: odds ratio, CI: confidence interval, SNP: single-nucleotide polymorphism

ethnic people. Our results also showed that rs705702 in the *RAB5B/SUOX* was associated with PCOS risk, while other loci were not found to be significantly associated with PCOS risk. The different genotype distributions might reflect differences in genetic background, and therefore gene variants might be associated with different relative risks in different populations. Although a previous study demonstrated that these SNPs were associated with PCOS in Han Chinese women, the current study showed that these SNPs were not involved in the pathogenesis in Xi'an Han Chinese women. These different results indicated that there is different genetic background between these SNPs and PCOS.

rs705702 is located at 12q13.2 between the RAB5B and SUOX genes. RAB5B, PCOS GWAS candidate, is a Rab-GTPase, also thought to be involved in endocytosis and receptor recycling and could, therefore, be a molecule interacting with the DENN domain (20,21,22) and has also been reported to involve PI3K, PKB, and MAPK/ERK components (23). RAB5B is a small GTPase that plays a role in early endosome formation and is required for the endocytic pathway that mediates the transport of clathrin-coated vesicles from the plasma membrane to the early endosome. RAB5B is a member of the RAS oncogene family and SUOX, sulfite oxidase, is a homodimeric protein enzyme localized to the intermembrane space of mitochondria, which catalyzes oxidation of sulfite to sulfate, the final reaction in the oxidative degradation of the sulfur amino acids cysteine and methionine. rs705702 was not associated with PCOS in the current study and was only nominally significant in previous European studies (13). Shi et al (7) found that rs705702 in *RAB5B/SUOX* was associated with PCOS in the Han Chinese study and in our current study, we also showed that it was associated with PCOS in Xi'an Han Chinese people. As to other loci, in our study, we found no significant correlations with PCOS. However, in the GWAS study, it was reported that rs13405728 (*LHCGR*), rs3802457 (*C9orf3*), rs1894116 (*YAP1*), rs2272046 (*HMGA2*), rs4784165 (*TOX3*), rs2059807 (*INSR*), and rs6022786 (*SUMO1P1*) were associated with PCOS risk in Han China (7). In addition, rs13405728 was shown to have an association with PCOS in Hui Chinese people (12). rs3802457 was shown to be related to PCOS in a Dutch study (4). As to other loci, an association with PCOS risk has not been reported in studies from Europe. Therefore, the ultimate role of this variant in PCOS pathogenesis remains to be determined.

To conclude, our study has, for the first time, described an association between SNPs in RAB5B and PCOS risk in a group composed of Han individuals of Xi'an China. Large well-designed and population-based studies are warranted to confirm these findings which may well have implications for the etiology of PCOS.

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Table 4. Association between rs705702 and Polycystic ovary syndrome risk

SNP ID	Model	Genotype	Controls	Cases	OR (95% CI)[a]	OR (95% CI)[b]	p[a]	p[b]
rs705702	Codominant	A/A	194 (64.9%)	159 (55.8%)	1	1	0.048	0.048
		A/G	91 (30.4%)	103 (36.1%)	1.38 (0.97-1.96)	1.46 (1.01-2.12)		
		G/G	14 (4.7%)	23 (8.1%)	2.00 (1.00-4.02)	1.89 (0.91-3.96)		
	Dominant	A/A	194 (64.9%)	159 (55.8%)	1	1	0.025	0.018
		A/G-G/G	105 (35.1%)	126 (44.2%)	1.46 (1.05-2.04)	1.52 (1.07-2.16)		
	Recessive	A/A-A/G	285 (95.3%)	262 (91.9%)	1	1	0.092	0.17
		G/G	14 (4.7%)	23 (8.1%)	1.79 (0.90-3.55)	1.66 (0.80-3.42)		
	Over-dominant	A/A-G/G	208 (69.6%)	182 (63.9%)	1	1	0.14	0.079
		A/G	91 (30.4%)	103 (36.1%)	1.29 (0.92-1.83)	1.38 (0.96-1.99)		
	Log-additive	---	---	---	1.40 (1.07-1.83)	1.42 (1.07-1.89)	0.014	0.014

p-value ≤0.05 indicates statistical significance;

OR: odd ratio; CI: confidence interval, SNP: single-nucleotide polymorphism

p[a]: p-values were calculated from two-sided chi-square tests or Fisher's exact tests for either genotype distribution.

p[b]: p-values were calculated by unconditional logistic regression adjusted for age & body mass index.

Ethics

Ethics Committee Approval: Second Affiliated Hospital, Xi'an Jiatong University during 2009 to 2013, Informed Consent: Written informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ming Liu, Design: Ying Sun, Tianbo Jin, Data Collection or Processing: Yi Yuan,

Analysis or Interpretation: Hua Yang, Tian Feng, Literature Search: Jingjie Li, Yongri Ouyang, Writing: Ying Sun.

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Original Article

Development and Validation of a Pediatric Endocrine Knowledge Assessment Questionnaire: Impact of ac Pediatric Endocrine Knowledge Assessment Questionnaire Intervention Study

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ABSTRACT

Objective: While there is general agreement that patient education is essential for compliance, no objective tools exist to assess knowledge in children and parents of children with endocrine disorders. We aimed to design and validate a Pediatric Endocrine Knowledge Assessment Questionnaire (PEKAQ) for congenital hypothyroidism, Hashimoto's thyroiditis, isolated growth hormone deficiency, Graves' disease, and congenital adrenal hyperplasia. We evaluated baseline knowledge of children and parents of children with these disorders and assessed impact of educational intervention.

Methods: At baseline, 77 children (12-18 years) and 162 parents of children 1-18 years participated in this prospective intervention study. Educational handouts for five targeted disorders were designed. Following one-on-one educational intervention, 55 children and 123 parents participated. Baseline and post-intervention knowledge scores were compared using McNemar's test.

Results: Adequate multi-rater Kappa measure of agreement was achieved for children's (0.70) and parent's (0.75) PEKAQs. Flesch Reading Ease Score for both PEKAQs (15 questions each) was 65. Post-intervention, significantly higher proportion of parents and children answered majority of questions correctly ($p<0.05$). Sixteen percent more parents and 22% more children knew their diagnosis correctly ($p<0.05$). Significant improvement was noted among all participants regarding reason for treatment, steps to take in a situation of missed dose, exercise and diet with these disorders, and long-term prognosis. Parent's knowledge score was an independent predictor of child's score.

Conclusions: To our knowledge, this is the first validated PEKAQ that can be used widely in pediatric endocrinology clinics. We noted significant improvement in knowledge of children and parents of children with endocrine disorders.

Keywords: Adolescent, pediatric endocrinology, thyroid, patient education

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Endocrine disorders commonly require adherence to lifelong hormone replacement therapy. Incomplete understanding by patient and/or caregiver of the importance of following prescribed treatment is considered a primary barrier to adherence. Educational programs have been shown to provide knowledge to patients and enable them to take better care of their chronic disorders. However, there are currently no validated questionnaires to assess patient knowledge of pediatric endocrine disorders.

WHAT THIS STUDY ADDS?

We developed and validated the first ever known Pediatric Endocrine Knowledge Assessment Questionnaire (PEKAQ) for five most common pediatric endocrine disorders. We designed effective teaching tools for these five disorders. Using PEKAQ and the teaching tools, we made significant improvement in knowledge of children and parents of children with these disorders. We describe an effective education model that might guide development of future programs.

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Introduction

The most common pediatric endocrine disorders, with the exception of type 1 diabetes mellitus, include congenital hypothyroidism, Hashimoto's thyroiditis, isolated growth hormone deficiency (GHD), Graves' disease, and congenital adrenal hyperplasia (CAH) (1,2,3,4,5). Endocrine disorders commonly require lifelong hormone replacement therapy. Adherence to treatment is crucial for improved prognosis in a developing child (6). The goal of achieving treatment adherence in pediatrics is hindered by several barriers including lack of adequate time for physician-patient interaction, form and palatability of medication, complexity of medication schedule and implementing it without interrupting a child's routine (7). However, incomplete understanding by patient and/or caregiver of the importance of following prescribed treatment is considered a primary barrier by most clinicians and researchers (7,8,9,10,11,12). Not surprisingly, overall treatment adherence rate for the pediatric population is 50% (ranging 11-93%) (13), and may be significantly lower for chronic disorders.

Educational programs have been shown to provide knowledge to patients and enable them to take better care of their chronic disorders (14,15,16). Effectiveness of such programs is best evaluated by assessing knowledge of patients before and after educational intervention. While there are several health-related quality of life surveys for pediatrics (17,18,19,20), there are currently no validated questionnaires to assess patient knowledge of pediatric endocrine disorders, except two questionnaires developed by King et al (21) and Dunn et al (22) for CAH and type 1 diabetes mellitus, respectively, though the latter was not developed specifically for children.

We sought to develop and validate a single Pediatric Endocrine Knowledge Assessment Questionnaire (PEKAQ) for congenital hypothyroidism, Hashimoto's thyroiditis, isolated GHD, Graves' disease, and CAH. We also aimed to design effective teaching tools for these disorders. Using PEKAQ, we aimed to analyze improvement in knowledge of patients and identify factors underlying this improvement. If the effectiveness of this model is established, it may give directions for future clinic-based educational intervention programs.

Methods

Designing the Pediatric Endocrine Knowledge Assessment Questionnaire

Preliminary twenty-one multiple-choice questions were developed regarding diagnosis, treatment, self-care, sick-day management, and prognosis of the five targeted endocrine disorders. Questions raised during research personnel's encounters with patients, online parent forums, and frequently asked questions on websites related to these disorders were included. Quality-of-life related questions were not included. Each question had five answer choices, with one correct answer for each disease. A 'do not know/unsure' option was included. However, incorrect and 'do not know/unsure' answers are reported together for purpose of analysis. Question number 4 was the only open-ended question: 'Please write names of medications that you/your child takes for the endocrine disorder'. Respondents were allowed to look up names on their medication bottles.

Two questionnaires, one each for children and parents, were designed. Both questionnaires had the same questions and were designed to be easily comprehensible by children 12-18 years and parents or legal guardians of children 1-18 years. Ambiguous, leading, and hypothetical questions were avoided (23,24). Demographic data including the patients' age, sex, and disease duration were collected.

Validating the Pediatric Endocrine Knowledge Assessment Questionnaire

Delphi technique was employed to validate the PEKAQs (25), wherein an expert panel comprising of 20 pediatric and adult endocrinologists at Detroit Medical Center, Michigan was formed. The questionnaires were sent to each panel member through an online survey. Each member was asked to evaluate the degree to which they thought each question reflected the knowledge required by children/caregivers living with these endocrine disorders to understand and effectively manage the disorder. They were then asked to rank on a five-point Likert scale whether they agreed or disagreed that the question should be included and whether they thought the multiple-choice answers were suitable and parallel. If they thought a question should not be included as it was, they were asked to indicate if they thought the item should be included if it was modified. A space was provided for any modifications and/or comments (21).

Study Design

The study was conducted at Children's Hospital of Michigan (CHM) Pediatric Speciality Center and Etkin Speciality Center (satellite clinic of CHM). Initially, a list of all children 1-18 years who had a pre-existing diagnosis of one of the five targeted endocrine disorders and who were being followed by a pediatric endocrinologist at either of the two centers was prepared. From this list, all patients coming for their visits to the clinic during the duration of study were invited to participate. Written informed consent was obtained from participating parents and from parents or legal guardians of participating children younger than 18 years. Oral assent was obtained from children aged 12 years and written assents were obtained from children aged 13-17 years.

Exclusion criteria included new consultation visit, patients older than 18 years, transient congenital hypothyroidism, participants unable to stay for the entire duration of the education session, unable to read the PEKAQ (blind, non-English

speaking/reading), and presence of other chronic disorders (type 1 diabetes mellitus, Down's and Turner' syndrome, celiac disease). The study was approved by the Institutional Review Board of Children's Hospital of Michigan.

Administering the Pediatric Endocrine Knowledge Assessment Questionnaire

Baseline PEKAQ was administered during participants' endocrine clinic visit. PEKAQ was scored, with each correct answer worth 1 point and each incorrect or 'do not know/ unsure' answer worth zero point. This was immediately followed by the educational session as detailed below. At their follow-up clinic visit in 3-6 months, PEKAQ was again administered to participants by the same research team. On an average, each participant spent 9-12 minutes completing the questionnaire. The questionnaire was completed in presence of research personnel to avoid discussion between parent and child. Once the participants had completed their response, the questionnaire was checked for any missed item which was then requested to be completed.

Educational Intervention

A face-to-face educational intervention session of about 10-15 minutes was developed for each disorder. Each participating child and his/her parent were educated simultaneously. If both parents were present, one parent was requested to volunteer for participation.

Reader-friendly, attractive, and informative handouts were designed separately for congenital hypothyroidism, Hashimoto's thyroiditis, isolated GHD, Graves' disease, and CAH. Each handout contained information regarding diagnosis, pathogenesis, treatment, self-care, sick-day management, and prognosis. Information was obtained from literature review on PubMed and Up-to-date, and the websites of the Pediatric Endocrinology Society, Pediatric Endocrinology Nursing Society, American Academy of Pediatrics, Mayo Clinic, National Institutes of Health, CAH Research Education and Support, and American Thyroid Association.

Members of the research team were trained to impart education in an interactive and consistent pattern. Participants were given ample opportunity to ask questions. Subsequently, these handouts were given to participants as take-home material.

Statistical Analysis

Data were managed on an Excel spreadsheet. All entries were checked for keyboard error. Descriptive statistics and knowledge assessment scores were computed as arithmetic mean and standard deviation. Comparison was made between the pre- and post-intervention variables using paired t-test for normally distributed continuous variables, Wilcoxon-singed-rank test for skewed continuous variables, and McNemar's test for categorical variables. Multiple linear regression analysis was applied to test the effect of current age, sex, age at diagnosis, duration of diagnosis, and corresponding parent's score on child's knowledge score. A p-value <0.05 was considered statistically significant. The IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. was used for data analysis.

Results

Readability of Pediatric Endocrine Knowledge Assessment Questionnaires

Readability of the PEKAQs was assessed by Flesch Reading Ease score which is based on the average number of syllables per word and words per sentence (9). The Flesch Reading Ease score for both PEKAQs was 77 (reading grade level of 6 or above). The Flesch Reading Ease score for each of the five educative handouts was 55-64 (reading grade level of 6-7 or above).

Responses from Delphi survey were anonymously collected; data were coded and analyzed. After deleting questions with low agreement between the experts' panel, 15 questions were retained in both the questionnaires. The multi-rater Kappa measure of agreement was 0.70 for children's questionnaire and 0.75 for parents' questionnaire. Kappa measurement of 0.70 or above indicates adequate inter-rater agreement.

Sample Characteristics

Of the 162 parents in pre-intervention survey, 79% (n=128) were mothers (Table 1). Post-intervention survey was completed by 123 parents. Among children, 77 participated in pre-intervention survey and 55 completed post-intervention survey. The average age at endocrine diagnosis of participating children was 8.9±4.2 years. Data analysis reported in Tables 2, 3, 4 and Figure 1 includes only those participants who completed pre- as well as post-intervention survey. Reasons for attrition in post-intervention survey included not showing up for follow-up, discontinuation of growth hormone therapy or Grave's disease treatment, and moving to a different geographical region, thus changing providers.

Parents: Assessment of Baseline Knowledge

Percentage of parents who answered each PEKAQ question correctly at baseline is given in Table 2. Notably, at baseline, 26.8% parents did not know the correct name of their child's endocrine disorder and only 63.4% parents knew the reason or beneficial effect of treating their child's endocrine disorder. Almost 80% parents were not aware of the toxic effects of their child's endocrine medicine. Less than 70% parents had a sick-day plan and 64.2% knew what should be done in case the child forgets to take his/her medicine at the right time. One-fifth of parents did not know correctly the duration of treatment required, frequency of follow-up at the endocrinology clinic, and reasons for wearing medical alert pendant/bracelet.

Parents: Impact of Pediatric Endocrine Knowledge Assessment Questionnaire Educational Intervention

Following educational intervention, 10 out of 15 questions were answered correctly by a significantly higher proportion of parents (p<0.05) (Table 2). Specifically, 15.4% more parents correctly knew the endocrine diagnosis of their child (p=0.000) and 36.6% more parents were knowledgeable in recognizing toxic side-effects of their child's endocrine medicine (p=0.000). Significant improvement in knowledge was observed regarding reason for treatment, steps to take in situations of missed doses, exercise and diet with these disorders, long-term prognosis, and benefit of medical alert pendant/bracelet (p<0.05 for all).

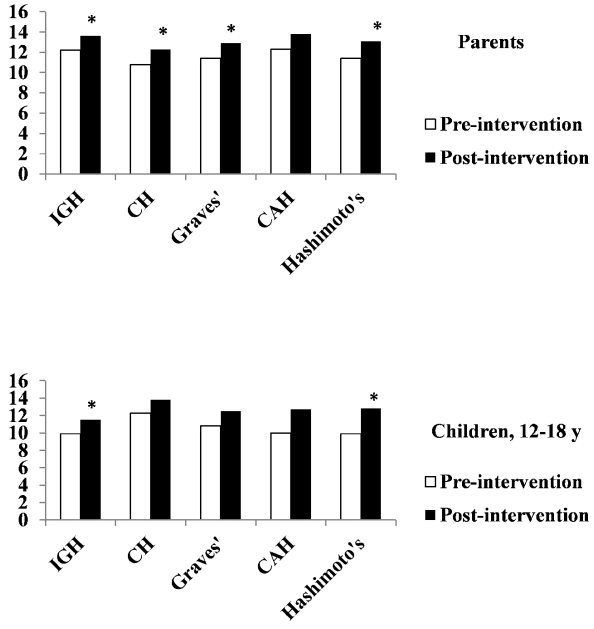

Figure 1. Mean knowledge scores before and after educational intervention by endocrine disorder *p-value <0.05
IGH: isolated growth hormone, CH: congenital hypothyroidism, CAH: congenital adrenal hyperplasia

Children: Assessment of Baseline Knowledge

Table 3 shows the percentage of children who answered PEKAQ questions correctly at baseline. During the pre-intervention survey, 69.1% children knew correctly their own endocrine diagnosis and only 25.5% knew toxic effects of their medicines. Nearly half of the children did not know what should be done if they forget to take their medicine at the right time. About 65% children knew that with their endocrine diagnosis, they do not have dietary restrictions and should consume a well-balanced diet. One-fourth of the participating children were not aware of long-term prognosis of their disorder and if that will affect their education, job, life span, or fertility.

Children: Impact of Pediatric Endocrine Knowledge Assessment Questionnaire Educational Intervention

After educational intervention, a significantly higher proportion of children answered 7 questions out of 15 correctly (p<0.05) (Table 3). About 22% more children knew correctly their endocrine diagnosis (p=0.002) and 25% more children knew correctly what they should do if they forget to take their medicine at the scheduled time (p=0.007). Further, 89.1% children post-intervention compared to 65.5% pre-intervention recognized that their underlying endocrine disorder does not require any dietary restrictions (p=0.004).

Predictors of Knowledge Assessment Scores in Parents and Children

Among parents, during pre- and post-intervention surveys, none of the variables including age, sex, age at diagnosis, and duration of diagnosis were found to be independent predictors of knowledge score (Table 4). However, among children, after adjusting for age, sex, and duration of diagnosis, the

Table 1. Characteristics of participants

	Parents		Children	
	Pre-intervention	**Post-intervention**	**Pre-intervention**	**Post-intervention**
Participants (n)	162	123	77	55
Age (y)[a]	40.2±9.1	39.8±9.3	14.1±1.8	14.3±1.8
Sex (n)	M=31; F=131	M=24; F=99	M=7; F=40	M=28; F=27
Relation to patient (n)	Mother=128	Mother=97	NA	NA
	Father=31	Father=24	NA	NA
	Other[b]=3	Other[b]=2	NA	NA
Age at diagnosis (y)[a,c]	6.4±4.8	6.2±4.8	8.9±4.2	8.7±4.5
Duration of diagnosis (y)[a,c]	4.3±3.6	4.1±3.7	5.4±4.2	5.6±4.6

n: number, y: years, M: male, F: female, NA: non applicable
[a]Mean ± standard deviation
[b]Grandmother, aunt
[c]For their corresponding child

Table 2. Proportion of parents (%) who answered Pediatric Endocrine Knowledge Assessment Questionnaire questions correctly before and after educational intervention

Questions	Before n=123		After n=123		
	Correct	Incorrect	Correct	Incorrect	p[a]
1. Endocrine condition for which your child is being seen today is…	73.2	26.8	88.6	11.4	0.000[a]
2. Your child's endocrine condition involves ____ gland.	92.7	7.3	95.1	4.9	0.508
3. In this condition, the body makes too much/less of ___ hormone.	77.2	22.8	84.6	15.4	0.078
4. Names of your child's endocrine medications are…	95.1	4.9	91.9	8.1	0.125
5. It is important to take the medicine ___ time of the day.	99.2	0.8	98.4	1.6	1.000
6. The medicine regulates your child's…	63.4	36.6	75.6	24.4	0.011[a]
7. If your child forgets to take the medicine, he/she should…	64.2	35.8	83.7	16.3	0.001[a]
8. If your child takes too much of his/her medicine, it will cause…	21.1	78.9	57.7	42.3	0.000[a]
9. With proper treatment, your child should be able to exercise…	87.0	13.0	95.9	4.1	0.003[a]
10. Children with this endocrine condition should eat…	87.8	12.2	94.3	5.7	0.039[a]
11. If your child gets sick with an infection, you should…	65.9	34.1	74.0	26.0	0.184
12. Children with this endocrine condition require treatment for…	79.7	20.3	89.4	10.6	0.002[a]
13. If properly treated, your child can have a regular...	87.0	13.0	94.3	5.7	0.022[a]
14. Regular follow-up with endocrinology is recommended every…	78.9	21.1	90.2	9.8	0.013[a]
15. Main reason for wearing a medical alert pendant or bracelet is…	75.6	24.4	91.1	8.9	0.002[a]

PEKAQ: Pediatric Endocrine Knowledge Assessment Questionnaire
[a]p-value <0.05

Table 3. Proportion of children 12-18 y (%) who answered Pediatric Endocrine Knowledge Assessment Questionnaire questions correctly before and after educational intervention

Questions m	Before n=55		After n=55		
	Correct	Incorrect	Correct	Incorrect	p[a]
1. Endocrine condition for which you are being seen today is…	69.1	30.9	90.9	9.1	0.002[a]
2. Your endocrine condition involves ____ gland.	81.8	18.2	89.1	10.9	0.219
3. In this condition, the body makes too much/too less of _ hormone.	78.2	21.8	89.1	10.9	0.180
4. Names of your endocrine medications are…	81.8	18.2	83.6	16.4	1.000
5. It is important to take the medicine ___ time of the day.	94.5	5.5	100.0	0.0	NA[b]
6. The medicine regulates your…	60.0	40.0	74.5	25.5	0.134
7. If you forget to take the medicine at the right time, you should…	50.9	49.1	76.4	23.6	0.007[a]
8. If you take too much of your medicine, it will cause…	25.5	74.5	45.5	54.5	0.013[a]
9. With proper treatment, you should be able to exercise…	83.6	16.4	89.1	10.9	0.453
10. Children with this endocrine condition should eat…	65.5	34.5	89.1	10.9	0.004[a]
11. If you get sick with an infection, you should…	60.0	40.0	83.6	16.4	0.004[a]
12. Children with this endocrine condition require treatment for…	65.5	34.5	74.5	25.5	0.302
13. If properly treated, you can have a regular...	74.5	25.5	80.0	20.0	0.508
14. Regular follow-up with endocrinology is recommended every…	60.0	40.0	87.3	12.7	0.001[a]
15. Main reason for wearing a medical alert pendant or bracelet is...	67.3	32.7	87.3	12.7	0.007[a]

PEKAQ: Pediatric Endocrine Knowledge Assessment Questionnaire, NA: non applicable
[a]p-value <0.05
[b]Computed only for a p x p table, where p must be greater than 1

Table 4. Multiple linear regression for knowledge scores in parents and children

Variables	Before PEKAQ		After PEKAQ	
	β-coefficient	95% CI[a]	β-coefficient	95% CI[a]
Parents				
Age	-0.007	-0.071 to 0.057	0.055	-0.164 to 0.274
Sex	-0.659	-1.800 to 0.440	-0.473	-4.200 to 3.300
Age at diagnosis[b]	0.032	-0.228 to 0.291	0.450	-0.438 to 1.340
Duration of diagnosis[b]	0.045	-0.226 to 0.315	0.499	-0.425 to 1.422
Children				
Age	0.399	0.022 to 0.776[a]	0.306	-0.124 to 0.737
Sex	-0.397	-1.730 to 0.937	-0.458	-1.983 to 1.067
Duration of diagnosis	0.009	-0.161 to 0.180	0.018	-0.177 to 0.213
Corresponding parent's score	0.368	0.010 to 0.725[a]	0.761	0.642 to 0.880[a]

PEKAQ: Pediatric Endocrine Knowledge Assessment Questionnaire
[a]p-value <0.05
[b]For their corresponding child

corresponding parent's score emerged as an independent predictor of children's knowledge score during both pre- and post-intervention surveys. In the pre-intervention survey, age was a positive predictor of knowledge score in children; this was not a statistically significant finding during the post-intervention survey, indicating that the intervention made greater improvement in younger children as compared to older children.

Impact of Pediatric Endocrine Knowledge Assessment Questionnaire Educational Intervention by Endocrine Diagnosis

Following PEKAQ educational intervention, parents of children with isolated GHD, congenital hypothyroidism, Graves' disease, and Hashimoto's thyroiditis showed statistically significant improvement in knowledge of these disorders ($p<0.05$) (Figure 1). In children, improvement was noted in those with isolated GHD and Hashimoto's thyroiditis ($p<0.05$). Sample size of children with congenital hypothyroidism, Graves' disease, and CAH was too small to allow statistically meaningful analysis.

Discussion

In this study, we have designed and validated an effective model to assess and educate children and parents of children with congenital hypothyroidism, Hashimoto's thyroiditis, isolated GHD, Graves' disease, and CAH. To our knowledge, this is the first validated PEKAQ. We have highlighted marked gaps in knowledge of study participants about their endocrine disorders. Encouraging results were observed in post-intervention survey, with significant improvement in knowledge in the majority of participants.

Studies assessing knowledge of children with endocrine disorders are rare (26,27), with no report that we could find, on impact of educational intervention. The PEKAQs in our study were designed specifically for rapid and reliable knowledge assessment in pediatric endocrine patients during clinic visits. In a randomized controlled trial, Sahlqvist et al (28) reported that shortening a relatively lengthy questionnaire significantly increased the response rate. Rosenfeld and Bakker (9) used a 134-question survey to identify key factors that influence compliance in patients receiving growth hormone therapy. Using a 22-question survey, Smith et al (27) reported that 60% of their patients had limited understanding about their growth hormone treatment. King et al (21) developed a 22-question survey to assess knowledge of families living with CAH. We retained 15 questions in the PEKAQs to minimize item non-response rate while allowing efficient measurement of study parameters.

At the first visit with a pediatric endocrinologist, it is often challenging for families to assimilate news of a lifelong diagnosis (29). Focus of the initial visit is to put patient and parents at ease and help them understand the basics of management. Because we believe this visit might be too overwhelming for them, we enrolled participants at follow-up visits.

Previously, a clear relationship has been reported between knowledge of children treated with growth hormone and their degree of compliance and acceptance of treatment (26,30). In our study, at baseline only two-thirds of the participants knew their diagnosis correctly, partly due to several synonyms used for same disorder (Hashimoto's thyroiditis, acquired hypothyroidism, chronic lymphocytic thyroiditis, autoimmune thyroiditis) or medical acronyms (CAH) for these disorders. Understanding the reason for treatment was noted in only two-thirds of the participants, which is a significant factor in impeding compliance. The improvement in post-intervention knowledge in our study was likely due to the involvement of dedicated research personnel who spent one-on-one time with participants.

Parents play a critical role in management of pediatric chronic disorders (8,31). In fact, we found parents' knowledge assessment score to be a significant independent predictor of children's knowledge score. It is therefore paramount to ensure parental understanding of their child's disorder, ability to recognize toxic effect of medications, and ensure healthy lifestyle. Through this study, parents were educated about continuing endocrine medications in the event of an acute sickness as well as carrying and administering intramuscular corticosteroid for children with CAH, which can be a life-saving intervention. An important highlight of the educative handouts was to communicate to the parents the need to treat their child like any other child without the endocrine disorder, as much as possible. Information about what to expect when beginning a new treatment was also provided.

As of September 2012, 81% of U.S. adults use the internet and, of those, 72% have looked online for health information in the past year (32). The National Institutes of Health recommends that patient education material be written at or below the 6th grade reading level in order to be most effective and understood (33). However, Barnes and Davies (34) reported a mean reading grade level of 13 for 63 online and paper materials on thyroid nodule evaluation and management. Most of these materials had 'extensive or serious shortcomings', which lead to uncalled anxiety and misconceptions (35,36). With limited patient encounter time, there is not enough opportunity for all questions to be addressed. A comprehensive yet succinct education module designed in our study will aptly fill that gap. We selected questions following careful review of 'frequently asked questions' on various online forums, common concerns expressed by families during their clinic visits, and queries from their primary care providers.

Commonly, the information about endocrine diagnosis in clinical practice is directed towards parents, partly due to the age of the child at initiation of treatment and also because parents are the primary caregivers. Middle school children are capable of understanding their illness and treatment (37). High school children are more likely to think in terms of impact of their disorder on their daily activities (including diet and physical activity), role of treatment, prognosis, and impact on their future relationships including questions regarding fertility, lifespan, and genetics. Our educational handouts were designed to address each of these concerns. Children are afraid to ask questions or tell a physician if they do not understand something. In the present study, children were encouraged to ask questions, share their concerns, and be more involved in their management. A 'do not know/unsure' option was included, because it is preferable for the participants to recognize that they are 'unsure' of the answer, than for them to 'think' they know the correct answer when they are in fact incorrect.

We did not find duration of diagnosis as a significant factor in predicting knowledge score of participants. This finding was supported by King et al (21) who found no statistical relationship between the length of time since diagnosis of CAH and knowledge score (p=0.591). However, in that study, individuals whose total score was <25 (maximum score 44) were generally family members of children diagnosed with CAH more than 10 years ago. This indicates that education is an ongoing process which should address the evolving needs of the family. No significant difference was found between the scores of mothers as compared to fathers in our study, a finding supported by previous studies (21).

The study had certain limitations. Questions related to quality of life were not included as it was felt that such issues could not be assessed adequately in this type of questionnaire. Socioeconomic status, parental education level, and ethnicity data were not collected. Only English speaking/reading participants could be enrolled. Patient population will be considerably more racially and ethnically diverse over the next several decades (38), and efforts are needed to translate these tools and disseminate health information in multiple languages. Long-term follow-up to evaluate changes in attitudes, practices, and outcomes of our study participants was not conducted as part of this study. Future studies to create educational modules for disorders with multi-hormone involvement are required.

In conclusion, we developed and validated a PEKAQ that will be suitable for use in conjunction with education in pediatric endocrinology clinics. The PEKAQ designed in this study is the first tool for this purpose, to our knowledge. It will be invaluable in assessing parental and patient understanding of their disorder and identifying deficits that can be addressed through education. Significant improvement in knowledge of children and parents of children with chronic endocrine disorders was shown in this study. Our education model might guide development of future programs. It is important to start the education process early in the course of management, include adolescents in discussions, and integrate input of pediatric endocrinologists, pediatricians, family physicians, and nurse educators.

Acknowledgments

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Ethics

Ethics Committee Approval: Institutional Review Board at Childrens Hospital of Michigan, Informed Consent: Written informed consent was obtained from participating parents and from parents or legal guardians of participating children younger than 18 years.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Nidhi Gupta, Marwan Zidan, Chandra Edwin, Design: Nidhi Gupta, Marwan Zidan, Chandra Edwin, Data Collection or Processing: Nidhi Gupta, Kathleen Moltz, Amita Adhikari, Colleen Buggs-Saxton, Hanaa Zidan, Dania Abushanab, Chandra Edwin, Analysis or Interpretation: Nidhi Gupta, Marwan Zidan, Aida Lteif, Chandra Edwin, Literature Search: Nidhi Gupta, Writing: Nidhi Gupta, Marwan Zidan, Aida Lteif, Chandra Edwin, Kathleen Moltz, Amita Adhikari, Colleen Buggs-Saxton, Hanaa Zidan.

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Original Article

The ^{13}C-Glucose Breath Test for Insulin Resistance Assessment in Adolescents: Comparison with Fasting and Post-Glucose Stimulus Surrogate Markers of Insulin Resistance

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ABSTRACT

Objective: To evaluate the use of the ^{13}C-glucose breath test (^{13}C-GBT) for insulin resistance (IR) detection in adolescents through comparison with fasting and post-glucose stimulus surrogates.

Methods: One hundred thirty-three adolescents aged between 10 and 16 years received an oral glucose load of 1.75 g per kg of body weight dissolved in 150 mL of water followed by an oral dose of 1.5 mg/kg of U-^{13}C-Glucose, without a specific maximum dose. Blood samples were drawn at baseline and 120 minutes, while breath samples were obtained at baseline and at 30, 60, 90, 120, 150, and 180 minutes. The ^{13}C-GBT was compared to homeostasis model assessment (HOMA) IR (≥p95 adjusted by gender and age), fasting plasma insulin (≥p90 adjusted by gender and Tanner stage), results of 2-h oral glucose tolerance test (OGTT), insulin levels (≥65 μU/mL) in order to determine the optimal cut-off point for IR diagnosis.

Results: ^{13}C-GBT data, expressed as adjusted cumulative percentage of oxidized dose (A% OD), correlated inversely with fasting and post-load IR surrogates. Sexual development alters A% OD results, therefore individuals were stratified into pubescent and post-pubescent. The optimal cut-off point for the ^{13}C-GBT in pubescent individuals was 16.3% (sensitivity=82.8% & specificity=60.6%) and 13.0% in post-pubescents (sensitivity=87.5% & specificity=63.6%), when compared to fasting plasma insulin. Similar results were observed against HOMA and 2-h OGTT insulin.

Conclusion: The ^{13}C-GBT is a practical and non-invasive method to screen for IR in adolescents with reasonable sensitivity and specificity.

Keywords: ^{13}C-glucose breath-test, insulin resistance, oral glucose tolerance test, adolescents

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The ^{13}C-glucose breath test (^{13}C-GBT) is an accurate and reliable method to detect glucose metabolism disorders in adults, however, the use of this technique to assess insulin resistance (IR) in pediatric individuals is still under examination.

WHAT THIS STUDY ADDS?

The ^{13}C-GBT was evaluated in a large population that included individuals with different body mass indexes and stages of pubertal development, furthermore, the A% oxidized dose was compared against IR surrogates in both fasting and post-load scenarios. This protocol suggests cut-off points to identify IR with reasonable sensitivity and specificity.

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Introduction

Type 2 diabetes mellitus (T2DM) is a prevalent chronic disease and represents a grievous public health problem (1). This condition is initially asymptomatic, but it is a definite risk factor for cardiovascular disease, nephropathy, and neuropathy. Morbidity- and mortality-related T2DM decreases with adequate metabolic control, therefore, an early diagnosis is imperative (2). Insulin resistance (IR) is defined as a state in which a normal or elevated insulin level produces an attenuated biological response and constitutes a physiopathological basis for development of T2DM (3). IR is associated to a sedentary lifestyle and an unbalanced diet - risk factors commonly observed in adolescence. Thus, adolescence is a critical period of life for diagnosis and initiation of lifestyle intervention in at-risk individuals (4,5,6).

The gold standard to diagnose IR is the hyperinsulinemic euglycemic clamp (HEC) because it provides a direct, dynamic, and accurate assessment (7); nonetheless, this is an expensive and highly invasive method, unfeasible for standard clinical pediatric practice (8). Surrogate IR markers are frequently used to detect IR in ordinary settings, such as the homeostasis model assessment (HOMA-IR) which is based on fasting plasma glucose and insulin concentrations (9) and the Matsuda and DeFronzo (10) insulin sensitivity index (ISI-Composite) derived from plasma glucose and insulin levels throughout an oral glucose tolerant test (OGTT). These measurements have a moderate to good correlation with the HEC technique but remain invasive and poorly reproducible (11). The development of practical and non-invasive screening tests to detect IR is imperative (12).

