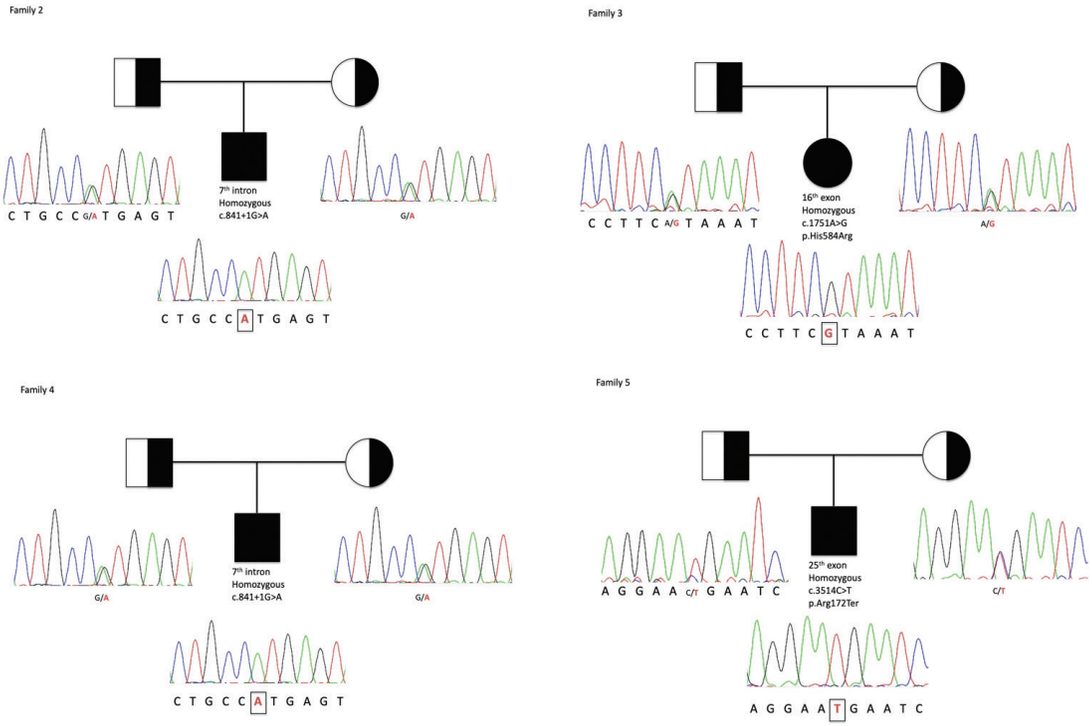
JCRPE

Journal of Clinical Research in Pediatric Endocrinology

September 2021 | volume 13 | issue 3 | www.jcrpe.org | ISSN: 1308-5727

E-ISSN: 1308-5735



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Official Journal of
Turkish Pediatric Endocrinology
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Journal of Clinical Research in Pediatric Endocrinology

Volume: 13 Issue: 3 September 2021 Page: 251-369

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Özgün Basım Tanıtım San. Tic. Ltd. Şti. Yeşilce Mah. Aytekin Sok. Oto Sanayi Sitesi No: 21 Kat: 2 Seyrantepe Sanayi, Kağıthane, İstanbul, Turkey
Phone: +90 212 280 00 09
Certificate Number: 48150
Date of printing: September 2021
ISSN: 1308-5727
E-ISSN: 1308-5735

JCRPE Journal of Clinical Research in Pediatric Endocrinology
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DOI: 10.4274/jcrpe.galenos.2020.2020.0091

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Traditional and New Methods of Bone Age Assessment-An Overview

Monika Prokop-Piotrkowska[1], Kamila Marszałek-Dziuba[1], Elżbieta Moszczyńska[1], Mieczysław Szalecki[2], Elżbieta Jurkiewicz[3]

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Abstract

Bone age is one of biological indicators of maturity used in clinical practice and it is a very important parameter of a child's assessment, especially in paediatric endocrinology. The most widely used method of bone age assessment is by performing a hand and wrist radiograph and its analysis with Greulich-Pyle or Tanner-Whitehouse atlases, although it has been about 60 years since they were published. Due to the progress in the area of Computer-Aided Diagnosis and application of artificial intelligence in medicine, lately, numerous programs for automatic bone age assessment have been created. Most of them have been verified in clinical studies in comparison to traditional methods, showing good precision while eliminating inter- and intra-rater variability and significantly reducing the time of assessment. Additionally, there are available methods for assessment of bone age which avoid X-ray exposure, using modalities such as ultrasound or magnetic resonance imaging.

Keywords: Maturation, children, radiographs, deep learning, neural networks

Introduction

Maturation Indicators

The processes of growth and maturation in children are usually correlated, but they cannot be treated as one process as they may not be linear and may proceed at different paces. Due to numerous disturbances, such as growth hormone (GH) deficiency, deficiency of thyroid hormones or delayed puberty, but also sometimes in healthy children, the chronological age (CA) doesn't match the biological age. This is because they are regulated by various factors, which include genes and nutrition, but also include many hormones, including GH, insulin-like growth factor-1, sex hormones and adrenal steroids such as cortisol, dehydroepiandrosterone, and testosterone (1,2). In paediatric endocrinology, it is especially important to assess the child's growth and puberty in relation to biological age, rather than CA. Thus, clinicians have been looking for a good marker of maturation rate in children for decades (3). Age at menarche is a solid biological indicator of maturity, but it is a one-off event and relates to only half of the population (3). Dentists, mainly orthodontists, use dental age judged using the Demirjian or Willems scale in daily practice but this practice has not been established as a reliable tool for other clinicians (3,4,5). Sexual characteristics, such as that made by assessment of position on the Tanner scale, are useful only in the adolescent period and are very subjective. The only biological indicator of maturity, which is available from birth to adulthood, is bone age (BA) (3).

Bone Age

In paediatric endocrinology, BA is an important tool used in the clinical assessment of patients, mainly those suffering from growth and puberty disorders. Many parameters correlate better with BA than with CA including height velocity, menarche, muscle mass and bone mineral mass (6). Delayed BA is typical for GH deficiency, constitutional delay of growth, hypothyroidism, malnutrition and chronic

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Conflict of interest: None declared
Received: 08.05.2020
Accepted: 15.10.2020

J Clin Res Pediatr Endocrinol
2021;13(3):251-262

illness (6,7). On the other hand, BA is advanced in many conditions that include precocious puberty and congenital adrenal hyperplasia, when there is a prolonged elevation of sex steroid levels (6,7,8). BA may be also marginally advanced in cases of overweight children, children with tall stature or premature adrenarche (1,6,8). In genetic overgrowth syndromes, for example Sotos syndrome, Beckwith-Wiedemann syndrome and Marshall-Smith syndrome, BA is usually significantly advanced (6). In all cases it is important to remember that advancement or delay of BA in relation to CA is a slow process, thus BA may not be altered in the case of examinations performed shortly after the first manifestations of a disorder and should be assessed in a temporal manner (7).

What is more, BA is used in forensic and legal medicine to estimate CA, for example in asylum seekers or unaccompanied minors without documents. In such cases an adequate assessment of age using precise methods is crucial. The consequences of incorrect assessment of a child as an adult may result in more restricted access to education, medical care or other forms of support provided for children (9).

This article considers different methods of BA assessment from the perspective of a paediatrician or paediatric endocrinologist (Table 1).

Traditional Methods

Although there have been attempts to assess BA by examinations of specific bones, such as the clavicle or iliac bone (Risser sign) (10,11,12,13,14,15), in paediatrics and paediatric endocrinology, the established way to obtain BA is by performing a radiograph of the hand and wrist of the non-dominant hand. Assessment of development of the bones can be performed in the traditional, manual way or using one of the automated methods. The manual method involves a comparison of obtained radiograph with radiographs in atlases. The manual methods can be divided into two groups depending on the type of atlas – holistic or analytic.

The first atlases were published shortly after the discovery of X-rays in 1895. In 1898, John Poland published the first one: "skiagraphic atlas showing the development of bones of the wrist and hand" (16). In his atlas, he depicted skiagraphs (positive reprints) of hand radiographs of 19 British children, aged between 1 and 17 years, with an attached description of each radiograph (16). However, the two most important publications in this field were issued in 1959 by Greulich and Pyle (17) and in 1962 by Tanner, Whitehouse and Healy (18).

