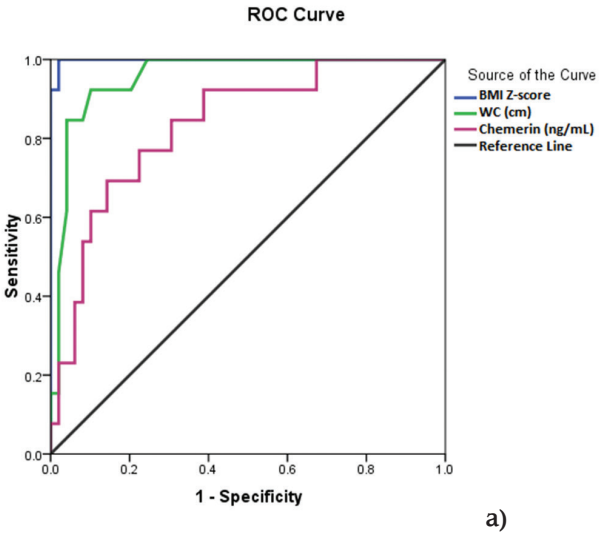
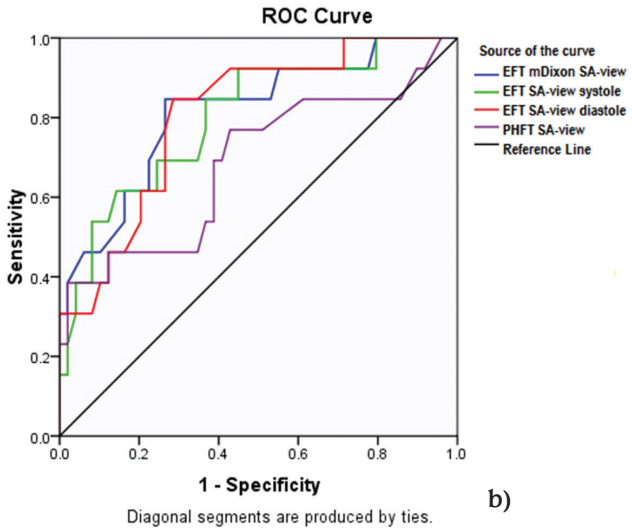
JCRPE

Journal of Clinical Research in Pediatric Endocrinology

December 2021 | volume 13 | issue 4 | www.jcrpe.org | ISSN: 1308-5727

E-ISSN: 1308-5735





a) Receiver operating characteristic (ROC) curve for prediction of metabolic syndrome from BMI Z-score, WC and serum chemerin in girls with Turner syndrome.

b) ROC curve for prediction of metabolic syndrome from epicardial fat thickness sequences and perihepatic fat thickness in girls with Turner syndrome

Epicardial and Perihepatic Fat as Cardiometabolic Risk Predictors in Girls with Turner Syndrome: A Cardiac Magnetic Resonance Study

Salem NA et al.

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Official Journal of
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Printing at:
Türkocağı Cad No:3/A Türkiye Gazeticiler Cemiyeti Altı
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Phone: 0212 527 61 81 (pbx)
Certificate Number: 48150
Date of printing: December 2021
ISSN: 1308-5727
E-ISSN: 1308-5735

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REVIEW

DOI: 10.4274/jcrpe.galenos.2021.2021.0060

J Clin Res Pediatr Endocrinol 2021;13(4):370-374

Care and Support of Children with Type 1 Diabetes at School: The Turkish Experience

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Abstract

Diabetes care at school has recently appeared on the agenda of international diabetes organizations, the basic principles of which have been newly determined. The aim of this review was to summarize the activities and output of the Diabetes at School Program - a program that has been delivered in Turkey for the last 10 years - and to focus on different aspects of Diabetes Care at School through a national model. Recently, a detailed set of national regulations, including the basic principles proposed by the International Society for Pediatric and Adolescent Diabetes and the experience in Turkey, was prepared and has come into force. The future agenda includes giving priority to socio-economically disadvantaged regions, provision of an Individual Treatment Plan at School for each child with diabetes and ensuring that each school has an action plan for the care of children with diabetes. We believe that if all countries have programs and structured national regulations similar to the Diabetes at School Program, this will enable significant progress in the level of care delivered to children with diabetes.

Keywords: Type 1 diabetes, children, school, program

Introduction

Once a child of school-age is diagnosed with type 1 diabetes (T1D), this becomes a problem and a concern not only for the child, but, as might be expected, also for the whole family. The first problem encountered by parents and caregivers is the difficulties associated with organizing their child/children's school life. Today, in almost all countries, there is a significant gap between the arrangements needed to maintain the routines of diabetes care such as insulin therapy, nutrition and carbohydrate counting, management of emergencies including hypoglycemia, correction boluses to keep glucose in the target range, and so on and the capabilities and facilities available at school. This challenge has persisted since 2009 when Lange et al (1) described this gap as "disturbing facts" in their article (2).

In countries like Turkey, this gap has long been one of the most important problems in diabetes care, and families, especially mothers, bear the burden of diabetes care at school (3). However, in addition to achieving the goal of having a 'Time in Range' proportion of 70% and above, a goal which has become important in recent years, it is also a priority to organize a plan that integrates diabetes care into school in such a way as to increase the school performance, including mathematics test scores, of children with T1D (4,5).

In recent years the International Society for Children and Adolescent Diabetes (ISPAD) and the International Diabetes Federation (IDF) have focused on diabetes care at school. The consensus document published by ISPAD in 2018 has been an important step in this regard (6,7). Based on this

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Conflict of interest: None declared
Received: 03.03.2021
Accepted: 19.05.2021

document, recommendations of other diabetes-related organizations and expert opinions, the principles of diabetes care at school are summarized in Table 1 (2,8,9). This article aims to share the evolution of the "Diabetes at School Program" that has been operational in Turkey for 10 years and to emphasize and develop the latest recommendations on diabetes care at school.

The Diabetes at School Program: History and Main Activities

In Turkey there are approximately 20,000 children under the age of 18 living with T1D. It has been estimated that at least 15,000 of them are of school age (10). As in many countries, it is known that children with diabetes face significant issues at school and that the knowledge of teachers and school staff about T1D and daily treatment routines is insufficient (11,12). Moreover, poor knowledge of T1D and delays in diagnosis due to other reasons are still important problems, both in school and out. Consequently, the frequency of diabetic ketoacidosis at the time of diagnosis is around 50%, and this rate can reach as high as 80% in the eastern region of Turkey (13,14). To address all these problems, the School Diabetes Program was initiated in 2010 as a part of the national diabetes program (3,15). The program was developed as a joint protocol initiated by the Turkish Society of Pediatric Endocrinology and Diabetes in co-operation with the Ministry of Education and Ministry of Health. Since 2010, a training meeting has been organized in schools nationwide every World Diabetes Day. These educational meetings are attended by teachers, students and, in some schools, parents. A presentation and two films explaining diabetes and childhood obesity with a total running time of 24 minutes are downloaded from the website and watched by the participants. A video to raise public awareness was also prepared and broadcast on popular TV channels over a period of three months. The video shows the symptoms of diabetes in children, how a delay in diagnosis of diabetes may cause a severe clinical picture, that children with diabetes can lead a normal life like their peers and participate in all activities, and the importance of a support from teachers and other members of society (16).

Table 1. The principles of diabetes care at school based on ISPAD recommendations (6)

Proper diabetes management at school is a prerequisite for successful school performance and avoiding medical complications.
It is of importance to provide blood glucose levels within the target range during school hours, and daily blood glucose targets should not vary with a change in environment.
It should be ensured that children with T1D participate in school life on an equal basis with their peers.
Families cannot be expected to remain in school all day long to monitor blood glucose and administer insulin injections. The requirements for glucose monitoring and insulin administration should be met by someone other than a family member.
Immediate treatment of hypoglycemia should be provided.
Healthy eating options should be provided in the schools.
The pediatric endocrinology and diabetes centers should cooperate with the family to prepare an "Individual Treatment Plan for School" document for each child with T1D and this document should be delivered to the school administration with an official letter. This document should be updated annually.
All the school staff, including teachers, administrators, and school nurses should be trained to support the implementation of daily diabetes care routines.
There should be legislation for diabetes care at school, detailing the principles, responsibilities, and duties at a national level.
In order to provide the transition of a child with T1D to a self-management process in diabetes care, there should be a supportive plan appropriate for her development, and support should be provided in the school environment.
There should be an action plan in the school for every child with T1D and the school administration should designate the school nurse, if any, or another school staff member as being responsible for coordination.
The value of close cooperation between the child and stakeholders such as the school nurse, teachers, school administration, family, and the diabetes center where the child is monitored should always be kept in mind.

ISPAD: International Society for Children and Adolescent Diabetes, T1D: type 1 diabetes

The program includes training for teachers and recommendations on how to make schools friendlier places for children with diabetes, as well as guidelines for preventing obesity and diabetes. The program's goals are: Raising awareness of T1D through schools; early diagnosis; decreasing the frequency of diabetic ketoacidosis; better care for school children with T1D; promotion of healthy life choices; and raising awareness of obesity. The target groups are primarily those teachers with children with T1D in their classes as well as other teachers, members of the Children Diabetes Teams and municipal authorities and school administrators. The initial activities included: prominent display of the poster "Does my child have diabetes?" in 60,000 schools nationwide; distribution of educational leaflets on childhood diabetes; and online presentations were made available for teachers. Instructions were sent out to municipalities nationwide. With this program, pediatric endocrinology clinics across the country began sending program-branded letters to the teachers of children with T1D after they were discharged from hospital. The letters have been sent to the local health care units as well as the schools since 2018. Additionally, two short films and a website were prepared for teachers (16). The films are shown in schools every 14th November (World Diabetes Day) (17).

In terms of nationwide school activities, a training platform had been created on the website to access educational material. On this platform, there are films, brochures and posters about diabetes and obesity. The film about diabetes

describes what it is like for a child living with T1D using the own words. Particular emphasis was given to difficulties experienced in school and the expectations of children with T1D. The website is www.okuldadiyabet.com and this is a free, online education platform where all the above-mentioned materials (Presentations, Films, Brochures, Posters) are available to download (16).

The program was updated in 2017 and the 'Task and Responsibility Document for Diabetes Care at School' came into force (18). This document covers the duties and responsibilities of families, school authorities, teachers, school nurses and pediatric endocrinology centers regarding diabetes care. With this new protocol, glucagon injection in the case of severe hypoglycemia was defined as a task for teachers, with the consent of the family. Additionally, a 'Guide for school exams for children with T1D', 'Individualized Diabetes Management Plan', and 'School Action Plan for children with T1D' were prepared and sent to schools.

In the last two years, there has been an emphasis on training for teachers and the presentations used in the training have been updated with new developments. Within the framework of teacher training, meetings between children with T1D and their families, teachers and diabetes teams have been organized in the larger cities of Turkey. These meetings have been held in Gaziantep, Adana, Samsun, Ankara, İstanbul and Eskişehir, and so far, they have been very effective.

The instructions were published in the Journal of Announcements of the Ministry of Education on October 14, 2020. Finally, 'The Diabetes at School Program 10th Year Thank You Video' was prepared and shared in schools and on social media platforms (19).

School Nursing and Diabetes Care in School

The "Diabetes at School Program for School Nurses Istanbul Meeting" was held on 14 June 2019 and the meeting was attended by approximately 250 school nurses (most of whom were from İstanbul), members of the Public Health Nursing Association School Health Nursing commission, academician nurses working in the field of public health and child health, and mothers of children with T1D. The main messages of the meeting are summarized below.

- There are about 20,000 children with T1D in Turkey; these children spend most of their time at school; they are at risk of having impaired glucose control, particularly during school hours. School nurses play an important role in providing support to children with diabetes.

- Recently, there have been significant advances in the diagnosis and treatment of T1D. In particular, the use of continuous glucose monitoring systems and subcutaneous continuous insulin infusion (pump) systems that provide remote glucose monitoring facilitate diabetes management in children and reduce the burden on children with diabetes and their families. School nurses play a key role in the use of diabetes technologies in school for children under the age of 12.

- The school nursing system, which is important for improving school health, is not sufficiently supported in Turkey.

- There is a need for official arrangements defining school nurses' employee personal rights, duties and responsibilities and their position vis-à-vis the law.

- Priority should be given to the establishment of a Turkey-specific "School Nursing System", similar to that in developed countries.

Regulations for Diabetes Care in the School Environment

In Turkey's legal system, the ministries' own practices are regulated by directives prepared in the context of national laws. The most significant achievement of the Diabetes at School Program is the publication of the official regulations entitled "care and support of children with T1D at school" in October 2020 by the Ministry of National Education. These regulations were prepared by the National Pediatric Endocrinology and Diabetes Association and the document was then finalized by the officials of the Ministry of Education and Health, finally becoming legislation of the Ministry of National Education (18).

The main features of the new regulations are shown below.

- It provided regulations regarding the care of children with diabetes in the school environment in accordance with the recommendations of international organizations (including the International Child and Adolescent Diabetes Association) for the first time in Turkey.

- It contained the most up-to-date knowledge and experience concerning T1D.

- The duties of school staff, including teachers, families and diabetes teams, were described.

- The rights of children with diabetes, and in particular, the right to be treated like their peers, were guaranteed.

- The regulations consist of 18 items in total and detailed arrangements are given in Table 2.

These regulations have been sent by the Ministry of National Education to all school administrators. Our next task will be to improve the lives of children with diabetes in all schools

Table 2. The 18 main topics of national regulations for diabetes care at school setting

The principles of diabetes care in the school setting
Preparing an "individual treatment plan at school" for every student with T1D
Preventing families from waiting unnecessarily at schools to administer insulin injections or carry out other tasks
Providing appropriate places for insulin injections
Support for insulin injection and glucagon administration
Support for the use of diabetes technologies in the school setting
Training of school staff concerning diabetes
Obtaining consent from school staff for the administration of insulin and glucagon when needed
Management of emergencies, especially hypoglycemia
Nutrition at school
Exercise, relationships with peers and participation in school life
Exams and other situations
The responsibilities and duties of parents or caregivers
The responsibilities and duties of the school administration
The responsibilities and duties of teachers
The responsibilities and duties of school nurses
The responsibilities and duties of diabetes teams
Collaboration

T1D: type 1 diabetes

in Turkey and to work with an understanding that includes teachers in teams that support diabetes management. A distance learning module (which will be available online in 2021), consisting of short videos and texts providing practical information was prepared for teachers, school nurses, school administrators, other school staff and families. It is planned that this module will be placed in the distance learning infrastructure of the Ministry of National Education and that teachers and school staff who have students with diabetes in their schools will complete the "Diabetes Care in School" course and receive a certificate.

Conclusion

Strengthening diabetes care in schools in Turkey and elsewhere, while taking into account the needs and perspective of children with diabetes (qualitative research is needed on this issue) is still a work in progress (2,6). Although there are successful programs in countries such as Canada, Australia, Sweden and Slovenia, there are only a few countries where diabetes care at school is defined as a national program (7,20,21,22,23). The most important feature of the program in Turkey is that it is a sub-program of the National Diabetes Program, co-ordinated by the National Pediatric Endocrinology and Diabetes Association, but is essentially fully owned by the Ministries of National Education and Health. In this context, community health nurses affiliated with the Ministry of Health were provided to support many schools which did not have school nurses, and regional education programs were organized for this purpose. Again, within the scope of this program a 14-minute video explaining Diabetes in Children and Diabetes Care at School continues to be shown every year on World Diabetes Day, 14th November.

Undoubtedly, despite the efforts of the last 10 years, problems continue in terms of awareness of T1D and providing the necessary support to children with diabetes during school hours. In a recent country-level study involving 55,677 teachers, school administrators and school nurses, 73% of the participants reported that they had heard about the Diabetes at School Program, 75% stated that their knowledge had increased thanks to this program, and 50% stated that their self-confidence had increased. The results of this study showed that nurses and the staff who had students with T1D in their classrooms/schools (women in particular), had higher scores (24). However, lower scores were noted in the northern and eastern regions of Turkey, where even in schools which had students with T1D, school staff did not have enough knowledge to determine carbohydrate count and bolus doses, and efforts were needed to continue a positive attitude change. Based on the results of this study and evaluations of the program, a nationwide distance education module for the Diabetes at School training has been prepared and it is planned to train all teachers, especially school staff who have students with diabetes in their schools, and to give a "participation certificate" to those who have completed this module.

Diabetes care at school should be delivered in support of families who say "I am actually his pancreas" (25), reducing their burden and maintaining the quality of uninterrupted diabetes care and should be the priority of all staff, especially pediatric diabetes teams. While the last consensus document of ISPAD on diabetes care at school is an important contribution to progress, the next step should be to recommend the "Diabetes at School Program" and the Regulations for Diabetes Care at School to all countries.

Acknowledgments

We would like to thank Alan J. Newson for English language editing of our manuscript.

Ethics

Peer-review: Internally peer-reviewed.

Authorship Contributions

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2021.0305

J Clin Res Pediatr Endocrinol 2021;13(4):375-383

Bisphenol A Exposure in Exclusively Breastfed Infants and Lactating Women: An Observational Cross-sectional Study

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What is already known on this topic?

Bisphenol A (BPA) is a known endocrine disruptor. Exposure to BPA throughout breastfeeding may impair health. There is no data concerning the exposure of Turkish exclusively breastfed infants to BPA.

What this study adds?

This is the first study to estimate exclusively breastfed infants' daily BPA exposure and BPA risk index in a Turkish population. BPA was measured in human milk samples and the median concentration of free BPA in milk was 0.63 µg/L. Exclusively breastfed infants' estimated exposure was below the temporary tolerable daily intake limit of 4 µg/kg body weight/day. There was a weak negative correlation between exclusively breastfed infants' BPA exposure and their current body weight.

Abstract

Objective: Bisphenol A (BPA) is a known endocrine disruptor and free BPA will interact with estrogen. BPA is also fat soluble and will therefore contaminate breast milk. The European Food Safety Authority has set a limit for temporary tolerable daily intake of 4 µg/kg body weight/day in breastfeeding infants. The aim of this study was to measure human milk BPA concentrations in Turkish women and thus exclusively breastfed infants' exposure to BPA.

Methods: Healthy, postnatal, exclusively breastfeeding women were recruited and breast milk samples were collected. Free BPA concentration was analyzed in the milk samples using competitive enzyme-linked immunosorbent assay. Participants' demographic characteristics and nutritional habits were investigated through face-to-face interviews using a detailed questionnaire.

Results: Eighty women participated. Median milk free BPA level was 0.63 µg/L. There was no statistically significant association between maternal body mass index, birth type, parity, infant birth week, infant birth weight, and human milk BPA concentration. Nevertheless, there was a significant association between human milk BPA level and consumption of fast-food and carbonated drinks ($p = 0.022$ and $p = 0.018$, respectively). Exclusively breastfed infants' mean BPA exposure was 0.0099 ± 0.0079 µg/kg bw/day. There was a moderate negative significant correlation between infant BPA exposure and infant current body weight ($r = 0.327$, $p = 0.003$).

Conclusion: BPA exposure in exclusively breastfed infants was within accepted limits and the current dietary exposure level of infants in this cohort was safe.

Keywords: Bisphenol A, breastfeeding, exposure, lactation, maternal exposure

Introduction

Bisphenol A (BPA) is a human-made chemical compound used in the production of polycarbonate (PC) plastics, such as food packaging materials, and epoxy resins which are used to coat the inside of food cans and water storage tanks (1). The primary route for BPA contamination of food is direct contact with packages, and BPA penetrates foods rapidly (2). Almost all dietary free BPA, which is absorbed

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Conflict of interest: None declared
Received: 14.12.2020
Accepted: 25.02.2021

through the gastrointestinal system, is metabolized in the liver, and then conjugated with glucuronide. In a healthy population, conjugated BPA (BPA-glucuronide) is mostly excreted through the urine (3,4).

However, free (unconjugated) BPA is biologically active and a known endocrine disruptor; free BPA has been detected in human samples, especially in lipid-rich biofluids such as breast milk (5,6). When evaluating exposure to endocrine disrupting chemicals, exposure to free BPA should be considered (7). Free BPA is rapidly metabolized, has a short half-life (< 6 hours), and excretion can be measured as it will normally be completely excreted within 24 hours of ingestion, although BPA can also accumulate in the human body. BPA exposure may be of particular concern for fetal and neonatal development. This is because fetal/neonatal and childhood liver has inadequate metabolic enzymic capacity to inactivate BPA via conjugation. This inability to safely metabolize ingested BPA will lead to relatively high free BPA concentrations in urine and plasma in toddlers compared to adults (8,9). Fetuses and neonates are sensitive to perturbation by hormone-like chemicals and early-life exposure to low-dose BPA can alter the epigenetic mechanism (10). These epigenetic changes may increase the risk of developing adult-onset diseases.

BPA has been measured in a range of human biological fluids, such as serum, urine, saliva (11,12), human milk, and colostrum (5). The European Food Safety Authority (EFSA) has published an expert opinion on the safety of BPA and has assigned a new threshold value of 4 µg/kg of body weight/day for temporary tolerable daily intake (t-TDI) (12).

Human milk is the optimal food for human infants and babies and contains a considerable amount of essential nutrients. Accordingly, exclusive breastfeeding is recommended by the World Health Organization up to six months and breastfeeding in conjunction with appropriate complementary foods up to two years of age or beyond (13). Human milk contains proteins, lipids, and carbohydrates. In addition, chemical contaminants such as persistent organic pollutants, polychlorinated biphenyls, polybrominated diphenyl ethers, dichlorodiphenyltrichloroethane, and dichlorodiphenyl-dichloroethylene isomers may also be present. Furthermore, human milk composition will vary depending on maternal diet, genetics, lactation stage, breastfeeding practice, maternal and infant health status, and environmental exposure (14). Diet is considered the primary route of BPA exposure and accounts for the majority of estimated DI of BPA per body weight among the general population, including infants and children (12). Exclusively breastfed infants BPA exposure can be assessed by measuring maternal human milk BPA concentration (15).

In vitro studies have suggested that postnatal BPA exposure impacted sperm production and reproductive success (16), and caused genomic damage, and significant alterations in liver enzymes and lipid profile (17). The aim of this study was to measure maternal human milk BPA levels in a cohort of Turkish women and thus, the exposure of their exclusively breastfed infants to BPA and the BPA exposure risk index. Furthermore, the association between breast milk BPA concentration and participants' baseline characteristics, such as nutritional habits, preferred cuisine and food packaging types would be investigated.

Methods

Study Setting and Participants

This study was conducted at Hacettepe University İhsan Doğramacı Child Hospital, Social Pediatrics Department in Ankara province, between August 2018-December 2018. The study protocols were approved by the Ethics Board and Commission of Hacettepe University. Exclusively breastfeeding women, aged between 19-40 years, and their healthy 1-3 month-old infants attending for routine health checks were approached to participate. All participants gave informed consent before being included in the study. Exclusion criteria were: smokers; multiple pregnancies; gestational diabetes; diabetes mellitus; other chronic disease; and those who regularly used medicines or took vitamin/mineral supplements or used alcohol. Any woman who might have had occupational exposure to BPA was also excluded.

The questionnaire was completed by face-to-face interview. The first section of the questionnaire recorded demographic and anthropometric data about the mother and her infant. Mothers' weight and height were measured, and body mass index (BMI) was calculated by dividing weight in kg by height in meters squared (m^2) (18). Infants' weight and height were measured at the hospital by trained nurses. The second section of the questionnaire investigated daily culinary behaviour, nutritional habits, and packaging for purchased food. All participants provided 5 mL mature human milk (hindmilk) samples directly from the nipple into a BPA-free, sterile tube. Samples were stored at low temperature (-80 °C) until the day of analysis.

Investigation of human milk free-BPA levels was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) (EuroProxima, Holland) according to manufacturers instructions. The detection limit of the ELISA kit was between 0.2 and 10 ng/mL. The EuroProxima BPA ELISA is validated for water and milk

samples using sample preparation procedures developed and validated in cooperation with the RIKILT (Wageningen, the Netherlands) (19).

Estimation of Infant Daily Milk Consumption

Exclusively breastfed infants BPA exposure was calculated by estimating the mean ingestion of human milk based on each infant's age in months and combining this with the concentration of BPA measured in breast milk. Precise measurement of exclusively breastfed infants' daily human milk consumption is very challenging. Therefore, a human milk intake average constant value was used. Maternal milk production of an exclusively breastfeeding woman between 1- and 6-months averages to approximately 750 to 800 mL/day (20). Thus, it was assumed that infants daily ingestion would be approximately 800 mL of human milk (21). Both the daily infant intake of BPA in breastmilk and the risk index (RI) was determined for human milk samples, as previously described (15):

$$DI\ (ng/kg\ bw/day) = \frac{Milk\ Consumption\ (mL/day) \times BPA\ concentration\ (ng/mL)}{Infant\ body\ weight\ (kg)}$$

$$RI = \frac{DI\ (ng/kg\ bw/day)}{Tolerable\ Daily\ BPA\ Intake\ (\mu g/kg\ bw/day)}$$

Statistical Analysis

Two critical assumptions were made when manipulating measurements below the detection limit. Firstly, because the number of data points below the detection limit value was less than 15% of all data, human milk BPA concentrations below the limit of detection (LOD) were assigned a value equal to the LOD divided by the two. In the case of the ELISA kit used, the LOD was 0.2 ng/mL. Samples were analyzed according to standards specified by the U.S. Environmental Protection Agency. Analytical data were very carefully reviewed to ensure quality control (22). Human milk BPA data in this cohort were normally distributed. Secondly, different countries have used different approaches for assigning values to measurements below the LOD. However, the Canadian Health Measures Survey and the Korean National Environmental Health Survey both used a value of LOD/2 for measures below the LOD. Thus, we believe adopting this approach in this study was justified (23). Statistical analysis was performed to compare distribution among groups. Data sets were assessed for normality of distribution and appropriate comparative tests were used depending on dta set distribution. The independent sample t-test was used with parametric data while the Mann-Whitney U and Kruskal-Wallis tests were used for non-parametric data. Correlation analysis was performed using the Pearson coefficient test for non-parametric data and the Spearman coefficient test for parametric data. Statistically, significance was set at $p < 0.05$. Data management and analysis were performed using Statistical Package for the Social Sciences version 23 (IBM Inc., Armonk, NY, USA).

Results

Subjects

In total 80 women who were exclusively breastfeeding their babies were recruited to the study. The baseline characteristics of the participants are presented in Table 1. The mean ± standard deviation of maternal age and BMI was 28.88 ± 5.17 years and 26.41 ± 4.28 kg/m^2, respectively. All participants reported being healthy and not using any medicines regularly. All infants were singleton, born at full-term with normal neonatal outcome, and they were healthy

Table 1. Characteristics of mothers and their infants

Features	n (%)	Human milk BPA level (µg/L)	p
Mothers		**Median (P25-P75)**	
Age (years)			
19-28	40 (50%)	0.5792 (0.3432-0.7878)	0.554*
29-40	40 (50%)	0.6457 (0.3127-0.8033)	
BMI (kg/m^2)			
18.5-24.9	35 (43.75%)	0.6464 (0.4136-0.8388)	0.521***
25.0-29.9	30 (37.5%)	0.5686 (0.2779-0.7674)	
30.0-34.9	9 (11.25%)	0.6790 (0.4524-1.0234)	
35.0-39.9	6 (7.5%)	0.4684 (0.2153-0.7204)	
Birth type			
Normal spontaneous Vaginal delivery	40 (50%)	0.6667 (0.3607-0.8180)	0.225*
Caesarean	40 (50%)	0.5711 (0.3044-0.7464)	
Parity			
0	38 (47.5%)	0.6857 (0.4891-0.8224)	0.095**
≥1	42 (52.5%)	0.5328 (0.2711-0.7181)	
Infants			
Birth week (week)			
Boy	40 (50%)	0.6518 (0.3430-0.8114)	0.513**
Girl	40 (50%)	0.5738 (0.3044-0.7559)	
Total	n = 80 (100%)		

P25: 25th percentile (P25) and P75: 75th percentile (P75).
BPA: bisphenol A, BMI: body mass index

according to parental report and had no complications during pregnancy or delivery.

Bisphenol A Exposure in Exclusively Breastfeeding Infants

The median value of free-BPA concentrations in breast milk was 0.63 ng/mL, and the mean ± SD was 0.49 ± 0.37 ng/mL. Therefore, the estimated mean exposure of exclusively breastfed infants to free-BPA was 0.0099 ± 0.0079 µg/kg bw/day, and the geometric mean was 0.0073. In this study, estimated free-BPA exposure levels were in the range of 0.0008-0.489 (Table 2). Furthermore, exclusively breastfed infants mean RI was calculated as 0.002 ± 0.0019 and the geometric mean was 0.0018 (Table 2).

There was no statistically significant correlation between human milk free-BPA concentration and infant birth week, birth weight, body weight (see Table 3). However, there was a weak negative correlation between infant BPA exposure and infant body weight ($r = -0.327$, $p = 0.003$). However, there was again no correlation between infant BPA exposure and infant birth week and infant birth weight.

The exposure risk index for these infants based on the estimated human milk-free BPA concentration was calculated (Figure 1). We identified a direct high positive correlation between exclusively breastfed infants' BPA exposure and BPA risk index ($r = 0.990$; $p < 0.05$).

Nutritional Habits and Human Milk Bisphenol A Levels

To identify sources of human milk BPA contamination, a nutritional survey of participating women was undertaken. The findings are summarized in Table 4, below. Culinary equipment such as kettles, drinking water storage bottles, and baking moulds play are used on a daily basis in most homes. Analysis did not reveal any significant association between human milk BPA level and culinary equipment. In terms of nutritional habits, the only significant association to emerge was that between breast milk BPA concentration and fast-food consumption. Within the fast-food consumption group, women who consumed fast food once a month had higher BPA levels than participants who did not consume fast food ($p = 0.02$) but the BPA concentration was also higher than in women who consumed fast food twice a month. There was no correlation between human milk BPA level and the packaging type used for drinking water or vegetable oil. However, a significant association emerged between human milk BPA level and packaged carbonated drinks ($p = 0.018$). When pairwise comparison was made participants who did not consume carbonated beverages had higher human milk BPA level than those who did consume carbonated drinks either from glass or polyethylene terephthalate (PET) bottles ($p = 0.042$ and $p = 0.020$, respectively).

Table 5 shows the distribution of parameters regarding human milk free-BPA and comparison with other studies.

Table 2. Infants BPA exposure level and risk index due to human milk BPA concentration

	Ingested human milk free-BPA level (µg/kg bw/day)					
	$\bar{x}$ (SD)	GM	S_x	Median	Min	Max
Exclusively breastfed infants BPA exposure	0.0099 (0.0079)	0.0073	0.0008	0.008	0.0008	0.489
RI	0.002 (0.0019)	0.0018	0.0002	0.002	0.0002	0.0122

$\bar{x}$: mean, SD: standard deviation, GM: geometric mean, S_x: standard error, Min: minimum, Max: maximum, RI: risk index, BPA: bisphenol A

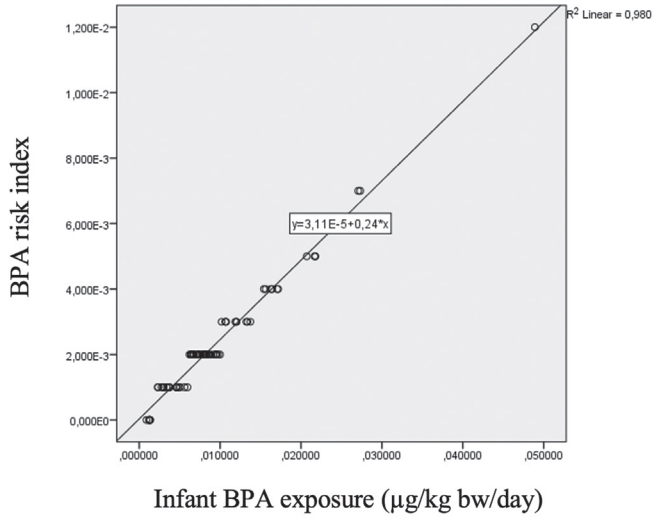

Figure 1. Association between infants BPA exposure and BPA risk index

BPA: bisphenol A

Table 3. Association between human milk BPA concentration, infant BPA exposure and birth week, birth weight, and infant current body weight

BPA	Infant birth week		Infant birth weight		Infant current body weight	
	r	p	r	p	r	p
Human milk free-BPA concentration (µg/L)	-0.010[b]	0.932[b]	0.071[a]	0.530[a]	0.000[a]	0.997[a]
Infant BPA exposure (µg/kg bw/day)	-0.024[b]	0.836[b]	0.035[b]	0.759[b]	-0.327[b]	**0.003[b*]**

[a]Pearson correlation analysis ($p < 0.05$); bSperman correlation analysis ($p < 0.05$); [b*]Sperman correlation analysis ($p < 0.01$).
BPA: bisphenol A

The median concentration value of human milk free-BPA found in this study was with that reported in studies from Japan and the USA. Concentrations reported from Spain and Canada were around a sixth of the value found in this Turkish population while the value reported from a South Korean population was much higher than that found in any other study shown here.

Table 4. Association between the human milk BPA level (µg/L) and culinary materials, nutritional habits, and packaged information

	Human milk BPA level (µg/L)		
	N	Median (P25-P75)	p
Culinary equipment			
Water heater			
Steel teapot	50	0.6482 (0.3180-0.7951)	
Steel kettle	18	0.6122 (0.2904-0.8392)	0.989
Plastic kettle	12	0.5919 (0.4385-0.7038)	
Water bottle			
Plastic	25	0.6450 (0.4136-0.7858)	
Glass	55	0.5172 (0.2766-0.8351)	0.451
Baking moulds			
Teflon	42	0.5973 (0.3359-0.7354)	
Glass (heat resistance)	14	0.5993 (0.3352-1.0234)	
Silicon	9	0.7078 (0.2554-0.8557)	0.937
Granit	15	0.6412 (0.2538-0.7858)	
Nutritional habits			
Main meal consumption in a day			
2 times a day	25	0.615 (0.29-0.79)	0.976
3 times a day	55	0.641 (0.34-0.79)	
Fast-food consumption			
Once a month	22	0.7536 (0.6035-0.9238)	
Twice a month	9	0.5152 (0.2716-0.8123)	**0.022**
Not consumed	49	0.5124 (0.2603-0.7101)	
Instant- packaged meal consumption			
No	58	0.6301 (0.2956-0.7726)	0.714
Yes	22	0.6103 (0.4255-0.8392)	
Canned food consumptions (tuna fish, soup, corn, peas)			
No	60	0.6431 (0.3374-0.8180)	0.764
Yes	20	0.5713 (0.3044-0.7512)	
Canned beverage consumptions			
No	65	0.6152 (0.3195-0.7632)	0.315
Yes	15	0.6501 (0.4544-1.1269)	
Packaged ınformation			
Drinking-water			
PET plastics	30	0.6631 (0.3950-0.7994)	
Carboy (recycling code = 7)	26	0.6518 (0.3359-0.7588)	0.678
Tap	24	0.5327 (0.2683-0.9536)	
Carbonated drinks			
PET plastics	62	0.5696 (0.2975-0.7607)	0.018
Canned	9	0.6450 (0.5541-0.8297)	
Glass	4	0.4414 (0.1509-0.6723)	
Not consumed	5	1.0818 (0.8723-1.3696)	
Vegetable oil			
PET plastics	37	0.5632 (0.3185-0.7820)	
Canned	36	0.6438 (0.3484-0.8224)	**0.788**
Glass	6	0.7409 (0.2544-0.7990)	
Not consumed¥	1	-	

¥Because the "no consumed" sample size was 1, it was not included in the statistical assessment.
PET: polyethylene terephthalate, BPA: bisphenol A

Discussion

In this study free-BPA level in human milk samples were measured in Turkish lactating women using an ELISA method. Total BPA is calculated from the sum of free BPA and BPA glucuronide (27). Moreover, the exposure of exclusively breastfed infants to BPA was estimated and the BPA exposure risk index was calculated. The implications of these findings are discussed below.

Participants were healthy mothers with 1-3 month-old, exclusively breastfed, healthy infants. Exclusively breastfed infants estimated breast milk consumption was assumed to be 750-800 mL/day (20). BPA is reported to be incorporated into human milk (28) and is subsequently naturally transferred to the infant via breastfeeding (5). Thus exposure of exclusively breastfed infants to BPA is inevitable. The adverse outcome of BPA exposure varies according to the exposure dose and term (29).

BPA is metabolized in humans through the activity of uridine diphosphate glucuronosyltransferase (UGT) enzymes, which gradually rises from the age of around 3-6 months to 10 years and then reaches normal adult levels. Infants between 1-3 months of age do not have sufficient UGT activity to metabolize BPA effectively (30). A recently developed ELISA method kit for the measurement of BPA has high sensitivity and comparatively low cost compared to other methods for measuring BPA in biological samples (28,31).

As conjugated BPA is not an active biologic form, both conjugated and free BPA concentration should be determined together (25). Studies have measured both free and total BPA levels in human milk (6,28,32). The primary BPA exposure source is dietary (12,33), but dietary habits vary considerably from society to society so countries should determine local BPA exposure risks in their own populations. The study presented here is one of the first investigations to assess the impact of BPA exposure and risk index for exclusively breastfed Turkish infants. The mean BPA exposure value in our cohort was 0.0099 µg/kg bw/day. The EFSA suggested that the limit of BPA exposure during the first three months for exclusively breastfed infants should be 0.2 µg/kg bw/day (12). Furthermore, we found a BPA exposure risk index value of 0.002 for exclusively breastfed infants based on the EFSA's BPA TDI value. The RI value is less than one so that there is no BPA exposure risk among exclusively breastfed infants (15). The correlation between human milk

Table 5. Distribution of the parameters and comparison with other studies for free BPA concentration of human milk

			Free-BPA (ng/mL)				
Country	**(n)**	**Method**	**n > LOD***	**X (S)**	**Median**	**Min-Max**	**Reference**
Turkey	80	ELISA	71 (88.75%)	0.49 (0.37)	0.63	< LOD*-1.9	This study
Japan	23	HPLC	23 (100%)	-	0.61	0.28-0.97	Sun et al (24)
USA	21	UHPLC-MS/MS	13 (62%)	3.13	0.68	< 0.22-10.8	Zimmers et al (8)
Spain	120	HPLC-MS/MS	92 (77.4%)	0.15 (4.8)	0.10	< LOD-41	Dualde et al (6)
Canada	278	GC-MS/MS	46 (16.5%)	0.11	0.10	< 0.036-2.3	Cao et al (25)
Korea	100	LC/MS/MS	100	-	6.60	0.65-29.9	Yi et al (26)

BPA: bisphenol A, LOD: Limit of detection (0.2-10 ng/mL), ELISA: enzyme-linked immunosorbent assay, HPLC: high-performance liquid chromatography, MS: mass spectrometry, Min: minimum, Max: maximum

BPA level and infant birth week, weight, and current body weight were not statistically significant (Table 3). Casas et al (34) did not find a statistically significant association between BPA exposure during pregnancy and fetal growth parameters. Intriguingly, we found a moderate negative correlation between BPA exposure in the infants studied and infant current body weight (Table 3). This suggests that an increase in BPA exposure may have a negative effect on weight gain. Normally after delivery, when lactation begins, estrogen levels start to decrease (35), but elevated levels of BPA may inhibit lactation. Kasper et al (36) demonstrated an association between maternal BPA exposure and decreased breastfeeding at one month postpartum. As presented in Figure 1, a strong positive linear relationship was found between estimated exposure of the infants to BPA and their BPA risk index. This is because the only source of BPA for exclusively breastfed infants is contamination of their mother's milk. Research has shown that the primary source of BPA exposure is diet (37,38). Thus, pregnant and lactating women should carefully consider their diet.

The effect of different types of culinary equipment, nutritional habits and food packaging was investigated in the current study. The EFSA has reported that estimated BPA exposure caused by PC plastic water kettles ranged from 2.0 to 3.2 ng/kg bw/day (12). Moreover, plastic kettle use has been identified as a critical exposure route for BPA (39). It should be noted that the highest values are observed in adults, due to their higher consumption of coffee and tea. However, in the present study there was no significant difference in BPA exposure associated with metal or PC kettles. This association may be explained by lower coffee and tea consumption among lactating women than in other adults.

Interestingly, mothers who consumed fast food once a month had significantly higher BPA breast milk concentrations than women who reported never eating fast food. Somewhat confusingly, mothers who ate fast food twice a month also had similar breastmilk BPA concentrations to those who never ate fast food but the sample sizes for the twice-a-month consumers was small. In a cross-sectional study among the U.S population, Zota et al (40) showed that fast-food consumption did not appear to be a BPA exposure source (40). In addition, the scientific opinion of the EFSA was that PC plastic food packaging did not pose a health risk to consumers of any age group, including unborn children, infants, and adolescents (12). Differences in exposure due to fast food consumption could be due not only to frequency of consumption but also preferred diet for fast food stuffs and the local regulations concerning the packaging of fast foods. For example, it has been reported that the concentration of BPA in a hamburger was 10.9 ng/g (41).

PC plastics, which contain BPA are used in reusable water carboys (42). Considerable amounts of BPA (approximately 0.15 µg/L) have been reported to leach from PC bottles within the first 24 hours of storage (43). If PC carboys are stored at or below room temperature, BPA water levels are expected to be normal. PC carboys are widely used for water storage because of the properties of PC, that is clear and rigid plastic (44). In our study, human milk BPA level did not change based on the mothers' preferred storage material for drinking water. According to the Food and Drug Administration, scientific evidence has supported the safety of BPA for the currently confirmed utilization in food containers and packaging (45).

In this cohort, women who reported carbonated drinks from glass bottles had significantly higher human milk BPA levels than either those who did not consume carbonated beverages or those who drank carbonated drinks from PET bottles ($p = 0.042$ and $p = 0.020$, respectively). This rather intriguing result may be due to the specific ingested carbonated drinks and would also be a function of consumption quantity and frequency, which was not investigated. Previous studies have reported BPA concentrations in canned carbonated drinks and plastic bottled water. The BPA concentrations in canned carbonated drinks were between 83-340 ng/L. And two canned carbonated beverages BPA concentrations was detected below the limit of quantitation (46).

Previous studies investigating human milk free-BPA levels are summarized in Table 5. The current results are consistent with previous researches (8,47) although there are discrepant results reported by other studies (6,25,26), they are consistent with some other published studies (8,24). Firstly, a possible explanation for these differences could be related to the applied analysis method in each study, as suggested by Yi et al (26). There are methodological differences between studies so that detection limits vary between studies. Studies utilizing high-performance liquid chromatography (HPLC) would have a wider detection range than those using LC-tandem mass spectrometry (LC-MS/MS) method. However, studies using HPLC with a fluorescence detector (HPLC/FLD) analysis would have a lower sensitivity than LC-MS/MS analysis. Secondly, the primary BPA exposure source is nutritional habits (12), and these will vary from country to country (33). Another possible explanation for variability in results could be that dietary habits change over time and from region to region within countries.

Study Limitations

Several limitations of this study should be noted. Firstly, human milk samples were only collected from each woman at one time point. Unfortunately, a single day sample collection is likely to introduce bias because a single sample collection may not reflect BPA exposure precisely. Possible solutions for this would be to collect two or more non-consecutive samples to estimate the normal mean human milk BPA level (48). A further solution would be to have a very large cohort. Secondly, used an ELISA method for measuring BPA concentrations in samples whereas most other studies have used chromatographic methods instead of ELISA for determining the BPA level of samples of human milk. However, ELISA can be used for screening purposes, and it is an inexpensive method (49). Furthermore, we did not consider the seasonal difference while collecting human milk samples. We collected the first sample in August and the last in November. BPA may leach into foods and beverages from packaging or storage containers, especially when heated to high temperatures as may happen in the summer months (50).