The ^{13}C-glucose breath test (^{13}C-GBT) has been shown to be an accurate and reliable method to identify glucose metabolism disorders in adults (13,14). The ^{13}C-GBT consists of ingestion of a ^{13}C-glucose dose used as a tracer to label exhaled CO_2 together with an oral load of non-labeled glucose to challenge insulin-dependent tissues. Patients with impaired glucose metabolism will have a reduced amount of exhaled $^{13}CO_2$; this represents an indirect measurement of glucose oxidation via Krebs cycle (13,15). Lewanczuk et al (13) have shown that the ^{13}C-GBT is effective to assess insulin sensitivity in obese individuals with T2DM. Recently, our group demonstrated that the ^{13}C-GBT is a reproducible method to identify glucose metabolism defects also in adults without T2DM (14). We also established that the ^{13}C-GBT is a valid method for screening for metabolic syndrome in adolescents (16).

The ^{13}C-GBT has been extensively studied in adults. However, the use of this method to evaluate IR in pediatric individuals has been insufficiently explored. The purpose of this study was to assess the use of the ^{13}C-GBT for IR detection in adolescents through comparison with fasting and post-glucose stimulus IR surrogates.

Methods

This cross-sectional study was conducted in the Medical Nutrition Research Unit of the Mexican Social Security Institute in Mexico City, Mexico. The protocol was approved by the Ethics Committee of the said institution (R-2010-3603-35). One hundred thirty-three apparently healthy adolescents aged between 10 and 16 years assented to participate in the study. Informed consent was provided by the parents or by the accompanying adult. Exclusion criteria were: current chronic disease, diagnosed T2DM or presence of a capillary blood glucose level of ≥126 mg/dL, the use of medications that affect glucose metabolism, and fever in the last 48 hours.

Procedures

Voluntary participants arrived to the Medical Nutrition Research Unit with their parents or legal guardian at 8:00 am after an 8-hour fast. Anthropometric measurements (weight, height, and body mass index) were obtained. Subjects received an oral glucose load of 1.75 g per kg of body weight up to a maximum dose of 75 g (ACS reagent; Sigma-Aldrich, St. Louis, MO) dissolved in 150 mL of water, followed by a dose of 1.5 mg/kg of universally labeled ^{13}C-glucose (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) mixed in 50 mL of water, without a specific maximum dose. Blood samples were drawn at baseline and 120 minutes through an antecubital venipuncture. Breath samples were obtained at baseline and at 30, 60, 90, 120, 150, and 180 minutes in 10 mL Exetainer® test tubes (Labco Limited, UK), because in a previous protocol, ^{13}C-GBT results had the highest reproducibility when measured at 180 minutes (14).

Biochemical Determinations

Plasma glucose levels were quantified using an enzymatic method (YSI 2300 Stat Plus™ glucose analyzer; YSI Inc., Yellow Springs, OH), and plasma insulin levels were measured by radioinmmunoassay employing a commercial kit (Millipore, Billerica, MA). The coefficients of variation (CV%) for glucose and insulin were 3.9% and 7.5%, respectively.

As an IR surrogate, the HOMA-IR was calculated with the following formula (9):

HOMA-IR=[fasting glucose (mg/dL) *fasting insulin (μU/mL)]/405.

Breath CO_2 Measurements

Carbon 13 in breath samples was determined with an isotope ratio mass spectrometer BreathMat Plus (Finnigan, Bremen, Germany; CV <1%). Breath test data were expressed as cumulative percentage of oxidized dose at 180 minutes (A% OD) as described previously (17).

Statistical Analysis

Data analysis was performed with the SPSS software (version 19; SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to assess data distribution. Data are presented as mean ± standard deviation or median (minimum-maximum) for normal or non-normal distribution, respectively. ANOVA test with Tukey post-hoc analysis or Kruskall-Wallis tests with Mann-Whitney U-test were used for the comparison between groups according to data distribution. Linear multiple regression models were used to summarize associations of insulin resistance surrogates (HOMA-IR, fasting plasma insulin and 2-h OGTT insulin) with sexual development (Tanner stage) and gender. Correlation coefficients were determined with Pearson's or Spearman's analyses according to data distribution. To determine the optimal cut-off point for IR diagnosis through the ^{13}C-GBT, several receiver-operating characteristic (ROC) curves were constructed with a 95% confidence interval.

Results

A total of 133 adolescents (62 females and 71 males) living in Mexico City were enrolled during 2011. Mean age was 13 years, weight and abdominal circumference values ranged from 34 to 113 kg and from 63 to 129 cm, respectively. Body mass index (BMI) presented a median of 23 (15.6 to 37.8 kg/m^2), and this parameter was used to classify individuals into three groups according to the child growth standards established by the World Health Organization (18), namely, lean (BMI between p3 and p85, 42.1%), overweight (BMI >p85, 14.3%) and obese (BMI >p97, 43.6%). Data describing the study sample and the statistically significant differences between the subgroups are summarized in Table 1.

The following parameters had statistically significant differences among the three subgroups: weight, BMI, abdominal circumference, fasting plasma insulin, and HOMA-IR (19). When contrasting lean versus obese and overweight versus obese individuals, 2-h OGTT insulin and A% OD at 180 minutes differed significantly. The comparison of lean versus overweight and lean versus obese subjects revealed that the 2-h OGTT glucose was substantially different. Finally, fasting plasma glucose achieved a statistically relevant difference only between lean and obese individuals.

Three multiple regression models with three different IR surrogates were used to determine the influence of Tanner stage and gender on ^{13}C-GBT; IR was defined as HOMA-IR ≥p95 reference score adjusted by gender and age (19), fasting plasma insulin ≥p90 reference score adjusted by gender and Tanner stage (20), and 2-h OGTT insulin ≥65 µU/mL (21). Gender does not substantially alter ^{13}C-GBT when co-analyzed with HOMA-IR (β=0.8; p=0.361), fasting plasma insulin (β=1.0; p=0.239), and 2-h OGTT insulin (β=1.4; p=0.131). In contrast, it was established that Tanner stage modifies ^{13}C-GBT when co-evaluated with HOMA (β=-2.1; p=0.017), fasting plasma insulin (β=-1.9; p=0.034), and 2-h OGTT insulin (β=-2.1; p=0.017), therefore, in subsequent analyses, the sample was stratified into pubescent (Tanner stages 2 and 3) and post-pubescent (Tanner stages 4 and 5) individuals. Of the total sample, 46.6% were classified as pubescent and 53.4% as post-pubescent.

A Spearman's rank correlation coefficient revealed that BMI, HOMA-IR, fasting plasma insulin, 2-h OGTT insulin, and 2-h OGTT glucose were inversely associated to A% OD at 180 minutes (Table 2). In contrast, fasting plasma glucose did not achieve statistical significance.

Several ROC curves were plotted to determine the optimal cut-off points for the A% OD at 180 minutes according to different IR surrogates as described previously (Figure 1). Diagnostic attributes of the ^{13}C-GBT for each cut-off point are presented in Table 3. In pubescent and post-pubescent individuals, the ^{13}C-GBT rendered the highest accuracy when compared to fasting plasma insulin. With said parameter, in pubescent individuals, an A% OD at 180 minutes ≤16.3% diagnoses IR with a sensitivity of 82.8%, a specificity of 60.6%, a positive predictive value (PPV) of 64.9%, and a negative predictive value (NPV) of 80.0%. In post-pubescent subjects, an A% OD at 180 minutes ≤13.0% indicates IR with a sensitivity of 87.5%, a specificity of 63.6%, a PPV of 41.1%, and a NPV of 94.6%.

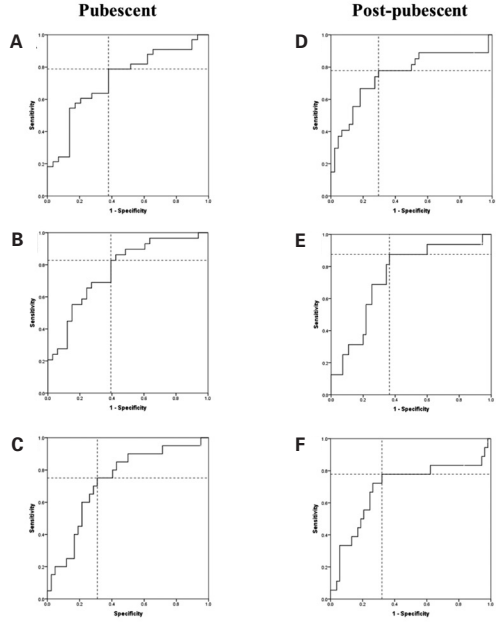

Figure 1. Receiver operating characteristic curves evaluating the sensitivitiy and specificity of the ^{13}C-glucose breath test using different criteria to define insulin resistance. A-C: Performance in pubescent individuals. D-C: Performance in post-pubescent individuals. A, D= HOMA-IR ≥p95 reference score; B, E= fasting plasma insulin ≥p90 reference score; C, F: plasma insulin at the 2-hour time point of an oral glucose tolerance test ≥65 µU/mL

Table 1. Inter-group comparison of the study group according to body mass index

Characteristic	All (n=133)	Lean (n=56)	Overweight (n=19)	Obese (n=58)
Age (years)[b]	13 (9-16)	14 (12-16)	13 (11-15)	13 (9-16)
Weight (kg)[a,b,c]	58 (34-113)	49 (34-73)	59 (44-80)	69.5 (46-113)
Height (m)	1.6 (1.4-1.8)	1.6 (1.4-1.8)	1.6 (1.5-1.8)	1.6 (1.4-1.8)
BMI (kg/m2)[a,b,c]	23 (15.6-37.8)	20 (15.6-23)	22.8 (19.3-26)	27.6 (22.3-37.8)
AC (cm)[a,b,c]	86 (63-129)	76 (63-93)	86 (79-102)	95 (81-129)
FPG (mg/dL)[b]	85.6±7.9	83±7.4	86.7±7.9	87.8±7.8
2-h OGTT PG (mg/dL)[a,b]	97±16.4	89.1±13.2	100.2±14.5	103.4±16.7
FPI (µU/mL)[a,b,c]	14.2 (4.5-43.6)	9.7 (4.5-27.6)	12.1 (7.3-26.4)	19.4 (6.4-43.6)
2-h OGTT PI (µU/mL)[b,c]	45.6 (9.9-266.9)	30.1 (9.9-147.2)	43.4 (11.9-136.6)	69.1 (18.8-266.9)
HOMA-IR[a,b,c]	2.9 (0.8-9.7)	2 (0.8-5.6)	2.8 (1.5-5.4)	4.3 (1.3-9.7)
A% OD (%)[b,c]	14.1±5.4	17.6±4.6	15±3.7	10.5±4.2

Data presented as mean standard deviation or median (minimum-maximum). The comparison between groups was made by either ANOVA test with Tukey post hoc analysis or a Kruskall-Wallis test with Mann-Whitney U-test comparison between groups ± standard deviation or median (minimum-maximum).
[a]: Lean subjects group versus overweight subjects group, p<0.05;
[b]: Lean subjects group versus obese subjects group, p<0.05;
[c]: Overweight subjects group versus obese subjects group, p<0.05.
BMI: body mass index, AC: abdominal circumference, FPG: fasting plasma glucose, 2-h OGTT PG: plasma glucose at the 2-hour time point of an oral glucose tolerance test, FPI: fasting plasma insulin, 2-h OGTT PI: plasma insulin at the 2-hour time point of an oral glucose tolerance test, HOMA-IR: homeostasis model assessment for insulin resistance, A% OD: adjusted percentage of oxidized ^{13}C-glucose dose

Table 2. Correlation of the adjusted percentage of oxidized ^{13}C-glucose dose at 180 minutes with different insulin resistance surrogates

	BMI	HOMA-IR	FPI	2-h OGTT PI	FPG	2-h OGTT PG
All	-0.659	-0.473	-0.473	-0.413	-0.164*	-0.381
Pubescent	-0.649	-0.531	-0.508	-0.443	-0.279	-0.426
Post-pubescent	-0.664	-0.492	-0.481	-0.415	-0.161*	-0.330

(Spearman's rank correlation coefficient of the adjusted percentage of oxidized ^{13}C-glucose dose at 180 minutes with different insulin resistance surrogates and glucose metabolism markers, p<0.05).
BMI: body mass index, HOMA-IR: homeostasis model assessment for insulin resistance, FPI: fasting plasma insulin, 2-h OGTT PI: plasma insulin at the 2-hour time point of an oral glucose tolerance test, FPG: fasting plasma glucose, 2-h OGTT PG: plasma at the 2-hour time point of an oral glucose tolerance test; *non-significant association

Table 3. Diagnostic attributes of the ^{13}C-glucose breath test (according to different cut-off points for the oxidized ^{13}C-glucose dose at 180 minutes)

	A% OD	Sensitivity	Specificity	PPV	NPV	Accuracy	p
Pubescent							
HOMA-IR ≥p95	≤16.0%	78.9%	62.1%	70.3%	72.0%	71.0%	0.003
FPI ≥p90	≤16.3%	82.8%	60.6%	64.9%	80.0%	76.6%	0.000
2-h OGTT PI ≥65 µU/mL	≤14.6%	75.0%	69.0%	53.6%	85.3%	71.0%	0.004
Post-pubescent							
HOMA-IR ≥p95	≤13.0%	77.8%	70.5%	61.8%	83.8%	73.2%	0.000
FPI ≥p90	≤13.0%	87.5%	63.6%	41.1%	94.6%	73.6%	0.004
2-h OGTT PI ≥65 µU/mL	≤12.6%	77.8%	67.9%	45.2%	90.0%	70.4%	0.016

HOMA-IR: homeostasis model assessment for insulin resistance, FPI: fasting plasma insulin, 2-h WSOGTT PI: plasma insulin at the 2-hour time point of an oral glucose tolerance test, A% OD: adjusted percentage of oxidized ^{13}C-glucose dose at 180 minutes, PPV: positive predictive value, NPV: negative predictive value

Discussion

The purpose of this study was to explore the use of the ^{13}C-GBT to identify IR in adolescents with different BMIs. Even though the use of ^{13}C-GBT has been extensively described in adults, to our knowledge, definite cut-off points for pediatric populations have not yet been established. In this study, we compare the ^{13}C-GBT with fasting plasma insulin, HOMA-IR, and 2-h OGTT insulin to propose several cut-off points for IR diagnosis. Similar values of sensitivity, specificity, PPV, and NPV were observed among the different IR surrogates contrasted with the ^{13}C-GBT. Our results are similar to those described by Ibarra-Pastrana et al (22) in Mexican adults, where the ^{13}C-GBT rendered a sensitivity of 80% and a specificity of 67.4% when compared to HOMA-IR. Moreover, the cut-off points proposed in this article are comparable with the ones recommended for the detection of metabolic syndrome in adolescents (sensitivity=81.5% and specificity=66.7%) in a recent study published by our group (16).

The ^{13}C-GBT is based on the premise that subjects with impaired insulin sensitivity have a reduced glucose uptake in response to insulin; consequently, the A% OD at 180 minutes is diminished in subjects with IR. Indeed, the ^{13}C-GBT correlates inversely with fasting and post load IR surrogates. Similar associations were described by Jetha et al (15) who compared the ^{13}C-GBT with fasting plasma insulin ($r=-0.51$, $p<0.01$), HOMA-IR ($r=-0.51$, $p<0.01$) and 2-h OGTT insulin ($r=-0.040$, $p<0.05$) in 39 pre-pubescent obese individuals.

Interestingly, even though the A% OD at 180 minutes is inversely associated to BMI, HOMA-IR, fasting plasma insulin, 2-h OGTT insulin and 2-h OGTT, fasting plasma glucose is not statistically correlated, probably because fasting plasma glucose is affected tardily in the pathogenesis of T2DM (23), this supports employing the ^{13}C-GBT for early IR detection.

Undoubtedly, a weakness in this protocol is that the ^{13}C-GBT was not contrasted against the HEC which is considered the gold standard to assess insulin sensitivity, therefore, the suggested cut-off points may undervalue or overestimate the true diagnostic performance of the ^{13}C-GBT. However, the HEC is highly invasive and relatively unsuitable for pediatric subjects, thus, the ^{13}C-GBT was compared against commonly used IR surrogates. Future studies to validate the sensitivity and specificity of the ^{13}C-GBT for IR in adolescents are warranted.

The evaluation of the ^{13}C-GBT in a substantial sample with a wide BMI spectrum is a definite strength of this study. Even though obesity is strongly associated to IR, it is now accepted that physically lean subjects may have defective insulin sensitivity and are candidates for IR screening (24,25). Another valuable asset is that the proposed cut-off points for the ^{13}C-GBT were stratified according to sexual development, since it has been established that sexual hormones influence glucose homeostasis (26,27).

Considering the increasing evidence regarding the existence of noxious metabolic disorders at young ages, the epidemiological relevance of T2DM, the long-term complications associated to impaired glucose homeostasis, and the knowledge that lifestyle interventions in individuals with IR are effective to prevent or delay the onset of T2DM (28), the development of methodologically feasible procedures to screen for IR in large populations is fundamental for the implementation and assessment of public health strategies to face the T2DM epidemic. The use of non-invasive methods to detect IR is particularly appealing for pediatric populations, where pain and emotional stress associated to venipuncture are continuing concerns (29). Moreover, T2DM is recognized as a progressive disease; in fact, a young age at onset of T2DM is associated with a higher incidence of macrovascular complications (30), therefore, early IR detection is distinctly relevant in pediatric subjects. The non-invasive nature of the ^{13}C-GBT and its reasonable diagnostic performance render this method suitable to perform IR screening in large pediatric populations.

Acknowledgments

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Ethics

Ethics Committee Approval: This protocol was approved by the Ethics Committee of the Mexican Social Security Institute (registry number: R-2010-3603-35) in Mexico City, Mexico, Informed Consent: Informed consent was provided by the parents or by the accompanying adult.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Jorge Maldonado-Hernández, Design: Jorge Maldonado-Hernández, Azucena Martínez-Basila and Alejandra Salas-Fernández, Data Collection or Processing: Azucena Martínez-Basila and Alejandra Salas-Fernández, Analysis or Interpretation: Mónica I. Piña-Aguero, José R. Navarro-Betancourt and Mariela Bernabe-García, Literature Search: Mariela Bernabe-García, Writing: Mónica I. Piña-Aguero and José R. Navarro-Betancourt.

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Original Article

Lower Plasma Ghrelin Levels are Found in Women with Diabetes-Complicated Pregnancies

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ABSTRACT

Objective: To evaluate the associations of glycemic control and gestational age with ghrelin and proinsulin levels in cord blood and mothers' peripheral blood during pregnancy.

Methods: This is a cross-sectional comparative study of twenty-four pregnant women with gestational diabetes (GD), 18 with type 2 diabetes mellitus (T2DM), and 36 without diabetes, as well as their neonates. Levels of proinsulin, ghrelin, and glycated hemoglobin A1c (HbA1c) were measured from maternal blood during the last week before caesarian delivery and in neonatal umbilical cord blood samples.

Results: Mothers with GD and T2DM had significantly lower ghrelin levels compared to the healthy mothers ($p<0.001$). Maternal proinsulin was lower in women with GD than in women without diabetes ($p<0.001$). Proinsulin was significantly elevated in the neonates of women with GD and in women with HbA1c ≥6.5% ($p<0.001$). However, maternal ghrelin levels were higher ($p=0.031$) and neonate proinsulin levels lower in the pre-term offspring of mothers with GD ($p=0.033$). There was a negative correlation between HbA1c levels and birth weight ($r=-0.407$, $p<0.001$).

Conclusion: Ghrelin levels were lower in pregnant women with diabetes, although pre-term birth appeared to reverse this trend in GD. Proinsulin levels were also low in pregnant women with diabetes and even lower in pre-term vs. at-term births. Both ghrelin and proinsulin levels were lower in pregnant women with diabetes and HbA1c of <6.5%. Thus, ghrelin participates in the adaptation to the caloric imbalance of diabetic pregnancy and may play a similar role in pregnancy-related complications, since high ghrelin concentrations may be necessary for normal fetal development.

Keywords: Proinsulin, ghrelin, diabetes, hyperglycemia, neonates

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Although ghrelin and proinsulin can regulate several metabolic pathways, few studies have evaluated these hormones in mothers with diabetes and their neonates.

WHAT THIS STUDY ADDS?

Our results indicate that pregnant women with gestational or type 2 diabetes had significantly lower ghrelin levels, compared to non-diabetic pregnant women. However, pregnant women with gestational diabetes had significantly lower proinsulin levels, compared to non-diabetic pregnant women. Thus, ghrelin participates in the adaptation to the caloric imbalance of diabetic pregnancy and may play a similar role in pregnancy-related complications, since high ghrelin concentrations may be necessary for normal fetal development.

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Introduction

Ghrelin helps modulate appetite and regulate several metabolic pathways. This peptide hormone is mainly produced in the stomach, although small amounts are also produced in the hypothalamus, kidney, heart, pancreatic cells, and placenta (1). Ghrelin has a role in both endocrine (e.g., the release of prolactin and adrenocorticotrophic hormone) and non-endocrine (e.g., stimulating gastric acid secretion and intestinal motility) functions (2,3). Ghrelin has two molecular forms: acylated ghrelin (acylated at the serine-3 residue) and des-acyl ghrelin. Women with gestational diabetes (GD) have lower levels of acylated ghrelin, which may reflect the inhibitory effect of insulin on ghrelin secretion (4). Although the role of ghrelin in fetal adaptation to intrauterine malnutrition is incompletely understood, there are studies which indicate a strong correlation between acylated and total ghrelin levels as a parameter for neonatal ghrelin regulation (5,6,7). Nevertheless, high ghrelin concentrations appear to be necessary for normal fetal development and this requires an optimal uterine environment that is free from hyperglycemia (8).

The relationships between ghrelin levels and various anthropometric and biochemical measurements remain controversial (9). Diabetes may also affect ghrelin concentrations, as plasma ghrelin levels are thought to decrease during hyperglycemia and hyperinsulinemia, although studies of ghrelin levels in pregnant women with diabetes are scarce (10).

Proinsulin is synthesized by the early embryo, before the differentiation of the pancreas. Proinsulin stimulates cardiogenesis and prevents apoptosis during neurulation (11). As sugars easily cross the placenta, the fetal pancreas responds to hyperglycemia by increasing insulin production. This process leads to fetal hyperinsulinemia, which affects carbohydrate, protein, and fat metabolism and is associated with an increased risk of metabolic diseases in adulthood (10,12). Furthermore, similar to insulin and insulin-like growth factor 1, proinsulin has an impact as a growth factor and this property may be associated with the higher incidence of congenital defects among children of diabetic mothers (11,13,14,15,16). Hyperglycemia can also induce metabolic damage by causing beta cell injury (17). Although long-term follow-up studies on this issue are lacking, alterations in maternal metabolism are reported to be associated with pancreatic islet hyperplasia, changes which may have long-term consequences for the fetus (18).

In this present study, using glycated hemoglobin A1c (HbA1c) levels, we evaluated the association of glycemic control with ghrelin and proinsulin concentrations in umbilical cord blood and maternal peripheral blood. We also evaluated the associations between maternal and neonatal ghrelin and proinsulin levels in preterm and term deliveries. Our hypothesis was that pregnant women with GD or type 2 diabetes mellitus (T2DM) would be more likely to have neonates with decreased ghrelin and increased proinsulin concentrations compared to the women without diabetes, which might be a risk factor for pre-term delivery.

Methods

This cross-sectional comparative study evaluated women with type 2 or GD and their offspring according to the American Diabetes Association criteria (19). We included 78 pregnant women and their neonates in the study. Of the women, 42 (53.8%) were diabetics. A group consisting of 36 (46.2%) healthy women and their offspring served as controls. The study and control groups were recruited from among consecutive pregnant women who were covered by our social security system and who had attended scheduled visits at the Hospital of Gynecology Pediatrics 3A (UMAE, National Medical Center "La Raza") over an 11-month period. The exclusion criteria for mothers were type 1 diabetes mellitus (T1DM), pregnancy complications (e.g. pre-eclampsia), arterial hypertension, chronic renal or hepatic disease, cardiac failure, arrhythmia, cardiomyopathy, receiving steroids within 24 h after delivery, and serious maternal or fetal complications during the birth process. Also excluded were neonates born vaginally, those with an Apgar score <6 at 1 minute, with a short umbilical cord (unable to take blood sample), with sepsis, or with meconium in the amniotic fluid. The study was approved by the Ethics and Research Committee of the Mexican Social Security Institute and complied with the tenets of the Declaration of Helsinki. All participants provided written informed consent.

A blood sample was obtained from each mother during the last week before caesarian delivery and after a 12-hour fast and was used to measure plasma HbA1c, ghrelin, and proinsulin concentrations. Immediately after birth, a 10-mL cord blood sample was obtained for determination of total ghrelin and proinsulin concentrations. After the neonate became stable, supine body length (in millimeters, taken on a hard horizontal surface from crown to heel), unclothed weight (in grams), and cephalic perimeter (in centimeters) were measured. Gestational age was determined using the Capurro evaluation system (20). Infant weight was classified as adequate-, low-, or high-for-gestational-age according to the Lubchenco tables (21).

Whole blood HbA1c levels were determined using ion exchange high-performance liquid chromatography (normal range: 4-6%). The untreated samples were stored in aliquots at -80 °C until analysis. Total ghrelin and proinsulin concentrations were determined via radioimmunoassay using reagents from Millipore Corporation (MA, USA). The total ghrelin test has a sensitivity of 93 pg/mL, with intra-

and inter-assay coefficients of variation (CVs) of 8.0% and 9.5%, respectively. The proinsulin test has a sensitivity of 2 pMol/L (normal fasting range: 7.9±1.5 pMol/L) and CVs of 5.0% and 10.1%, respectively.

Definitions

Maternal HbA1c levels of ≥6.0% are generally considered indicative of inadequate glycemic control, according to the American Diabetes Association recommendations (19), while levels <6.5% are considered adequate (22). Therefore, we defined maternal hyperglycemia as a plasma HbA1c level of ≥6.5%. Any infant born before the completion of 37 weeks of gestation was classified as pre-term and those born at 37-42 weeks as a term infant. The infants were defined as small-for-gestational-age if they were below the 10th percentile for body weight, appropriate-for-gestational-age if between the 10th and 90th percentiles, and large-for-gestational-age if above the 90th percentile in weight (21).

Statistical Analysis

Analysis of variance or the Kruskal-Wallis test was used to compare anthropometric values and ghrelin, proinsulin, and HbA1c levels. A Pearson correlation coefficient test was used for data with a normal distribution and Spearman correlation coefficient for data with a non-normal distribution. Student's t-test was used to compare parametric variables and the Mann-Whitney U test was used to compare the non-parametric variables, proinsulin levels in neonates of mothers with or without diabetes, proinsulin levels in pre-term and at-term neonates, and according to metabolic control. All analyses were performed using Statistical Package for the Social Sciences software (version 15; SPSS Inc., Chicago, IL, USA). The "General Lineal Models" module was used in lineal regression to adjust for the confounding factors of body mass index (BMI), age, disease duration, and HbA1c at the end of pregnancy. A p-value of <0.05 was considered significant.

Results

Table 1 shows the characteristics of the mothers and neonates. Among the mothers with diabetes, 57.1% (24/42) had GD and 42.9% (18/42) had T2DM. The durations of type 2 and GD were 29.8±23.4 months (range: 4-84 months) and 3.4±2.1 months (range: 1-8 months), respectively. Among women with diabetes, 54.8% (23/42) had an HbA1c value of <6.5%, 19% (8/42) were of normal weight, 50% (21/42) were overweight, and 31% (13/42) were obese. Dietary management was prescribed for 52.4% (22/42) of these women, while insulin therapy was necessary for 37.5% (n=9) of women with GD and for 61.1% of women with T2DM (n=11). Only 8.3% (3/36) of the non-diabetic mothers were overweight. It should be noted that one of the women without diabetes had an HbA1c of 6.2%. Nevertheless, both her fasting glucose (95 mg/dL) and insulin (12.7 μU/mL) levels were within normal ranges, and in the follow-up, she was not diagnosed as a diabetic in view of the principles suggested by Metzger et al (22).

Of infants born to mothers with type 2 or GD, 64.3% (27/42) were male and 45% (19/42) were pre-term. Of these neonates, 4.7% (2/42) were of low-for-gestational-age birthweight, 69% (29/42) of normal birthweight, and 26.3% (11/42) of high-for-gestational age birthweight. All neonates from non-diabetic mothers were born at term with normal birthweights and 55.6% (20/36) were male.

There was a significant difference in proinsulin levels between neonates who were born to mothers with or without diabetes (p<0.001) as well as between neonates from mothers with an HbA1c level of <6.5% or ≥6.5% (p<0.001) (Table 1).

Pregnant women with gestational or T2DM had significantly lower plasma ghrelin levels compared to women without diabetes (p<0.001). This difference remained significant after adjusting for BMI, age, disease duration, and HbA1c levels at the end of pregnancy. Maternal ghrelin concentrations were significantly higher in women without diabetes vs. women with diabetes (p=0.013). Ghrelin levels were also high in women with HbA1c levels <6.5% (vs. HbA1c of ≥6.5%) (p=0.01) (Table 2).

While maternal ghrelin levels were lower in the presence of diabetes, when only women with diabetes were evaluated, maternal ghrelin concentrations were higher in women who had pre-term deliveries (vs. term), and especially so in women with GD (p=0.031). The same trend was observed for women with T2DM, although the differences were not statistically significant (Table 3). There was no difference in proinsulin levels between women with and without diabetes. Pregnant women with T2DM and at-term birth had significantly higher proinsulin concentrations vs. pre-term birth (p=0.027), although this trend was not significant in women with GD (p=0.63).There was no difference in ghrelin concentrations between neonates who were born to mothers with or without diabetes. Furthermore, ghrelin levels were not modified by a maternal HbA1c level ≥6.5% or by pre-term birth. Nevertheless, negative correlations were observed between HbA1c concentration and birth weight (r=–0.407, p<0.001), ghrelin concentrations and birth weight among term neonates (r=–0.270, p=0.039), and between maternal ghrelin and neonatal ghrelin levels (r=–0.328, p=0.034).

Neonates born to mothers with diabetes had significantly higher proinsulin levels, regardless of glycemic control [adequate glycemic control (p<0.001) and inadequate glycemic control (p=0.026)]. Elevated proinsulin levels were observed in neonates born to women with T2DM and with an HbA1c value of <6.5% (177.6 pMol/L) and in neonates born to women with GD and an HbA1c value of ≥6.5% (80.9 pMol/L) (data not shown).

Table 1. Characteristics of the study sample

		No diabetes	Gestational diabetes	Type 2 diabetes mellitus	
		n=36	**n=24**	**n=18**	**p-value**
Mothers	Age, years	26.6±5.3 (17-36)	32.2±6.7 (18-44)	33.0±6.7 (20-43)	**<0.001**
	Pre-gestational weight, kg	60.6±3.4 (55-67)	71.6±12.4 (55-107)	67.4±11.9 (44-96.8)	**<0.001**
	Height, m	1.62±2.6 (158-168)	1.57±6.2 (145-169)	1.55±0.7 (145-170)	**<0.001**
	BMI, kg/m^2	22.9±1.2 (20.9-25.5)	28.8±4.5 (22.4-40.7)	27.8±4.4 (20.0-41.3)	**<0.001**
Obstetrics	Previous macrosomic pregnancy, n (%)	**0**	9 (37.5)	1 (5.6)	**<0.001**
	Previous GD, n (%)	**0**	2 (8.3)	1 (5.6)	0.240
	Previous congenital malformations, n (%)	**0**	3 (12.5)	3 (16.7)	0.058
	Pulmonary maturity inducers, n (%)	**0**	2 (8.3)	1 (5.6)	0.240
	Cord around the neck, n (%)	**0**	2 (8.3)	4 (22.2)	**0.016**
Neonates	Weight, kg	3.440±0.509 (3.000-3.985)	3.127±0.697 (1.720-4.630)	3.268±0.527 (2.380-4.060)	0.110
	Height, cm	50.11±0.62 (49-52)	49.56±3.30 (40-55)	49.97±2.11 (45.5±56)	0.777
	Gestational age, weeks	39.52±2.69 (38-40)	36.62±2.01 (31-39)	36.0±3.04 (29-40)	**<0.001**
	Low birth weight, kg	n=0	n=2 (8.4%) 2.220±0.226 (2.060-2.380)	n=0	---
	Normal birth weight, kg	n=36 (100%) 3.440±0.250 (3.000-3.985)	n=17 (70.8%) 2.971±0.525 (1.720-3.700)	n=12 (66.7%) 2.977±0.367 (2.380-3.540)	**<0.001**
	High birth weight, kg	n=0	n=5 (20.8%) 4.020±0.412 (3.550-4.630)	n=6 (33.3%) 3.851±0.194 (3.600-4.060)	0.394
Laboratory results	Maternal total ghrelin levels, pg/mL*	438.5 (350-534.5)	273 (110-523.8)	239.2 (127.5-359.9)	**<0.001**
	Neonatal total ghrelin levels, pg/mL*	888.9 (558.5-1,244.4)	872.1 (637-1,198.7)	831.8 (733.2-1,005.4)	0.406
	Maternal proinsulin levels, pMol/L*	16.8 (11.8-21.1)	13.5 (9–38.3)	10.9 (7.7-15.9)	0.055
	Neonatal proinsulin levels, pMol/L*	20.1 (12.7-32.8)	72.8 (22.9-358.2)	32.7 (18.8-334.4)	**<0.001**
	Maternal HbA1c, %	5.5±0.20 (5–6.2)	6.4±1.6 (3.9–10.6)	6.3±1.3 (4.3-9.2)	**0.031**

BMI: body mass index, GD: gestational diabetes, HbA1c: glycated hemoglobin. Data are expressed as mean ± standard deviation, or *median, with (interquartile range). p-values were calculated using analysis of variance or the Kruskal-Wallis test, and p-values of <0.05 (bold) were considered significant. Neonatal ghrelin and proinsulin samples were taken from umbilical cord blood.