Greulich-Pyle Atlas

'The Radiographic Atlas of Skeletal Development of the Hand and Wrist' by Greulich and Pyle (17) (GP) has been widely recognized and is used in many centers currently. This atlas was created based on radiographs of hands of paediatric patients referred to endocrinologists William Walter Greulich and Sarah Idell Pyle by paediatricians between the years 1931-1942. These patients were Caucasian children from a generally upper middle class background, living in Cleveland, Ohio, United States (19,20). This atlas consists of separate reference images for boys and girls aged 0-18 (boys) or 0-19 years (girls) in various intervals (3 months-1 year). Images are accompanied by an explanation of the gradual age-related changes in the bones at a given age and separate BAs calculated for each bone. Due to the natural variability of the BA of different bones in one individual, in some bones, it is often more or less advanced than the standard it is intended to represent. For example, a radiograph representing the age of 3 years 6 month (42 months) includes a 36-month first metacarpal and a 54-month lunate (17). BA is calculated by comparing the non-dominant wrist radiographs of the subject with the nearest matching reference radiographs provided in the atlas. Thus this method is termed a holistic method. Figure 1 presents GP atlas.

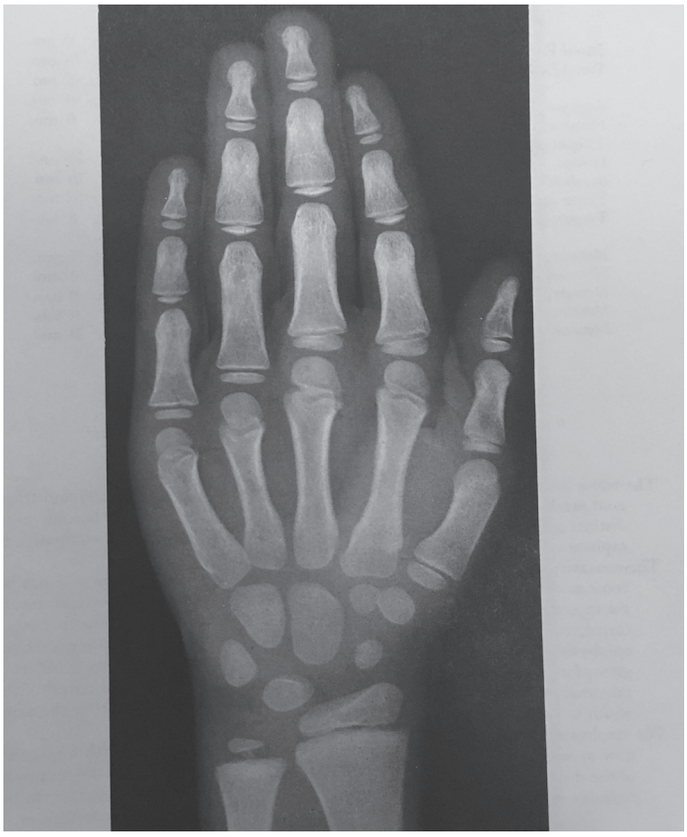

Figure 1. Greulich-Pyle atlas

GP is the most popular method among clinicians and radiologists, as the assessment by GP is relatively quick and easy to learn. Although widely used, this method has significant drawbacks. BA assessment (BAA) using GP shows high inter- and intra-observer variability. In addition given the reference population used in GP, this method may not be an appropriate, universal tool for use in various populations.

BAA by GP is very subjective and the standard error on a single determination in inter-observer studies ranges from 0.45 to 0.83 years (21,22,23,24,25). There is no standardization in how the bones are weighted. Depending on a rater, in clinical practice one may assign different weight to different bones, some raters may ignore the carpals and others may assign even half weight to the carpals during the assessment. Raters using the carpals reduce their importance at higher maturity but again not in a standardized manner (24).

It has been reported that currently boys and girls develop secondary sex characteristics earlier than decades ago in United States (26,27). Thus current use of the GP atlas, even in a similar population to the original source, may not be as precise as when it was created.

What is more, it has been proven that correlation of BA with CA, and consequently the applicability of GP, depends on ethnic origin (28,29). According to a recent meta-analysis it has been proven that in African females, in comparison to GP standards, BA is significantly advanced. Conversely, in Asian males, BA is significantly delayed between 6 and 9 years of age and significantly advanced at 17 years (28). This should be taken into consideration while assessing BA in these populations using the GP atlas.

There is an online version of GP uploaded by Brazilian Instituto Mineiro de Endocrinologia (28).

Tanner-Whitehouse Atlas

The second most popular tool for BA assessment is the Tanner-Whitehouse atlas (TW). The first version of TW was created in 1962 based on 2600 radiographs collected in the1950s and 1960s of British children coming from average socio-economic class (18). It was later updated in 1983 to Tanner-Whitehouse 2 (TW2) and in 2001 the latest updated version was published - Tanner-Whitehouse 3 (TW3). These updates have attempted to adjust for the secular trends that influence the relationship between the total bone maturity score and BA (30). In several countries standardized TW methods have been created which change the relationship between the total maturity score and BA to make it suitable for different ethnic groups (31,32,33).

TW2 is an analytic or scoring method and it is based on the maturity levels of 20 regions of interest (ROI) in different bones of the hand and wrist. The level of development of each ROI is labeled as a given stage, which is then converted to a numerical score. A total maturity score is calculated by adding the scores of the ROIs and it is matched with the age of boys and girls separately.

The TW method is considered to be more objective than the holistic GP method and to also exhibit higher reproducibility than GP. Bull et al (21) reported that the intra-observer variation was greater using GP than TW (95% confidence interval, -2.46 to 2.18 vs -1.48 to 1.43, respectively). However, assessment using the TW method is more time-consuming. In a study performed by King et al (34) the average time required for TW assessment was calculated as 7.9 min. vs. 1.4 min. in the case of GP assessment. In this study the intra-observer variation between GP and TW assessment was also found to be insignificant (the average spread of results was 0.74 years for TW and 0.96 years for the GP). It should be noted that the sample size assessed by King et al (34) was considerably smaller than that assessed by Bull et al (21). A comparison of GP and TW methods is presented in Table 1 (Table 2).

Other Atlases

The FELS method was developed in 1988 using 13,823 serial radiographs of the left hand-wrist of boys and girls in

Table 1. Bone age assessment methods

	Manual	Automatic
Radiograph	- Greulich-Pyle Atlas (17)	- CASAS (55),
	- Tanner-Whitehouse Atlas (30)	- BoneXpert (71,72)
	- FELS Method (36)	- AI methods (97-109)
	- Gilsanz and Ratib Atlas (37)	
MRI	- Pediatric Hand MR Scanner (45,46)	- Method of Štern et al (51)
	- Method of Tomei et al (48)	
	- Method of Hojreh et al (49)	
USG	- Femoral head cartilage thickness (44)	- BonAge (40)
	- Risser's stage (45)	

MRI: magnetic resonance imaging, USG: ultrasonography

Table 2. Comparison of Greulich-Pyle and Tanner-Whitehouse methods

Atlas	Greulich-Pyle	Tanner-Whitehouse
Advantages	- Widely recognised	
	- BAA relatively quick	- Latest version from 2001
	- Easy to learn	- Higher reproducibility than Greulich-Pyle
Disadvantages	- High intra- and inter-rater variability	- BAA time consuming
	- Not applicable to some populations	
	- One version since 1959	

BAA: bone age assessment

the Fels Longitudinal Study performed by William Cameron Chumlea, Alex F. Roche and David Thissen from two universities in Kansas and Ohio, US (35). It is based upon maturity indicators that represent radiographic features that occur during the maturation of every child (35). The set of maturity indicators is analysed with a computer program that provides the BA and the standard error for that assessment (35). However, the FELS method has not gained wide recognition.