Conclusion

Exposure to BPA is a concern because of the possible health effects, and it plays a role in the pathogenesis of several endocrine disorders, including obesity, and asthma and neurobehavioural disturbances. The present study extends our knowledge of BPA exposure among breastfeeding Turkish women and their infants. The BPA exposure of exclusively breastfed infants positively correlates with human milk BPA concentrations. These results suggest that BPA exposure of exclusively breastfed Turkish infants was far below the EFSA tolerable BPA level of 4 (µg/kg bw/day) and we suggest that exclusively breastfed Turkish infants have negligible BPA exposure risk. It is thought that current regulatory restrictions on BPA use have reduced exposure levels. However, the possibility of low dose BPA exposure cannot be ruled out. Further, larger, well-designed national studies are warranted to confirm and extend these findings.

Ethics

Ethics Committee Approval: This research study was ethically approved on July 24, 2018, by the Hacettepe University Clinical Research Ethical Board with project number GO 18/715 and decision number GO 15/715-33.

Informed Consent: All procedures performed in this study involving human participants followed the institutional and national research committee's ethical standards and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: Seda Çiftçi, Sıddıka Songül Yalçın, Gülhan Samur.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2021.0169

J Clin Res Pediatr Endocrinol 2021;13(4):384-390

Vitamin D Deficiency Prevalence in Late Neonatal Hypocalcemia: A Multicenter Study

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**on behalf of Neonatal Study Group*

What is already known on this topic?

Late neonatal hypocalcemia (LNH) occurs after the first 72 hours and the most common causes include excessive phosphate intake, hypomagnesemia, hypoparathyroidism, and vitamin D deficiency.

What this study adds?

Maternal vitamin D deficiency was found to be the most common cause of LNH in our study. Due to the immaturity of regulating factors of parathyroid hormone (PTH) and calcium, serum intact PTH levels may not reach expected levels and serum phosphorus levels may remain high in vitamin D deficient neonates, posing a diagnostic dilemma by mimicking primary hypoparathyroidism.

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Conflict of interest: None declared
Received: 14.09.2020
Accepted: 15.04.2021

Abstract

Objective: Late neonatal hypocalcemia (LNH) is a common metabolic problem associated with hypoparathyroidism, high phosphate intake and vitamin D deficiency, often presenting with seizures. In this cross-sectional study, we aimed to evaluate the role of vitamin D deficiency in LNH in Turkey and to describe the characteristics of affected newborns.

Methods: Conducted with a cross-sectional design and with the participation of 61 neonatal centers from December 2015 to December 2016, the study included term neonates with LNH (n = 96) and their mothers (n = 93). Data were registered on the FAVOR Web Registry System. Serum samples of newborns and mothers were analyzed for calcium, phosphate, magnesium, albumin, alkaline phosphatase, intact parathyroid hormone (iPTH) and 25 hydroxyvitamin D [25(OH)D] levels.

Results: The median (range) onset time of hypocalcemia was 5.0 (4.0-8.0) days of age, with a male preponderance (60.4%). The median (range) serum 25(OH)D levels of the neonates and their mothers were 6.3 (4.1-9.05) and 5.2 (4.7-8.8) ng/mL, respectively. The prevalence of vitamin D deficiency (< 12 ng/mL) was high in both the neonates (86.5%) and mothers (93%). Serum 25(OH)D levels of the infants and mothers showed a strong correlation ($p < 0.001$). While the majority (93.7%) of the neonates had normal/high phosphorus levels, iPTH levels were low or inappropriately normal in 54.2% of the patients.

Conclusion: Vitamin D deficiency prevalence was found to be high in LNH. Efforts to provide vitamin D supplementation during pregnancy should be encouraged. Evaluation of vitamin D status should be included in the workup of LNH.

Keywords: Vitamin D deficiency, hypocalcemia, infant, newborn

Introduction

Neonatal hypocalcemia is defined as a total serum calcium (Ca) level of less than 7.5 mg/dL and ionized calcium (Ca^{2+}) level of less than 4.4 mg/dL in term newborns. Neonatal hypocalcemia may manifest as signs of neuromuscular hyperexcitability (irritability, jitteriness, tetany, laryngospasm, and seizures), apnea, cyanosis, feeding problems, and/or cardiac rhythm disturbances (1,2). The causes of neonatal hypocalcemia are classified by the time of onset. Early neonatal hypocalcemia occurs within the first three days after birth, with an exaggeration of normal decline in serum Ca concentration in the first two postnatal days (2). Late neonatal hypocalcemia (LNH) occurs after the third day of birth, with the main causes being a high phosphate intake, hypoparathyroidism, parathyroid hormone (PTH) resistance, hypomagnesemia, and perinatal vitamin D deficiency. Neonatal vitamin D levels depend on maternal vitamin D status, breast-feeding, and sunlight exposure (3,4). The risk factors for maternal vitamin D deficiency include habitation at high altitude, darker skin pigmentation, and excessive skin coverage with clothing. Most studies conducted in developing countries, including Turkey, have shown a high prevalence of hypovitaminosis D in newborns with a direct relationship with maternal vitamin D levels (5,6,7,8,9,10,11). Maternal vitamin D deficiency results in a poor transplacental transmission of vitamin D during pregnancy and reduced storage in newborns, leading to reduced intestinal Ca absorption, reduced ionized Ca concentrations, and secondary hyperparathyroidism, which in turn decreases urinary Ca loss, increases bone Ca reabsorption, normalizes serum Ca levels, but also decreases renal phosphate reabsorption (12,13). The reduced serum phosphate concentration is mainly responsible for the development of rickets, by impairing apoptosis of hypertrophic chondrocytes in the growth plate. In newborns and early infancy, a high metabolic demand for Ca, PTH resistance, and/or inadequate PTH response may result in hypocalcemia before radiological evidence of rickets (14). There are reports, albeit few in number, reporting hypocalcemia as a manifestation of vitamin D deficiency (15,16).

The aim of this study was to describe the clinical and biochemical characteristics of neonates with LNH, and to determine the relationship between LNH and maternal vitamin D deficiency in Turkey.

Methods

This cross-sectional, nationwide study was carried out between December 2015 and 2016 with the participation of 61 centers, and involved term neonates with LNH and their mothers. The study was endorsed by the Turkish Neonatology Society.

A case recording form (CRF), designed by two pediatric endocrinologists and a neonatologist (G.S.K., S.H., A.G.), was used to collect demographic data: maternal history including maternal clothing, lifestyle, and use of vitamin D supplements during pregnancy; anthropometric and clinical findings of the neonates; and laboratory findings of the newborns and their mothers. The CRF was uploaded to the FAVOR Web Registry System website. The study data were entered into the system over a 12-month period (December 11, 2015-2016) by the participating researchers. Data entered into the registry were also checked for consistency by a research assistant (G.S.K.). A total of 98 newborns with LNH and their mothers were registered in the database.

The babies enrolled in our study were those who presented with hypocalcemia or who were hospitalized for other reasons and developed hypocalcemia during their follow-up. Hypocalcemia in asymptomatic babies was identified through routine biochemical tests during the hospital stay.

Inclusion criteria were term newborns born at 37-42 weeks of gestation and the presence of LNH, defined as total serum Ca level of less than 7.5 mg/dL after the first 72 hours of birth. Registered data were reviewed in terms of the following exclusion criteria: a birthweight of less than 2,000 g and the presence of maternal diabetes; neonatal asphyxia; sepsis; malabsorption; renal insufficiency; liver disease; or use of anticonvulsants. Neonates fed with breast milk and/or formula were enrolled in the study.

Maternal and newborn venous blood samples were drawn at presentation to measure serum levels of total Ca, phosphate (PO_4), magnesium (Mg), albumin, alkaline phosphatase (ALP), intact PTH (iPTH), and 25 hydroxyvitamin D [25(OH)D]. Serum Ca, P, ALP, and iPTH levels were measured on the same day; for 25(OH)D, the blood supernatant samples after centrifugation were stored at -80 °C until the time of assay in a single laboratory using an enzyme immunoassay method (IDS Immunodiagnostic Systems Ltd, Boldon, UK).

According to the Global Consensus Recommendations, maternal and neonatal 25(OH)D levels of less than 12 ng/mL (30 nmol/L) were considered to be indicative of vitamin D deficiency (17). The inter- and intra-assay coefficients of variation were 6.7% and 8.7%, respectively. The reference range for total Ca was 8.7-10.4 mg/dL in mothers (adult women). The normal range of inorganic phosphate was 5.2 to 8.4 mg/dL for infants 0-5 months of age and 2.5 to 4.5 mg/dL for adult women. The upper normal limits for ALP were 420 IU/L and 130 IU/L for infants and non-pregnant women, respectively (18). The normal range for iPTH was defined as 5-65 pg/mL.

Anterior fontanelle evaluation was performed using the following technique. The anteroposterior diameter (AB) was determined as the length and the transverse diameter (CD) as the width. The average of the longitudinal and transverse dimensions was recorded as the mean fontanelle size ([AB + CD]/2). Neonates with mean fontanelle size above 97th percentile were determined as having large fontanelle (19).

Regular vitamin D intake during pregnancy was accepted as 1,200 IU/day vitamin D starting from 12th week of pregnancy, irrespective of vitamin D status, as stated in Turkish Ministry of Health Support Program (20).

The consistency and appropriateness of the diagnoses and enrolment were reviewed by two of the authors (G.S.K, A.G.). After exclusion of two neonates and five mothers due to the lack of centrifuged serum samples for the 25(OH)D measurement, the final analyses included 96 newborns with newly-diagnosed late hypocalcemia and their 93 mothers.

Written informed consent was obtained from parents of the newborns, and the study protocol was approved by the Kocaeli University Ethics Committee (report number: KOU KAEK 2015/322).

Statistical Analysis

Data were processed using Statistical Package for the Social Sciences, version 20 (IBM Inc., Armonk, NY, USA). The data for normality of distrubition was assesed. Numerical variables were expressed as medians and are presented as interquartile range (IQR) and frequencies (percentages). The association between the maternal and neonatal 25(OH)D levels was evaluated by the Spearman's correlation coefficient. A p value of less than 0.05 was considered to be significant.

Results

Among the 96 neonates, which included 38 (39.6%) girls and 58 (60.4%) boys, with newly diagnosed late hypocalcemia, the median (IQR) age of onset was 5.0 (4.0-8.0) days (Table 1). Thirty-three (34.4%) neonates presented with the symptoms of hypocalcemia, with convulsion being the most common symptom (n = 18). There was a seasonal pattern of presentation, with increased incidence in winter and spring months (65.6%). The majority of mothers (n = 74, 77.1%) used covered clothing and spent most of their time indoors. Only 20.4% of the mothers received regular vitamin D supplements during pregnancy.

Table 2 shows the laboratory findings of neonates with LNH. The median (IQR) serum Ca level was 6.9 (6.4-7.2) mg/dL, serum phosphorus 7.2 (6.1-8.5) mg/dL, serum ALP 197 (142.5-279.2) IU/L, and serum iPTH 69.1 (37.4-106) pcg/mL. The median (IQR) serum vitamin D level was 6.3 (4.1-9.05) ng/mL, with 83 (86.5%) neonates having vitamin D deficiency, defined as a serum 25(OH)D level <12 ng/mL. Serum phosphorus levels were normal/high in all but six neonates, and iPTH levels were high in 44 neonates (45.8%).

The maternal median (IQR) serum vitamin D level was 5.2 (4.7-8.8) ng/mL, and 93% of the mothers were vitamin D deficient (Table 3).

All but three mothers with vitamin D deficiency had neonates who also had vitamin D deficiency. A strong positive correlation was found between maternal and neonate serum 25(OH)D levels ($r = 0.513$, $p < 0.001$) (Figure 1).

Table 1. Demographic and clinical characteristics of 96 neonates with late neonatal hypocalcemia

Characteristics (n = 96)	Value*
Age at onset of hypocalcemia, (days)	5 (4-8)
Sex	
Male	58 (60.4)
Female	38 (39.6)
Season of birth time	
Winter (December-February)	27 (28.1)
Spring (March-May)	36 (37.5)
Summer (June-August)	14 (14.6)
Autumn (September- November)	19 (19.8)
Living area	
Urban	49 (51)
Rural	47 (49)
Maternal clothing	
Covered	74 (77.1)
Not covered	22 (22.9)
Maternal vitamin D supplementation during pregnancy	19 (20.4)
Regular	4 (4.3)
Irregular	70 (75.3)
None	
Symptom (+)	33 (34.4)
Convulsion	18 (18.8)
Poor sucking reflex	17 (17.7)
Exaggerated startle	11 (11.5)
Lethargy	7 (7.3)
Vomiting	3 (3.1)
Cyanosis	2 (2.1)
Apnea	1 (1)
Large anterior fontanelle	6 (6.3)
Gestational age (weeks)	38 (37-39)
Birth weight (g)	3305 (2976-3640)
Apgar score	
1-minute	8 (7-8)
5-minute	9 (9-10)

*Values are presented as number (%), median (interquartile range), or number only.

Table 2. Biochemical parameters of 96 neonates with late neonatal hypocalcemia

Variable	Median (IQR)		n (%)
Calcium (mg/dL)	6.9 (0.9)		
Phoshorus (mg/dL) (normal: 5.2-8.4)	7.2 (2.4)	Low Normal High	6 (6.3) 63 (65.6) 27 (28.1)
ALP (IU/L) (normal: 145-420)	197 (136.7)	Normal High	87 (90.6) 9 (9.4)
iPTH (pg/mL) (normal: 5-65)	69.1 (68.6)	Low Normal High	2 (2.1) 50 (52.1) 44 (45.8)
25(OH)D (ng/mL) (deficiency < 12 insufficiency 12-20 sufficiency > 20)	6.3 (4.9)	Deficiency Insufficiency Sufficiency	83 (86.5) 5 (5.2) 8 (8.3)

ALP: alkaline phosphatase, iPTH: intact parathyroid hormone, 25(OH)D: 25 hydroxyvitamin D, IQR: interquartile range, n: number

Table 3. Biochemical parameters in mothers of term neonates with late neonatal hypocalcemia

Variable	Median (IQR)		n (%)
Calcium (mg/dL)	8.9 (0.9)		
Phoshorus (mg/dL) (normal: 2.5-4.5)	3.9 (0.87)	Normal High	71 (88.8) 9 (11.3)
ALP (IU/L) (normal: < 150)	126 (60.1)	Normal High	57 (70.4) 24 (29.6)
iPTH (pg/mL) (normal: 10-65)	65.5 (56.9)	Low Normal High	1 (1.1) 43 (49.4) 43 (49.4)
25(OH)D (ng/mL) (deficiency < 12 insufficiency 12-20 sufficiency > 20)	5.2 (4.1)	Deficiency Insufficiency Sufficiency	80 (93) 4 (4.7) 2 (2.3)

ALP: alkaline phosphatase, iPTH: intact parathyroid hormone, 25(OH)D: 25 hydroxyvitamin D, IQR: interquartile range, n: number

Discussion

This is the first cross-sectional, multi-center study of term neonates with late hypocalcemia to investigate the relationship between maternal vitamin D status and LNH in Turkey. Maternal and neonatal vitamin D deficiency was found to be related with neonatal late hypocalcemia. Although infants can be protected from severe hypocalcemia thanks to a nationwide program of free supplementation of 400 U/day vitamin D for infants, the problem of LNH will continue unless pregnant women are provided with and consistently use vitamin D supplementation (21).

The prevalence of inadequate vitamin D status is high among pregnant and lactating women around the world (22,23,24). Women who wear covered clothing, live at high altitudes, and do not have an adequate exposure to sunlight are especially at risk for vitamin D deficiency (14).

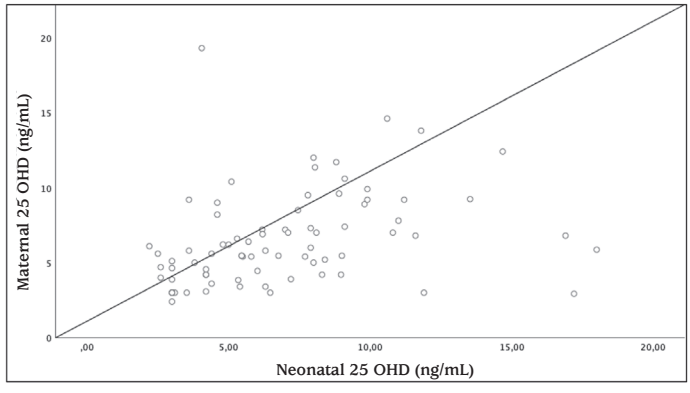

Figure 1. Correlation between maternal and neonatal serum 25 hydroxyvitamin D levels

In a study from Turkey, in lactating women and their babies a serum 25(OH)D level below 11 ng/mL was accepted as severe vitamin D deficiency, 11-25 ng/mL as moderate

deficiency, and a value >25 ng/mL as normal. Severe vitamin D deficiency was found in 27% of the mothers, and moderate deficiency in 54.3%. Severe vitamin D deficiency was detected in 64.3% of the neonates, and moderate deficiency in 32.9% (24).

An Indian study by Mehrotra et al (5) in 2010 found that 90% of neonates with hypocalcemic convulsions and 89% of their mothers had vitamin D deficiency. Furthermore, a significant correlation was found between the serum 25(OH) D levels of the mothers and infants.

Hatun et al (15) examined the medical records of infants with vitamin D deficiency and/or nutritional rickets in Turkey and found that 79% of the infants whose mothers were also vitamin D deficient had presented with hypocalcemic seizures. In the present study, vitamin D deficiency was diagnosed in 86.5% of the neonates with late hypocalcemia and in 93% of their mothers and, consistent with many previous reports, there was a significant correlation between maternal and newborn 25(OH)D levels (5,10).

As a known risk factor for vitamin D deficiency, covered clothing was seen in 77.1% of the mothers. Another study conducted in Turkey reported similar results (15). In a study carried out in Iran, where the vast majority of the population are Muslim and where women are required to veil themselves, among 100 neonates diagnosed with hypocalcemia, the prevalence of maternal vitamin D deficiency was 74% (9).

According to the Global Consensus Recommendations, the basic approach to prevent LNH is to supplement women with vitamin D of at least 600 U/day particularly over the last three months of pregnancy. However, data from an earlier national study showed that 25(OH)D levels can be normalized only by the administration of 2000 U of vitamin D per day during pregnancy (25).

In the current study, the majority of late hypocalcemic babies were born in the winter and spring months, which is consistent with a previous study from Korea that included 17 term newborns with LNH (26). This may be explained by limited sunlight exposure for mothers whose late pregnancy periods take place during the winter and spring months, leading to low maternal and neonatal vitamin D levels. We observed a male preponderance, which was previously reported by an American study of 78 full-term neonates with transient hypocalcemia, as well as by a study from the United Kingdom involving hypocalcemic neonates and children due to vitamin D deficiency (27,28).

In our study, despite the high prevalence of vitamin D deficiency (86.5%), large anterior fontanelle was observed in a small percentage of neonates (6.3%). Since reduced serum phosphate concentration is mainly responsible for the skeletal findings of rickets, we can speculate that, regardless of the amount, continuous transplacental transfer of Ca and phosphorus to the fetus might have limited the clinical and biochemical manifestations of vitamin D deficiency in these neonates. Elevated PTH level is an important cause of decreased phosphorus reabsorption. However, in neonates and young infants, PTH resistance and/or inadequate PTH response may result in hypocalcemia before skeletal findings occur.

Vitamin D deficiency is associated with biochemical disruption to Ca homeostasis, resulting in a typical constellation of hypocalcaemia, hypophosphatemia, and elevated levels of alkaline phosphatase and PTH. Other studies have documented that vitamin D deficiency triggers PTH release in adults, children, and older infants. However, in newborns and young infants, this feedback loop appears not to occur, and hypovitaminosis D coexisted with blunted PTH response and/or PTH resistance (29,30,31,32,33). In our study, baseline iPTH concentrations were increased in only 45.8% of neonates, and serum phosphorus levels were normal/high in all but five patients. Do et al (26) reported that, in 17 neonates with late-onset hypocalcemia secondary to vitamin D deficiency, iPTH levels were not remarkably elevated except in one case. Similar results in neonates were described by Maghbooli et al (34), who reported elevated serum iPTH levels in only 10% of vitamin D deficient neonates at birth. Late maturation of the parathyroid axis is thought to be a main cause of transient neonatal hypocalcemia, as suggested by the low or inappropriately normal PTH levels and high phosphorus levels in these infants. We also observed biochemically normal/high phosphorus levels in hypocalcemic neonates, although hyperparathyroidism is present, reflecting a possible PTH resistance. In the literature, some studies report PTH resistance in bone, secondary to vitamin D deficiency (15).

One of the basic points that we would like to emphasize is that a secondary increase in PTH level as a response to hypocalcemia (progress from stage 1 to stage 2) may not take place in vitamin D deficiency in early infancy. Thus, a decreased Ca level and normal or elevated serum phosphorus level in the presence of low and/or inappropriately normal iPTH level may be detected, mimicking hypoparathyroidism. This puzzles clinicians, and pseudohypoparathyroidism is considered for cases where PTH level is high. We would also like to stress that, in some vitamin D deficient cases, serum phosphorus levels may be normal or high, despite an increase in PTH level, due to secondary PTH resistance. Thus vitamin

D deficiency should be kept in mind in the differential diagnosis of pseudohypoparathyroidism. Elevated serum PTH concentration in the face of hypocalcemia and normal/high serum phosphate indicates an element of end organ resistance to PTH, mimicking pseudoparahypothyroidism. It was postulated in experimental rats that vitamin D depletion made them unresponsive to PTH. The vitamin D-depleted hypocalcemic rats failed to show elevation of serum Ca or a phosphaturic effect to injected PTH extracts, which could be corrected by addition of a small dose of vitamin D. The end organ resistance observed in vitaminD deficiency could result from down regulation of PTH/PTHrP receptor (35). Rao et al (36) observed an impaired phosphaturic response but normal urinary cAMP excretion to PTH in vitamin D deficient infants, similar to the picture in pseudohypoparathyroidism. The response to PTH was restored to normal following vitamin D and Ca supplementation, suggesting that in the presence of vitamin D deficiency and/or hypocalcemia, the renal tubules are resistant to the action of PTH.

The aim of our study was to determine the prevalence of vitamin D deficiency in babies with LNH, to draw attention to the importance of checking perinatal vitamin D levels when investigating the cause of LNH and to highlight that vitamin D deficiency may have biochemical findings leading to clinical confusion with other etiologies. Although the majority of the babies and their mothers included in our study was vitamin D deficient, we tried to be cautious not to indicate vitamin D deficiency as the sole reason for hypocalcemia in these babies. We wanted to emphasize that the prevalence of vitamin D deficiency is high in babies with hypocalcemia and it should be kept in mind as one of the leading possible causes. Cases of pseudohypoparathyroidism due to G protein receptor defects are known to present with symptoms after the newborn and infancy period (37). The cases that may be confused with vitamin D deficiency in the neonatal period are mainly of transient pseudohypoparathyroidism due to PTH receptor immaturity. In these cases, serum 25-OHD levels are not expected to be low. In addition, delay in both PTH release and the maturation in PTH receptors may be associated with neonatal vitamin D deficiency, which leads to biochemical findings, such as hyperphosphatemia and symptomatic hypocalcemia (15).

Study Limitations

A potential limitation of the study was the small number of the patients despite the participation of many centres. It is, however, difficult to recruit a large number of neonates with hypocalcemia in intensive care units due to the exclusion criteria including the presence of maternal diabetes, neonatal asphyxia, malabsorption, renal insufficiency, liver disease, or use of anticonvulsants and the concerns of parents about giving approval for taking blood samples from their babies. In addition, ELISA which was used in this study to measure vitamin D levels is not the gold standard test because of interference with vitamin D metabolites.

Conclusion

This study provides further evidence highlighting the need to maintain adequate vitamin D status in pregnancy. LNH has a close association with perinatal vitamin D deficiency. Thus the first step of a diagnostic work-up for LNH should be to measure serum 25(OH)D levels.

Ethics

Ethics Committee Approval: The study were approved by the Kocaeli University Ethics Committee (report number: KOU KAEK 2015/322).

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Gülcan Seymen-Karabulut, Ayla Günlemez, Ayşe Sevim Gökalp, Şükrü Hatun, Design: Gülcan Seymen-Karabulut, Ayla Günlemez, Ayşe Sevim Gökalp, Şükrü Hatun, Data Collection or Processing: Fatma Kaya Narter, Mehmet Mutlu, Şebnem Kader, Demet Terek, Deniz Hanta, Emel Okulu, Leyla Karadeniz, H. Gözde Kanmaz Kutman, Ayşegül Zenciroğlu, Özmert M. A. Özdemir, Dilek Sarıcı, Muhittin Çelik, Nihat Demir, Özden Turan, Kıymet Çelik, Fatih Kılıçbay, Sinan Uslu, Sara Erol, Sabahattin Ertuğrul, İlkay Er, Hasan Tolga Çelik, Merih Çetinkaya, Filiz Aktürk-Acar, Yakup Aslan, Gaffari Tunç, Ömer Güran, Ayşe Engin Arısoy, Analysis or Interpretation: Gülcan Seymen-Karabulut, Ayla Günlemez, Literature Search: Gülcan Seymen-Karabulut, Ayla Günlemez, Şükrü Hatun, Writing: Gülcan Seymen-Karabulut, Ayla Günlemez, Şükrü Hatun.

Financial Disclosure: This study was supported by Kocaeli University Scientific Research Fund.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2021.0230

J Clin Res Pediatr Endocrinol 2021;13(4):391-399

Is Bioavailable Vitamin D Better Than Total Vitamin D to Evaluate Vitamin D Status in Obese Children?

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What is already known on this topic?

There is conflicting evidence about the frequency of vitamin D deficiency in obese children. In these studies, vitamin D deficiency was evaluated by measuring total vitamin D level. It is also known that vitamin D binding protein (VDBP) concentration and VDBP polymorphisms affect vitamin D level, although once again, the evidence is contradictory.

What this study adds?

While there was no difference in terms of total vitamin D, free and bioavailable vitamin D levels were lower in the obese group in winter. In addition, VDBP and parathyroid hormone concentrations were found to be higher. Concentration of VDBP and VDBP polymorphism had no effect on total vitamin D level.

Abstract

Objective: Free hormones are biologically more active in target tissues. Thus, measurement of vitamin D taking into account bioavailability and free vitamin D may be preferable, especially when evidence is contradictory, as in obese children. In order to assess bioavailablity and free vitamin D, using a previously reported formula, vitamin D-binding protein (VDBP) level was measured and VDBP polymorphisms were also evaluated because of variations in binding affinity.

Methods: Eighty-four obese and 78 healthy children were included. Anthropometry, calcium, phosphorus, alkaline-phosphatase, parathyroid hormone (PTH), 25 hydroxyvitamin D [25(OH)D], bioavailable-free vitamin D, and VDBP concentration and polymorphism were evaluated in the whole group.

Results: Obese girls had significantly higher PTH than normal weight girls ($p = 0.001$). Regardless of gender, obese children had significantly higher concentrations of VDBP ($p = 0.008$) and PTH ($p = 0.002$). When samples taken in winter were analyzed, PTH and VDBP were found to be higher and bioavailable and free vitamin D lower in the obese group. There was no difference in terms of total vitamin D between groups during the winter season.

Conclusion: While total, free, and bioavailable vitamin D in the obese group was similar to the control group in autumn, free and bioavailable vitamin D in the winter was lower in the obese than the control group. In addition, PTH was higher in the obese group in both autumn and winter. Therefore, more research is needed to evaluate the variability of free and bioavailable vitamin D according to body habitus, season and the effect any differences may have.

Keywords: 25 hydroxyvitamin D, bioavailable vitamin D, free vitamin D, vitamin D binding protein, polymorphism, obesity

Introduction

Vitamin D is a pre-pro-hormone and some of its main functions are the regulation of calcium metabolism and bone homeostasis, although many more effects of vitamin D have been reported. Obesity, the frequency of which has been increasing in recent years, is considered a risk factor for vitamin D deficiency. It has been reported that vitamin

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Conflict of interest: None declared
Received: 08.10.2020
Accepted: 23.04.2021

D deficiency in obesity may be due to rapid metabolic clearance of vitamin D, large distribution volume and decreased bioavailability (1,2). Vitamin D synthesis starts in the skin with sunlight exposure and thus a further reason for vitamin D deficiency in obesity may be decreased cutaneous synthesis (3). Therefore, a seasonal difference is normally observed in vitamin D levels.

25 hydroxyvitamin D [25(OH)D] is highly lipophilic and thus requires a carrier serum protein to facilitate action in the target cell. Around 85-90% of circulating 25(OH)D is bound to vitamin D-binding protein (VDBP) and 10-15% albumin-bound. Less than 1% of the circulating hormone is in free form (4,5). According to the free hormone hypothesis, only hormones separated from the binding protein can enter the cell and have a biological effect (6). Bioavailable vitamin D, which is not bound to VDBP, is known to be biologically more active in the target tissues (7). VDBP concentration and VDBP polymorphism can both affect vitamin D sufficiency as both factors will change the affinity of binding to vitamin D. It has also been suggested that there may be a VDBP effect on 25(OH)D-related intacrine responses (8). In most of the studies published to date investigating vitamin D deficiency, total vitamin D level has been measured. Some researchers have hypothesized that this total vitamin D level does not reflect the biologically active vitamin D available to the organism (5,8). It has been suggested that the evaluation of hormonal activity and sufficiency by measuring bioavailable vitamin D will be much more reliable. The aim of this study was to evaluate whether vitamin D level was different when comparing an obese group of children with a normal weight group of children and to compare factors affecting total, bioavailable, and free vitamin D levels in both groups.

Methods

The study was performed at the pediatric endocrinology outpatient clinic of a single hospital between September 2018 and March 2019. Informed consent was taken from the families of volunteers participating in the study. The Ethics Review Board of Zekai Tahir Burak Women's Health Training and Research Hospital approved the study protocol (approval number: 16/2018, dated: 06.03.2018). The study was conducted as a University of Health Sciences' Scientific Research Coordination Unit (project number: 2018/040).

Eighty-four obese children [body mass index (BMI) $>95^{th}$ percentile] and 78 healthy children (BMI between 15^{th} and 85^{th} percentiles) without additional systemic diseases and drug-free were included in the study. Children who received vitamin D in the six months prior to the study were not included. Prepubertal cases were not included in the study. Pubertal staging was done according to Tanner in all cases included. In girls breast stage ≥2 and in males testicular volume ≥4 mL were defined as pubertal.

A SECA scale (SECA, Hamburg, Germany) and a Harpenden stadiometer (Holtain Ltd., Crymych, UK) were used to measure weight and height, respectively. Anthropometric data for the Turkish population, such as height, weight and BMI, are available in an online database (www.ceddcozum.com) (9).

Calcium, phosphorus, alkaline phosphatase (ALP), parathyroid hormone (PTH), 25(OH)D, VDBP level and VDBP polymorphism were examined in both obese and healthy groups. In addition, fasting blood glucose and insulin, hemoglobin A1c (HbA1c), total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels were measured only in the obese group. All measurements were made after a 10-hour fasting period. Glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, calcium, phosphorous, ALP, AST, ALT and albumin levels were measured with ready-made kits in auto-analyzer system (Beckman Coulter, Inc. USA). Serum insulin and PTH levels were measured with colorimetric method using ready-made kits. (Siemens Healthcare GmBH, Germany). HbA1c levels were measured with a ultraviolet detector using the high-performance liquid chromatography method based on the pirinciple of ion exchange chromatography.

Serum Vitamin D Measurement

Serum 25(OH)D analyses by liquid chromatography-tandem mass spectrometry were performed with a commercial kit from Recipe (Munich, Germany). That is traceable to the NIST 972a standard reference material.

VDBP Measurement

Blood samples taken from the obese and healthy control groups were centrifuged at 3000 g for 10 minutes. Separated serum was stored at -80 °C in Eppendorf tubes until analysis. All samples were melted simultaneously. Analysis was performed using commercial kits [Cloud-Clone Corp. (USCN Life Science Kit Inc.) (product number: SEB810HU) (1304 Langham Creek Dr, Suite 226, Houston, TX 77084, USA)] on an enzyme-bound immune-absorbent (ELISA) automated analyzer (Biotek, USA). This method used polyclonal VDBP antibodies. The intra-test coefficient variability (CV) of the kit was $<10\%$ and the inter-test CV was $<12\%$.

Bioavailable vitamin D was calculated using the previously reported formula (4). The formula is a mathematical

calculation model that includes VDBP and albumin binding constant.

$$\text{Free vitamin D} = \frac{\text{Total 25(OH)D}}{1 + (6x10^5 \text{ x albumin}) + (7x10^8 \text{ x VDBP})}$$

Bioavailable vitamin D = (D Free) + ($D_{albumin}$) = (D-free). [$K_{albumin}$. (Alb) + 1]

Polymorphism Genotyping

However, the most common polymorphisms of the VDBP gene, which are rs4588, rs7041 and rs587776830, were studied because genotypic differences in this protein may cause significant variations in binding affinity and serum concentration.

For VDBP polymorphism, samples separated into EDTA tubes were stored at -20 °C. DNA was isolated with a magnetic bead method (MagPurix, Zinexts, Taiwan). PCR amplification was performed using in-house designed primers. Amplicons were checked by 2 % agarose gel electrophoresis. Sequencing was done by a next generation sequencing method using Miseq-Illumina equipment (Illumina, San Diego, CA, USA) in accordance with manufacturer's instructions. Data was evaluated by IGV 2.3 (Broad Institute) software (https://igv.org/).

Statistical Analyses

Descriptive statistics for continuous variables (characteristics) are presented as mean and standard deviation (SD) for normally distributed variables, as median and minimum-maximum for data not normally distributed; while categorical variables are shown as count and percent. Normality assumption of the continuous variables was tested with Kolmogov-Smirnov test. ANOVA or Student's t-test was used for comparison of means in normally distributed characteristics. However, for non-normally distributed variables, Mann-Whitney U test was performed for two groups and Kruskal-Wallis test was performed more than two groups. For determination of linear relationships among the continuous variables, Pearson correlation analysis was carried out in each group. In addition, chi-square test was also performed to determine the relationships between categorical variables. Statistical significance level was considered as 5% and Statistical Package for the Social Sciences, version 22 (IBM Inc., Armonk, NY, USA) was used for all statistical computations.

Results

There was no difference between the groups in terms of age and gender and pubertal stage ($p = 0.886$, $p = 0.309$ and $p = 0.051$, respectively). In the obese group, height SD score (SDS), weight, weight SDS, BMI, and BMI SDS were significantly higher than the control group (see Table 1). When obese and control subjects were compared without gender discrimination, VDBP and PTH levels were found to be statistically higher in the obese group. Total, bioavailable and free vitamin D levels were similar in both groups. In addition, when normal weight and obese girls were compared, PTH values were found to be higher in the obese group (Table 1). Also, VDBP values were found to be non-significantly higher in obese girls ($p = 0.057$). No differences were detected between obese and normal weight males in these parameters. In addition, when VDBP level was compared according to the pubertal stage, there was no difference ($p = 0.180$).

In 34.5% (n = 29) of the obese group and 39.7% (n = 31) of the control group, 25(OH)D level was below 12 ng/mL and was considered deficient. In the obese group, PTH and HDL cholesterol were higher, and phosphorus, free and bioavailable vitamin D were lower in the deficient group (Table 2). Comparison of the whole obese group with the subgroup of controls (n = 11) with normal vitamin D [25(OH)D > 20 ng/mL and PTH < 65 pg/mL] is shown in Table 3. In this comparison, total, free, bioavailable vitamin D and phosphorus were lower and PTH was higher in the obese group.

Sixty-five of the obese cases were included in the study in the autumn and 19 in the winter season while 26 of the control cases were included in the study in the autumn and 52 in the winter. When only the cases included in the winter period were analyzed, PTH and VDBP were found to be higher and bioavailable and free vitamin D lower in the obese group (Table 4). There was no difference in terms of total vitamin D between groups during the winter. In autumn, phosphorus and ALP were found to be lower while PTH was higher in the obese group. When the obese group was stratified by season total, bioavailable and free vitamin D were lower, and VDBP was higher in the obese group in winter. Similarly, on stratification by season in the control group P, ALP, total, bioavailable and free vitamin D were found to be lower in the winter.

VDBP polymorphism distribution was similar in the obese and control groups (Table 5). In addition, calcium metabolism parameters were compared within the rs587776830, rs7041, and rs4588 genotype subgroups. There were 12 subgroups in rs587776830 genotype group and there was no difference between the groups in terms of Ca, P, ALP, PTH, total vitamin D, free vitamin D, bioavailable vitamin D and VDBP (respectively $p = 0.457$, $p = 0.786$, $p = 0.706$, $p = 0.897$, $p = 0.125$, $p = 0.200$, $p = 0.239$, and $p = 0.722$). Also, as indicated in Table 6, no difference was found in the

rs7041 and rs4588 group in terms of calcium parameters, notably including VDBP.

In the whole group, there was positive correlation between VDBP and PTH (p = 0.009; r = 0.20), negative correlation between VDBP rs4588 polymorphism and PTH (p = 0.04; r = -0.15), negative correlation between PTH (p = 0.01; r = -0.20) and total 25(OH)D, negative correlation between bioavailable vitamin D and PTH (p = 0.001; r = -0.24). Free vitamin D positively correlated with P, ALP, total 25(OH)D and bioavailable vitamin D (p < 0.001; r = 0.30;

Table 1. Anthropometric and laboratory characteristics by gender in the obese and control groups

	Female			Male			
	Obese (n = 54)	**Control (n = 54)**	**p value**	**Obese (n = 30)**	**Control (n = 24)**	**p value**	**p* value**
Age (years) Median (min-max)	13.3 (9-17.8)	12.3 (9-17.9)	0.265#	13.0 (9.9-17.8)	14.4 (11-17.8)	0.096	0.886#
Height (cm)	158.8 ± 7.6	152.2 ± 10.2	**0.001†**	159.2 ± 21.7	165.2 ± 12.5	0.236	0.208†
Height SDS	0.6 ± 1.08	-0.1 ± 1.08	**0.001†**	0.42 ± 0.85	0.05 ± 1.13	0.193	0.001†
Weight (kg)	77.9 ± 15.2	45.2 ± 9.4	**0.001†**	85.9 ± 20.5	57.8 ± 12	**0.001**	**0.011†**
Weight SDS	3 ± 0.98	-0.2 ± 0.8	**0.001†**	2.4 ± 0.8	-0.05 ± 0.78	**0.001**	**0.001†**
BMI (kg/m²)	30.7 ± 4.5	19.2 ± 2.1	**0.001†**	32.1 ± 5.1	20.9 ± 2.1	**0.001**	**0.001†**
BMI SDS	2.7 ± 0.6	-0.1 ± 0.6	**0.001†**	2.4 ± 0.6	-0.04 ± 0.64	**0.001**	**0.001†**
Calcium (mg/dL)	10.02 ± 0.29	10.01 ± 0.37	0.885†	10.05 ± 0.32	10.14 ± 0.28	0.281	0.804†
Phosphorus (mg/dL)	4.3 ± 0.64	4.4 ± 0.7	0.597†	4.3 ± 0.5	4.4 ± 0.8	0.636	0.579†
Alkaline phosphatase (U/L)	178 ± 90.1	209.3 ± 125.6	0.141†	225.3 ± 80.5	220.2 ± 118.7	0.858	0.687†
Albumin (g/dL)	4.6 ± 0.2	4.5 ± 0.2	0.689†	4.6 ± 0.24	4.7 ± 0.32	0.202	0.766†
Parathyroid hormone (pg/mL)	71 ± 29.5	54.3 ± 19.6	**0.001†**	69.8 ± 31.8	59.3 ± 28.5	0.207	**0.002†**
25(OH)D (ng/mL)	16 ± 8.5	14.5 ± 7	0.303†	18.3 ± 7.4	15 ± 7.1	0.103	0.083†
Free vitamin D (pg/mL)	10.1 ± 6.4	9.6 ± 4.8	0.616†	11.5 ± 6.9	9.9 ± 4.6	0.355	0.705†
Bioavailable vitamin D (ng/mL)	4.4 ± 2.8	4.1 ± 2.1	0.630†	4.9 ± 2.9	4.3 ± 1.9	0.414	0.743†
VDBP (mg/L) Median (min-max)	61.1 (43.1-228.2)	52.8 (43.2-181.9)	0.057#	67.0 (43.2-161.4)	49.7 (43.2127.9)	0.077	**0.008#**

BMI: body mass index, SDS: standard deviation (SD) score, Min-Max: minimum-maximum, VDBP: vitamin D binding protein, 25(OH)D: 25 hydroxyvitamin D.
†Student's t-test was used and mean±SD values are given.
#Mann-Whitney U test was used and median (min-max) values were given.
*Comparison of obese and control group regardless of gender.

Table 2. Comparison of metabolic parameters of patients with vitamin D deficient and sufficient in obese group

	25(OH)D < 12 ng/mL (n = 29)	**25(OH)D ≥12 ng/mL (n = 55)**	**p value**
Calcium (mg/dL)	10.0 ± 0.3	10.0 ± 0.3	0.487†
Phosphorus (mg/dL)	4.0 ± 0.6	4.4 ± 0.6	**0.022†**
Alkaline phosphatase (U/L)	168.9 ± 96.8	208.7 ± 82.7	0.066†
Albumin (g/dL)	4.6 ± 0.2	4.5 ± 0.2	0.249†
Parathyroid hormone (pg/mL)	79.7 ± 32.0	65.8 ± 28.2	**0.043†**
Free vitamin D (pg/mL)	4.9 ± 2.0	13.6 ± 6.2	**0.000†**
Bioavailable vitamin D (ng/mL)	2.1 ± 0.9	5.8 ± 2.7	**0.000†**
VDBP (mg/L) Median (min-max)	71.2 (43.2-197.5)	60.2 (43.1-228.2)	0.202#
Glucose (mg/dL)	90.3 ± 8.8	92.1 ± 10.4	0.415†
Insulin (mIU/mL)	23.0 ± 13.5	22.9 ± 15.3	0.968†
Cholesterol (mg/dL)	165.9 ± 28.8	157.9 ± 30.0	0.240†
LDL cholesterol (mg/dL)	100.1 ± 25.9	93.9 ± 25.1	0.298†
HDL cholesterol (mg/dL)	44.2 ± 8.3	40.2 ± 6.6	**0.030†**
Triglycerides (mg/dL)	107.5 ± 44.0	118.6 ± 48.9	0.296†

25(OH)D: 25 hydroxyvitamin D, SD: standard deviation, VDBP: vitamin D binding protein, LDL: low density lipoprotein, HDL: high density lipoprotein.
†Student's t-test was used and mean±SD values were given.
#Mann-Whitney U test was used and median (minimum-maximum) values were given.

p = 0.008; r = 0.20, p < 0.001, r = 0.86; p < 0.001; r = 0.99, respectively), and negatively correlated with PTH and VDBP (p = 0.001; r = -0.25; p < 0.001; r = -0.47). In addition, negative correlation between bioavailable vitamin D and HDL cholesterol (p = 0.04; r = -0.21), positive correlation between PTH and fasting blood glucose and fasting insulin (p < 0.01; r = 0.39; p = 0.04; r = 0.22, respectively) were detected in the obese group.

Discussion

Although there have been many studies examining the relationship between obesity and vitamin D in children and adults, there are very few studies evaluating bioavailable vitamin D, free vitamin D and VDBP levels and VDBP polymorphism together in obese and healthy controlS (2,10,11,12,13,14). Our study is the first to evaluate total, bioavailable, free vitamin D and VDBP levels and VDBP polymorphism in obese and healthy children.

In our study, total 25(OH)D, bioavailable vitamin D and free vitamin D levels were similar in both the obese and control groups. However, considering the seasons separately, when the obese group was compared to the control group, total vitamin D was similar, bioavailable and free vitamin D levels were low, and VDBP and PTH levels were high in winter.