Table 2. Total ghrelin and proinsulin levels in the mothers and infants according to glycated hemoglobin levels

	No diabetes	Diabetes		*p-value	Ł p-value
	n=36	**HbA1c <6.5% n=23**	**HbA1c ≥6.5% n=19**	**All three groups**	**≥6.5% vs. <6.5%**
Maternal total ghrelin, pg/mL	438.5 (350-534.5)	247.4 (44.7-403.6)	264.39 (186.3-531.6)	**0.013**	**0.010§**
Neonatal total ghrelin, pg/mL	888.9 (558.5-1,244.4)	918.1 (639.6-1,176.1)	791.7 (667.0-1,019.6)	0.635	0.760§
Maternal proinsulin, pMol/L	16.8 (11.8-21.1)	11.3 (8.9-18.9)	14.4 (5.7-23.7)	0.055†	0.076**
Neonatal proinsulin, pMol/L	20.1 (12.7-32.8)	36.1 (22.4-380.3)	49.7 (14.4-315.3)	**<0.001†**	**<0.001****

*Test compared the groups with no diabetes, with diabetes and hemoglobin A1c of <6.5%, and with diabetes and hemoglobin A1c of ≥6.5%.
ŁTest compared the groups with hemoglobin A1c of <6.5% and ≥6.5%. Data are median (interquartile range), and analyzed using ANOVA, †the Kruskal-Wallis test, §Student's t-test, or **Mann-Whitney U test. p-values of <0.05 (bold) were considered significant. Neonatal ghrelin and proinsulin samples were taken from umbilical cord blood

Table 3. Total ghrelin and proinsulin levels among women with diabetes and their offspring in preterm and term births

		n (%)	Pre-term births median (IQR)	n (%)	Term births median (IQR)	p-value
Gestational Diabetes	Maternal total ghrelin, pg/mL	12 (50)	450.8 (268.7-600.1)	12 (50)	127.9 (26.5-367.8)	**0.031***
	Neonatal total ghrelin, pg/mL	12 (50)	732.1 (538.9-1,320.9)	12 (50)	983.0 (678.5-1,198.7)	0.646*
	Maternal proinsulin, pMol/L	12 (50)	18.9 (8.9-55.5)	12 (50)	13.5 (9.2-19.9)	0.630**
	Neonatal proinsulin, pMol/L	12 (50)	29.2 (16.4-113.5)	12 (50)	248.7 (42.5-525.5)	**0.033****
Type 2 diabetes mellitus	Maternal total ghrelin, pg/mL	7 (39)	247.3 (202.1-304.2)	11 (61)	203.2 (45.3-398.2)	0.520*
	Neonatal total ghrelin, pg/mL	7 (39)	792.0 (700.6-918.1)	11 (61)	909.5 (744.1-1,019.6)	0.367*
	Maternal proinsulin, pMol/L	7 (39)	7.7 (5.6-10.6)	11 (61)	15.4 (9.2-20)	**0.027****
	Neonatal proinsulin, pMol/L	7 (39)	23.8 (12.9-36.1)	11 (61)	87.0 (20.2-395.4)	0.328**
All diabetes	Maternal total ghrelin, pg/mL	19 (45)	304.2 (246.5-531.6)	23 (55)	163.6 (33.7-398.2)	**0.019 ***
	Neonatal total ghrelin, pg/mL	19 (45)	791.7 (563.3-1,036.5)	23 (55)	925.3 (713.3-1,084.4)	0.505*
	Maternal proinsulin, pMol/L	19 (45)	9.9 (7.6-33.7)	23 (55)	15.4 (9.2-20.0)	0.536**
	Neonatal proinsulin, pMol/L	19 (45)	28.3 (14.4-64.7)	23 (55)	126.4 (22.4-405.7)	**0.029****

IQR: interquartile range. p-values were calculated using *Student's t-test or **the Mann-Whitney U test; p-values of <0.05 (bold) were considered significant. Neonatal ghrelin and proinsulin samples were taken from umbilical cord blood.

Discussion

The findings of this study support the concept that ghrelin affects the adaptive response to caloric imbalance. In this context, diabetic pregnancy can involve a positive or negative caloric balance, although women with T2DM typically have a caloric surplus. Our data show that women with gestational or T2DM had significantly lower plasma ghrelin concentrations at term compared to the non-diabetic controls. However, this difference was not observed for women with pre-term neonates, which may indicate that this is an obstetric complication that is caused by ambient stress and/or caloric deficiency. This interaction may explain the partial correction of low ghrelin plasma concentrations in women with pre-term birth compared to women with diabetes. Our results also suggest that ghrelin participates in the adaptation to the caloric imbalance of diabetic pregnancy and may play a similar role in pregnancy-related complications. Few reports have evaluated plasma ghrelin concentrations in women with diabetes and their offspring, although Kos et al (23) found lower plasma ghrelin levels at the end of pregnancy in women with T1DM. However, this finding was not replicated by Hehir et al (24), and Lappas et al (25) reported lower plasma ghrelin concentrations in women with GD, with persistence of this abnormality at 12 weeks post-partum predicting incident maternal diabetes. Aydin et al (26) found transitory low ghrelin levels in women with GD, although the levels normalized at 2 weeks post-partum. The same trend was observed in pregnant women with pre-gestational T2DM, although their ghrelin concentrations remained low compared to the control group. Our results extend the available evidence and indicate that maternal ghrelin concentrations decrease during pregnancy in women with type 2 or GD.

The low ghrelin concentrations during diabetic pregnancy may be related to maternal or placental factors. Insulin resistance is a common feature of T2DM that is exacerbated during pregnancy and is usually associated with decreased ghrelin secretion (27). The placenta also plays an important role in maintaining the appropriate circulating levels of maternal ghrelin during the later gestational stages. Therefore, diabetic pregnancy is a cause of endothelial dysfunction and premature placental aging, which may result in abnormal placental ghrelin secretion.

Our observation that pre-term birth partially reverses low ghrelin concentrations in pregnant women with diabetes is relevant, as maternal ghrelin concentrations do not vary significantly during a normal pregnancy (28). Interestingly, high ghrelin concentrations have been detected in the cord blood of pre-term and small-for-gestational-age infants (5,7). One study evaluated children of women with T1DM and reported that cord blood ghrelin concentrations negatively correlated with birth weight and that female infants had higher ghrelin concentrations. We also observed that weight-for-gestational-age negatively correlated with serum ghrelin in at-term neonates, which is similar to a previous report of weight-for-gestational-age being negatively correlated with neonatal serum ghrelin levels (29). However, another study reported that ghrelin levels did not differ between pre-term and at-term neonates (30). Nevertheless, maternal ghrelin at the end of a pregnancy is not correlated with fetal birth weight or placental weight (23), although there are no data regarding maternal serum ghrelin concentrations in pre-term neonates. These data suggest that maternal ghrelin may help control fetal growth, and ghrelin may be needed for fetal adaptation to abnormal uterine conditions, such as hyperglycemia (31).

Additionally, abnormal ghrelin levels in the newborn may have long-term consequences in the regulation of appetite and weight (32).

Reports have consistently indicated that diabetes in pregnant women increases neonatal proinsulin concentrations, regardless of birth weight. For example, the fetal pancreas would respond to maternal hyperglycemia by increasing insulin production and subsequently cause beta cell hyperplasia in the islets of Langerhans (33,34). Although the effect of maternal diabetes on the conversion of proinsulin to insulin in the fetus is not known, our findings confirm reports which indicate that proinsulin levels are higher in neonates born to diabetic mothers (35). Proinsulin levels may also be a risk marker for the development of diabetes, metabolic syndrome, arterial hypertension, dyslipidemia, and other metabolic diseases (36).

The present study has several limitations. First, the sample size was small, so the conclusions may not be definitive. Secondly, approximately half of the mothers were receiving insulin, and this treatment heterogeneity may be a confounding factor. We also did not have the means, in this paper, to evaluate the differences in age and BMI of the subjects, which might be considered a confounding factor, as suggested by Tschop et al (37). However, both age and BMI were considered in the logistic regression. Also, we did not collect data regarding acylated ghrelin (the active form), although both acylated and unacylated ghrelin levels are altered by diabetes (38,39). Finally, we cannot compare ghrelin levels with those in other studies, due to the heterogeneity of measurement methodology.

In conclusion, ghrelin levels were lower in pregnant women with diabetes, although pre-term birth appeared to reverse this trend in GD. The proinsulin concentrations in pregnant women with diabetes were generally low, and this was particularly true among pregnant women with T2DM and pre-term birth (vs. at-term birth). Finally, among pregnant women with diabetes, proinsulin and ghrelin concentrations were lower in women with HbA1c of <6.5%. These data appear to indicate that ghrelin and proinsulin concentrations in pregnant women and their offspring depend on the type of maternal diabetes, gestational age at birth, and the degree of maternal glycemic control.

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Ethics

Ethics Committee Approval: The study was approved by the Ethics and Research Committee of the Mexican Social Security Institute, complied with the tenets of the Declaration of Helsinki, Informed Consent: All participants provided written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Rita Angélica Gómez-Díaz, Design: Rita Angélica Gómez-Díaz, Data Collection or Processing: Monica P. Gómez-Medina, Eleazar Ramírez-Soriano, Lucio López-Robles, Analysis or Interpretation: Renata Saucedo, Adan Valladares-Salgado, Literature Search: Arturo Zarate, Adan Valladares-Salgado, Writing: Rita Angélica Gómez-Díaz, Carlos A. Aguilar-Salinas, Niels H. Wacher.

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Original Article

Pseudohypoparathyroidism Type 1A-Subclinical Hypothyroidism and Rapid Weight Gain as Early Clinical Signs: A Clinical Review of 10 Cases

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ABSTRACT

Objective: To evaluate the clinical signs and symptoms that would help clinicians to consider pseudohypoparathyroidism (PHP) type 1A as a diagnosis in a child.

Methods: A retrospective review of the medical records of children diagnosed by erythrocyte Gsα activity and/or *GNAS1* gene study and followed-up for PHP type 1A. Clinical and biochemical parameters along with epidemiological data were extracted and analyzed. Weight gain during infancy and early childhood was calculated as change in weight standard deviation score (SDS), using the French growth reference values. An upward gain in weight ≥0.67 SDS during these periods was considered indicative of overweight and/or obesity.

Results: Ten cases of PHP type 1A were identified (mean age 41.1 months, range from 4 to 156 months). In children aged ≤2 years, the commonest clinical features were round lunar face, obesity (70%), and subcutaneous ossifications (60%). In older children, brachydactyly was present in 60% of cases. Seizures occurred in older children (3 cases). Short stature was common at all ages. Subclinical hypothyroidism was present in 70%, increased parathormone (PTH) in 83%, and hyperphosphatemia in 50%. Only one case presented with hypocalcemia. Erythrocyte Gsα activity tested in seven children was reduced; *GNAS1* gene testing was performed in 9 children. Maternal transmission was the most common (six patients). In three other cases, the mutations were de novo, c.585delGACT in exon 8 (case 2) and c.344C>TP115L in exon 5 (cases 6&7).

Conclusion: Based on our results, PHP type 1A should be considered in toddlers presenting with round face, rapid weight gain, subcutaneous ossifications, and subclinical hypothyroidism. In older children, moderate mental retardation, brachydactyly, afebrile seizures, short stature, and thyroid-stimulating hormone resistance are the most suggestive features.

Keywords: Pseudohypoparathyroidism, subclinical hypothyroidism, early obesity, early signs

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Pseudohypoparathyroidism type 1A is a rare heterogeneous disorder characterized by multiple end-organ resistance to hormones that share Gs protein-coupled receptors as signaling pathway. Age at its diagnosis is extremely variable.

WHAT THIS STUDY ADDS?

This study analyzes the clinical and biochemical presenting features to identify those that should raise clinicians' suspicion and lead to the diagnosis work-up.

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Introduction

Pseudohypoaparathyroidism (PHP) type 1A is a rare genetic disorder with autosomal dominant transmission and parental imprinting, characterized by the target-organ unresponsiveness to hormones that share Gs protein-coupled receptors as the signaling pathway. Globally, PHP constitutes a heterogeneous group of disorders that have in common resistance to the action of parathyroid hormone (PTH) (1,2,3). Its prevalence has been estimated, in Japan, around 3.4 cases per million population (4).

The entity is classified into two main types: type 1 and type 2. Type 1 PHP is, in turn, divided into three subtypes 1A, 1B, and 1C, each with different pathogenesis, phenotype, and genetic findings. Overall, the three PHP type 1 subtypes result from the heterozygous loss-of-function mutation in exon 1-13 of the gene encoding Gsα or from imprinting abnormalities in the stimulating G protein, the α-subunit (Gsα) of which constitutes the signaling protein for several hormones [PTH, thyroid-stimulating hormone (TSH), gonadotropins, and glucagon] and neurotransmitters actions (2,3).

Typically, patients with PHP type 1A demonstrate laboratory findings of resistance to PTH, TSH, gonadotropins, and growth hormone-releasing hormone (GHRH), and decreased Gsα activity (≤50%) (1,2,5).

PHP type 1A patients present with typical features termed albright hereditary osteodystrophy (AHO), a constellation of short stature, round face, brachydactyly, brachymetacarpy, centripetal obesity, subcutaneous ossifications, and variable degree of mental or developmental delay (1,2,3).

From the molecular standpoint, AHO results from a heterozygous mutation of the *GNAS* gene encoding the G-stimulatory subunit (Gsα) located at chromosome 20q13.2. This mutation leads to a loss of expression or function of Gsα which impairs the transmission of stimulatory signals to adenylate cyclase with limitation of cyclic AMP generation necessary for hormone action (6). Consequent to the *GNAS* gene being subject to imprinting, AHO patients show variable hormone unresponsiveness: those with mutations on maternally inherited alleles manifest multiple hormone resistance (PTH, TSH, gonadotropins, GHRH, and glucagon) leading to PHP type 1A, while patients with mutations on paternally inherited alleles have phenotypic features of AHO without hormonal resistance (pseudopseudoparathyroidism) in consequence to Gsα expression from the paternal allele being normally suppressed because of imprinting in hormone-target tissues (6,7).

PHP type 1A is often diagnosed late due to the high variability in the age at which its characteristic features become clinically apparent (8,9).

In early infancy, features may be subtle and extremely variable ranging from a classical round face, subcutaneous ossifications, seizures, subclinical hypothyroidism to mild delay in acquisition of milestones (8,10,11,12,13), while other manifestations such as brachymetacarpia, short stature, and mental retardation tend to become apparent relatively late in childhood. Diagnosing PHP type 1A in early life relies, therefore, on strong clinical expertise and a high index of suspicion.

We reviewed a regional case series of 10 children diagnosed with PHP type 1A to identify the early signs and symptoms that might suggest a diagnosis of PHP in children.

Methods

We conducted a retrospective regional search of all patients diagnosed with PHP type 1A in all Pediatrics Departments of the Poitou-Charentes region in France, via each Hospital's medical informatics department. From each medical record of identified cases, we considered only those with a confirmed diagnosis either by erythrocyte Gsα activity studies performed as previously described (5,14,15), or by molecular biology studies of *GNAS1* gene (performed by commercial laboratories), or both. We extracted from each identified medical record, data on date and place of birth, birth weight (BW), birth length (BL), family history of short stature and/or relevant medical history, patient's phenotypic features, age at which the first significant symptoms and signs became apparent, and the age at which the diagnosis was made.

Each patient's gain in body weight during infancy (0 to 2 years) and early childhood (3 to 6 years) was calculated as changes in weight standard deviation score (SDS) using the French growth reference data (16). Body mass index (BMI) was calculated by dividing body weight in kilogram by height in meter squared and then compared against the same French reference (expressed as 5^{th}, 50^{th}, 85^{th} and 95^{th} centiles). Upward gain in weight ≥0.67 SDS during infancy or early childhood was considered indicative of overweight, as previously reported (17). Similarly, an increase in BMI at/or above 85^{th} centile during the same periods was considered as indicative of overweight, and a BMI at/or above 95^{th} as indicative of obesity.

We also extracted from each medical record the results of biochemical parameters (thyroid function tests, Gsα activity test, molecular gene study results, growth hormone (GH) test results when performed, luteinizing hormone, follicle stimulating hormone results, etc.) as well as data on final diagnosis and management. Additional data on patients' outcome were collected when available.

The study received approval from the Local Ethics Committee.

Results

We identified 10 cases of PHP type 1A with a mean age at diagnosis of 41.1 months (range from 4 to 156 months). There were 5 boys and 5 girls. Patients' characteristics, clinical signs at presentation, and family medical history are summarized in Table 1. The family history was positive for AHO in mothers of 4 patients (cases 1, 5, 6, 7). Six mothers were short with a mean height of 145.8 cm (<-3.13 SDS) (cases 1, 2, 5, 6, 7, 10). Birth data were available for all 10 children, the mean gestational age being 38.5 weeks (range 37 to 40 weeks).

The study population's mean BW was 2873±607.09 g, with a mean BL of 42.15±14.92 cm. Mean BW was lower in boys in comparison to girls [2838 g (-1.67 SDS) versus 2904 g (-1.0 SDS), respectively] (p=0.85). Girls were, however, shorter at birth, with a mean BL of 45.7 cm (-2.05 SDS) versus 48.25 cm (-0.90 SDS) in boys (p=0.55). Moreover, 4 out of 5 girls were small for gestational age for BL.

Our study population's mean BMI was 21.7±3.94 kg/m^2. At the time of diagnosis, seven children were overweight, with BMI above 85th centile (16), their mean age was 14.6 months (range 5 to 32 months). Rapid weight gain calculated as an upward increase in body weight ≥0.67 SDS during infancy was noted in six children (cases 1, 2, 3, 5, 6, 8); in one case (patient 9), rapid gain in weight was recorded during early childhood. This gain in weight in early life was suggestive of early overweight and/or obesity in most children.

In children aged less than two years, the predominant clinical signs were obesity (70%) and subcutaneous ossifications (60%) diagnosed at a mean age of 17.3 months (range 5 months to 5 years), whereas in older children, brachydactily was present in 60% of cases (age at diagnosis 96.5 months-range 50 to 156 months).

As shown in Table 2, erythrocyte Gsα activity was studied in six patients and the results were frankly suggestive of PHP in all these cases (activity <85%).

GNAS1 gene molecular testing was performed in 9 patients and the results revealed *de novo* mutations in three cases (c.585delGACT in exon 8) (case 2) and (c.344C>TP115L in exon 5) (cases 6&7). In the remaining children, mutations were maternally inherited. The location of different mutations identified in our patients is shown in Table 2.

Table 1. Patients' characteristics, ages, signs at diagnosis, and family history

	1	2	3	4	5	6	7	8	9	10
Year of birth	2008	2000	2007	1994	2003	2005	2009	2008	1997	1984
Gender	Female	Male	Female	Male	Female	Male	Female	Male	Male	Female
Family h/o	AHO (Mother)	148 cm (Mother)	No	NA	147 cm (Mother)	AHO (Mother)	AHO (Mother)	No	NA	145 cm (Mother)
BW kg (SDS)	3.05 (-0.60)	1.86 (-4.63)	2.81 (-1.23)	3.22 (-0.51)	3.09 (-0.50)	2.89 (-1.51)	2.45 (-2.18)	3.32 (-0.21)	2.90 (-1.41)	3.14 (-.037)
Weight (kg) at follow-up	11.350 (14 mo) (>97 P*)	11.000 (12 mo) (> 97 P*)	11.200 (12 mo) (>97 P*)	32.500 (9 yrs) (>97 P*)	10.250 (9 mo) (>97 P*)	7.615 (4 mo) (>97 P*)	4.535 (3 mo) (Mean)	9.500 (5 mo) (>97 P)*	25.00 (5 yrs) (>97 P*)	55.00 (11 yrs) (>97 P*)
Upward weight gain (SDS)	+1.14 SDS	+1.38 SDS	+1.62 SDS	+2.19 SDS	+1.82 SDS	+2.14 SDS	-2.48 SDS	+4.21 SDS	+5.75 SDS	+6.67 SDS
BL cm (SDS)	46 (-1.46)	46 (-2.0)	46 (-1.9)	-	47.5 (-1.05)	48 (-1.0)	44 (-3.0)	50 (mean)	49 (-0.5)	45 (-2.44)
At diagnosis										
Age yrs	0.11	2.6	1.0	9.0	0.8	0.4	0.7	1.3	5.0	13
BMI kg/m^2	21.6	20.5	21.6	19.5	23.2	19.4	18.5	21.09	19.4	32.2
Round face	Yes	No	Yes	No	Yes	No	Yes	Yes	Yes	Yes
Brachydactyly	-	-	-	9.0 yrs	6.0 yrs	5.0 yrs	-	-	5.0 yrs	13 yrs
Mental delay	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
S/C (age)£	1.0 yr	1.0 yr	0.5 yr	No	0.6 yr	No	No	0.9 yr	5.0 yr	No
Seizures (age)£	-	2.8 yrs	-	9 yrs	-	-	-	-	5 yrs	-

BW: birth weight, BL: birth length, SDS: standard deviation score, BMI: body mass index, S/C: subcutaneous calcifications, NA: not available, £: age at which diagnosed, P*: percentile

Table 2. Biochemical characteristics and *GNAS* gene study results of the patients

	1	2	3	4	5	6	7	8	9	10
Gender	Female	Male	Female	Male	Female	Male	Female	Male	Male	Female
Age at diagnosis (months)	11	30	12	108	8	3	7.5	15	60	156
Calcium (N 2.25-2.60 mmol/L)	N	1.18	N	2.54	2.08	N	N	N	1.58	2.41
PTH N 15-65 pg/mL)	69.9	157	88	181	73	120	62	340	423	57
Phosphatemia (N 0.80-1.45 mmol/Ll)	N	2.65	N	2.54	2.11	N	N	1.88	2.58	1.53
Subclinical hypothyroidism (age at diagnosis) (months)	6	30	5	168	3	1	3	6 days	1.5	156
TSH (0.3-5.0 mU/L)	N	5.6	12.6	13.2	6.09	12.0	11.0	26.0	27.0	7.8
fT_4 (N 10-19 pmol/L)	-	10.6	11.3	9.60	7.40	12.0	-	14.90	7.0	11.0
Gαs activity (N 85-115%)	61%	44%	NT	62%	62%	NT	NT	NT	70%	47%
GNAS1 mutation	c.565_568delGACT	c.585delGACT	c.688G>T	D343G	Q35X	c.344C>TP115L	c.344C>TP115L	c.1025G>CR342P	NT	c.427insTCC
	Exon 7	Exon 8	Exon 9	Exon 12	Exon 1	Exon 5	Exon 5	Exon 12	-	Exon 5
Transmission	Maternal	*De novo*	Maternal	-	Maternal	*De novo*	*De novo*	Maternal	-	NT

PTH: parathyroid hormone, TSH: thyroid-stimulating hormone, fT_4: free thyroxine

Biochemically, four children presented with hypocalcemia (cases 2, 4, 5, and 9), their mean age at diagnosis was 69.3 months (range 8 to 108 months). Only one of these patients was aged <2 years (case 5), confirming that this abnormality has a progressive onset. Serum PTH levels were increased in 83% of our patients before the age of two years, but this early rise was not associated with hypocalcemia. Mean age at diagnosis of rise in PTH in our study population was 27.8 months (range 1 to 108 months). Hyperphosphatemia was diagnosed in 50% of cases. Subclinical hypothyroidism [high TSH with normal or low free thyroxine (fT_4)] was present in 70% of the cases and was diagnosed at a mean age of 38 months (range 6 days to 168 months).

Seizures occurred in older children over age two years (cases 2, 4, and 9) at a mean age of 5.5 years.

GH status was tested only in one patient (case 6), in whom results were in favour of GH deficiency (peak serum GH <20 mU/L). None of our older patients was diagnosed with hypergodotropic hypogonadism, the younger patients were, unfortunately, not all tested.

Discussion

This study aimed at describing those early clinical features that should lead clinicians to consider PHP type 1A as a potential diagnosis in a child.

The commonly reported clinical features in children with PHP are lunar face (70%), short stature (80%), obesity (up to 90%), brachymetacarpy (70%), subcutaneous ossifications (42%), and a variable degree of mental retardation (64%) (17,18). All these features are rarely present together in a given patient in the early stage of the disorder which is often the reason why the diagnosis of PHP type 1A is made more difficult and often delayed.

Our results seem to indicate that the presenting clinical features characteristic of PHP type 1A are age-dependent. Taken together with those previously reported (8), it clearly appears that PHP type 1A has a bimodal clinical and biological presentation. Clinical features such as lunar face, short stature, subcutaneous ossifications, and obesity were most predominant signs in toddlers (age <2 years) and should raise suspicion. It is of note that in some patients, the search for subclinical ossifications may sometimes require X-ray investigation, as suggested by Elli et al (19,20).

Seizures as an inaugural manifestation reported by others (21) occurred in only three children whose mean age was 5.5 years (cases 2, 4, and 9) in our series. Despite being the primary biochemical abnormality in PHP type 1A, hypocalcemia with its various clinical expressions (muscle weakness, seizures, etc.) occurs secondary to the rise in PTH. An elevated PTH level, however, is rare in early infancy. Moreover, even in toddlers with raised serum PTH levels, hypocalcemia and seizures were found to be rare, with

delayed clinical expression. The reasons for this delayed occurrence has recently been provided in a recent study by Turan et al (22) who were able to demonstrate, in knockout mice model, that manifestations of PTH resistance caused by maternal loss of Gαs develop after early postnatal life. On the other hand, the silencing of the paternal Gαs allele in proximal renal tubules is delayed in onset and gradual. This delay in imprinting of *GNAS* in proximal renal tubules leads to delay in PTH resistance and also to the clinical and biochemical expression of its associated manifestations such as hypocalcemia and seizures (22). We, additionally, speculate that vitamin D and/or calcium supplementation in toddlers, as practiced in some countries, could also play a role in delaying the occurrence of hypocalcemia in some patients.

Brachydactyly defined by the shortening of the metacarpals 3, 4, and 5, although typical of PHP, is a progressive sign (23) and is also a non-specific finding in the general population (1). When present, this sign was apparent in only 60% of our patients, all of whom were aged 5 years and above (mean 7.6 yrs at diagnosis), as also reported earlier by Fernandez-Rebollo et al (9). The fact that we did not calculate the metacarpophalangeal pattern profile in all our patients, as suggested by Poznanski et al (23), could have led to an underestimation of the true prevalence of this typical sign of PHP (23,24).

Obesity is a common finding in children with PHP type 1A. We evaluated the variation in body weight in our study population by comparing the gain in weight during infancy and early childhood and found that in most of our patients (7/10, 70%), that upward weight gain was greater than ≥0.67, a rise that is considered an early marker of obesity (17,25). Our findings corroborate previous reports (26,27) and indicate that in unexplained early obesity in a child, PHP type 1A should be considered. It is noteworthy that two of our patients (cases 1 and 3) were brought to medical attention with rapidly increasing body weight as the main complaint at the age of six months (BMI 21.43 kg/m^2) and three months (BMI 22.6 kg/m^2), respectively.

Long et al (25) have reported that obesity is more severe in patient when the mutant allele is inherited from the mother. When the mutant allele is paternal in origin, as it is in pseudopseudohypoparathyroidism, obesity is often not present or it is less severe.

As this study was retrospective, we were not able to perform additional tests to ascertain the precise origin of the mutant allele and hence analyze the possible correlation between the mutant allele and the degree of obesity.

With the pathognomonic signs of PHP becoming more apparent at different ages, our findings confirm that this disorder is a heterogeneous disease with variable clinical presentation.

Biochemically, the earliest features suggestive of PHP type IA in our population were subclinical hypothyroidism characterized by high TSH with normal or low fT_4 present in 70% of cases, followed by increased PTH (83% of cases) and hyperphosphatemia (50% of cases). These findings were similar to those reported by others (9).

Moreover, two patients in this study (cases 8 and 9) had presented in the neonatal period with prolonged jaundice associated with increased TSH [26 mUI/L and 27 mUI/L, respectively (Normal range 0.3-5.0 mU/L)].

In spite of subclinical hypothyroidism being an important feature in the mode of presentation of PHP type 1A, none of the patients had been detected by the systematic post-natal congenital hypothyroidism screening. Whether lowering the TSH screening cut-off point would have detected affected children is difficult to ascertain in a retrospective study like this one. Langham et al (28) have recently reported that lowering TSH screening cut-off point to >6 mU/L as practiced at Great Ormond Street Hospital in London enabled their team to diagnose up to 36% of children with transient hypothyroidism who were subsequently treated. Further prospective studies addressing the ideal TSH screening cut-off point that would detect the largest number of children with thyroid pathology, including those with PHP is awaited.

As previously reported by Riepe et al (29), we overall found the association of early subclinical hypothyroidism with rapid gain in weight and more or less subcutaneous ossifications to be the most prominent clinical features. Our results suggest, therefore, that a child with subclinical hypothyroidism and a rapid increase in body weight should be investigated for PHP.

The diagnosis of this disorder is based on clinical and biochemical findings, as well as on the molecular study of *GNAS* gene. Molecular biology study results were available for nine of our patients. Known heterozygous mutations were found in six children (cases 1,3,4,5,8, and 10) in exons 1,5,7,9, and 12; in four patients for whom maternal DNA study had been performed, identical mutations to those found in their children were present (cases 1, 3, 5, 8). In three other children (cases 2, 6, and 7), mutations were *de novo*-c.delGACT in exon 8 (case 2) and c.344C>TP115L in exon 5 (cases 6 and 7, brother, sister, and their mother).

Lastly, we looked into the existence of a possible correlation between the genotype/phenotype and the age at onset of clinical landmarks of PHP type IA in patients with various mutations detected in our patients; we could not find any specificity and/or significant difference. No difference was found between our patients and those carrying similar mutations as published in the literature either (9).

In conclusion, PHP type 1A is a rare and complex condition, but it has some clinical features that should

raise suspicion. Diagnosing this disorder can be tricky as characteristic clinical and biochemical parameters do not follow a similar chronological pattern in all patients. Based on our results, one should take into account two different periods each with its related signs, namely, round face, rapid weight gain, subclinical hypothyroidism, and subcutaneous calcifications in toddlers, and moderate mental retardation, brachydactyly, afebrile seizures (hypocalcemia), short stature, and TSH resistance in older children. Patients presenting with these associations should be screened for PHP type IA and close clinical follow-up organized thereafter.

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Ethics

Ethics Committee Approval: The study received approval from the Local Ethics Committee, Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Simon Kayemba-Kay's, Design: Simon Kayemba-Kay's, Data Collection or Processing: Simon Kayemba-Kay's, Cedric Tripon, Analysis or Interpretation: Simon Kayemba-Kay's, Cedric Tripon, Anne Heron, Literature Search: Simon Kayemba-Kay's, Cedric Tripon, Writing: Simon Kayemba-Kay's, Peter Hindmarsh.

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Original Article

The Effect of Congenital and Postnatal Hypothyroidism on Depression-Like Behaviors in Juvenile Rats

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ABSTRACT

Objective: The aim of this study was to investigate depression-like behaviors of juvenile rats with congenital and postnatal hypothyroidism.

Methods: Twenty-seven newborn rat pups were used. First, 6-month-old Wistar Albino female rats were impregnated. Methimazole (0.025% wt/vol) was given to dam rats from the first day of pregnancy until postnatal 21 days (P21) to generate pups with congenital hypothyroidism (n=8), whereas in the postnatal hypothyroidism group (n=10), methimazole was given from P0 to P21. In the control group (n=9), dam rats were fed ad libitum and normal tap water. Offspring were fed with breast milk from their mothers. The behavioral parameters were measured with the juvenile forced swimming test (JFST). The procedure of JFST consisted of two sessions in two consecutive days: the 15-minute pre-test on day 1 and the 5-minute test on day 2.

Results: Increased immobility and decreased climbing duration were observed in both congenital and postnatal hypothyroidism groups. Decreased swimming duration was detected in the postnatal hypothyroidism group. Both hypothyroidism groups had a lower body weight gain compared with the control group, while the congenital hypothyroidism group had the lowest body weight.

Conclusion: Our results showed that hypothyroidism had negative effects on depression-like behavior as well as on growth and development. Both congenital and postnatal hypothyroidism caused an increase in immobility time in JFST. New studies are required to understand the differing results on depression-like behavior between congenital and postnatal hypothyroidism.

Keywords: Congenital hypothyroidism, depression, forced swimming test, rats

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Hypothyroidism has been recognized as an important cause of depression. Adult rats with hypothyroidism have showed increased immobility time in forced swimming test (FST).

WHAT THIS STUDY ADDS?

This is the first study that explores behavioral patterns of juvenile rats with congenital or postnatal hypothyroidism in FST.

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Introduction

The interaction between thyroid hormones and neurobehavioral alterations has been reported in previous studies (1,2). In mammals, this interaction begins in fetal life and continues throughout life. Thyroid hormones play an essential role in the maturation of the central nervous system by increasing trimonoaminergic neurotransmitters and mediating the formation of neuronal branching and synapses (3). A number of studies have highlighted that fetal and neonatal hypothyroidism have a negative effect on the neurodevelopment process (4,5). Retarded locomotor ability as well as hyperactivity were reported as a consequence of experimental hypothyroidism in developing rats (6,7,8). In clinical trials, hypothyroidism and elevated thyroid-stimulating hormone (TSH) levels have been shown to lead to depression in adult patients (9,10,11,12). In a child and adolescent sample, withdrawal, anxiety/depression, mental problems, attention problems and aggressive behavior subscale scores were found to be significantly higher in the congenital hypothyroidism group in which treatment was started at an early age compared with a control group (13). However, the relationship between depression and congenital hypothyroidism is debatable.

The purpose of this study was to investigate the depression-like behaviors of juvenile rats with congenital and postnatal hypothyroidism.

Methods

The study has been approved by Adnan Menderes University Animal Experiments Local Ethics Committee for the ethical care and use of animals in research and was conducted on 6-month-old Wistar Albino female rats (210-250 g weight) and their 27 pups provided by Adnan Menderes University Medical Faculty Experimental Animal Laboratory. All animal care and experimental procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals 1985.

The rats were mated with males for fertilization. Vaginal smears were performed for the determination of pregnancy. When semen was detected in the vaginal smears, rats were thought to be pregnant. Pregnant rats were divided into 3 groups.

Group 1 [methimazole (MMI)-induced prenatal hypothyroidism group]: MMI (0.025% wt/vol) was given daily in drinking water to pregnant rats from the first day of pregnancy until postnatal 21 days (E0 to P21) to generate pups with congenital hypothyroidism (n=8). All pups were fed with breast milk from their lactating mothers.

Group 2 (MMI-induced postnatal hypothyroidism group): The pregnant rats were fed ad libitum with water during pregnancy. MMI (0.025% wt/vol) was given daily in drinking water to dam rats from birth. Offspring were fed with breast milk from their mothers to generate postnatal hypothyroidism (n=10).

Group 3 (Control group): The pregnant rats were fed ad libitum and normal tap water without MMI from E0 to P21. Rat pups were fed with breast milk from their lactating mothers (n=9). Pups were kept in the same cage with their own dams until P21.

The observers were blind to the treatment. The last day, pups were weighed.

The rats were placed separately in restricted plastic cages under artificial lighting from fluorescent lamps, with a 12-h light photoperiod and a 12-h dark photoperiod. The room temperature was set at 25 °C constant heat and 45%-55% humidity rates.

MMI (SC-205747A, Santa Cruz Biotechnology, Inc., Dallas, TX) (0.025% wt/vol) was prepared daily and administered via the peroral route in drinking water. This protocol and dosage of MMI administration are typically used for the production of congenitally hypothyroid rats (14,15,16).

Juvenile forced swimming test (JFST) was administered as described by Reed et al (17). On the first day of the experiment, the rats were placed one by one in a tank 40 cm in height, 25 cm in diameter containing water of 23 °C. The animals were left to spend 15 minutes inside the water. The rats were then placed back in their cages and dried. The water in the tank was changed at each animal replacement. Twenty-four hours after this familiarization, the JFST was performed. A high-resolution camcorder (Samsung HMX-QF30 full HD) recorded the 5 minutes following the first minute of contact with the water. The rats were placed back in their cages after they were taken from the tank and dried. The presented procedure was applied to all animals in an identical way. Then climbing, swimming, and immobility durations were determined via the camcorder recordings by a researcher blind to the treatment groups. Modified scoring criteria for juvenile rats were applied (17). 5-minute durations were uniformly divided into 5-second duration intervals and the type of interval was determined according to the dominant activity in the interval (18,19,20). The duration in seconds of each activity exhibited by the experiment animal during the JFST was determined by multiplying the number of the intervals of the corresponding activity type by 5.

JFST was performed to all pups to investigate the depression-like behaviors. The procedure of JFST consisted of two sessions in two consecutive days: the 15-minute pre-test on day 1 and the 5-minute test on day 2 (24 h later). The behavioral parameters analyzed were duration of immobility, swimming, and climbing. After the swimming test was terminated, intracardiac blood samples were collected under anesthesia with 50 mg/kg ketamine and 10 mg/kg xylazine.

Serum concentrations of free 3,5,3′,5′-tetraiodothyronine [free thyroxine (fT_4)] and free 3,5,3′-triiodothyronine (fT_3) were measured with electrochemiluminescence immunoassay (ECLIA) by using commercial kits.

Statistical Analysis

SPSS 20.0 for Windows packaged program was used to analyze the data (21). Suitability for the normal distribution was evaluated by Kolmogorov-Smirnov test. Data were expressed as mean ± standard deviation (SD). The normally-distributed data were analyzed using a one-way analysis of variance (ANOVA). Behavior patterns (swimming and climbing) were analyzed using ANOVA. The Kruskal-Wallis (KW) H test was used as a non-parametric test for immobility duration. A two-tailed p-value <0.05 was considered statistically significant.