In 2005 a digital atlas created by Vicente Gilsanz and Osman Ratib (GR) was published. It consists of artificially created, idealised images of hands and wrists, specific for age and sex. These images were produced by an analysis of the size, shape, morphology and density of ossification centres of 522 hand radiographs from healthy Caucasian children from Los Angeles, US (50% girls and 50% boys). Each image includes typical characteristics of development for each of the ossification centres (36). The images are of better quality and precision in comparison to GP. Another advantage is the regular spacing of the images at 6-monthly intervals from the ages of 2 to 6 years and yearly intervals from the age of 7 to 17 years (37). In one study the GR atlas was compared to GP and it was concluded that they were comparable in terms of precision. Yet again, however, the study was performed on a small number of examinations (38).

Ultrasound Assessment

Other imaging modalities, which have developed considerably over the years, now offer some advantages over the ubiquitous radiograph for assessment of BA. One of these is ultrasound (USG), the major advantage of which is that it does not expose the patient to any ionizing radiation, important when patients receive sequential assessment of BA. Some studies have been performed to establish different methods of BAA, including by performing USG (39).

A result of one of these trials is BonAge® (Sunlight Medical Ltd, Tel Aviv, Israel) which consists of a device that performs an ultrasonographic examination and software that calculates the BA on the basis of this examination (19,40,41,42,43). BonAge® measures the ossifying cartilage structures of the wrist as an ultrasonic wave passes through the subject's distal radius and ulnar epiphysis. According to the producer, BonAge® provides on-the-spot, easy-to-read, immediate results, without exposing children and adolescents to ionizing X-ray radiation, and moreover, it is objective and safe (40). The time of the examination is approximately five minutes although this can prove problematic in the smallest children (41).

Several studies have been performed to assess the precision of this instrument. Mentzel et al (41) and Shimura et al (42) concluded that the results of BonAge® examinations correlate closely with BA evaluated conventionally using the GP or TW2 method. However, in a more recent study performed by Khan et al (43) on a bigger number of patients it was shown that BonAge® tended to over read delayed BA and under read advanced BA and the authors concluded that ultrasonographic assessment should not yet be considered a valid replacement for radiographic BAA.

There has also been a report of ultrasonographic assessment of the thickness of anterior femoral head cartilage, which correlates strongly with the child's CA and BA, standing height and body weight, according to the authors of the study (44). Ultrasonic examination of ossification of the iliac crest apophysis, (Risser's sign), was also studied and it presented with high accuracy, specificity and sensitivity in comparison to hand X-ray examination and GP assessment (45).

Although the majority of the authors of these studies conclude that USG methods investigated are of good accuracy in comparison to hand X-ray, USG-based BAA is rarely used in daily practice. This may be because the examination needs to be performed by a trained specialist or there is a need for a specific device. In both cases, it takes more time to perform than an X-ray. Taking into consideration that most studies investigating the utility of USG in BAA were performed on relatively small groups of patients, the clinical utility of USG examination is as

yet unproven. Isolation of the forearm allows for minimal radiation exposure and the radiation during hand X-ray is very low (0.0005 mSv). However, in the future, USG may be an advantageous method that may allow total elimination of children's exposure to ionizing radiation during BAA.

Magnetic Resonance Imaging Assessment

The first research in the field of BAA using magnetic resonance imaging (MRI) was performed in 2007 to find a tool suitable to establish the age of male football players without unnecessary radiation exposure (29). Since in some Asian and African countries registration at birth is not compulsory, age determination is crucial to prevent participation in the incorrect age group (29).

In 2012 Terada et al (46) reported a technique for BAA based on MRI examination. BA was determined using an open, compact, newly designed MR imager optimized for evaluation of a child's hand and wrist and it was scored by two raters using the TW system adapted for the Japanese population. Evaluation of this method was performed on a group of 93 healthy Japanese children and a strong positive correlation with BA and CA was demonstrated. What is more, the intra-and inter-rater reproducibility rates were significantly high (46). Another study from the same authors was performed in 2014 to improve the performance of this method (47). This was conducted on a group of 88 healthy children with three raters assessing BA and it confirmed the reliability and validity of this method (47). However, a disadvantage of MRI is that it requires a relatively long time to be performed (2 min and 44 sec), therefore it may not be suitable for the youngest children, due to body movement.

Another study was performed by Tomei et al (48) and this was published in 2014. They performed hand and wrist MRIs on 179 healthy children aged 11-16 years old and analyzed the correlation with CA. It was concluded that BAA with MRI was feasible and showed good inter-observer reproducibility (48).

In 2017 the results of another study were published regarding the use of MRI in BAA. Hojreh et al (49) performed hand MRI and X-ray examinations in 50 healthy volunteers and 10 patients, all of whom were adolescents (aged 15 ± 2 years and 13.5 ± 2.6 years, respectively) and assessed both examinations according to GP criteria. This study concluded that the correlation between estimated patients' ages on radiographs assessed by GP and MRI was high with the average estimated age difference between the MRIs and radiographs being $-0.05/-0.175$ years. However larger, multicenter studies are necessary to confirm the usefulness of this method. There have also been attempts to automate the BAA using MRI instead of radiography (50,51). The comparison of RTG, USG and MRI methods is presented in Table 3.

Automated Techniques

Due to the problems associated with BAA when using traditional methods, such as inter- and intra-observer variability and the fact that it is time-consuming, a need emerged for new, objective tools that would provide immediate results. As Computer-Aided Diagnosis (CAD) has emerged and has started to be used in clinical practice, one obvious procedure, which would be suitable for adaptation to CAD was BAA, and BA was one of the first radiologic examinations to be automated. This is not recent, however. The first trials of CAD in BAA date back to 1989 when a semi-automated system called HANDX was introduced by Michael and Nelson (52). More recently, work on a system which is based on assessment of phalangeal regions of interest (PROI) was published by Pietka et al (53) in 1991. In this method, the PROI were detected and the lengths of the distal, middle, and proximal phalanx were measured

Table 3. Comparison of radiographic, ultrasonographic and magnetic resonance imaging-based methods

Method	Radiograph	Ultrasonography	Magnetic resonance imaging
Advantages	- The most frequently used - Many recognised atlases - Easy to perform - Quick - Accessible - Doesn't require a radiologist to perform, only to assess - Automated methods available	- No X-ray exposure	- No X-ray exposure - Accuracy validated in studies - There are attempts to automate BAA using MRI
Disadvantages	- X-ray exposure	- Presence of radiologist required to perform - Time consuming - Only few studies on its accuracy	- Not easily accessible - Relatively time consuming (quicker than USG)

BAA: bone age assessment, USG: ultrasonography, MRI: magnetic resonance imaging

automatically. BA was estimated using the standard phalangeal length table, presented earlier by Garn et al (54).

CASAS

However, the first system to be used by different authors in studies was CASAS - a computerized image analysis system for estimating TW2 BA (55). This semi-automated system was introduced by Tanner and Gibbons in 1994 and it used the 13 bones of TW RUS system (radius, ulna and short bones) for BAA. These bones had to be located manually on the screen by a rater (correct positioning was assured by computer templates of each bone stage) and then automatic scoring was performed. Tanner and Gibbons (55) concluded that CASAS was more reliable and valid than manual TW RUS rating (56). Although other researchers have also reported that CASAS was useful and reliable (57,58), this system has not been widely adopted. The major drawback was that it took more time to estimate BA with CASAS than a manual TW assessment. In addition, difficulties with BAA in cases of abnormally shaped bones restricted the use of CASAS in some pathological conditions.

More recently there have been numerous approaches to BAA automation (58-71) and the most important ones are described below.

BoneXpert

This automated tool for BAA was created in 2008 by the Visiana company, based in Holte, Denmark (72,73,74). This computer program analyses BA automatically, in several steps. The first step is the definition of borders and intensity of the radiologic image of 13 points of interest of the same 13 bones used in the TW RUS system, that is the radius, ulna and 11 short bones. During this first step the system also defines if the picture is complete and of appropriate technical quality. In the next step, BA is assessed for each of the 13 bones separately. The last step is the transformation of the summary BA according to GP and TW criteria (72,73). Figure 2 presents BAA by BoneXpert. BAA is available for ages 2.5-19 years for boys and 2-18 years for girls (version 2.4.7.6.) (75). The data set used for the creation of this program consisted of 1678 hand radiographs of healthy Danish children and children from Belgium diagnosed with a range of disorders, such as Turner syndrome (73).