Table 3. The evaluation of calcium metabolism parameters in obese and control group

	Obese	Control	p value[#]
	Whole group (n = 84)	Vitamin D sufficient* (n = 11)	
Calcium (mg/dL)	10.05 (9.1-10.7)	10 (9.4-10.5)	0.619
Phosphorus (mg/dL)	4.2 (3.1-5.8)	4.8 (3.9-5.6)	**0.009**
Alkaline phosphatase (U/L)	198 (53-394)	222.5 (81-362)	0.367
Albumin (g/dL)	4.6 (4.1-5.3)	4.6 (4.3-4.9)	0.324
Parathyroid hormone (pg/mL)	64.6 (25-154.6)	48.4 (26.9-63.4)	**0.012**
25(OH)D (ng/mL)	16.4 (5.1-40.8)	25.9 (20.2-33.1)	**0.000**
Free vitamin D (pg/mL)	8.7 (1.2-35.3)	15.6 (7.8-24.5)	**0.011**
Bioavailable vitamin D (ng/mL)	3.7 (0.5-14.6)	6.8 (3.3-10.6)	**0.010**
VDBP (mg/L)	62.2 (43.1-228.1)	81.5 (43.2-181.9)	0.935

25(OH)D: 25 hydroxyvitamin D, VDBP: vitamin D binding protein.
*The cases with 25(OH)D > 20 ng/mL and PTH < 65 pg/mL were defined as sufficient.
[#]Mann-Whitney U test was used and median (minimum-maximum) values were given.

Table 4. Comparison of calcium metabolism parameters in obese and control groups by seasons

	Autumn			Winter				
	Obese (n = 65)	Control (n = 26)	p value[#]	Obese (n = 19)	Control (n = 52)	p value[#]	p value[†]	p value[*]
Calcium (mg/dL)	10.1 (9.1-10.7)	10.2 (9.4-10.8)	0.226	9.9 (9.3-10.4)	10 (9.3-10.8)	0.705	0.291	0.139
Phosphorus (mg/dL)	4.3 (3.1-5.6)	4.8 (3.6-5.9)	**0.001**	4.1 (3.2-5.8)	4.1 (2.8-5.7)	0.805	0.425	**0.000**
Alkaline phosphatase (U/L)	198 (56-394)	274.5 (73-563)	**0.004**	194 (53-318)	139 (42-491)	0.851	0.248	**0.001**
Albumin (g/dL)	4.6 (4.1-5.3)	4.5 (4.2-5.1)	0.561	4.6 (4.2-4.9)	4.5 (4-5.2)	0.829	0.552	0.685
Parathyroid hormone (pg/mL)	61.7 (25-149.4)	45.8 (20.9-95.8)	**0.004**	85.8 (26.7-154.6)	57.1 (4.9-153.6)	**0.007**	0.060	0.059
25(OH)D (ng/mL)	17.8 (5.6-40.8)	16.9 (11.3-36.3)	0.477	10.4 (5.1-24.5)	11.5 (4.8-30.7)	0.483	**< 0.001**	**0.000**
Free vitamin D (pg/mL)	11.2 (2.5-35.3)	12.8 (7.0-24.5)	0.288	4.4 (1.2-14.0)	7.5 (2.2-17.2)	**0.008**	**< 0.001**	**0.000**
Bioavailable vitamin D (ng/mL)	4.6 (1.2-14.6)	5.4 (3.0-10.6)	0.199	1.92 (0.5-6.2)	3.3 (0.94-7.1)	**0.006**	**< 0.001**	**0.000**
VDBP (mg/L)	57.3 (43.1-228.2)	54.2 (43.2-140.6)	0.580	86.9 (46.6-197.5)	51.2 (43.3-181.9)	**< 0.001**	**0.001**	0.672

25(OH)D: 25 hydroxyvitamin D, VDBP: vitamin D binding protein.
[#]Mann-Whitney U test was used and median (minimum-maximum) values are given.
[†]Comparison of the obese group by seasons.
*Comparison of the control group by seasons.

While there was no difference in vitamin D and VDBP in autumn, PTH levels were higher in the obese group. Also in the obese group, while total, free, and bioavailable vitamin D were lower in winter compared to autumn, VDBP was higher and there was no difference in PTH level. Similarly, in the control group, total, bioavailable and free vitamin D were lower in winter than in autumn. In the controls VDBP and PTH did not differ between autumn and winter. We observed that all forms of vitamin D in obese and healthy children were lower in winter than in autumn.

In a study investigating the seasonal variability of vitamin D and PTH in obese children, vitamin D was found to be higher in summer than in autumn and winter in the obese and control groups. In addition, seasonal variability was not detected in the obese group in terms of PTH, while it was found to be high in the control group in autumn and spring months (3). Seasonal variation in vitamin D was evaluated in a study conducted in the UK that included 223 obese, overweight and normal weight adults (2). The level of vitamin D in the obese and overweight groups was found to be lower than in normal weight in autumn and spring, but similar to those of normal weight in winter. Also, it has been concluded that the synthesis of vitamin D in the skin is similar in obese and normal weight individuals. One study reported in 2015, with 63 obese and 21 healthy children aged 4-15 years, that total 25(OH)D was lower in the obese group than in the non-obese group, but no difference was found between bioavailable vitamin D and the PTH levels, regardless of the season (11). In our study, in the obese group vitamin D levels were similar to the control group and PTH was higher than the control group in autumn. In winter, the total vitamin D level in the obese group was similar to the control group, free and bioavailable vitamin D was lower than the control group, and VDBP and PTH were higher than the control group.

In the present study, Ca, P, ALP and albumin levels were similar in the obese and healthy control group, whereas PTH levels were higher in the obese group. Vitamin D deficiency is known to be associated with decreased calcium absorption and increased PTH (5,15). PTH is also thought to be a useful indicator of the biological significance of low vitamin D level (16). In a study involving 595 female adult patients, 25(OH)D, PTH and VDBP levels were measured and VDBP Gc phenotyping was performed. Similar to our study, an inverse relationship was found between 25(OH) D and PTH. The patients with Gc1-1 phenotype and 25(OH)

Table 5. Vitamin D binding protein polymorphism distributions in obese and control groups

SNP ID	Genotype	Obese n (%)	Control n (%)	p value[#]
VDBP rs587776830				0.543
VDBP *rs7041*				0.363
	TT	12 (14)	16 (20)	
	TG	42 (50)	31 (40)	
	GG	30 (36)	31 (40)	
	T allele	66 (39)	63 (40)	
	G allele	102 (61)	93 (60)	
VDBP *rs4588*				0.256
	CC	56 (67)	46 (59)	
	CA	22 (26)	29 (37)	
	AA	6 (7)	3 (4)	
	C allele	134 (80)	121 (78)	
	A allele	34 (20)	35 (22)	

SNP: single nucleotide polymorphism, VDBP: vitamin D binding protein
[#]Pearson χ^2 test was used.

Table 6. The evaluation of calcium metabolism parameters according to the genotypes in whole group

	rs7041				***rs4588***		
	GG (n = 61)	**TT (n = 28)**	**TG (n = 73)**	**p value**	**CC (n = 102)**	**CA (n = 51)**	**p value**
Calcium (mg/dL)	10.05 ± 0.36	10.1 ± 0.27	10 ± 0.31	0.273[†]	10.03±0.33	10.05±0.32	0.967[φ]
Phosphorus (mg/dL)	4.3 ± 0.6	4.3 ± 0.7	4.3 ± 0.7	0.796[†]	4.3±0.7	4.2±0.6	0.283[φ]
Alkaline phosphatase (U/L)	206.1 ± 103.7	223.9 ± 117.8	193.4 ± 105.2	0.416[†]	201.7±104.9	195.3±104.5	0.747[φ]
Albumin (g/dL)	4.5 ± 0.3	4.6 ± 0.2	4.6 ± 0.21	0.455[†]	4.5±0.2	4.6±0.2	0.304[φ]
Parathyroid hormone (pg/mL)	66.43 ± 31.1	61.88 ± 27	61.66 ± 24.9	0.568[†]	65.75±27.6	57.46±25.4	0.248[φ]
25(OH)D (ng/mL)	15.8 ± 7.9	15.8 ± 7.9	15.8 ± 7.5	0.992[†]	16.5±8.2	14.9±6.7	0.381[φ]
Free vitamin D (pg/mL)	10.4 ± 5.8	9.3 ± 5	10.3 ± 6	0.808[†]	10.7±6.1	9.5±5.2	0.337[φ]
Bioavailable vitamin D (ng/mL)	4.43 ± 2.47	4 ± 2.2	4.4 ± 2.6	0.856[†]	4.6±2.6	4.13±2.3	0.367[φ]
VDBP (mg/L) Median (min-max)	52.8 (43.2-181.2)	71.0 (43.2-153.2)	57.3 (43.1-228.2)	0.111[#]	56.9 (43.1-228.2)	56.5 (43.1-197.4)	0.422[λ]

SD: Standard deviation, 25(OH)D: 25 hydroxyvitamin D, VDBP: vitamin D binding protein, min-max: minimum-maximum
[†]One-way ANOVA was used and mean±SD values are given.
[#]Kruskal Wallis test was used and median (min-max) values are given.
[φ]Student's t-test was used and mean±SD values are given.
[λ]Mann-Whitney U test was used and median (min-max) values are given.

D < 40 nmol/L had higher PTH levels but when all patients were included in the evaluation, in terms of PTH, it has been reported that there was no difference according to Gc phenotypes (17). In our study, a negative correlation between VDBP rs4588 polymorphism and PTH suggested that VDBP polymorphisms may affect PTH levels.

Our findings showed that the VDBP level was significantly higher in the obese group than in the control group. VDBP is the major serum transport protein of vitamin D (18). VDBP also facilitates the transport of 25(OH)D to tissues and regulates its bioavailability (19). In our study, total 25(OH)D, bioavailable and free vitamin D levels were similar between the two groups, while PTH and VDBP were increased in the obese group. There was no difference between the groups in terms of pubertal stages. In a study conducted in 15 obese and 15 normal weight adults between the ages of 20-35, the VDBP level was reported to be higher in the obese group (18). In a study performed in 2014 comparing 43 obese and 43 normal-weight women aged 22-45 years VDBP levels were found to be higher in the obese group (20). It has been stated that high estrogen levels in obese women increase the hepatic production of VDBP. However, in our study, there was no difference in VDBP by gender in the whole group. Similarly, in another study conducted in adults, VDBP and PTH were higher in the obese group (14). In our study, the positive correlation between VDBP and PTH in the whole group, independent of vitamin D, suggested that these two variables may be related to each other.

The binding affinity of 25(OH)D to VDBP can change according to VDBP polymorphism and its level may be affected (8). In our study, the distribution of VDBP polymorphism in the obese group was similar to the control group. According to polymorphisms, there was no difference in terms of 25(OH)D, bioavailable vitamin D, free vitamin D and VDBP. Contrary to our results, in many studies, it has been shown that total vitamin D, free vitamin D and VDBP levels are lower in the Gc2 genotype (21,22,23,24,25,26). The reason why there was no difference in our study may be due to the low number of our cases compared to other studies or it has been examined in different ethnicity.

In our study, a negative correlation was found between total 25(OH)D, bioavailable, free vitamin D and PTH. Similarly, in the literature it was reported that a negative correlation was found between PTH and total 25(OH)D and bioavailable vitamin D in a study investigating cases with chronic renal failure between the ages of 5-21 years (27). In another study examining 94 dialysis patients in adulthood, a negative correlation was found between bioavailable vitamin D and PTH, while no correlation was found between total 25(OH) D and PTH (28). In another study of adults, it was reported that a negative correlation was found between total vitamin D and free vitamin D and PTH in women and men, and, in women only, a negative correlation was found between bioavailable vitamin D and PTH (14).

There are publications showing that vitamin D status is associated with many diseases, such as obesity, insulin resistance (IR), diabetes, dyslipidemia, atherosclerosis and cancer, in addition to regulating intestinal calcium absorption and bone homeostasis. In our study, negative correlations between bioavailable vitamin D and HDL cholesterol, and positive correlations between fasting blood glucose, fasting insulin and PTH were found in the obese group. There are studies reporting negative correlation between total vitamin D level and fasting glucose levels (12), positive correlation between total and free vitamin D and insulin sensitivity, and negative correlation with homeostasis model assessment of IR (29), and negative correlation between free vitamin D and fasting blood glucose (30). There are also studies reporting positive correlation between VDBP and total cholesterol, LDL cholesterol and triglycerides (31). In light of these data, it is apparent that vitamin D and PTH play an effective part in metabolic balance.

Study Limitations

The dietary calcium and vitamin D intakes could not be evaluated. The clothing characteristics, skin pigmentation and the time spent outside were not investigated in any study subjects.

Conclusion

It was found that total, bioavailable and free vitamin D levels in the obese group were similar to the control group. Our results suggest that VDBP level and VDBP polymorphism may have a direct effect on PTH regulation. However, when the obese and control groups in winter were compared, there was no difference in total vitamin D, while free and bioavailable vitamin D was lower and PTH and VDBP was higher in obese children. More research is needed to explain the variability of total, free and bioavailable vitamin D according to seasons.

Acknowledgements

We wish to express our gratitude to the parents and the patients who participated in this study.

Ethics

Ethics Committee Approval: The Ethics Review Board of Zekai Tahir Burak Women's Health Training and Research

Hospital approved the study protocol (approval number: 16/2018, dated: 06.03.2018).

Informed Consent: Informed consent was taken from the families of volunteers participating in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Gülin Karacan Küçükali, Şervan Özalkak, Serdar Ceylaner, Zehra Aycan, Şenay Savaş Erdeve, Concept: Gülin Karacan Küçükali, Zehra Aycan, Şenay Savaş Erdeve, Design: Gülin Karacan Küçükali, Zehra Aycan, Şenay Savaş Erdeve, Data Collection or Processing: Gülin Karacan Küçükali, Özlem Gülbahar, Şervan Özalkak, Hasan Dağlı, Serdar Ceylaner, Analysis or Interpretation: Özlem Gülbahar, Hasan Dağlı, Serdar Ceylaner, Şenay Savaş Erdeve, Literature Search: Gülin Karacan Küçükali, Şenay Savaş Erdeve, Writing: Gülin Karacan Küçükali, Şenay Savaş Erdeve.

Financial Disclosure: The study was conducted as a University of Health Sciences' Scientific Research Coordination Unit (project number: 2018/040).

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0020

J Clin Res Pediatr Endocrinol 2021;13(4):400-407

Urinary NGAL is a Potential Biomarker for Early Renal Injury in Insulin Resistant Obese Non-diabetic Children

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What is already known on this topic?

Neutrophil gelatinase-associated lipocalin (NGAL) is a newly described biomarker used for detecting acute renal injury. NGAL was also defined as an early renal injury biomarker in type 2 diabetes mellitus. Type 2 diabetes has an insidious clinical course. Microalbuminuria has significant limitations in determining disease progression. Therefore, identification and validation of new biomarkers for early diagnosis of kidney injury may help to predict nephropathy and progression.

What this study adds?

Obese children with normoalbuminuric insulin resistance (IR) without diabetes have higher urinary NGAL levels than those with no evidence of IR and are at risk for early renal damage. NGAL may be a marker of early renal damage in obese IR children before type 2 diabetes develops.

Abstract

Objective: Neutrophil gelatinase-associated lipocalin (NGAL) is one of the new biomarkers for detecting acute renal injury. There are studies showing the relationship between NGAL and renal injury in obese children. The aim of this study was to investigate whether urinary levels of NGAL, kidney injury molecule-1, and serum cystatin C are increased in insulin resistance (IR) patients before the development of diabetes.

Methods: Cross-sectional, case-controlled study that included non-diabetic obese children and adolescent patients with IR and a non-diabetic obese control group with no IR, who attended a tertiary center pediatric endocrinology outpatient clinic between 2016-2018. Those with diabetes mellitus and/or known renal disease were excluded. NGAL and creatinine (Cr) levels were evaluated in the morning spot urine from all participants. Serum renal function was evaluated.

Results: Thirty-six control and 63 IR patients were included in the study, of whom 68 (68.7%) were girls. The mean age of all participants was 13.12 ± 2.64 years and no statistically significant difference was found between the two groups in terms of age or gender distribution. Median (range) spot urinary NGAL (u-NGAL) values in the IR group were significantly higher at 26.35 (7.01-108.7) ng/mL than in the control group at 19.5 (3.45-88.14) ng/mL (p = 0.018). NGAL/Cr ratio was also significantly higher in the IR group compared to the control group (p = 0.018).

Conclusion: Obese pediatric patients with IR were shown to have elevated levels of u-NGAL, a marker of renal injury. u-NGAL examination may show early renal injury before development of diabetes.

Keywords: NGAL, renal injury, child, KIM-1, insulin resistance

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Conflict of interest: None declared
Received: 27.01.2021
Accepted: 07.05.2021

Introduction

The impact of insulin resistance (IR) and obesity on chronic kidney disease has been reported (1,2,3,4). Obesity is an important driver of microvascular dysfunction (MVD) (5). The relationship between hyperglycemia and MVD is bidirectional and can be considered a vicious circle. Experimental data suggest that hyperglycemia may cause microvascular disease (6). MVD contributes to IR and the onset of type 2 diabetes mellitus (T2DM) (5) with a higher prevalence of comorbidities in youth (5,6,7). In addition, a reduction in hyperglycemia is associated with delay of onset and reduced progression of nephropathy. MVD precedes nephropathy (8,9,10).

The standard noninvasive diagnostic test currently used in clinical practice to predict the onset and monitor the progression of diabetic nephropathy is microalbuminuria measurement. However, this is a sign of early glomerular damage rather than a marker for susceptibility to it. Microalbuminuria has significant limitations in determining disease progression because of the observation that some type 1 diabetes mellitus (T1DM) patients revert to normoalbuminuria without treatment (11). Also, studies suggest that tubulointerstitial injury may precede the appearance of glomerulopathy in diabetic nephropathy (12,13). Therefore, identification and validation of new biomarkers for early diagnosis of kidney injury may help to predict nephropathy and progression (14). Biomarkers of tubular injury, such as urinary neutrophil gelatinase-associated lipocalin (NGAL), urinary kidney injury molecule 1 (KIM-1), serum cystatin C, urinary IgG, and transferrin have been investigated in pediatric and adult patients with T2DM (14,15,16,17,18). NGAL has been shown to be an early renal injury biomarker in type 2 DM (19). The aim of the present study was to investigate whether levels of these biomarkers, suggesting early renal damage, are elevated in obese children with IR before the development of diabetes.

Methods

Participants

This single-center, cross-sectional, case-control study included children aged between 7-18 years, who attended Manisa Celal Bayar University Hospital, Pediatric Endocrinology and Pediatric Outpatient Clinics with the complaint of obesity, between January 1, 2016, and May 31, 2018. Patients were divided into two groups: those with IR (IR group) and those without IR (control group).

Children with type 1 diabetes or obesity with a syndrome (Prader-Willi syndrome, Laurence Moon Biedl syndrome, etc.) or endocrinological or metabolic pathologies, or on dietary supplementation were excluded. Children with infection, kidney or other systemic diseases were also excluded from the study. None of the participants were using antihypertensive and/or lipid-lowering drugs.

Clinical and Laboratory Evaluation

All obese/overweight patients underwent a thorough physical examination and routine laboratory evaluation, including obesity screening tests, urinalysis, and urinary culture. Obesity screening tests included measurement of blood thyroid stimulating hormone (TSH), free thyroxine (fT4), fasting glucose, fasting insulin, lipid profile and estimation of homeostasis model assessment-IR (HOMA-IR). These assessments were all performed by a single, specially-trained clinical researcher. Demographic information was collected and urinary tract abnormalities and urinary tract infections were investigated.

The children and their families were informed about the study and written informed consent was obtained from participants. The Local Ethics Committee (Manisa Celal Bayar University/2015-20478486-217) approved the study in accordance with the Declaration of Helsinki.

Classification of Patients

Body mass index (BMI) was calculated using the standard formula; weight (in kg) divided by square of height in meters (m^2). BMI standard deviation score (SDS) and BMI percentiles were calculated using age and gender-specific norms published by Neyzi et al 2006 (20). Obesity was defined as BMI ≥95th percentile, and overweight was defined as BMI ≥85th for age and sex (21).

IR was evaluated according to the HOMA-IR index, which was calculated using the following formula: [fasting insulin (mU/mL) x fasting glucose (mg/dL)/405] (22). Cut-off values for different stages were prepubertal >2.5 and pubertal >4 (23).

Prediabetes was defined according to hemoglobin A1c (HbA1c) in the range 5.7-6.4% or fasting plasma glucose levels 100-126 mg/dL and/or two-hour plasma glucose levels 140-199 mg/dL following an oral glucose tolerance test (OGTT) (24).

Testing for diabetes was done by measuring HbA1c, with an HbA1c >6.5% concurrent with a random glucose level >200 mg/dL or fasting plasma glucose >126 mg/dL indicating diabetes. Alternatively, by performing an OGTT, with a post-OGTT 2-hour plasma glucose level >200 mg/dL also indicating diabetes (24). Patients with diabetes were excluded.

Blood pressure was taken with the appropriate cuff, systolic blood pressure, and diastolic blood pressure were measured twice, after a ten-minute rest, using the right arm and a calibrated sphygmomanometer and the mean of these two BP values were calculated. Hypertension was defined as a value above the 95th percentile for age and height, according to the National Health and Nutrition Examination Survey (25).

An ambulatory blood pressure monitoring (ABPM) device was applied on the same day. ABPM protocol was performed by a single investigator (Ç.Ö.). A validated recorder (Contec ABPM50, Germany) was used to measure BP at 20-min intervals from 8 AM to 11 PM and at 30-min intervals from 11 PM to 8 AM. The most appropriate original standard cuff was selected depending upon the individual's non-dominant arm. The participants were instructed to follow their usual daily activities, to avoid strenuous exercise and shower, to remain still with the forearm extended during measurements, to note the time when they went to bed and arose, and to detach the device 24 hours later. Measurements with systolic BP <240 and >70 mm Hg, diastolic BP <140 and >40 mm Hg, and diastolic BP $<$ systolic BP were accepted as valid (26). ABPM data with <30 valid daytime BP measurements and/or <12 nighttime measurements were not included (27).

Microalbumin levels were measured in a 24-hour urine sample obtained from all participants. Microalbuminuria was defined as a urinary albumin 30-300 mg/24 hours. Microalbuminuric patients were examined three times. If one result was negative, it was subsequently classified as normoalbuminuria (28).

Laboratory Measurements

All blood samples were collected in the morning after at least 8 hours of fasting for measurements of complete blood count and biochemical parameters, including obesity screening tests, urea, creatinine (Cr), and cystatin C. Obesity screening tests included fasting glucose, fasting insulin, HOMA-IR, TSH, fT4, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride measurements. Estimated glomerular filtration rate (eGFR) was calculated using the Schwartz formula (29).

The morning spot urine samples (at least 10 mL) were collected from all patients and centrifuged (3000 rpm for five minutes), and the supernatants were frozen at -80 °C until analysis. Urinary NGAL (u-NGAL) was measured by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (Human NGAL Platinum ELISA Kit, eBioscience, Vienna, Austria). Urine samples were diluted according to the kit package insert. u-NGAL levels were obtained by multiplying the results by 1000 (dilution factor). The kit sensitivity is 6.5 pg/mL. The average intra-assay precision CV value of the kit was calculated as 4.0%. KIM-1 levels in human urine samples were analyzed using the Human KIM-1 ELISA Test Kit (BioAssay Works, Ijamsville, USA). Urine samples were diluted according to the kit package insert. The obtained results were multiplied by the dilution factor (a six-fold multiplication).

Statistical Analysis

The sample size was calculated using the power analysis method; an effect size of 0.70 and a power of 0.80 (alpha 0.05) required a sample of 63/36 people for a case/control study.

Comparison of study variables was first made between patients with IR and control group. Between-group comparison for categorical variables was performed by using the χ^2 test, or Fisher's exact tests. All data were tested for normality using the Kolmogorov-Smirnov test or Shapiro-Wilks test. Mann-Whitney U test was used for comparison of non-normally distributed continuous and nonparametric variables, while the t-test was used for normally distributed variables. Univariate correlation analysis was performed between IR participants and healthy controls using the Spearman test. Statistical analyses were performed using Statistical Package for Social Sciences, version 15.0 (IBM Inc., Chicago, IL, USA). A $p<0.05$ was considered statistically significant.

Results

In this study, 99 obese and overweight children/adolescents were evaluated, of whom 95 (96%) were classified as obese and the remaining four were overweight. The median (range) age of the participants was 13 (7-18) years and 68 (68.7%) were female. Patients were divided into two groups based on the presence of IR or not. The group with IR consisted of 63 participants (male/female: 17/46) with a median (range) age of 13.9 (7-18) years. The group without IR consisted of 36 obese controls (male/female: 14/22) with a median (range) age of 12.1 (8-17) years. These two groups were similar in terms of age, gender and BMI (Table 1). Prediabetes was significantly more common in the IR group than the control group (20 vs. 3; $p=0.008$). There was no difference between the IR group and the control group in terms of prevalence of hypertension (17 vs. 9, respectively; $p=0.908$). Comparison of ABPM data between the two

Table 1. Sociodemographic characteristics, laboratory findings, urinary NGAL, urinary KIM-1 and serum cystatin C levels in obese insulin resistance group and control group

	Insulin resistance group (n = 63)	Control group (n = 36)	p
Age (years)	13.9 (7-18)	12.1 (8-17)	0.061
Gender (M/F) n (%)	17/46 (26.9/73.1)	14/22 (38.8/61.2)	0.263
BMI (kg/m^2)	29.8 (17-42)	28.1 (20-39)	0.063
SBP (mmHg)	115 (80-150)	115 (90-160)	0.923
DBP (mmHg)	70 (50-100)	70 (50-100)	0.587
Prediabetes n (%)	20	3	0.008
Fasting glucose (mg/dL)	86 (67-105)	84 (70-100)	0.486
Fasting insulin (mUI/L)	30.30 (17-60)	16.05 (8-21)	< 0.001
HOMA-IR	6.06 (4-14)	3.3 (2-4)	< 0.001
Blood urea (mg/dL)	21.7 (13-36)	24.0 (15-34)	0.402
Blood creatinine (mg/dL)	0.56 (0.1-1)	0.5 (0.1-1)	0.717
Uric acid (mg/dL)	5 (1.60-9.60)	4.85 (3.20-7.10)	0.155
eGFR (Schwartz) (mL/min/1.73 m^2)	119.5 (83.50-231)	120.2 (70.7-178.1)	0.608
Triglycerides (mg/dL)	114 (44-389)	96 (42-257)	0.167
Total cholesterol (mg/dL)	165 (103-254)	149.5 (91-219)	0.062
HDL-c (mg/dL)	45.1 (32-79)	47.05 (26-68)	0.417
LDL-c (mg/dL)	92 (19-187)	78.5 (34-131)	0.077
TSH (mIU/L)	2.5 (1.05-5.8)	2.55 (0.95-5.6)	0.954
Urinary NGAL (pg/mL)	26.35 (7.01-108.7)	19.5 (3.45-88.14)	0.018
NGAL/creatinine ratio (pg/mg)	0.27 (0.05-1.58)	0.16 (0.01-1.5)	0.018
KIM-1 (pg/mL)	0.84 (0-2.09)	0.85 (0-6.18)	0.789
KIM-1/creatinine ratio	0.01 (0-0.03)	0.008 (0-0.06)	0.570
Serum cystatin C (mg/L)	0.82 (0.28-1.0)	0.84 (0.7-1.0)	0.154
Cystatin-C eGFR median	93.7 (72.2-170.4)	90.75 (71.5-110.5)	0.138
Urinary protein/creatinine (mg/g)	0.04 (0.02-0.16)	0.04 (0.02-0.61)	0.994
Microalbuminuria (mg/24 hours)	6 (0-29)	6.8 (0-29.9)	0.252

BMI: body mass index, DBP: diastolic blood pressure, HDL-c: high density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment of insulin resistance, InsT0^1: fasting insulin, KIM-1: kidney injury molecule-1, LDL-c: low density lipoprotein cholesterol, NGAL: neutrophil gelatinase-associated lipocalin, SBP: systolic blood pressure, M/F: male/female, eGFR: estimated glomerular filtration rate, TSH: thyroid stimulating hormone

groups resulted in similar hypertension rates and nighttime BP SDS values. None of the subjects with hypertension were found to have a secondary cause. The percentages of systolic and diastolic dipping and the rate of non-dippers were similar between the two groups. None of the participants had leukocytosis, neutrophilia, thrombocytosis, elevated Cr or abnormal eGFR levels or hypothyroidism. The remaining laboratory analyses are shown in Table 1.

Median urinary microalbumin levels were similar in both groups (p = 0.252). Urinary KIM-1, KIM-1/Cr ratio, and serum cystatin C levels were similar between the IR and control groups. However median (range) u-NGAL levels and NGAL/Cr ratios were significantly greater in the IR group compared to the control group at 26.35 (7.01-108.7) vs. 19.5 (3.45-88.14) ng/mL (p = 0.018) and 0.27 (0.05-1.58) vs. 0.16 (0.01-1.5) (p = 0.018), respectively (see Table 1).

Discussion

In daily practice, measurement of microalbuminuria is used to screen and monitor renal injury in diabetes. This study investigated whether the prediction of renal injury is possible at the IR stage using biomarkers, such as serum cystatin C, urinary KIM-1, and u-NGAL, before diabetes has developed. The results suggest that NGAL measurements may be used as an early biomarker for renal injury in obese patients with IR in the absence of diabetes and microalbuminuria. However, the other biomarkers examined, serum cystatin C and urinary KIM-1, did not differ between the IR group and the non-IR controls in this study.

The increasing global rates of obesity in children and adolescents is strongly associated with IR, which in turn is associated with some conditions, including T2DM (30). Patients with youth-onset T2DM are at considerable risk for diabetic nephropathy and. eventually, renal failure in young

adulthood due to microvascular complications (31,32,33). As the onset of T2DM may be insidious in many cases, the real duration of the disease is often not known. This may be one of the reasons why there is a poorer correlation between albumin excretion rate and disease duration in T2DM compared to T1DM (30). At T2DM diagnosis, many patients already have microalbuminuria. Screening and monitoring of microalbuminuria should begin at the time of diagnosis and continue annually (30). Compared to T1DM, in young-onset T2DM microalbuminuria was observed more frequently, with earlier and rapid progression to diabetic nephropathy, due to IR (7,32,34,35,36,37). Our findings support this study results. IR may be the starting point of diabetic nephropathy. Our results suggest the presence of tubular kidney damage, as evidenced by elevated NGAL levels, in obese children with IR. Based on this finding, patients may need to be screened for biomarkers of tubulopathy at the IR stage, before T2DM develops. Experimental studies have shown that reduced insulin sensitivity and hyperinsulinemia are important factors leading to renal injury (38). It is accepted that insulin mediates renal function, primarily at the tubular level, as specific binding of insulin is greatest in the thick ascending limb and distal convoluted tubules (39). There is also evidence that insulin acts in the proximal tubules (40). A number of experimental studies have shown that hyperinsulinemia led to decreased nitric oxide levels, increased transforming growth factor-β1 and insulin-like growth factor-1 levels, and endothelin-1 production, resulting in increased oxidative stress (38,41,42,43,44). These mechanisms may explain the increased NGAL found in our IR group, which also had significantly elevated levels of fasting insulin compared to the control group.

NGAL is a member of the lipocalin family. Several studies suggest that NGAL might be a marker for a variety of conditions associated with lipid metabolism, such as obese-inflammation-induced metabolic syndrome (MetS), IR, disrupted glucose and lipid metabolism or endothelial dysfunction (45). It has been shown that NGAL is released from injured renal tubular cells in acute kidney injury before a decrease in the GFR can be detected (46). Furthermore, u-NGAL can be used as an early biomarker of diabetic nephropathy (47). The results of a meta-analysis, which also included pediatric studies, suggest that NGAL in urine can be considered a valuable biomarker for early detection of diabetic nephropathy in the normoalbuminuric stage. It is accepted that the pathophysiology and progression of diabetic nephropathy are associated with both glomerular and tubular interstitial damage, and it has been shown that, in the absence of glomerular proteinuria, tubular dysfunction can even precede glomerular injury and, thus, microalbuminuria. In recent studies, NGAL concentration was found to be increased in patients with T2DM, with or without albuminuria in subclinical tubular damage (14,19,48,49). However, most childhood studies have investigated the relationship between NGAL and tubular damage in T1DM (50,51,52,53,54). Also, it was shown that normal range albuminuria does not exclude nephropathy in children with T1DM (55). The present study is the first to demonstrate a relationship between renal tubular damage and NGAL in children with IR before T2DM development.

In adult T2DM patients, urinary KIM-1 levels were found to be a useful biomarker of renal impairment (56,57,58). However, in a study of obese children, urinary KIM-1 was shown to not be associated with renal injury (59). Similarly, studies in T2DM adults have demonstrated the clinical utility of measuring cystatin C as a useful marker of early renal impairment. In children cystatin C levels were shown to be elevated in obese subjects with MetS compared to those without MetS (60,61). The similarity in median urinary KIM-1 and serum cystatin C levels in the IR and control groups, while finding significant differences in median u-NGAL concentrations, suggest that NGAL is an earlier marker of renal effects in IR associated with pediatric obesity.

Study Limitations

There are some limitations to our study. The number of participants in the control group was low. The reversibility of the observed renal effects by treatment was not investigated. It was also not possible to estimate the duration of IR, and therefore its possible relationship to changes in the measured biomarkers in affected subjects. The gold standard for assessment of renal damage is a biopsy, but performance of renal biopsy in this study was not ethically acceptable.

Conclusion

In obese children with normoalbuminuric IR without T2DM, u-NGAL levels were significantly higher than in obese children without IR. This suggests that the former are at risk of early renal damage. To the best of our knowledge, this is the first pediatric study showing evidence of tubular damage, indicated by elevated NGAL in children with IR. We suggest that, because of the insidious onset of T2DM, screening for renal damage should be performed from the IR stage and urinary NGAL may be a useful marker for this. Further studies are needed to investigate the relationship between u-NGAL and definitive measures of renal damage, such as renal biopsy, in children with IR.

Acknowledgments

This study was funded by Scintific Research Institution of Manisa Celal Bayar University.

Ethics

Ethics Committee Approval: The Local Ethics Committee (Manisa Celal Bayar University/2015-20478486-217) approved the study in accordance with the Declaration of Helsinki.

Informed Consent: The children and their families were informed about the study and written informed consent was obtained from participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Semra Şen, Deniz Özalp Kızılay, Concept: Semra Şen, Deniz Özalp Kızılay, Design: Semra Şen, Deniz Özalp Kızılay, Data Collection or Processing: Semra Şen, Deniz Özalp Kızılay, Fatma Taneli, Çınar Özen, Pelin Ertan, İpek Özunan, Raziye Yıldız, Betül Ersoy, Analysis or Interpretation: Semra Şen, Deniz Özalp Kızılay, Literature Search: Semra Şen, Deniz Özalp Kızılay, Çınar Özen, Writing: Semra Şen, Deniz Özalp Kızılay.

Financial Disclosure: This study was funded by Scientific Research Institution of Manisa Celal Bayar University.

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0030

J Clin Res Pediatr Endocrinol 2021;13(4):408-417

Epicardial and Perihepatic Fat as Cardiometabolic Risk Predictors in Girls with Turner Syndrome: A Cardiac Magnetic Resonance Study

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What is already known on this topic?

Turner syndrome (TS) patients are at high risk of cardiometabolic disorders.

What this study adds?

TS girls displayed adverse cardiometabolic profile during late childhood and adolescence. Cardiac magnetic resonance-derived epicardial fat-thickness (EFT) and perihepatic fat-thickness are emerging cardiometabolic risk predictors in TS patients. Excess EFT, rather than total body adiposity, may contribute to altered metabolic profile among lean patients with TS.

Abstract

Objective: Turner syndrome (TS) patients are at high risk of cardiometabolic disorders. Cardiometabolic risk factors are more commonly related to visceral rather than total body adiposity. Adipocytokines have been explored as a potential link between obesity and obesity-related cardiometabolic dysfunction. This study explored the validity of epicardial fat-thickness (EFT) and perihepatic fat-thickness (PHFT) measurement as cardiometabolic-risk predictors in TS-girls in relation to standard obesity-indices and metabolic syndrome (MetS) components.

Methods: Forty-six TS girls and twenty-five controls (10-16 years) were subdivided into two age-groups (10 to less than 13 and 13-16). Participants were assessed for body mass index (BMI) Z-scores, waist circumference (WC), total-fat mass (FM) and trunk-FM by bioimpedance-technique, EFT and PHFT by cardiovascular magnetic resonance, lipid-profile, homeostasis model assessment of insulin resistance (HOMA-IR), and serum chemerin. MetS was defined according to International Diabetes Federation criteria.

Results: Overweight/obesity and MetS were detected in 45.7% and 37% of TS-girls respectively. BMI Z-score, WC, total-FM, trunk-FM, EFT and PHFT values were significantly higher in TS-age groups compared to age-matched control groups, being more pronounced in the older group when TS-girls had been exposed to estrogen. Dyslipidemia, higher HOMA-IR, chemerin, EFT and PHFT values were observed in lean-Turner compared to BMI-Z-matched controls. EFT and PHFT were significantly correlated with chemerin and several components of MetS. EFT at a cut-off-value of 6.20 mm (area under the curve = 0.814) can predict MetS in TS-girls.

Conclusion: TS-girls displayed an adverse cardiometabolic profile during late childhood and adolescence. EFT and PHFT are emerging cardiometabolic risk predictors in TS-patients. Excess EFT rather than total body adiposity may contribute to altered metabolic profile among lean-Turner patients.

Keywords: Epicardial fat, perihepatic fat, metabolic syndrome, Turner syndrome

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Conflict of interest: None declared
Received: 28.01.2021
Accepted: 19.05.2021

Introduction

Childhood obesity represents a major public-health crisis that has persistently increased in incidence throughout recent decades at an alarming rate (1). Metabolic syndrome (MetS) is a clustering of co-incident cardiometabolic risk factors, which predict adverse cardiovascular outcomes in adulthood (2).

Turner syndrome (TS) is one of the most common chromosomal disorders in females caused by complete or partial deficit of the second X-chromosome (3). Current epidemiological evidence indicates that children and adolescents with TS are susceptible to a wide spectrum of cardiometabolic risk factors compared to age-matched controls including: higher obesity-indices; impaired glucose metabolism; and atherogenic lipid profile (4,5,6,7).

According to the adipose tissue expandability hypothesis, excess visceral fat (within/or surrounding viscera) together with relatively less subcutaneous adipose tissue, elicit a state of chronic low-grade inflammation that increases the risk of cardiovascular diseases (8). Currently, visceral fat deposition has been identified as an emerging marker of cardiovascular risk (9). Epicardial adipose tissue, the visceral fat reservoir of the heart, is enclosed between the pericardium and the myocardium layers and secrets several adipocytokines, some of which are mediators of inflammation (10).

Cardiovascular magnetic resonance (CMR) imaging is considered the standard reference for epicardial adipose tissue quantification (11). Epicardial fat thickness (EFT) shows good correlation with visceral abdominal fat, components of MetS, and severity of cardiovascular diseases (12,13).

Chemerin, is an adipocytokine that modulates glucose and lipid metabolism in adipocytes (14). Chimerin has displayed strong associations with MetS components (15), and with EFT in adults with coronary artery disease (16). Thus, it may form an integral link between obesity and obesity-related cardiometabolic dysfunction (17).

Therefore, the aim was to explore for the first time the validity of EFT and perihepatic fat thickness (PHFT) as cardiometabolic risk predictors in girls with TS in relation to standard obesity-indices and components of MetS.

Methods

This case-control study included forty-six girls with TS (age range: 10-16 years) and twenty-five age-matched healthy girls. Girls with TS were recruited sequentially between September 2018 and November 2019 during their routine visits to the Pediatric Endocrinology Clinic at Mansoura University Children's Hospital. The study was approved by the Ethics Committee of Mansoura Faculty of Medicine-Institutional Research Board (code no. R.20.04.800). Informed consent was obtained from the parents of all participants included in the study.

Girls with TS follow a uniform protocol for recombinant human growth hormone (rGH) therapy; thirty-six girls were receiving rGH (0.05 mg/kg/day) while ten girls in the cohort had stopped rGH at a mean age of 14.4 ± 0.8 year as height velocity < 3 cm/year. Estrogen replacement therapy (ERT), oral ethinylestradiol at initial dose of 2 mg/day, was initiated for girls who had exhibited no clinical signs of spontaneous puberty by 14 years. Seven girls had spontaneous puberty at a mean age of 13.9 ± 0.4 years. TS girls having chronic comorbidities, thyroid dysfunction, congenital/acquired heart diseases or currently receiving medications, other than rGH and/or ERT, were excluded from the study.

Clinical Evaluation

Anthropometric measurements, including weight, height, and waist circumference (WC), were obtained by a trained nurse according to standardized techniques. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Height and BMI Z-scores were calculated using reference data for Egyptian children and adolescents (18). In girls with TS, BMI Z-score was corrected for patient's height age to adjust for the effect of short stature (19). Girls with TS were classified based on WHO BMI Z-score cut-offs into a "lean-group" [BMI Z-score $\leq +1$ standard deviation (SD)] or an "overweight/obese-group" (BMI Z-score $> +1$ SD) (20). Systolic and diastolic blood pressure (SBP/DBP) was measured by standard technique (21).

Participants were classified according to Tanner breast scale into pre-pubertal (stage 1), early-puberty (stages 2-3) and late-puberty (stages 4-5). Girls with TS were subdivided into two groups; pre-pubertal (10-to less than 13 years) and pubertal (13-16 years) groups, the latter group included both early-and late-puberty stages. The controls were subdivided into two groups; early-puberty (10-to less than 13 years) and late-puberty (13-16 years) groups.

Definition of Metabolic Syndrome

In the TS group, MetS was diagnosed according to the 2007 International Diabetes Federation (IDF) pediatric definition for MetS (2), with the exception of blood pressure for which "elevated blood pressure" was defined according to the 2017 Clinical Practice Guideline for Screening and Management of High Blood Pressure in Children and Adolescents (21). MetS was diagnosed by central adiposity (WC $\geq 90^{th}$ percentile for age and gender) and the presence of at least two of the

remaining four criteria which are: Fasting blood glucose ≥100 mg/dL (5.6 mmol/L); triglycerides levels ≥150 mg/dL (1.7 mmol/L); HDL-c level ≤40 mg/dL (1.03 mmol/L); and SBP and/or DBP ≥90th percentile for age, gender and height percentile.

Body Fat Composition Evaluation

Total-fat mass (FM; kg) and trunk-FM (kg), a marker of central (abdominal) adiposity, were measured by a bioimpedance technique using a Tanita BC-418MA body composition analyzer (Tanita Corp., Tokyo, Japan).

Biochemical Evaluation

Blood samples were collected in the morning, after a 12-hour overnight fast. Sera were stored at -20 °C. Total cholesterol and triglycerides were measured by colorometric kit (Spinreact, Girona, Spain), and HDL-c was measured by colorometric kit (Human Diagnostics, Wiesbaden, Germany). Serum chemerin (ng/mL) was measured using an enzyme-linked immunosorbent assay-Sandwich technique (SUN RED, Shanghai, China; cat. no. 201-12-1436). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as FBG (mg/dL) × fasting insulin (mIU/L)/405.