Results

In this study, increase in duration of immobility was observed in the postnatal hypothyroidism group compared with control and congenital hypothyroidism groups (KW H test, χ^2=14.347, df=2, p=0.001; mean ranks: control=8.39, congenital=11.06, postnatal=21.40) (Figure 1).

There was a statistically significant difference in swimming duration between the groups as determined by one-way ANOVA [F (2.24)=7.438, p=0.003]. A Tukey post-hoc test revealed that swimming duration was significantly lower in the postnatal hypothyroidism group (50.5±45.4 s, p=0.002) compared to the congenital hypothyroidism group (Figure 2).

In terms of climbing duration, it was lower in the congenital (144.3±32.9 s) and postnatal (99±44.2 s) hypothyroidism groups than in the control group (196.6±48.1 s) (one-way ANOVA [F (2.24)=12.391, p<0.001, Tukey post-hoc test; postnatal x control p<0.001, congenital x control p=0.048]. There was no statistically significant difference between the congenital and postnatal hypothyroidism groups (p=0.085) (Figure 3).

Blood levels of fT_3 and fT_4 were lower in the congenital and postnatal groups when compared with the control group, as expected (Table 1). There were statistically significant differences within the groups in weight as an indicator of growth/development (p<0.001) (Table 2).

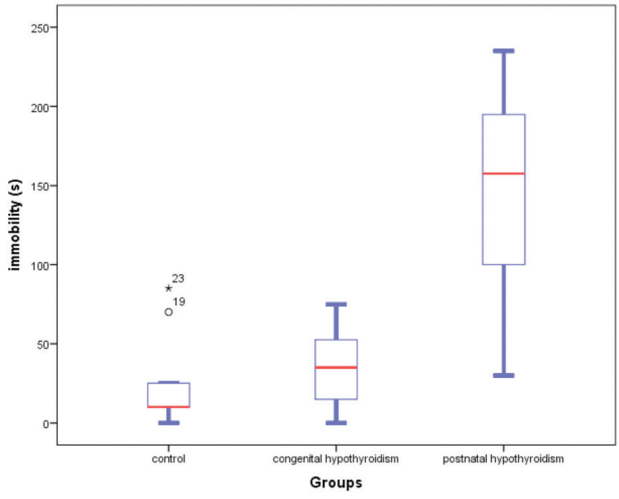

Figure 1. Duration of immobility

Discussion

The forced swimming test is a method that has been accepted and widely used in the assessment of depression-like behavior in rodents (22,23). Prolongation of immobility time in the FST is the main indicator of depression-like behavior (24). In the present study, statistically significantly increased immobility and decreased climbing duration in both congenital and postnatal hypothyroidism groups and decreased swimming duration in the postnatal hypothyroidism group was detected. Consistent with our findings, it has been reported that adult rats with hypothyroidism created using propylthiouracil or hemi/total thyroidectomy, showed increased immobility and

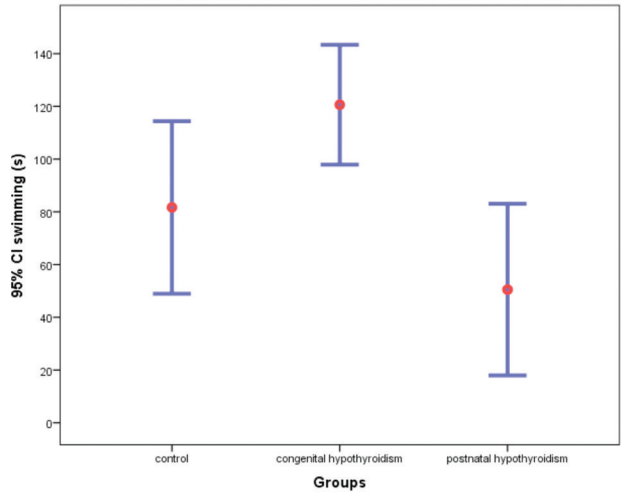

Figure 2. Duration of swimming

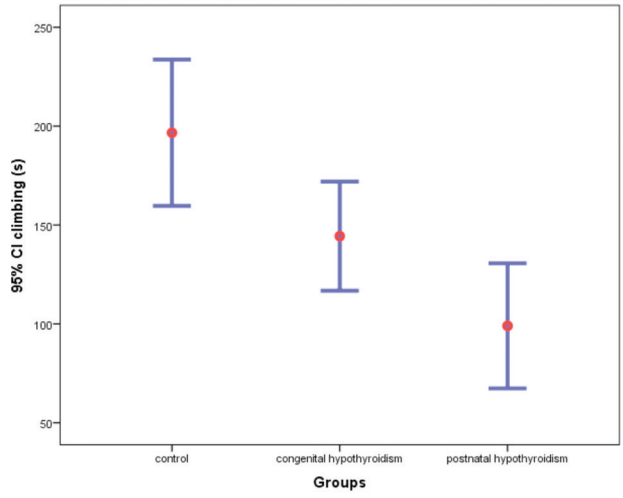

Figure 3. Duration of climbing

Table 1. Blood levels of free triiodothyronine and free thyroxine in the 3 groups

	Controls		Congenital hypothyroidism		Postnatal hypothyroidism	
	Dam (n=1)	Pups (n=9) (mean)	Dam (n=1)	Pups (n=8) (mean)	Dam (n=1)	Pups (n=10) (mean)
fT_3 (pg/mL)	1.85	2.5	<1	<0.4	<1	<0.4
fT_4 (ng/dL)	0.8	0.81	<1	<0.4	<1	<0.4

fT_3: free triiodothyronine, fT_4: free thyroxine

Table 2. Body weight of the animals in the 3 groups

	Controls (Mean ± SD)	Congenital hypothyroidism (Mean ± SD)	Postnatal hypothyroidism (Mean ± SD)	p-value
Body weight (g)	63.4±3.5	31.3±3.5	40.8±2.7	0.001*

*One-way ANOVA (F (2.24)=221.276, p<0.001. Tukey post-hoc test; control x congenital p<0.001, control x postnatal p<0.001, congenital x postnatal p<0.001
SD: standard deviation

decreased climbing time in FST (25,26,27,28,29). Additionally, Ge et al (27) reported reduction in swimming time in both clinical and subclinical hypothyroid rats. In contrast, Yu et al (30) and da Conceicao et al (31) reported decreased immobility time in adult rats with hypothyroidism. As there is a paucity of research evaluating depression-like behavior in juvenile rats with hypothyroidism, we were not able to compare our findings with other studies.

Alterations in both the hypothalamic-pituitary-thyroid and hypothalamic-pituitary-adrenal (HPA) axis have been shown in depression models in rats and in humans (32,33). Stress-induced hyperactivity of the HPA axis, including increased availability of corticotropin-releasing hormone and cortisol may affect the amygdala and hippocampus and may lead to decreased serotonergic neurotransmission (34). Thus, the HPA axis is thought to be the final common pathway in the pathogenesis of depression (35). Montero-Pedrazuela et al (28) suggested that a depressive-like behavior in adult-onset hypothyroidism in rats had a relationship with an impairment of hippocampal proliferation. Interestingly, in our study it was observed that the postnatal hypothyroidism group had the longest immobility time. Johnson et al (36) found that duration of hypothyroidism had different effects on the HPA axis in rats. According to their study, short-term hypothyroidism was associated with increased pituitary corticotroph responsiveness to corticotropin-releasing hormone in contrast with long-term hypothyroidism (36). We speculate that when an imbalance of thyroid hormone homeostasis is acquired, duration of exposure to hypothyroidism would be a possible reason for the different findings in behavioral tests.

There are many animal studies suggesting that thyroid hormones influence norepinephrine and serotonin levels which play crucial role in depression pathogenesis (31,37,38,39,40). A recent study, which aimed to explore the underlying mechanism of a link between thyroid and serotoninergic system, suggested that the lateral habenula might play a role in depression-like behavior in rats with hypothyroidism (29). Hassan et al (41) observed that there was a significant decrease of plasma dopamine, norepinephrine, and serotonin levels in young and adult rats with hypothyroidism. A similar decrease in platelet serotonin concentration was also reported in a study conducted on hypothyroid patients (42). Congenital hypothyroidism leads to a lower developmental quotient and delay in psychomotor development as well as to high depression/anxiety scores in clinical samples (13,43,44,45).

Determination of lower fT_3 and fT_4 blood levels in the congenital and postnatal hypothyroidism groups compared with the control group provided an evidence of hypothyroidism. This finding is expected to be accompanied by an increase in TSH levels. Our failure to analyze the rat-specific TSH is the most important limitation of this study.

Thyroid hormones play an important role in growth, development, and neurodevelopmental processes. It has been reported that brain and bone growth and sexual maturation are more affected in rats with thyroid hormone deficiency which has an onset in fetal life (4). Both hypothyroidism groups had a lower body weight gain compared with the control group, while the congenital hypothyroidism group had the lowest body weight. It is thought that growth and development are more affected due to earlier onset of thyroid hormone deficiency in congenital hypothyroidism.

In conclusion, to our knowledge, this is the first study that explores behavioral patterns of juvenile rats with congenital or postnatal hypothyroidism in JFST. Our results showed that hypothyroidism had negative effects on depression-like behavior as well as on growth and development. In both congenital and postnatal hypothyroidism groups, increased immobility time in JFST was observed. We found that in juvenile rats, postnatal hypothyroidism was more likely to cause a depression-like behavior, while congenital hypothyroidism affects mainly the growth and development processes. New studies are required in order to understand the differing results in depression-like behavior between subjects with congenital and postnatal hypothyroidism.

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Ethics

Ethics Committee Approval: Adnan Menderes University Animal Experiments Local Ethics Committee, 21.04.2014 64583101/2014/047.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Erdoğan Özgür, Hatice Aksu, Design: Erdoğan Özgür, Data Collection or Processing: Erdoğan Özgür, Börte Gürbüz Özgür, Gökhan Cesur, Analysis or Interpretation: Börte Gürbüz Özgür, Gökhan Cesur, Hatice Aksu, Literature Search: Erdoğan Özgür, Gökhan Cesur, Hatice Aksu, Writing: Erdoğan Özgür.

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Original Article

Vitamin D Deficiency in Pediatric Fracture Patients: Prevalence, Risk Factors, and Vitamin D Supplementation

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ABSTRACT

Objective: Although vitamin D levels are not routinely monitored in pediatric fracture patients, identification of children with a vitamin D deficiency may be clinically relevant because of the potential role of vitamin D in fracture healing. This study aimed to determine the prevalence of vitamin D deficiency in a pediatric fracture population and to identify risk factors for deficiency.

Methods: All pediatric patients (<18 years) who were treated for a fracture of the upper or lower extremity from September 2012 to October 2013 in the outpatient setting of a level one trauma center were included in this cross-sectional study. Vitamin D deficiency was defined as a serum calcidiol <50 nmol/L. Potential risk factors for vitamin D deficiency were analysed using multivariable logistic regression analysis.

Results: A total of 108 boys (58%) and 79 girls, of a mean age 11.1 years (standard deviation 3.9), who had undergone 189 fractures were included in the study. Sixty-four children (34%) were vitamin D deficient. Of those with follow-up measurements, 74% were no longer deficient after supplementation. Vitamin D status did not influence the occurrence of complications during fracture treatment. Independent risk factors for vitamin D deficiency were older age, season (spring), and a non-Caucasian skin type.

Conclusion: Clinicians who treat children with a fracture should inform patients and parents on vitamin D supplementation. Vitamin D measurement and supplementation may be needed for children with a non-Caucasian skin type or for those who present with a fracture during spring months.

Keywords: Vitamin D, vitamin D deficiency, child, infant, fracture, fracture healing

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Vitamin D plays a role in the complex cellular processes of fracture healing, studies that address risk factors for vitamin D deficiency, the clinical effects of vitamin D deficiency or supplementation on fracture healing are scarce and inconclusive.

WHAT THIS STUDY ADDS?

Vitamin D deficiency is relatively common in a pediatric fracture population. For children with a non-Caucasian skin type or presentation with a fracture during spring, clinicians might consider vitamin D measurement and supplementation.

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Introduction

Up to 60% of boys and 40% of girls undergo a fracture during childhood (1,2,3,4,5). Increased participation in competitive sports and the relative under-mineralization of the skeleton during the early phase of the pubertal growth spurt may contribute to the high fracture rate (6). Vitamin D deficiency is considered a global health problem (7). The prevalence in healthy European children varies between 8% and 95% depending on risk factors such as geographical location, sun exposure, skin type, vitamin D supplementation, or the presence of obesity (7,8). Vitamin D is essential for bone mineralization and maintenance of bone quality through its vital role in the regulation of calcium and skeletal homeostasis (9). Although vitamin D plays a role in the complex cellular processes of fracture healing, studies that address risk factors for vitamin D deficiency, the clinical effects of vitamin D deficiency, or the question of supplementation on fracture healing are scarce and inconclusive (9,10).

Low bone mineral density is a risk factor for fractures (11). Infants with severe vitamin D deficiency, such as is present in rickets, have a tendency towards increased fracture rates (12,13). The possible relationship between vitamin D deficiency and the occurrence of fractures in pediatric ages has not yet been established (14,15,16,17). A recent study showed that a lower vitamin D status is associated with fractures requiring surgery, but not with the occurrence of fractures (18). Although the prevalence of vitamin D deficiency in children in the general population has been well described (7,8), the prevalence of vitamin D deficiency in the pediatric fracture population is less often reported with a wide variation ranging from 8% to 47% (Table 1) (14,15,17,18,19,20,21).

The primary aim of the present study was to determine the prevalence of vitamin D deficiency in a pediatric population who had undergone a fracture in the upper or lower extremity. The second aim was to identify the risk factors for vitamin D deficiency in this patient group.

Methods

Approval for this cross-sectional study was obtained from the Medical Ethics Review Committee of our institution (P12.058). The study included all consecutive pediatric patients (age <18 years) who were treated for a fracture of the upper or lower extremity between 1 September 2012 and 1 October 2013 in the outpatient clinic of our level 1 trauma center. According to Dutch law, children aged 16 years or older are considered able to give informed consent themselves for study participation. For children of 12 to 16 years consent from both the child and the parents is needed before inclusion. In children younger than 12 years only consent of the parents is necessary. In this study, conservatively treated children and/or their parents received study information and were asked to provide written informed consent in the plaster cast room approximately one week after the fracture. In cases who required surgery, children and/or their parents were asked to provide written informed consent before the intervention.

A blood sample was taken during the first follow-up visit after the fracture incident. The serum concentration of 25-hydroxyvitamin D was measured using an Electro Chemo Luminescence Immuno Assay from Roche Diagnostics (Modular E170). In the literature, there is no consensus on the adequate vitamin D levels and this may explain the inconsistency of reported data related to the effect of vitamin D deficiency on occurrence of hyperparathyroidism, metabolic bone disease, and hypocalcaemia. The American Academy of Pediatrics, the Pediatric Endocrine Society, and the Institute of Medicine all consider a serum concentration vitamin D >50 nmol as sufficient/normal (22,23,24). Also, according to their recommendations, a minimum serum concentration of 50 nmol/L should also be maintained or should be the target value in case of supplementation. Serum concentrations below 50 nmol/L were defined as deficient by the Endocrine Society Clinical Practice Guidelines (25). Based on these definitions and target values, we defined vitamin D deficiency as a serum 25-hydroxyvitamin D level <50 nmol/L (20 ng/mL) in our study. Patients with low vitamin D levels were referred to a pediatrician for further assessment, supplementation according to the schedule presented in Table 2, and follow-up.

Body mass index (BMI) was determined according to gender and age. Classification of being underweight, having a normal weight, or being overweight or obese was based on the BMI distribution for Dutch boys and girls in 2009 (26). Month of fracture was categorized into autumn (September, October, November), winter (December, January, February), spring (March, April, May), and summer (June, July, August) months.

The children and/or their parents completed a questionnaire on potential risk factors for vitamin D deficiency including medical history, medication, sun exposure, and vitamin D usage prior to the fracture (27). In the questionnaire, daily UV-radiation exposure was defined as the average number of hours spent outdoors between 10:00 am and 03:00 pm (27,28,29). Skin type was determined using the Fitzpatrick scale (30). According to this scale, individuals are classified as type 1: pale white skin, always burns, never tans; type 2: white skin, burns easily, tans minimally; type 3: white skin, burns moderately, tans uniformly; type 4: light brown/moderate brown skin, burns minimally, always tans well; type 5: brown skin, rarely burns, tans profusely; type 6: dark brown to black skin, never burns.

Complications concerning fracture healing were documented as follows: refracture, epiphysiodesis, malunion, delayed union, and non-union which occurred within 6 months after the fracture.

Statistical Analysis

Patient characteristics are presented as means and standard deviations (SD) or as percentages. Patient groups were compared using the student's t-test for continuous variables and the chi-squared test or Fisher's exact test for categorical data, as appropriate. Patient characteristics with

a univariable association (p≤0.10) with vitamin D deficiency were combined in a multivariable logistic regression analysis to identify independent risk factors for these conditions. The strength of selected risk factors was expressed as the adjusted odds ratio (OR) with its corresponding 95% confidence interval (CI). Statistical analysis was performed with Statistical Package for the Social Sciences software version 20 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). A p-value of <0.05 was considered statistically significant.

Results

A total of 587 children with fractures (40% located in the distal forearm) were found to be eligible for the study. Of these, 352 (60%) were boys and 235 were girls. The mean age of the group was 10.2±4.1 years. Of these children, 187 (31.9%) agreed to participate in the study and provided written informed consent. In the study group, 108 were boys (58%) and 79 girls. The mean age of the group was 11.1±3.9 years (Table 3). Together they sustained 189 fractures, of which, the most frequent (43%) were distal forearm fractures (Figure 1). Most of the fractures were treated conservatively (n=161; 85%). The majority of the fractures which required surgery were treated with K-wires (14/28) or Elastic Stable Intramedullary Nailing (6/28). Of the 187 children, 73 (39%) had previously sustained a fracture (Table 2).

The number of patients receiving medication was 23 (18%). These medications were predominantly antiallergic drugs (salbutamol and/or salmeterol /fluticasone) and drugs for attention-deficit hyperactivity (methylphenidate) or diabetes (insulin). Most patients (n=163, 88%), had a Caucasian skin type (Fitzpatrick skin type 1, 2, or 3). Vitamin D supplements were used by 24 (13%) patients mostly as a component in a multivitamin preparation. Although recommended in the Netherlands, only 4 of the 5 children younger than four years were on vitamin D supplements, and only one of the 22 children with a dark skin type (4 or 5) aged 4 years and older was receiving vitamin D supplementation.

The blood sample for determination of serum 25-hydroxyvitamin D levels was taken at a median time of 8 days after the fracture (range 0-69 days). With a mean of 64.9 (SD 27), a total of 123 children (66%) had a 25-hydroxyvitamin D level ≥50 nmol/L and 64 children (34%) were vitamin D deficient (25-hydroxyvitamin D <50 nmol/L).

Potential risk factors (univariable p≤0.10) for vitamin D deficiency were higher age, non-Caucasian skin type, and season (winter and spring) (Table 3). In the older age groups we found more vitamin D deficiency: 16% for children younger than 10 years, 46% in age group 10-16 years and 41% in children aged 16-18 years (p=0.001). A potentially protective factor against vitamin D deficiency was a holiday with high sun exposure within the previous month. Combined in the multivariable logistic regression model, all these factors were shown to be independent risk/protective factors for vitamin D deficiency.

Of 64 children with vitamin D deficiency who were referred to the pediatrician, 51 actually visited the pediatrician (Figure 2). No clinical, biochemical, or radiological signs of rickets were found in any of these children. Osteopenia was diagnosed with a dual-energy X-ray absorptiometry scan in one of the two children with celiac disease. All 51 children were treated according to the protocol shown in Table 1. In 39 of them, the serum 25-hydroxyvitamin D measurement was repeated after 4 months; 29 (74%) were no longer vitamin D deficient (Figure 2). No vitamin D intoxication occurred in any of the supplemented children.

The mean follow-up period in the 160 conservatively treated patients was 6.1 weeks (range 1-59 weeks). During the cast immobilization which lasted on average 3.7 weeks, no complications occurred. In 3 of the 160 children, a refracture occurred respectively within one month, after 6 weeks, and after 5 months after removal of the cast. In these 3 children, the initial 25-hydroxyvitamin D levels were 119, 39, and 23 nmol/L, respectively. Only in the last patient, the vitamin D level was determined at the second presentation and was shown to have reached a sufficient level. The occurrence of complications after cast immobilization was not related to the initial vitamin D status in this cohort. The 28 children with a surgically treated fracture had an average follow-up of 15.4 weeks (range 1-42 weeks). In 21 children, the fixation material was removed according to the treatment protocol. In the surgically treated group, all fractures healed without complications within 6 months after treatment.

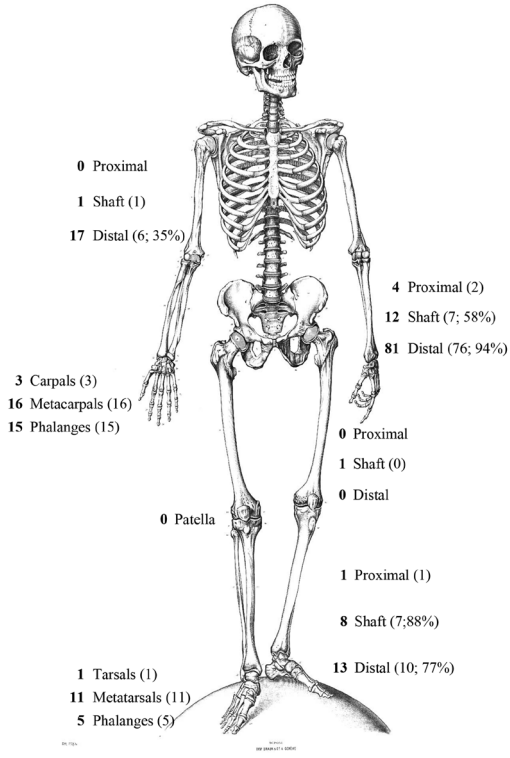

Figure 1. Fracture location and treatment of 189 fractures. The **bold** numbers indicate the number of fractures. The number (%) of conservatively treated fractures are indicated between parentheses

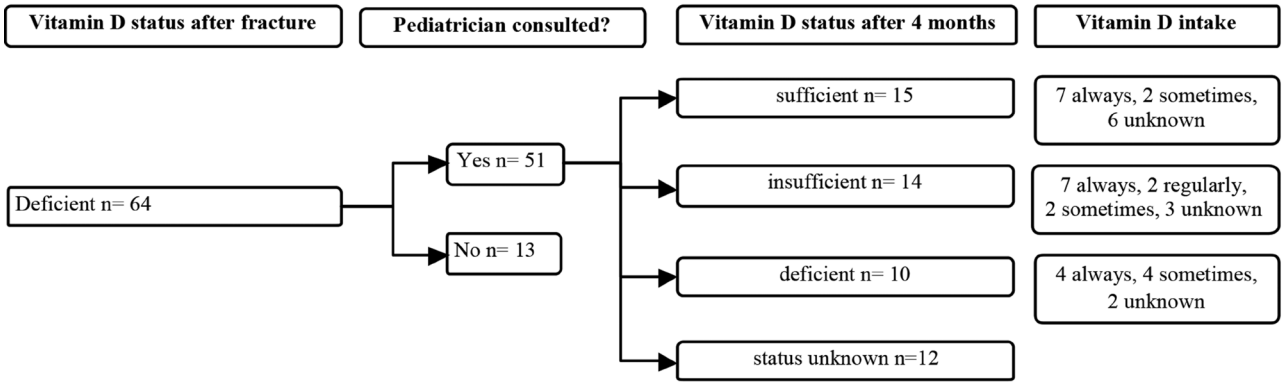

Figure 2. Flow chart of follow up of children with a vitamin D deficiency, vitamin D supplementation and results after supplementation

Table 1. Reported prevalence rates of vitamin D deficiency (calcidiol <50 mol/L) in pediatric fracture patients

Study	Year	Included fracture population	Prevalence of vitamin D deficiency
Schilling et al (15)	2011	118 children younger than 2 years old	8%
Ceroni et al (20)	2012	100 children with a fracture of the upper or lower extremity	12%
Minkowitz et al (18)	2015	369 children	18%
Contreras et al (17)	2014	100 children with a fracture	20%
Olney et al (21)	2008	68 children with two or more fractures in the past	21%
James et al (19)	2013	213 children with a fracture of the upper extremity	24%
Rayn et al (14)	2012	76 African-American children with a forearm fracture	47%

Table 2. Schedule for supplementation of children with vitamin D deficiency (25-hydroxyvitamin D <50 nmol/L)

Children younger than 1 year		Children older than 1 year	
Clinical, biochemical, or radiological signs of rickets?		Clinical, biochemical, or radiological signs of rickets?	
No	Yes	No	Yes
1,000 IU vitamin D per day for 4 weeks followed by 400 IU (10 µg) vitamin D per day for 3 months	50 0000 IU vitamin D per day for 4 weeks followed by 400 IU (10 µg) vitamin D per day for 3 months	2000 IU vitamin D per day for 4 weeks followed by 400 IU (10 µg) vitamin D per day for 3 months	50 000 IU vitamin D per day for 8 weeks followed by 400 IU (10 µg) vitamin D per day for 3 months

Discussion

The results of this study show that 34% of the pediatric fracture patients had vitamin D deficiency. However, no patient had the clinical signs of rickets. Higher age, a non-Caucasian skin type, and spring season were independent risk factors for vitamin D deficiency. After four months of treatment with vitamin D, 74% of the children who initially had vitamin D deficiency were no longer vitamin D deficient.

In the literature, the prevalence of vitamin D deficiency in the pediatric fracture population is reported to be between 8% and 47% (Table 1). Inclusion to our study was not limited to certain age groups, type of treatment, skin type, or fracture location. Therefore, the observed frequency of 34% for vitamin D deficiency probably also reflects the prevalence in the general pediatric fracture population. Schilling et al (15) found a far lower incidence of 8% vitamin D deficiency in 118 children younger than 2 years with a fracture. This low prevalence may be age- and country-dependent due to the recommendation of the American Academy of Pediatrics to supplement vitamin D in the very young children (31). As no children younger than 2 years were present in our series, we could not compare these data with our results. Ryan et al (14) examined 76 African-American children with a forearm fracture and found 47% to be vitamin D deficient. The inclusion of only children with a dark skin type, a risk factor for having a vitamin D deficiency, probably explains why they found so many more vitamin D deficient children in their population compared to our pediatric population. This obvious variation in vitamin D deficiency prevalence clearly reflects the presence or absence of certain risk factors. The five

Table 3. Univariable association and multivariable logistic regression analyses of patient characteristics/risk factors for vitamin D deficiency. Results are presented as numbers (%) unless indicated otherwise

				Vitamin D deficiency			Risk factor	
Characteristic			**Total n=187**	**Yes n=64**	**No n=123**	**p**	**OR**	**Adjusted OR (95% CI)**
Gender	Boy		108 (58)	41 (38)	67 (62)	0.21		
	Girl		79 (42)	23 (29)	56 (71)			
Age, years*			11.1 (3.9)	12.3 (3.4)	10.4 (4.1)	0.002	1.15	(1.04 - 1.26)
BMI	Underweight		33 (19)	9 (27)	24 (73)	0.67		
	Normal weight		118 (68)	42 (36)	76 (64)			
	Overweight/Obese		23 (13)	8 (35)	15 (65)			
Skin-type	Caucasian (type 1, 2, 3)		163 (88)	49 (30)	114 (70)	0.001		Reference
	Non-Caucasian (type 4, 5, 6)		23 (12)	15 (65)	8 (35)		4.71	(1.68 - 13.2)
Fracture history	Fracture	Yes	73 (39)	25 (34)	48 (66)	1.00		
		No	114 (61)	39 (34)	75 (66)			
Use of medication	Any	Yes	23 (12)	5 (22)	18 (78)	0.18		
		No	164 (88)	59 (36)	105(64)			
Use of vitamin D	Vitamin D supplements	Yes	24 (13)	6 (25)	18 (75)	0.31		
		No	163 (87)	58 (36)	105 (64)			
Sun exposure	Number of hours/day*		1.80 (0.9)	1.65 (0.9)	1.83 (0.9)	0.20		
	Sun vacation in previous month	Yes	20 (11)	1 (5)	19 (95)	0.004	0.11	(0.01 - 0.88)
		No	166 (89)	62 (37)	104 (63)			Reference
Season of fracture	Summer		53 (28)	11 (21)	42 (79)	0.02		Reference
	Autumn		44 (24)	12 (27)	32 (73)		1.70	(0.59 - 4.90)
	Winter		37 (20)	16 (43)	21 (57)		2.64	(0.95 - 7.39)
	Spring		53 (28)	25 (47)	28 (53)		3.15	(1.23 - 8.11)

*Mean (standard deviation)
OR: odds ratio, CI: confidence interval, BMI: body mass index

children with a dark skin type (type 5 or 6) who were included in our study were indeed all vitamin D deficient. Olney et al (21) retrospectively identified children with a history of two or more fractures and found a vitamin D deficiency prevalence of 21% in this group. The results of James et al (19) were limited to children with an upper extremity fracture and showed a vitamin D deficiency in 24%. Ceroni et al (20) included 100 adolescent (between 10 and 16 years) patients with upper- or lower-limb fractures and found that 12 (12%) were vitamin D deficient. We documented a prevalence of 46% vitamin D deficiency in 98 children between 10 and 16 years. Ceroni et al (20) only included surgically treated children in their series and measured the vitamin D concentration at once after storage, which may explain the difference in prevalence. Similar to our study, Contreras

et al (17) did not limit inclusion to the study to age or fracture location, although they did not report the patients' skin type. Minkowitz et al (18) included all fracture locations in a population aged between 2 and 18 years. These two authors, respectively, reported vitamin D deficiency in 20% and 18% of their subjects. The seeming differences in prevalence between our and other studies may have resulted from seasonal differences and differences in geographical distribution or latitude (27,32), but also from differences in characteristics of the study populations. It should be noted that in our study, only 12% of the children had a non-Caucasian skin type and our study population tended to be more close to adolescence with a mean age of 11.1 years.

To our knowledge, only James et al (19) and Minkowitz et al (18) described risk factors for vitamin D deficiency in a pediatric fracture population. Although not tested in a multivariable analysis, they also described a significant effect of skin type on the serum concentration of vitamin D. In contrast to our results, age and season did not seem to affect the serum calcidiol level in the study of James et al (19). Contreras et al (17) merged the fracture group with the non-fracture group and described risk factors for an insufficient vitamin D concentration (<75 nmol/L). They also found a higher prevalence of insufficiency in non-Caucasian children, as well as in children presenting in the winter and spring. Some established risk factors for vitamin D deficiency in a nonfracture population have been reported in the literature. One of these is obesity (22). Because vitamin D is a fat-soluble vitamin, a higher dose of vitamin D supplementation in obese children is recommended (22). Although some studies have identified obesity as a risk factor for vitamin D deficiency, we did not find this relation in our pediatric fracture population.

Results of studies in many countries and also national data on Dutch children indicate that prevalence of vitamin D deficiency is not expected to differ in children with or without a fracture (14,17,18,20,21,33,34). Thus, routine vitamin D measurement in children with a fracture should be avoided. The prevailing advice of the National Health Councils also render routine vitamin D measurement in children with a non-Caucasian skin type unnecessary. The Dutch Health Council advises daily vitamin D supplementation of 400 IU in all children up to four years in order to prevent rickets (35). In children of four years and older, the Health Council advises standard daily vitamin D supplementation with 400 IU in children with a light skin type (Fitzpatrick skin type 1, 2, or 3) who have insufficient daily sun exposure (<15 min between 11:00 am and 03:00 pm) and in all children with a dark skin type (Fitzpatrick skin type 4, 5, or 6) (35). This dose is consistent with estimated average requirement described by the Institute of Medicine (24). The Endocrine Society Clinical Practice Guideline recommends a higher (600-1000 IU) daily dose for children at risk for vitamin D deficiency. Our results indicate that these recommendations are poorly implemented; only 1/22 children with a dark skin type aged ≥4 years had received vitamin D supplementation prior to the study. And we identified a non-Caucasian skin type as an independent risk factor for vitamin D deficiency, a result also reported by others (17,18,19). The overall awareness of the importance of an adequate vitamin D status in these children and knowledge of the advice of the Health Council (supplementation with 400 IU per day in the risk population) should become part of the fracture treatment protocol.

A limitation of our study was the low participation rate. The most commonly stated reasons given by children and their parents for non-participation were fear of blood collection and increased time spent in the hospital. The low participation rate may have introduced a selection bias, but the included group of children seemed representative based on the available information of all eligible children regarding age, gender, fracture location, and seasonal distribution. Although blood samples were obtained as soon as possible after the fracture had occurred, this took place up to two months after initial trauma and this delay in some patients may have resulted in a less accurate information on the vitamin D status at the time of injury. Another limitation was that data concerning fracture healing were obtained retrospectively, with all well-known shortcomings of retrospective data acquisition.

In conclusion, this study has shown that one in three children with a fracture can be vitamin D deficient. Nevertheless, routine vitamin D measurement in children with fractures is not recommended. The results of our study also show that higher age, a non-Caucasian skin type, and spring season are risk factors for vitamin D deficiency in pediatric fracture patients. Clinicians who treat children with a fracture should inform the patient and their parents about the prevailing advice regarding vitamin D supplementation and also note the presence of potential risk factors. Vitamin D measurement and supplementation can be considered in children with a non-Caucasian skin type and in those who present with a fracture during spring months.

Ethics

Ethics Committee Approval: Approval for this cross-sectional study was obtained from the Medical Ethics Review Committee of our institution (P12.058), Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Erwin A. Gorter, Wilma Oostdijk, Pieta Krijnen, Inger B. Schipper, Design: Erwin A. Gorter, Wilma Oostdijk, Pieta Krijnen, Inger B. Schipper, Data Collection or Processing: Erwin A. Gorter, Abraham Felius, Analysis or Interpretation: Erwin A. Gorter, Wilma Oostdijk, Abraham Felius, Pieta Krijnen, Inger B. Schipper, Literature Search: Erwin A. Gorter, Wilma Oostdijk, Abraham Felius, Pieta Krijnen, Inger B. Schipper, Writing: Erwin A. Gorter, Wilma Oostdijk, Abraham Felius, Pieta Krijnen, Inger B. Schipper.

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Original Article

Effect of Cytokine Signaling 3 Gene Polymorphisms in Childhood Obesity

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ABSTRACT

Objective: Although polymorphisms in suppressor of cytokine signaling 3 (*SOCS3*) was reported to be related to obesity, Metabolic syndrome (MS), and type 2 diabetes mellitus in various adult studies, there is a lack of data in children. In this study, we examined eight reported polymorphisms of *SOCS3* in obese Turkish children and adolescent with and without MS and compared the results with that of controls.

Methods: One hundred and forty eight obese and 63 age- and sex-matched control subjects were enrolled in the study. Obesity classification was carried out according to body mass index. World Health Organization and National Cholesterol Education Program criteria were used for the diagnosis of MS. Genotyping procedure was carried out by polymerase chain reaction and Sanger sequencing protocol.

Results: The frequency of rs2280148 polymorphism was significantly higher in obese subjects with MS than in the control group, whereas the frequency of rs8064821 polymorphism was significantly higher in obese subjects with MS than in obese children without MS.

Conclusion: The significant associations of certain *SOCS3* polymorphisms with obesity parameters in both MS and MS -related insulin resistance, hypertension, and fatty liver suggest that polymorphisms in this gene may play a role in the pathogenesis of MS and also that they can be potentially used as a marker for attenuated or aggressive disease.

Keywords: Cytokine signaling, polymorphism, obesity, children

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

There is a relation of Metabolic syndrome (MS) with leptin and insulin levels.

WHAT THIS STUDY ADDS?

Suppressor of cytokine signaling polymorphisms also affect MS, obesity, and morbid obesity.