To date several papers have been published that verify the reliability and precision of BAA using BoneXpert in comparison to GP in different populations (Table 4). In European populations, studies have been conducted among healthy children from the Netherlands (405 patients), German children with short stature (1,097 patients), precocious or early puberty (116 patients), congenital adrenal hyperplasia (100 patients) and with various other endocrinological disturbances (514 patients) (75,76,77,78,79). Moreover, there was a study conducted with 1100 healthy American children from four different ethnic groups (Caucasian, African American, Asian and Hispanic) (22) and another on 515 eutrophic, overweight and obese children from Brazil (80). Research into the validity of BoneXpert has also been performed in Asian populations, including a study on 397 healthy children from Shanghai, China (81), in a large population of 6026 healthy children from five different cities in China (82) and among Japanese children, using 185 radiographs from 22 healthy children and 284 radiographs from 22 patients diagnosed with GH deficiency (83).

What is more, studies have confirmed the validity of BAA via BoneXpert in groups of children suffering from different disorders, including juvenile idiopathic arthritis (84), in severely disabled children (85) and, as previously noted, children with short stature (76), precocious puberty (77) and congenital adrenal hyperplasia (78). All these studies conclude that BoneXpert is a suitable tool to perform BAA, it is faster than traditional methods and eliminates rater variability. However, it should be noted that one of the authors of most of these studies is a person connected to the commercial activity of Visiana company, the producer of BoneXpert.

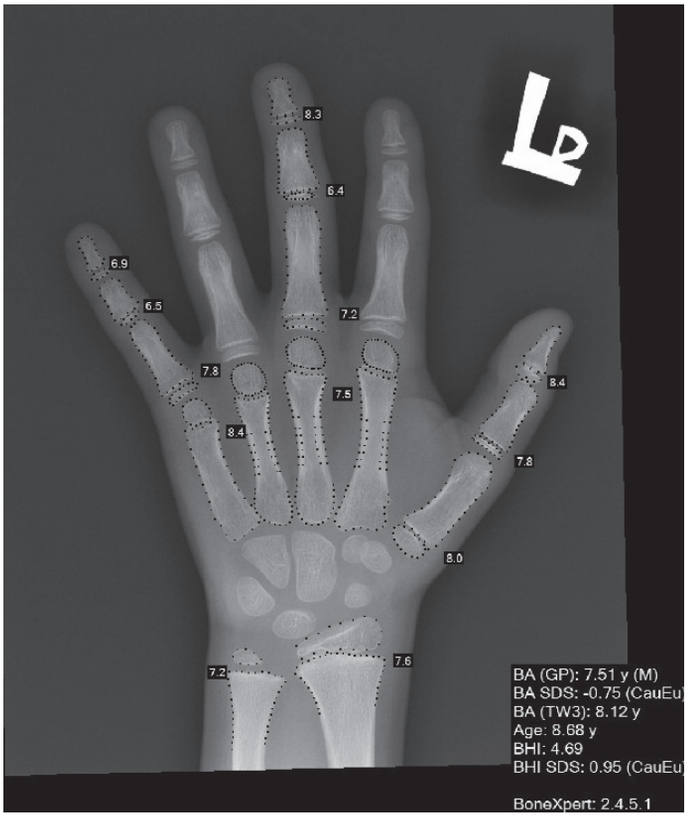

Figure 2. Bone age assessment by BoneXpert

Table 4. Studies assessing the validity of BoneXpert vs. the Greulich-Pyle method

Study			Population		Validity claimed
Author	Year	Size	Origin	Health status	
Van Rijn et al (75)	2009	405	Netherlands	Healthy	Yes
Martin et al (76)	2008	1097	Germany	Short stature	Yes
Martin et al (77)	2011	116	Germany	Precocious or early puberty	Yes
Martin et al (78)	2013	100	Germany	Congenital adrenal hyperplasia	Yes
Booz et al (79)	2020	514	Germany	Various endocrinological disturbances	Yes
Thodberg and Sävendahl (22)	2010	1100	American (4 ethnic groups)	Healthy	Yes
Artioli et al (80)	2019	515	Brasil	Healthy, overweight and obese	Yes
Zhang et al (81)	2016	397	Shanghai	Healthy	Yes
Zhang et al (82)	2013	6026	China	Healthy	Yes
Martin et al (83)	2010	44	Japan	Healthy, deficiency of growth hormone	Yes
Anink et al (84)	2014	69	Netherlands	Juvenile idiopathic arthritis	Yes
Mergler et al (85)	2016	95	Netherlands	Severely disabled	Yes

BoneXpert has several critical limitations. BA is not identified directly, the prediction depends on the relationship between CA, which is an input to the system, and BA (62). The system is brittle and will reject radiographs when there is excessive noise, in one study it rejected 4.5% of individual bones (81). Finally, until recently BoneXpert did not take the carpal bones into consideration, although in younger children they contain discriminative features. This has been changed in the latest version - BoneXpert 3.0 released in September 2019 - which now does include carpal bones in the analysis.

An additional feature that BoneXpert offers is measurement of a parameter called the Bone Health Index (BHI) (86), which is a unique parameter. BHI is a measurement of bone mass counted as a function of cortical thickness of three central metacarpals and their width and length. The program also automatically calculates standard deviation (SD) values for BHI, based on cohort data of Caucasian children (86). There are several research studies on the comparison of BHI values and traditional methods of bone mass measurement. In one study BHI was compared to dual-energy X-ray-absorption (DXA) and peripheral quantitative computed tomography (pQCT) in a cohort of paediatric patients from paediatric endocrine or paediatric oncology outpatient clinics and it was concluded that BHI values showed a strong positive correlation with DXA readings and total bone mineral density, as assessed via pQCT, also positively correlated with the BHI (87,88). In another study on a group of patients with juvenile idiopathic arthritis, BHI measured by BoneXpert was correlated to measurements of bone mineral density by DXA, however, the correlation of Z-scores of bone mineral density measured by the two methods was weaker (89). The authors of these studies noted that a significant advantage of using BHI, in comparison to DXA or pQCT, was that radiation exposure was lower and in low-risk peripheral areas. Also, BHI has already been used in research studies of BA in patients with juvenile idiopathic arthritis (89). There is an extension to BoneXpert, known as digital X-ray radiogrammetry (DXR). DXR measures the cortical bone thickness in the shafts of the metacarpals and has been shown to be effective in the assessment of hand bone loss caused by rheumatoid arthritis (90).

Another advantage of BoneXpert is a prediction of the final height of a child (91,92), which is a vital element of clinical assessment of a child with short stature. Methods in current routine use take into consideration BAA using traditional methods – GP or TW. The variability of these assessments is the main reason for the variability of predicted final height. When BAA derived from BoneXpert is used, it is possible to predict final height in an objective, precise way. This program takes into consideration sex, CA, height and BA of a child in order to predict their final height. One can also add the height of parents and height at menarche to obtain even more reliable outcome. It is also compulsory to classify the child into one of nine population groups, five within the Caucasian ethnicity, Asian Chinese, Asian American, Hispanic and African American. The result of these calculations is accompanied by an SD value and the true height values will be within the indicated range with 68% probability (93). This method's accuracy has been validated in a clinical study (91).

Artificial Intelligence and Machine Learning

New possibilities of automating BAA emerged with the use of artificial intelligence (AI) and machine learning, especially the specific type of machine learning known as deep learning. The most popular use a convolutional neural network (CNN), which has already found application in areas such as detection of patterns of interstitial lung disease on CT imaging (94) or segmenting the vascular network of the human eyes on fundus photographs (95). In recent years there has been tremendous progress in this field and there have been numerous publications reporting the automation of BAA using CNN (96-108).