Cardiac Magnetic Resonance Imaging

The measurements of EFT and PHFT were performed by a single expert technician who was blinded to the study groups. CMR was performed using a 1.5 T Tesla MR imaging (MRI) machine (Ingenia, Philips, Netherland). Electrocardiogram gated cine steady state free precession (SSFP) images were acquired in short axis-view (SA) (slice thickness 5 mm, slice gap -1 mm, TR = 3.2 ms, TE = 1.6 ms, matrix 175/352, FOV = 350 mm^2, slices 25), while modified Dixon (mDixon) sequence was obtained in SA plane (slice thickness 5 mm, slice gap -2.5 mm, TR = 5.9 ms, TE = 0.0 ms, matrix 151/320, FOV = 400 mm^2, slices 92). Image analysis was performed by a single radiologist (N.B.) who was also blinded to the study groups. Images were transferred to workstation (extended MR Workspace 2.6.3.5, Philips medical systems, Netherland). Maximum EFT was measured opposite the right ventricular free wall in the following sequences; m-Dixon (SA-view) and cine SSFP (SA-view) at end systole and end diastole. Maximum PHFT was measured in sub-diaphragmatic region during cine SSFP (SA-view) (Supplementary Figure 1).

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences, version 23.0 (IBM Inc., Chicago, IL, USA). Categorical data are presented as number and percent, and chi-square test or Fisher exact test were used as appropriate for comparison of two groups. Continuous data were tested for normality using one-sample Kolmogorov-Smirnov test, then data presented as mean ± SD for parametric data and median (minimum-maximum) for non-parametric data. Two groups were compared using Student's t-test for parametric data and Mann-Whitney U test for non-parametric data. Pearson and Spearman correlation analysis were used to correlate parametric and non-parametric data respectively. Receiver operating characteristic (ROC) curves were constructed to analyze the discriminative power of EFT, and PHFT to predict MetS among girls with TS. Areas under curves (AUCs) and 95% confidence interval, optimal cut-off-values with relevant specificity, sensitivity, and accuracy were determined. Statistical significance was set at $p < 0.05$.

Results

The study included two age-matched groups, Turner-group (n = 46) with a mean age of 13.14 ± 3.15 years and a control-group (n = 25) with a mean age of 12.17 ± 3.02. Based on karyotype results, two groups were identified; 45,X group (n = 24) and non-45,X group (n = 22), the latter group including isochromosome 46,X,i[Xq10] (n = 12), deletion 46,X,del(Xp-) (n = 4), and different forms of mosaicism (n = 6). No significant differences were detected in cardiometabolic variables between 45,X and non-45,X groups ($p > 0.05$) (Supplementary Table 1).

Girls with TS in the pre-pubertal (10-13 years) group displayed significantly higher total-FM, trunk-FM, serum cholesterol, HOMA-IR and EFT values compared to age-matched controls. Girls with TS in the pubertal (13-16 years) group displayed similar significant differences to the pre-pubertal group with the addition of significantly higher BMI Z-score, WC, serum triglycerides, and PHFT values compared to age-matched control groups (Table 1).

Based on BMI status, the overall prevalence of overweight/obesity in girls with TS was 45.7% (21/46). When the age groupings were considered separately this was 8/26 (30.8%) in the pre-pubertal group and 13/20 (65%) within pubertal group. Overweight/obese Turner-group were significantly older and had significantly higher BMI Z-score, WC, total-FM, trunk-FM, HOMA-IR, serum cholesterol and triglycerides, EFT and PHFT values compared to both lean-Turner and control groups. Interestingly, lean-Turner had significantly higher HOMA-IR, serum cholesterol, and EFT values compared to controls, although BMI Z-score, total-FM, and trunk-FM were significantly lower in lean-Turner compared to age-matched controls (Table 2).

Based on IDF criteria (2), the prevalence of MetS in TS girls was 37% (17/46). Girls with TS were then sub-classified into a "MetS-group" (n = 17) and a "non-MetS group" (n = 29) and compared. All girls in the MetS-group were obese, while in the non-MetS group, twelve girls (41.4%) were overweight/obese. BMI Z-score, WC, total-FM, trunk-FM, HOMA-IR, cholesterol, triglycerides, EFT and PHFT were significantly higher in the MetS-group compared to the non-MetS and control groups. Although non-MetS and control groups were matched for BMI Z-score and WC, non-MetS group displayed significantly higher total-FM, trunk-FM, HOMA-IR, cholesterol and EFT compared to control girls (Table 3).

Serum chemerin levels were significantly higher in TS participants compared to age-matched control-groups; in overweight/obese-Turner compared to lean-Turner ($p = 0.014$) and control ($p < 0.001$) groups; in the MetS-group compared to the non-MetS ($p = 0.044$) and control ($p < 0.001$) groups; and in non-MetS and lean-Turner groups compared to controls. Serum chemerin was positively correlated with age, BMI Z-score, WC, total-FM, trunk-FM, HOMA-IR, triglycerides, EFT and PHFT in TS (Table 4). However, these correlations were not evident in the control group (Supplementary Table 2).

EFT and PHFT were positively correlated, EFT at different sequences including; m-Dixon (SA-view) and cine SSFP (SA-view) at end systole and end diastole were positively correlated with age, BMI Z-score, WC, HOMA-IR, triglycerides, total-FM, and trunk-FM, while PHFT showed similar correlations but was not correlated with triglycerides (Table 4).

Regarding prediction of MetS among TS girls, EFT in mDixon SA-view with a cut-off-value of 6.20 mm had the highest discriminative power among CMR-derived parameters (AUC = 0.814) (84.6% sensitivity; 73.5% specificity), while PHFT with a cut-off-value of 5.15 mm had the lowest discriminative power (AUC = 0.685). AUC of EFT at mDixon SA-view was comparable to those of standard cardiometabolic risk factors; BMI Z-score (AUC = 0.998), WC (AUC = 0.955), HOMA-IR (AUC = 0.899), and triglycerides (AUC = 0.885). Finally, serum chemerin of more than 250 ng/mL can predict MetS in girls with TS with AUC = 0.834, 76.9% sensitivity and 77.6% specificity (Table 5 and Figure 1a, 1b).

Interestingly, Supplementary Figure 1 represents CRM imaging of a lean Turner girl aged 15 years and 2 months (height age-adjusted BMI Z-score = 0.9), although lean, the

Table 1. Clinical, body composition, biochemical and cardiovascular magnetic resonance parameters among Turner syndrome and control age-groups

	Turner group (n = 46)		Control group (n = 25)		Test of significance	
	10-13 years (n = 26)	13-16 years (n = 20)	10-13 years (n = 10)	13-16 years (n = 15)	p1	p2
Clinical parameters						
Median BMI Z-score	0.43 (-1.1, 4)	1.5 (-1.5, 3.1)	0.9 (-0.6, 1.0)	0.7 (0.0, 1.0)	0.549	0.031*
Mean WC (cm)	66.69 ± 8.82	76.75 ± 7.79	66.54 ± 5.51	72.71 ± 2.69	0.960	0.042*
Body composition parameters						
Median total body FM (kg)	7.80 (5.5-17.4)	13.45 (7.2-28.9)	5.10 (3.43-8.6)	10.0 (5.4-13.8)	0.022*	0.019*
Median trunk FM (Kg)	3.20 (2.5-7.9)	7.15 (3.4-15.9)	2.20 (0.7-3.8)	4.20 (0.6-6.5)	0.010*	0.025*
Biochemical parameters						
Median HOMA-IR	2.11 (0.96-5.43)	4.27 (0.92-8.26)	1.66 (0.79-2.01)	1.48 (1.06-2.23)	0.047*	0.001*
Mean cholesterol (mg/dL)	145.6 ± 26.7	176.8 ± 35.5	121.5 ± 9.5	136.3 ± 11.1	0.011*	< 0.001*
Mean triglycerides (mg/dL)	95.5 ± 27.5	111.8 ± 40.5	80.4 ± 10.7	78.1 ± 13.8	0.088	0.041*
Mean HDL (mg/dL)	54.61 ± 13.73	55.66 ± 13.07	56.83 ± 11.68	49.57 ± 5.25	0.139	0.242
Median chemerin (ng/mL)	249.4 (108.5-388.5)	353.5 (109.3-630.3)	128.5 (35.5-136)	131.0 (119-175)	0.033*	0.008*
CMR parameters						
Mean EFT-SA mDixon (mm)	5.26 ± 1.65	7.64 ± 1.80	4.68 ± 0.51	5.18 ± 1.17	0.294	0.002*
Mean EFT-SA systole (mm)	6.35 ± 1.64	9.19 ± 2.04	4.55 ± 0.51	5.17 ± 0.63	0.002*	< 0.001*
Mean EFT-SA diastole (mm)	3.79 ± 1.14	4.89 ± 1.61	2.38 ± 0.31	2.94 ± 0.47	0.001*	0.004*
Mean PHFT (mm)	5.62 ± 1.86	6.31 ± 2.37	4.57 ± 0.38	4.99 ± 1.06	0.134	0.023*

Data presented either as mean ± SD or median (minimum-maximum).
*Significant difference ($p < 0.05$).
p1: Turner group vs. control group (10-13 years); p2: Turner group vs. control group (13-16 years).
BMI: body mass index, CMR: cardiovascular magnetic resonance, EFT: epicardial fat thickness, FM: fat mass, HOMA-IR: homeostasis model assessment of insulin resistance, HDL: high density lipoprotein, SA: short axis view, PHFT: perihepatic fat thickness, WC: waist circumference, SD: standard deviation

results of EFT in different sequences and PHFT exceeded the established cut-off values derived from ROC analysis (Table 5).

Discussion

In the current study and for the first time, the clinical relevance of CMR-derived EFT and PHFT as cardiometabolic risk predictors in girls with TS in relation to standard obesity-indices and components of MetS was investigated.

Data on the prevalence of MetS in pediatric and adult TS cohorts are limited. In the current study, the prevalence of overweight/obesity and MetS were 45.7% (21/46), and 37% (17/46) respectively. In adult TS, Calcaterra et al (22) reported a prevalence of MetS of 4.7% (4/85), whereas Álvarez-Nava et al (23) reported a prevalence of overweight/obesity and MetS to be 40% (35/88) and 49% (43/88), respectively. In a pediatric TS cohort (n = 19), O'Gorman et al (5) found that 7/19 girls met one criterion for MetS, 8/19 met two criteria, but none fulfilled the diagnosis of MetS. The discrepancy in the prevalence of MetS may mostly be related to ethnic differences in rates of obesity and MetS components and different criteria used to define MetS. In the current study it was observed that, although Turner girls in the non-MetS group were age, BMI Z-score and WC-matched with control girls, they had significantly higher total-FM, trunk-FM, HOMA-IR, total cholesterol and EFT values. These findings can be explained because 41.4% of girls in the non-MetS group were overweight/obese.

However, although BMI Z-score, total-FM, and trunk-FM values were significantly higher in the control group compared to lean-Turner, interestingly, the lean-Turner group had significantly higher HOMA-IR, total cholesterol, and EFT values compared to control girls, together with the significant associations between EFT and PHFT and triglycerides and HOMA-IR. These observations support the utility of visceral adipose tissue deposition measurement from traditional measurements of body adiposity, and also highlight that visceral fat, rather than subcutaneous adipose tissue, is more metabolically active and has a key role in the development of different cardiometabolic risk factors (8).

Similar associations between EFT and cardiometabolic risk factors and with increased carotid intima-media thickness

Table 2. Clinical, body composition, biochemical and cardiovascular magnetic resonance parameters in girls with Turner syndrome according to BMI status compared to control group

	Turner group (n = 46)		Control group (n = 25)	Test of significance		
	Overweight/obese (n = 21)	Lean (n = 25)		p1	p2	p3
Clinical parameters						
Mean age (years)	14.46 ± 2.26	12.04 ± 3.41	12.17 ± 3.02	0.007*	0.879	0.006*
Median height Z-score	-3.65 (-4.8--2.1)	-2.89 (-5.0--1.3)	0.90 (-1.5-1.7)	< 0.001*	< 0.001*	0.165
Median BMI Z-score	2.15 (1.1-4.0)	-0.15 (-1.7-1.0)	0.70 (0.0-1.0)	< 0.001*	< 0.001*	< 0.001*
Mean WC (cm)	78.81 ± 6.66	63.72 ± 7.96	66.02 ± 7.28	< 0.001*	0.292	< 0.001*
Body composition parameters						
Median total body FM (kg)	12.00 (4.6-31.9)	4.50 (2.4-14.0)	7.60 (2.6-10.8)	< 0.001*	< 0.001*	< 0.001*
Median trunk FM (kg)	4.70 (2.1-15.9)	1.60 (0.6-5.5)	3.20 (1.2-6.5)	0.001*	< 0.001*	< 0.001*
Biochemical parameters						
Median HOMA-IR	4.15 (1.82-8.26)	2.29 (0.92-4.71)	1.48 (0.35-2.23)	< 0.001*	0.001*	0.004*
Mean total cholesterol (mg/dL)	185.33 ± 39.33	155.16 ± 15.49	117.04 ± 10.35	< 0.001*	0.010*	0.007*
Mean triglycerides (mg/dL)	116.52 ± 35.79	74.72 ± 9.24	80.04 ± 10.97	< 0.001*	0.161	0.007*
Men HDL (mg/dL)	52.90 ± 14.07	51.84 ± 13.73	51.80 ± 10.11	0.759	0.991	0.797
Median chemerin (ng/mL)	344.90 (109.3-630.3)	221.70 (45.0-290.1)	119.00 (20.4-175.0)	< 0.001*	< 0.001*	0.014*
CMR parameters						
Mean EFT-SA mDixon (mm)	7.24 ± 2.04	5.69 ± 2.12	4.64 ± 0.97	< 0.001*	0.031*	0.016*
Mean EFT-SA systole (mm)	8.71 ± 2.73	6.54 ± 2.22	4.58 ± 0.77	< 0.001*	< 0.001*	0.005*
Mean EFT-SA diastole (mm)	4.88 ± 1.76	3.27 ± 1.05	2.87 ± 0.65	< 0.001*	0.053	0.013*
Mean PHFT (mm)	6.97 ± 2.66	5.11 ± 1.14	4.99 ± 1.06	0.001*	0.702	0.003*

Data presented either as mean SD or median (minimum-maximum).
*Significant difference ($p < 0.05$).
p1: overweight/obese TS vs. control; p2: Lean TS vs. control; p3: overweight/obese TS vs. Lean TS.
BMI: body mass index, CMR: cardiovascular magnetic resonance, EFT: epicardial fat thickness, FM: fat mass, HOMA-IR: Homeostasis model assessment of insulin resistance, HDL: high density lipoprotein, SA: short axis view, PHFT: perihepatic fat thickness, WC: waist circumference, SD: standard deviation

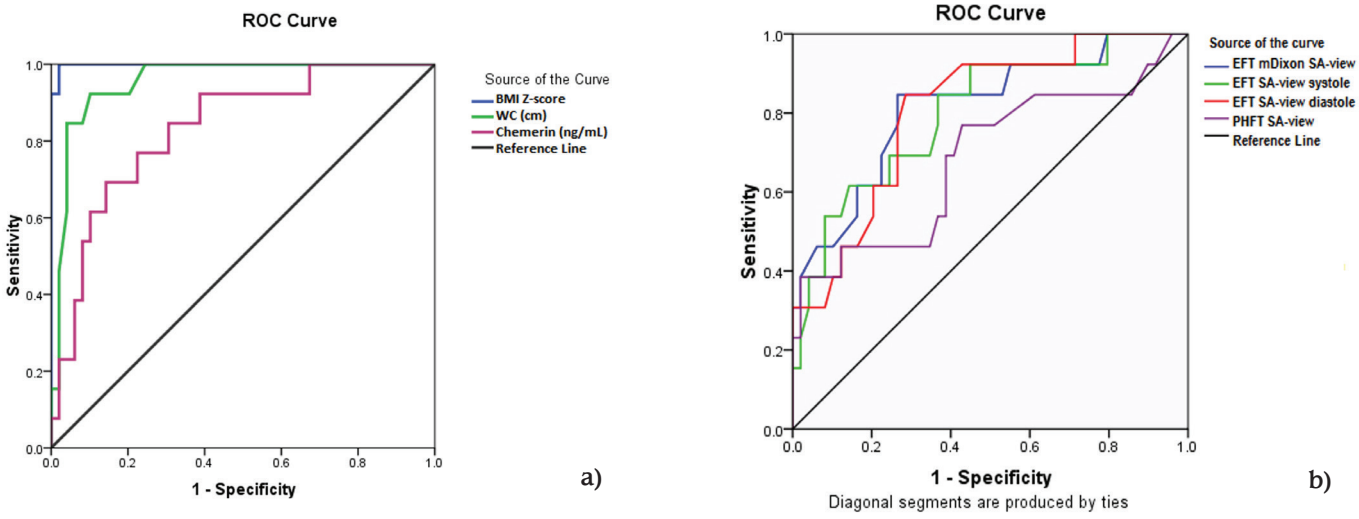

Figure 1. a) Receiver operating characteristic (ROC) curve for prediction of metabolic syndrome from BMI Z-score, WC and serum chemerin in girls with Turner syndrome. b) ROC curve for prediction of metabolic syndrome from epicardial fat thickness sequences and perihepatic fat thickness in girls with Turner syndrome

BMI: body mass index, WC: waist circumference, ROC: receiver operating characteristic, EFT: epicardial fat thickness, SA: short axis view

in obese children and adolescents were reported (24,25,26). In previous studies conducted among TS cohorts, O'Gorman et al (5) have demonstrated a significant increase in MRI-derived subcutaneous adipose tissue with no significant differences in MRI-derived intra-myocellular lipid measured by MRI or in bioimpedance-derived body-FM between young TS girls and age-and BMI Z-score-matched controls, while Ostberg et al (27) demonstrated increased intrahepatocellular lipids using MRI in adult TS cohorts.

ROC analysis to assess the validity of EFT and PHFT in prediction of MetS in girls with TS revealed that EFT measured in an mDixon SA-view with a cut-off-value of 6.20 mm had the highest discriminative power (AUC = 0.814), while PHFT had the lowest discriminative power (AUC = 0.685) among CMR-derived parameters. The results of EFT aproached those of standard cardiometabolic risk factors and also that of serum chemerin (AUC = 0.834).

Currently, there is no consensus for EFT cut-off values and most previous studies yielded different EFT cut-off values, mostly related to differences in samples, such as age, ethnicity and degree of obesity. Abaci et al (28) reported an EFT cut-off value of 4.1 mm for prediction of IR (90% sensitivity and 61% specificity), while Okyay et al (29) reported an EFT cut-off value of 4.35 mm for prediction of MetS (61.7% sensitivity; 79.2% specificity) in obese children. However, there is almost no data available concerning PHFT measurements and no reported specific cut-off value for PHFT in the literature.

Interestingly, Supplementary Figure 1 shows the CRM imaging of a lean Turner girl aged 15 years and two months with a height/age-adjusted BMI Z-score of 0.9. Although she was lean, the results of the EFT at different sequences and PHFT exceeded the established cut-off values derived from ROC analysis in the current study.

Currently, two opposite phenotypes have been reported which are the "metabolically healthy obese" and the "metabolically obese but normal-weight" (30). The underlying mechanism of such apparent dissociation is not fully understood. However, advances in non-invasive imaging techniques are making significant inroads, allowing understanding of the fundamental contribution of visceral adiposity and fat distribution in such phenotypes, which potentially mediate their metabolic effects through adipocytokine production. Therefore, excess EFT rather than total-FM may explain the altered metabolic profile among the lean-Turner group with a potential role of chemerin as an adipocytokine.

The results of the current study revealed higher serum chemerin levels in overweight/obese-Turner compared to lean-Turner and control groups, and also in the MetS-group compared to non-MetS and control groups. It is worth noting that serum chemerin was significantly higher in non-MetS than in controls, despite being matched for age, BMI Z-scores, and WC. This could be due to 41.4% of girls in the non-MetS group being overweight/obese. However, serum chemerin levels were significantly higher in lean-Turner than control girls, despite control girls having higher total-FM, and trunk-FM. The significantly increased EFT and PHFT in lean-Turner compared to control group together with significant correlation between chemerin and EFT and PHFT may point to the greater contribution of visceral fat

(EF and PHF) rather than subcutaneous fat as a source of circulating chemerin.

In addition, positive correlations between chemerin and BMI Z-score, WC, FM, HOMA-IR, triglycerides, EFT, and PHFT were evident in the TS group but not in the control group, supporting the possible role of chemerin in mediating metabolic derangement in TS patients and indicating that serum chemerin may have increased in association with a pathological increase in body mass and excess body adiposity. Similarly, in previous studies, serum chemerin displayed strong associations with components of MetS (15), and with EF-volume (16). Thus, chemerin may form a pivotal link between obesity and obesity-related cardiometabolic dysfunction (17). In a recent study, chemerin levels were significantly increased in girls with TS compared with age- and BMI-matched controls but was not correlated with age, BMI Z-score, or any of the metabolic parameters including; fasting blood glucose, fasting insulin, HOMA-IR, triglyceride, and non-HDL (31).

In the current study, young TS patients displayed increased risk for overweight/obesity and for adverse cardiometabolic profile, whereas the metabolic derangements (high cholesterol and HOMA-IR), unfavorable body composition (increased total-FM and trunk-FM) and increased visceral adiposity (EFT) start to be evident in girls with TS in pre-pubertal (10-13 years) group, while older TS girls in the pubertal (13-16 years) group who had been eventually exposed to estrogen displayed similar abnormalities in addition to higher BMI Z-scores, WC, serum triglycerides, and PHFT values compared to age-matched control groups. Similarly, in a recent longitudinal study, metabolic comorbidities were found to start in childhood, increasing the risk for cardiovascular disease across the Turner patient's lifespan (7). These findings reinforce the importance of annual screening for cardiometabolic risk factors and early counseling regarding healthy nutrition and active lifestyle in young TS girls (3).

Study Limitations

The small sample size of the current TS cohort precluded reliable evaluation of cardiometabolic profile, EFT and PHFT of different karyotypes groups and the cross-sectional design precluded the evaluation of the beneficial/or adverse effects of rGH and ERT in the context of cardiometabolic

Table 3. Clinical, body composition, biochemical and cardiovascular magnetic resonance parameters among Turner syndrome subgroups (with and without metabolic syndrome) and control group

	Turner syndrome (n = 46)		Control (n = 25)	Test of significance		
	MetS group (n = 17)	Non-MetS group (n = 29)		p1	p2	p3
Clinical parameters						
Mean age (years)	15.26 ± 1.77	13.86 ± 2.07	13.45 ± 2.04	0.012	0.515	0.046
Median BMI Z-score	2.53 (1.9, 4.0)	0.54 (-1.5, 2.1)	0.68 (0.2, 0.9)	< 0.001*	0.607	< 0.001*
Mean WC (cm)	82.00 ± 4.30	68.46 ± 7.85	66.82 ± 5.51	< 0.001*	0.867	< 0.001*
Body composition parameters						
Median total body FM (kg)	13.70 (11.2-31.9)	5.35 (3.4-14.0)	4.80 (2.6-5.1)	< 0.001*	0.017*	< 0.001*
Median trunk FM (kg)	5.70 (3.4-15.9)	2.50 (0.6-7.2)	1.75 (1.2-2.2)	< 0.001*	0.013*	< 0.001*
Biochemical parameters						
Median HOMA-IR	5.81 (2.35-8.26)	3.13 (0.92-4.31)	1.48 (0.35-2.23)	< 0.001*	< 0.001*	0.003*
Mean total cholesterol (mg/dL)	183.53 ± 34.87	154.50 ± 18.97	118.68 ± 9.89	< 0.001*	< 0.001*	0.029*
Mean triglycerides (mg/dL)	131.92 ± 29.09	92.16 ± 33.39	79.58 ± 11.64	< 0.001*	0.096	0.001*
Mean HDL (mg/dL)	50.07 ± 13.86	53.25 ± 14.22	54.16 ± 10.28	0.346	0.809	0.518
Median chemerin (ng/mL)	378.80 (115.7-630.3)	245.95 (108.5-335.7)	104.50 (20.4-166.9)	< 0.001*	< 0.001*	0.044*
CMR parameters						
Men EFT-SA mDixon (mm)	7.87 ± 2.07	6.22 ± 1.86	4.90 ± 0.82	< 0.001*	0.004	0.019*
Mean EFT-SA systole (mm)	9.06 ± 2.75	6.72 ± 1.98	4.78 ± 0.63	< 0.001*	< 0.001*	0.042*
Mean EFT-SA diastole (mm)	5.16 ± 1.98	4.14 ± 1.13	2.59 ± 0.46	0.001*	< 0.001*	0.053
Mean PHFT (mm)	7.31 ± 3.03	5.67 ± 1.53	5.37 ± 0.89	0.042*	0.452	0.034*

Data presented either as mean ± SD or median (minimum-maximum).
*Significant difference ($p < 0.05$).
p1: MetS vs. control; p2: non-MetS vs. control; p3: MetS vs. non-MetS.
BMI: body mass index, CMR: cardiovascular magnetic resonance, EFT: epicardial fat thickness, FM: fat mass, HOMA-IR: homeostasis model assessment of insulin resistance, HDL: high density lipoprotein, MetS: metabolic syndrome, SA: short axis view, PHFT: perihepatic fat thickness, WC: waist circumference, SD: standard deviation

profile, EFT and PHFT. Nevertheless, our study is the first to identify the validity of EFT and PHFT as cardiometabolic risk predictors in TS patients. Thus it is to be hoped that this study will provide a stimulus for future larger studies.

Conclusion

Girls with TS display adverse cardiometabolic profile during late childhood and adolescence. CMR-derived EFT and PHFT are emerging tools for assessment of cardiometabolic risk and for prediction of MetS in a high-risk population, such as TS patients. There is an evident need to establish specific cut-off values for EFT and PHFT and when this has been achieved, it will improve the subsequent utility of EFT and PHFT as screening tools and follow-up markers for cardiometabolic risk in high-risk populations, such as girls with TS.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Mansoura Faculty of Medicine-Institutional Research Board (code no. R.20.04.800).

Table 4. Correlation analyses of serum chemerin, epicardiac fat thickness and perihepatic fat thickness with clinical, biochemical, and body composition parameters in Turner syndrome group

		Serum chemerin	PHFT	EFT-SA mDixon	EFT-SA systole	EFT-SA diastole
Age	r	0.508	0.406	0.621	0.622	0.369
	p	<0.001*	0.005*	<0.001*	<0.001*	0.012*
BMI Z-score	r	0.368	0.417	0.343	0.373	0.251
	p	0.012*	0.004*	0.020*	0.011*	0.093
WC	r	0.425	0.535	0.442	0.505	0.347
	p	0.003*	<0.001*	0.002*	<0.001*	0.018*
Total body FM	r	0.483	0.378	0.450	0.477	0.399
	p	0.001*	0.010*	0.002*	0.001*	0.006*
Trunk FM	r	0.431	0.326	0.363	0.393	0.340
	p	0.003*	0.027*	0.013*	0.007*	0.021*
HOMA-IR	r	0.652	0.358	0.430	0.372	0.306
	p	<0.001*	0.014*	0.003*	0.011*	0.039*
Triglycerides	r	0.500	0.157	0.344	0.268	0.228
	p	0.011*	0.296	0.019*	0.072	0.127
Chemerin	r	-	0.448	0.535	0.443	0.394
	p	-	0.002*	<0.001*	0.002*	0.007*
PHFT	r	-	-	0.494	0.491	0.416
	p	-	-	<0.001*	0.001*	0.004*

*Significant correlation, r: regression coefficient.
BMI: body mass index, EFT: epicardial fat thickness, FM: fat mass, HOMA-IR: homeostasis model assessment of insulin resistance, SA: short axis view, PHFT: perihepatic fat thickness, WC: waist circumference

Table 5. ROC curves for the traditional cardiometabolic risk factors, serum chemerin, epicardial fat thickness and perihepatic fat thickness in the diagnosis of metabolic syndrome in girls with Turner syndrome

	AUC	95% CI	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
BMI Z-score	0.998	0.99-1.0	1.97	92.3	98	92.3	95.8	94.6
WC (cm)	0.955	0.905-1.0	76.50	92.0	90.8	75	95.2	86.4
HOMA-IR	0.899	0.819-0.979	3.32	91.5	77.6	66.7	92.8	81.1
Triglycerides (mg/dL)	0.885	0.799-0.972	94.0	92.1	69.4	63.1	92.8	78.4
Chemerin (ng/mL)	0.834	0.715-0.952	250.1	76.9	77.6	62.5	85.7	75.6
EFT-SA mDixon (mm)	0.814	0.677-0.951	6.20	84.6	73.5	64.7	90	78.4
EFT-SA systole (mm)	0.800	0.662-0.938	6.15	83.8	63.3	55	88.2	70.3
EFT-SA diastole (mm)	0.807	0.681-0.933	3.55	80.9	71.4	61.1	89.4	75.7
PHFT (mm)	0.685	0.502-0.868	5.15	72.7	57.1	50	82.3	64.8

AUC: area under the curve, CI: confidence interval, PPV: positive predictive value, NPV: negative predictive value, BMI: body mass index, EFT: epicardial fat thickness, HOMA-IR: homeostasis model assessment of insulin resistance, SA: short axis view, PHFT: perihepatic fat thickness, WC: waist circumference, ROC: receiver operating characteristic

Informed Consent: Informed consent was obtained from the parents of all participants included in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Nanees A. Salem, Nihal M. Batouty, Wafaa Laimon, Design: Nanees A. Salem, Nihal M. Batouty, Wafaa Laimon, Data Collection or Processing: Nanees A. Salem, Nihal M. Batouty, Wafaa Laimon, Analysis or Interpretation: Nanees A. Salem, Nihal M. Batouty, Ahmed M. Tawfik, Donia M. Sobh, Basma Gadelhak, Shimaa R. Hendawy, Wafaa Laimon, Literature Search: Nanees A. Salem, Nihal M. Batouty, Ahmed M. Tawfik, Donia M. Sobh, Basma Gadelhak, Shimaa R. Hendawy, Wafaa Laimon, Writing: Nanees A. Salem, Nihal M. Batouty.

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

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Click for Supplementary Figure 1, Supplementary Table 1 and Table 2 access link: http://glns.co/6gg7o

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0033

J Clin Res Pediatr Endocrinol 2021;13(4):418-425

Precocious Puberty in Boys: A Study Based on Five Years of Data from a Single Center in Northern China

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What is already known on this topic?

The etiology of precocious puberty (PP) in boys is diverse.

What this study adds?

Although previous studies have shown that the majority of central PP (CPP) cases in boys are pathological, our findings showed a high prevalence of idiopathic CPP in Northern China.

Abstract

Objective: To evaluate the clinical features and etiology of precocious puberty (PP) in Chinese boys.
Methods: In this study, data from boys who were referred for evaluation of PP from 2015 to 2020 at a tertiary hospital in Northern China were retrospectively analyzed.
Results: Eighty-two boys were diagnosed with PP from 2015 to 2020. Sixty-two patients (75.6%) were diagnosed with central PP (CPP), and twenty patients (24.4%) were diagnosed with peripheral PP (PPP). In the CPP group, forty-nine cases were classified as idiopathic CPP, and thirteen patients had pathogenic CPP. The top three causes of PPP were congenital adrenal hyperplasia, germ cell tumors and familial male-limited PP.
Conclusion: The etiology of PP in males is diverse. The majority of CPP cases in Chinese boys are idiopathic rather than organic.
Keywords: Male, precocious puberty, etiology

Introduction

Precocious puberty (PP) in boys refers to the development of secondary sexual characteristics before nine years of age. The prevalence of PP is approximately 10 times lower in boys than in girls (1). Female PP is mostly idiopathic, while male PP may be due to pathological factors. However, there are fewer studies about male than female PP, and the male studies have tended to include fewer patients. The proportion of organic central PP (CPP) in males is 26~64% (2,3,4). Over 15 years in South Korea, Lee et al (3) reported that 38% (27/71) of male PP cases were organic CPP, and the majority were due to intracranial diseases or tumors. Topor et al (4) reported all cases of male CPP seen at a US pediatric hospital for 10 years; almost two-thirds of the boys were overweight/obese, and more than 60% had neurogenic CPP. Neurofibromatosis type 1 (NF1) was the most common diagnosis. In one Polish study, 16 boys were referred for hospital evaluation because of pubertal signs; 50% were diagnosed with precocious adrenarche, four with central nervous system (CNS) tumors, two with congenital adrenal hyperplasia (CAH), and one each with a Leydig cell tumor and idiopathic CPP (5).

Few studies have analyzed peripheral PP (PPP) and CPP together, and most studies have focused solely on either

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Conflict of interest: None declared
Received: 01.02.2021
Accepted: 19.05.2021

male CPP or male PPP. We did not focus exclusively on CPP or PPP because central and peripheral manifestations sometimes overlap. In fact, males with PPP can present with CPP because of complexity. Studies from different countries and regions seem to support the role of pathological factors in males with PP, but the etiology is not the same. Data on male PP are still insufficient. Therefore, this retrospective study of male PP diagnosed at tertiary hospitals in northern China was conducted and the clinical manifestations and etiology were analyzed.

Methods

Subjects and Clinical Assessment

This retrospective study included patients who were evaluated for PP between 2015 and 2020 at the Department of Endocrinology, Children's Hospital Capital Institute of Pediatrics, Beijing, China. PP was defined as the onset of secondary sexual characteristics before 9 years of age, and the medical records of each patient were complete (6). Patients who had previously been diagnosed at other hospitals and those with variants of PP, such as premature adrenarche, were excluded from the analysis.

The clinical, laboratory, radiological, and molecular study data (if needed) were evaluated for all patients. Chinese growth charts for children were applied to interpret growth data, which were described as standard deviation (SD) score values (7). The diagnosis of CPP was made based on a clinical evaluation, which included taking a detailed history of the patient and his caregivers, followed by a physical examination and Tanner staging. Testicular volume was assessed using an orchidometer. All boys included in this study underwent blood collection for the measurement of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone (T). Bone age was assessed by a single pediatric endocrinologist and a radiologist, using the comparative method of Greulich and Pyle. Cranial/pituitary magnetic resonance imaging (MRI) was mandatory in all cases, and cerebrospinal fluid (CSF) sampling was required when a germ cell tumor (GCT) was strongly suspected. A diagnosis of CPP was confirmed via elevated LH response in a gonadotropin-releasing hormone (GnRH) analog stimulation test using subcutaneous triptorelin at a dose of 0.1 mg/m^2, to a maximum of 0.1 mg (Ferring, Hoechst, Frankfurt, Germany). CPP was defined as peak LH $>$ 7 IU/L and bone age at least 1 year greater than chronological age (8).

CAH was diagnosed based on elevated 17-hydroxyprogesterone (17-OHP), progesterone and adrenocorticotropic hormone; decreased cortisone, with or without hyperkalemia and hyponatremia; and a computed tomography (CT) or ultrasound scan suggesting adrenal hyperplasia (9). Patients with CNS GCTs were diagnosed through histopathology and/or elevated CSF human chorionic gonadotropin (hCG) levels (normal: $<$ 0.1 IU/L) and the appearance of an intracranial mass on MRI (10). Patients with hypothalamic hamartomas (HH) were diagnosed with CPP, either isolated or in combination with seizures, and their MRI scans showed a mass either located adjacent to hypothalamic structures (i.e., in a parahypothalamic position) or infiltrating the hypothalamus (11). Familial male-limited PP (FMPP) was diagnosed in boys who had a family history of PP, normal hCG levels and a constitutively activated mutant LH receptor (LHCGR) leading to elevated T synthesis (12). Leydig cell tumors, optic pathway tumors (OPT) and choriocarcinomas were diagnosed using histopathological proof. The study protocol was approved by the Children's Hospital Capital Institute of Pediatrics Ethics Committee (SHERLL2020003, date: 14.01.2020).

The boys were divided into two groups: the CPP (gonadotropin-dependent) group and the PPP (gonadotropin-independent) group.

Molecular Analysis

Genomic analysis of LHCGR was performed by direct sequencing. Genomic DNA was extracted from peripheral blood lymphocytes. The coding regions and intron-exon boundaries of LHCGR were amplified by polymerase chain reaction (PCR). The pathogenicity of each variant was interpreted according to American College of Medical Genetics and Genomics standards and categorized (13). PolyPhen-2 and SIFT were used in silico prediction analysis.

Statistical Analysis

Statistical analyses were conducted using Statistical Package for the Social Sciences, version 22.0 (IBM Inc., Armonk, NY, USA). The results were expressed as the mean $\pm$ SD, and t-tests for independent samples were applied when normally distributed. Non-parametric data were presented as median and the interquartile range, analyzed by the Mann-Whitney U test. P values below 0.05 were regarded as statistically significant.

Results

Eighty-seven boys were admitted to our hospital for the evaluation of pubertal development. After clinical and laboratory evaluation of all patients except those excluded with PA (n = 5), a total of 82 (94%) boys were diagnosed with PP. The patients' age at the time of referral ranged from

0.92 to 10.5 years; 73.5% were over 7 years old at the time of their first visit, 20.7% were 3 to 6 years old, and 7% were under 3 years old (Figure 1). The main complaints included penis and/or testis enlargement (n = 45, 54.8%), growth acceleration (n = 45, 54.8%), the development of pubic and/or axillary hair (n = 39, 47.6%), voice change (n = 10, 12.2%), breast mass (n = 5, 6.1%), acne (n = 4, 4.9%), and erection/ejaculation (n = 2, 2.4%).

Sixty-two boys (75.6%) were diagnosed with CPP, and 20 boys were diagnosed with PPP (24.4%). The average age at evaluation of the PPP group was much lower than that of the CPP group (5.50 ± 2.23 years vs 8.83 ± 2.19 years, $p < 0.001$), while the T levels did not differ significantly (median: 8.03 nmol/L vs 8.21 nmol/L, $p = 0.781$). The difference between bone age and chronological age in PPP patients was significantly greater than that in CPP patients (4.44 ± 2.41 years vs 2.26 ± 1.31 years, $p = 0.001$). Descriptive data are listed in Table 1 and Table 2.

In the CPP group, 49 boys were diagnosed with idiopathic CPP (ICPP, 79%). Thirteen boys were diagnosed with pathogenic CPP (21%), including all cases of HH and post bone marrow transplant (PBMT), three cases of CAH, one case of OPT and one undetermined case (Figure 2).

Hypothalamic Hamartomas

Five patients were diagnosed with HH (0.92 ~ 8.17 years). The imaging findings were assessed by two radiologists (Figure 3A). The patients' basal LH levels (median: 5.07 IU/L, range:1.18 ~ 22.40 IU/L) were consistent with the diagnosis of CPP. Four cases were of the "parahypothalamic type", in which the hamartoma is attached only to the floor of the third ventricle or tethered to the floor by a peduncle, and two of the patients presented with gelastic seizures. One case was of the "intrahypothalamic type", in which the hamartoma is involved or enveloped by the hypothalamus

Table 1. Comparison between CPP and PPP groups. The values in brackets indicate the 25th and 75th percentiles

	CPP	PPP	p value
N (%)	62 (75.6)	20 (24.4)	
Age at evaluation (years)	8.83 ± 2.19	5.50 ± 2.23	< 0.001
Testis volume (mL)	10.67 ± 5.30	5.63 ± 4.45	< 0.001
Hight Z-score	0.22 ± 0.94	-0.81 ± 0.94	< 0.001
Weight Z-score	0.21 ± 0.97	-0.85 ± 0.62	< 0.001
BMI Z-score	0.09 ± 1.02	-0.53 ± 0.67	0.015
BLH (IU/L)	5.07 (3.13,6.37)	0.19 (0.10,0.29)	< 0.001
PLH (IU/L)	21.82 (15.48,27.70)	1.41 (0.38,3.30)	< 0.001
BFSH (IU/L)	3.73 (2.71,4.77)	0.74 (0.14,0.95)	< 0.001
PFSH (IU/L)	5.37 (3.83,6.86)	1.58 (1.34,3.53)	< 0.001
T (nmol/L)	8.03 (4.91,13.37)	8.21 (3.56,14.57)	0.781
BA-CA (year)	2.26 ± 1.31	4.44 ± 2.41	0.001

BMI: body mass index, BLH: basal luteinizing hormone, PLH: peak LH, BFSH: basal follicle stimulating hormone, T: testosterone, BA-CA: bone age minus chronological age, CPP: central precocious puberty, PPP: peripheral precocious puberty

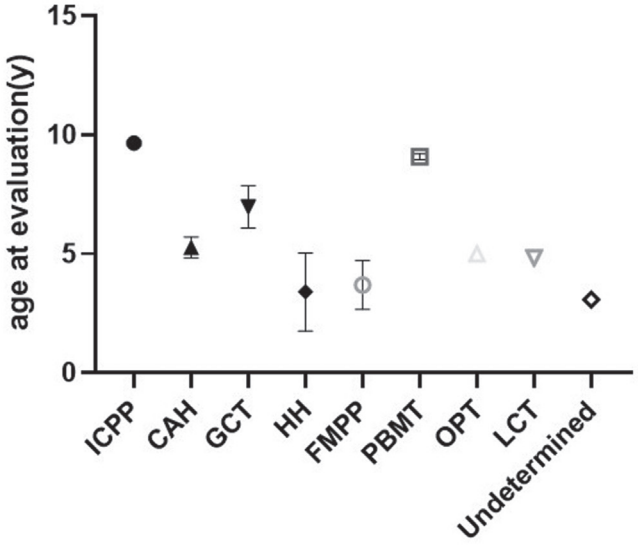

Figure 1. The age at evaluation by etiology

ICPP: idiopathic central precocious puberty, CAH: congenital adrenal hyperplasia, GCT: germ cell tumor, HH: hypothalamic hamartoma, FMPP: familial male limited precocious puberty, PBMT: post bone marrow transplant, OPT: optic pathway tumor, LCT: Leydig cell tumor

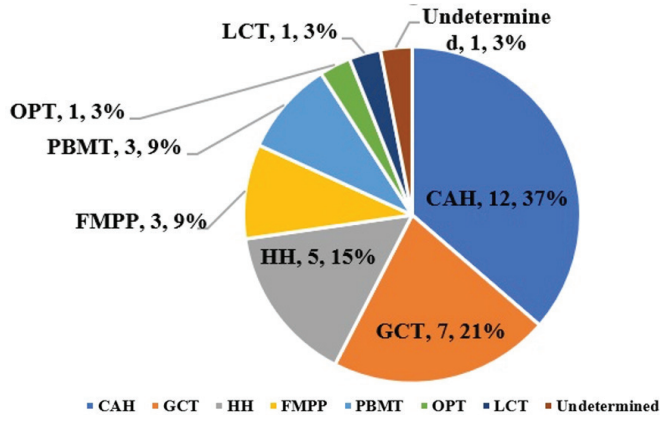

Figure 2. Etiologic spectrum of boys with pathologic precocious puberty

ICPP: idiopathic central precocious puberty, CAH: congenital adrenal hyperplasia, GCT: germ cell tumor, HH: hypothalamic hamartoma, FMPP: familial male limited precocious puberty, PBMT: post bone marrow transplant, OPT: optic pathway tumor, LCT: Leydig cell tumor

and the tumor distorts the third ventricle; this patient also presented with gelastic seizures.

Post Bone Marrow Transplant

Three patients (one each of Fanconi anemia, myelodysplastic syndrome and aplastic anemia) received chemotherapy and bone marrow transplantation. Busulfan/cyclophosphamide (BU/CY) was used as the conditioning regimen for myelodysplastic syndrome and aplastic anemia. The patient with aplastic anemia took *Epimedium* for at least 6 ~ 7 months after transplantation. The patient with Fanconi anemia had a conditioning regimen including BU/fludarabine + antithymocyte globulin + TBI. PP occurred 1 ~ 3 years after allogeneic hematopoietic stem cell transplantation. All three patients presented with CPP.