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Introduction

Obesity [Online Mendelian Inheritance in Man® (OMIM) #601665] is a multifactorial disease arising from the interaction between genetic and environmental factors (1). The incidence of obesity in childhood has been increased significantly during the recent years. Possible role of genetic and epigenetic factors involved in the pathophysiology of obesity are not fully known yet. Especially the relation of these factors with the severity of the disease in childhood has rarely been studied. It was previously shown that childhood obesity leads to atherosclerosis in later life, which starts as a chronic inflammatory process related to elevated cytokine levels (2).

Cytokine functions are regulated by suppressor of cytokine signaling proteins (SOCS). SOCS proteins are negative regulators of the JAK/STAT pathway and inhibit cytokine signaling (3). It is considered that SOCS proteins can attenuate signaling by inhibiting JAK activity or by promoting protein degradation (3,4). The human *SOCS3* maps to chromosome 17q25.3 and consists of two exons spanning 2.729 nucleotides. The coding sequence in exon 2 (total of 2.401 nucleotides) comprises 678 nucleotides. Some of the single nucleotide polymorphisms (SNPs) on *SOCS3* were previously shown to be related to obesity and type 2 diabetes mellitus in adults although the exact functional mechanisms are unclear (5,6,7,8). It has been reported that excessive secretion of *SOCS3* may cause insulin resistance and play a role in hepatic fatty acid synthesis (8,9). The association between previously reported SNPs on *SOCS3* and the parameters of childhood obesity with or without Metabolic syndrome (MS) is not well-known.

The aim of the present study is to determine the possible relations and prevalence of eight SNPs (-1044 C>A, rs12059, rs1061489, rs17849241, rs2280148, rs8064821, rs12953258, and rs4969169) defined previously on *SOCS3* gene in obese and extremely obese children with or without MS.

Methods

The Selection of Obese and Control Children and Adolescents

In the current study, 148 (66 female, 82 male; age range, 8 to 16.4 years) children and adolescents followed in Gazi University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology Clinic with a diagnosis of obesity were enrolled. Sixty-three (34 female, 29 male; age range, 8.5 to 18 years) sex- and age-matched healthy children and adolescents were enrolled as the control group. Volunteer subjects in both groups who met the inclusion criteria were selected randomly. Approval for the study was obtained from the Ethics Committee of Gazi University Faculty of Medicine, and informed consent was obtained from parents. Obese subjects only with diabetes, hypertension, hyperlipidemia, hypothyroidism, Cushing's syndrome, severe chronic disease, acute illness, as well as genetic and metabolic diseases and syndromes were excluded from the study. The control group consisted of non-obese subjects without any chronic disease or infection.

Anthropometric and Biochemical Evaluations

All anthropometric measurements were performed in the morning with underclothes and without shoes. Body height and weight were measured twice, and the mean values were recorded. Body height was measured with a Harpenden stadiometer and approximated to the nearest 0.1 cm. Individuals were weighed twice using a portable digital scale, and these values were also approximated to the nearest 0.1 kg; remeasurement was performed if the first two measurements differed by >0.2 kg. Body mass index (BMI) was calculated by dividing weight in (kilograms) by height in meters squared. Children with a BMI at or above the 95 percentile for their age and gender were classified as obese (10). A BMI of 40 or above was considered to indicate severe obesity (morbid obesity) (11). By a tape measure, waist circumference was measured from belly pit with loose belly and hip circumference was measured around the great trochanters while the patient is upright. Waist/hip ratio was calculated and recorded. The method used in measuring the waist circumference and the percentile values were obtained from the study of Hatipoglu et al (12). Blood pressure was measured while the patient was seated after a 30-minute rest. Blood pressure levels above the 95th percentile for age, gender, and height was defined as hypertension (13). Detailed family histories were obtained and physical examinations were performed. World Health Organization and National Cholesterol Education Program criteria for the diagnosis of MS were utilized in this study (14,15). Venous blood samples were collected into tubes containing no anticoagulant. The tubes were centrifuged (4000 rpm) at room temperature for 10 min to separate the serum, and the serum samples were stored at -80 ^{0}C until analysis. Leptin, insulin, fasting plasma glucose (FPG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations were measured. Serum insulin, TC, HDL-C, TG, and FPG measurements were performed by using the Abbott-Aeroset autoanalyzer (Chicago, IL, USA) with original kits. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald equation. Serum leptin levels were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (DRG International Inc., NJ, USA). The inter-assay and intra-assay coefficients of variation for this method were 6.6% and 4.6%, respectively. Homeostasis model assessment for insulin resistance (HOMA-IR) was used to estimate the insulin resistance in our population (15,16). The HOMA-IR index was calculated using the following formula, [FPG (mmol/L)×fasting serum insulin (mU/mL)]/22.5. Obese patients in the study group were divided into two groups as insulin resistant [IR (+)] if their HOMA-IR values were above

3.16 and non-insulin resistant [IR (-)] if their HOMA-IR values were lower than 3.16 (15).

Liver steatosis was evaluated using ultrasonographic examination and classified according to the criteria defined by Saverymuttu et al (17). Ultrasonographic examination of the liver was performed by an experienced radiologist, using a high-resolution B-mode ultrasonography system (General Electric LOGIQ 500, convex 3-5 MHz). The radiologist was masked to all clinical and biochemical characteristics of subjects.

Genotyping

Genomic DNA was extracted from peripheral blood using NucleoSpinR Blood kit (MN GmbH, Germany), according to the manufacturer's protocol. Four polymerase chain reaction (PCR) primer sets were designed for the amplification of SNP regions. For PCR amplification, SuperHoTTaq (Bioron GmbH, Germany) was used as Taq polymerase and the reaction was carried out with an annealing temperature of 60 ^{0}C. The final concentrations of reagents in PCR were as follows: 1.5 mM of MgCl2, 0.2 UM of each primer, and 0.2 mM of each dNTP. A duplex PCR was performed (two tubes for each sample), each with a volume of 25 U. After the confirmation of PCR by agarose gel electrophoresis, two duplex PCR tubes were combined to get ready for genotyping. Genotyping of the 8 SNPs in all 211 samples was carried out by using SNaPshotR Multiplex Kit (Applied Biosystems Inc, USA), according to manufacturer's protocol. SNPs were confirmed by direct DNA sequencing. Primer sets used in this study can be sent upon request.

Statistical Analysis

Statistical Package for the Social Sciences for Windows v.11.5 (Chicago, IL, USA) was used in the statistical analysis. Genotype and allele frequencies in *SOCS3* polymorphism were compared separately between groups. Descriptive statistics are given as mean ± standard deviation, frequency, and percentage. The groups were compared using the t-test or Mann-Whitney U test, as appropriate. Logistic regression analysis was used to calculate the odds ratios and 95% confidence interval values. A p-value of <0.05 was considered statistically significant.

Results

Totally eight SNPs (-1044 C>A, rs12059, rs1061489, rs17849241, rs2280148, rs8064821, rs12953258, and rs4969169) in *SOCS3* were examined. Four of the examined SNPs (-1044 C>A, rs12059, rs1061489, and rs17849241) were not detected in any of the individuals.

Obese Group vs. Control Group

Biochemical and anthropometric features of the groups are given in Table 1. Except TC and LDL-C levels, significant difference was found in other parameters between control and obese groups (p<0.05). For the genotype and allele frequencies, no significant difference was found between control and obese groups (p>0.05) (Table 2).

Control Group vs. Morbid Obese Group

Except TC and LDL-C levels, significant difference was found between control and morbid obese groups (p<0.05). For the genotype and allele frequencies, no significant difference was found between control and morbid-obese groups (p>0.05) (Table 3).

Morbid Obese vs. Non-Morbid Obese Groups

Except waist circumference, systolic blood pressure, TC, HDL-C, LDL-C, and TG levels, significant difference was found between morbid obese and non-morbid obese group for the other parameters studied (p<0.05). Although genotype frequencies of polymorphisms in subjects with morbid obesity were not different from non-morbidly obese patients, A-allele carrier frequency was significantly higher than C allele carrier frequency at rs2280148 SNP locus in morbidly obese subjects (Table 4).

Control Group vs. Obese Patients with Metabolic Syndrome

Of the148 obese patients, 16 children (10.8%) had MS. Except TC and LDL-C levels, significant difference was found between controls and obese patients with MS (p<0.05). The AC genotype frequency was higher in obese patients with MS than control group at rs2280148 SNP locus (p<0.05). On the contrary, AA genotype frequency at the same locus was significantly higher in control group than obese children with MS (p<0.05) (Table 5).

Table 1. Features of obese, morbid obese, and control groups

Groups		n	Age (years)	BMI (kg/m^2)	PFG (mg/dL)	Insulin (mIU/dL)	Leptin (ng/dL)	HOMA-IR	TG (mg/dL)	TC (mg/dL)
Control	F	34	14.04±2.12	19.77±1.18	84.38±10.53	10.74±2.54	8.06±7.09	2.22±0.58	146.29±36.12	167.50±33.35
	M	29	14.29±2.45	20.63±1.29	84.69±10.13	11.03±2.51	7.22±6.61	2.27±0.53	142.45±39.11	160.21±39.46
Obese	F	66	12.23±2.25	30.76±5.76	88.61±8.08	18.79±8.57	30.07±14.97	4.17±2.12	129.12±66.72	166.46±39.81
	M	82	12.49±1.87	30.43±5.26	88.26±6.74	16.60±8.17	24.65±9.16	3.61±1.76	132.47±62.15	174.08±43.62
Morbid obese	F	9	14.79±0.61	42.92±1.92	89.22±7.76	21.72±8.41	48.55±19.64	4.86±2.15	144.78±58.66	151.84±19.47
	M	8	13.47±1.77	43.74±3.54	90.63±12.60	26.50±13.48	25.70±13.38	5.72±2.63	135.63±65.57	174.63±39.92

BMI: body mass index, PFG: plasma fasting glucose, TG: triglyceride, TC: total cholesterol, HOMA-IR: homeostasis model assessment for insulin resistance, F: female, M: male

Table 2. Genotype distributions and estimates of the polymorphisms in control and obese groups

SOCS3 marker	Control (n=63)	Obese (n=148)	p-value	Estimated genotype odds ratio*	Allele	p-value	Estimated minor allele odds ratio*
rs8064821 G>T							
GG	40 (63%)	99 (67%)	0.634	1.162 (0.627-2.152)			
GT	21 (33%)	42 (28%)	0.472	0.792 (0.420-1.494)	G,T	0.826	0.943 (0.557-1.595)
TT	2 (3%)	7 (5%)	0.609	1.514 (0.306-7.499)			
rs12953258 C>A							
CC	50 (79%)	124 (84%)	0.440	1.343 (0.634-2.845)			
AC	13 (21%)	24 (16%)	0.440	0.744 (0.351-1.577)	C,A	0.463	0.767 (0.337-1.560)
AA	0 (0%)	0 (0%)					
rs2280148 A>C							
AC	4 (6%)	20 (14%)	0.134	2.305 (0.754-7.042)			
AA	59 (94%)	128 (86%)	0.134	0.434 (0.142-1.326)	A,C	0.146	2.210 (0.740-6.603)
CC	0 (0%)	0 (0%)					
rs4969169 C>T							
CC	52 (83%)	125 (84%)	0.729	1.150 (0.523-2.528)			
CT	11 (23%)	23 (11%)	0.729	0.870 (0.396-1.913)	C,T	0.740	0.881 (0.416-1.866)
TT	0 (0%)	0 (0%)	0				

*Exact 95% confidence interval

Table 3. Genotype distributions and estimates of the polymorphisms in control and morbid obese groups

SOCS3 marker	Control (n=63)	Morbid obese (n=17)	p-value	Estimated genotype odds ratio*	Allele	p-value	Estimated minor allele odds ratio*
rs8064821G>T							
GG	40 (63.5%)	12 (70.6%)	0.586	1.380 (0.432-4.413)			
GT	21 (33.3%)	4 (23.5%)	0.439	0.615 (0.179-2.120)	G,T	0.774	0.866 (0.323-2.317)
TT	2 (3.3%)	1 (5.9%)	0.602	1.906 (0.162-22.374)			
rs12953258 C>A							
CC	50 (79.4%)	13 (87.5%)	0.796	0.845 (0.236-3.027)			
AC	13 (20.6%)	4 (23.5%)	0.796	1.183 (0.330-4.239)	C,A	0.808	1.159 (0.352-3.813)
AA	0 (0%)	0 (0%)					
rs2280148 A>C							
AC	4 (6.3%)	2 (11.8%)	0.452	1.967 (0.329-11.773)			
AA	59 (93.7%)	15 (88.2%)	0.452	0.508 (0.085-3.044)	A,C	0.461	1.906 (0.334-10.876)
CC	0 (0%)	0 (0%)					
rs4969169 C>T							
CC	52 (82.5%)	13 (76.5%)	0.569	0.688 (0.188-2.512)			
CT	11 (17.5%)	4 (23.5%)	0.569	1.455 (0.398-5.315)	C,T	0.590	1.394 (0.414-4.688)
TT	0 (0%)	0 (0%)					

*Exact 95% confidence interval

Table 4. Genotype distributions and estimates of the polymorphisms in obese and morbid obese groups

SOCS3 marker	Obese (n=131)	Morbid obese (n=17)	p-value	Estimated genotype odds ratio*	Allele	p-value	Estimated minor allele odds ratio*
rs8064821 G>T							
GG	87 (66.4%)	12 (70.6%)	0.731	1.214 (0.402-3.663)			
GT	38 (29%)	4 (23.5%)	0.753	0.231 (0.231-2.457)	G,T	0.840	0.909 (0.357-2.312)
TT	6 (4.6%)	1 (5.9%)	0.812	1.302 (0.147-11.519)			
rs12953258C>A							
CC	111 (84.7%)	13 (76.5%)	0.385	0.586 (0.173-1.979)			
AC	20 (15.3%)	4 (23.5%)	0.385	1.708 (0.505-5.770)	C,A	0.406	1.613 (0.517-5.037)
AA	0 (0%)	0 (0%)					
rs2280148 A>C							
AC	18 (13.7%)	2 (11.8%)	0.823	0.837 (0.176-3.971)			
AA	113 (86.3 %)	15 (88.2%)	0.823	1.195 (0.252-5.668)	A,C	0.000	0.084 (0.020-0.356)
CC	0 (0%)	0 (0%)					
rs4969169 C>T							
CC	112 (85.5%)	13 (76.5%)	0.334	0.551 (0.163-1.871)			
CT	19 (14.5%)	4 (23.5%)	0.334	1.814 (0.535-6.153)	C,T	0.355	1.705 (0.544-5.348)
TT	0 (0%)	0 (0%)	0				

*Exact 95% confidence interval

Table 5. Genotype distributions and estimates for the polymorphisms of cases between control and metabolic syndrome

SOCS3 marker	Control (n=63)	Metabolic syndrome (n=16)	p-value	Estimated genotype odds ratio*	Allele	p-value	Estimated minor allele odds ratio*
rs8064821G>T							
GG	40 (63.5%)	7 (43.8%)	0.151	0.447 (0.147-1.361)			
GT	21 (33.3%)	7 (43.8%)	0.4372	0.556 (0.509-4.758)	G,T	0.080	2.116 (0.904-4.955)
TT	2 (3.2%)	2 (12.5%)	0.129	4.357 (0.564-33.651)			
rs12953258C>A							
CC	50 (79.4%)	15 (93.8%)	0.178	0.390 (0.471-32.304)			
AC	13 (20.6%)	1 (6.2%)	0.178	0.256 (0.310-2.124)	C,A	0.201	0.280 (0.035-2.227)
AA	00 (0%)	00 (0%)					
rs2280148 A>C							
AC	4 (6.3%)	4 (25%)	0.027	4.917 (1.077-22.447)			
AA	59 (93.7%)	12 (75%)	0.027	0.203 (0.045-0.929)	A,C	0.083	2.643 (0.845-8.265)
CC	00 (0%)	00 (0%)					
rs4969169 C>T							
CC	52 (82.5%)	15 (93.8%)	0.265	3.173 (0.379-26.599)			
CT	11 (17.5%)	1 (6.2%)	0.265	0.315 (0.380-2.642)	C,T	0.285	0.337 (0.042-2.713)
TT	00 (0 %)	00 (0%)					

*Exact 95% confidence interval

Table 6. Features of obese children with metabolic syndrome and without metabolic syndrome

Obese groups	Sex	n	Age (years)	BMI (kg/m^2)	FPG (mg/dL)	Insulin (mIU/dL)	Leptin (ng/dL)	HOMA-IR	TG (mg/dL)	TC (mg/dL)
With Metabolic syndrome	F M	59 73	12.22±2.22 12.30±1.87	30.58±5.58 29.85±4.82	88.41±8.31 88.62±6.49	17.60±7.92 14.71±5.83	30.57±14.81 24.49±9.30	3.89±1.96 3.23±1.35	110.90±40.33 117.44±46.51	167.91±40.72 175.35±45.21
Without Metabolic syndrome	F M	7 9	12.25±2.72 14.00±1.05	32.20±7.48 35.06±6.64	90.29±6.02 85.33±8.34	28.80±7.73 31.90±8.63	25.85±16.82 25.95±8.31	6.49±2.06 6.68±1.73	282.71±41.31 254.44±29.05	154.26±30.75 163.82±27.02

BMI: body mass index, PFG: plasma fasting glucose, TG: triglyceride, TC: total cholesterol, HOMA-IR: homeostasis model assessment for insulin resistance, F: female, M: male

Table 7. Genotype distributions and estimates for the polymorphisms of cases between with and without metabolic syndrome

SOCS3 marker	Without Metabolic syndrome (n=132)	With Metabolic syndrome (n=16)	p-value	Estimated genotype odds ratio*	Allele	p-value	Estimated minor allele odds ratio*
rs8064821G>T							
GG	92 (69.7%)	7 (43.8%)	0.037	0.338 (0.118-0.971)			
GT	35 (26.5%)	7 (43.8%)	0.149	2.156 (0.746-6.226)	G,T	0.023	2.395 (1.104-5.192)
TT	5 (3.8%)	2 (12.5%)	0.121	3.629 (0.643-20.472)			
rs12953258 C>A							
CC	109 (82.6%)	15 (93.8%)	0.252	3.165 (0.398-25.174)			
AC	23 (17.4%)	1 (6.2%)	0.252	0.216 (0.040-2.513)	C,A	0.240	2.395 (1.104-5.192)
AA	0 (0 %)	0 (0%)					
rs2280148 A>C							
AC	16 (12.1%)	4 (25%)	0.155	2.417 (0.695-8.405)			
AA	116 (87.94 %)	12 (75%)	0.155	0.414 (0.119-1.439)	A,C	0.083	2.643 (0.845-8.265)
CC	0 (0%)	0 (0%)					
rs4969169 C>T							
CC	110 (83.3%)	15 (93.8%)	0.277	3.000 (0.377-23.902)			
CT	22 (16.7%)	1 (6.2%)	0.277	0.333 (0.042-2.656)	C,T	0.286	0.349 (0.046-2.639)
TT	0 (0%)	0 (0%)	0				

*Exact 95% confidence interval

Obese Children with Metabolic Syndrome vs. Those without Metabolic Syndrome

Although BMI, waist circumference, blood pressure, fasting insulin, HOMA-IR value, and TG levels of children with MS were significantly higher than the values of those without MS, HDL-C levels were found to be significantly lower ($p<0.05$). No significant differences were found between the two groups for FPG, leptin, LDL-C, and TC levels (Table 6). The GG genotype frequency at rs8064821 locus was significantly higher in non-MS obese group than MS obese group (Table 7).

The Association of Genotypes with Risk Factors of Obesity

Insulin resistance was observed in 78 (52.8%) obese patients. There was no significant difference between insulin resistant, non-insulin resistant obese, and control groups according to genotype and allele frequency (data not shown). However, mean insulin levels in obese cases carrying the GT genotype were significantly higher than those of individuals having the GG genotype at rs8064821 SNP locus ($p<0.01$).

Ultrasonographic examination revealed moderate or severe fatty liver in 58 (39%) of obese subjects. The GG genotype frequency at rs8064821 locus of "non-fatty liver" group was significantly higher than "fatty liver" group among obese group. In addition, GT genotype frequency in obese subjects with fatty liver was significantly higher ($p<0.05$) (data not shown).

Discussion

SOCS is a family of intracellular proteins that negatively regulates cytokine signaling by interacting with cytokine receptors and signaling proteins. SOCS genes, especially

SOCS3, display tissue-specific function and are expressed in many tissues and immune regulator cells. Of these, SOCS1 and *SOCS3*, expressed in beta cells, regulate the IFN-γ signaling pathway (18). These findings suggest that the SOCS molecules are implicated in the development of autoimmunity or allergy in human diseases (19).

In obese German children, two SNPs (-1044 C>A, rs12953258) in *SOCS3* were analyzed and no difference was reported (9). Four (-1044 C>A, rs12059, rs1061489, rs17849241) of the eight polymorphisms were not detect in our population. We did not detect a significant difference between obese and control groups in terms of genotype and allele distribution of rs12953258 polymorphism and this was in agreement with the previous findings of Hölter et al (9). However, allele frequencies between morbidly obese and obese subjects were different. Accordingly, the frequency of A allele carrier at rs2280148 SNP locus in morbidly obese was significantly higher than C allele carrier frequency compared to obese group. This finding suggests that A allele carrier status at rs2280148 SNP locus may be a risk factor or a marker for morbid obesity. In recent years, it has been shown that the increase in the prevalence of obesity, may also lead to an increase in the prevalence of MS (20). *SOCS3* polymorphisms may play a role in the development of MS components. In particular, SOCS proteins, by affecting insulin and cytokine signaling, play an important role in the pathogenesis of MS (21). However, there is not sufficient data to comment about the functional relation. Sixteen of our cases (10.8%) had MS. Although the frequency of AC genotype at rs2280148 SNP locus was higher in children with MS than in the control group, the frequency of AA genotype was significantly lower. This suggests that the presence of AC genotype at rs2280148 SNP locus may be a risk factor for MS, while the AA genotype seems to be a marker of uncomplicated obesity. In addition, the frequency of GG genotype at rs8064821 locus in obese patients without MS was found to be significantly higher than that in obese subjects with MS. Again, the presence of GG genotype at this locus is associated with an uncomplicated disease.

Increased cytokine levels in obese patients are suggested to be associated with insulin resistance seen in these patients. In the recent years, it has been proposed that SOCS proteins, especially *SOCS3*, have been extensively studied in the context of the regulation of insulin signaling (22). At least three mechanisms used by SOCS, leading to the inhibition of insulin signaling, have been revealed: 1- competition for binding to the activated insulin receptor (IR), 2- degradation of IR substrate (IRS) proteins, and 3- inhibition of IR tyrosine kinase activity (23). Decreased glucose levels have been detected in SOCS1 knockout mice, and cells derived from these mice exhibited increased insulin signaling (8). In another study, increased SOCS1 and *SOCS3* were determined in insulin resistant obese animals (21). Three SNPs (rs4969169, rs12953258, and rs8064821) were studied in 2777 normal white twin women in the UK and no direct relation was detected between insulin sensitivity measures and leptin and serum lipids. The authors stated that *SOCS3* polymorphisms alone do not play a fundamental role in the regulation of body weight in adults (24). Our results also do not support a major role for *SOCS3* variants in body weight regulation in our female population. In a Danish study on 360 healthy young adults, rs12953258 polymorphism in *SOCS3* was associated with insulin sensitivity (9). In the present study, the insulin sensitivity index in AA individuals was significantly higher than that in heterozygous carriers. In our study, there was no significant difference between obese with (78 cases) or without insulin resistance groups in terms of examined polymorphisms. However, there was a significant difference between subjects with GG and GT genotypes at rs8064821 polymorphism locus regarding insulin levels. Accordingly, the average insulin levels in the GT genotype carriers were higher than that of the GG genotype carriers. These results suggest that the presence of GT genotype in rs8064821 polymorphism locus may be one of the insulin-related factors in our society, and it is important to follow up the children with the related genotype till adulthood because development of insulin resistance is an ongoing process. In addition, similar to the study on twin women (24), we did not detect any relation between *SOCS3* polymorphisms and leptin, TG, and TC levels.

Fatty liver is commonly seen in obesity. Ueki et al (21) demonstrated that over-expression of cytokine signaling suppressors in mice liver (especially, SOCS1and *SOCS3*) might increase the levels of sterol regulatory element-binding transcription factor 1c (SREBP-1c) protein which has a key role in fatty acid synthesis in the liver. SOCS1 and *SOCS3* inhibition in the latter study increased insulin sensitivity, decreased SREBP-1c to normal levels, and ameliorated fatty liver and hypertriglyceridemia dramatically in obese diabetic mice. Therefore, reducing the expression of SOCS proteins in the liver may be useful in diabetes, in obesity-related MS, and in the treatment and prevention of fatty liver (21). Fatty liver was seen in 58 obese individuals in our study. The frequency of GG genotype at rs8064821 SNP locus was significantly higher in subjects without steatosis than in those with steatosis. The frequency of GT genotype was significantly higher in obese patients with fatty liver. These results suggest that, the presence of GT genotype may be a risk factor; conversely, presence of GG genotype shows a decreased probability of liver involvement. The presence of GG genotype at rs8064821 SNP locus was significantly higher in obese patients without hypertension, and the presence of GT genotype was significantly higher in hypertensive obese patients. Similarly, the presence of GT genotype may be a risk factor, while the presence of GG genotype shows a decreased risk for hypertension in obese patients. As far as we know, this is the only data available in children. For the prevention, early

diagnosis, and treatment of metabolic complications, waist circumference measurement is recommended in children with central obesity (25). In obese, there is a significant relation of waist circumference with leptin, insulin, HOMA-IR, and BMI, however, no significant relation has been detected between blood lipids and FPG levels. No significant relation was detected between waist circumference values and *SOCS3* polymorphisms and this was also not previously investigated. Like insulin, leptin is a key hormone involved in the regulation of energy balance and glucose homeostasis. Development of resistance to the action of this hormone which can occur with age, obesity, and inflammation appears to have a primary role in the pathogenesis of obesity and type 2 diabetes mellitus. SOCS family of proteins is now thought to have a role in the development of leptin resistance owing to their ability to inhibit leptin signaling pathway (26). Leptin signaling cascade may be the possible underlying mechanism of leptin resistance in obese patients. In fact, we detected a significant increase in leptin levels of the obese patients compared to that of the control group; conversely, we did not determine any significant difference between leptin levels and *SOCS3* polymorphisms.

In conclusion, some SNPs in *SOCS3* might be an important marker of attenuated or aggravated disease in childhood and adolescent obesity. To our knowledge, this is the first study that investigates the possible associations between the presence of *SOCS3* polymorphisms and several risk factors for obesity. *SOCS3* and in particular the high incidence of the variances of genotype rs8064821 and rs2280148SNPs in obese patients with MS may be important markers of insulin resistance, hypertension, or fatty liver, and more aggressive treatment of obesity may be considered in the presence of these polymorphisms. One of the weak points of our study is the number of the cases, which we think is not sufficient to make a proposal. It is obvious that more population-based genetic studies are needed to reveal the accuracy of this finding.

Ethics

Ethics Committee Approval: Gazi University Ethics Committee of Medicine Faculty, 2008, Informed Consent: It was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Mehmet Boyraz, Peyami Cinaz, Design: Mehmet Boyraz, Ediz Yeşilkaya, Fatih Ezgü, Aysun Bideci, Korkut Ulucan, Data Collection or Processing: Mehmet Boyraz, Peyami Cinaz, Analysis or Interpretation: Mehmet Boyraz, Ediz Yeşilkaya, Fatih Ezgü, Aysun Bideci, Haldun Doğan, Korkut Ulucan, Peyami Cinaz, Literature Search: Mehmet Boyraz, Korkut Ulucan, Writing: Mehmet Boyraz, Korkut Ulucan.

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Original Article

Prevalence of Vitamin D Deficiency and Calcium Homeostasis in Saudi Children

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ABSTRACT

Objective: Vitamin D deficiency (VDD) and vitamin D insufficiency (VDI) are significant health problems all over the world. The aim of this study was to determine the prevalence of VDD and VDI in children and adolescents residing in 8 provinces in the Kingdom of Saudi Arabia and to also investigate calcium homeostasis in these subjects.

Methods: A cross-sectional study was conducted in 2110 participants aged between 6 and 15 years. Information on socio-demographic status, anthropometric measurements, knowledge about vitamin D, color of the skin, dietary intake, sun exposure experience, smoking, and physical activity were collected through a questionnaire given to the parents of all subjects. The subjects were divided into three groups as vitamin D deficient, vitamin D insufficient, and vitamin sufficient according to their blood level of vitamin D [VDD ≤25 nmol/L (25 hydroxy vitamin D), VDI >25-50 nmol/L, and VDS >50 nmol/L].

Results: VDD was highly prevalent in this group of children. 95.3 of the subjects had either VDD (45.5%) or VDI (49.9%). The prevalence rate of VDD combined with VDI was higher in females (97.8%) compared to males (92.8%) ($p<0.001$). Only 1.6% had significant hypocalcaemia. Children with dark skin had lower concentrations of vitamin D and higher concentrations of parathormone. A positive correlation was observed between 25 hydroxy vitamin D level and serum calcium, inorganic phosphate, and alkaline phosphatase concentrations.

Conclusion: The results showed a high prevalence of VDD and VDI in Saudi children with significantly higher prevalence in girls. These findings necessitate the set-up of a national program for vitamin D supplementation and health education for this vulnerable group.

Keywords: Vitamin D, vitamin D deficiency, vitamin D insufficiency, parathyroid hormone levels, calcium, inorganic phosphate

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Vitamin D deficiency in children in Saudi Arabia was reported in previous small studies in single cities in the country.

WHAT THIS STUDY ADDS?

Herein, we present our large-scale study on prevalence of vitamin D deficiency and calcium homeostasis in Saudi children and adolescents covering almost the whole country.

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Introduction

Vitamin D is an important steroid hormone with endocrine, paracrine, and autocrine effects. It is produced endogenously in the skin by exposure to ultraviolet rays or can be taken from exogenous sources such as some food items and vitamin D preparations (1,2,3).

Vitamin D has a crucial role in enhancing physiological functions both in skeletal and extra-skeletal tissues. Its vitamin D deficiency (VDD) and vitamin D insufficiency (VDI) are associated with many acute and chronic illnesses including disorders of calcium (Ca) metabolism, autoimmune diseases, some cancers, type 2 and type 1 diabetes mellitus, cardiovascular disease, and infectious diseases (4,5,6).

Vitamin D is primarily synthesized in the skin after exposure to ultraviolet radiation (UVR) and less than 10% is derived from dietary sources. The quantity of vitamin D synthesized in the skin depends on the angle of the sun rays and thus on latitude, season, time of the day and duration of exposure. It is highest when the sun is in its zenith. However, sun exposure does not lead to any vitamin D3 production in the skin during most of the winter at latitudes above and below ~33 degrees North and South. Other factors influencing cutaneous vitamin D production adversely are increased skin pigmentation, aging, and the topical application of sunscreens (7,8). Food items which naturally contain vitamin D in significant amounts are very limited (9).

VDD is diagnosed when 25 hydroxy vitamin D [25(OH) vitamin D] is ≤25 nmol/L, while VDI is defined as a 25(OH) vitamin D level of >25-50 nmol/L. 25(OH) vitamin D >50 nmol/L is considered sufficient, with 75-150 nmol/L being the preferred range (10,11,12). The 2011 Institute of Medicine (IOM) committee, in agreement with the Lawson Wilkins Pediatric Endocrine Society, targeted a serum value for 25(OH) vitamin D of at least 50 nmol/L as meeting the needs of nearly all children as well as those of adults (13,14). Hypovitaminosis D is prevalent in the Middle East North Africa region (MENA) and in the Arab gulf countries (15). In these countries, a lack of population-based studies, as well as gaps in studies in infants, pre-pubertal children, and in adolescents hinder the development of region-specific guidelines and constitute a major obstacle to impact this chronic and most often subclinical disease (13,16,17,18).

The aim of this study was to determine the prevalence of VDD and VDI in a large cohort of Saudi children and adolescents in relation to their Ca homeostasis. Moreover, the study assessed Ca homeostasis parameters and factors associated with VDD, including age, skin color, and body mass index (BMI).

Methods

The study was approved by the King Abdullah International Medical Research Center (KAIMRC) and conducted during the years 2013-2014. It included 2110 apparently healthy male and female children (1013 male, 1097 female) aged 6-15 years. The subjects were recruited from primary, intermediate, and secondary schools of the Western, Central, and Eastern regions of Saudi Arabia. These schools represent all educational levels in the country. Due to limited resources, no participants from the Northern and the Southern regions of the country could be included in this survey.

Individuals with renal, liver, and gastrointestinal disease, as well as those on any form of drug treatment with possible effect on bone metabolism (e.g. corticosteroids, anticonvulsants, and/or thyroid hormones) were excluded.

Trained health workers helped in data collection and blood extraction. Parental consent was obtained ahead of time and covered all ethical issues related to the questionnaire and the study. All parents were given a questionnaire developed in Arabic language by the investigators and pretested and coded before the actual field work. The parents were also given a covering letter that explained the objectives of the study and included information (telephone number and emails) on the investigators for any inquires. The questionnaire included questions on socio-demographic state, anthropometric measurements, knowledge about vitamin D and VDD, skin color, dietary intake, sun exposure, smoking, and physical activity.

All participants underwent a general physical examination. Body weight to the nearest 0.1 kg was measured using a standard balance beam, and body height to the nearest 0.1 cm was measured using the Harpenden stadiometer (Holtain Ltd, Ales, UK). BMI was calculated as weight (kilograms) divided by height squared (square meters). Waist circumference was measured using anthropometric tape by determining the distance midway between the iliac crest and the lowest rib with the subject standing.

Blood samples were collected from the subjects in the 3 different regions throughout the academic year, which includes part of the summer and other seasons. Specimens were collected during the day, from 09:00 o'clock to 12:00 noon. In all samples, the serum was immediately separated and the samples were protected from light and stored at -70 °C. The samples were then sent in ice to a central laboratory (at King Abdulaziz Medical City in Jeddah). The specimens were analyzed in the central laboratory using the same method (chemiluminescence immunoassay) within 2 weeks of blood collection.

The cutoff points of the IOM for vitamin D levels, namely, ≤25 nmol/L for deficiency, >25-50 nmol/L for insufficiency, and >50 nmol/L for sufficiency were used in the analysis of the data (10-12). Intact parathyroid hormone (PTH), Ca, inorganic phosphate (PO_4), alkaline phosphatase (ALP), and creatinine were determined in Architect machine (ABBOTT laboratories, Wiesbaden, Germany). Chemiluminescent Microparticle Immuno Assay (CMIA) was used for quantification of intact PTH in serum and plasma.

The sensitivity of the chemiluminescence immunoassay is <3.0 pg/mL and intra- and inter-assay CV percentages were 6.1% and 3.4% at a level of 69 pg/mL, respectively.

The outcome variable VDD was used as continuous variable and categorical variable and was divided into three categories according to the level of 25(OH) vitamin D, as given above.

Statistical Analysis

To describe our study population, we used frequencies and absolute numbers for categorical variables and mean ± standard deviation values. Median and inter-quartile range values were used for continuous variables. Association between two categorical variables were assessed using the chi-squared test or the Fisher exact test when the data are sparse in one or more category. Associations between continuous variables were examined using either student's t-test for unpaired samples or one-way analysis of variance (ANOVA), as appropriate. Linear regression equation was used to investigate possible relations between the different variables. For all statistical tests, a p-value of <0.05, two tail probability was accepted as significant. We used the Statistical Package for Social Sciences version 19 for data analysis.

Results

In this cross-sectional study of a large cohort of children of ages 6 and 15 years, the overall prevalence of combined VDD and VDI was 95.3% and that of vitamin D sufficiency only 4.7%. The prevalence in females (97.8%) was significantly higher than in males (92.8%). VDD [25(OH) vitamin D ≤25 nmol/L] was detected more frequently in females (63.9%) than in males (25.6%) (Table 1). VDD was more prevalent in the older age groups (47.2%) than in the younger groups (29.9%) as shown in Table 1.