In 2017 Radiological Society of North America (RSNA) conducted a challenge to assess BA from paediatric hand radiographs (RSNA Pediatric Bone Age Machine Learning Challenge 2017), as part of efforts to spur the creation of AI tools for radiology (109,110). The goal of the RSNA 2017 Machine Learning Challenge was to develop an algorithm which can most accurately determine BA using a validation set of paediatric hand radiographs. The results were evaluated by determining the mean difference and the mean absolute difference (MAD) between the performance of each system and the mean of all reviewers' estimates. The company 16 Bit were placed first in the competition with a MAD of 4.265 months and concordance correlation coefficient of 0.991 (111). The training data set available for competitors contained 12612 images from two American hospitals with a minimum age of 1 month, maximum age of 19 years and mean (SD) age of 10 years and 7 months (3 years 6 months) (111). Their Paediatric Bone Age Calculator is freely available on the website 16Bit.ai, although it is provided with the rider that the application is strictly for demonstration purposes and should not be used for clinical decision making (111). However, this tool has already been validated by a group of Canadian researchers, who compared its results to BAA using the GP atlas in a group of 213 male and 213 female patients and found that the differences between BA assessed by these two methods was not statistically significant (median difference was 0.33 years) and concluded that the tool created by 16 Bit is suitable for clinical use (112).

Another attempt to automate BAA using CNN was described in 2016 by Spampinato et al (113). They compared performance of several approaches, ranging from existing, off-the-shelf CNN, through existing pre-trained CNN (with general imagery) and fine-tuned programs to custom, trained from scratch only on BA radiographs (113). All of these CNNs were tested on the same, public data set, the *Digital Hand Atlas Database System*, provided in 2007 by Gertych et al (114). This atlas includes 1391 digitized, left-hand radiographs from evenly distributed, normally developed children of Caucasian, Asian, African-American and Hispanic origin, both male and female, with an age range from 1 to 18 years. Spampinato et al (113) conclude that the best performance was observed with BoNet, which was an original, new CNN trained from scratch specifically to assess hand radiographs (114).

Another study in this area deserving attention, as it is especially thorough and methods used have been precisely described, concerns a system called the Fully Automated Deep Learning System for BAA, which was created in 2017 by a group of researchers from Massachusetts General Hospital, Harvard Medical School. They used a pre-trained, fine-tuned CNN to create a new tool for BAA, using a large number of hand radiographs that included 4278 for females and 4047 for males but excluded children aged 0-4 years (115). This system calculates BA and provides a result as a number with representative picture and presents four more pictures of BA +1, +2, -1, -2 years. Thus the radiologist can verify the result and compare it with the closest ones. It achieved an accuracy of 57.32% and 61.4% for the female and male cohorts on held-out test images. Female test radiographs were assigned a BAA within 1 year 90.39% of the time and within 2 years 98.11% of the time. Male test radiographs were assigned 94.18% within 1 year and 99.00% within 2 years. It should be noted that this system does not reject malformed images (115). These authors also compared the BAA performance of a cohort of paediatric radiologists with and without the assistance of their tool for automatic BAA (116). They concluded that AI improves the radiologist's performance for BAA by increasing accuracy and decreasing variability and root mean squared error. The best results were achieved when radiological assessment was assisted by AI and this was better than using AI alone, a radiologist alone, or a pooled cohort of experts (116).

A comparison of chosen AI methods and BoneXpert is presented in Table 5. Due to the small number of radiographs in training and validating data sets, all the systems based on CNNs used data augmentation (increasing the number of radiographs by rotating the pictures, adding noise, etc.). In some studies authors tested more than one type of CNN. In these studies the CNN with the best performance is presented in the table.

Conclusion

For clinicians, especially paediatric endocrinologists, it is very important to assess BA as precisely as possible to be able to make the right diagnosis and monitor closely the development of a child, the progress of a disease or effects

Table 5. Comparison of artificial intelligence methods and BoneXpert

Name of tool/ author	BoneXpert (72)	Spampinato et al (113)	Bilbily and Cicero (111)	Lee et al (115)	Van Steenkiste et al (107)	Liu et al (102)
Year of creation/ last update	2008/2019	2016	2017	2017	2018	2019
Method	Conventional (non-deep) Machine Learning	CNN BoNet	CNN (pre-trained Inception V3)	CNN (pre-trained GoogLeNet)	CNN (pre-trained VGGNet)	CNN (pre-trained VGGNet)
Input	Radiograph, race, CA and gender	Radiograph, race and gender	Radiograph and gender	Radiograph and gender	Radiograph and gender	Radiograph and gender
Data set (no. of radiographs)	1 678*	1 391 (Digital Hand Atlas)	12 611 (RSNA Challenge)	8 325	12 611 (RSNA Challenge)	1 391 (Digital Hand Atlas)
Age range (years)	2.5-19 for boys 2-18 for girls	0-18	1-19	5-18	1-19	0-18
Reported accuracy (MAD in months)	4.5 (4th place in RSNA) challenge)	9.6	4.265 (1st place in RSNA) challenge)	11.16 (females) / 9.84 (males)**	6.8	8.28

*Validation on numerous groups of patients healthy and with various conditions and of various ethnic origin (Table 2).
**Result reported in RMSE (root mean square error) instead of MAD.
MAD: mean absolute difference, CNN: convolutional neural network, CA: chronological age, RSNA: Radiological Society of North America

of treatment. The traditional methods used to date have very significant drawbacks. These drawbacks include being highly time consuming, having a high inter- and intra-rater variability, making comparison of chronologically sequential examinations of one patient difficult and the need to possess a physical copy of the atlas. The new automated BAA techniques provide instant results, eliminate inter- and intra-rater variability and all only need access to the software. Much research in this field is currently underway and the results are very promising. Most of the programs described herein have been validated in clinical studies, in comparison to traditional BAA and they show very good precision while possessing the benefits of automated BAA systems. There are already some widely available options for clinical use, including BoneXpert and the Paediatric Bone Age Calculator from 16Bit.ai. It is to be expected that these automated tools will continue to gain acceptability and widespread usage, making the traditional atlas-based BAA a thing of the past.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Elżbieta Jurkiewicz, Mieczysław Szalecki, Elżbieta Moszczyńska, Monika Prokop-Piotrkowska, Design: Elżbieta Jurkiewicz, Mieczysław Szalecki, Elżbieta Moszczyńska, Monika Prokop-Piotrkowska, Literature Search: Monika Prokop-Piotrkowska, Kamila Marszałek-Dziuba, Writing: Monika Prokop-Piotrkowska, Kamila Marszałek-Dziuba.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2020.0184

J Clin Res Pediatr Endocrinol 2021;13(3):263-268

Pre-treatment Neutropenia in Children and Adolescents with Autoimmune Hyperthyroidism

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What is already known on this topic?

Untreated hyperthyroidism can cause neutropenia and the prevalence in adults is reported to range between 14-18%. To our knowledge, there is currently no data on this in children and adolescents.

What this study adds?

This study describes the prevalence of pre-treatment neutropenia in autoimmune hyperthyroidism in children and adolescents at presentation, which was found to be 16%. Pre-existing neutropenia in the study population resolved in the short term and did not worsen with thionamides, while no other thionamide-treated patient developed drug-associated neutropenia.

Abstract

Objective: Neutropenia can occur in untreated autoimmune hyperthyroidism (AIH) or in association with treatment with the anti-thyroid drug, methimazole (MMI). Starting MMI in children and adolescents with AIH and pre-existing neutropenia could thus be worrisome. The aim was to describe the prevalence of neutropenia in pediatric AIH, prior to antithyroid drug therapy and to assess the effect of antithyroid drugs on neutrophil count.

Methods: Patients with AIH attending a pediatric endocrinology clinic were retrospectively reviewed. Absolute neutrophil count (ANC) data at presentation and during anti-thyroid treatment for up to 24 weeks was collected. AIH was defined as elevated free thyroxine (fT4) or free tri-iodothyronine (fT3), suppressed thyroid stimulating hormone, and positive thyroid autoantibodies. Neutropenia was defined as ANC < 1500 cells/µL.

Results: Thirty-one patients (71% female) were included with a median interquartile range (IQR) age of 14.71 (11.89-17.10) years. Neither fT4 nor fT3 levels correlated with ANC at presentation ($r_s = 0.22$, $p = 0.24$ and $r_s = 0.13$, $p = 0.54$, respectively). 26/31 (84%) had normal baseline ANC. None developed neutropenia with thionamides. 5/31 (16%) had baseline neutropenia (median ANC 1,200/µL; IQR 874-1200). Four of these five started MMI at diagnosis while one was started on propranolol only but MMI was started one week later. All five normalized ANC within 24 weeks.