The etiology of CPP in one 3-year-old boy remains unknown. He had enlarged testes shortly after birth, and he was admitted to our department because of extremely large, symmetrical testes (25 mL). His blood test results showed an LH peak of 4.17 IU/L, an FSH peak of 28.3 IU/L and extremely high T (17.15 nmol/L). He had a long face, large ears, white skin color and mild mental retardation. A testicular biopsy revealed normal findings. The MRI findings and hCG and 17-OHP results were normal. The results of a fragile X test were negative. Whole-exome sequencing or microarray examination showed no expected typical mutations in the *KISS1, KISS1R, MKRN3* or *DLK1* genes.

In the PPP group, CAH (n = 9), CNS GCT (germinomas n = 6 and choriocarcinoma n = 1) and FMPP (n = 3) were the most common causes (45%, 35% and 15%, respectively). We also diagnosed a boy with a Leydig cell tumor (n = 1, 5%) (Figure 2).

Tumor Causes

In all, nine boys had tumors (aged 2.25 to 10.0 years), including seven boys with CNS GCTs, one boy with a Leydig cell tumor (chief complaint: testis mass on the left side; laboratory examinations consistent with PPP) and one boy with an OPT (chief complaints: blurred vision and growth acceleration; laboratory examinations consistent with CPP), all of which were diagnosed by histopathology. Among patients with GCTs (Figure 3B), six showed moderately elevated hCG in serum (0.1 ~ 40.6 IU/L) and/or CSF (20.5 ~ 32.3 IU/L); their tumor sites were suprasellar (n = 3) or in the basal ganglia (n = 3). One 2.5-year-old boy presented with rapid pubertal development; testing showed remarkably elevated hCG (10,000 IU/L). hCG in patients with GCTs was significantly elevated, and the other patients had hCG levels that were below the lower limit of detection. MRI of one boy revealed an approximately 5.6 × 6.7 × 12.8 mm mass in the pineal gland (Figure 3C), and biopsy showed choriocarcinoma. CT scans of the abdomen, pelvis and chest were normal in all of the GCT patients. In the seven GCT patients, although their testicular volumes varied from 4 mL to 8 mL, all of the GnRH analog stimulation tests revealed a prepubertal response to gonadotropin.

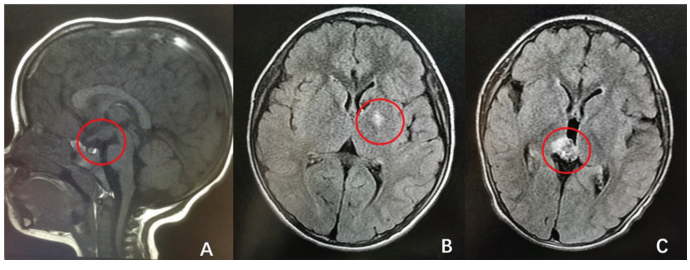

Figure 3. Pathological brain magnetic resonance imaging from three representative cases: Hypothalamic hamartoma (A), basal ganglia germ cell tumor (B), and choriocarcinoma (C)

Familial Male Limited Precocious Puberty (OMIM #176410)

LHCGR gene screening was performed in three unrelated boys (patients coded A, B and C) (1.67 to 4.41 years old) (Figure 4). In patients A and B, sequencing analysis of the *LHCGR* gene revealed a heterozygous C-to-T transition at codon 577 (c.1730C > T), and this transition led to the substitution of threonine with isoleucine at codon 577 (p.T577I). The two patients' mothers were heterozygous for the p.T577I mutation, while neither patient's father carried the mutation. Patient C had a heterozygous T-to-C transition at codon 576 (c.1726T > C) of the *LHCGR* gene, and this transition led to the substitution of phenylalanine with leucine at codon 576 (p.F576L). Patient C's father (who was also a patient) was heterozygous for the p.F576L mutation, while the mother did not carry the mutation. These two variants were predicted to be deleterious/damaging by both PolyPhen-2 and SIFT.

Congenital Adrenal Hyperplasia (PPP combined with CPP)

All 12 boys (aged 3.25 to 7.67 years) were diagnosed with a 21-hydroxylase deficiency. All presented with penis enlargement and three also presented with premature pubarche. Nine boys presented with PPP, and three patients presented with CPP. The evaluation age of the CAH-PPP group was slightly lower than that of the CAH-CPP group (5.03 ± 1.42 vs 6.00 ± 1.86 years, p = 0.360). The 17-OHP levels were higher than the rest of groups. but showed no significant difference. In the CAH-CPP group, one patient presented with CPP before the administration of hydrocortisone, and the other two patients presented with CPP at six months and one year after treatment. The testicular volumes of the latter two were both 2 mL before treatment but increased to 4 mL and 5 mL, respectively,

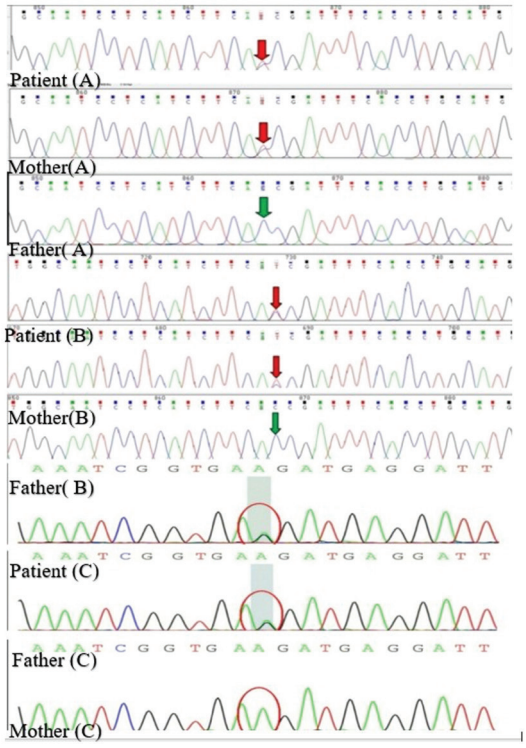

Figure 4. Mutation analysis of the *LHCGR* gene. In patient A and B, sequencing analysis of the *LHCGR* gene revealed a heterozygous C to T transition at the codon 577 (c.1730C > T), both from the mother. Patient C had a heterozygous T to C transition at the codon 576 (c.1726T > C) of the *LHCGR* gene, from his father (who was also a patient)

after treatment. The basal value of LH increased from 0.1 IU/L to 0.4 IU/L and 5.0 IU/L, respectively.

Discussion

Of the 62 boys who were diagnosed with CPP in our department, 79% were diagnosed with ICPP. No pathological factors were found during the follow-up. Such a high proportion of ICPP is quite different from what has been reported in other countries. Studies have shown that 50-70% of boys with CPP have identifiable pathological

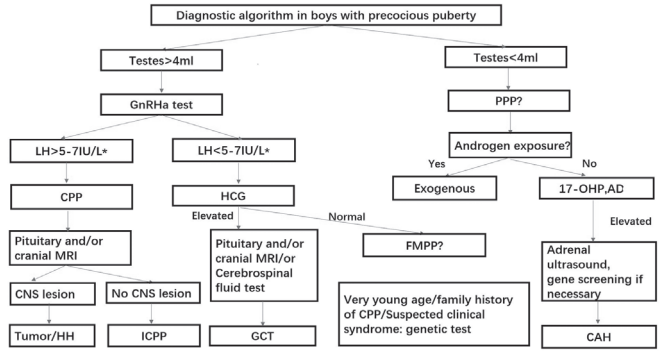

Figure 5. Algorithm for etiological spectrum

CAH: congenital adrenal hyperplasia, GCT: germ cell tumor, HH: hypothalamic hamartoma, FMPP: familial male limited precocious puberty, MRI: magnetic resonance imaging

Table 2. Comparison between different subgroups of pathogenic precocious puberty

	CAH	GCT	HH	FMPP	PBMT	OPT	LCT	Undetermined
N (%)	12 (14.6)	7 (8.5)	5 (6.1)	3 (3.7)	3 (3.7)	1 (1.2)	1 (1.2)	1 (1.2)
Age at evaluation (year)	5.27 ± 1.51	6.99 ± 2.69	4.09 ± 3.23	3.69 ± 1.78	9.19 ± 0.63	5.00	4.83	3.08
Testis volume (mL)	3.32 ± 1.45	5.71 ± 2.93	7.60 ± 3.21	6.33 ± 1.53	7.33 ± 2.51	6	4	25
Hight Z-score	-0.53 ± 0.75	-0.61 ± 1.02	-1.70 ± 1.47	-1.56 ± 1.40	1.02 ± 0.16			
Weight Z-score	-0.78 ± 0.79	-0.51 ± 0.72	-1.03 ± 1.08	-1.19 ± 0.64	1.12 ± 1.52			
BMI Z-score	-0.85 ± 0.84	-0.08 ± 0.31	0.33 ± 0.45	-0.09 ± 0.78	0.53 ± 2.17			
BLH (IU/L)	0.25	0.10	5.07	0.1	5.17	7.98	0.10	0.52
(min-max)	0.10-1.50	0.10-0.17	1.18-22.4	0.1-0.13	2.04-5.77			
PLH (IU/L)	2.42	0.24	19.22	1.6	33.32		0.55	4.17
(min-max)	0.10-24.19	0.10-1.02	15.04-21.52	1.23-2.43	11.00-40.42			
BFSH (IU/L)	0.98	0.13	3.85	0.18	6.43	11.30	0.31	15.9
(min-max)	0.10-6.8	0.10-0.74	2.29-6.98	0.11-0.74	1.72-11.88			
PFSH (IU/L)	3.45	0.36	6.62	1.42	4.90		1.54	28.3
(min-max)	0.10-5.8	0.15-2.14	3.85-10.93	1.41-1.44	1.47-4.97			
T (nmol/L)	4.29	19.34	15.94	9.70	10.69	19.9	13.3	17.2
(min-max)	1.87-53.80	8.72-28.34	5.60-35.70	6.38-9.99	3.20-11.18			
ACTH (pg/mL)	213	42	31	78	73	19	23	51
(min-max)	111-604	15-129	18-101	39-79	46-87			
Cor (ug/mL)	3.0	10.2	8.4	17.2	10.6	3.11	10.7	7.9
(min-max)	0.1-14.2	4.5-16.0	3.9-16.6	7.7-17.7	6.7-11.8			
17-OHP (ng/mL)	39.22	2.00	2.52	1.51	2.23	0.34	1.94	1.29
(min-max)	25-249	1.10-4.40	1.78-2.92	1.23-2.11	1.92-2.42			
BA (years)	11.5 ± 2.0	9.6 ± 3.1	6.9 ± 3.7	7.6 ± 1.7	13.0 ± 1.0	5.0	7.0	5.0

BMI: body mass index, BLH: basal luteinizing hormone, PLH: peak LH, BFSH: basal follicle stimulating hormone, T: testosterone, ACTH: adrenocorticotropic hormone, Cor: cortisol, 17-OHP: 17-hydroxyprogesterone, BA: bone age, OPT: optic pathway tumor, CAH: congenital adrenal hyperplasia, GCT: germ cell tumor, HH: hypothalamic hamartoma, FMPP: familial male limited precocious puberty, PBMT: post bone marrow transplant, LCT: Leydig cell tumor, min-max: minimum-maximum

changes (6). The majority (64%) of boys with CPP in a ten-year study in Boston had organic CPP (4). A study from Saudi Arabia reported that organic CPP accounted for 50% of CPP cases in boys (14). This discrepancy may be caused by several factors. Previous studies have suggested that there is a downward trend in the age of puberty onset in boys (15,16). The same trend may have occurred in our patients. Increased awareness may be another cause of the increased number of ICPP diagnoses. At the same time, genetic background could play a role, as suggested by the difference in neurofibromatosis rates: neurofibromatosis was the main cause of CPP in the Boston study but was not found in a single case in our study.

Other than ICPP, HH was the most common etiology of CPP in our cohort. Lesions or malformations of the CNS are usually considered first in the differential diagnosis of organic CPP. The main risk factors for a physical CNS etiology include young age and male sex (17,18). The most common CNS lesion associated with pathogenic CPP is HH, which is a rare congenital non-neoplastic CNS lesion containing mature tissue in a heterotopic location (19). For most symptomatic patients with HH associated with CPP and/or gelastic seizures, the relationship between the neuroradiological findings and clinical presentation has not been fully investigated (20,21). In our study, 41.6% of patients with pathogenic CPP had HH. However, in the US, 64% of boys with CNS-CPP had NF I (4). This suggests that the etiology of CPP differs among different races.

We also found that in addition to the usual etiology, a relatively rare but not negligible cause of male CPP is post BMT. Most studies suggest that gonadal impairment is an important side effect in transplanted patients, and busulfan conditioning regimens and TBI are associated with gonad damage (22,23). Interestingly, three patients developed CPP after BMT. A Korean study suggested that the application of cyclophosphamide may be related to PP (3). In our study, only two patients' conditioning regimens included cyclophosphamide. One of our patients had a long history of using *Epimedium*, which has the effect of enhancing sexual function in mice (24). In addition to chemotherapy and herbal remedies, cranial irradiation might cause damage to gamma-aminobutyric acid–secreting neurons, which could lead to premature activation of GnRH neurons (25). Further studies are needed to determine whether BMT and irradiation could be associated with male CPP.

The spectrum of male PPP is complex. In our study, CAH, GCT and FMPP were the three most common etiologies of PPP. CAH is the predominant etiology in boys with PPP (26). In a six-year retrospective study of PP cases at a tertiary hospital in Southern China, 38% were due to CAH (27). CAH was also the most common etiology of organic PP in our study, occurring in 12 patients. Three patients with CAH developed CPP. Secondary CPP may complicate CAH. First of all, chronic hyperandrogenemia may trigger the activation of the hypothalamic-pituitary axis, leading to CPP (28,29) and hydrocortisone treatment reduces androgen levels and activates gonadotropins. The decrease in sex steroids during treatment of the primary underlying disorder causes activation of the precociously matured hypothalamic GnRH pulse generator via feedback mechanisms, resulting in secondary CPP (30). CPP can occur either before or after treatment. This combination of PPP and CPP in patients with CAH is worthy of wider clinical recognition and attention, and better clinical management of patients is needed.

Usually, patients with hCG-secreting GCTs present with signs of increased intracranial pressure and diabetes insipidus, and PPP is less common than other manifestations (31). In a study by Atay et al (32) that excluded CAH, hCG-secreting tumors, Leydig cell tumors and adrenocortical tumors played equally important roles in the etiology of PPP. All of our patients had a significant increase in blood and/or CSF hCG. hCG-secreting GCTs can cause PP in boys by stimulating Leydig cells to secrete T, consistent with previous studies almost exclusively in boys (33). Although seven of our patients had PPP, it has been reported that some boys with hCG-secreting GCTs near the hypothalamus may actually develop CPP (34).

FMPP is an autosomal-dominant form of PPP in males. FMPP is a very rare disorder, affecting approximately nine individuals per million (35). The *LHR* gene, located on human chromosome 2p21, encodes a transmembrane receptor expressed on the cell membranes of testicular Leydig cells (36). Affected males usually begin pubertal development by 1 ~ 4 years of age, with rapid growth and progressive virilization, and the same characteristics were observed in the present study (37). The most common mutation reported in the literature in the *LHCGR* gene is a D578G missense mutation (38). In our study, PCR product sequencing revealed a heterozygous adenosine-guanine transition at nucleotide 1730 in codon 577 in two boys; this mutation in *LHCGR* has been identified before (39). One patient was found to have a heterozygous mutation in *LHCGR*, causing an F576L substitution within Transmembrane region 6 of LHCGR. Although this mutation has not been previously reported, given that it occurs in a mutation hotspot in a patient with a family history, and the variants were predicted to be damaging, we suspect that it is also pathogenic. All three boys presented with PPP, but as seen with CAH, if the diagnosis was delayed, CPP could also be present at the first visit.

Different studies have shown different disease spectra. Based on these five years of data and a review of the literature, we propose a diagnostic algorithm (summarized in Figure 5) (32). Zou et al (27) reported that 23% of boys with PPP in Southern China had a history of exogenous hormone intake, such as contraceptives, whereas not a single case due to exogenous hormone intake was found among our patients. Testicular tumors were also common in the study by Zou et al (27) (19%) but very rare in our study. Genetic and dietary differences may be among the reasons for these discrepancies. We did not find any cases of McCune-Albright syndrome or hypothyroidism, which have been reported in other studies (40). In addition to untreated primary hypothyroidism, very rare syndromes, such as Prader-Willi syndrome and Sotos syndrome, can also result in PP (41). In addition, some etiologies remain undetermined despite complex and detailed investigation.

Study Limitations

This study had several limitations. The enrollees were treated at a single medical center, and the data on male PP could vary from that found in other centers. In the ICPP group, further studies are needed to examine the possible contribution of genetic factors. Sequencing and genetic studies are undertaken increasingly often in affected individuals, thereby resulting in a declining number of "idiopathic" cases.

Conclusion

The etiology of PP in boys is diverse. In our cohort of Chinese males, CPP was mainly idiopathic, and HH was the most common etiology of pathogenic CPP. CAH and GCTs accounted for the etiology of 80% of PPP cases. It should be borne in mind that PPP patients may become CPP patients, and the diagnosis of some cases remains challenging.

Ethics

Ethics Committee Approval: The study protocol was approved by the Children's Hospital Capital Institute of Pediatrics Ethics Committee (SHERLL2020003, date: 14.01.2020).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept – Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: Liu Ziqin, Li Xiaohui, Chen Xiaobo.

Financial Disclosure: Supported by the Beijing Municipal Administration of Hospitals Incubating Program (PX2020055).

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0047

J Clin Res Pediatr Endocrinol 2021;13(4):426-432

Investigating the Efficiency of Vitamin D Administration with Buccal Spray in the Treatment of Vitamin D Deficiency in Children and Adolescents

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What is already known on this topic?

The main purpose of vitamin D therapy is to optimize serum 25-hydroxyvitamin D [25(OH)D] concentrations to improve bone homeostasis and decrease the risk of osteopenia and osteoporosis. Daily or weekly oral drops or a single large dose, either orally or through injection are used more frequently in the treatment of vitamin D deficiency. There are limited numbers of studies that have evaluated the effectiveness of buccal spray against other modes of vitamin D delivery in the treatment of vitamin D deficiency in children and the results of these studies are conflicting.

What this study adds?

Vitamin D3 supplementation with buccal spray and oral drops is equally effective in terms of raising serum 25(OH)D concentrations in short-term treatment of vitamin D deficiency.

Abstract

Objective: The aim of this study was to evaluate the efficiency of a buccal spray form of vitamin D compared to single oral dose (stoss therapy) and oral drops therapy in the treatment of vitamin D deficiency.

Methods: Ninety healthy children and adolescents (3-18 years) with vitamin D deficiency [serum level of 25-hydroxyvitamin D (25(OH)D) < 12 ng/mL] were randomized to receive vitamin D3 buccal spray (2000 U, n = 30, group 1) for six weeks, oral drops (2000 U, n = 30, group 2) for six weeks and a single oral dose (300 000 U) vitamin D3 (n = 30, group 3). Serum calcium, phosphorus, alkaline phosphatase, parathyroid hormone and 25(OH)D levels of the patients were measured at baseline and after the treatment on the 42nd day.

Results: All three groups had a significant increase in serum 25(OH)D concentrations ($p < 0.001$). In group 1, baseline mean 25(OH)D was 8.0 ± 0.41 ng/mL, which rose to 22.1 (17.8-28.2) ng/mL after treatment with a mean increase of 15.6 ± 1.3 ng/mL. Similarly in group 2, baseline, post-treatment and mean increase in 25(OH)D concentrations were 7.9 ± 0.45 ng/mL, 24.4 (20.6-29.6) ng/mL and 17.3 ± 1.1 ng/mL while for group 3 these values were 7.6 ± 0.47 ng/mL, 40.3 (29.4-58.4) ng/mL and 34.3 ± 3.2 ng/mL, respectively.

Conclusion: We conclude that vitamin D3 supplementation with buccal spray and oral drops is equally effective in terms of raising vitamin D concentrations in short-term treatment of vitamin D deficiency.

Keywords: Vitamin D, buccal spray, 25-hydroxyvitamin D, oral drops

Introduction

Vitamin D is a pro-hormone for active intestinal calcium (Ca) absorption, and it plays a major role in maintaining Ca and phosphorous homeostasis and skeletal integrity (1). Deficiency of vitamin D leads to rickets, the failure of mineralization of growing bone in children and osteomalacia in adults (1). Meanwhile, it has been reported that vitamin D deficiency may be associated with chronic diseases such as cardiovascular diseases, diabetes, hypertension and autoimmune diseases, among others (2,3,4,5,6,7,8,9,10). So, treatment of vitamin D deficiency and thus, maintenance

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Conflict of interest: None declared
Received: 25.02.2021
Accepted: 19.05.2021

of 25-hydroxyvitamin D [25(OH)D] concentrations in the normal range, as advised by several expert committees to provide optimal tissue health, is very important.

Numerous reported consensus reports on vitamin D therapy have been published by many organizations around the world (11,12,13,14,15). In these consensus reports, different treatment algorithms are recommended for vitamin D deficiency in healthy children with or in those with chronic diseases, such as celiac disease, inflammatory bowel diseases, and cystic fibrosis. For healthy children, different treatment regimens, such as daily, weekly or a single dose (stoss) with cumulative vitamin D dose ranging from 84,000 to 600,000 IU are recommended (11,12,13,14,15). The recommended treatment duration of daily or weekly treatment regimens can range from 6 to 12 weeks. In these treatment protocols, vitamin D is usually given as cholecalciferol (vitamin D3) rather than ergocalciferol (vitamin D2), and as oral low-dose long-term therapy or oral/intramuscular high-dose injection (stoss therapy). However, both treatment protocols have their own disadvantages. Although low-dose long-term therapy varies, depending on the dose, the treatment duration can be up to three months. This situation often causes problems in compliance with treatment. In addition, in cases of malabsorption, such as in patients with celiac disease, a problem occurs in the dose adjustment required for the desired serum level. Recently, novel treatment modalities have been developed for the treatment of vitamin D deficiency, including an oral spray, soft capsule, gels, and gums (16). Most of the studies comparing different vitamin D treatment modalities were conducted with adults (1,17,18), and there are limited studies conducted in children (19,20). In most of these studies, in which capsule, drop and spray forms of vitamin D were compared, it was shown that different treatment modes did not have superiority to each other (16,17,18), but in one study the oral spray form was reported to be more effective (1). These studies are heterogeneous in terms of treatment dose and duration, population age, study design and health status, which make it difficult to draw assumptions from the results. Therefore, in this study, we aimed to evaluate the efficiency of the buccal spray form of vitamin D compared to single oral dose (stoss therapy) and oral drops therapy in the treatment of vitamin D deficiency.

Methods

Study Population

This study was conducted in children diagnosed with vitamin D deficiency aged between 3-18 years old who were treated in University of Health Sciences Turkey, Dr. Behçet Uz Children's Hospital between January-March 2020. The exclusion criteria were: hepatic or renal failure; uncontrolled hypothyroidism or hyperthyroidism; systemic inflammatory or malignant disease; vegan diet; or had a confirmed diagnosis of a malabsorptive condition including ulcerative colitis, Crohn's disease or steatorrhea. In addition, patients using medication known to influence vitamin D metabolism (bisphosphonates, glucocorticoids and anticonvulsants) and those who had been on a sun holiday in the 30 days prior to baseline measurements or those planning a sun holiday during the time of the study, or using medication known to affect bone metabolism were also excluded. Women who were pregnant or attempting to become pregnant during the study period were also excluded. The Local Ethics Committee approved the study (University of Health Sciences Turkey, Dr. Behçet Uz Children's Hospital, Clinical Research Ethics Committee, İzmir; approval number: 2018/17-12), and written informed consent was obtained from all individuals involved.

Baseline Data Collection

Age, sex, height and weight of all cases were evaluated. A Harpenden stadiometer with sensitivity of 0.1 cm was used for measurement of height. Body weight measurement was performed using a scale with sensitivity of 0.1 kg (SECA, Hamburg, Germany). All measurements made by the same person. The patients took off their shoes and wore light clothes before the measurement. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters (m^2). BMI percentiles and Z-scores were determined by using reference data for Turkish children, according to age and sex (21,22). Children with a BMI equal to or greater than the 95th percentile were considered obese.

Baseline fasting blood samples including serum Ca, phosphorus (P), alkaline phosphatase (ALP), 25(OH)D, albumin, blood urea nitrogen (BUN), serum creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were collected. Serum 25(OH)D levels and parathyroid hormone (PTH) were measured by the electrochemiluminescence method. This assay was carried out through quantitative determinations of total 25(OH)D in serum samples using a standard kit available for the Abbott Architect system, (Abbott Laboratories, IL, USA). Serum Ca, P, ALP, albumin, BUN, serum creatinine, ALT, and AST were also measured in duplicate and assessed using an Architect C system biochemistry analyzer (Abbott Laboratories, IL, USA).

In this study, the following classifications were used: Serum vitamin D level > 20 ng/mL is regarded as "sufficient", < 12

ng/mL is regarded as deficient, and 12-20 ng/mL is regarded as "insufficiency" (11,23). Written and signed consent was obtained from the parents of the participants < 12 years old who met the criteria and agreed to participate in the study, and from both parents and children in those older than 12 years.

Patients with vitamin D deficiency who met the inclusion criteria were included in each group sequentially. A total of 90 patients (30 patients in each group) were included in the study between January 2020 and March 2020. The first group (group I) was treated with buccal spray (Wellcare vitamin D3, 1 puff equals 1000 U), the second group (group 2) was treated with vitamin D-containing drops (Devit3 oral drop, 1 drop is approximately133 U) and the third group (group 3) was treated with a single oral dose of vitamin D from an ampoule (Devit3 ampoule). In group 1, patients received 2000 IU/day (two puffs) for six weeks; while in group 2 patients received 2000 IU/day (15 drops) for six weeks. The patients in group 3 were treated with a single dose of vitamin D (300,000 IU single oral dose). All participants kept a record of the intake time and the amount of the medication they used. They were told that if they forgot to take the drops or spray, they would take the missing dose when they remembered. Blood samples of all three groups were obtained and analysed at the end of the treatment period of six weeks (42nd day).

Statistical Analysis

Statistical analyses of the data were performed using Statistical Package for the Social Sciences, version 20.0 (IBM Corp., Armonk, NY, USA). Distribution of data was evaluated using the Kolmogorov-Smirnov test. For comparison of more than two groups, one-way ANOVA or Kruskal-Wallis test were used as appropriate for the distribution of the data. If a significant difference was found in the comparison of more than two groups, Mann-Whitney U test with Bonferroni correction or Tukey test were performed as a post-hoc test to determine where the differences truly originated. In the comparison of two dependent groups (for pre- and post-treatment measurements), paired t-test or Wilcoxon test were performed according to distribution of the parameters. The chi-square test was used to compare categorical variables. Spearman's rho correlation was used to identify the associations between BMI standard deviation score (SDS), post-treatment serum 25(OH)D levels and the amount of increase in 25(OH)D levels. Categorical data were expressed as frequency (%), while numerical data were expressed as median (25-75th percentile) or mean ± standard deviation. A value of $p < 0.05$ was considered significant.

Results

The mean ages were 12.1 ± 4.1, 10.8 ± 3.6, 11.9 ± 3.9 years in groups 1, 2 and 3, respectively ($p > 0.05$). Of the 90 participants, 29 (32.2%) were male, and 61 (67.8%) were female. Sex distribution in each group was as follows: group 1 11 (36.7%) male and 19 (63.3%) female while in groups 2 and 3 there were 9 (30%) male and 21 (70%) female participants. The anthropometric and demographic data of participants are shown in Table 1. There was no difference in sex, age, weight, height, weight SDS, height SDS, or BMI SDS between the three groups ($p > 0.05$).

Baseline and post-treatment Ca, P, ALP, PTH, 25(OH)D values are shown in Table 2. All participants were normo-calcemic, therefore none of them received Ca supplementation. There was no statistically significant difference between the three groups in terms of the baseline or post-treatment Ca, P, ALP, PTH. In contrast, both the post-treatment levels of serum 25(OH)D and the degree of increase in serum 25(OH)D levels after treatment were significantly higher in group 3 ($p < 0.001$).

When the baseline and post-treatment values of the parameters were compared, while serum 25(OH)D levels increased and PTH levels decreased in all three groups

Table 1. Anthropometric and demographic data of participants

Parameters	Group 1	Group 2	Group 3	p
Sex, n (%) Male Female	30 11 (36.7%) 19 (63.3%)	30 9 (30%) 21 (70%)	30 9 (30%) 21 (70%)	0.816[a]
Age (years)	12.1 ± 4.1	10.8 ± 3.6	11.9 ± 3.9	0.411[b]
Weight (kg)	45.2 (30.6-56.0)	40.6 (29.8-56.6)	47.2 (28.2-56.4)	0.823[c]
Height (cm)	153.1 (134.8-160.3)	148.6 (126.1-159.5)	149.1 (123.8-160.0)	0.873[c]
Weight SDS	-0.05 ± 0.24	0.02 ± 0.20	-0.13 ± 0.17	0.868[b]
Height SDS	-0.40 ± 0.19	-0.12 ± 0.18	-0.31 ± 0.18	0.561[b]
BMI SDS	0.13 ± 0.22	0.72 ± 0.21	-0.50 ± 0.18	0.815[a]

Data are presented as mean ± standard deviation or median (25-75th percentiles), [a]chi-square, [b]One-way ANOVA, [c]Kruskal-Wallis.
BMI: body mass index, SDS: standard deviation score

($p < 0.05$) and serum ALP levels decreased in group 1 only ($p < 0.05$), no statistically significant change in serum Ca and P levels were found between groups.

At the end of the treatment, 20 (66.7%) patients in group 1, 26 (86.7%) patients in group 2 and 27 (90%) patients in group 3 had normal (> 20 ng/mL) serum 25(OH)D levels ($p = 0.044$). In the remaining patients, serum 25(OH)D levels were in the insufficient range of 12-20 ng/mL. Six cases in group 1, and 3 cases in each of group 2 and 3 were obese ($p = 0.421$). Serum 25(OH)D level at the end of treatment was sufficient in 4 of 6 obese patients in group 1, in all obese patients in group 2, and in 2 of 3 obese patients in group 3 ($p > 0.05$). There was no correlation between the amount of increase in 25(OH)D level and BMI SDS in groups 1, 2 and 3 ($p > 0.05$).

Discussion

In the current study, a single oral dose treatment of 300,000 IU was superior to 2000 IU of oral drop vitamin D3 daily for six-weeks or 2000 IU of buccal spray vitamin D3 daily for six weeks treatments in increasing serum 25(OH)D levels. Moreover, oral drop and buccal spray treatments were found to be similarly effective in raising serum vitamin D levels. In three groups with similar baseline serum 25(OH) D levels, however, the proportion of patients with normal serum 25(OH)D levels (> 20 ng/mL) at the end of treatment was lower with buccal spray treatment (66.7%) compared to oral drops (86.7%) or 300 000 IU oral single dose (90%) treatments. Malabanan et al (24) reported that vitamin D supplementation using 50,000 IU weekly for eight weeks was successful in the treatment of vitamin D deficiency in older children and adolescents. In another study conducted in healthy infants and young children with hypovitaminosis D, patients were divided into three different groups that received either 2,000 IU oral vitamin D2 daily, 50,000 IU vitamin D2 weekly or 2,000 IU vitamin D3 daily, and these three regimens were compared. All three treatment regimens were applied for six weeks and were shown to

Table 2. Baseline and post-treatment laboratory characteristics of patients

Parameters	Baseline values	p	Post-treatment values	p
25(OH)D (ng/mL)				
Group 1 (Buccal spray) n = 30	8.0 ± 0.41		22.1 (17.8-28.2)[d]	
Group 2 (Oral drops) n = 30	7.9 ± 0.45	0.852[a]	24.4 (20.6-29.6)[d]	< 0.001[b]
Group 3 (Oral stoss) n = 30	7.6 ± 0.47		40.3 (29.4-58.4)[d,f]	
25(OH)D > 20 ng/mL				
Group 1 (Buccal spray) n = 30	0 (0%)		20 (66.7%)	
Group 2 (Oral drops) n = 30	0 (0%)	-	26 (86.7%)	0.044[c]
Group 3 (Oral stoss) n = 30	0 (0%)		27 (90%)	
Serum PTH (pg/mL)				
Group 1 (Buccal spray) n = 30	60.1 (47.0-71.2)		51.4 (36.2-65.7)[d]	
Group 2 (Oral drops) n = 30	60.0 (54.0-69.6)	0.273[b]	47.1 (35.1-61.7)[d]	0.585[b]
Group 3 (Oral stoss) n = 30	53.7 (37.3-76.1)		50.4 (34.3-68.6)[d]	
Serum ALP (U/L)				
Group 1 (Buccal spray) n = 30	176.0 (81.2-229)		140.5 (71.7-216.5)[c]	
Group 2 (Oral drops) n = 30	192.5 (102.7-240.7)	0.590[b]	188.5 (108.2-254)	0.118[b]
Group 3 (Oral stoss) n = 30	186.5 (71.0-240.0)		165.0 (70.0-234.5)	
Serum calcium (mg/dL)				
Group 1 (Buccal spray) n = 30	9.9 (9.7-10)		9.8 ± 0.05	0.928[a]
Group 2 (Oral drops) n = 30	9.9 (9.6-10.1)	0.234[b]	9.8 ± 0.07	
Group 3 (Oral stoss) n = 30	10.0 (9.7-10.5)		9.8 ± 0.06	
Serum phosphorus (mg/dL)				
Group 1 (Buccal spray) n = 30	4.3 (4.0-4.8)		4.4 (4.0-5.0)	
Group 2 (Oral drops) n = 30	4.5 (4.1-5.1)	0.789[b]	4.6 (4.4-5.1)	0.405[b]
Group 3 (Oral stoss) n = 30	4.4 (3.7-4.9)		4.4 (4.0-4.8)	
Change in 25(OH)D (ng/mL)				
Group 1 (Buccal spray) n = 30		-	15.6 ± 1.3	
Group 2 (Oral drops) n = 30	-		17.3 ± 1.1	< 0.001[a]
Group 3 (Oral stoss) n = 30			34.3 ± 3.2[e]	
Change in 25(OH)D (%)				
Group 1 (Buccal spray) n = 30		-	214 (110-294)	
Group 2 (Oral drops) n = 30	-		224 (137-334)	< 0.001[c]
Group 3 (Oral stoss) n = 30			445 (221-727)	

Data were presented as mean ± standard deviation or median (25-75th percentiles), [a]One-way-ANOVA, [b]Kruskal-Wallis test, [c]chi-square, [d]Wilcoxon test ($p < 0.05$); comparison variables between baseline and post-treatment value, [e]Tukey test ($p < 0.05$); post-hoc test for ANOVA, [f]Mann-Whitney U test with Bonferroni correction ($p < 0.017$); post-host test to determine the predominance for non-parametric three group comparisons.
PTH: parathyroid hormone, ALP: alkaline phosphatase

give equivalent results in the short-term treatment of hypovitaminosis D among healthy infants and young children (25). Pappa et al (26) found that both 2,000 IU of daily vitamin D3 and 50,000 IU of weekly vitamin D2 were superior to 2,000 IU of daily vitamin D2, all taken orally for six weeks, in raising serum 25(OH)D concentration in young patients with inflammatory bowel disease and vitamin D insufficiency. When all these studies and the current study are evaluated, an inference can be made that 2,000 IU oral vitamin D3 per day, 50,000 IU oral weekly treatment for 6-8 weeks and 300,000 IU oral single dose treatment are effective in the treatment of vitamin D deficiency.

There are studies suggesting that new treatment modalities, such as buccal spray, are as effective as oral drops in this treatment (1,16,17,18,19). Satia et al (1) compared the absorption of vitamin D3 through the oral route by comparing buccal spray and gelatin capsule in healthy adults and patients with malabsorption disease. All participants in groups were randomized to receive either the vitamin D3 buccal spray (2 sprays, each of 500 IU) or soft gelatin capsule containing vitamin D3 (1000 IU) for 30 days. After the completion of the 30-day treatment, all participants were given a 30-day washout. In the second period, crossover was performed so that those participants who had received the buccal spray formulation in period I received the soft gelatin capsule formulation in period 2 and *vice versa*. In this study, the superiority of vitamin D3 delivery via buccal spray compared to capsules in both healthy subjects as well as in patients with intestinal malabsorption syndrome was reported. On the other hand, the trial had limitations regarding the washout duration. Todd et al (17) compared the efficacy of vitamin D3 liquid capsules and oral spray solution in increasing wintertime total 25(OH)D concentrations in a randomized, open-label, cross-over trial in healthy adults. Twenty-two healthy adults received 3000 IU (75 µg) vitamin D3 daily, for four weeks in either capsule or oral spray form. After 10 days wash-out period, participants received the other treatment for four weeks. They demonstrated that oral spray vitamin D3 was just as effective as capsule supplementation in increasing total serum 25(OH)D concentrations in the healthy adult population. Penagini et al (19) demonstrated that vitamin D3 supplementation with buccal spray and oral drops was equally effective in the short-term treatment of vitamin D deficiency in a population of children with neurodisabilities. In this study, patients received vitamin D3 buccal spray 800 IU/daily (n = 12) and a second group received oral drops 750 IU/daily (n = 12) for three months during winter. Williams et al (18) conducted a randomized, placebo-controlled, three-arm parallel design study in healthy volunteers to compare the rate of change of vitamin D status in response to vitamin D3 (3000 IU/day) supplementation in capsule and sublingual spray preparations over a six week period. They suggested that sublingual vitamin D spray was an effective mode of delivery for supplementation in a healthy population and the capsule and spray were equally efficacious. When all these studies are considered, only Satia et al (1) advocated the superiority of buccal spray vitamin D against the other modes of delivery in increasing serum 25(OH)D concentrations.

Recent systematic reviews demonstrated that the administration of vitamin D3 by buccal spray did not differ from other supplementation methods in increasing serum plasma 25(OH)D levels (16,27). However, the small number of randomized controlled trials and the high degree of clinical heterogeneity of study populations did not allow for any reliable conclusions to be drawn from the results (16). In the study of Unsur (20), in which evaluated infants received 400 IU/day vitamin D supplementation as oral drops or buccal spray form during the first year of life, it was reported that the serum 25(OH)D levels measured at the age of one year were higher and the frequency of vitamin D deficiency was lower in infants using buccal spray than those using oral drops. In the current study, the group receiving stoss vitamin D had a significantly higher mean increase than both groups receiving buccal spray or oral drops. However, there was no significant difference in terms of increase in 25(OH)D levels between the group receiving buccal spray and oral drops. It was notable that when 25(OH)D levels of the three groups were evaluated at the end of the treatment, the proportion of patients with normal 25(OH)D level in the buccal spray group was smaller than in the oral drops group despite the same dose and duration (66.7% vs 86.7%). This suggests that in cases with a 25(OH)D level of < 12 ng/mL, 2000 IU/day 6-week spray therapy may be insufficient.

It is well recognized that there are various factors that affect the effectiveness of vitamin D therapy other than the route of administration or dose (14). Dark skinned children, reduced sunlight exposure due to constant use of sunscreens or lifestyle factors, covering clothing for religious or cultural reasons, chronic illness, obesity, malabsorption syndromes, drugs such as anticonvulsants, systemic glucocorticoids, antiretroviral therapy, and systemic antifungals can all affect the success of treatment (14). The Institute of Medicine does not take BMI into account in recommendations for vitamin D treatment, however the Clinical Practice Guidelines by the Endocrine Society recommend obese subjects be given two to three times more vitamin D to satisfy their body's vitamin D requirement. In a study in adults, it was shown that supplementation efficiency is associated with

BMI. In participants with normal body weight a greater change in serum 25(OH)D level was observed (28). Ekwaru et al (29) recommended 2- to 3-times higher vitamin D supplementation for obese subjects and 1.5 times higher for overweight subjects relative to normal weight subjects. Although the association between vitamin D deficiency and obesity and obesity-related diseases has been confirmed by numerous studies, the existence of a causal relationship is still unclear. In the current study, no significant relationship between obesity and the success of vitamin D therapy was found, although only 12 of 90 (13.3%) subjects were obese in our study.

Study Limitations

Our study has some limitations that should be acknowledged. The first limitation was the small sample size. In defence of this, the current study was conceived as a pilot study to assess the three different modes of vitamin D administration, buccal spray, oral drops and oral stoss vitamin D. The second limitation of our study was that the vitamin D binding protein (VDBP) status was unknown in all patients. Genetic variants not only affect vitamin D metabolism, but also affect the phenotype of the VDBP with different affinities to 25(OH)D and 1,25-(OH)2 D3 (23). Genetic polymorphisms of DBP can also alter the protein concentration in blood (30). Furthermore, assessment of VDBP polymorphisms may be useful to adjust treatment in individuals with an insufficient response to vitamin D supplementation. Genetic factors may be taken into account in the future design of personalized supplementation. Additionally, while all patients were living at the same latitude, the impact of intake of vitamin D containing foods, duration of breastfeeding, clothing, and exposure to sunlight were not considered. Finally, the patients' compliance to treatment (especially those receiving daily oral or buccal vitamin D treatment) was evaluated on the basis of self-reporting and most of the patients were adolescents and it is well-known that low adherence to treatment at this age is very common, which may have skewed the results, especially in groups 1 and 2.

Conclusion

The results of this study show that a single dose 300,000 IU vitamin D3 formulation was able to increase mean serum vitamin D3 concentration significantly compared to 2000 IU/day for six weeks given either by buccal spray or oral drops in both healthy children and adolescents. Vitamin D3 supplementation with buccal spray and oral drops was equally effective in terms of raising vitamin D concentration in the short-term treatment of vitamin D deficiency. However, in cases with a baseline serum level of 25(OH) D <12 ng/mL, treatment with 2000 IU/day for six weeks given by buccal spray may be insufficient to normalize serum 25(OH)D in a significant proportion of patients.

Ethics

Ethics Committee Approval: The Local Ethics Committee approved the study (University of Health Sciences Turkey, Dr. Behçet Uz Children's Hospital, Clinical Research Ethics Committee, İzmir; approval number: 2018/17-12).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Özlem Nalbantoğlu, Sezer Acar, Gülçin Arslan, Özge Köprülü, Behzat Özkan, Concept: Özlem Nalbantoğlu, Sezer Acar, Behzat Özkan, Design: Özlem Nalbantoğlu, Sezer Acar, Gülçin Arslan, Behzat Özkan, Data Collection or Processing: Özlem Nalbantoğlu, Sezer Acar, Analysis or Interpretation: Özlem Nalbantoğlu, Sezer Acar, Özge Köprülü, Behzat Özkan, Literature Search: Özlem Nalbantoğlu, Sezer Acar, Özge Köprülü, Behzat Özkan, Writing: Özlem Nalbantoğlu, Sezer Acar, Özge Köprülü, Behzat Özkan.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0056

J Clin Res Pediatr Endocrinol 2021;13(4):433-438

Molecular Diagnosis of Monogenic Diabetes and Clinical/Laboratory Features in Turkish Children

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What is already known on this topic?

Monogenic diabetes is a heterogeneous group of disorders, accounting for just 1-6% of all diabetes. Variants in *HNF1A, HNF4A*, and *GCK* accounts for most *MODY*-monogenic diabetes cases. Patient numbers and information are limited on less common causes of monogenic forms of diabetes.

What this study adds?

This study is the first Turkish multicenter genetic study of patients with molecularly diagnosed monogenic diabetes. This study determined the clinical and laboratory features, the admission characteristics and distribution of monogenic diabetes in childhood. The distribution in this cohort was in disagreement with the previously published distribution.

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Conflict of interest: None declared
Received: 07.03.2021
Accepted: 08.07.2021

Abstract

Objective: Monogenic diabetes is a heterogeneous disease that causes functional problems in pancreatic beta cells and hyperglycemia. The aim of this study was to determine the clinical and laboratory features, the admission characteristics and distribution of monogenic form of diabetes in childhood in Turkey.