As shown in Table 2, comparisons between two age groups (6-12 years versus 13-15 years) showed that the younger group had a higher mean 25(OH) vitamin D level (33.1±12.2 nmol/L) compared to the older group (27.6±11.4 nmol/L). Circulating PTH concentrations were significantly higher in the older group.

Table 1. Prevalence of vitamin D deficiency and vitamin D insufficiency in the study group by gender and age group

Gender and age	Number	≤25 nmol/L	25-50 nmol/L	>50 nmol/L
Gender*				
Males	1013	259 (25.6%)	681 (67.2%)	73 (7.2%)
Females	1097	701 (63.9%)	372 (33.9%)	24 (2.2%)
Age group#				
6-12 years	204	61 (29.9%)	123 (60.3%)	20 (9.8%)
13-15 years	1906	899 (47.2%)	930 (48.8%)	77 (4.0%)
Total	2110	960 (45.5%)	1053 (49.9%)	97 (4.6%)

*Chi-square test, p<0.05, p-value is significant for all three levels of 25(OH) Vitamin D
#Chi-square test, p<0.05, p-value is significant for all three levels of 25(OH) Vitamin D

Serum Ca and PO_4 concentrations did not differ significantly between the two groups. None of the children in the young group had hypocalcemia defined as a serum Ca level of <2.1 nmol/L, while 0.8% of the older group had hypocalcemia.

Children with VDD (≤25 nmol/L) had significantly higher PTH concentrations and lower PO_4 levels compared to those with higher 25(OH) vitamin D concentrations. Serum Ca levels did not differ between the two groups. Lower serum PO_4 and higher PTH levels were observed in patients indicative of the presence of a compensatory response to low 25(OH) vitamin D levels (Table 3).

The degree of skin darkness of all children and adolescents was assessed and divided into 3 grades where 1=light (white), 2=brown, and 3 is black (Tables 4, 5). Comparison between the different groups according to their skin color revealed that children with black skin (group 3) had significantly lower 25(OH) vitamin D and higher PTH and ALP levels compared to those with lighter skin (ANOVA, p<0.001).

In this study, 47.1% of children and adolescents were not receiving any supplements of vitamin D, 16.5% were taking no

Table 2. Relationship between age groups and selected variables of calcium homeostasis

Variable	Age 6-12 years n=184 Mean (standard deviation)	Age 13-15 years n=1829 Mean (standard deviation)	p-value#
AST (IU/L)	20.1 (7.0)	16.3 (7.3)	0.049
ALT (IU/L)	4.9 (4.0)	4.6 (5.4)	0.914
GGT (IU/L)	14.6 (8.1)	14.8 (7.3)	0.013
Urea nitrogen (mmol/L)	3.8 (1.0)	3.7 (1.0)	0.018
Creatinine (µmol/L)	53.1 (7.1)	59.7 (17.5)	0.044
Albumin (g/L)	42.5 (2.0)	44.0 (2.8)	0.576
PTH (pg/mL)	53.1 (32.7)	62.0 (48.5)	0.019
Calcium (mmol/L)	2.39 (0.1)	2.4 (0.1)	0.090
Phosphate (mmol/L)	1.6 (0.2)	1.5 (0.2)	0.110
ALP (IU/L)	238.5 (58.9)	219.6 (102.2)	0.715
BMI	18.0 (4.2)	21.5 (6.9)	0.979
25(OH) vitamin D (nmol/L)	33.1 (12.2)	27.6 (11.4)	0.001

#Independent student's t-test
AST: aspartate transaminase, ALT: alanine transaminase, GGT: gamma glutamyl transpeptidase, PTH: parathyroid hormone, ALP: alkaline phosphatase, BMI: body mass index, 25(OH) vitamin D: 25 hydroxy vitamin D

milk or milk products, while 8.6% were taking milk less than once weekly. In 29% of the cohort, no exposure to the sun at any time was reported (Table 5) and 27% of the subjects were reported to wear complete covering clothes including the face and hands. In those who reported being exposed to the sun, 52% reported exposure at less efficient times for UV rays (early morning and late afternoon) and 10% reported use of sun-screening creams.

Correlation analyses revealed that 25(OH) vitamin D levels correlated significantly with Ca level (r=0.15, p<0.001) (Figure 1) and negatively with PTH (Figure 2), age, and BMI. A positive correlation was observed between PTH and ALP levels (r=0.23, p<0.001) (Figure 3).

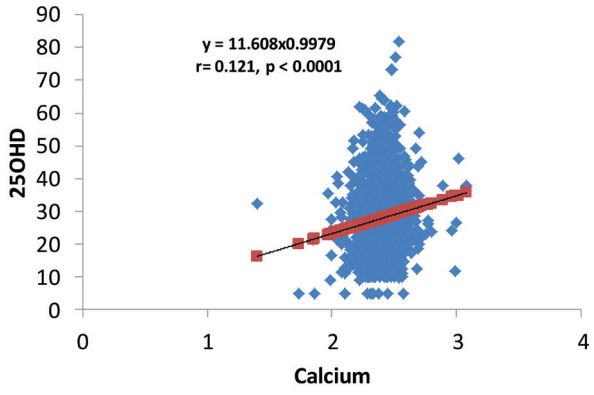

Figure 1. Regression of Vitamin D level on calcium concentration in population

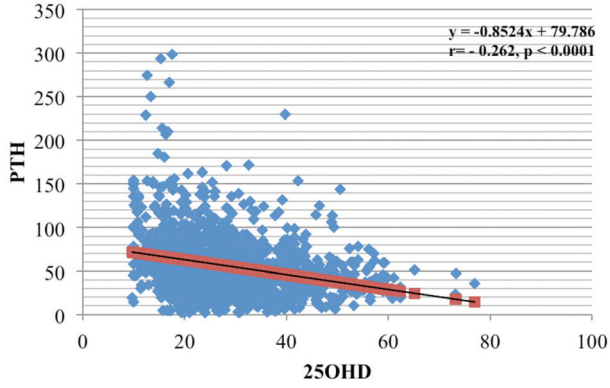

Figure 2. Regression of Vitamin D on parathyroid hormone level in population <15 years

Table 3. Calcium homeostasis in children according to vitamin D level

Variable	25(OH) Vitamin D ≤25 n=960 Mean (standard deviation)	25(OH) Vitamin D >25 n=1150 Mean (standard deviation)	p-value#
AST (IU/L)	16.0 (6.8)	17.4 (7.8)	0.001
ALT (IU/L)	4.3 (4.9)	4.9 (5.6)	0.914
GGT (IU/L)	14.3 (7.3)	15.2 (7.5)	0.013
Urea nitrogen (mmol/L)	3.5 (0.9)	4.0 (1.0)	0.018
Creatinine (µmol/L)	58.4 (17.3)	59.6 (16.5)	0.044
Albumin (g/L)	43.9 (2.5)	43.8 (3.0)	0.576
PTH** (pg/mL)	72.0 (56.8)	52.0 (35.0)	0.019
Calcium (mmol/L) (mmol/L)	2.39 (0.1)	2.4 (0.1)	0.09
Phosphate (mmol/L)	1.3 (0.2)	1.5 (0.2)	0.011
ALP (IU/L)	209.5 (105.9)	232.1 (91.0)	0.715
BMI	21.9 (8.0)	20.6 (5.4)	0.979
Age (years)	13.1 (1.6)	12.7 (1.8)	0.002

#Independent student's t-test
AST: aspartate transaminase, ALT: alanine transaminase, GGT: gamma glutamyl transpeptidase, PTH: parathyroid hormone, ALP: alkaline phosphatase, BMI: body mass index, 25(OH) vitamin D: 25 hydroxy vitamin D

Table 4. Prevalence of vitamin D deficiency according to age, gender, region, skin color, and exposure to the sun

Variable		25(OH) Vitamin D (nmol/L)	
		≤25	>25
Region*	Western	310 (41.4)	438 (58.6)
	Eastern	313 (51.2)	298 (48.8)
	Central	337 (44.9)	414 (55.1)
Gender*	Male	259 (25.6)	754 (74.4)
	Female	701 (63.9)	396 (36.1)
Sun* exposure	Yes	626 (43.4)	817 (56.6)
	No	298 (51.0)	286 (49.0)
Exercise*	Yes	268 (35.1)	495 (64.9)
	No	380 (54.2)	321 (45.8)
Vitamin* supplements	Yes	521 (49.3)	536 (50.7)
	No	393 (41.6)	552 (58.4)
Skin color*	White	300 (44.8)	369 (55.2)
	Brown	635 (45.7)	755 (54.3)
	Black	7 (87.5)	1 (12.5)
BMI*	<95th %	862 (44.6)	1072 (55.4)
	≥95th %	88 (58.7)	62 (41.3)
Age*	6-12	61 (29.9)	143 (70.1)
	13-15	899 (47.2)	1007 (52.8)

*Chi-square test, p-value <0.05
BMI: body mass index (abnormally high >95th % for age and sex)

Table 5. Relationship between vitamin D and other biochemical data according to skin color

Skin color variable	White n=669 Mean (standard deviation)	Brown n=1390 Mean (standard deviation)	Black n=8 Mean (standard deviation)	p-value#
AST (IU/L)	16.5 (7.8)	16.7 (6.8)	17.7 (4.8)	0.803
ALT (IU/L)	4.5 (4.8)	4.6 (4.7)	4.6 (4.9)	0.960
GGT (IU/L)	14.5 (6.8)	14.8 (7.3)	24.4 (16.3)	0.001
Urea nitrogen (mmol/L)	3.7 (1.0)	3.8 (1.0)	2.7 (0.6)	0.015
Creatinine (µmol/L)	58.5 (7.2)	59.4 (20.1)	56.2 (8.1)	0.525
Albumin (g/L)	43.9 (2.9)	43.8 (2.7)	44.6 (2.5)	0.587
PTH (pg/ml)	56.4 (37.2)	62.7 (50.3)	136.1 (141.4)	0.001
Calcium (mmol/L)	2.4 (0.1)	2.4 (0.1)	2.4 (0.1)	0.377
Phosphate (mmol/L)	1.5 (0.2)	1.5 (0.2)	1.5 (0.4)	0.177
ALP (IU/L)	208.4 (99.4)	226.8 (98.1)	270.3 (108.5)	0.001
25(OH) vitamin D (nmol/L)	28.6 (12.1)	28.1 (11.3)	20.8 (13.8)	0.127

#Analysis of variance (ANOVA test)
AST: aspartate transaminase, ALT: alanine transaminase, GGT: gamma glutamyl transpeptidase, PTH: parathyroid hormone, ALP: alkaline phosphatase, BMI: body mass index, 25(OH) vitamin D: 25 hydroxy vitamin D

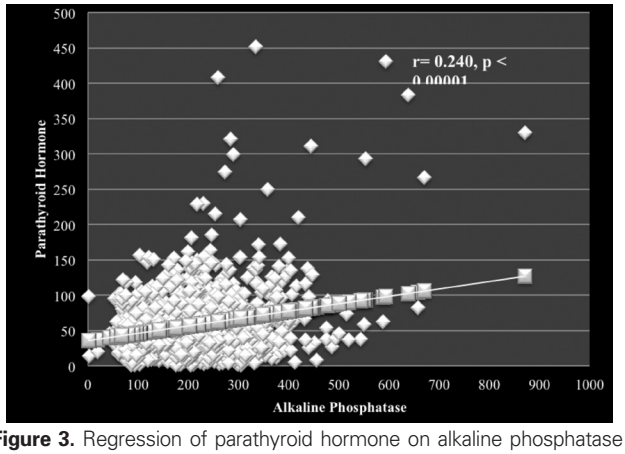

Figure 3. Regression of parathyroid hormone on alkaline phosphatase concentrations in population <15 years

Discussion

Vitamin D is critical for Ca homeostasis and for mineralization of the skeleton, especially during periods of rapid growth, namely, growth in infancy, childhood, and pubertal period. Without vitamin D, only 10-15% of dietary Ca and about 60% of phosphorus are absorbed. The active form, 1,25-dihydroxy vitamin D [1,25-$(OH)_2D_3$] markedly increases the efficiency of intestinal Ca and phosphorus absorption. Serum levels of 25(OH) vitamin D below 50 nmol/L are associated with a significant decrease in intestinal Ca absorption. In children, adolescents, and adults, this is associated with increased PTH and decreased insulin-like growth factor 1 (IGF-1) secretion. Serum levels of 25(OH) vitamin D are directly related to bone mineral density with a maximum density achieved when the 25(OH) vitamin D level reaches 100 nmol/L (40 ng/mL) or more. Severe and/or prolonged VDD is associated with impaired linear growth and the development of many skeletal disorders including rickets, osteomalacia, and fractures. In addition, many extra-skeletal disorders have been associated with VDD. An increasing body of evidence also shows the extra-skeletal benefits of vitamin D, such as those on the immune system, fuel metabolism, cardiovascular system, and cancer. In addition, associations with decreased mortality have been reported (13, 17,18,19,20,21,22,23,24,25,26,27,28,29).

The results of this study showed a very high prevalence of VDD and VDI in the majority of children and adolescents between 6 and 15 years of age in a sunny country such as the Kingdom of Saudi Arabia. Several large-sample populations-based studies as well as smaller studies also revealed a high prevalence of hypovitaminosis D and of rickets in infants (0.5% of Saudis <2 years) and adolescents in Saudi Arabia (21,22,23,24,25,30).

While rickets is almost eradicated in western populations, its prevalence remains unacceptably high in Asia, Africa, and the Middle East and resurgence is also registered in ethnic minority groups in some Northern European countries despite its plentiful sunshine. Such findings are explained by the prevalence of specific risk factors for hypovitaminosis D in this region. These include lifestyle factors, namely lack of sunlight exposure (because of very hot weather) (29% of our cohort) or exposure during the wrong time of the day (52% of our cohort) and inadequate use of vitamin D supplements (47% of our cohort) are well recognized major determinants of circulating 25(OH) Vit D levels.

Many studies reported significant lack of sun exposure and lack of vitamin D supplementation in the Arab Gulf region (26,27,28,29,31,32). A study conducted in Riyadh city which included 808 Saudi children and 561 adults of both genders showed that subjects who had a sun exposure of <20 min and who were of dark skin had the highest prevalence of VDD (33). In our study, data on duration of sun exposure, time of exposure, clothing, using sun protection substances were also collected. These factors working independently or interacting with each other will be analyzed in reference to skin color in future papers.

It has been reported that genetic factors may contribute to up to 50% of inter-individual variability in serum 25(OH)

vitamin D levels and in 27% of Saudi patients (32). The study done by Baroncelli et al (34) on 98 rachitic children conducted in Egypt and Turkey showed that VDD and rickets in the Middle East countries was determined, in addition to nutritional and environmental factors, also by genetic predisposition factors. They found that vitamin D receptor genotypes may predispose to rickets by increased frequency of the F allele.

In our study and also in other studies, females had more prevalent VDD compared to males. In adolescents and adults, this finding may be due to the more restricted outdoors activity of the females and the conservative clothing they wear that prevent exposure to the sun rays. In addition, lack of awareness of the importance of sun exposure for bone health and for cosmetic reasons (avoiding darkening of their skin) including using sun-screening agents are other important factors.

A very recent study in children and adolescents from Iran to determine the reference intervals for vitamin D and other 12 biochemical indices showed the cutoff level for VDD to be 50 nmol/L (35). We also used this cutoff point in our study and found a high prevalence of VDD in our population despite the sunshine all the year around, a finding which can be associated with the influence of other interacting risk factors that need to be investigated in further studies.

The majority of the children with VDD in our cohort, even those with severe VDD [25(OH) vitamin D <25 nmol/L], had normal serum Ca, PO_4, and ALP levels. The development of clinical manifestations of VDD rickets and osteomalacia depends on many factors apart from the severity and duration of the VDD. A potent adaptation process, mediated by the PTH and the IGF-1 modifies the clinical and radiological manifestations of VDD (12,13). Therefore, overt cases of rickets and osteomalacia represent only the tip of the iceberg of patients with severe VDD and may indicate a defect in adaptation. It is also noteworthy that when clinical rickets develops, the entire process occurs rapidly, within a few months. This potent adaptation process, brought about by increased PTH secretion, explains the normal Ca level of our black children and adolescents despite their significantly low 25(OH) vitamin D levels. In addition, this same adaptation process explains the relatively low incidence of florid rickets and osteomalacia in the presence of a high prevalence of VDD. In support of this view, the children in our cohort with 25(OH) vitamin D <25 nmol/L values have significantly higher PTH and lower PO_4 levels (due to the phosphaturic effect of PTH), to maintain their Ca levels within the normal range. Consequently, VDD in adolescents may be asymptomatic and go undetected. These patients usually present with vague manifestations including pain in weight-bearing joints (back, thighs, calves), difficulty in walking, running and/or climbing stairs, getting up from a squatting position, and muscle cramps. Facial twitches and carpo-pedal spasms are less frequent symptoms. Due to the demineralization of bones, deformities such as triradiate pelvis, lordosis, and/or genu valgus or varus may develop. These manifestations may go unnoticed for long periods. In severe and prolonged deficiency, vertebral compression fractures and fractures of the long bones may occur. Moreover, VDD can be misdiagnosed as fibromyalgia, chronic fatigue syndrome, or simply depression in adolescents (13,36,37).

In conclusion, VDD is highly prevalent in Saudi children, both in females and males. Most of the cases are asymptomatic or may present with vague and non-specific symptoms. These data urge pediatricians and physicians to have a higher degree of clinical suspicion for VDD and to screen all the patients with non-specific musculoskeletal pain by measuring 25(OH) vitamin D level. Food fortification with vitamin D and health education in schools and media to improve sun exposure appear to be very important steps to correct this prevalent deficiency and prevent its short- and long-term deleterious consequences. In addition, regular screening of children for VDD and initiation of treatment at an early stage are important measures for prevention of the undesirable consequences of VDD.

Ethics

Ethics Committee Approval: King Abdullah International Medical Research Center (KAIMRC), Jeddah, Saudi Arabia 2012-2013, Informed Consent: It was taken.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar, Design: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar, Data Collection or Processing: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar, Analysis or Interpretation: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar, Literature Search: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar, Writing: Adnan M. Al Shaikh, Bahaa Abaalkhail, Ashraf Soliman, Ibrahim Kaddam, Khalid Aseri, Yousef Al Saleh, Ali Al Qarni, Ahmed Al Shuaibi, Waleed Al Tamimi, Abdel Moniem Mukhtar.

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Case Report

Complex Glycerol Kinase Deficiency and Adrenocortical Insufficiency in Two Neonates

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ABSTRACT

Contiguous gene deletions of chromosome Xp21 can lead to glycerol kinase deficiency and severe adrenocortical insufficiency (AI) in a male newborn among other problems. We describe our experience with two such patients who presented with dysmorphic facies, AI, and pseudo-hypertriglyceridemia. Both infants had normal serum 17-hidroxyprogesterone levels, and adrenal glands could not be observed with ultrasonography. Creatine kinase and triglyceride levels were measured to elucidate the etiology of adrenal hypoplasia and were above normal limits in both cases. Both patients required steroid and salt supplementation. They were both found to have Xp21.2 deletions (*DMD, NR0B1, GK, IL1RAPL1*). We conclude that AI in the context of other genetic abnormalities should prompt chromosomal investigations in the absence of another unifying explanation.

Keywords: Deletions, X-chromosome, glycerol kinase, adrenal insufficiency

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Complex glycerol kinase deficiency (CGKD) typically develops from partial deletion of the Xp21 chromosomal locus involving the genes responsible for glycerol kinase deficiency, adrenal hypoplasia congenita, Duchenne muscular dystrophy, and others causing various developmental defects.

WHAT THIS STUDY ADDS?

CGKD is a rare disorder. We reported our experience in two neonates with CGKD.

Introduction

Complex glycerol kinase deficiency (CGKD) is a contiguous gene deletion syndrome which is inherited as an X-linked trait. CGKD typically develops from partial deletion of the Xp21 chromosomal locus involving the genes responsible for glycerol kinase deficiency (GKD), adrenal hypoplasia congenita (AHC), Duchenne muscular dystrophy (DMD), and others causing various developmental defects. Symptoms are related to the extent of the deletion and may present early in life. The diagnosis is based

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on clinical and laboratory findings. With genetic analyses, it is possible to confirm the diagnosis by demonstrating gene deletion at Xp21 locus and the female carrier can be identified (1,2,3). We describe our experience with two such patients.

Case Reports

Case 1

A 36-day-old male infant was brought to the hospital for difficulty to feed, vomiting, and weight loss. He was delivered at term and with no complications via normal vaginal delivery to a 36-year-old mother. There was no parental consanguinity; however, the second child of this couple had similar findings to our case and had died at 7 months of age because of muscle disease. The birth weight of our patient was 3200 g, but at the time of presentation, his weight was only 2700 g (<3rd percentile). His length was 54 cm (50th percentile) and head circumference was 38 cm (25th-50th percentile). The infant was hypotonic, lethargic, and appeared to be malnourished and dehydrated. His skin was hyperpigmented, with pigmentation being more pronounced in the areola of the breasts and in the scrotum (Figure 1). He had dysmorphic facial features (Figure 2). Initial laboratory tests revealed the following serum levels: glucose: 57 mg/dL, sodium: 128 mEq/L, potassium: 8.6 mEq/L, serum cortisol: 12.6 µg/dL, adrenocorticotropic hormone (ACTH): >2000 pg/mL, 17-hydroxyprogesterone (17-OHP): 0.79 ng/mL. Based on these findings, the patient was considered to have partially compensated adrenocortical insufficiency. Fluid and electrolyte therapy along with hydrocortisone and fludrocortisone replacement at proper doses were initiated. The patient, who improved with treatment, was investigated for etiology. The adrenal gland could not be visualized by ultrasonography. Serum creatine phosphokinase (CPK) and triglycerides were investigated to evaluate complex glycerol kinase (GK) deficiency and were measured as 5758 U/L (normal range: 68-580) and 1193 mg/dL(normal range: 35-110), respectively. With urinary organic acid analysis using gas chromatography-mass spectrometry, the patient's urinary glycerol excretion was 4847.6 mmol/mmol creatine (normal range: 0-40) (Figure 3). Routine peripheral lymphocyte chromosome analysis result was 46,XY. Comparative genomic hybridization (CGH) showed a deletion involving all coding seqeunces of the *GK* gene. The deletion included part of the *DMD* gene, the entire *NR0B1* gene, and part of the *IL1RAPL1* gene (Figure 4). On the 51th day of hospitalization, the patient was discharged with oral hydrocortisone, fludrocortisone, and salt supplementation.

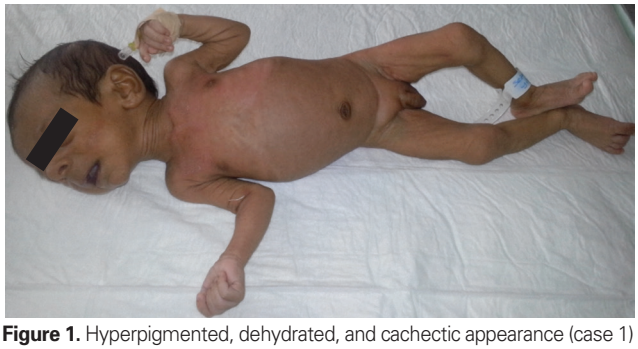

Figure 1. Hyperpigmented, dehydrated, and cachectic appearance (case 1)

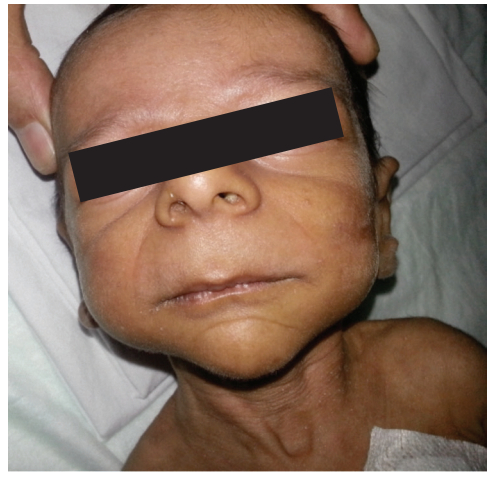

Figure 2. Dysmorphic facial features characterized by midfacial hourglass appearance, hypertelorism, long philtrum, rounded palpebral fissures (case 1)

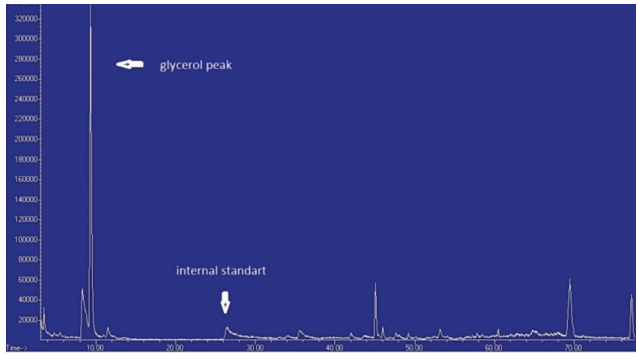

Figure 3. Glycerol peak against internal standards observed in urinary organic acid analysis (case 1)

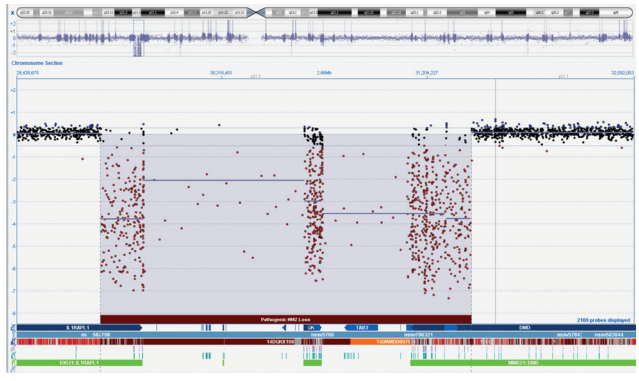

Figure 4. The deletion involving all coding seqeunces of the *GK* gene, including part of the *DMD* gene, the entire *NR0B1* gene, and part of the *IL1RAPL1* gene (case 1)

Case 2

A male infant delivered at term at another facility to a 33-year-old primigravida was brought to medical attention on the 18th postnatal day for reduced breastfeeding, vomiting, and weight loss. There was no history of parental consanguinity. There were no similar cases in the pedigree. His birth weight was 3100 g, but at presentation, his weight was 2400 g (<3rd percentile). His length was 52 cm (25th-50th percentile) and head circumference 37 cm (50th percentile). The infant had dysmorphic facial features and was dehydrated (Figure 5). Laboratory values included serum glucose: 52 mg/dL, sodium: 124 mmol/L, potassium: 7.4 mmol/L, ACTH: 628 pg/mL, cortisol: 20.6 µg/dL, 17-OHP: 6.04 ng/mL. The adrenal glands could not be visualized by ultrasonography. Fluid and electrolyte therapy along with hydrocortisone and fludrocortisone replacement were initiated. Serum triglyceride level was 761 mg/dL, CPK was 28.134 U/L, and CK-MB was 592 U/L (normal range: 0-25). Routine karyotype was consistent with normal 46,XY constitution; however, CGH showed a 3.88 Mb deletion encompassing part of the *DMD* gene (exon 45 extending through 3' end) and three additional disease-associated genes (*NR0B1, GK, and IL1RAPL1*) (Figure 6).

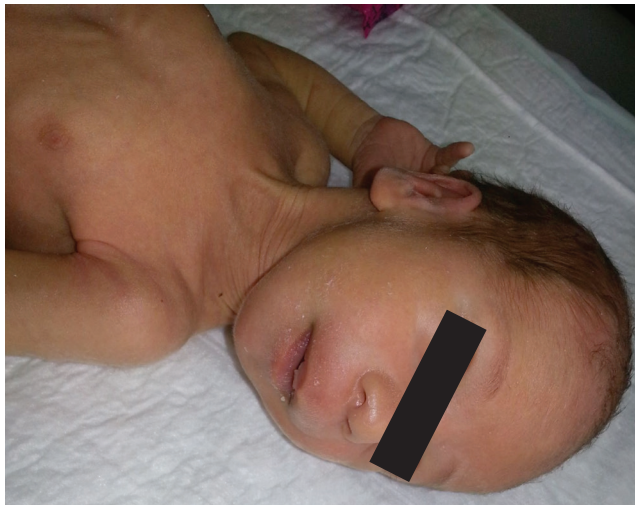

Figure 5. Dysmorfic facial features characterized by prominent forehead, rounded palpebral fissures, expanded and flattened ear lobes and long philtrum (case 2)

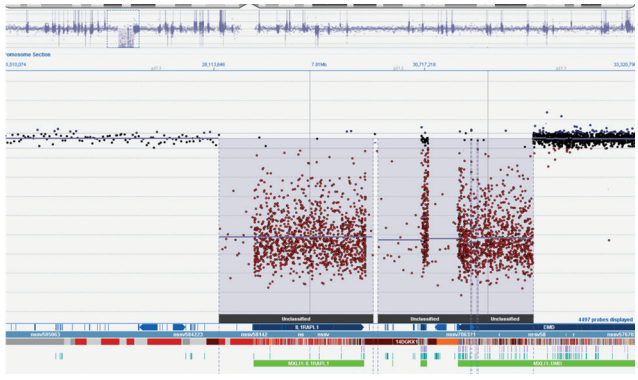

Figure 6. The deletions involving DMD, NR0B1, GK, and IL1RAPL1 genes (case 2)

The patient was discharged with oral hydrocortisone, fludrocortisone, and salt supplementation when 42 days old.

Informed consent was obtained from the parents of the two children studied for further investigation. DNA was extracted by standard methodology and CGH.

Discussion

Both patients presented here were diagnosed with at least partially compensated primary adrenal insufficiency due to the lack of adequate elevation in cortisol levels, despite increasing ACTH levels, and presence of dehydration, hyponatremia, hyperpotassemia, and hyperpigmentation.

Congenital adrenal hyperplasia (CAH) is the most common cause of primary adrenal insufficiency. However, 17-OHP values below <10 ng/mL during the neonatal period rule out CAH (4). CAH is also associated with large adrenal glands at ultrasonography (5). The findings in our patients were supportive of AHC, as seen in patients with mutations or deletions of the *DAX-1* (*NROB1*) gene at the X chromosome (6,7), defects of *steroidogenic factor 1* gene at the 9q33 chromosome (8), and IMAGe syndrome (9). In X-linked AHC, deletions of the *DAX-1* gene may occur along with deletions of adjacent genes at the Xp21 locus. In some cases, this may be accompanied by the deletion of the gene encoding dystrophin, leading to DMD. Other cases involve deletion of the GK leading to GKD. Thus, AHC manifestations vary depending on the site and extent of the deletion (2). When GKD is accompanied by DMD or AHC or both, this is called CGKD (1).

Creatine kinase and triglyceride levels were measured to elucidate the etiology of AHC and were above normal limits in both cases. Glycerol is measured as triglyceride in routine laboratory tests. Thus, elevated levels of triglycerides in these cases are not described as hypertriglyceridemia but, as a more precise term, as "pseudo-hypertriglyceridemia" (10). Although glycerol is not an acidic compound, glyceroluria can usually be detected with urinary organic acid measurements using gas chromatography-mass spectrometry (1). There was a glycerol peak in the urinary organic acid assay in case 1. With these findings, both of our cases were considered as CGKD with coexisting AHC, DMD, and GKD.

Deletions and mutations in the DMD, AHC (NROB1), and *GK* genes at locus Xp21 can be demonstrated by genetic analysis in CGKD. CGH showed a deletion involving all coding seqeunces of the *GK* gene, the deletion included part of the DMD gene, the entire *NR0B1* gene, and part of the *IL1RAPL1* gene in the first patient. CGH also showed a deletion encompassing part of the DMD and three additional disease-associated genes (*NR0B1, GK, and IL1RAPL1*) in case 2.

Patients with concurrent AHC, DMD, and GKD have characteristic facial features. These include prominent forehead and eyebrows, depressed nasal root and bridge, which together give an "hourglass" appearance to the midfacial region. Other facial characteristics are hypertelorism, rounded palpebral fissures, esotropia, wide and flattened ear lobes, and downturned corners of the mouth (11).

In conclusion, AHC and CGKD should be considered in male neonates with dysmorphic features presenting with adrenal crisis. Performing genetic analysis such as CGH is helpful in finalizing the diagnosis and predicting prognosis by determining the location and magnitude of deletions as well as in detection of female carriers.

Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Sabriye Korkut, Osman Baştuğ, Margarita Raygada, Nihal Hatipoğlu, Selim Kurtoğlu, Mustafa Kendirci, Charalampos Lyssikatos, Constantine A. Stratakis, Design: Sabriye Korkut, Osman Baştuğ, Margarita Raygada, Nihal Hatipoğlu, Selim Kurtoğlu, Mustafa Kendirci, Charalampos Lyssikatos, Constantine A. Stratakis, Data Collection or Processing: Sabriye Korkut, Osman Baştuğ, Nihal Hatipoğlu, Selim Kurtoğlu, Analysis or Interpretation: Sabriye Korkut, Osman Baştuğ, Margarita Raygada, Nihal Hatipoğlu, Selim Kurtoğlu, Mustafa Kendirci, Charalampos Lyssikatos, Constantine A. Stratakis, Literature Search: Sabriye Korkut, Osman Baştuğ, Margarita Raygada, Nihal Hatipoğlu, Selim Kurtoğlu, Mustafa Kendirci, Charalampos Lyssikatos, Constantine A. Stratakis, Writing: Sabriye Korkut, Osman Baştuğ, Margarita Raygada, Nihal Hatipoğlu, Selim Kurtoğlu, Mustafa Kendirci, Charalampos Lyssikatos, Constantine A. Stratakis.

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Case Report

A Novel Missense Mutation in the CLPP Gene Causing Perrault Syndrome Type 3 in a Turkish Family

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ABSTRACT

Perrault syndrome (PRLTS) is a heterogeneous group of clinical and genetic disorders characterized by sensory neuronal hearing loss in both sexes and premature ovarian failure or infertility in females. Neurological and hearing loss symptoms appear early in life, but female infertility cannot be detected before puberty. Spastic limbs, muscle weakness, delayed puberty and irregular menstrual cycles have also been observed in PRLTS patients. Mutations in five genes, i.e. *HSD17B4, HARS2, CLPP, LARS2,* and *C10orf2*, have been reported in five subtypes of PRLTS. Here, we report a milder phenotype of PRLTS in a Turkish family in which two affected patients had no neurological findings. However, both were characterized by sensory neuronal hearing loss and the female sibling had secondary amenorrhea and gonadal dysgenesis. Genome-wide homozygosity mapping using 300K single-nucleotide polymorphism microarray analysis together with iScan platform (Illumina, USA) followed by candidate gene Sanger sequencing with ABI 3500 Genetic Analyzer (Life Technologies, USA) were used for molecular diagnosis. We found a novel missense alteration c.624C>G; p.Ile208Met in exon 5 of the *CLPP* at chromosome 19p13.3. This study expands the mutation spectrum of *CLPP* pathogenicity in PRLTS type 3 phenotype.

Keywords: Secondary amenorrhea, Perrault syndrome, *CLPP*

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Mutations in five genes -*HSD17B4, HARS2, CLPP, LARS2,* and *C10orf2*- have been reported in five subtypes of Perrault syndrome.

WHAT THIS STUDY ADDS?

We found a novel missense alteration c.624C>G; p.Ile208Met in exon 5 of the *CLPP* at chromosome 19p13.3. This study expands the mutation spectrum of *CLPP* pathogenicity in Perrault syndrome type 3 phenotype.

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Introduction

Perrault syndrome (PRLTS) is a rare autosomal recessive disorder leading to pure gonadal dysgenesis in affected females (46,XX) and sensorineural hearing loss (SNHL) or deafness in males. Ovarian dysfunction ranges from absent or streak gonads to primary ovarian insufficiency defined as cessation of menses before age 40 years (1). Central nervous system findings have also been reported with this syndrome. Neurologic features described in some affected women include developmental delay, intellectual disability, cerebellar ataxia, and motor and sensory peripheral neuropathy (1).