Conclusion: In this cohort, 16% of AIH patients had neutropenia at presentation, but this resolved in the short term and did not worsen with thionamides. Thionamides may be used with caution in these patients with close monitoring of blood counts.

Keywords: Hyperthyroidism, neutropenia, thionamides, methimazole, agranulocytosis

Introduction

Autoimmune hyperthyroidism (AIH) is the most common cause of hyperthyroidism in the pediatric population, and methimazole (MMI) is the anti-thyroid drug (ATD) of choice (1). Agranulocytosis is a known rare, but serious, adverse reaction to ATD, and has been reported to occur in 0.1-0.5% of patients with Graves' disease after initiation of therapy (2).

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Conflict of interest: None declared
Received: 29.07.2020
Accepted: 12.11.2020

However, untreated hyperthyroidism *per se* can cause hematologic abnormalities, including anemia, leukopenia, thrombocytopenia, or rarely pancytopenia. This has been reported several times in the literature, generally in the adult population. Most of these patients were treated with thionamides, with blood counts improving after achievement of euthyroidism (3,4,5,6,7,8,9,10). In particular, the presence of baseline neutropenia in a patient presenting with hyperthyroidism could be worrisome for the clinician, given the concern for potential agranulocytosis as a side effect of ATDs.

A prospective study by Aggarwal et al (6) found that among newly diagnosed adult patients with Graves' disease (n = 206), 14.1% had pre-treatment neutropenia. The study also found that neutrophil counts increased after treatment with antithyroid drugs and that this was related to a reduction in thyroid hormone levels. To our knowledge, there is currently no pediatric data on this subject.

Objectives

The objectives of this study were to describe the prevalence of neutropenia in children and adolescents with AIH prior to treatment with antithyroid drugs and to assess the effect of antithyroid drugs on the neutrophil count.

Methods

This was a retrospective study of patients diagnosed with AIH who were seen at the Pediatric Endocrinology clinic from January 1, 2005 to May 31, 2019. Inclusion criteria were those who have never received ATD, those who had been off ATD for more than three months due to non-compliance, those who succeeded initially in being tried off ATD for more than three months but went into relapse afterwards, and those who underwent radioactive iodine (RAI) therapy or surgical thyroidectomy but went into relapse at least three months after these procedures. Group A was the control group and consisted of those who were not found to have neutropenia at baseline and Group B consisted of those with neutropenia at baseline. Those for whom pretreatment data were unavailable were excluded.

Data up to 24 weeks after treatment was collected. To be diagnosed with AIH, patients must have had elevated free thyroxine (fT4) [normal range (NR): 0.7-1.5 ng/dL] or free tri-iodothyronine (fT3) (NR: 1.71-3.71 pg/mL), suppressed thyroid stimulating hormone (TSH) (NR: 0.4-4.5 mIU/L), and at least one positive thyroid autoantibody Neutropenia was defined as absolute neutrophil count (ANC) < 1,500 cells/µL (11). Data collected included patient age, sex, ethnicity, presenting signs and symptoms, levels of TSH, fT4, fT3, anti-thyroglobulin (anti-TG) antibody, anti-thyroperoxidase (anti-TPO) antibody, TSH receptor antibody (TRAb), thyroid-stimulating immunoglobulin (TSI), complete blood count including white blood cell with differential count, red blood cell count, platelet count, as well as levels of anti-neutrophil cytoplasmic antibodies (ANCA). Patients were divided into two group based on baseline ANC. Group A had normal baseline ANC while Group B had neutropenia.

The study protocol was reviewed and approved by the New York University (NYU) School of Medicine's Institutional Review Board as Exempt Category 4 (study # i19-00627). As this was a retrospective chart review which did not make use of identifiable health information and provided no more than minimal risk to the subjects, informed consent was not necessary, and a request for waiver of authorization to use identifiable health information for research was approved by the NYU Faculty of Medicine's Institutional Review Board in accordance with 45 CFR.164.512.

Statistical Analysis

This was primarily a qualitative study that looked at the prevalence of pre-treatment neutropenia in newly diagnosed patients with pediatric AIH. Statistical analysis was done to find whether fT4 or fT3 correlated with pre-treatment ANC. None of the variables had normally distributed data, thus a Spearman correlation test was performed using the SciPy library program (https://www.scipy.org/). Median values for age, fT4, fT3 and ANC were obtained using the same program.

Results

A total of 31 patients with AIH were included. Six patients were excluded due to absence of pre-treatment data. All patients were newly diagnosed and had never received ATD. Median age was 14.71 years (0.02-19.2) and 22 (71%) were females. Ethnicity was: 48% Hispanic (n = 15); 32% Asians (n = 10); 10% Caucasians (n = 3); 6% African Americans (n = 2); and 3% Middle Eastern (n = 1). All had positive thyroid antibodies: five (16%) with TRAb/TSI only, four (13%) with anti-TPO and/or anti-TG, nine (29%) with TRAb/TSI and either anti-TPO or anti-TG, and nine (29%) were positive for all three. Four (13%) only had TSI measured and were positive. Family history of thyroid disease was present in 15 out of 29 (51.7%) with no data available in two. Neither fT4 nor fT3 levels significantly correlated with ANC at presentation ($r_s = 0.22$, $p = 0.24$ and $r_s = 0.13$, $p = 0.54$, respectively) (Table 1, Figures 1 and 2).

In the cohort 26/31 patients (Group A, 84%) had normal baseline ANC (median 3,800/µL; 2,100-9,700) and 77% (n = 20) were females. In Group A median fT4 was 3.47 ng/dL (1.5-10.12) and fT3 12.2 pg/mL (4.6-29.8) (n = 23). None of these patients developed neutropenia after starting thionamides.

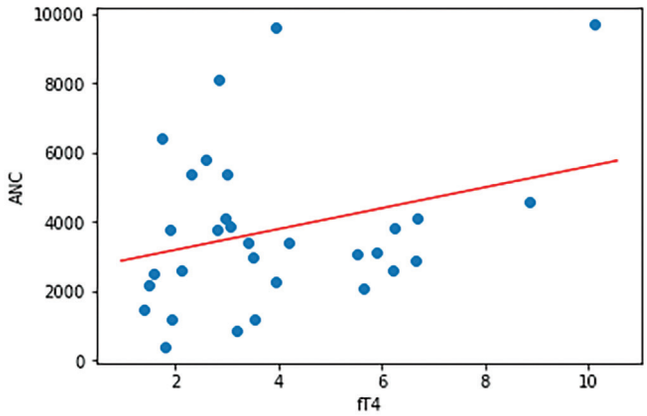

Figure 1. Lack of correlation between pre-treatment free thyroxine and absolute neutrophil count

ANC: absolute neutrophil count, fT4: free thyroxine

Five out of 31 patients (16%) had baseline neutropenia (median 1,200/µL; 400-1,458) and constituted Group B of whom 40% (n = 2) were females. Median fT4 was 1.92 ng/dL (1.4-3.55) and fT3 8.3 pg/mL (6.1-19) (n = 3). In 4/5 patients, MMI was started at diagnosis. The other patient started on propranolol only but MMI was introduced one week later. All

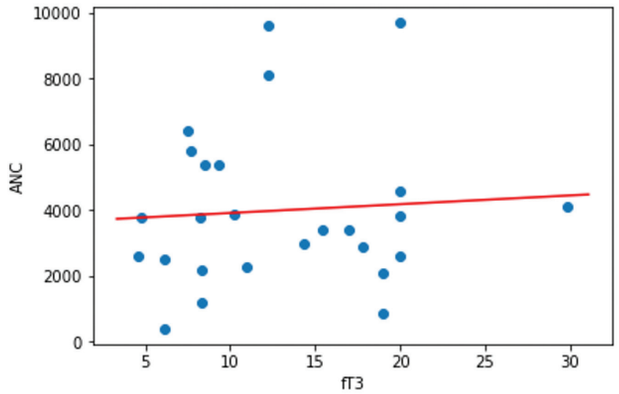

Figure 2. Lack of correlation between pre-treatment free tri-iodothyronine and absolute neutrophil count