Methods: Patients aged 0-18 years, who were molecularly diagnosed with monogenic diabetes, and consented to participate, were included in the study.

Results: Seventy-seven (45.6%) female and 92 male cases with a mean age of 8.18 ± 5.05 years at diagnosis were included. 52.7% of the cases were diagnosed with monogenic diabetes by random blood glucose measurement. The reason for genetic analysis in 95 (56.2%) of cases was having a family member diagnosed with diabetes under the age of 25. At the time of diagnosis, ketone was detected in urine in 16.6% of the cases. Mean hemoglobin A1c on admission, fasting blood glucose, fasting insulin, and c-peptide values were 7.3 ± 2.1%, 184.9 ± 128.9 mg/dL, 9.4 ± 22.9 IU/L, 1.36 ± 1.1 and ng/L respectively. GCK-MODY was found in 100 (59.2%), HNF1A-MODY in 31 (18.3%), and variants in *ABCC8* in 6 (3.6%), *KCNJ11* in 5 (3%), *HNF4A* in 2 (1.2%), and *HNF1B* in 2 (1.2%).

Conclusion: Recent studies have indicated HNF1A-MODY is the most frequent of all the MODY-monogenic diabetes cases in the literature (50%), while GCK-MODY is the second most frequent (32%). In contrast to these reports, in our study, the most common form was GCK-MODY while less than 20% of cases were diagnosed with HNF1A-MODY.

Keywords: Monogenic diabetes, early-onset diabetes, next-generation sequencing, *GCK, HNF1A*

Introduction

Monogenic diabetes is a monogenic, clinically and genetically heterogeneous form of diabetes. This includes neonatal diabetes mellitus, maturity onset diabetes of the young (MODY) and rare diabetes-associated syndromes due to defects in beta cell function. The disease may be inherited within families as a dominant, recessive, or non-Mendelian trait or may present as a spontaneous case due to a *de novo* variant. Well over 40 different genetic subtypes of monogenic diabetes have been identified to date, each having a typical phenotype and a specific pattern of inheritance (1). The acronym MODY was used to highlight its firm hereditary basis (2).

MODY is estimated to account for only about 1-2% of all cases attributed to diabetes, and is usually initially misclassified as type 1 or type 2 diabetes (3,4). Previously, 13 MODY subtypes were identified, and recently a 14th subtype, which is caused by a heterozygous variant in the *APPL1* gene, has been added to the list. All subtypes have in common a primary defect in insulin secretion associated with pancreatic beta cell dysfunction (5). Heterozygous variants in the *GCK* (glucokinase) (MODY 2), *HNF1A* (hepatocyte nuclear factor 1 alpha) (MODY 3), and *HNF4A* (hepatocyte nuclear factor 4 alpha) (MODY 1) genes are the most frequent, and together they account for over 95% of the known genetic causes of MODY. The relative frequencies of MODY subtypes show variations across countries because of the use of different selection criteria for patients for genetic testing (6).

The aim of the study was to determine the clinical and laboratory features, the admission characteristics, and distribution of monogenic diabetes in childhood in Turkey.

Methods

Subjects

In this multicenter genetic study, the data of participants were cross-sectionally analyzed. A nation-wide, web-based, CEDD-NET Data System (http://cedd.saglik-network.org/) was used for data collection between March 2016 and April 2017. Written informed consent was obtained from all subjects, and the study was approved by Gülhane Military Medical Academy, Ethics Committee on 17.02.2016 (number: 50687469-1491-191-16/1648-409).

A total of 169 patients, aged 0-18 years, who were molecularly diagnosed with monogenic diabetes were enrolled. The suspected clinical diagnoses of monogenic diabetes were based on: (1) The early onset of diabetes (< 25 years of age); (2) negative pancreatic autoantibodies; (3) persistently detectable C-peptide; and (4) low or no insulin requirement 3 years after the diagnosis or dominant inheritance or incidental hyperglycemia.

Clinical data of the patients, including gender, age at diagnosis, and family history of diabetes such as diabetic complications and treatments, were obtained through medical records during hospitalization. Laboratory data at diagnosis, including blood glucose, insulin, C-peptide, glycated hemoglobin A1c (HbA1c), and autoantibodies were also collected.

Genetic Studies

To confirm the diagnosis of monogenic diabetes, genetic tests were performed in various genetic laboratories and entered onto the statistics program by each center participating in

the study. Sequence variants were analyzed by VarSome (7), and classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (8). Variants evaluated as "pathogenic" and "likely pathogenic" according to ACMG criteria were included in the study.

Statistical Analysis

Data analysis was performed with IBM-Statistical Package for the Social Sciences for Windows, version 21.0, software (IBM Inc., Armonk, NY, USA). Descriptive statistics of the clinical and laboratory findings were expressed as mean ± standard deviation, number, and percentage. Since the number of patients in most subgroups were few and not homogeneously distributed, comparative statistics were not performed in order to avoid type 2 statistical errors.

Results

A total of 169 patients, 92 males (54.4%) and 77 females (45.6%), were included in the study. The mean age of the patients at diagnosis was 8.18 ± 5.05 years. The mean birth weight was 3110 ± 660 g and the mean gestation week was 38.5 ± 2.1 weeks. Presenting complaints included: incidental diagnosis in 89 patients (52.7%); polyuria and polydipsia in 50 (29.6%); family history of diabetes in 14 (8.3%); weight loss in 5 (3%); and obesity in 2 (1.2%). Five patients (3%) were diagnosed with ketoacidosis and 12 patients (7.1%) with ketosis at initial evaluation. No patient had additional features, such as renal cysts.

Initial treatment consisted of diet alone in 93 patients (55%), diet and insulin treatment in 62 (36.7%), and diet and an oral anti-diabetic drug in 13 (7.7%). Oral anti-diabetic drugs given to the patients at their follow-ups included; sulfonylurea in 34 (20.1%), metformin in 10 (5.9%), and one (0.6%) received repaglinide.

Genetic Findings

The distribution of the variants was: *GCK* (MODY 2) in 100 patients (59.2%); *HNF1A* (MODY 3) variants in 31 patients (18.3%); *ABCC8* variants in 6 patients (3.6%); *KCNJ11* variants in 5 patients (3%); *INS* in 4 patients (2.4%); *HNF4A* (MODY 1) in 2 patients (1.2%); *BLK* (MODY 11) in 2 patients (1.2%); *HNF1B* (MODY 5) in 2 patients (1.2%); *NEUROD1* (MODY 6) in one patient; and *CEL* (MODY 8) in one patient. Genetic diagnosis was not specified in 15 cases and further genetic analysis is planned (Figure 1).

The reason for requesting genetic analysis in 95 (56.2%) of the cases was having a family member diagnosed with diabetes under the age of 25, while in 89 (52.2%) of the cases was autoantibody negativity at the time of diagnosis, and in 15 (8.9%) was that diabetes was diagnosed before 6 months of age.

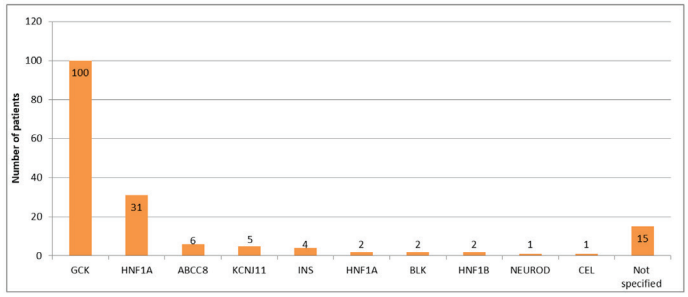

Figure 1. The distribution of the variants causing monogenic forms of diabetes

Characteristics of Patients with GCK Variants

In patients with a *GCK* variant (41 females and 59 male), mean age at diagnosis was 8.55 ± 4.66 years, mean birth weight was 3160 ± 460 g, and mean gestation week was 38.9 ± 1.2. Anthropometric data and laboratory characteristics of GCK-MODY patients at baseline are summarized in Table 1. Almost 67% of the cases with GCK-MODY were diagnosed by random blood glucose measurement. None of the patients had ketonemia/ketonuria, ketoacidosis, or dyslipidemia at the time of diagnosis or during follow-up. Of all the patients, 91 had impaired fasting glycemia (IFG), and nine had impaired glucose tolerance (IGT). Forty (40%) of the patients had typical family history of diabetes in three generations. Sixty-two (62%) of the patients' parents had been diagnosed before the age of 25 years. Nineteen (19%) of the patients' mothers were diagnosed during pregnancy. Initial treatment consisted of diet alone in 74 patients (74%), diet and insulin treatment in 19 (19%), and diet and an oral anti-diabetic drug in 7 (7%). Oral anti-diabetic drugs were started in patients at their follow-ups including sulfonylurea in 13 patients (13%) and metformin in 5 (5%).

Characteristics of Patients with *HNF1A* Variants

In patients with a *HNF1A* variant, including 17 females (54.8%) and 14 males, the mean age at diagnosis was 10.1 ± 3.6 years, mean birth weight was 2870 ± 630 g, and mean gestational week at birth was 38.4 ± 2.52. Anthropometric data and laboratory characteristics of HNF1A-MODY patients at baseline are summarized in Table 2. Nine (29%) of the patients had ketonemia/ketonuria, but none of the patients had ketoacidosis at the time of diagnosis or during follow-up. Only 6% of the cases were diagnosed with HNF1A-MODY by random blood glucose measurement. Thirteen (54.8%) of the patients had typical family history of diabetes in three generations. Twenty-three (74.2%) parents had been diagnosed before the age of 25

Table 1. Anthropometric and laboratory characteristics of GCK-MODY patients (n = 100)

Age at time of study, (years)	12.24 ± 5.29
Age at the time of diagnosis, (years)	8.55 ± 4.66
Female/male, (n)	41/59
Body mass index (kg/m^2)	17.3 ± 3.31
Birth weight, (g)	3160 ± 467
Gestational age at birth, (weeks)	38.9 ± 1.2
Fasting blood glucose, (mg/dL)	127.8 ± 30.7
Fasting insulin, (IU/L)	8.95 ± 24.15
HbA1c, (%)	6.40 ± 0.48
C-peptide at the time of diagnosis, (ng/mL)	1.39 ± 1.05

HbA1c: hemoglobin A1c

Table 2. Anthropometric and laboratory characteristics of HNF1A-MODY patients (n = 31)

Age at time of study, (years)	14.04 ± 3.43
Age at the time of diagnosis, (years)	10.1 ± 3.6
Female/male, (n)	17/14
Body mass index (kg/m^2)	19.56 ± 4.15
Birth weight, (g)	2870 ± 630
Gestational age at birth, (weeks)	38.4 ± 2.52
Fasting blood glucose, (mg/dL)	218 ± 111
Fasting insulin, (IU/L)	10.5 ± 7.3
HbA1c, (%)	8.5 ± 2.5
C-peptide at the time of diagnosis (ng/mL)	1.61 ± 0.99

HbA1c: hemoglobin A1c

years. Five mothers were diagnosed during pregnancy. Initial treatment consisted of diet alone in 9 patients (29%), diet and insulin treatment in 19 (61.3%), and diet and an oral anti-diabetic drug in 3 (9.7%). Oral anti-diabetic drugs were initiated during patients' follow-ups including sulfonylurea in 34 patients (20.1%), metformin in 10 (5.9%), and one (0.6%) received repaglinide.

Characteristics of Patients with Variants in Other Genes

In the present study, *ABCC8* variants were found in 6 patients (3.6%), *KCNJ11* variants in 5 patients (3%), *INS* in 4 patients (2.4%), *HNF4A* (MODY 1) in 2 patients (1.2%), *BLK* (MODY 11) in 2 patients (1.2%), *HNF1B* (MODY 5) in 2 patients (1.2%), *NEUROD1* (MODY 6) in one patient, and *CEL* (MODY 8) in one patient. The genetic diagnosis was not specified in 15 cases.

The reason for requesting genetic analysis in patients with *ABCC8* variants was: being diagnosed before 6 months of age in three cases (50%); having a family member diagnosed with diabetes under the age of 25 in two cases; and diagnosis of non-obese type 2 diabetes in one. Two of the patients had diabetic ketosis at initial evaluation, while the other four did not. The aforementioned two were on sulphonylurea therapy, while the other four continued on the prior insulin regimen.

The reason for requesting genetic analysis in all the patients with *KCJN11* variants was because the patients were diagnosed before 6 months of age. Two of these patients were diagnosed with diabetic ketoacidosis. Sulfonylurea treatment was administered to all the patients and the treatments were continued during their follow-up.

The reason for requesting genetic analysis in *INS* was due to diabetes being diagnosed before 6 months of age. Three of these patients were diagnosed with diabetic ketosis, and all these patients were given insulin and continued this therapy. The patients with *HNF4A* (MODY 1) were diagnosed with IGT without ketone in urine. After the genetic results of the patients were obtained, the initial insulin therapy was terminated, and oral anti-diabetic treatment was initiated. The two patients with *BLK* (MODY 11) were diagnosed with diabetes due to polyuria and polydipsia. One of the patients had diabetic ketosis and, therefore, no response to sulfonylurea treatment so the initial treatment was replaced with insulin therapy. The other patient with the *BLK* variant had no ketosis and responded to sulfonylurea treatment. One of the two patients with *HNF1B* variant was diagnosed with diabetes due to polyuria and polydipsia at the age of 12.7 years with an HbA1c of 6.8% and insulin treatment was deemed unnecessary during the follow-up. As for the other patient who was diagnosed with diabetes due to polyuria and polydipsia with ketosis at the age of 8.1 years and an HbA1c of 11.2%, insulin therapy was necessary throughout the follow-up period. The patient with *NEUROD1* (MODY 6) had the initial complaint of polyuria with IFG. BMI of the patient was 18.51 kg/m^2 and HbA1c, at the time of diagnosis, was 5.6%. The patient did not need additional treatment, since normoglycemia was achieved on diet therapy. The patient with *CEL* (MODY 8) variant was diagnosed by random blood test measurement (HbA1c 8.7%) and sulfonylurea treatment was initiated. After one-year of oral anti-diabetic therapy, since the HbA1c value had risen to 10.8%, insulin treatment was deemed necessary.

Discussion

The relative frequencies of monogenic forms of diabetes show variations according to the countries where the studies took place. For example, MODY 3 is the most common subtype in the United Kingdom, The Netherlands, Denmark, and Norway, but MODY 2 is the most common in Germany, Austria, Poland, the Czech Republic, Italy, Greece, and Spain (6). In our study, MODY 2 was the most frequently detected

subtype (59.2%). These differences may be explained by a variety in ethnicity and the use of different selection criteria for patients for genetic testing (6).

GCK-MODY is one of the most common subtypes of monogenic diabetes in the pediatric diabetes clinic and its clinical phenotype is remarkably homogeneous among patients (9). The patients show non-progressive mild hyperglycemia from birth. Their HbA1c is mildly elevated but usually below 7.5% (10). In agreement with recent studies, the mean HbA1c value of our GCK-MODY patients was 6.4%. Because blood glucose does not deteriorate significantly over time, this subtype of monogenic diabetes is rarely associated with complications of diabetes and patients do not generally require any treatment (11). In most cases, diet alone is sufficient to achieve metabolic control (12). However, due to incompatibility with diet therapy in our cohort, oral anti-diabetic drugs (18%) and insulin regimen (19%) were added to the treatment of patients.

HNF1A-MODY is the most common form of monogenic diabetes that results in familial symptomatic diabetes (13). Almost 94% of the cases were diagnosed as HNF1A MODY with a symptomatic finding in recent reports. Over time, fasting hyperglycemia and osmotic symptoms (polyuria, polydipsia) present but patients rarely develop ketosis because some residual insulin secretion persists for many years (14). In our study, 29% of the patients had ketosis and the treatment consisted of insulin treatment or an oral anti-diabetic drug in addition to diet therapy in 71% of these patients. The development of chronic complications of diabetes is related to the degree of metabolic control (14). None of our patients developed complications.

GCK (MODY 2), *HNF1A* (MODY 3), and *HNF4A* (MODY 1) genes are responsible for many genetic causes of monogenic diabetes (6). The other monogenic forms of diabetes have been shown to represent together only a small proportion (<5%) of cases, but molecular confirmation of the diagnosis prevents unnecessary insulin treatment in patients with monogenic diabetes and improves both metabolic control and quality of life (15). Sulfonylurea treatment was started for all our patients with *KCJN11* variants and this treatment was continued during their follow-up. One-third of the patients with *ABCC8* variants continued sulfonylurea treatment alone, while all INS-MODY patients were started on insulin therapy and continued with this therapy. In a single-center study on monogenic diabetes conducted in Turkey, half of the patients with the *ABCC* variant and the patient with the *KCJN11* were receiving sulfonylurea therapy, while the patient with the *INS* variant was on insulin regimen (16).

Family history is crucial for requesting genetic analysis for monogenic diabetes and evidence such as diagnosis of diabetes under the age of 25 years in at least one family member, autosomal dominant inheritance pattern through at least three generations, or the existence of at least two first-degree relatives with diabetes should raise suspicion of monogenic diabetes (17). In our study, having a family member diagnosed with diabetes under the age of 25 was the most frequent reason for requesting genetic analysis (56.2%). These prediction models show high sensitivity, although with relatively low positive predictive values that result in even higher proportions of variant-negative cases. However, use of these models leads to diagnosis of the more rare monogenic forms of diabetes and to distinguish monogenic diabetes from diabetes type 1 or type 2, to avoid the unnecessary insulin or sulfonylurea treatment, which may severely affect the patient's health.

Study Limitations

Detection of all case data for the whole country was incomplete and naturally some findings were not declared. Moreover, laboratory and genetic tests were analyzed in different centers. There may also be bias in terms of case determination in different centers. Finally, lack of the standardized approach in the management of diabetes was one of the limitations of the study.

Conclusion

This is the first Turkish multicenter genetic study of children with monogenic diabetes. In this study we tried to determine distribution, and clinical and laboratory features of monogenic diabetes. Our study showed that random blood glucose measurement had an important place in diagnosis and the most common reason leading to genetic analysis was having a family member diagnosed with diabetes at a young age. Moreover, the study results may contribute to a better understanding of the pathogenesis of the most common subtypes of monogenic diabetes and to a more personalized approach to patients' treatment, follow-up, and genetic counselling.

Acknowledgement

We appreciate Pediatric Endocrinology and Diabetes Association and Erkan Sarı; Mikair Gerens and Can Celiloğlu for their contribution.

Ethics

Ethics Committee Approval: The study was approved by Gülhane Military Medical Academy, Ethics Committee at 17.02.2016 (number: 50687469-1491-191-16/1648-409).

Informed Consent: Written informed consent was obtained from all participants or their parents/guardians.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Şükran Darcan, Concept: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Şükran Darcan, Design: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Şükran Darcan, Data Collection or Processing: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Yılmaz Kor, Erdal Eren, Özlem Korkmaz, Merih Berberoğlu, Gülay Karagüzel, Eren Er, Ayhan Abacı, Olcay Evliyaoğlu, Emine Demet Akbaş, Edip Ünal, Semih Bolu, Özlem Nalbantoğlu, Ahmet Anık, Meltem Tayfun, Muammer Büyükinan, Saygın Abalı, Gülay Can Yılmaz, Deniz Kör, Elif Söbü, Zeynep Şıklar, Recep Polat, Şükran Darcan, Analysis or Interpretation: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Şükran Darcan, Literature Search: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Eren Er, Şükran Darcan, Writing: Damla Gökşen, Ediz Yeşilkaya, Samim Özen, Eren Er, Şükran Darcan.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2021.0010

J Clin Res Pediatr Endocrinol 2021;13(4):439-445

Qualitative Parental Perceptions of a Paediatric Multidisciplinary Team Clinic for Prader-Willi Syndrome

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What is already known on this topic?

Prader-Willi syndrome is a complex, multisystemic, neurodevelopmental genetic disorder. Clinical symptoms vary with age and include infantile hypotonia, hyperphagia, excessive weight gain, endocrine dysfunction, behavioural problems and psychiatric issues. Guidelines recommend a multidisciplinary team (MDT) approach to provide a multi-faceted approach to manage the diverse symptoms.

What this study adds?

Medical and social care access varies greatly, and no family had previously accessed an MDT. Parents valued the connection with the specialist clinical team and with other families. Parents perceived an MDT clinic to be an efficient way to manage appointments and receive integrated timely support.

Abstract

Objective: This preliminary review was conducted to inform the design of a new service to support families with children with Prader-Willi syndrome (PWS). Families were invited to attend a pilot clinic at a hospital outpatient department, comprising appointments with a multi-disciplinary team (MDT).

Methods: Following the clinic, families (n = 6) were invited to take part in semi-structured qualitative interviews that were audio-recorded, transcribed and analysed using thematic analysis.

Results: Families reported that the clinic offered enhanced support in the following categories: integrated care; professional input; signposting to social support (respite and financial); connection with the wider PWS community; and behavioural support.

Conclusion: This is the first paper that documents the parental perspective of an MDT clinic for children with PWS. The families felt an MDT clinic was superior to current care, offering more convenient access to an enhanced service, which would provide integrated and consistent care for their children's diverse, challenging and changing needs.

Keywords: Paediatric, Prader-Willi syndrome, multi-disciplinary team, qualitative

Introduction

Prader-Willi syndrome (PWS) is a neurodevelopmental genetic disorder caused by a lack of expression of the q11-q13 region on paternal chromosome 15 and has three genetic subtypes (1). The incidence of PWS is approximately 1 in 15,000 people (1). Clinical symptoms vary with age and the earlier symptoms include infantile hypotonia, failure to thrive, short stature, hypogonadism and other endocrine dysfunctions. Symptoms developing later include hyperphagia and excessive weight gain, if left uncontrolled. PWS is also associated with behavioural

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Conflict of interest: None declared

Received: 10.03.2021

Accepted: 14.08.2021

problems such as tantrums, self-harm and psychiatric issues (1,2,3,4,5).

The multifaceted nature of this disorder provides challenges to clinicians, and medical care alone may leave needs unmet (6). A multidisciplinary team (MDT) clinic has been recommended as it provides a patient-centred, biopsychosocial approach to treatment (3,6). In an MDT clinic patients are seen by a wider range of health professionals equipped to support with behaviour, diet and community connections, and attending an MDT clinic has been shown to improve mortality and morbidity (6).

MDT clinics for PWS are not widespread across the UK. There is no specialist MDT clinic that can be accessed by the patients in the South West of EnglandAs part of a funding bid to initiate a clinic in this region of England, a single pilot MDT PWS clinic was conducted. Due to the low prevalence of PWS (5), the clinic would support families across a wide geographical area. To ensure the future clinic was designed around patients' needs, the parents who attended this pilot clinic were asked to take part in a qualitative review that discussed the needs of their family and their perceptions of the clinic. Whilst several recent articles have reported that an MDT is the best model of care for children with PWS and their families from a health professional point of view (3,6,7), this preliminary work, for the first time, presents the parents' perspective of an MDT PWS clinic.

Methods

Experimental Subjects

Families (n = 6) were selected from the regional database of children with PWS and confirmed their willingness to attend a pilot clinic. Participant characteristics are detailed in Table 1. On invitation, parents were informed about the opportunity to provide feedback. On arrival, a member of the clinical team introduced the clinic and the research team. All six sets of parents agreed to participate in the review and were consented by the research team. The families at current care varied but typically included a large number of appointments with a range of health professionals, with some accessing support through their child's special education school and some through endocrine or weight management clinics, and others via PWS charities. One family had disengaged with their current NHS PWS care.

Interviews were conducted by EH (researcher) and JC (researcher) who were external to the clinical team and accompanied by RA (social worker). Interviews took place on a single day, in an outpatient ward of a large community hospital, where the clinic would likely be held if funded. Interviews were carried out in a private appointment room, adjacent to the clinical team. Interviews were semi-structured (See Appendix 1 for interview schedule). Three interviews took place prior to the clinic appointments; five took place after, with clinic scheduling allowing two families to be interviewed both before and after the clinic appointments, resulting in eight interviews in total. All interviews were audio recorded using a dictaphone. The duration of each interview was between 10 and 25 minutes.

Data Analysis

Interviews were transcribed *verbatim* by external, approved services and were anonymised. Thematic analysis was used to code the transcripts independently by two researchers due to its applicability in this type of study (8). The same two then met to refine coding as part of the iterative analysis process. Transcripts were re-read and recoded with amendments. Sub-themes and overarching themes were decided upon collaboratively.

The service review had approval from the Patient Experience and Involvement Team at University Hospitals Bristol and all interviewees provided informed consent.

Interventions

The clinic itself comprised three appointments. First, families were seen by the consultant paediatric endocrinologist, a weight management nurse specialist, and a paediatric endocrine nurse specialist. The clinic also included a consultant adult psychiatrist with an interest in PWS as a voluntary observer. Secondly, patients were seen by a clinical psychologist and a dietician together. Finally, patients were seen by the social worker, who also participated in the clinic interviews.

Main Outcome Measures

The interviews sought to explore parents' experience of the MDT clinic compared with their previous care and understand the areas of greatest need for families. They sought to engage parents in the design of both the structure and the content of the clinic, and thus feedback was requested to facilitate co-design.

Results

Each of the identified themes, shown in Figure 1, will be presented in turn along with illustrative quotes in Table 2.

Integrated Support

Overall, parents perceived the clinic to both enhance their access to support and be an improved delivery mode

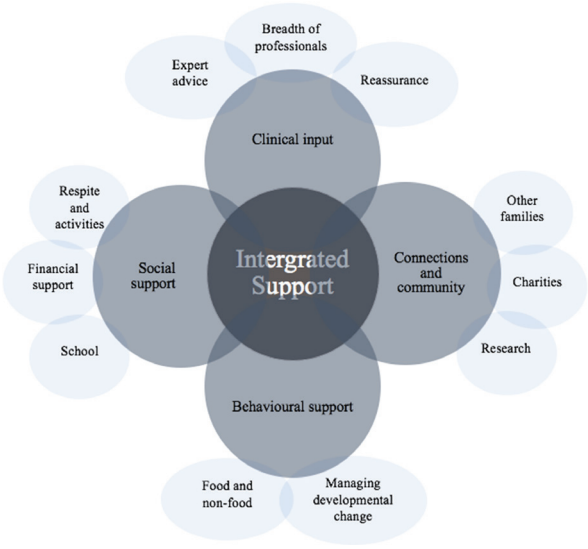

Figure 1. Families experienced integrated support from an MDT led clinic

MDT: multidisciplinary team

when compared to current care. Whilst families valued appointments with the endocrinologist - the typical care received by most families - they felt the MDT approach to be superior. Importantly, parents felt that an MDT clinic would enable a more "joined-up" approach to their care, facilitating collaborative, coordinated strategies without lengthy referral times (Table 2, 1a).

The clinic offered families a *"one-stop-shop"* reducing the disruption and time-off school caused by multiple appointments. This was beneficial when considering their children's need for routine and gave parents the freedom to better manage other life commitments (Table 2, 1b). Whilst families acknowledged the sometimes-lengthy travel time to reach the clinic, parents felt it was acceptable to facilitate access to this breadth of support (Table 2, 1e).

Clinical Input

Access to Health Professionals

Families typically had, or had previously had, frequent contact with a wide range of medical professionals but there was a large disparity in access to services. Some special schools were reported to host clinics but this access was not available to all families. Most noted they only had regular contact with an endocrinologist, therefore access to other health professionals at a single clinic was praised (Table 2, 2a).

Families felt that it was beneficial to see every staff member present at the clinic. In addition, families recommended that the inclusion of speech and language teams (Table 2, 2b), physiotherapists, orthotics, and creative therapies

Table 1. Participant characteristics

	Patients (n = 6)	Parents (n = 9)
Characteristics	**N**	**N**
Female	5	6
Ethnicity		
Caucasian	6	9
Age		
< 5	3	
5-11	1	
12 +	2	

would enhance the service further. Parents discussed how the inclusion of a play-worker would improve the impact of the clinic by providing a distraction for younger patients and supervising older children, thus reducing the need for parental supervision. One example of a benefits of this would be that parents of older children would be able to converse more candidly with clinicians about difficulties without these discussions taking place in front of the child (Table 2, 2c).

Expert Advice

Some parents were highly informed about best-practice in other clinics in the UK and internationally and were keen to ensure their child had the same access to current, top quality care (Table 2, 2d). They had participated in these interviews in part, to ensure staff connected with, and replicated the programmes running elsewhere and recommended that staff work collaboratively with charities to access specialist training (Table 2, 2e).

Reassurance

Other families explained that the greatest benefit to attending a specialised PWS clinic was to be able to "check in" with professionals, to ensure they were doing everything they could for their child. This reassurance renewed their sense of strength as parents, restoring their energy to maintain the levels of care required (Table 2, 2f).

Behavioural Support

Parents felt strongly about pro-actively managing children's behavioural problems and felt that the pilot clinic had already given them helpful strategies to implement.

Food and Non-food Management

Parents explained that as behavioural problems were often triggered by food, seeing the dietitian and psychologist together enabled them to fully explore the relevant issues (Table 2, 3a). Strategies for wider behaviour management

Table 2. Illustrative quotations

Integrated support	(1a) To have those professionals together, that is a success in itself, because they communicate then. The fact that the dietician is in the same place, if there's an issue with the weight, we can go straight in and see the dietician." (Mother to Belinda, after) (1b) "We had so many appointments all the time. That, when you are trying to run your life, as well, it is a lot just to fit all that in. So, to be able to come to one place and to see all these different people, that's been really good" (Mum to Freya, after) (1d) "I was saying, "I hope it's worth the trip this time," because we didn't really know what to expect. Yes, if it was something like this, it would be definitely worth the trip" (Mother of Freya, after)
Professional input	
Breadth of professionals	(2a) "Yes, so we go to endocrinology. We don't see a dietician anymore; we don't see physio. She has speech through school" (Mother of Belinda, after) (2b) "Language and communication would be good. We did see speech and language at school, but we haven't seen her for about a year" (Mother of Abigail, before) (2c) "To have play leaders here or play workers, because obviously there is stuff that you want to talk about, you can't talk with your child present, but to have a play team available in these clinics […] a lot of things are really big triggers for her at the moment, and I imagine for others with Prader-Willi of a similar age, they'd struggle. Sometimes anyway even when she was younger, you don't necessarily want to say the really bad stuff in front of your own child" (Mother of Alice, after)
Expert advice	(2d) ""Why don't we have that? I'd love access to something like that." So, I think this is ideal" (Mother of Belinda, before) (2e) "The best people to speak to really are the Prader-Willi Association, because over the years we've had loads of training and conferences from them" (Mother of Alice, after)
Reassurance	(2f) "Feeling that you can go back, having some questions answered that, maybe, have been making us feel like we're not doing the best job sometimes, to then being able to get some support with that and then go home and start to feel better again and like we're ready to tackle that" (Mother of Freya, after)
Behavioural support	
Food and non-food management	(3a) "When she steals food, I was talking about it feels wrong to discipline her, because she can't help it, but at the same time, I want her to know that she shouldn't be stealing food. So, just being able to talk, and the fact that they [psychologist and dietitian] were in the room together really helped" (Mother of Belinda, after) (3b) "She [mother] can't even… well, she's powerless… she's glued at home with him, […] And it has been like that where it's kicked off, he's had to be restrained, everything […] It's quite sad, isn't it?" (Father of Jason, before)
Managing developmental milestones	(3c) "I'm very much aware that things are going to get harder as she gets older and I want to be proactive rather than reactive. I want to be on the ball and the more I can do to learn and to meet people, and just the more I can do to be prepared, the better." (Mother of Belinda, after) (3d) "It's going to be quite a big shift when she goes to school in September, especially with the behavioural stuff. You know, if they come to us and say, "She's been doing this today," it's like, "Okay, we don't know how to do this, we don't know what to tell them", because we know how to deal with it when it's us but not when she's left" (Father of Freya, after)
Social support	
Respite and activities	(4a) "But we do need… The thing is what I struggle with is getting him doing activities, because there's nothing around my way for disability… children with disabilities, and, basically, if there is […] but they want £30 a day, and there's no… You know, that's the reason why he can't" (Mother of Jason, before) (4b) "…my mum died, so we don't even have my mum. Another lady who used to help a lot has got Alzheimer's and obviously I can't rely on that family because they've got enough of their own woes" (Mum of Alice, after)
Financial support	(4c) "There's nothing for him. Well, there is, but you've got to pay for it" (Mother of Jason, before) (4d) "The biggest thing, really, is we've got DLA [disability learning allowance] due through now and I just don't know how to word stuff, so that's really frustrating for us. We do feel that she is entitled to it." (Mother of Sarah, before)
School	(4e) "School are brilliant. Yes, school are fantastic. They do lots of clinics and the dentist comes to the school as well so […] No, I do''t think she'd cope anywhere else. It's the best facility for her, it really is" (Parent of Abigail, before) (4f) "The school have just fobbed us off […] they haven't even put the lunch boxes out of sight […] I mean they won't go to the toilet with her because they say they haven't got enough staff and they won't want a one-to-one, they don't encourage it, they say it's not healthy for the child because they get too attached" (Mother of Sarah, before)
Connections and community	
Connections to other families	(5a) "It's not like you can talk to the school mums, like I would with my other children. I can't say, "Oh, is he doing this and that? […] To me, that is the most useful, because other parents that've done it- which is why I think it would nice today, if I get see other parents in the waiting room, it's just, again, another reassurance that we're all in the same boat and we're doing what we can" (Mother of Belinda, before) (5b) "But it's always scary seeing the adults and stuff who have it, because it's looking into the future, before we are ready. But the future is always changing, the research is always changing…" (Mother of Freya, after)
Connections to PWS charities	(5c) "I'm always ringing them up, PWS to ask for- when it comes to things like- do you know? […] Obviously they know what they're talking about these people." (Father of Jason, after) (5d) "The PWSA, the charity, or the FPWR the charity, they could be useful, kind of thing, liaising. So, they maybe a representative for them here" (Parent of Freya, after)
Connection to research	(5e) "Obviously, the conferences are either alternate years or really random and far away, but to be able to offer here some of the expertise locally to us, that would be really good" (Mother of Alice, after) (5f) "Any new research, happy to have that. That would be really good. Any clinic trials, I'm happy for her to be involved in trials if there are any that she would be suitable for" (Parent of Abigail, before)

FPWR: Foundation for Prader-Willi Research

were also valued, particularly the parents of the older children who sought help for difficulties with violent outbursts, which had previously escalated to require police involvement in one case. They had previously refused offers of assistance, but they now felt they needed support to manage and were willing to accept this from the pilot clinic (Table 2, 3b).

Managing Developmental Milestones

The families reported that the consistency of the clinic would enable them to feel more supported throughout times of change (Table 2, 3c). Parents valued having clinical input on adjustments such as moving schools or their child progressing to independent living and also felt this expert input made them feel more equipped to share this knowledge with other key caregivers (Table 2, 3d).

Social Support

The inclusion of a social worker was integral to the family's experience of the clinic. Many families were juggling their child's care needs with the support of their wider families, without having access to the full range of support available to them.

Respite and Activities

Families were often not receiving formalised support packages. Therefore, for those who were not able to pay, children had little access to extra-curricular activities or social time with peers (Table 2, 4a). Parents of the older children specifically raised this "The two main things are respite and activities for him" (Mother of Child E, after). When children did attend activities, the parents reported being required to stay with their child, thus negating any respite effect and giving them little time for themselves or other family needs. Some families were occasionally supported by informal respite time with grandparents or friends. However, this was felt to be non-sustainable (Table 2, 4b).

Financial Support

Finances were a perceived barrier to improving the child's wellbeing, independence and making dietary change (Table 2, 4c). Families were not always aware of the extent of the support available to them, and how to access it. The social worker was able to support with this, and families saw this as an asset to the clinic (Table 2, 4d).

School

School was a polarising experience for the families. Some parents reported schools being extremely supportive, typically those at special educational needs schools. These families had access to a wider range of support and additional health care facilities (Table 2, 4e).

Other families reported their child's school to be unsupportive, offering little in the way of additional assistance. These families perceived the prospect of the clinic's nurse and social worker aiding mediations with schools as an advantage.

Connections and Community

There was variability in knowledge about PWs across the families interviewed, and also the extent to which they were connected to other services and families.

Connections to Other Families of Children with PWS

Some families reported feeling isolated from others with PWS. Those who had engaged in either in-person or online support groups reported them to be a beneficial source of camaraderie and advice, as well as allowing parents to give back and support others. The clinic was felt to be beneficial in offering further opportunities to meet other families, regardless of their current level of connection (Table 2, 5a).

It is important to note that one family expressed that they had had concerns prior to the clinic about meeting older children with PWS due to an apprehension of experiencing what their life may be like in the future (Table 2, 5b).

Connection to PWS Specific Charities

The advice from, and connection to, PWS charities including Prader-Willi Syndrome Association (PWSA) and Foundation for Prader-Willi Research (FPWR) were highly valued. Even one family that refused most help, regularly contacted charities for advice (Table 2, 5c). Parents felt that having a representative from these organisations at the clinic would be beneficial (Table 2, 5d).

Research

Families sought to stay informed with the latest developments but feared that they would miss out due to the complex wording of academic works, and the geographical and cost barriers to attending conferences. Parents felt that having a professional who could summarise what recent research findings mean for their family would be advantageous (Table 2, 5e). Families were willing for their children to take part in research and were keen to support developments in PWS treatment and understanding (Table 2, 5f).

Discussion

Parents in this preliminary study felt that the MDT clinic facilitated the holistic care required to manage their child's diverse needs. The clinic was perceived to be a potential hub for their child's care (7), a sounding-board where families could share concerns and keep up-to-date with developments. Families felt a sense of apprehension about what the future held, knowing that their child's condition and thus the challenges they faced would vary with age (7). By having consistent appointments, potentially every six months (6), throughout their child's life, families were

optimistic that the clinic could offer sustainable management that would enable concerns to be pre-empted (7). As access to specialist care is currently not universally accessible (PWSA UK, 2019) this clinic would facilitate equal access to all in the region, regardless of geography or finances.

Families understood the MDT clinic to enable integrated care, with enhanced communication and reported coming-away with tangible, implementable actions, without lengthy referral times. Families in this review were more concerned about treatment outcomes involving health and social integration, and less directly concerned about weight. Centres of excellence for PWS care have been suggested to support socialisation by including family-based therapeutic options, liaising with schools and developing education health and care plans (6,9). This clinic goes further with an integrated social worker to implement links between healthcare, education, respite and activities to help their children thrive (10). Notably, the collaboration between the psychologist and dietician was valued, addressing the need to manage behavioural difficulties alongside the relationship with food (11). Parents suggested that other specialists who should attend the proposed MDT would be speech and language team members and specialists to help with concerns with, and these suggestions are consistent with guideline recommendations (3,6,7).

Every family commented on how they valued meeting other families. Whilst clinics may not perceive peer support to be the primary function of this kind of appointment, other UK clinics do list this as an aim for their clinic (12). As families very much appreciated these relationships, help in accessing relevant charities and networks to obtain further connection would be valuable.

The clinic was considered to be practical and worked logistically. Long journey times were considered worthwhile to receive this standard of care. The MDT condenses some children's extensive calendar of appointments, reducing disruption; particularly important when the importance of routine (11,12) and the high prevalence of autism or autism-like characteristics in children with PWS is considered (13). Whilst this long appointment was preferable, in the interest of quality, privacy and attention, families voiced the importance of a play-worker to support their child during possibly lengthy appointments.

The views expressed may be transferable to other similar regions where families do not have access to an MDT. Should the clinic trial the MDT approach as their core offering, this would open opportunities to both quantitatively and qualitatively evaluate patient outcomes in a larger trial. An economic evaluation of the cost-effectiveness of the service may also provide insightful, and important data outcomes at this point.

Study Limitations

It is important to note that all-but-one of these families were previously engaged with treatment. Thus, further work with families who are currently disengaged with care would help to create a service that has broader appeal. In order to maintain research impartiality, researchers were external, and the clinical team, with the exception of the social worker, were not involved in interviews. However, as the interviews took place in the same setting and researchers had an in-depth understanding of PWS (14,15,16), this division may not have been absolute and may have influenced responses.

Conclusion

Families felt the experience of an MDT clinic was superior to visiting the endocrinologist alone, enabling them to address issues around social support and behaviour in addition to health. They felt the sustained presence of a specialist clinic offered the support needed to feel competent in pro-actively meeting their child's needs.

Acknowledgements

The authors wish to thank the families who contributed to the writing of this paper, and the clinical staff members who ran the trial clinic, enabling this article to be conducted.

Ethics

Ethics Committee Approval: This work is a service article and thus no ethical approval was required. The service article had approval from Patient Experience and Involvement Team at University Hospitals Bristol and all interviewees provided informed consent to their participation and the publication of the results. All names used in this work are pseudonyms to protect the patient's anonymity.

Informed Consent: The service review had approval from the Patient Experience and Involvement Team at University Hospitals Bristol and all interviewees provided informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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Financial Disclosure: This work received no external funding. This article was conducted by researchers funded by the GW4, MRC and the NIHR Bristol Biomedical research centre, Nutrition theme. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0107

J Clin Res Pediatr Endocrinol 2021;13(4):446-451

Clinical Management in Systemic Type Pseudohypoaldosteronism Due to *SCNN1B* Variant and Literature Review

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What is already known on this topic?

Pseudohypoaldosteronism is a life threatening disease due to serious salt loss. Differential diagnosis from other adrenal insufficencies is important because the treatments are different. Patient compliance is difficult due to the need for excessive amounts of oral treatments.

What this study adds?

We present a patient with a difficult diagnostic process due to hypertension. A novel variant resulting in a premature stop codon was detected in the patient. Clinical and laboratory features of all published cases with *SCNN1B* variant are reviewed.

Abstract

Systemic pseudohypoaldosteronism (PHA) is a rare, salt-wasting syndrome that is caused by inactivating variants in genes encoding epithelial sodium channel subunits. Hyponatremia, hyperkalemia, metabolic acidosis, increased aldosterone and renin levels are expected findings in PHA. Clinical management is challenging due to high dose oral replacement therapy. Furthermore, patients with systemic PHA require life-long therapy. Here we report a patient with systemic PHA due to *SCNN1B* variant whose hyponatremia and hyperkalemia was detected at the 24th hour of life. Hyperkalemia did not improve with conventional treatments and dialysis was required. He also developed myocarditis and hypertension in follow-up. Challenges for diagnosis and treatment in this patient are discussed herein. In addition, published evidence concerning common features of patients with *SCNN1B* variant are reviewed.

Keywords: Systemic pseudohypoaldosteronism, hyponatremia, hyperkalemia, metabolic acidosis, epithelial sodium channel, *SCNN1B*

Introduction

Aldosterone is a mineralocorticoid hormone that provides sodium absorbtion and potassium secretion. Sodium crosses the apical membrane in the principal cells in kidney and enters the epithelial cell through the ion selective epithelial sodium channel (ENaC). Potassium is also secreted into the tight epithelium in the kidney. The ENaC is located in the apical membranes of sensitive tissues, such as the distal nephron, distal colon, salivary and sweat glands and creates a rate-limiting step in sodium reabsorption (1,2). ENaC is a heteromultimeric protein consisting of three subunits, α, β, γ (3). ENaC subunits are encoded by the *SCNN1A* gene on chromosome 12p13, and the *SCNN1B* and *SCNN1G* genes on chromosome 16p12.2-p12.1.