Pathogenic alterations in five genes have been reported in five subtypes of PRLTS. PRLTS type 1 is caused by mutations in *HSD17B4* gene at chromosome 5q23.1 (2) and PRLTS1 patients may present with hearing loss, ovarian dysgenesis leading to female infertility, male infertility, ataxia, and peripheral neuropathy (2,3,4). PRLTS type 2 is caused by mutations in HARS2 at chromosome 5q31.3 and is characterized by deafness in both males and females and gonadal dysgenesis in female patients only (5). PRLTS type 3 is caused by mutations in *CLPP* gene at chromosome 19p13.3 (6,7). *PRLTS3* patients may present with progressive hearing loss, female infertility and premature menopause, microcephaly, epilepsy, growth and mental retardation (6,7). PRLTS type 4 is caused by mutations in *LARS2* gene at chromosome 3p21.31and is characterized by hearing loss and premature ovarian failure (8). PRLTS type 5 is caused by mutations in *C10orf2* gene at chromosome 10q24.31 (9). PRLTS5 patients may present with progressive ataxia, axonal neuropathy, hyporeflexia, abnormal eye movements, progressive hearing loss, and ovarian dysgenesis (9).

Here, we report the clinical and molecular investigations of two PRLTS patients from a Turkish family (Figure 1).

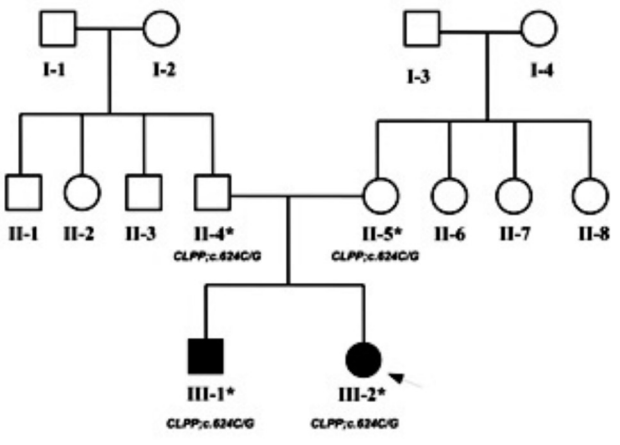

Figure 1. Pedigree of the parents showing autosomal recessive mode of inheritance in the affected individuals. The index patient is indicated with an arrow. The asterisk (*) indicates the samples that were validated by Sanger sequencing with their respective genotypes below each symbol

Case Reports

Patient 1

The patient was a 16-year-old girl (III-2) who presented with secondary amenorrhea. She was attending a special school for hearing-impaired students. The parents were both healthy and non-consanguineous but came from the same village. There were no dysmorphic findings or evidence of other systemic disease in the physical examination. Her weight was 51 kg (25p), height was 160 cm (25-50p), axillary hair was present, pubic hair was at stage 5, and breast development was bilaterally at stage 3 according to the Tanner staging. Neurologic examination was normal. Pelvic ultrasonography revealed a uterus of 8x12x50 mm in size, but ovaries could not be detected. Whole blood count, renal functions, liver functions, as well as glucose and electrolyte levels were within normal ranges, while hormone studies revealed hypergonadotropic hypogonadism. Luteinizing hormone was 20.7 mIU/mL, follicle stimulating hormone was 63.8 mIU/mL, and estradiol was 15 pg/mL. The karyotype was 46,XX. Adrenal steroid levels and thyroid functions were also normal. Hormone replacement treatment with estrogen was initiated. The patient was suspected to have PRLTS because of gonadal failure in association with bilateral sensorineural deafness. Repeated neurologic examination was normal as well as the brain magnetic resonance imaging.

Patient 2

He was the 21-year-old brother (III-1) of our first patient. He was invited to the clinic because of his hearing loss and a sibling with the clinical diagnosis of PRLTS. He had also attended a school for hearing-impaired students. Physical examination revealed no dysmorphic findings. He was in Tanner stage 5 of puberty with a height and weight of 170 cm [-0.95 standard deviation score (SDS)] and 75 kg (+0.36 SDS), respectively. Neurologic examination was normal. However, he was under the supervision of a psychiatrist and receiving risperidone because of attention-deficit disorder.

Genetic Analysis

Homozygosity Mapping

Genome-wide homozygosity mapping on four family members (unaffected parents and the two affected siblings) was performed using 300K single-nucleotide polymorphism (SNP) microarray (HumanCytoSNP12.2 chip) along with iScan platform (Illumina, USA). We found that a region on 19p13.3 was homozygous in the two affected individuals and was heterozygous in the two parents (Figure 2). This 2 Mb region (chr19:5469832-7472041) contained 64 genes including *CLPP* according to human genome map (Annotation release 105 http://www.ncbi.nlm.nih.gov/projects/mapview/).

Sanger Sequencing

Genomic sequence of the wild-type *CLPP* gene (ENSG00000125656) was obtained from Ensembl Genome Browser (www.ensembl.org). The six coding exons including exon-intron boundaries were polymerase chain reaction amplified with the primers sets (in Table 1) and sequenced with ABI3500 Genetic Analyzer according the manufacturer's instructions (Life Technologies, USA). We found a novel homozygous transversion alteration, cytosine to guanine, in exon 5 at nucleotide 624 (c.624C>G) of *CLPP* gene causing alteration of isoleucine to methionine at 208 amino acid position (p.Ile208Met). Both parents were heterozygous (carriers) for this variant confirming the autosomal recessive inheritance of *PRLTS3* phenotype in this family (Figure 3). Sequencing of 100 unaffected healthy individuals (200 chromosomes) excluded the probability of neutral polymorphism of the variant (*CLPP*, c.624C>G) identified in our patients. Computational prediction software (SIFT, Polyphen-2 and Mutation Taster) declared this alteration as protein damaging. Furthermore, this variant (chr19:6366337C>G) had not been listed in 1000 human genome (http://browser.1000genomes.org/) in 60,706 individuals in the Exome Aggregation Consortium (http://exac.broadinstitute.org/) databases.

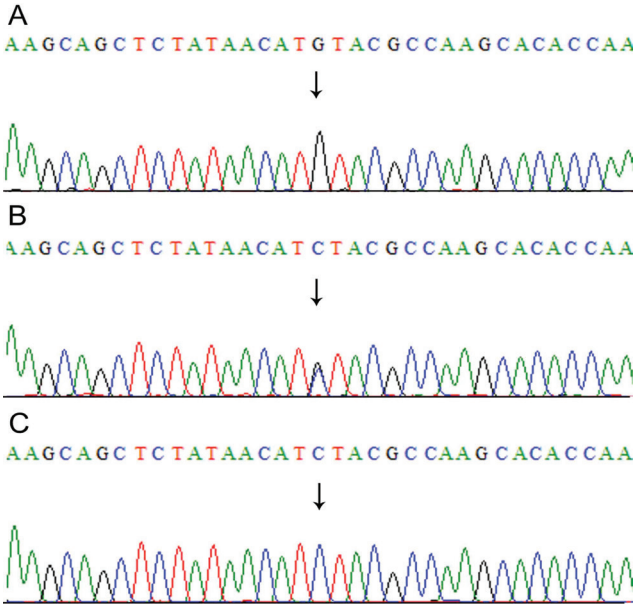

Figure 3. Sequencing analysis of the *CLPP* gene exon 5 showing C to G transversion at nucleotide position 624 (c.624C>G) in the affected patients (A), heterozygous carrier parents (B), and unaffected siblings or healthy controls (C)

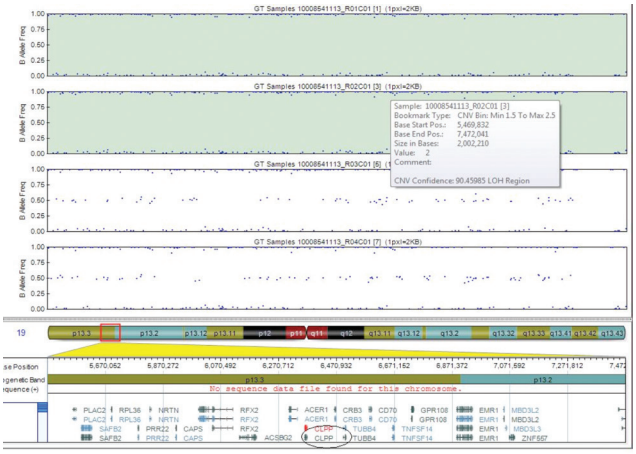

Figure 2. Single-nucleotide polymorphism microarray analysis showing a common region of homozygosity in the affected individuals flanking *CLPP* gene on chromosome 19p13.3

Discussion

Genetic analysis of PRLTS remained unresolved until the first gene was discovered in 2010 (2). Since then, several familial and sporadic cases have been reported, of which, the majority was of European descent (2,5,8). *PRLTS3* gene was identified in three Pakistani families (6) and we also screened a family from Saudi Arabia very recently (7). To date, only four mutations including one splicing (c.270+A>G) and three missense (c.433A>C; p.Thr145Pro, c.440G>C; p.Cys147Ser, c.685T>G; p.Tyr229Asp) have been identified in the *CLPP* gene (6,7). Here, we present, for the first time, a novel *CLPP* alteration in a Turkish family.

Table 1. List of primers along with the annealing temperature used for polymerase chain reaction amplification of the six coding exons of *CLPP* gene

No	Primer name	Primer sequence (5'-3')	Annealing
1	CLPP_1-2F	GGACTCGAACTGGAGACTCTAAA	62.9 °C
2	CLPP_1-2R	TTAAGAGCCGAGGAGCAGAG	60.5 °C
3	CLPP_3F	CTTCCTGGTTCCCTGACC C	61.7 °C
4	CLPP_3R	ACGCTCTGCACCCTTTCCCA	62.5 °C
5	CLPP_4F	CCAGGTTTAGGAGATGGAAT	56.4 °C
6	CLPP_4R	TGTCTAGACCCTGTCCTGAT	58.4 °C
7	CLPP_5F	AGCCCACCAGCCTCAAAC	58.4 °C
8	CLPP_5R	CATCCCAGAGAACGATCCAG	60.5 °C
9	CLPP_5R2	GCCCTGAAAGTCCGCAGGG	63.8 °C
10	CLPP_6F2	GACCCAGACCTGGCCCTG	63.0 °C
11	CLPP_6R2	TCCAAGCCCAGCAACAAGGG	62.5 °C
12	CLPP_6R3	CCACATGATTCTGGAGAGGAG	61.3 °C

The *CLPP* enzyme is a 277 amino acid-long peptidase which works in the presence of ATP and magnesium cleaving of larger proteins to smaller peptides (10,11,12). Accumulation of CLPX, mtDNA, and inflammatory factors in tissues have been observed in mice mutants due to *CLLP* loss of function leading to infertility, hearing loss, and growth retardation (13). A similar mechanism might be involved in humans with *PRLTS3* carrying *CLPP* alterations (6,7).

Molecular diagnosis of PRLTS is efficiently performed through genome-wide SNP microarray for linkage analysis followed by candidate gene sequencing or by directly stepping into whole exome sequencing. These methods can either be utilized individually (2,5,6,8,9) or by combing the two strategies (7). Exome analysis has the advantage of finding causative variants more efficiently compared to candidate gene screening in rare genetic disorders (14,15). However, genome-wide SNP microarray genotyping or array comparative genomic hybridization (CGH) has the advantage of finding out the chromosomal aberrations (16), which may not be possible through whole exome analysis alone. The SNP microarray can also exclude known PRTLS candidates to pin point a single region of homozygosity in ethnically isolated populations (6,17). Here, we found the genome-wide SNP microarray analysis followed by candidate (*CLPP*) gene sequencing as a successful strategy for identifying the causative variant underlying *PRLTS3* in an isolated Turkish family.

In the clinical diagnosis of PRLTS, SNHL and neurological abnormalities both in males and females and female ovarian dysgenesis are considered key findings (1,18). Amenorrhea, gonadal dysgenesis, and SNHL were present in our index patient. However, all these signs may not be detected in younger patients (7). For example, patients with ovarian failure may present with lack of female sexual characteristics, or with primary or secondary amenorrhea. In such cases, pathogenic variants in various causative genes involved in ovarian dysgenesis could be of help in precise diagnosis (19,20). Autoimmunity is also considered as one of the important exclusion factors in patients with ovarian insufficiency, especially in secondary amenorrhea cases (21,22). Congenital disorders of adrenal and gonadal steroidogenesis are also rare causes of ovarian failure (23). Similarly, hearing loss is reported to be present in approximately 50% of women with Turner syndrome (18). For this reason, girls with delayed puberty or amenorrhea with low estrogen and raised gonadotropins need to be investigated either by karyotyping or array CGH analysis to exclude abnormalities of the X chromosome (20,24,25,26).

In addition to SNHL and ovarian insufficiency, neuromuscular abnormalities (spastic diplegia, dysarthria, titubation of the head, hyporeflexia, sensory neuropathy, demyelinating polyneuropathy, cerebellar ataxia, nystagmus, ophthalmoplegia, ptosis, seizures), developmental abnormalities (microcephaly, delayed motor and mental development, learning disabilities), and dysmorphic findings (pes cavus, pes equinovarus, contracted heel cords, atypical facial features, short neck) were found to be associated with PRLTS1 (2,3,27,28,29). These features were not observed in our cases. Previously, we and others reported that short stature, microcephaly, seizures, moderate learning difficulties, and truncal and cerebellar ataxia with signs of lower limb spasticity may occur in *PRLTS3* (6,7). Neurologic disabilities, which started by the 18th month and worsened through years, were defined in two siblings with a *CLPP* mutation in Pakistani and Saudi families (8), but were not observed in our patients. The PRLTS5 patients are also characterized by progressive ataxia, axonal neuropathy, hyporeflexia, and abnormal eye movements as previously reported in Japanese patients (9), but these symptoms were also not observed in our cases.

Our primary clinical diagnosis in our patients, due to absence of neurological findings, pointed to either PRTLS2 or PRLTS4. However, after establishing the molecular diagnosis of *CLPP* pathogenicity, we concluded that p.Ile208M might have caused a milder *PRLTS3* phenotype in our cases. On the other hand, it must be remembered that all the features may not always be prominent in *PRLTS3* patients. For example, a previously reported Pakistani family, with splice donor-site mutation (c.270+A>G in CLPP), had only hearing loss with neither brain involvement nor any other associated abnormality (6). The clinical features of *PRLTS3* are also age-dependent as described previously (7). Furthermore, we assume that hearing defect and ovarian dysgenesis without neurological findings might be a specific association with our mutation. Marlin et al (30) reviewed 34 cases from 15 families and reported hearing defect and ovarian dysgenesis without neurological findings. Our analyses encourage *CLPP* screening in such cases.

In conclusion, PRLTS is clinically diagnosed with the presence of primary ovarian failure in association with SNHL, and sometimes, with neuromuscular involvement. Clinical presentation is quite variable since the onset of all components may take time to appear. Gonadal insufficiency is not usual in boys and is noted only after pubertal age in girls. For these reasons, this syndrome should be suspected in patients presenting with unexplained neurologic findings and SNHL. The definite diagnosis of the type of PRLTS can be made only by molecular analyses since the clinical features may overlap. Patients with an identified mutation in the genes associated with PRLTS should be followed up in terms of clinical findings even if they are asymptomatic. Our patients were unique for having a novel mutation in *CLPP* gene, which leads to a mild form of *PRLTS3* without any neurologic involvement. The genome-wide SNP microarray genotyping or array CGH followed by candidate gene sequencing may be used as a useful tool for PRTLS-causative variant identification in ethnically or geographically isolated familial cases.

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Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authors Contributions

Concept: Fatma Dursun, Musharraf Jelani, Heves Kırmızıbekmez, Design: Musharraf Jelani, Fatma Dursun, Hussein Sheikh Ali Mohammoud, Data Collection and Processing: Hussein Sheikh Ali Mohammoud, Noreen Karim, Analysis and Interpretation: Noreen Karim, Muhammad Naeem, Literature Research: Muhammed Naeem, Heves Kırmızıbekmez, Writing: Musharraf Jelani, Fatma Dursun, Heves Kırmızıbekmez.

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Case Report

A Novel Homozygous Mutation in the KCNJ11 Gene of a Neonate with Congenital Hyperinsulinism and Successful Management with Sirolimus

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ABSTRACT

Congenital hyperinsulinism (CHI) is the most common cause of neonatal persistent hypoglycemia caused by mutations in nine known genes. Early diagnosis and treatment are important to prevent brain injury. The clinical presentation and response to pharmacological therapy may vary depending on the underlying pathology. Genetic analysis is important in the diagnosis, treatment, patient follow-up, and prediction of recurrence risk within families. Our patient had severe hypoglycemia and seizure following birth. His diagnostic evaluations including genetic testing confirmed CHI. He was treated with a high-glucose infusion, high-dose diazoxide, nifedipine, and glucagon infusion. A novel homozygous mutation (p.F315I) in the *KCNJ11* gene, leading to diazoxide-unresponsive CHI, was identified. Both parents were heterozygous for this mutation. Our patient's clinical course was complicated by severe refractory hypoglycemia; he was successfully managed with sirolimus and surgical intervention was not required. Diazoxide, nifedipine, and glucagon were discontinued gradually following sirolimus therapy. The patient was discharged at 2 months of age on low-dose octreotide and sirolimus. His outpatient clinical follow-up continues with no episodes of hypoglycemia. We present a novel homozygous p.F315I mutation in the *KCNJ11* gene leading to diazoxide-unresponsive CHI in a neonate. This case illustrates the challenges associated with the diagnosis and management of CHI, as well as the successful therapy with sirolimus.

Keywords: Congenital hyperinsulinism, newborn, persistent hypoglycemia, sirolimus

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Congenital hyperinsulinism is a genetic disorder characterised by challenges associated with the diagnosis and management.

WHAT THIS STUDY ADDS?

This case has a novel homozygous p.F315I mutation in the *KCNJ11* gene unresponsive to diazoxide therapy but successfully treated with sirolimus before pancreatectomy.

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Introduction

Congenital hyperinsulinism (CHI) is a complex heterogeneous genetic condition caused by unregulated insulin secretion from pancreatic β-cells. The inappropriate release of insulin leads to persistent severe hypoketotic hypofattyacidemic hypoglycemia and most of the cases present in the neonatal period (1). The prevalence of the disorder is 1 in 40000-50000 live births, increasing to 1:2500 in consanguineous populations (2). Infants are usually macrosomic at birth and require a high glucose infusion rate (GIR) (3). The molecular basis of CHI involves defects in key genes controlling complex mechanisms of insulin secretion. Thus far, mutations in nine genes have been identified and broadly classified into channelopathies and metabolopathies (4). The former are attributed to adenosine triphosphate (K_{ATP})-sensitive potassium channel genes (*ABCC8, KCNJ11*) and metabolopathies regulate different pathways (*GLUD1, GCK, HNF4A, HNF1A, SLC16A1, UCP2, HADH*). The most common forms affect K_{ATP} channel genes and are predominantly recessive mutations (5,6). There are three histological forms: focal CHI (FCHI) or β-cell adenomatosis, diffuse CHI (DCHI), and atypical CHI (6,7,8,9).

Early recognition, diagnosis, and initiation of immediate management are important in CHI. Maintenance of a normoglycemic state and prevention of permanent brain damage are the principal aims of the treatment (10). Once CHI is confirmed, diazoxide given in a dose of 10-15 mg/kg/d is the drug of choice in medical management (1,5). Fluid retention and hypertrichosis are common side effects of diazoxide, and most centers use chlorothiazide concurrently (1,5). Nifedipine has been used (0.5-2 mg/d), however, the vast majority of patients fail to respond to nifedipine (10). Most of the diazoxide-unresponsive patients have recessive mutations in the *ABCC8* or *KCNJ11* gene (11). Octreotide may be added to the treatment in doses of 10-50 µg/kg/d. Glucagon in doses of 1-20 µg/kg/h should be used for acute management or in combination with octreotide (10).

The response to diazoxide therapy plays a key role in the management of CHI. Because of inactivating mutations in the *ABCC8* and *KCNJ11* genes, diazoxide is often ineffective in DCHI and focal forms. The vast majority of patients with DCHI undergo a near-total pancreatectomy, resulting in diabetes and exocrine pancreatic insufficiency (12,13). The indications for surgery include medically unresponsive DCHI and confirmed FCHI by fluoro-18-L-dihydroxyphenylalanine positron emission tomography/computed tomography (^{18}F-DOPA-PET/CT) (5,10,13). Additional therapy with diazoxide, octreotide, and/or frequent feedings may be required postoperatively (14,15).

We present a male infant who was diagnosed with CHI and had a novel homozygous p.F315I mutation in the *KCNJ11* gene leading to diazoxide-unresponsive CHI. Both parents were heterozygous for this mutation, and the patient was successfully managed with sirolimus therapy.

Case Report

This male infant was born to a healthy 26-year-old mother at $37^{4/7}$ weeks of gestation via cesarean section with Apgar scores 8 and 9 at first and fifth minutes. The infant weighed 4190 g (large for gestational age) at birth. The parents were second cousins and there was no history of a similar condition or of diabetes mellitus in the family. The patient was diagnosed as a case of persistent hypoglycemia and was given GIR up to 14 mg/kg/min and prednisolone 2 mg/kg/d. Serum levels were: insulin 42.5 µU/mL, cortisol 10.9 mg/dL, growth hormone 32.1 ng/mL, and C-peptide 2.7 ng/mL at the time of hypoglycemia (blood glucose level 32 mg/dL). The patient experienced hypoglycemic attacks despite the use of diazoxide (10 mg/kg/d) and octreotide (10 µg/kg/d) and was referred to our unit at age 21 days.

On admission, his physical examination was unremarkable except for macrosomia. The results of his laboratory analysis including a hemogram, acute phase reactants, arterial blood gases, biochemical and urinary evaluations were within normal limits. Echocardiography revealed an atrial septal defect and mild septal hypertrophy. The patient was administered enteral nutrition with breast milk at 2-h intervals. Despite intensive therapy (diazoxide 25 mg/kg/d, chlorothiazide 1 mg/kg/d, octreotide 40 µg/kg/d, glucagon infusion 10 µg/kg/h, and nifedipine 1 mg/kg/d), his hypoglycemia persisted until 1 month of age. As ^{18}F-DOPA-PET/CT was unavailable, we could not determine the histological form of the disorder. DNA samples were sent to the United Kingdom (Exeter Clinical Laboratory, Exeter, UK) for mutation analysis.

We considered sirolimus therapy before pancreatectomy when the patient was 35 days old. Parental consent and approval of off-label use of the drug from the Turkish Ministry of Health Ethics Committee were obtained. His glucose levels increased following oral sirolimus therapy in a dose of 0.5 mg/m^2/d. We stopped diazoxide, hydrochlorothiazide, nifedipine, and glucagon infusion gradually, and his octreotide dose was decreased to 5 µg/kg/d. The sirolimus dose was adjusted according to the serum level. He was discharged at 2 months of age with pre-feed home blood glucose monitoring on low-dose octreotide (5 µg/kg/d) twice-daily subcutaneous injections and sirolimus (0.3 mg/m^2/d). His outpatient clinical follow-up continued without hypoglycemia. At the time of this report, the patient was 5 months of age, there were no abnormal findings on his neurological or physical examination and cranial magnetic resonance imaging revealed normal findings according to his age.

Genetic analysis revealed a novel homozygous mutation in the *KCNJ11* gene (p.F315Ic.943T>A); the parents were heterozygous for this mutation. This mutation had not been previously identified in over 3000 patients with CHI or with neonatal diabetes referred to the Exeter Clinical Laboratory for testing.

Discussion

CHI is a heterogeneous disorder caused by mutations in nine key genes regulating insulin secretion. The *ABCC8* and *KCNJ11* genes (both localized on chromosome 11p15.1) encode the two components of the K_{ATP} channel: the pore-forming inward rectifier potassium channel subunit (KIR6.2) and the regulatory subunit sulfonylurea receptor 1 (SUR1) (1,2,3,4,5,6). Loss-of-function mutations in these genes are present in the homozygous or compound heterozygous state and may be dominantly acting. Recessive inactivating mutations in the *ABCC8* and *KCNJ11* genes constitute the most common and severe forms of CHI. Approximately 300 different mutations in *ABCC8* and 30 mutations in *KCNJ11* account for 36.3% of all cases (12,16,17). Genetic analysis in our patient revealed a novel homozygous mutation in the *KCNJ11* gene (p.F315I) leading to diazoxide-unresponsive DCHI.

Although the molecular mechanisms of FCHI and DCHI are different, their clinical presentations appear to be similar. DCHI is inherited in an autosomal recessive manner in most cases, whereas FCHI is sporadic (12,16). It is well known that patients with homozygous recessive or compound heterozygous mutations in the *ABCC8* or *KCNJ11* gene present with DCHI. They are usually medically unresponsive and account for 60-70% of all cases. Therefore, our diazoxide-unresponsive patient may have DCHI. Paternally inherited mutations in the *ABCC8* or *KCNJ11* gene and a concomitant loss of the maternal 11p allele (11p15.1-11p15.5) result in focal pancreatic lesions (6,9,10,12,16).

The management of diazoxide-unresponsive DCHI constitutes a major therapeutic challenge. Because of abnormal activation of the mammalian target of rapamycin (mTOR) pathway in several neoplasms, including insulinoma, mTOR inhibitors have been increasingly recognized as a treatment option in patients with CHI (18,19). Senniappan et al (20) described four infants with severe CHI unresponsive to maximal doses of diazoxide (20 mg/kg/d) and octreotide (35 µg/kg/d). They reported that all patients showed a clear glycemic response to sirolimus, but one patient required a small dose of octreotide to maintain normoglycemia. Abraham described a neonate with CHI caused by a homozygous ABCC8 mutation, who was unresponsive to diazoxide and octreotide (21). He reported achievement of euglycemia by using sirolimus therapy postoperatively. Kara et al (22) treated a newborn having CHI with sirolimus, but discontinued it due to hepatic and renal failure. Another report describes an 8-year-old boy with severe CHI due to a biallelic heterozygous ABCC8 mutation who exhibited a drastic improvement with sirolimus (23). Sirolimus therapy appears to be a feasible alternative to subtotal pancreatectomy, either alone or in combination with somatostatin analogues for selected patients with no contraindications. Sirolimus allows the discontinuation of intravenous dextrose, glucagon infusion, and octreotide. In our patient, following sirolimus therapy, 'we discontinued diazoxide, thiazide, glucagon, and glucose infusion and decreased the dose of octreotide.

The adverse effects of mTOR inhibitors, such as everolimus and sirolimus, include stomatitis, increased risk of infection, immunosuppression, abnormalities in renal function, fatigue, and pneumonitis. Transient elevations of aminotransferase levels have been reported. Patients with CHI who receive sirolimus therapy must be monitored regularly to assess glycemic control and adverse events (20,24). During the short follow-up period, we did not observe any adverse effects caused by sirolimus.

Patients with CHI should be closely followed to monitor the efficacy of treatment and complications related to medications and underlying disease. These patients should record their blood glucose levels using a home glucometer. Blood glucose level, diet, growth, and side effects of medications should be regularly evaluated (2,25,26). It is clear that early severe hypoglycemic events may cause poor neurological outcomes, such as psychomotor retardation, cognitive deficit, epilepsy, and cerebral palsy. Neonatal onset of CHI is usually more severe and requires regular screening, detection, and appropriate management by a pediatric neurologist (27,28,29). We trained the mother of our patient in home glucose monitoring and arranged visits with a pediatric endocrinologist and neurologist following discharge. Our patient had normal neurodevelopment at 5 months of age.

In conclusion, we described the case of a neonate with a novel homozygous *KCNJ11* mutation leading to diazoxide-unresponsive DCHI. This case illustrates the pitfalls and challenges associated with the treatment of CHI, as well as the successful therapy with sirolimus.

Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

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Case Report

A p.(Glu809Lys) Mutation in the WFS1 Gene Associated with Wolfram-like Syndrome: A Case Report

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ABSTRACT

Wolfram-like syndrome (WFSL) is a rare autosomal dominant disease characterised by congenital progressive hearing loss, diabetes mellitus, and optic atrophy. The patient was a boy with the juvenile form of diabetes mellitus and findings which clinically matched the symptoms of Wolfram syndrome. At the age of 3 1/4 years, diabetes mellitus was diagnosed in this boy who also had severe psychomotor retardation, failure to thrive, a dysmorphic face with Peters anomaly type 3 (i.e. posterior central defect with stromal opacity of the cornea, adhering stripes of the iris, and cataract with corneolenticular adhesion), congenital glaucoma, megalocornea, severe hearing impairment, a one-sided deformity of the auricle with atresia of the bony and soft external auditory canal, non-differentiable eardrum, missing os incus, hypothyreosis, and nephrocalcinosis. Molecular-genetic examinations revealed a de novo mutation p.(Glu809Lys) in the *WFS1* gene. No mutations were detected in the biological parents. The mutation p.(Glu809Lys) in the *WFS1* gene is associated with WFSL.

Keywords: Wolfram syndrome, genotype, phenotype

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The Wolfram syndrome (WFS, Online Mendelian Inheritance in Man 222300), also known as the DIDMOAD syndrome (diabetes insipidus, early-onset diabetes mellitus, progressive optic atrophy, and deafness) is mostly associated with a recessive mutation in the *WFS gene 1* (*WFS1*), rarely in the *WFS2*. A dominant mutation in the *WFS1* gene was described in connection with sensorineural hearing loss, deafness, and optic atrophy: Wolfram-like syndrome (WFSL). Variable clinical symptoms, rare occurrence, and molecular complexity complicate the diagnosis and the genotype-phenotype correlation of the disease.

WHAT THIS STUDY ADDS?

The novelty of the data and their impact on the field: the p.(Glu809Lys) mutation in the *WFS1* gene is associated with the occurrence of the WFSL.

Introduction

Wolfram syndrome (WFS, OMIM 222300) is a rare genetic disease with a prevalence of cca. 1:710.000 (1). The disease is also known as the DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy, deafness). Most of the individuals afflicted with this disease have the recessive mutation in the *WFS* gene 1 (*WFS1*, 4p16.3) (1,2) and rarely in the *WFS* gene 2 (*WFS2*) (3). A dominant mutation in the *WFS1* gene was also described in connection with sensorineural hearing loss, deafness, and optic atrophy [Wolfram-like syndrome (WFSL)] (4).

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Case Report

Our patient was a boy whose clinical findings matched those reported for the Wolfram syndrome. At the age of 3 1/4 years, the patient was diagnosed to have diabetes mellitus along with severe psychomotor retardation, failure to thrive, a dysmorphic face with Peters anomaly (PS) type 3 (posterior central defect with stromal opacity of the cornea, adhering stripes of the iris, and cataract with corneolenticular adhesion), congenital glaucoma, megalocornea, hypothyreosis, nephrocalcinosis, severe hearing impairment, a one-sided deformity of the auricle with atresia of the bony and soft external auditory canal, a non-differentiable eardrum, and a missing os incus. At presentation, the patient did not have optic atrophy or diabetes insipidus. The proband's karyotype was 46,XY, 9qh+.

We performed sequencing analysis of the coding exons and adjacent intron regions of the *WFS1* gene and of the *B3GALTL, CYP1B1, PITX2,* and *PAX6* genes associated with PS. The analysis for the WFS1 gene was complemented by multiple ligation-dependent probe amplification (SALSA MLPA P163 GJB-WFS1, MRC-Holland) identifying potential deletions/duplications. Apart from standard polymorphisms, the only potentially causal mutation was found to be a heterozygous missense mutation in the *WFS1* gene, namely, c.2425G>A, p.(Glu809Lys). This mutation was analysed in the proband's family, namely, in both parents and siblings of the proband-twin B. The mutation was not detected in any of the family members and the final diagnosis was WFSL caused by a de novo mutation c.2425G>A, p.(Glu809Lys). This mutation localised in the exon 8 of *WFS1* was not reported in databases (http://databases.lovd.nl/whole_genome/genes or http://exac.broadinstitute.org/) and was described as a likely pathogenic finding in http://www.ncbi.nlm.nih.gov/clinvar/variation/215413/. *In silico* analysis of this mutation using prediction programs PolyPhen-2 and SIFT showed probable damaging and damaging effects, respectively.

Discussion

In 2014, Matsunaga et al (1) described a proband with WFS and c.2425G>A, p.(Glu809Lys) mutation in the *WFS1* gene. The patient suffered from diabetes mellitus, optic atrophy, deafness, and mental disorder. In the same year, Lee et al (5) mentioned a proband with c.2425G>A, p.(Glu809Lys) mutation in the *WFS1* gene. The proband suffered from cataract, hypotonia, sensorineural deafness, and diabetes mellitus. The second causal mutation was not determined in either patient. Follow-up molecular genetic examinations of the family members had apparently not been carried out in these cases. We believe that molecular genetic examination in the members of the family of a proband with one mutation in the *WFS1* gene is important.

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Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Dagmar Prochazkova, Design: Dagmar Prochazkova, Lenka Fajkusova, Data Collection or Processing: Zuzana Hruba, Petra Konecna, Jarmila Skotakova, Analysis or Interpretation: Dagmar Prochazkova, Lenka Fajkusova, Literature Search: Dagmar Prochazkova, Lenka Fajkusova, Writing: Dagmar Prochazkova.

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Case Report

A Case of Vitamin D-Dependent Rickets Type 1A with a Novel Mutation in the Uzbek Population

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ABSTRACT

Vitamin D-dependent rickets type 1A (VDDR-1A) (Online Mendelian Inheritance in Man #264700) is a rare, autosomal recessively inherited disorder due to inactivating mutations in *CYP27B1*. It is characterized by early onset of rickets with hypocalcemia. We aimed to describe the clinical and laboratory findings in a VDDR-1A case and to report a novel homozygote truncating mutation NM_000785.3 c.403C>T (p.Q135*) in *CYP27B1* which to our knowledge is the first described mutation in the Uzbek population. The patient was admitted with tetany at the age of 12 months. He was a healthy Uzbek boy until 9 months of age when he had a seizure due to hypocalcemia. Vitamin D treatment was given orally in Turkmenistan (no data available for dose and duration). The patient was the product of a consanguineous marriage. His brother had died with hypocalcemia and pneumonia. At physical examination, anthropometric measurements were within normal limits; he had caput quadratum, enlarged wrists, and carpopedal spasm. Blood calcium, phosphorus, alkaline phosphatase, and parathormone (PTH) levels were 5.9 mg/dL, 3.5 mg/dL, 987 IU/L, and 182.8 pg/mL (12-72), respectively. Radiological findings included cupping and fraying of the radial and ulnar metaphyses. Renal ultrasound revealed nephrocalcinosis (grade 1). Despite high serum PTH and 25-hydroxyvitamin D3 levels, 1,25-dihydroxyvitamin D3 level was low, suggesting a diagnosis of VDDR-1A. The patient was treated with calcium carbonate and calcitriol. DNA sequencing revealed a novel homozygous mutation of NM_000785.3 c.403C>T (p.Q135*) in *CYP27B1*. VDDR-1A is a rare disorder which needs to be considered even in countries where nutritional vitamin D deficiency is still common.

Keywords: 25-hydroxyvitamin D 1-α hydroxylase, the *CYP27B1* gene, vitamin D-dependent rickets type 1, calcitriol

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Vitamin D-dependent rickets type 1A (VDDR-1A) is a rare, autosomal, recessively inherited disorder due to inactivating mutations in CYP27B1. It is characterized by early onset of rickets with hypocalcemia. In different ethnic groups, several mutations (homozygous or compound heterozygous) have been identified. Some studies reported that there is a good genotype-phenotype correlation in VDDR-1A. However, the patients carrying the mutations which can totally abolish the enzyme activity can have mild symptoms. Patients with VDDR-1A are usually treated with alfacalcidol or calcitriol.

WHAT THIS STUDY ADDS?

VDDR-1A is a rare disorder. We report here our clinical and treatment experience and a novel mutation in the *CYP27B1* gene which as far as we know is the first described mutation in the Uzbek population.