ANC: absolute neutrophil count, fT3: free tri-iodothyronine

Table 1. Baseline characteristics of patients with autoimmune hyperthyroidism

	n	Total	Group A n = 26 (84%)	Group B n = 5 (16%)
Age in years	31	14.71 (0.02-19.2)	14.6 (0.02-19.2)	16.58 (4.8-18.75)
Sex	31			
Males		9 (29%)	6 (23%)	3 (60%)
Females		22 (71%)	20 (77%)	2 (40%)
Ethnicity	31			
Hispanic		15 (48%)	14 (54%)	1 (20%)
Asian		10 (32%)	7 (27%)	3 (60%)
Caucasian		3 (10%)	3 (12%)	0
African American		2 (6%)	1 (4%)	1 (20%)
Middle Eastern		1 (3%)	1 (4%)	0
Thyroid antibodies	31			
TRAb/TSI only		5 (16%)	4 (15%)	1 (20%)
anti-TPO or anti-TG only		4 (13%)	3 (12%)	1 (20%)
TRAb/TSI + anti-TPO or anti-TG		9 (29%)	8 (31%)	1 (20%)
All three		9 (29%)	8 (31%)	1 (20%)
Only TSI measured (positive)		4 (13%)	3 (12%)	1 (20%)
Family history of thyroid disease	29	15 (52%)	13/24 (54%)	2/5 (40%)
fT4 (N: 0.7-1.5 ng/dL)	31	3.20 (1.4-10.12)	3.47 (1.5-10.12)	1.92 (1.4-3.55)
fT3 (N: 1.71-3.71 pg/mL)	26	11.60 (4.6-29.8)	12.2 (4.6-29.8)*	8.3 (6.1-19)**
ANC (cells/µL)	31	3,400 (400-9,700)	3,800 (2,100-9,700)	1,200 (400-1,458)

Median (range) or n (%).
*Group A: 23/26 patients had fT3 levels.
**Group B: 3/5 patients had fT3 levels.
TSI: thyroid-stimulating immunoglobulin, fT4: free thyroxine, fT3: free tri-iodothyronine, ANC: absolute neutrophil count, TRAb: thyroid stimulating hormone receptor antibody, anti-TPO: anti-thyroperoxidase, anti-TG: anti-thyroglobulin

five cases normalized their ANC within 24 weeks and 4/5 cases had normal ANC by four weeks. However one of these four developed a transient drop in ANC at week nine which again normalized by 21 weeks; the reason for this transient decrease could not be elucidated, as it occurred despite normalizing fT4 and fT3, and was not associated with an increase in MMI dose or any other documented stressor. The remaining patient continued to have neutropenia after 12 weeks of therapy but had normalized by 24 weeks. In addition, 3/5 patients were checked for ANCA and all were negative (Figure 1 and Table 2).

Discussion

In this study, 16% of pediatric patients with AIH were found to have neutropenia prior to initiating treatment with ATDs, similar to studies in adults which have reported prevalences between 14-18% (6,12). Most patients in this study who had pre-treatment neutropenia (3/5 patients) had ANC >1,000 cells/μL, while one had moderate neutropenia (874 cells/μL) and one had severe neutropenia (400 cells/μL). In an adult study by Aggarwal et al (6), 14.1% of patients with Graves' disease (n=206) had pretreatment neutropenia, although the study used an ANC cutoff of <2000 cells/μL; mean ANC in these neutropenic patients was 1,600±300 cells/μL. Aggarwal et al (6) found that neutrophil counts in patients with pre-treatment neutropenia increased after initiation of ATDs and euthyroidism in these patients was achieved after a median time period of 55 days. The increase in ANC was found to be related to a reduction in thyroid hormone levels. In our study, neither fT4 nor fT3 levels were found to correlate with ANC at baseline. Although Aggarwal et al (6) used an ANC cutoff of <2,000 cells/μL, in pediatrics, neutropenia is conventionally defined as an ANC <1,500 cells/μL, so we opted to use this cutoff in our study (11). Gangadharan et al (13) reported a case of a 13 year old boy with Graves' disease and a pre-treatment ANC of <1,500 cells/μL. The child was treated with propranolol with Lugol's iodine and neutrophil counts improved after 16 days of treatment, upon which carbimazole was started.

Neutropenia has been reported to occur in less than 0.3% of adults with Graves' disease upon starting MMI, but the prevalence in children is unclear (14,15). In a study by Rabon et al (15), the pediatric patients who developed neutropenia after starting MMI (9 out of 251) had mild neutropenia, with none having an ANC <1,000 cells/μL. Rivkees et al (14) studied 100 consecutively treated pediatric patients with Graves' disease, of whom two developed moderate neutropenia (500 and 750 cells/μL). These studies looked at the overall rates of adverse events with MMI and did not go into particulars as to how the patients with neutropenia were managed, but generally, the patients who developed adverse effects with MMI stopped the medication and underwent definitive therapy, such as surgery or RAI. MMI-associated agranulocytosis is thought to be due to either direct toxicity when the drug is oxidized by neutrophils to reactive metabolites, or to immune-mediated mechanisms, which may include ANCA; these are generally believed to be idiosyncratic reactions (16,17,18). However, a large study by Takata et al (19) found that agranulocytosis was significantly more common in those who received a higher daily dose of MMI (0.8% in those who received 30 mg, n=2087; 0.3% in those who received 15 mg, n=2,739; p<0.001), suggesting that there could be a dose-dependent mechanism. In our study, none of the patients who had a normal ANC at baseline (Group A) developed neutropenia after treatment with thionamides. Patients who had pre-treatment neutropenia (Group B) were started on standard doses of MMI (Table 2), and none of them developed severe agranulocytosis with treatment.

Several studies have reported not only neutropenia but pancytopenia as a complication of poorly controlled Graves' disease (3,4,5,7,8,10,20,21,22,23,24) which responded to

Table 2. Characteristics, treatment regimen, and absolute neutrophil count response in those with pre-treatment neutropenia

	Age (years), sex	Ethnicity	Baseline ANC (cells/uL)	Baseline fT4 (ng/dL)	Baseline fT3 (pg/mL)	Treatment		ANC response				
						MMI (mg daily dose)	Propranolol (mg TID)	2-4 wks	4-8 wks	8-12 wks	12-20 wks	20-24 wks
Patient 1	18.6, M	A	1458	1.4	-	5*	-	-	1900	1800	1900	1500
Patient 2	16.6, M	AA	874	3.2	>19	20	20	1800	1500	1300	1700	-
Patient 3	4.8, F	A	400	1.79	6.1	2.5**	1.25**	4170	3850	5370	2270	-
Patient 4	18.7, M	H	1200	1.92	8.3	20	-	-	-	1400	-	1700
Patient 5	14.7, F	A	1200	3.55	-	30	-	2600	3800	2500	-	-

*MMI x 1 month then PTU 100 mg BID.
**Propranolol monotherapy x 1 week before adding MMI.
A: Asian, AA: African American, H: Hispanic, M: male, F: female, ANC: absolute neutrophil count, fT4: free thyroxine, fT3: free tri-iodothyronine, wks: weeks, MMI: methimazole

ATDs, with or without RAI. In one case report (10), although pancytopenia improved with ATDs, there was a recurrent increase in thyroid hormone levels and pancytopenia after four months, upon which subtotal thyroidectomy was performed. The patient's pancytopenia then resolved with good control of thyroid function.