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Conflict of interest: None declared

Received: 23.05.2020

Accepted: 11.08.2020

Pseudohypoaldosteronism (PHA) is a salt wasting syndrome that develops due to variants in the mineralocorticoid receptor (MR) or ion channels in the kidney tubules. The estimated incidence of this rare disease is between 1/47,000 and 1/80,000, and its prevalence is < 1/1,000,000 (4,5,6). PHA1 is divided into renal (PHA1A) and systemic (PHA1B) forms depending on mutation in the *NR3C2* gene that codes the MR or in the *SCNN1A, SCNN1B* and *SCNN1G* genes that code ENaC subunits, respectively. In the systemic form, there is serious salt loss from the lung, colon, sweat and salivary glands, besides the kidney, and the symptoms begin in the neonatal period. Systemic PHA, which is inherited in an autosomal recessive fashion, results in life-threatening hyponatremia, hyperkalemia and metabolic acidosis. Plasma renin and aldosterone levels increase significantly, indicating end organ resistance. Treatment requires high doses of sodium replacement and potassium-lowering approaches. In this article, the clinical management of a patient with PHA due to an *SCNN1B* variant and the common features of patients with *SCNN1B* variants are presented.

Case Report

The male patient was born at term by normal vaginal delivery with a birth weight of 3600 grams and was followed up in the neonatal intensive care unit due to respiratory distress. It was learned that hyponatremia and hyperkalemia were detected (Na 118 mEq/L, K 8 mEq/L) at the 24th hour of hospitalization. He received 6x1 g salt, 4x2 g/kg calcium polystyrene sulfonate, 12x1 mL/kg 8.4% $NaHCO_3$ and 12x4 mL/kg 3% NaCl treatment during this period. He was subsequently referred to our clinic because of lack of response to treatments at the age of 1 month and 15 days. On physical examination, his weight was 4000 grams [-1.32 standard deviation score (SDS)], height was 55 cm (-0.34 SDS), and he did not have hyperpigmentation and any abnormality on genital examination. His daily weight gain was insufficient. His blood pressure was high at 110/80 mmHg (95th percentiles for systolic and diastolic blood pressure for this age group are 94/46 mmHg). There was third degree consanguinity between the parents. His brother had died at the age of seven days with hyponatremia and hyperkalemia. On admission, Na concentration was 123 mEq/L, K 7.1 mEq/L, blood pH 7.12, and HCO_3 was 10.8 mmol/L. Echocardiography was normal. He was diagnosed with congenital adrenal hyperplasia. Hydrocortisone and fludrocortisone treatments were started. Calcium gluconate, glucose-insulin infusion, $NaHCO_3$ infusion, and salbutamol inhalation were administered for hyponatremia and hyperkalemia. Despite these interventions, hyponatremia persisted. Anti-hypertensive treatment was started for hypertension (0.1 mg/kg/day amlodipine). Oral 2x0.5 grams of salt was also added to the treatment and the dose was gradually increased. The laboratory findings at admission to our clinic were: 17-hydroxyprogesterone 1.22 ug/L, dehydroepiandrosterone sulfate 241.8 µg/dL, total testosterone 215.6 ng/dL, adrenocorticotropic hormone 255 pg/mL, cortisol 43.8 µg/dL, renin 16.3 ng/mL/hour (NR = 2.4-37), aldosterone 6.4 µg/L (NR = 0.065-0.86), urine Na 134 mmol/L, urine K 2 mmol/L (when blood Na 123 mEq/L and blood K 7.1 mEq/L). The transtubular potassium gradient (TTKG) was 1.3, indicating very low renal potassium excretion. Based on the laboratory test results, hydrocortisone and fludrocortisone treatments were discontinued. Urinalysis, urine culture and renal ultrasonography were normal. During this process, hypertension continued. The diagnosis of systemic PHA was considered given that the patient was admitted with hyponatremia and hyperkalemia in the neonatal period, with high aldosterone level, increased urinary Na excretion, and decreased K excretion. When hyperkalemia did not respond to conventional treatments, including a trial of calcium polystyrene sulfonate at 1 g/kg/dose in four doses, peritoneal dialysis was required. After three days of peritoneal dialysis, K decreased to 4.18 mEq/L. Electrolyte values of the patient were kept in the normal range with 6x1 g of oral salt and 4x3 g of anti-potassium treatment. The patient had fever during the follow up and despite subsequent normalization of body temperature, tachycardia persisted. The patient was diagnosed with myocarditis due to an increase in acute phase reactants, troponin I level and electrocardiographic findings. Myocarditis findings regressed on the tenth day. However, the cause of hypertension could not be explained and was thought to be related to the salt treatment. Then his blood pressure returned to normal ranges and amlodipine and propranalol treatments were discontinued on the fourteenth day. The Sanger sequencing analysis of the *SCNN1A* gene, which is the most common gene to carry pathogenic variants in systemic PHA type 1B, was found to be normal. In subsequent Illumina MiSeq sequencing, a homozygous c. 978 C > A (p.Tyr326Ter) variant was detected in the sixth exon of the *SCNN1B* gene (NM_000336). After oral salt (6x1 g) and antipotassium (4x3 g) administration, the patient had normal electrolyte values and was discharged. One month after discharge, during a period of infection, the patient had to be hospitalized again due to the loss of oral intake and salt wasting crisis. At the last follow-up, the patient was seven months old, his weight was 7.3 kg (-1.3 SDS), height was 68 cm (-0.83 SDS) and blood pressure was 80/35 mmHg (50th percentile). His growth and development was

appropriate for the age with the current treatments (6x1 g oral salt, 4x3 g calcium polystyrene sulfonate and 4x2 mL $NaHCO_3$). Clinical follow-up continues.

Discussion

Systemic PHA type 1 is a rare life-threatening disease. Clinical manifestations are similar to other adrenal gland insufficiencies, such as congenital adrenal hyperplasia, hypoaldosteronism, and secondary PHA. The clinical presentation is characterized by insufficient weight gain, vomiting, and dehydration (4,6,7,8). Our patient had normal genitalia with hyponatremia and hyperkalemia. Hydrocortisone and fludrocortisone treatments were started until adrenal androgen results were obtained. Since adrenal hormone levels were normal in the follow-up, the patient was diagnosed with PHA1B. In the differential diagnosis of our patient, transient aldosterone resistance secondary to urinary tract infection was also considered (4), and this diagnosis was ruled out when urinalysis, urine culture and renal ultrasonography were normal. Elevated aldosterone level accompanying hyponatremia and hyperkalemia supported resistance to aldosterone in the kidney and directed the treatment of our patient.

To date, more than 40 variants have been reported in the genes encoding ENaC subunits (9), and these variants have most often been found in the gene encoding the alpha subunit. Eleven variants have been reported in the gene encoding the beta subunit (Table 1). Consequently, we first investigated the *SCNN1A* gene encoding the alpha subunit, but when no pathogenic variant was found, the *SCNN1B* gene was sequenced. In this patient, a novel c.978 C > A (p.Tyr326Ter) homozygous variant was detected, presumably leading to a premature stop codon in the *SCNN1B* gene. This variant has not been previously reported in the literature or in the GnomaD database including genome data from healthy individuals, and is anticipated to be a pathogenic change by Variant Taster, one of the *in silico* assessment tools used to predict pathogenicity of a variant. In addition, this change has been considered pathogenic according to the criteria of American College of Medical Genetics guidelines 2015 (PVS1, PM2, PP3).

The *SCNN1B* gene, consisting of 13 exons, encodes a transmembrane protein with two transmembrane segments with 640 amino acids (Figure 1) (7). The detected variant is located in the extracellular region of the protein, and, it is predicted to cause loss of function by creating an early stop codon, presumably resulting in nonsense mediated decay at the mRNA level.

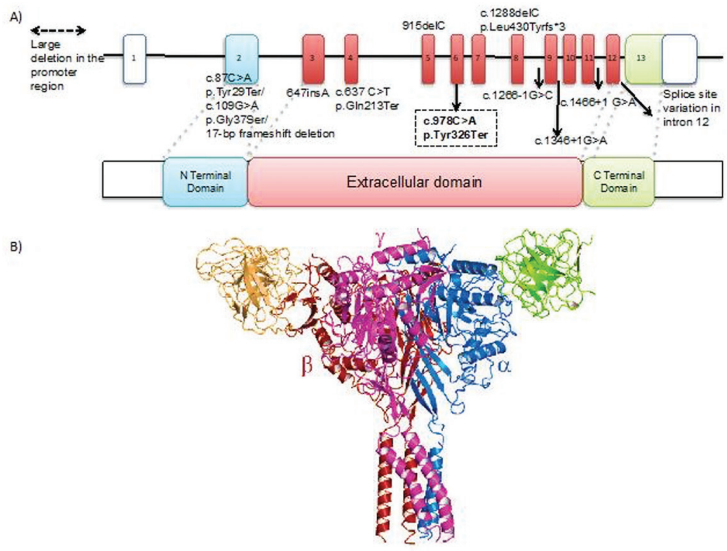

Figure 1. A) 3-D structure of the SCNN1B protein (PDB 6BQN) black arrow indicates the mutated position on the protein. B) Close up view of the tyrosine aminoacid at the 326th position

To date, 11 variants with PHA due to *SCNN1B* variants have been reported (Table 1). These cases were diagnosed in infancy with classical findings. On follow-up, two case died due to salt wasting crisis (6,8). In four cases, there were skin manifestations, such as dry skin, severe eczema, bullous dermatitis and hidradenitis suppurativa (7,10,11). Recurrent lung infections developed in four cases (7,11,12,13) and gastrostomy was required in four cases due to the salt wasting (8,10,11,13). Pulmonary hypertension developed in one case during follow-up (11). Apart from the case presented here, no other developed viral myocarditis and hypertension was encountered during attacks.

Both urgent and long-term treatments of PHA involve many challenges. Hyperkalemia can be life threatening due to the risk of cardiac arrhythmia. In the literature and similar to our case, there have been previous reports of peritoneal dialysis to correct hyperkalemia (7,14,15,16). Cases requiring gastrostomy due to difficulties in continuing oral treatment have also been reported (4,5,8,10,11,13,17). Patients with PHA are prone to pulmonary infections due to a decrease in sodium-dependent fluid absorption in the lungs. Rapid decompensation may occur during episodes of infection or when oral intake is impaired. After the electrolyte balance was achieved in the follow-up of our patient, salt wasting reccurred due to intervening viral myocarditis and, during this period, treatments were given via nasogastric tube.

Conclusion

Systemic PHA is a challenging disease to manage, with severe salt wasting that starts in the neonatal period. In

Table 1. Clinical and laboratory features of this and other cases with SCNN1B mutation in the literature thoma

	Gender	Age of diagnosis	Na meq/L	K meq/L	Aldosteron ng/dL	Renin ng/mL/h	Genetic	Treatment	Current age	Additional findings	Clinical follow-up
Our case	M	1 month 25 days	123	7.1	640	16.3	c.978C > A (p.Tyr326Ter) (E6) Homozygous	NaCl, $NaHCO_3$, sodium polystyrene sulphate	7 months old	Myocarditis and hypertension	Once experienced salt wasting crisis during infection, now his development is appropriate for age
Chang et al (18)	-	19 days	133	8.2	*	-	c.109G > A (p.Gly37Ser) (E2) Homozygous	-	-	-	-
Kerem et al (21)	F	-	-	-	-	-	Two frame shift variations 647insA (E3)/915delC (E5) Compound heterozygous	-	18 years	High serum IgE concentration, normal spirometry and chest radiography	
Thomas et al (12)	-	4 days	127	10.2	1.281	235.5	Large homozygous deletion in the promoter region of of βENaC	NaCl, $NaHCO_3$, sodium polystyrene sulphate	7 years old	Recurrent lung infection	There is a decrease in the frequency of lung infections
Saxena et al (8)	M	7-8 days	< 120	8.5-10.5	> 1440	-	1669 + 1G > A splice site mutation in intron 12, Homozygous	NaCl, kayexalate	-	-	Recurrent salt-wasting crisis in newborn and infancy, gastrostomy was required, his growth and development was normal but he died at the age of 6.5 year after cardiac arrest
Edelheit et al (13) and Hanukoglu et al (20)	M	6 days	135	5.9	39.6-248.7	> 50	1669 + 1G > A splice site mutation in intron 12, Homozygous	NaCl, kayexalate	7 years old	Persistent clear nasal discharge, frequent lower respiratory infections and failure to thrive	Recurrent salt-wasting crisis, gastrostomy was performed at 14 months of age
Belot et al (10)	F	6 days	126	6.8	1.627	1335	c.637 C > T (p.Gln213Ter) (E4), Homozygous	-	3 years old	Bullous dermatitis	During follow-up, gastrostomy was opened, normal development at the age of 3 but still experiencing diarrhea and respiratory distress attacks
Dogan et al (19)	M	3 days	125	9	946	140	c.1266-1G > C splice site mutation in intron 8, Homozygous	NaCl, $NaHCO_3$, sodium polystyrene sulphate	3,5 years old	Vomiting, poor feeding	Short stature, decrease in dehydration attacks and in hospitalization with age

Nobel et al (11)	F	2-3 weeks	135	5,1	2.800	190	c.1288delC (p.Leu430 Tyrfs*3) (E9)/c.1466 + 1 G > A splice site mutation in intron 11 Compound heterozygous		32 years	Myalgia, hidradenitis suppurativa, pulmoner hypertension	Continuous hospitalization from 2 weeks to 2 years and NaCl, $NaHCO_3$ and potassium chelation support with gastrostomy tube up to 3.5 years old, recurrent episodes of chronic bronchitis during childhood
Cayir et al (6)	M	9 days	106	11,8	317.5	98.2	c.87C > A (p.Tyr29Ter) (E2)/ c.1346 + 1G > A splice site mutation in intron 9 Compound heterozygous	NaCl, sodium polystyrene sulphate	-	-	During the follow up had seven salt wasting crisis and died in the last crisis at 6 months of age
Gopal-Kothandapani et al (7)	F	1 day	128	7.8	64.7	-	c.1542 + 1G > A splice site mutation in intron 12	NaCl, $NaHCO_3$, sodium resonium	8 years old	Severe eczema	Recurrent electrolyte imbalances
Gopal-Kothandapani et al (7)	M	8 days	113	11	600	-	17-bp frameshift deletion in exon 2	NaCl, $NaHCO_3$, sodium resonium	14 years old	Dry skin	Peritoneal dialysis need at the first application, frequent lung infection

Plasma aldosterone concentration stated as 1 g/L (1-95) in the article.
Nomenclature of the variations are written as in the original publications.

this disease, life-threatening arrhythmias can be seen due to recurrent salt wasting and severe hyperkalemia. PHA1B can be confused with congenital adrenal hyperplasia. If a patient with hyperkalemia has hyponatremia, elevated urinary sodium excretion and low TTKG, mineralocorticoid resistance/deficiency should be considered. Treatment compliance is difficult due to the need for high dose oral salt and anti-potassium treatment. Long-term follow-up and treatment of these patients should careful, as the patients are frequently non-compliant with treatment and the frequency of rapid decompensation is high, especially during periods of infection.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

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

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DOI: 10.4274/jcrpe.galenos.2020.2020.0065

J Clin Res Pediatr Endocrinol 2021;13(4):452-455

A Rare Cause of Hyperinsulinemic Hypoglycemia: Kabuki Syndrome

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What is already known on this topic?

Kabuki syndrome (KS) patients with the *KMT2D* gene mutations develop symptoms like facial dysmorphism, neonatal feeding problems, kidney anomalies, and skeletal malformations, whereas KS patients with the *KDM6A* mutations have a higher risk of hyperinsulinemic hypoglycemia (HH).

What this study adds?

The presented case is very rare because the infant had a mutation in *KMT2D* but presented with HH.

Abstract

Kabuki syndrome (KS) is a disease characterized by distinctive facial features, skeletal anomalies and delay in neuromotor development. KS 1 is an autosomal dominant condition caused by mutations in the *KMT2D* gene, whereas KS 2 is an X-linked disorder caused by mutations in the *KDM6A* gene. In the majority of KS patients who present with hypoglycemia, *KDM6A* is the defective gene. A 9-month old girl was admitted to our emergency department due to a seizure. On physical examination, hypotonia, mild facial dysmorphism, brachydactyly of the 5th finger, prominent finger pads and pansystolic murmur were detected. A fasting glucose tolerance test was performed the next day due to her history of hypoglycemia, but she had convulsions at the fifth hour of the test. Her serum glucose was 24 mg/dL, insulin 1.94 mIU/L, C-peptide 0.94 ng/mL, growth hormone 11 ng/mL, anti-insulin antibody 4.2 IU/mL, cortisol 19.8 µg/dL, and adrenocorticotropic hormone 9.3 pg/mL. A diagnosis of hyperinsulinemic hypoglycemia was considered. Given the abnormalities, genetic analysis for congenital hyperinsulinism, including the genes causing KS was performed. A heterozygous frameshift mutation (c.2579del, p.Leu860Argfs*70) was detected in the *KMT2D* gene. Epilepsy and other neurological symptoms may be seen in KS patients and in some of these the neurological symptoms are the result of hypoglycemia. In such cases, the detection and prevention of hypoglycemia can help prevent the progression of neurological symptoms. We suggest considering the diagnosis of KS for patients with hypoglycemia and dysmorphic features, even if the patient does not manifest all features of KS.
Keywords: Diazoxide, hyperinsulinemic hypoglycemia, Kabuki syndrome, *KMT2, KDM6A*

Introduction

Kabuki syndrome (KS) is a rare congenital syndrome first described by Niikawa et al (1) and Kuroki et al (2) in 1981. Its prevalence is 1:32,000 in Japan, and 1:86,000 in Australia and New Zealand (3,4). In approximately 85% of KS patients, mutations in either *KMT2D* gene (previously known as *MLL2*) located at 12q13.13 (autosomal dominant) or *KDMGA* gene located at Xp11.3 (X-linked dominant) were identified (5,6,7,8,9). There are five cardinal criteria for the diagnosis of KS: postnatal short stature, mild to moderate developmental delay/intellectual disability, distinctive facial features, skeletal abnormalities, and persistent fetal fingertip pads (3,10,11). Other symptoms, including hypoglycemia, congenital heart defects, congenital hypothyroidism, seizures, hypotonia, and gastrointestinal problems, might

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Conflict of interest: None declared
Received: 02.04.2020
Accepted: 10.08.2020

be seen infrequently (12). Here, we present a Turkish female infant with KS who also exhibited hyperinsulinemic hypoglycemia (HH).

Case Report

The patient was a female infant, born as the second child of healthy, non-consanguineous parents at 32 weeks of gestation by an uncomplicated cesarean section. Her birth weight was 2410 grams [+2.20 standard deviation score (SDS)]. She was hospitalized because of prematurity, respiratory distress syndrome and indirect hyperbilirubinemia. Hypoglycemia developed within the first few days of life due to hyperinsulinism (serum insulin 181.5 mIU/L and concurrent glucose 37 mg/dL). She was initially treated by intravenous glucose infusion (up to 15 mg/kg/min) and oral diazoxide. After achieving normoglycemia with oral feeding, diazoxide was eventually discontinued. Subsequently, neonatal transient hyperinsulinism was considered and no other treatment was initiated. She also had a ventricular septal defect (VSD) along with patent ductus arteriosus. The karyotype analysis was found to be 46,XX. After 45 days, she was discharged and given furosemide and captopril to address cardiac problems.

At the age of nine months, she was admitted to the pediatric emergency department because of somnolence after a seizure. Weight, height, and head circumference were 6760 grams (-1.93 SDS), 71 cm (-0.07 SDS), and 39.8 cm (-3.59 SDS), respectively. Hypotonia, pansystolic murmur, and facial dysmorphic features including wide forehead, arched and sparse eyebrows, ptosis, eversion of lateral third of inferior eyelids, and a short columella were noted. In addition, persistent fetal pads, and brachydactyly of fifth fingers were observed in the physical examination (Figure 1). Biochemical tests and electroencephalogram were found to be normal.

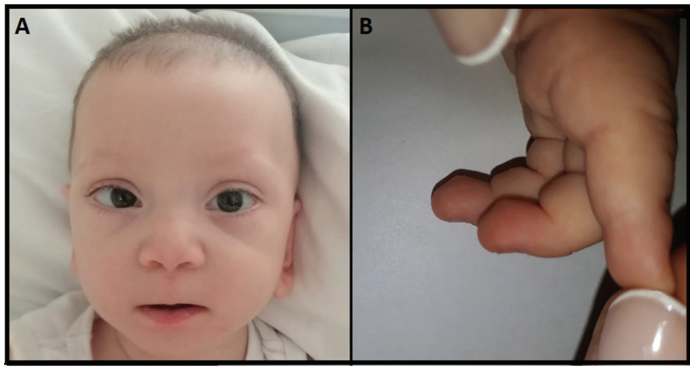

Figure 1. A) Facial appearance (wide forehead, arched and sparse eyebrows, ptosis, eversion of lateral third of inferior eyelids, short columella), B) Persistent fetal pads

A fasting glucose tolerance test was performed due to the history of hypoglycemia. She developed hypoglycemic convulsion at the fifth hour of the fasting test. The capillary blood glucose was 32 mg/dL. Critical blood and urine samples were collected. Hypoglycemia was treated by intravenous bolus injection of 0.2 g/kg dextrose, followed by an infusion at the rate of 8 mg/kg/min. The patient improved rapidly with this treatment. The samples collected at the time of hypoglycemia revealed normal blood count, electrolytes, liver, and kidney functions. Serum glucose was 24 mg/dL, insulin 1.94 mIU/L, C-peptide 0.94 ng/mL, growth hormone 11 ng/mL, anti-insulin antibody 4.2 IU/mL, cortisol 19.8 µg/dL, and adrenocorticotropic hormone 9.3 pg/mL. The levels of blood carnitine and acylcarnitines were normal and no ketone bodies were observed in the urinalysis. Skeletal radiographs, and abdominal ultrasound were normal. Based on the fasting test, HH was considered and diazoxide treatment was initiated at a dose of 3 mg/kg/day and that the dose was gradually increased to 15 mg/kg/day while tapering down dextrose. Feeding intervals of four hours, and dietary supplementation with uncooked cornstarch were recommended. The patient did not suffer from hypoglycemia under diazoxide therapy. The dysmorphic features, along with VSD and HH, suggested a possible diagnosis of KS. Genetic analysis was performed using a next-generation sequencing panel, including *KMT2D* and *KDM6A* genes, which revealed a heterozygous frameshift mutation in *KMT2D* gene (c.2579del, p.Leu860Argfs*70). The mutation had previously been reported to be associated with KS in a single case in ClinVar database, confirming the clinical diagnosis (13). The mutation was considered to be *de novo*, as the variant was not detected in the molecular genetic analysis of the parents. Informed consent was obtained from the parents for reporting genetic testing and publication of related data.

Discussion

KMT2D encodes a lysine-specific histone methyltransferase and is responsible for over 75% of KS cases (5,6,7,8). *KDM6A* encodes a histone demethylase and accounts for 5-8% of KS cases (9,14). KS patients with *KMT2D* gene mutations develop symptoms such as facial dysmorphism, neonatal feeding problems, kidney anomalies, and skeletal malformations (8), whereas KS patients with *KDM6A* mutations have a higher risk of HH (15).

Hyperinsulinism may manifest in various syndromes, especially Beckwith-Wiedemann, but it has rarely been observed in Kabuki, Sotos, Costello, Turner, Simpson-Golabi-Behmel, Ondine, Usher, Perlman and Timothy syndromes, and congenital disorders of glycosylation

(16,17,18,19,20,21,22). Although the frequency of neonatal hypoglycemia is 6.7%, HH is extremely rare (0.3%) in KS (10). A small cohort study indicated that the incidence of KS in neonates with HH may be around 1%. Moreover, this cohort reported that 45.5% of KS patients, who presented with HH, had *KDM6A* mutations. However, *KDM6A* mutations were detected in only 5-8% of all KS patients. These data support the fact that there is a higher risk of HH in KS patients with *KDM6A* mutations compared to those with *KMT2D* mutations (15). This makes the presented case unusual because the infant had a mutation in *KMT2D* but presented with HH, which was probably the cause of the seizure.

KS may not be easily identified in neonates and infants because the characteristic facial features may not yet have become distinct. Similar to our 9-month old patient, KS is usually diagnosed between 6 and 18 months of age. Although KS is very rare, it is important to consider KS in patients with HH to provide genetic counseling and determine the prognosis of hypoglycemia. In fact, in the case of our 9-month old patient, failure to diagnose KS during the neonatal period caused the clinicians to assume that her hyperinsulinism was transient. Therefore, diazoxide treatment was discontinued, which might have contributed to the severe re-occurrence of hypoglycemia, accompanied by seizures, loss of consciousness and worsening of neurological damage and of developmental problems. As seen in our case, it is not safe to completely stop diazoxide treatment before excluding any underlying genetic etiology. In the literature, it has been reported that diazoxide treatment may be needed until five years of age to maintain normoglycemia in KS patients with HH (11,23). Our patient is currently being continued on diazoxide with proper maintenance of glycemia. In addition, the 4-hour interval feeding has been maintained, including nocturnal feeds, to prevent hypoglycemia.

Conclusion

In conclusion, early detection and proper management of hypoglycemia would help to prevent progression of neurological symptoms and permanent sequelae in KS. KS should be considered in the differential diagnosis of infants with hypoglycemia and dysmorphic features, even if the patient does not manifest with all features of the syndrome.

Acknowledgement

Genetic testing was performed by the Department of Molecular Genetics at the University of Exeter Medical School, UK.

Ethics

Informed Consent: Our patient's mother and father gave informed consent for the genetic testing reported in this paper and for the publication of related data.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Onur Akın, Yılmaz Yıldız, Mina Mısırlıgil, Concept: Onur Akın, Yılmaz Yıldız, Mina Mısırlıgil, Design: Onur Akın, Mutluay Arslan, Data Collection or Processing: Sevinç Odabaşı Güneş, Mina Mısırlıgil, Analysis or Interpretation: Bülent Ünay, Literature Search: Mina Mısırlıgil, Writing: Yılmaz Yıldız, Mina Mısırlıgil.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0005

J Clin Res Pediatr Endocrinol 2021;13(4):456-460

Different Growth Responses to Recombinant Human Growth Hormone in Three Siblings with Isolated Growth Hormone Deficiency Type 1A due to a 6.7Kb Deletion in the *GH1* Gene

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What is already known on this topic?

Lack of growth response to recombinant human growth hormone (rhGH) in IGHD type 1A probably suggests underlying neutralising anti-GH antibody and alternative treatment strategies should be sought.

What this study adds?

IGHD type 1A is a rare cause of severe proportionate short stature and this is the first reported family from India. Three siblings with similar genetic abnormality demonstrated different growth responses to rhGH. An expected response in the first year of therapy was noticed in the eldest sister that waned rapidly after the first year. The second sibling demonstrated poor response from the beginning of therapy while the third sibling experienced an excellent response even after the third year of treatment.

Abstract

Isolated growth hormone deficiency (IGHD) type 1A is a rare, autosomal recessive disorder caused by deletion of the *GH1* gene and characterized by early onset severe short stature and typical phenotype. Lack of exposure to GH during fetal life often leads to formation of anti-GH antibody following exposure even the least immunogenic recombinant human GH (rhGH). Some patients with circulating anti-GH antibodies demonstrate lack of growth response to GH while others do not. However, the clinical significance of this antibody is unclear; hence testing is not routinely recommended. Three siblings, born of a consanguineous union, were referred with severe short stature. They were evaluated and IGHD was diagnosed in all of them. Genetic analysis revealed that all three had homozygous 6.7 Kb deletion in *GH1* gene, while their parents displayed a pattern of heterozygous 6.7 Kb deletions. rhGH was started at 10, 6 and 1.58 years of age, respectively. Growth and hormonal parameters were monitored throughout the course of treatment. The eldest sibling demonstrated expected growth velocity (9.5 cm/year) for the first year of rhGH that rapidly waned thereafter (2.5 cm/year). The youngest sibling experienced excellent growth response even after the third year (10.3 cm/year) while the middle sibling displayed sub-optimal response from rhGH initiation (6.3 cm/year). Change of rhGH brand did not work in the two elder sisters. Such a different growth response with rhGH in three siblings harbouring similar genetic abnormality has not been described previously.

Keywords: Isolated growth hormone deficiency type 1A, *GH1* gene, anti-growth hormone antibody

Introduction

Growth hormone deficiency (GHD) in children can present either as an isolated defect as in isolated GHD (IGHD) or in combination with one or more of the other pituitary hormone deficiencies, for example combined pituitary hormone deficiency. Defects in the growth hormone 1 (GH1) or growth hormone releasing hormone receptor *(GHRHR)* genes, involved in the control of growth hormone (GH) secretion, typically cause IGHD. IGHD is classified into three categories having different modes of inheritance:

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Conflict of interest: None declared
Received: 20.01.2020
Accepted: 24.08.2020

type 1 (autosomal recessive), type 2 (autosomal dominant) and type 3 (X-linked). Type 1 IGHD is further divided into two subtypes depending on severity: 1A (severe) and 1B (less severe). Type 1A IGHD is characterized by early onset severe short stature due to profound congenital GHD, a typical phenotype and an initial strong growth response following GH that is not infrequently followed by dramatic slowing of growth due to appearance of neutralizing anti-GH antibodies. As GH is not produced, even in fetal life, patients are immunologically intolerant to GH and frequently develop anti-GH antibodies when treated with any form of GH. Estimation of anti-GH antibody and mutational analysis are not yet component of routine care for patients with GHD in many countries due to lack of available laboratories, cost and utility of these tests in clinical practice. Early onset severe short stature, typical phenotype, undetected basal/stimulated GH, preserved pituitary functions without structural abnormality of the hypothalamo-pituitary area in the context of a typical family history is suggestive of *GH1* gene deletion.

Case Report

Three siblings case 1, case 2, and case 3 were referred for evaluation of severe short stature at the ages of 10 years, 6 years and 1.5 years, respectively. Born of a consanguineous union (Figure 1), all of them had cephalic presentation and were delivered vaginally at term. The birth weights were 3 kgs, 2.7 kgs and 2.8 kgs respectively. Other than prolonged neonatal jaundice in case 1, they had had uncomplicated perinatal periods. Motor milestones in case 1 and case 2 were slightly delayed. One of their siblings died immediately after birth due to unknown cause.

All of them had proportionate short stature, frontal bossing, depressed nasal bridge, mid facial crowding and high pitched voice without any midline defect. The rest of the systemic examination was unremarkable. The mid parental height was 145.35 cm with a standard deviation score (SDS) of -2.6. The auxologic parameters, expressed in cm and SDS according to Indian references, are summarized in Table 1. Sexual maturation rate in all of them was Tanner B1P1. Baseline investigations, including complete blood count, renal function tests, liver function tests, electrolytes, and urine and stool microscopy were normal. Hormonal and radiological evaluation is summarized in Table 1.

Genomic DNA was isolated from peripheral venous blood using the QIAGEN DNA extraction kit and following the manufacturer recommended method. Polymerase chain reaction (PCR) amplification of the whole *GH1* gene was performed using Velocity DNA polymerase (Bioline, USA, Cat. No.-BIO-21098) and oligonucleotide primers GH1F (5'-ccagcaatgctcagggaaag-3') and GH1R (5'-tgtcccaccggttgggcatggcaggtagcc- 3') (1). PCR mixtures were denatured for 2 minutes at 98 °C and submitted to 32 cycles at 98 °C for 30 seconds, 68 °C for 30 seconds, and 72 °C for 1 minute, followed by final extension at 72 °C for 10 minutes. The resulting PCR product (2700 bp) was visualized by agarose gel electrophoresis and ethidium bromide staining. Characterization of *GH1* gene deletion was performed according to the method of Vnencak-Jones et al (2), modified by Mone et al (3). Briefly, two homologous sequences flanking the *GH1* gene, and the fusion fragments resulting from different *GH1* gene deletions, were simultaneously amplified by PCR with the following primers: 5'-tccagcctcaaagagcttacagtc-3' (GH1_2F) and 5'-cgttttctctagtctagatcttcccagag-3' (GH1_2R). The resulting PCR fragments were digested overnight at 37 °C with *SmaI* restriction endonuclease (Cat. No.-RO141S, New England Biolabs, MA, USA) according to the manufacturer's protocol, and the digested products were visualized by ethidium bromide staining after electrophoresis on a 1% agarose gel.

GH1 gene PCR amplification yielded no product using three different genomic DNA samples of three probands as template, while her parents showed one amplicon of the expected size (Figure 2). This result was suggestive of *GH1* gene deletion in the patients. *SmaI* restriction enzyme digestion of PCR amplified two homologous sequences flanking the *GH1* gene, suggested that all three patients were carrying homozygous 6.7 Kb deletions, while their parents displayed a pattern of heterozygous 6.7 Kb deletion (Figure 2).

Recombinant human GH (rhGH) (Headon®, Manufacturer: M/s Shanghai United Cell Biotechnology Co.,Ltd. 1150 Guiqiao Road, China (Shanghai) Pilot Free Trade Zone, 201206, P.P.China; Imported and Marketed by: SUN Pharmaceutical Industries Ltd.) was started at 10, 6 and 1.58 years of age in case 1, 2 and 3 respectively. In addition, case 1 and case 2 were also put on 12.5 mcg of levothyroxine and thyroid stimulating hormone (TSH) values were kept below 2.5 mIU/L. The annual growth velocity (GV) data is summarized in Table 1. The dose of rhGH was gradually increased to 0.05 mg/kg/day. Due to poor response, the brand of rhGH was changed (Norditropin Nordilet®, Manufacturer: Novo Nordisk India Pvt Ltd, Plot No. 32, 47-50, EPIP Area, Whitefield, Bangalore - 560 066, India) after the second year of therapy in case 1 and 2 and treatment was ultimately stopped after the third year. The parents inadvertently stopped rhGH for seven months in case 3 after two years of therapy. Therapy was restarted and a height increase of 4.3 cm was observed in the subsequent five months (GV: 10.3 cm/year) (Figure

Table 1. Clinical characteristic and summary of investigations at baseline

	Case 1	Case 2	Case 3
Date of birth	11.10.2006	08.12.2010	15.05.2015
Chronological age	10 years	6 years	1.5 years
Height	77.5 cm	71.5 cm	55.5 cm
Height SDS	-9.2	-7.8	-8.7
Height age	18 months	9 months	3 months
Weight	8 kgs	8 kgs	4.1 kgs
Weight age	9 months	9 months	3 months
US: LS	1	1.1	1.18
Investigations			
Bone age (estimated by Greulich and Pyle method)	4.5 years	2 years	2 years
IGF-1 (ng/mL)	< 25 (Ref: 75-546)	34.4 (Ref: 53-250)	< 25 (Ref: < 25-258)
IGFBP-3 (µg/mL)			0.2 (Ref: 0.7-3.6)
Basal GH (ng/mL)	< 0.05	< 0.05	< 0.05
Clonidine stimulated GH (ng/mL)	All values < 0.05	All values < 0.05	All values < 0.05
fT4 (ng/dL) (at baseline)	1.31 (Ref: 0.8-1.8)	1.1 (Ref: 0.8-1.8)	1.42 (Ref: 0.9-2.4)
TSH (mIU/L)	6.37 (Ref: 0.7-6.4)	6.35 (Ref: 0.7-6.4)	3.35 (Ref: 0.7-6.4)
8:00 am cortisol (µg/dL)	9.4 **(Ref: 5-25)**	10.2 **(Ref: 5-25)**	7 **(Ref: 5-25)**
Post-synacthen peak cortisol (µg/dL)	22.1	19.8	18.2
MRI of hypothalamo-pituitary area	Small anterior pituitary, normal stalk and eutopic bright spot	Small anterior pituitary, normal stalk and eutopic bright spot	Small anterior pituitary, normal stalk and eutopic bright spot
Growth velocity after rhGH			
First year of therapy	9.5 cm	6.3 cm	13.5 cm
Second year of therapy	2.5 cm	2.4 cm	15 cm
Third year of therapy	1.5 cm	1.9 cm	4.9 cm (0.6 cm over first 7 months and 4.3 cm in next 5 months) (rhGH inadvertently stopped for first 7 months)

SDS: standard deviation score, TSH: thyroid stimulating hormone, US: upper segment, LS: lower segment, IGF: insulin like growth factor, IGFBP: IGF binding protein, GH: growth hormone, rhGH: recombinant human GH, fT4: free thyroxine, MRI: magnetic resonance imaging

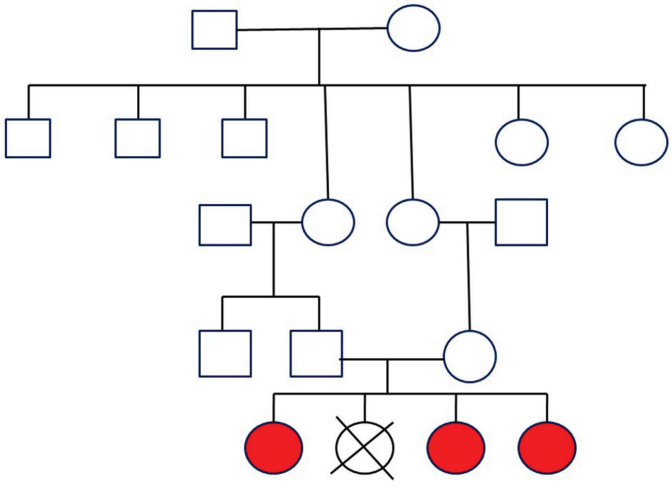

Figure 1. Family tree suggestive of autosomal recessive inheritance

3). Currently the youngest sibling is taller than the middle sibling (Figure 4).

Informed consent from the parents of the patients was taken for publication of these three cases.

Discussion

The frequency of *GH1* gene deletions in children with GHD is variable and deletions of different sizes have been described. The most frequently reported deletion size is 6.7 Kb, which is seen in 70-80 % of such cases. Other sizes reported include 7.0, 7.6, and 45 Kb. In addition, there have been reports of double deletions within the *GH* gene cluster located in the long arm of chromosome 17 (17q24.2) (4). Genetics and Neuroendocrinology of Short Stature International Study, a prospective, open-label, observational research program conducted in 30 countries at more than 800 study sites between 1999 and 2015 looked for mutations in *GH1* and *GHRHR* in 475 patients with IGHD of which 440 patients

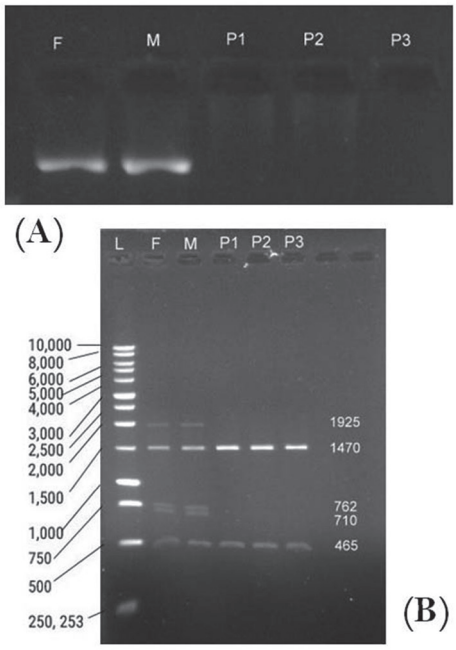

Figure 2. (A) *GH1* gene amplification (1.5% agarose gel electrophoresis, ethidium bromide staining). *GH1* gene polymerase chain reaction amplification yielded no product using the genomic DNA of probands as template (P1, P2, P3), while their parents (M, F) showed one band of the expected size (2,700 bp). (B) SmaI digestion (1% agarose gel electrophoresis, ethidium bromide staining). Fragment pattern was consistent with the father (F) and mother (M) being heterozygous carrier for the 6.7 Kb deletion, and patient 1 (P1), patient 2 (P2) and patient 3 (P3) are all homozygous for 6.7 Kb deletions. L: 1 Kb Ladder; F: Father; M: Mother; P1: Patient 1; P2: Patient 2; P3: Patient 3

had idiopathic GHD. *GH1* mutation was found in 23 of these 475 patients (4.8%) but only one patient (and one kindred) had a homozygous 6.7 Kb deletion and another had a 7.0 Kb deletion of the *GH1* gene (5).

Type 1A IGHD due to homozygous *GH1* deletion was first described in 1970 in three Swiss siblings with severe short stature and a particular phenotype, who subsequently developed high titers of anti-GH antibodies that interfered with growth response to pituitary-extracted GH (6). The widespread availability and use of rhGH has significantly reduced the frequency of development of these antibodies but has not eliminated it. In GH drug trials, measurement of anti-GH antibodies is a standard procedure and it's prevalence in children varies from 2% to 22% depending on aetiology and duration of follow-up. Most of the patients with type 1A IGHD have undetectable circulatory GH levels and subsequently develop anti-GH antibodies when exposed to rhGH. In a recently published retrospective study 13 GH-treated patients with either type 1A IGHD, neurosecretory dysfunction, bioinactive GH syndrome (without genetic confirmation) or constitutional delay of growth and

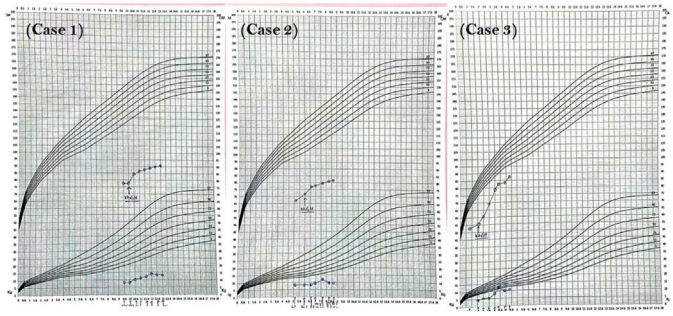

Figure 3. Growth charts (combined WHO 2006 MGRS and revised Indian Academy of Pediatrics 2015) of the three patients from the start of recombinant human growth hormone (rhGH) treatment. Note lack of growth response after 1st year of therapy in case 1 and case 2. No growth was also evident when rhGH was inadvertently stopped for seven months in the youngest sibling after 24 months of therapy

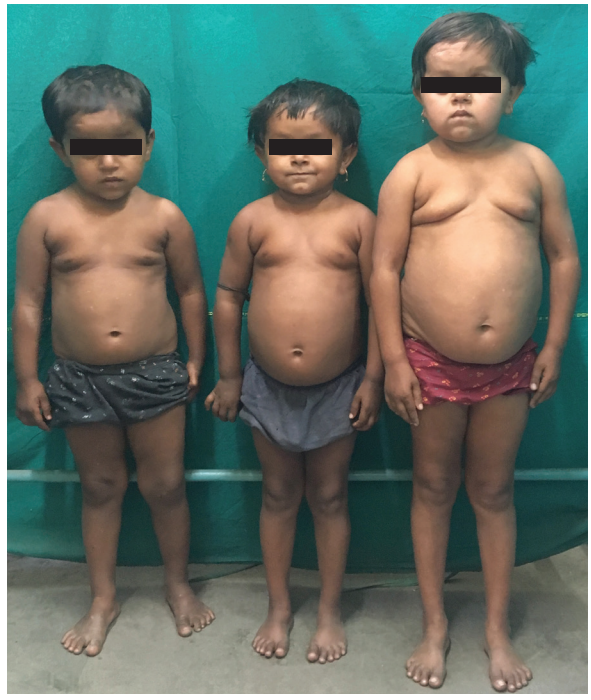

Figure 4. Current clinical profile of patients (from left to right: case 3, case 2, case 1). Note that the current height of the youngest sibling (case 3) is more than her elder sister (case 2)

puberty out of a cohort of 66 (19.7%) tested positive for these antibodies (7). The biological significance of anti-GH antibodies seems to be limited to some rare patients with very severe GHD with very high titres of neutralizing antibodies, encountered mostly in those with IGHD type 1A. Daily GH at the recommended doses typically accelerates growth in a GH-deficient child from a pre-treatment rate of 3-4 cm/year to 10-12 cm/year in the first year of therapy to 7-9 cm/year in the second and third years. Progressive waning of GH efficacy in all forms of GHD is poorly understood. Binder et al (7) observed an insufficient response to rhGH in one

sibling pair with IGHD type 1A while growth of a second sibling pair was unaffected, despite the fact that all tested positive for anti-GH antibodies. It has also been observed that despite having the identical genetic defect and similar anti-GH antibody titres, growth response to GH treatment may be quite heterogeneous, depending on the neutralizing effects of these antibodies (8,9). This is also evident in our cases, as the growth of the youngest sibling was unaffected which was in contrast to the other two. Though we could not estimate the anti-GH antibodies in these children due to non-availability of the test, the other possible causes of poor growth response to rhGH (poor compliance, incorrect injection techniques, subclinical hypothyroidism, excessive glucocorticoid therapy, prior irradiation of the spine epiphyseal fusion, coexisting systemic disease or alternate diagnosis of short stature) were confidently ruled out and the lack of response was attributed to anti-GH antibodies (10). TSH values in case 1 and case 2 were close to the upper reference limit and they were put on 12.5 mcg of levothyroxine to negate any possible detrimental effect of subclinical hypothyroidism on growth. Parents of the children were taught about the injection techniques and advised to administer injections themselves. Compliance to therapy was assured by the parents and cross checked with amount of rhGH used every month. Temporary cessation of rhGH therapy, changing the rhGH brand and recombinant human insulin like growth factor-1 instead of rhGH are the alternatives that have been proposed to optimise growth in such situation (11,12). However, these options are backed by poor quality evidence and change of rhGH brand did not work in our cases.