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Introduction

Vitamin D-dependent rickets type 1A (VDDR-1A) (Online Mendelian Inheritance in Man #264700) is an inborn error of vitamin D metabolism involving defective conversion of 25-hydroxyvitamin D3 [25(OH)D3] to the active form 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] by the enzyme 25(OH) D-1-hydroxylase (1). This type of rickets is characterized by hypotonia, weakness, growth failure, and hypocalcemic seizures in early infancy (1,2). The physical features, laboratory findings such as hypocalcemia with increased serum parathormone (PTH), and the radiological aspects of this condition mimic vitamin D deficiency; but typically, 25(OH)D3 levels are normal or elevated despite a low or low-normal serum 1,25(OH)2D3 level (1,2,3,4). Due to blockage of 25(OH) D-1-α-hydroxylase, treatment consists of supplementation with calcium and active forms of vitamin D (1,2).

VDDR-1A is an autosomal recessive disorder due to the mutation in the *CYP27B1* gene encoding 25(OH) D-1-α-hydroxylase, which catalyzes the hormonally regulated, rate limiting step in the bioactivation of vitamin D (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21). The *CYP27B1* gene is mapped on chromosome 12q14 (4). In different ethnic groups, several mutations (homozygous or compound heterozygous) have been identified in patients with VDDR-1A (3,5,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21). In some ethnic groups, certain mutations are more frequent (2,8,14,20). Some studies have reported that there is a good genotype-phenotype correlation in VDDR-1A (19). However, some patients carrying the mutations which can totally abolish the enzyme activity can present with mild symptoms. Additionally, partial remission during puberty may be observed more frequently in females than in males with the same mutation (8,9,19). These points lead one to speculate that there are other factors which contribute to the variations in degree of severity of the clinical and laboratory findings.

Herein, we report the clinical and laboratory findings in a case of VDDR-1A with a novel mutation NM_000785.3 c.403C>T (p.Q135*) in *CYP27B1* in a boy of Uzbek origin which as far as we know is the first mutation described in the Uzbek population.

Case Report

This male patient was admitted to our hospital with the clinical symptoms of tetany at the age of 12 months. He had his first seizure in Turkmenistan when he was 9 months old; at that time, he was found to be hypocalcemic which was attributed to vitamin D deficiency as he had never received vitamin D prophylaxis. Vitamin D treatment was given orally (no data are available regarding dose and duration of treatment). His parents brought the patient to Turkey in order to get a second opinion.

The patient was the product of a consanguineous marriage and both parents were of Uzbek origin. He teethed first at the age of 10 months. He had one healthy sister and an elder brother who had died at 12 months with a history of hypocalcemia and pneumonia.

Physical examination revealed carpopedal spasm and overactive tendon reflexes. Standard deviation score values

Table 1. Treatment and follow-up findings of the patient

Age (months)	Ca (mg/dL) (8.4-10.8)	P (mg/dL) (2.7-5.5)	ALP (IU/L) (60-525)	PTH (pg/mL) (12-72)	Urinary Ca/Cr ratio	Prescribed Treatment
12	5.9	3.5	987	182	0.08	Calcitriol 2x0.5 µg/dose Ca elementary 65 mg/kg/d
15	8.8	6	653	226.8	0.26	Calcitriol 3x0.25 µg/dose Ca elementary 65 mg/kg/d
18	9.3	6.2	374	77.5	0.42 (N:<0.4)	Calcitriol 3x0.25 µg/dose Ca elementary 50 mg/kg/d
21	10.4	6.8	243	6.81	0.54 (N:<0.4)	Calcitriol 2x0.5 µg/dose** Ca elementary 28 mg/kg/d
24	10.6	5.4	255	3.72	0.99 (N:<0.4)	Calcitriol 2x0.25 µg/dose Ca supplementation was stopped
31	9.6	5.7	266	69.91	0.1 (N:<0,38)	Calcitriol 2x0.25 µg/dose
35	9.3	5.2	262	46.7	0.1 (N:<0,38)	Calcitriol 2x0.25 µg/dose
43	10.4	6.3	318	26.55	0.36 (N:<0,38)	Calcitriol 1x0.25 µg/dose

*Calcitriol was given as 3x0.5 µg/dose by the mother

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

for height, weight, and head circumference were -1.83, -1.02, and 1.64, respectively. Caput quadratum and enlargement of the wrists were distinct. He had two central incisors. Blood calcium, phosphorus, and alkaline phosphatase levels were 5.9 mg/dL (1.4 mmol/L), 3.5 mg/dL (1.15 mmol/L), and 987 IU/L, respectively. Urine calcium/creatinine ratio was 0.006 [N:<0.4 (22)] in spot sampling. Serum levels of PTH (182.8 pg/mL, N:12-72) and 25(OH)D3 levels (125 µg/L) were high and $1,25(OH)_2D_3$ level (8.5 pg/mL, N:15-90 pg/mL) was low (Table 1). No abnormalities of acid-base metabolism and renal dysfunction were detected. Radiological findings included cupping and fraying of the metaphyseal regions of the radius and ulna. Renal ultrasonography revealed nephrocalcinosis of grade 1 (Figure 1). Clinical and laboratory findings suggested a diagnosis of VDDR-1A. Calcium carbonate (elementary calcium 75 mEq/kg/d) and calcitriol (1 µg/d) treatments were started to which the patient responded well by increasing his serum Ca level to the normal range. Laboratory findings and treatment of the patient in the follow-up are summarized in Table 1. Bone lesions were almost healed at the second month of the treatment (Figure 2).

A clinical diagnosis of VDDR-1A was made and a genetic analysis was performed to confirm the diagnosis. Sequence analysis of all coding regions and exon-intron boundaries were done by in-house designed primers using Sanger sequencing technique performed on ABI Prism 3130 GeneticAnalyser (Applied Biosystems, Inc., Foster City, CA, ABD) capillary electrophoresis system with standard protocols by using Big Dye Terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, ABD) and a novel mutation NM_000785.3 (*CYP27B1*): c.403C>T (p.Q135*) was described (Figure 3).

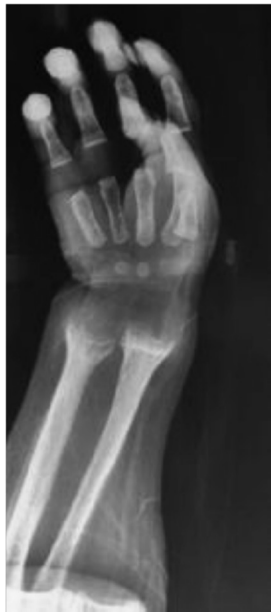

Figure 1. Radiological findings before treatment: cupping and fraying of the metaphyseal regions of ulna and radius (parents consented to the publication of these photos)

Mutation taster predicts this variant as a disease-causing mutation. This variant was screened in 200 healthy people and no mutation was detected in any. Other members of the patient's family (mother, father, and elderly sister) were heterozygous carriers for this novel mutation.

Discussion

VDDR-1A is an autosomal recessive disorder due to an inactivating mutation in the *CYP27B1* gene on chromosome 12q14 (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21). The *CYP27B1* gene encodes 25(OH) D-1-α-hydroxylase which catalyzes the hormonally regulated, rate limiting step

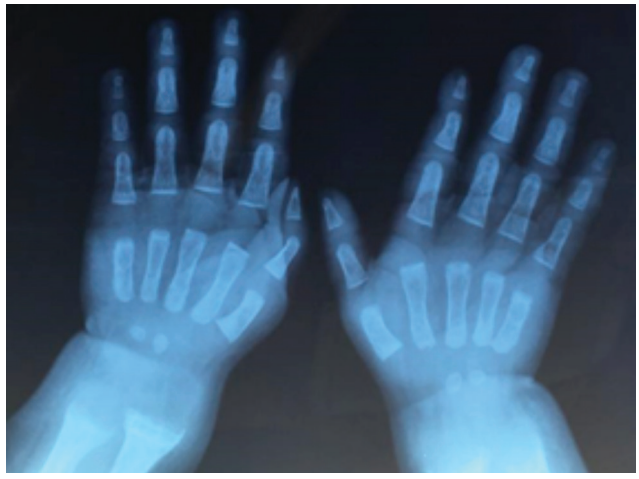

Figure 2. Radiological findings at the second month of the treatment: bone lesions were almost healed (parents consented to the publication of these photos)

Figure 3. Novel mutation p.Q135* (c.403 C>T) in the *CYP27B1* gene

Table 2. Clinical, laboratory, and genetic findings of our patient and of previously reported patients with CYP27B1 mutations in Turkish population

Mutation	Age	Clinical features	Ca mg/dL	P mg/dL	ALP IU/L	25(OH)D μg/L	1,25(OH)$_2$D$_3$ pg/mL	PTH pg/mL	Reference
Compound heterozygous c.1166G>A	13 months	NA	6.2 (↓)	3.7 (↓)	1042 (↑)	103 (↑)	55*	171	8
Homozygous 1319-1325dup CCCACCC	11.5 years	NA	7.6 (↓)	5.5	1730 (↑)	304 (↑)	44	NA	8
Homozygous 1319-1325dup CCCACCC	4 months	Hypocalcemic Seizure	6 (↓)	3.3(↓)	443 (↑)	32	UD	191.8 (↑)	19
Homozygous 1319-1325dup CCCACCC	4 month	Hypocalcemic Seizure	6 (↓)	5.4	1320 (↑)	51.3 (↑)	NA	102.6 (↑)	19
Homozygous 1319-1325dup CCCACCC	19 month	Failure to walk	9.1	1.7 (↓)	5254 (↑)	41.7	UD	516.7 (↑)	19
Homozygous 1319-1325dup CCCACCC	18 months	Failure to walk	9.1	1.61 (↓)	4441 (↑)	56	UD	340 (↑)	19
Compound heterozygous c.1079C>A	14 months	Bowed legs	7.5 (↓)	2.39 (↓)	5546 (↑)	108.2 (↑)	14 (↓)	376 (↑)	19
Homozygous 195+2T>G	18 months	Bowed legs Severe bone deformities Short stature	6.8 (↓)	3.5	496 (↑)	85.3 (↑)	14.2 (↓)	268.8 (↑)	19
Homozygous 195+2T>G	18 months	Bowed legs Severe bone deformities Short stature	6.1(↓)	3.5	2889 (↑)	142.2 (↑)	30*	176.6 (↑)	19
Homozygous 195+2T>G	12 months	Failure to thrive Inability to walk	8.9	1.8 (↓)	2190 (↑)	44	4.5 (↓)	938 (↑)	20
Homozygous 195+2T>G	26 months	Failure to thrive Inability to walk	7.1 (↓)	2.7	1850 (↑)	35	<2.1 (↓)	466 (↑)	20
Homozygous 195+2T>G	21 months	Failure to thrive Fractures	8.6	2.5	1825 (↑)	238 (↑)	14 (↓)	728 (↑)	20
Homozygous c.1022-1037del16	16 months	Inability to walk	8.5	3.4	1802 (↑)	40.44	3.2 (↓)	703.8 (↑)	20
Homozygous c.1022-1037del16	17 months	Failure to thrive Inability to walk	8.9	1.94(↓)	1523 (↑)	189 (↑)	9.1 (↓)	560 (↑)	20
Homozygous 1215+2T>A	21 months	Inability to walk	6.5 (↓)	2.9	1622 (↑)	125 (↑)	25	319	20
Homozygous 1215+2T>A	13 months	Failure to thrive Fractures Blue sclera	4.2 (↓)	3.5	684 (↑)	40	NA	284	20
Homozygous c.934_935delAC	13 months	Hypocalcemic seizure	6.5 (↓)	3.9	1100 (↑)	54	13 (↓)	555 (↑)	20
Homozygous c.403C>T	12 months	Hypocalcemic seizure	5.9 (↓)	3.5	987 (↑)	125 (↑)	8.5 (↓)	182 (↑)	**

Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, 25(OH)D: 25-hydroxyvitamin D, 1,25(OH)$_2$D$_3$: 1,25-dihydroxy vitamin D, PTH: parathyroid hormone, UD: undetectable

*Under calcitriol treatment

**Our patient

in the bioactivation of vitamin D (1,2,3,4). Due to blockage of this enzyme activity, normal or elevated 25(OH)D3 level, despite low or low-normal serum $1,25(OH)_2D_3$, is prominent in VDDR-1A which mimics clinically and radiologically vitamin D deficiency (1,2,3,5). There are no studies on the incidence of vitamin D deficiency in Turkmenistan where our patient lives, but there are reports from countries such as Turkey where nutritional rickets is still encountered (23). Our patient did not respond to vitamin D supplementation, but he was born to consanguineous parents and had an elder brother who was reported to have died of hypocalcemia and pneumonia. These 3 points raised suspicion and the low $1,25(OH)_2D$ levels concomitant with high 25(OH)D3 levels suggested a diagnosis of VDDR-1A, which was confirmed by identification of a novel homozygous mutation of p.Q135* (c.403 C>T) in the *CYP27B1*.

So far, more than 50 mutations have been identified in patients with VDDR-1A from various ethnic groups (3,5,6,7,8, 9,10,11,12,13,14,15,16,17,18,19,20,21). The Uzbek population is a Turkic people in Central Asia. Mutations, clinical and laboratory features of the previously reported cases with mutations in the Turkish population and the findings of our patient (presented in Table 2) indicate that age at presentation and clinical/laboratory findings show variations, even in patients with the same mutation. In our patient, the main clinical sign was hypocalcemic seizures. Also, he had hypocalcemia without hypophosphatemia. We identified a novel nonsense mutation c.403C>T (p.Q135*) in exon 3. Our patient was homozygous and other family members (mother, father, sister) were heterozygous for this novel mutation. As this mutation results in a truncating protein, it probably causes severe inactivation of the enzyme. As far as we know, this is the first mutation reported in the Uzbek population.

Patients with VDDR-1A are usually treated with alfacalcidol or calcitriol. Edouard et al (24) reported short- and long-term outcomes of calcitriol treatment in their patients. Calcitriol was started at a dose of 1.0 µg/d, given in two doses of 0.5 µg. Subsequently, the calcitriol dose was modified according to the results of biochemical analyses. Normal calcium and PTH levels without hypercalciuria were tried to be achieved. The median daily calcitriol dose was reduced to 0.50 µg/d (range 0.2-1.0 µg) after 3 months, to 0.25 µg/d (range 0.1-1.0 µg) after 1 year, and to 0.25 µg/d (range 0.1-0.5 µg) after two years of the treatment. Our patient was also treated with calcium supplementation and calcitriol of 1.0 µg/d, given in two doses. At the second month of the treatment, skeletal deformities were almost healed (Figure 2) and renal ultrasound was normal without nephrocalcinosis; but at the third month of treatment, serum phosphorus level increased to the upper normal limit and hypercalciuria was detected. The calcitriol dose was decreased to 0.75 µg/d given in three doses. At the ninth month of the follow-up, as calcitriol treatment was used in a dose higher than that prescribed (3x0.5 µg/dose instead of 3x0.25 µg/dose), serum PTH level decreased below normal ranges and urinary analysis revealed hypercalciuria. Over the next 13 months, the calcitriol dose was decreased to 0.5 µg/d, while the calcium supplementation was withdrawn at the third month of this tapering process. At the third year of follow-up, our patient was receiving 0.25 µg/d of calcitriol. His growth was normal, normocalcemia without hyperphosphatemia or nephrocalcinosis had been achieved (Table 1).

Although a rare disorder, VDDR-1A must be considered even in countries where vitamin D deficiency is still common. Genetic analyses are beneficial for early diagnosis of probable familial cases. The novel mutation NM_000785.3 c.403C>T (p.Q135*) causes a truncating protein probably associated with severe inactivity. We believe that this patient is the first case with this mutation reported in the Uzbek population.

Ethics

Informed Consent: It was taken.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu, Design: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu, Data Collection or Processing: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu, Analysis or Interpretation: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu, Literature Search: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu, Writing: Bahar Özcabı, Feride Tahmiscioğlu Bucak, Sevinç Jaferova, Çiğdem Oruç, Amra Adrovic, Serdar Ceylaner, Oya Ercan, Olcay Evliyaoğlu,

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Brief Report

A Critical Appraisal of Growth Hormone Therapy in Growth Hormone Deficiency and Turner Syndrome Patients in Turkey

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ABSTRACT

Early detection of abnormal growth, identification of the underlying cause, and appropriate treatment of the medical condition is an important issue for children with short stature. Growth hormone (GH) therapy is widely used in GH-deficient children and also in non-GH-deficient short stature cases who have findings conforming to certain indications. Efficacy of GH therapy has been shown in a multitude of short- and long-term studies. Age at onset of GH therapy is the most important factor for a successful treatment outcome. Optimal dosing is also essential. The aim of this review was to focus on challenges in the early diagnosis and appropriate management of short stature due to GH deficiency (GHD) and Turner syndrome. These are the most frequent two indications for GH therapy in Turkey approved by the Ministry of Health for coverage by the national insurance system.

Keywords: Growth hormone, growth hormone deficiency, Turner syndrome

Conflict of interest: None declared
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Introduction

Today, Turkey's population exceeds 78 million with children and adolescents constituting nearly one third of the total population (1). Being the best indicator of general health and well-being, appropriate monitoring of growth status and thus early identification of abnormal growth seems fundamental to health care in such a youthful population given the likelihood of management of underlying medical conditions, optimizing attainment of good health and normal adult height (2).

Accurate assessment and monitoring of growth in children on the basis of length or height according to age, weight, body mass index (BMI), and height velocity (HV) with respect to reference

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populations is of critical importance for early identification and proper evaluation of remediable conditions associated with a reduced growth rate and/or short stature. Early initiation of treatment in children with these conditions would possibly enable them to achieve their potential to reach an adult height within the normal population range (2,3).

The further below -2.0 standard deviations [SD] (2.5 percentile) an individual's growth falls, the more likely it is that there is an underlying pathological condition associated with short stature. The possibility to reach the genetically determined height potential also becomes more limited in such individuals (2,4).

According to data from the Turkish Demographic and Health Survey, an improvement was noted in the nutritional status of children over the years with a dramatic decrease in the frequency of undernutrition- and malnutrition-related growth failure (5). Additionally, the recent report of the Turkey Childhood Obesity Surveillance Initiative in 2013 stated that the frequency of children (aged 7-8 years) with a height standard deviation score (SDS) below -3 SD is 0.1% and that of those with a height below -2 SD is 2.3% (6). Nonetheless, despite this decline in the frequency of short children over the years, there are still several underlying causes of short stature other than those related to nutrition that need to be evaluated by pediatric endocrinologists.

Pituitary-derived human GH was first used as replacement therapy in a child with hypopituitarism (7). In subsequent years, with the development of the recombinant human GH (rhGH) and its introduction to clinical use, there has been a marked increase in the scope of GH treatment (8). Indications for GH therapy extended from replacement therapy in GH deficiency (GHD) to many diseases in which short stature is not secondary to GHD (9). Among several indications of GH treatment (10,11), GHD and Turner syndrome (TS) are the two most frequent conditions approved for GH treatment by the Turkish Ministry of Health. In this context, it must also be noted that under the Turkish national health system, all individuals are covered for their health expenditures, including GH treatment.

In Turkey, there are 73 pediatric endocrinology centers with a total number of 200 endocrinologists (according to 2015 data) (12). The Turkish Pediatric Endocrinology and Diabetes Society (TPEDS) is an active society that holds regular annual scientific meetings and that also organizes periodic educational conferences on growth disorders among other endocrine issues. TPEDS also has many publications including consensus reports and expert reports relating to the diagnosis and treatment of GH deficiency (13,14,15,16,17,18,19,20). Although there are many studies on growth disorders, early diagnosis of the underlying cause of short stature and especially the diagnosis of conditions that may benefit from GH treatment is still a challenge. The aim of this review is to focus on the early diagnosis and appropriate management of GHD and TS patients, with emphasis on optimum GH treatment, compliance to therapy, and transition to adult healthcare.

Growth Hormone Therapy in Children with Growth Hormone Deficiency

The diagnosis of GHD is usually based on the combination of auxological findings and poor growth velocity, confirmed by a low insulin-like growth factor-1 (IGF-1) concentration and the results of GH provocative testing using arginine, clonidine, glucagon, insulin, or L-dopa, with a peak GH cut-off set at 10 µg/L in at least two tests (21). In recent reports, it has been recommended that a cut-off peak level of 7 µg/L would be appropriate using the recent monoclonal GH antibodies (22).

Age at initiation of GH treatment has been shown to negatively correlate to response to therapy emphasizing the need of early diagnosis and treatment (23). In a multicenter study using the Turkish data in the KIGS database, 1008 cases with GHD were evaluated demographically and by treatment results of the first year. It was concluded that the diagnosis of GHD was late in these cases. Mean age (minimum-maximum) at onset of therapy was 11.3 (5.4-15.1) years in idiopathic GHD cases. Mean height SDS was -3.1 (-5.2 to -1.9) in these patients (24). Mean GH dose was 28 (21-34) µg/kg/d. The response expressed as delta height SDS was 0.6/1 year and 1.1/3 years of therapy which was within expected limits but at the lower end (25). To standardize the diagnosis and treatment of GHD in Turkey, TPEDS is working on new consensus guidelines for diagnostic procedures and treatment of Turkish children with GHD (26,27).

Several prevailing guidelines for the diagnosis and treatment of GHD in children have been published (23,28,29,30,31,32). However, differences in diagnostic procedures and treatment strategies among countries and even among centers in the same country continue to exist (33,34). The European Society of Pediatric Endocrinology (ESPE) recommends 25-35 µg/kg/d as starting doses of rhGH in GHD, while the dose recommended by the American Society of Pediatric Endocrinology is 43 µg/kg/d (32). In a recent study on current practice in diagnosis and treatment of GHD in childhood in Turkey, the most frequently used starting dose of rhGH was reported to be 25-30 µg/kg/d in prepubertal children and 30-35 µg/kg/d in pubertal children, consistent with ESPE recommendations (35).

In this survey, rhGH dose adjustment was primarily based on growth velocity as recommended by consensus publications with monitoring response and change in HV every 3-6 months. Cessation of rhGH therapy was primarily done according to HV and bone age advancement (35). There are different practices in European countries as well despite the published guidelines. In an audit from European

countries, the range of starting doses of GH in GHD patients was broad (11-50 µg/kg/d) and approximately 60% of the units from EU cenetrs preferred the dose of 30 µg/kg/d (36). Recent multicenter Italian study reported that median dose of GH was 33 µg/kg/d in GHD patients (37).

Growth Hormone Therapy in Children with Turner Syndrome

TS, the most common sex chromosome abnormality in females with an estimated prevalence of 1/2500 occurs as a result of partial or complete absence of one X chromosome, leading to a combination of characteristic phenotypic features including short stature (3).

The average adult height deficit in untreated women with TS is 20-21 cm compared to normal adults, with an average height of 147 cm (38). The therapeutical GH doses exceeding the physiological dose have been shown to improve growth velocity (39). However, the effect of GH therapy on the final height is substantially variable depending on several clinical and genetic factors such as polymorphisms related with GH receptor and/or *IGFBP3* gene, young age, and bone age delay at the start of GH treatment. GH dose, duration of GH treatment, maternal X chromosome origin, target height, and good first-year height response to GH treatment, use of oxandralone and weekly number of injections have also been shown to affect treatment results (40,41,42). Since the growth response is known to decrease over the years of GH treatment, not only the good first-year response to GH therapy but also maintenance of the good response has been considered necessary to be able to achieve final height. Hence, increments in GH dose in patients at risk of poor GH response have been considered to be effective in terms of cost and safety via generating better short- and long-term growth response (40,43).

Prompt initiation of GH treatment has been recommended in TS patients as soon as growth failure is demonstrated, even in infancy. Initiation of therapy at a young age, optimally while the child is still within normal length/height values for age, has been reported to improve the outcome, to decrease overall cost, and also to yield higher psychosocial benefits such as being closer to peers in height throughout life. With early treatment, puberty can also be more likely to be initiated at a normal age and GH therapy is likely to be terminated earlier (41).

Hence, early initiation of GH treatment (at a dose of 50 µg/kg/d) and induction of puberty at a normal physiological age were emphasized as important to achieve a taller adult stature (3). Younger age and tall height at GH therapy onset, tall parental heights, better first-year responsiveness to GH, long duration of therapy, and a high GH dose were reported amongst the factors predictive of taller adult height (44).

Data from a past study in Turkish TS patients revealed final height of non-GH treated TS cases to be 141.6 cm, which was 18.4 cm lower than average final height of women without TS (38).

A national survey with a multicenter design conducted in 2003 revealed that only 32% out of 367 TS patients received GH therapy. Of these, regular GH therapy was given to 72%. Advanced chronological age and/or bone age (35%) and lack of insurance benefits (61%) were the two most important factors in not initiating GH therapy (20).

Evaluation of the data of 70 TS patients registered from 11 centers in Turkey in the KIGS database who received GH in a dose of 33 (0.23/46) µg/kg/d subcutaneously, 6-7 times per week, with onset of therapy at age 12.5 (7.1/15.6) years revealed a non-significant increase in HV [6.3 (4.3/8.5) cm/year in the first year and 5.9 (3.6/8.7) cm/year in the second year]. Height SDS improved to -3.4 (-4.6/-2.2) in the first year and to -2.7 (-4.2/-1.6) in the 2nd year of therapy in the longitudinally followed TS patients (45). Thus, in Turkish children, there was a delay in both age at diagnosis of TS and age of onset of GH therapy as compared to other reports. The dose of GH used and the response were also lower (44). In an Italian multicenter study, median GH dose used for TS was 43 µg/kg/d (37). In another recent multicenter study, age at onset of GH therapy was 9.4±2.6 years, the dose of GH used was 50 µg/kg/d, and delta height SDS over 1 year was 0.4 SD (46). In a very recent study from Turkey, evaluation of 842 patients with TS from 35 centers revealed that mean age at diagnosis of TS was 10.5±4.8 years with initiation of rhGH therapy shortly after diagnosis, at 10.7±3.5 years (47). The number of GH treated patients was 615 in this cohort. The age at initiation of GH therapy in girls with TS improved in Turkey by years. We think that it is related to increased number of pediatric endocrinologist in Turkey and more frequent referral of patients with short stature to pediatric endocrinologists. The other important factor is continuous post-graduate education to pediatricians, family doctors, and pediatric endocrinologists. Last but not the least, almost all children under 18 years have insurance.

Evaluation of GH treatment response in TS patients either according to prediction models or HV target charts (48,49) will enable the clinician to evaluate the confounding factors and use optimum dose adjustment and also to be able to provide realistic information to the child and the parents.

Adherence to Growth Hormone Therapy

Long-term GH replacement therapy in GHD, starting at the time of diagnosis, typically from childhood throughout adolescence and into adulthood is recommended by the GH Research Society and the European Society of Pediatric Endocrinology (23,50). Accordingly, as in non–life-threatening chronic conditions, problems with adherence to GH therapy may be exacerbated due to long treatment duration along with factors specific to GH therapy such as the need for subcutaneous injections on a daily basis, inadequate training in device technique, as well as lack of immediate therapeutic benefits (50,51,52,53).

Data suggest that poor adherence is frequent among patients receiving GH therapy, despite the fact that continuous, long-term adherence is essential to achieve optimal therapeutic results with GH (54,55). Given the association of injection frequency with growth response, lower adherence to GH therapy has been associated with significantly reduced height velocities (53,55).

Data of 217 GH-naïve patients from 6 pediatric endocrinology clinics in Turkey revealed a decrement in adherence to GH therapy during a 12-month period (Figure 1). Excellent adherence ratio in the first 3 months was 88%, however it was 78% at the end of the first year. The poor adherence ratio was increased 3% to 7.5% in one-year period of treatment. In this study, patients with excellent and good adherence had better response to GH therapy. Also, growth velocity SDS was shown to correlate negatively with number of missed injections and positively with delta IGF-1 levels (51).

Hence, in patients who do not adhere to the prescribed GH therapy, there is a risk that they will not achieve the physical and psychological benefits of treatment and it is therefore important to consider non-adherence to contribute to variability in response to GH therapy and to be a possible cause in all cases of treatment failure.

In conclusion, we believe it is fair to state that the continuous education programs for medical doctors and other health workers conducted by the TPEDS, in addition to the several initiative efforts of our pediatric endocrinologist colleagues in Turkey towards improving the state regulations, have contributed much to the improvement and expansion of GH treatment in GHD and TS in Turkish children. However, we are still faced with challenges and barriers against early diagnosis and optimal dosing in Turkey. Continuous growth monitoring and correct evaluation of short stature or poor growth and referral to secondary or tertiary centers is mandatory for early diagnosis of the underlying etiology of short stature. Once indication of GH therapy is made, correct dosing and correct interpretation of growth velocity is important for optimum outcome.

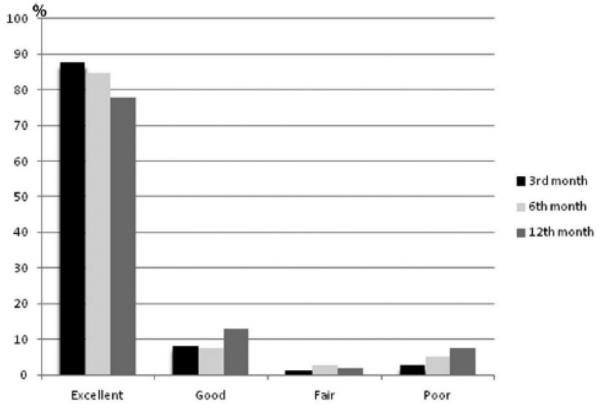

Figure 1. Percentage of adherence and decrement in adherence in the first year of growth hormone therapy (51)
Adherence segments based on percentage of doses omitted at each evaluation period: Excellent: 0%, good: 5%, fair: 5 to 10%, and poor: >10%
Adapted from Aydın BK, Aycan Z, Sıklar Z, Berberoğlu M, Ocal G, Cetinkaya S, Baş VN, Kendirci HN, Cetinkaya E, Darcan S, Gökşen D, Evliyaoğlu O, Sükür M, Baş F, Darendeliler F. Adherence to growth hormone therapy: results of a multicenter study. Endocr Pract 2014;20:46-51.

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Authorship Contributions

Concept: Feyza Darendeliler, Design: Feyza Darendeliler, Data Collection or Processing: Zehra Yavaş Abalı, Feyza Darendeliler, Olcay Neyzi, Analysis or Interpretation: Feyza Darendeliler, Olcay Neyzi, Literature Search: Zehra Yavaş Abalı, Feyza Darendeliler, Olcay Neyzi, Writing: Zehra Yavaş Abalı, Feyza Darendeliler, Olcay Neyzi.

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Wolcott-Rallison Syndrome with Novel EIF2AK3 Gene Mutation

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Dear Editor,

Wolcott-Rallison syndrome (WRS; Online Mendelian Inheritance in Man 226980) is an autosomal recessively inherited disorder characterized by neonatal insulin-dependent diabetes mellitus, skeletal dysplasia (epiphyseal dysplasia), acute hepatic and/or renal dysfunction, exocrine pancreatic insufficiency, neutropenia, developmental delay, and growth retardation (1,2). This syndrome is caused by mutations in the gene encoding eukaryotic translation initiation factor 2a kinase 3 (*EIF2AK3*), and to date, more than 60 cases have been reported (2,3).

A female Kurdish infant at 4 months of age had been diagnosed to have neonatal diabetes when admitted with an episode of diabetic ketoacidosis. Her parents were first-degree cousins. At diagnosis, laboratory findings (reference ranges) were as follows: glucose 492 mg/dL (70-105), C-peptide 0.001 ng/mL (0.9-4.3), insulin 0.2 µIU/mL (1.9-23), and HbA1c 15.2% (4.8-6.0). Her liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT)], thyroid stimulating hormone, thyroxine, blood urea nitrogen, and creatinine levels, and neutrophil count were in normal ranges. Type 1 diabetes-associated autoantibodies (islet cell antibody and glutamic acid decarboxylase antibody) were negative.

At the age of eight months, the patient was admitted because of acute hepatic failure (on treatment with a regimen of insulin detemir and insulin lispro injected three times a day). ALT and AST levels (822 U/L and 1559 U/L, respectively) were elevated with no hepatomegaly. Viral hepatitis markers were negative. Additionally, she had neutropenia. During follow-up, with supportive treatment, liver enzymes and absolute neutrophil count returned to normal. No clinical or biochemical evidence of exocrine pancreas insufficiency was observed.

At a routine visit at the age of 3 years and 5 months, her height was 90.9 cm (-1.53 standard deviation score) and weight was 11.3 kg (-2.45 standard deviation score). Her HbA1c was 8.69% while taking insulin detemir once a day and insulin lispro three times a day, with a total daily dose of insulin of 1.2 UI/kg. An X-ray survey showed osteopenia, generalized (proximal tibia, distal femur, and proximal phalanges) epiphyseal dysplasia, and tubulation deformities in the carpal bones and phalanges, but there were no abnormal findings in vertebral and pelvic bones (Figure 1).

A clinical diagnosis of WRS was corrected by the identification of a novel homozygous nonsense mutation (p.Q333) in exon 5 of the *EIF2AK3* gene. [University of Exeter Medical School (United Kingdom) with funding from the Wellcome Trust to Professors Andrew Hattersley and Sian Ellard].

We name all our *EIF2AK3* mutations according to the sequence reference AF110146.1. Patient's parents were heterozygous for this mutation (Figure 2).

Hepatic dysfunction is a typical feature of this syndrome presenting with hepatomegaly, elevated hepatic enzymes, and recurrent acute liver failure (4). Our patient had one temporary acute hepatic attack, and it has not recurred. Although developmental delay has been reported in this syndrome, our patient's development was normal.

The skeletal abnormalities of WRS are stated as progressive osteoporosis, osteopenia, and epiphyseal dysplasia (5). In our patient, osteopenia and generalized epiphyseal dysplasia were present.

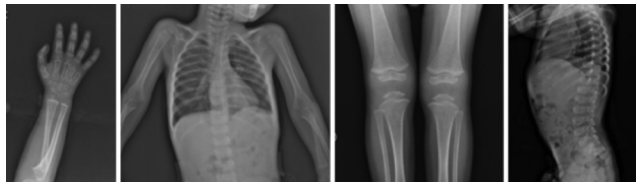

Figure 1. X-ray images show a generalized osteopenia, with epiphyseal dysplasia in the proximal tibia, distal femur, and proximal phalanges, additionally, tubulation deformities in the carpal bones and phalanges

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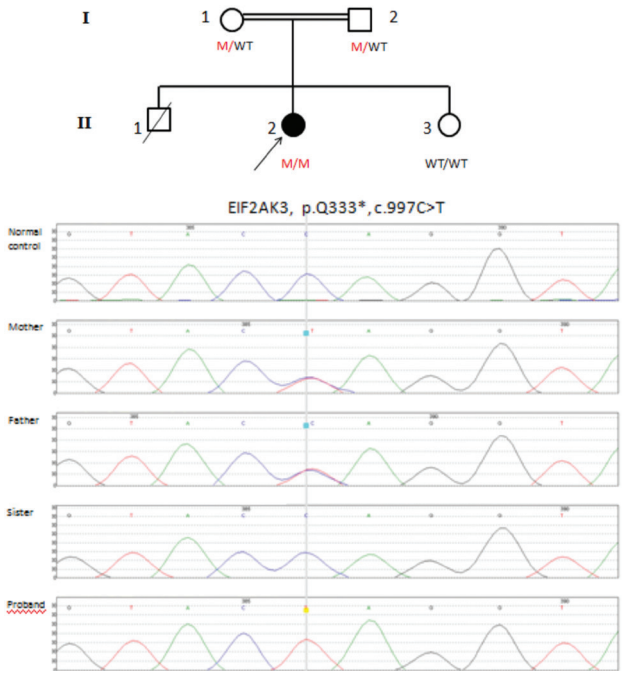

Figure 2. Electropherogram images for the mutation in the *EIF2AK3* gene and pedigree of the family members

In summary, the diagnosis of WRS should be considered in a neonatal or early infantile case of insulin-dependent diabetes mellitus with any of the accompanying features such as skeletal dysplasia, acute hepatic and/or renal failure, and neutropenia.

Keywords: Wolcott-Rallison syndrome, neonatal diabetes, epiphyseal dysplasia, EIF2AK3

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