Conclusion

Type 1A IGHD, the most severe form of inherited isolated GH deficiency, results form homozygous *GH1* gene deletion. Many children with this disease demonstarte insufficient growth response to rhGH secondary to development of neutralizing anti-GH antibody. However, there is significant inter-individual variation in growth response to rhGH even in siblings with identical genetic defect. Loss of response is unpredictable and noticed at variable point of time after initiation of therapy.

Ethics

Informed Consent: Informed consents were obtained from the parents of the children.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sayan Ghosh, Partha Pratim Chakraborty, Animesh Maiti, Concept: Sayan Ghosh, Partha Pratim Chakraborty, Biswabandhu Bankura, Animesh Maiti, Design: Sayan Ghosh, Partha Pratim Chakraborty, Data Collection or Processing: Sayan Ghosh, Partha Pratim Chakraborty, Analysis or Interpretation: Biswabandhu Bankura, Rajkrishna Biswas, Madhusudan Das, Literature Search: Sayan Ghosh, Partha Pratim Chakraborty, Writing: Partha Pratim Chakraborty.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0125

J Clin Res Pediatr Endocrinol 2021;13(4):461-467

The IGSF1 Deficiency Syndrome May Present with Normal Free T4 Levels, Severe Obesity, or Premature Testicular Growth

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What is already known on this topic?

Almost all individuals with IGSF1 deficiency have central hypothyroidism. Almost all individuals with IGSF1 deficiency show disharmonious pubertal development and macroorchidism in adulthood, but premature testicular growth is rare. Individuals with IGSF1 deficiency tend to be overweight, but extreme early-onset obesity has only been reported once.

What this study adds?

IGSF1 deficiency can present with free thyroxine levels above the lower limit of normal. Premature testicular growth without elevated serum testosterone can be a sign of IGSF1 deficiency. The extreme weight gain in this and a previous case suggests that this is part of the clinical spectrum of IGSF1 deficiency syndrome.

Abstract

Our objective was to further expand the spectrum of clinical characteristics of the IGSF1 deficiency syndrome in affected males. These characteristic include almost universal congenital central hypothyroidism (CeH) with disharmonious pubertal development (normally timed testicular growth, but delayed rise of serum testosterone), macroorchidism, increased body mass index (BMI), and decreased attentional control. In addition, a subset of patients show prolactin deficiency, transient partial growth hormone deficiency in childhood and increased growth hormone secretion in adulthood. We present a family in which the proband was diagnosed with CeH and low serum prolactin. Severe weight gain started at two years old, with a BMI of 42.3 at 13.9 years. Testicular enlargement (5-6 mL, 3.8-4.3 standard deviation score) started aged three years. A pathogenic variant was found in the *IGSF1* gene: c.3411_3412del, p.(Tyr1137*). His brother was referred for short stature at age 13 years and was diagnosed with CeH, normal serum prolactin and IGF-1, and disharmonious puberty. In four male relatives (the proband's brother and three cousins) with the variant (one adult), free thyroxine (fT4) was below the lower limit of the reference range in two, and just above this limit in the other two. Three were overweight or obese, adolescents had disharmonious pubertal development and the adult had profound macroorchidism. In conclusion, male hemizygous carriers of a pathogenic *IGSF1* variant can present with fT4 concentration above the lower limit of the reference range while severe early onset obesity or premature testicular growth are part of the phenotypic spectrum.

Keywords: IGSF1 deficiency, hypothyroidism, macroorchidism, obesity, prolactin

Introduction

Central hypothyroidism (CeH) is a rare disorder characterized by a low serum free thyroxine (fT4) concentration and inappropriately low or normal thyroid stimulating hormone (TSH) levels (1). Acquired CeH is often part of multiple pituitary deficiency, for example due to compressive lesions, cranial surgery or irradiation, or injury. Congenital CeH is usually part of multiple pituitary hormone deficiency, but can also be isolated; both forms can be caused by genetic defects (1,2).

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Conflict of interest: None declared
Received: 01.07.2020
Accepted: 29.09.2020

Usually, cases with the congenital form are not diagnosed shortly after birth, because most newborn screening programs in the world are based on the detection of elevated TSH values only. Even in the few countries and states where screening programs are based on a combination of TSH and T4, some cases of congenital CeH can remain undiagnosed for many years, because of borderline low T4 levels (3).

Recently, immunoglobulin superfamily, member 1 (IGSF1) deficiency (MIM #300888) emerged as a novel cause of congenital CeH (4), and is now considered the most prevalent cause of congenital CeH (5). *IGSF1* encodes a glycoprotein that is located on the membrane of various cell types and the transcript is abundantly expressed in the rodent pituitary gland and testis (4,6). Deficiency of this protein in the human male causes X-linked CeH, disharmonious pubertal development (normal timing of testicular growth, but delayed rise of testosterone) and macroorchidism. In a variable proportion of affected males other features are observed, including prolactin deficiency, partial and transient growth hormone (GH) deficiency in childhood, increased body mass index (BMI), and decreased attentional control (4,7,8,9,10,11,12,13,14,15,16,17,18,19). Mean follicle stimulating hormone (FSH) and GH secretion are increased in some affected males (20,21).

We present a male index case with CeH, premature testicular growth, and severe, early-onset obesity, associated with a hemizygous nonsense variant in *IGSF1* [c.3411_3412del, p.(Tyr1137*)], as well as variable phenotypes observed in four male relatives carrying the variant, including normal fT4 concentrations. The large variation in clinical and biochemical findings in affected males in this pedigree shows that the phenotype of IGSF1 deficiency is even broader than has so far been reported (4,7,8,9,10,11,12,13,14,15,16,17,18,19), and may include a serum fT4 concentration above the lower limit of the reference range, premature testicular growth and severe early-onset obesity.

Case Report

Proband

The proband was born full term after an uncomplicated vaginal delivery, with a birth weight of 4082 grams [1.1 standard deviation (SD) score (SDS)] and length of 52.1 cm (0.8 SDS) (22). He was admitted to the neonatal unit for hyperbilirubinemia four days after birth. Newborn screening using TSH and T4 showed CeH (T4 < 77.2 nmol/L and TSH < 20 mIU/L which are the cut-off limits in the available screening program). These results were confirmed by a blood draw at four weeks of age when the T4 was 52.9 nmol/L (reference range 58.1-154.8 nmol/L) and the TSH was 1.7 mIU/L (reference range 0.8-19.2 nmol/L). At seven weeks old, thyroid hormone replacement was started at 7.4 µg/kg/day. An extensive pituitary examination was performed, showing an undetectable serum prolactin level and normal serum levels of insulin-like growth factor 1 (IGF-1) and cortisol. No structural abnormalities were observed with magnetic resonance imaging of the pituitary.

At 2.4 years of age, he was obese (BMI 20.3 kg/m^2, 2.4 SDS) (22). At follow-up, normal linear growth and further excessive weight gain was noted with a maximum at 13.9 years of age, when weight was 125.2 kg with a BMI of 42.3 [163% of the 95th US percentile (22) and 4.2 SDS for a 1980 European reference (23), Figures 1A and 1B]. Thyroid hormone treatment was interrupted between the ages of 2.8 and 3.4 years due to proband's mother stopping medication, but has been administered since. Serum fT4 levels have been maintained in the lower half of the reference range (Table 1).

His testicular volume assessed with Prader orchidometer was 2 mL at 2.4 years (0.9 SDS) (24), but showed a remarkable enlargement to 5-6 mL at a follow-up visit at 3.4 years old (3.8-4.3 SDS) (Figure 2A), without pubic hair appearance or linear growth acceleration. At 6.3 years, a testicular ultrasound was performed, showing right and left testicular volumes of 6.3 and 5.8 mL [4.2 SDS, reference range at that age 0.3 to 1.1 mL (24)], still in the absence of pubic hair, and with undetectable plasma testosterone. A gonadotropin releasing hormone (GnRH) stimulation test showed a pre-pubertal pituitary response with baseline and peak luteinising hormone (LH) of 0.09 and 3 IU/L and a baseline and peak FSH of 0.95 and 16 IU/L. A borderline pubertal response to GnRH was first observed at 9.4 years old (baseline and stimulated serum LH of 0.3 and 7 IU/L; FSH of 2 and 17.9 IU/L), when his testicular size was 10-12 mL (3.5-3.8 SDS). However, plasma testosterone was still undetectable, and baseline and stimulated FSH secretion surpassed LH secretion. Further clinical and laboratory data are shown in Table 1. Reference data for serum testosterone in minipuberty (1-3 months) and Tanner stage were derived from the literature (25,26). A thyroid ultrasound at 12.5 years showed a symmetrically small gland, with an estimated volume of less than 1 mL for each lobe. Bone age was close to chronological age (27).

Genetic Analysis

Fragile X testing was negative, the methylation pattern of *SNRPN* was not suggestive of Prader-Willi syndrome, and the array comparative genomic hybridization (CGH) was normal. However, a pathogenic variant (nonsense mutation)

Table 1. Longitudinal clinical, laboratory, radiological and medication data in the proband

Age (years)	0.10	0.13	0.20	0.92	2.0	3.40	6.30	7.60	9.40	Reference range
Bone age (years)[a]							6.1	7.5		
Height SDS[b]					0.3	0.1	0.3	0.4	1.2	
BMI					20.4	22.5	25.1	27.3	28.9	
BMI SDS[b]					2.4	3.9	2.9	2.6	2.4	
Testicular volume (mL)[c]			2		2	5-6	8	8-10	10-12	
FT4 (pmol/L)		10.3[d]	29.6		11.6	10.0	15.4	12.9	14.2	11.6-20.6
TSH (mIU/L)	1.75	3.2	0.02	0.46		1.4	< 0.01		< 0.01	0.8-6.3
Prolactin (nmol/L)			3						< 1	2-18
Testosterone (nmol/L)			14.0[e]						< 0.2[f]	Age dependent
Levothyroxine dose (ug/kg/day)		7.4	5.5	2.7	1.5	Stopped 2.8-3.4 years	2.1	1.7	1.1	

[a]Assessed by the atlas of Greulich and Pyle (27).
[b]Based on Centers for Disease Control reference (22).
[c]Assessed by the Prader orchidometer.
[d]Sample taken before start of L-thyroxine treatment.
[e]For minipuberty (1-3 months) the reference is 7.2 ± 2.4 nmol/L (25).
[f]For Tanner stage 1 the reference range is < 0.3-0.5 nmol/L (27).
SDS: standard deviation score, BMI: body mass index, FT4: free thyroxine, TSH: thyroid stimulating hormone

in the *IGSF1* gene [NM_00117096.1: c.3411_3412del, p.(Tyr1137*)] was detected by Sanger sequencing (4,10,11). Segregation analysis showed that the mutation was also present in three female and five male family members (Figure 2B).

Relatives Carrying the *IGSF1* Variant

The proband was the second child of reportedly healthy parents of Italian descent (Figure 2B). Clinical and laboratory findings are shown in Table 2.

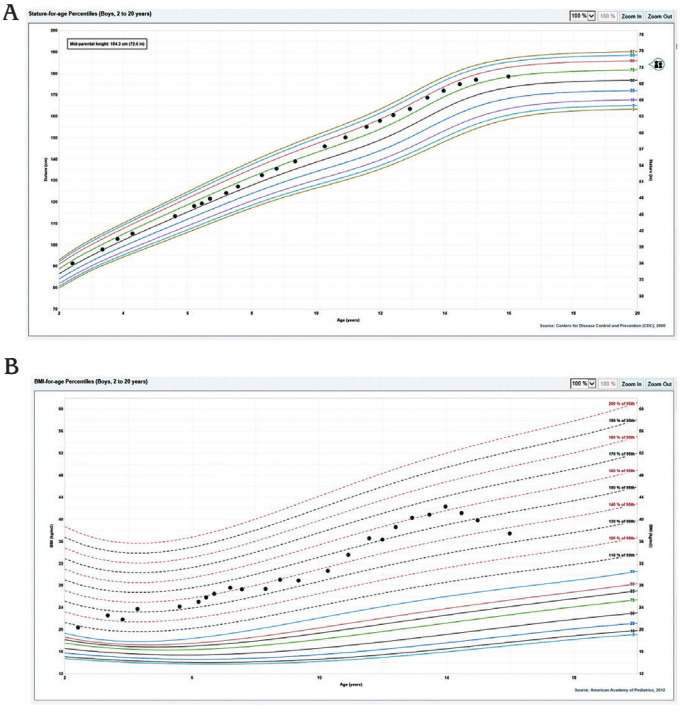

Figure 1. Height (panel A) and body mass index (panel B) for age of proband plotted on Centers for Disease Control charts (22)

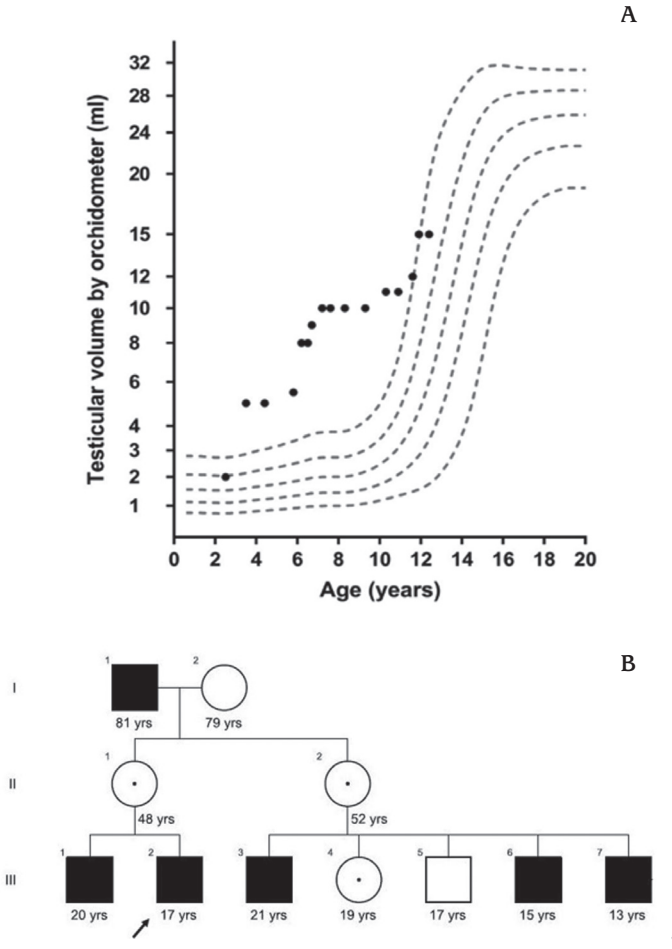

Figure 2. Panel A: Testicular growth as assessed by orchidometer in the proband plotted on reference charts according to Joustra et al (24). Panel B: Pedigree. The arrow indicates the proband. Solid squares indicate hemizygous carriers. Black dots signify heterozygous female carriers

The proband's older brother (case III-1) was seen at the clinic at the age of 13 years, after the proband's initial findings. He had a normal TSH and fT4 newborn screening result. At presentation, besides CeH with thyroid function test (TFTs) results of fT4 9.0 pmol/L (reference range 11.6-20.6) and TSH 1.54 mIU/L (reference range 0.5-4.3)], he had a low plasma testosterone level in contrast to a testicular size of 25-28 mL (2.1-2.3 SD). His serum prolactin and IGF-1 levels were normal, as was his height and BMI, and he did not have any manifestations of long-standing untreated hypothyroidism. He subsequently started levothyroxine at 1 µg/kg/day, which led to normalization of his fT4.

The first male cousin (case III-3, 20 years old) showed CeH, obesity, macroorchidism (50 mL), and attention issues at school. Initial TFTs showed a low fT4 of 9.7 pmol/L (reference range: 11.9-20.6) and inappropriately low TSH of 1.01 mIU/L. TFTs were repeated and showed a slightly higher fT4 (10.68 pmol/L), which was just above the lower limit of reference range of the fT4 assay in use at the time (10.55-22.78 pmol/L), with a concurrent TSH of 0.715 mIU/L. The patient did not start levothyroxine treatment.

The second affected male cousin (case III-6, 14.3 years old) showed a fT4 just above the lower limit of the reference range at 12 pmol/L (reference range: 11.9-20.6) with normal TSH levels, and obesity. At 14.3 years his testes were of appropriate size for age (10-12 mL, -0.8 to -0.4 SDS). On ultrasound, testicular volumes were 17 and 12 mL, (2.4 and 1.4 SDS, respectively) (24), which contrasted with the low plasma testosterone. The ultrasound also showed some peri-testicular fluid and microlithiasis.

The third affected male cousin (case III-7, 11.4 years old) also showed a serum fT4 just above the lower limit of the reference range at 12.7 pmol/L (reference range: 11.9-20.6 pmol/L) with normal TSH levels, and was also obese. He had difficulties at school with reading comprehension. At 11.4 years of age, his testicular volume (5-6 mL) was larger than expected for the pre-pubertal pubic hair Tanner stage.

The proband's mother and female cousin, who were heterozygous for the *IGSF1* variant, showed a serum fT4 of 12.9 pmol/L and 16.7 pmol/L, respectively (reference range: 11.9-20.6) Their menarcheal ages were 10 years and 11.5 years, respectively, and they both had normal menstrual cycles. Clinical data for one male patient and one female carrier were not available (cases I-1 and II-2).

For all patients discussed in this article, blood work-up and examinations were performed within the normal standard

Table 2. Clinical and laboratory findings in the proband and relatives carrying the pathogenic *IGSF1* variant (hemizygous in males, heterozygous in females)

	Proband	Brother	Mother	Cousin (M)	Cousin (F)	Cousin (M)	Cousin (M)	Reference range
Position in pedigree	III-2	III-1	II-1	III-3	III-4	III-6	III-7	
Sex	Male	Male	Female	Male	Female	Male	Male	
Age (years)[a]	15.9	13.7	43	20	18	14.3	11.4	
Birth weight SDS[b]	1.1	0.8	Unknown	1.3	1.8	2.1	2.1	
Tanner stage (P)	5	3	5	5	5	3	1	
Height SDS[b]	0.7	0.6	N/A	1.4	0.7	-1.5	1.2	
BMI	37.4	21.18	N/A	38.7	27.6	27	27.1	
BMI SDS[b]	2.6	0.73	N/A	2.5	1.4	1.7	2.1	
Testicular volume (mL)[c]	> 50 cc	25-28	N/A	50	N/A	10-12[d]	5-6	Age dependent
Developmental issues	Disruptive behavior at school	Attention deficit disorder	None	Focusing difficulties	None	None	Reading comprehension issues	
Free T4 (pmol/L)	See Table 1	**9.0**[e]	12.9	**9.7**	**16.7**	**12.0**	12.7	11.9-20.6[e]
TSH (mIU/L)	See Table 1	1.54	2.58	0.72	2.74	1.00	1.89	0.8-6.3
Prolactin (nmol/L)	< 1	15.25		12	24.5	9	21.5	5-45
Testosterone (nmol/L)	14.7[f]	2.5[g]	N/A	12.5[f]	N/A	3[g]		Dependent on age and Tanner stage

[a]Age at investigation.
[b]Based on Centers for Disease Control references (22).
[c]Assessed with Prader orchidometer.
[d]Ultrasonographic testicular volume of largest testis was 9.1 mL (0.87 SDS). Bilateral testicular microlithiasis was also noted.
[e]For patient III-1 the laboratory provided a reference range of 11.6-20.6 pmoL/L.
[f]For G5 the reference range is 12.2-21.3 nmol/L (26).
[g]For G3 the reference range is 3-11.2 nmol/L (26).
N/A: not applicable, SDS: standard deviation score, FT4: free thyroxine, TSH: thyroid stimulating hormone, SDS: standard deviation score, BMI: body mass index

of care. Informed consent (and assent, if appropriate) was obtained for all genetic testing that was completed. For patients below the age of 18 years, an informed assent and consent was obtained, as appropriate. For patients above 18 years of age, an informed consent was obtained. Limited data concerning the proband, his elder brother and his mother were previously published as part of a large case series (11), and did not include the proband's early obesity or very early testicular growth.

Discussion

Here, we describe a patient who presented initially with neonatal jaundice, CeH and low serum prolactin, and later showed severe obesity, premature testicular growth, disharmonious pubertal development, disruptive behavior at school, and macroorchidism. Genetic evaluation showed a pathogenic variant in the *IGSF1* gene [c.3411_3412del, p.(Tyr1137*)]. Further evaluation of the family showed that the proband's brother and three out of four male maternal cousins carried the same variant. Their fT4 levels were just below or, in three cases, just above the lower limit of the reference range at first or second testing. Macroorchidism was also present in two young adult male relatives. In the two adolescents, testicular volume was large compared to Tanner stage and plasma testosterone, consistent with disharmonious pubertal development. Serum prolactin was decreased only in the proband, compared with approximately 60% of patients in earlier reports (11). Birth weight was between 0.8 and 2.1 SDS in male carriers.

In this family, there are three clinical observations that are unusual when compared to previously reported families. First, in virtually all patients with IGSF1 deficiency reported to date, the serum fT4 concentration is decreased in combination with a normal or low serum TSH, while the occurrence of other clinical and laboratory features is more variable (4,7,8,9,10,11,12,14,16,17,18). Serum fT4 levels have been reported to be just above the lower limit of reference range (13,15) or fluctuating around it (19) in only two and one male patients, respectively. In the present report, we show that out of four male relatives of the proband, fT4 levels were just above the lower limit of normal in two of them, and fluctuated around the lower limit in a third case, suggesting that this may occur more often in males with IGSF1 deficiency than previously assumed.

Second, the start of testicular growth in the proband from two years of age was unusually early, possibly associated with relatively high serum FSH concentrations before and after stimulation by GnRH. A similarly early onset, but less extreme testicular growth (3 mL with the Prader orchidometer) with increased FSH levels has been recorded only once in a 3-year-old boy with a hemizygous *IGSF1* deletion (28). In adult male patients, 24-hour FSH secretion is generally increased, although within the normal range (11).

Third, although obesity has been observed in 21% of children and 17% of adult males (11), extreme early weight gain, as was present in the proband, is unusual. Only one previous report of a 2-year-old patient with IGSF1 deficiency showed such severe obesity (4.2 SDS) (16). In our report, obesity was also observed in three of the four proband's male relatives carrying the *IGSF1* variant. It is currently unknown why IGSF1 deficiency is associated with increased BMI, and in rare cases also with extreme body weight gain. One might speculate that intracellular thyroid hormone concentration or thyroid hormone dependent gene translation are defective in the adipose tissue of these patients. Alternatively, obesity may be caused by decreased fat-burning non-shivering thermogenesis given the role of thyrotropin releasing hormone (TRH) in the central regulation of brown adipose fat functioning and the decreased expression of the pituitary TRH receptor in *IGSF1* knockout mice (4). We have not been able to formally test signs of tissue hypothyroidism or thermogenesis.

The occurrence of other clinical features, such as relatively high birth weight, prolonged neonatal jaundice, as well as the variability of clinical features within one family, are consistent with previous reports (4,7,8,9,10,11,12,13,14,15,16,17,18,19). For example, birth weight was relatively high (0.8-2.1 SDS), in the upper half of what has been reported for other cohorts (9,11). Macroorchidism was seen in the three adult males and disharmonious puberty in the two adolescents, as reported in virtually all cases so far (11). Mild neurological findings, such as attention deficit and difficulties with reading and focusing, were present in three members of this family, in line with observations in a larger cohort (12).

The phenotypic differences within this family are intriguing, and this has been observed in all previously published families (11). In the proband, CGH and testing for Prader-Willi and fragile X syndrome were normal. In previously published families, whole exome/genome sequencing did not reveal other genetic defects that explain these differences. We speculate that polymorphisms or (variable penetrance of) epigenetic changes account for the phenotypic differences.

The pathophysiology of the IGSF1 deficiency syndrome is currently unknown, but may include reduced expression of the receptor for TRH and impaired TRH stimulation of thyrotropin secretion (29). The macroorchidism seen in these patients may be associated with increased FSH

secretion (20). Alternatively, the usual tri-iodothyronine-dependent pubertal increase in LH receptors and cessation of Sertoli cell proliferation could be affected in these patients (despite treatment with levothyroxine), causing a delay in pubertal testosterone rise in the presence of macroorchidism (30). The prolactin and GH deficiency may be related to effects on pituitary transcription factors, such as Pit-1 or TRH receptivity (2). The cause of the obesity seen in these patients has not been elucidated, and there seems to be no association between fT4 levels and the degree of overweight (11). Similarly, it is uncertain whether the issues with attentional control seen in these patients may be associated with timing of levothyroxine treatment (11).

Conclusion

In conclusion, this report demonstrates that male hemizygous carriers of a pathogenic *IGSF1* variant can present with fT4 levels above the lower limit of the reference range; that premature testicular growth without increased testosterone concentrations may be part of the spectrum of clinical features; and that severe early onset obesity may be part of the phenotypic spectrum. This would imply that testing for *IGSF1* should not only be considered in patients who have CeH of unknown cause and low prolactin, macroorchidism, or delayed puberty (11), but also when serum fT4 is slightly above the lower limit of normal and in cases with premature testicular growth or unexplained early-onset obesity. We endorse the advice to test family members in a pattern consistent with X-linked inheritance, if a proband is diagnosed with IGSF1 deficiency, because of the indirect evidence that levothyroxine treatment has a positive effect (11) and in order to obtain more information about the scope of the clinical and laboratory characteristics of this still enigmatic syndrome.

Ethics

Informed Consent: Informed consent (and assent, if appropriate) was obtained for all genetic testing that was completed.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Javier Aisenberg, Steven Ghanny, Aliza Zidell, Helio Pedro, Monique Losekoot, Concept: Javier Aisenberg, Steven Ghanny, Design: Javier Aisenberg, Steven Ghanny, Jan M. Wit, Sjoerd D. Joustra, Data Collection or Processing: Javier Aisenberg, Steven Ghanny, Aliza Zidell, Helio Pedro, Analysis or Interpretation: Javier Aisenberg, Steven Ghanny, Aliza Zidell, Helio Pedro, Jan M. Wit, Sjoerd D Joustra, Literature Search: Javier Aisenberg, Steven Ghanny, Jan M. Wit, Sjoerd D Joustra, Writing: Javier Aisenberg, Steven Ghanny, Jan M. Wit, Sjoerd D Joustra.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0160

J Clin Res Pediatr Endocrinol 2021;13(4):468-472

The Value of Telemedicine for the Follow-up of Patients with New Onset Type 1 Diabetes Mellitus During COVID-19 Pandemic in Turkey: A Report of Eight Cases

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What is already known on this topic?

The use of telemedicine systems reduce glycemic variability parameters including coefficient of variation and standard deviation values. Using telemedicine, the time in range may be achieved at recommended levels in children with type 1 diabetes (T1D).

What this study adds?

Good glycemic control can be achieved by negating the effect of the pandemic on accessibility of diabetic services with telemedicine in T1D.

Abstract

The current Coronavirus disease-2019 (COVID-19) pandemic has forced health care teams to look for alternative approaches to manage a great number of children with diabetes, not only in rural but also in urban locations. The aim was to assess the provision of information about follow-up of new-onset pediatric type 1 diabetes (T1D) patients, and to investigate the integration of telemedicine into routine clinical care in the long term. The changes in coefficient of variation (CV), standard deviation and percentages of time in range (TIR), time below range (TBR) and time above range were evaluated in eight children with new-onset T1D, diagnosed during the COVID-19 pandemic. The study period was two-months of follow-up using a telemedicine system. Median follow-up time was 51 (24-66) days. Two of the patients were using low glucose suspend system and six were on multiple daily injection therapy. Target TIR values were achieved in seven patients in the last televisit and, in line with recent guidelines, a TBR < 70 mg/dL (< 3.9 mmol/L) (level 1 hypoglycemia) of < 4% and a TBR < 54 mg/dL (< 3.0 mmol/L) (level 2 hypoglycemia) of < 1% were achieved in all patients. Seven patients achieved a CV of < 36% at their last televisit. Telemedicine as an alternative follow-up tool during unusual circumstances such pandemics, even in countries where it is not routinely used, could be beneficial to achieve optimum glycemic control in patients with new-onset T1D.

Keywords: Type 1 diabetes, telemedicine, COVID-19, technology, sensor augmented pump

Introduction

Telemedicine can be defined as "the remote delivery of healthcare services". It allows patients and physicians to communicate in real-time (1,2). The current Coronavirus disease-2019 (COVID-19) pandemic has forced health care teams to look for alternative approaches to manage a great number of children with diabetes, not only in rural but also in urban locations. Managing patients with new-onset type 1 diabetes (T1D) is acknowledged as a clinical challenge, and when this occurs during the current pandemic the challenges are magnified.

Data on the use of telehealth in patients with diabetes is encouraging (3). Telemedicine has been associated with improved cost-effectiveness and patient satisfaction (4). A study run with school children showed the benefits of telemedicine communication between the school nurse and

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Conflict of interest: None declared
Received: 21.07.2020
Accepted: 08.10.2020

the diabetes team, in addition to the children's regular care. The telemedicine group had lower hemoglobin A1c (HbA1c) which is a marker of metabolic control, improvement in reported quality of life and fewer hospitalizations/ emergency department visits (5). In studies conducted before the pandemic, telemedicine was more successful in adolescents with diabetes with longer duration of diabetes and with higher baseline HbA1c values (6).

In the early days of telemedicine implementing systems could incur high costs (7). Today, less costly smartphones and other devices make this technology accessible and cost-effective. The aim was to assess the provision of information about follow-up of new-onset pediatric T1D patients, and to investigate the integration of telemedicine into routine clinical care in the long term.

Case Report

When the first COVID-19 patient was diagnosed in Turkey on March 11, 2020, four new-onset T1D were in the hospital and they are included in the study. Additionally, during a two month period, nine patients were diagnosed during the COVID-19 pandemic and four of these were included in the study. Five patients were excluded: one did not continue with telemedicine; and four patients were still hospitalized when the manuscript was written. In total eight patients were included in this case series and characteristics of the patients are given in Table 1. Median follow-up time was 51 (24-66) days. Patients with continuous glucose monitoring (CGM)/flash glucose monitoring system (FGMS) were asked to share their glucose profile by using CareLink Personal software version 3.0 (Medtronic, Minneapolis, MN, USA) or FreeStyle LibreLink from homeland. Patients who did not use CGM/FGMS shared their daily self-monitoring of blood glucose (SMBG) measurements by either smartphones/ email. Insulin doses were adjusted by the same diabetes team.

When the cases were evaluated on an individual basis, two of the patients had been admitted with ketoacidosis and were changed to sensor augmented pump (SAP) with predictive low glucose suspend (PLGS) (Minimed 640 G®) after five days of multiple daily insulin (MDI) therapy because of family anxiety about hypoglycemia (case 2 and 3). MDI insulin treatment protocol included a fixed basal insulin administration, subcutaneously, once daily (insulin aspart or detemir) and rapid acting insulin administration (either insulin aspart/glulisin or lispro insulin) before meals with a dosing based on carbohydrate counting and blood sugar concentrations. Insulin doses of the patients are given in Table 2. In case 3, with ongoing education with telehealth, excellent glycemic metrics were achieved with a time in range (TIR) 96.3%, time below range (TBR) 0.5% and time above range (TAR) 3.2% by the second week. Cases 1 and 4 were admitted with severe diabetic ketoacidosis (DKA) and were started on MDI therapy and used FGMS Abbott FreeStyle Libre. Insulin doses were adjusted based on the outputs through televisits by the pediatric diabetologist. In case 4, after one month, she unfortunately stopped regular daily glucose sharing. At her last televisit, TIR decreased to

Table 1. Patient characteristics

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Age	6.4	9	10.7	13.7	2.3	7.4	12.6	8
Gender	M	M	M	F	F	M	M	F
Weight SDS	-0.38	0.47	2.42	0.63	-0.74	-1.13	0.73	-1.71
Height SDS	0.97	2.29	2.28	0.40	-0.66	2.0	1.25	
BMI SDS	-1.52	-1.9	1.94	0.43	-0.55	-5.16	0.27	
Diagnosis during COVID-19 pandemic	-	-	-	-	+	+	+	+
Clinical presentation	Severe DKA	Diabetic ketosis	Severe DKA	Severe DKA	Mild DKA	Severe DKA	Diabetic ketosis	Severe DKA
Co-morbidity	-	-	-	Hashimoto thyroiditis	-	-	Asthma depression	-
Anti-GAD (iU/mL)	>120	> 120	14.06	>120	>120	3.81	49.1	0.7
Anti-insulin autoantibody (U/mL)	2.12	3.6	-	3.06	10.44	2.75	2.56	2.37
Hospitalization (days)	25	27	10	15	7	6	8	12
Follow-up (days)	62	66	51	65	51	51	24	27
Insulin delivery method	MDI	CSII	CSII	MDI	MDI	MDI	MDI	MDI
HbA1c at diagnosis (%)	12.4	9.9	11.6	11.6	9.5	13.9	13.6	13.7
C-peptide (nmol/L)	0.180	0.52	0.23	0.3	0.1	0.1	0.46	0.52

SDS: standard deviation score, Anti-GAD: glutamic acid decarboxylase antibody, HbA1c: glycated hemoglobin A1c, M: male, F: female, COVID-19: Coronavirus disease-2019, BMI: body mass index, MDI: multiple daily insulin, DKA: diabetic ketoacidosis

61%, TAR increased to 36% with TBR 3%. We think that despite using FGMS, the deterioration of metrics may be due to early cessation of televisits.

Case 5 was a 2.3-year-old girl diagnosed with hyperglycemia and mild ketosis. As a result of her young age, and unpredictable eating habits and activity, use of a SAP-PLGS was recommended but the family declined due to the expense; SAP-PLGS is not covered by insurance in Turkey. Therefore, MDI treatment was initiated. Since the parents declined learning carbohydrate counting, she was discharged with an exchange meal plan after seven days of hospitalization. The parents also declined using any type of CGM due to financial issues and lack of insurance coverage. For the first 30 days, parents were encouraged to share SMBG measurements every day and family's education continued. Insulin doses were adjusted in consultation with the diabetes team. This patient had the worst glycemic control of the eight cases presented here. In the second week, she had a TIR of 48.7% but with ongoing education via telemedicine her TIR increased to 81.7% without any documented level 1 hypoglycemia. Cases 6 and 8 were diagnosed as severe DKA and after one day of pediatric intensive unit hospitalization, MDI treatment was started with SMBG. Case 7 was admitted to the hospital with ketosis and she was again treated with MDI therapy and SMBG.

Table 2. Total daily insulin doses (IU/kg) of patients at first 2 weeks and last 2 weeks

	Insulin doses (IU/kg)		
	Second week	First month	Last control
Case 1	0.44	0.50	0.60
Case 2	0.36	0.44	0.57
Case 3	0.38	0.37	0.41
Case 4	0.75	0.78	0.71
Case 5	0.91	1.08	1.07
Case 6	1.00	0.70	0.82
Case 7	1.43	1.32	1.23
Case 8	1.23	1.15	1.19

In all eight patients follow-up visits were scheduled every day for the first 14 days and then every week for the first two months and whenever needed. Glycemic variability (GV) index of the first 14 days after discharge and the last televisit are given in Table 3.

Discussion

In the late fall of 2019, Wuhan in China announced an outbreak of an infection, which was later designated COVID-19 by the World Health Organization (WHO) (8,9). COVID-19 was declared pandemic by WHO on March 11, 2020; the date on which the first positive case was detected in Turkey.

COVID-19 has required dramatic changes to our delivery of health care, some of which improved access and outcomes

Table 3. Glycemic variability and glucose metrics of patients at first 2 weeks and last 2 weeks

	TIR (%)			TAR >180 mg/dL (%)			TBR <70 mg/dL (%)			SD			CV (%)			Mean glucose (mg/dL)		
	2nd week	1st month	Last control	2nd week	1st month	Last control	2nd week	1st month	Last control	2nd week	1st month	Last control	2nd week	1st month	Last control	2nd week	1st month	Last control
Case 1	69	75	85	29	24	12	2	1	3	66.1	57.1	42.8	41.6	38.9	34.5	159.1	147	124
Case 2	79.2	86.7	79.6	19.5	6.7	12.6	1.2	6.6	0.7	43	35.1	36.9	30.1	25.6	25.3	143.1	137.4	146.2
Case 3	96.3	96.4	96	3.2	2.7	3.7	0.5	0.9	0.3	25	24.8	24.2	19.9	19.9	18.9	125.3	125	128
Case 4	82	81	61	9	14	36	9	5	3	40.3	42.6	51.1	33.5	31.6	31.1	135	135	159
Case 5	48.7	61.2	81.7	51.3	36.6	18.3	0	2.2	0	95.5	64.9	38.8	47	39.7	27.3	202.8	163.4	140.3
Case 6	64.7	82.1	89.6	27.7	15	8.8	7.6	2.9	1.7	62.6	44.2	45.8	42.5	33.7	32.6	147.3	131.1	140.4
Case 7	86.3	-	89.6	13.7	-	9.4	0	-	1	33.1	-	30.9	24.2	-	22.6	136.9	-	136.7
Case 8	69.6	-	69.8	29.4	-	29.2	1	-	1	56.3	-	59.2	36.6	-	42.1	153.6	-	140.3

TIR: time in range, TAR: time above range, TBR: time below range, SD: standard deviation, CV: coefficient of variation

for our patients with diabetes. The "Stay at Home" order for children <20 years in Turkey during COVID-19 pandemic has forced a majority of the diabetes teams to provide diabetes care remotely through telehealth when possible. Moreover, parents of many children with T1D postponed their appointments, due to anxiety about contracting COVID-19 in healthcare settings.

Digital platforms are places where diabetes teams and patients can meet virtually and share and discuss downloadable data from glucometers, CGMs and insulin pumps. Furthermore, with telehealth, SMBG data and insulin doses of MDI patients can be evaluated. Telehealth can be provided through teleconferencing, telephone, text messaging, and/or e-mail. This system can provide a good alternative to the physical, and perhaps risky, routine outpatient meeting (10). However, there are still a lot of areas in Turkey, and even in the USA, with no internet access. Healthcare through telemedicine depends on the availability of wireless network systems, smart phones and regular phones for both the healthcare providers and the patients and families. Since our case group had middle-high socioeconomic status, their access to health services using telemedicine was sufficient. In order to follow-up metabolic control of our patients through telehealth, we used email and WhatsApp and received SMBG, or PDF results of CGMS or SAP and calculated glucose metrics.

The number of daily blood glucose measurements was in the desired range with 7.1 ± 1.1 times/day. According to a consensus report, a TIR >70% is a recommended target for T1D (7). However, this target should be personalized and targets should be set according to the age of children. In a case report by Garg et al (11), TIR was 30% in a one-year-old patient with T1D diagnosed during the COVID-19 pandemic. Target TIR values were achieved in seven (87.5%) patients at the last televisit and, in line with recent consensus guidlines, a TBR <70 mg/dL (<3.9 mmol/L) (level 1 hypoglycemia) of <4% and a TBR <54 mg/dL (<3.0 mmol/L) (level 2 hypoglycemia) of <1% were achieved in all patients (7).

GV is a metric that provides an integrated picture of postprandial hyperglycemia and hypoglycemic episodes. GV has been hypothesized to be an independent risk factor for vascular disease, independent of HbA1c (12,13,14). Increased GV is consistently associated with mortality and is a consistent predictor of hypoglycemia, both in prospective studies and randomized clinical trials (15,16). For CV, a 36% threshold has been adopted as the primary metric to separate stable from unstable diabetes. Peters and Garg (17) reported CV values of 18% and 20.3% in two adult patients with T1DM diagnosed during COVID-19 pandemic. In our study, seven patients achieved a CV of <36% at their last televisit.

According to the Search for Diabetes in Youth study, which includes 1396 youths aged <20 years with newly diagnosed T1D, 28% had DKA at presentation (18). In our much smaller study this rate was 75%. Two of the patients had been admitted to the primary care physician and were misdiagnosed as upper respiratory tract infection and abdominal pain which may be due to lack of information of the physicians about pediatric diabetes, or their anxiety concerning COVID-19. All patients were negative for COVID-19 and the hospital stay of cases 5-8, whose diagnosis and treatment were after COVID-19 was reported in Turkey, were shortened to 8.2 ± 2.6 vs 19.2 ± 8.0 days in the patients already in hospital when the first COVID-19 case was reported. Some of the health education of cases 5-8 continued at home via telemedicine, but this difference seems to not effect glucose metrics.

Conclusion

In this study we reported managing diabetes remotely, especially in new-onset patients with T1D during this pandemic. With telehealth, optimum glycemic targets can be achieved in pediatric patients with new onset T1D. However, important limitations of telehealth systems include inability to perform a physical examination and there is no point-of-care testing available for accurate HbA1c measurement. If this new way of follow-up using telehealth is to continue after the pandemic is over, it may provide substantial improvements for patients who will no longer need to attend hospital as regularly, but can also provide the benefits of daily follow-up.

Ethics

Informed Consent: Written informed consent was obtained from all participants or their parents/guardians.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ferda Evin, Damla Gökşen, Concept: Ferda Evin, Design: Ferda Evin, Data Collection or Processing: Ferda Evin, Eren Er, Aysun Ata, Yasemin Atik Altınok, Analysis or Interpretation: Ferda Evin, Eren Er, Damla Gökşen, Aysun Ata, Arzu Jalilova, Literature Search: Ferda Evin Eren Er, Damla Gökşen, Samim Özen, Şükran Darcan, Günay Demir, Writing: Ferda Evin, Damla Gökşen.

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

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DOI: 10.4274/jcrpe.galenos.2021.e004

J Clin Res Pediatr Endocrinol 2021;13(4):473

DOI: 10.4274/jcrpe.2408

Güven A, Cebeci AN, Ellard S, Flanagan SE. Clinical and Genetic Characteristics, Management and Long-Term Follow-Up of Turkish Patients with Congenital Hyperinsulinism. J Clin Res Pediatr Endocrinol 2016;8:197-204. Epub 2015 Dec 18

The mistake has been made inadvertently by the author.

That line starting with No 8, of Table 2 on page 201 of the related article has been corrected by the author as below.

Incorrect line starting with No 8, in Table 2.

....

8	3.88	ABCC8	p.Gly1485Ala (c.4454G > A)	Novel missense	Heterozygous	p.Gly1485Ala (c.4454G > A)	N/N	No	3 months old/ diffuse	3.4	No treatment

Corrected line starting with No 8, in Table 2.

....

8	3.88	ABCC8	p.Gly1485Ala p.Ala726Thr (c.4454G > A c.2176G > A)	Two Novel missense	Heterozygous	p.Gly1485 Ala (c.4454G > A)	p.Ala726Thr (c.2176G > A;	No	3 months old/ diffuse	3.4	No therapy

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