The mechanism for pre-treatment hyperthyroidism-associated neutropenia has not been completely elucidated, but Kyritsi et al (25) found that out of 218 adult patients who presented to a hematology clinic with neutropenia, 43.6% had thyroid disease (including Graves' disease, Hashimoto's thyroiditis, patients who had undergone total thyroidectomy, nontoxic multinodular goiter, and antibody-negative subclinical hypothyroidism). Although patients who were undergoing treatment with ATDs were not excluded from the study, the authors did find that there was an inverse correlation between free T3 and the ANC ($r^2 = -0.274$, $p = 0.007$), suggesting a direct toxic effect of excess thyroid hormone to granulopoiesis. This is further supported by the fact that the subgroup of patients (n = 6) who had total thyroidectomy and subsequent iatrogenic medication-related hyperthyroidism had the lowest ANC, whereas those with non-toxic multinodular goiter (n = 18), who were euthyroid with no detectable antithyroid antibodies, had the highest ANC. In the same study, it was also found that CD4 + lymphocytes positively correlated with TSH levels ($r^2 = 0.16$, $p = 0.045$), but negatively with T4 levels ($r^2 = -0.274$, $p = 0.024$). The possible involvement of autoimmune anti-neutrophil antibodies causing hyperthyroidism-associated neutropenia has also been postulated (4,8). In our study, 3/5 patients who had pre-treatment neutropenia had ANCA checked and all three were negative.

ANCA has been associated with autoimmune neutropenia, albeit a direct causal relationship has not been established. ANCA has typically been associated with vasculitides, but neutrophil destruction by antineutrophil membrane antibodies may expose neutrophil proteinase 3 and neutrophil myeloperoxidase antigens to the circulation, thereby promoting ANCA formation. It has been suggested that a positive ANCA should prompt one to think of underlying toxin exposure or other autoimmune diseases (26). MMI-associated agranulocytosis is considered a form of transient acquired neutropenia (TAN) defined as neutropenia lasting < 3 months. The differential diagnoses for TAN include infection (especially viral infections), autoimmune neutropenia and drug-induced neutropenia (mainly anticonvulsants, sulfonamides, penicillins, antipsychotics and ATDs). Recombinant granulocyte colony stimulating factor is typically only used in these cases if the neutropenia is profound and associated with severe infection (11).

Thyrotoxicosis results in an increased response to catecholamines, particularly beta-adrenergic signaling, causing signs and symptoms such as palpitations, hypertension, and weight loss. The effects of thyroid hormone on alpha-adrenergic signaling is less clear (27). Increased beta-adrenergic activity as a result of thyrotoxicosis is less likely to be the cause of neutropenia since studies have shown that the effect of increased beta-adrenergic activity on bone marrow is actually to increase neutrophil production (28).

Study Limitations

Our study is limited by its retrospective nature, small sample size, and short duration of follow-up. Larger longitudinal studies would be of benefit to elucidate if and how the thyrotoxic state or autoimmune factors correlate with ANC in pediatric patients.

Conclusion

Around 16% of pediatric and adolescent patients with AIH may have pre-treatment neutropenia, but in the cohort described this resolved in the short term and did not worsen with thionamides. We suggest that MMI may be used with caution in these patients with close monitoring of blood counts.

Ethics

Ethics Committee Approval: The study were approved by the New York University Faculty of Medicine's Institutional Review Board as Exempt Category 4 (study # i19-00627).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept - Design- Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2020.0194

J Clin Res Pediatr Endocrinol 2021;13(3):269-275

Basal Serum Thyroxine Level should Guide Initial Thyroxine Replacement Dose in Neonates with Congenital Hypothyroidism

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What is already known on this topic?

High initial doses of sodium-levothyroxine (10-15 µg/kg/day) are recommended for rapid normalization of thyroid hormones and thyroid stimulating hormone, a marker of central nervous system hypothyroidism, for all infants with congenital hypothyroidism (CH).

What this study adds?

Our data suggest that standard high dose initial therapy in CH is not the only option. Adjusting the initial dose of thyroxine replacement to the basal serum free thyroxine level, with close follow-up in CH can be a valid strategy to provide target hormone levels while avoiding short-term iatrogenic hyperthyroxinemia.

Abstract

Objective: Initial high-dose sodium levothyroxine (Na-LT_4) (10-15 µg/kg/day) replacement for primary congenital hypothyroidism (CH) is recommended in guidelines. However, high-dose Na-LT_4 risks iatrogenic hyperthyroidism. The aim of this study was to investigate the normalizing effect of varying initial doses of Na-LT_4 on serum thyroid hormone levels.

Methods: Fifty-two patients were analyzed retrospectively. The patients were classified into mild (27/51.9%), moderate (11/21.1%) and severe (14/26.9%) CH, based on initial free thyroxine (fT_4) levels. Time taken to achieve target hormone levels was compared within groups.

Results: Initial mean Na-LT_4 doses for mild, moderate and severe disease were 6.9 ± 3.3, 9.4 ± 2.2 and 10.2 ± 2 µg/kg/day. Serum fT_4 levels reached the upper half of normal range (> 1.32 ng/dL) in a median of 16, 13 and 16 days in patients with mild, moderate and severe CH with the mean time from initial treatment to first control visit of 14.8 ± 6 days (range 1-36). There was no significant difference in terms of time to achieve target fT_4 hormone levels according to disease severity ($p = 0.478$). Seven (25.9%), eight (72.7%) and eight (57.1%) patients experienced hyperthyroxinemia (serum fT_4 > 1.94 ng/dL) in the mild, moderate, and severe CH groups at the first visit, respectively ($p = 0.016$).

Conclusion: Not all patients diagnosed with CH require high-dose Na-LT_4. Initial dose of Na-LT_4 may be selected on the basis of pre-treatment thyroid hormone levels. Some patients with moderate and severe CH, experienced iatrogenic hyperthyroxinemia even though the dose was close to the lower limit of the recommended range in guidelines. We suggest that lower initial doses may be appropriate with closer follow-up within the first week.

Keywords: Newborn screening, children, congenital hypothyroidism, Na-L thyroxine, dose

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Conflict of interest: None declared
Received: 15.08.2020
Accepted: 17.12.2020

Introduction

Congenital hypothyroidism (CH) is the most common endocrinological problem in newborns with an incidence of 1:2000-1:4000 and has a rising incidence, as reported in recent studies (1,2). Thyroid hormones are essential for brain development, especially prenatal neuronal differentiation, migration and proliferation, as well as postnatal myelinization. Thus a delay in diagnosis and treatment of permanent CH leads to irreversible brain damage and permanent neurodevelopmental defect (3,4).

Studies suggest that rapid normalization of thyroid hormones after birth, in infants with severe hypothyroidism may provide better cognitive outcomes (5). Therefore, guidelines for neonatal screening for CH recommend initial replacement doses of sodium levothyroxine (Na-LT_4) should be in the range 10-15 µg/kg/day to normalize serum thyroid hormone levels, and thus thyroid stimulating hormone (TSH) level, rapidly in order to achieve better outcomes (6). The guidelines also recommend the first follow-up visit should take place no more than 1-2 weeks after the initiation of Na-LT_4 treatment. However significant developmental improvement could not be shown for mild to moderate hypothyroidism using higher doses (7,8). Furthermore, recent data suggests a risk of iatrogenic hyperthyroxinemia may have a negative impact on behavior and attention in infants who are prescribed high initial doses (9,10,11). In light of this recent evidence, the latest European Society for Pediatric Endocrinology consensus guideline suggested, for the first time, that the initial dose of Na-LT_4 may be titrated with respect to initial hormone levels and disease severity (12).

In the current study, we aimed to investigate the effect of varying initial doses of Na-LT_4 on serum thyroid hormone levels in neonates and infants with primary CH.

Methods

Patients with primary neonatal CH diagnosed at Hacettepe University Medical Faculty, Division of Pediatric Endocrinology between January 2009 and January 2013 were included in the study. Patients, with a serum free thyroxine (fT_4) lower than 0.93 ng/dL (normal range: 0.93-1.71 ng/dL) and TSH higher than 10 mlU/L (normal range: 0.27-4.2 mIU/L) were considered to be primary CH. Patients with a gestational age of <37 weeks, history of severe underlying illness or central hypothyroidism, as well as those in whom treatment was delayed for three months were excluded from the study.

Patients' files were analyzed retrospectively to identify the etiology of CH and severity of hypothyroidism, as determined by initial fT_4 levels, as well as doses of Na-LT_4, and hormonal follow-up. Patients were grouped with respect to etiology, severity of CH, and initial dose of thyroxine (low vs high). Etiological classification of the study group relied on imaging as well as re-evaluation of thyroid hormones at three years of age after cessation of treatment.

Groups

(i) Patients were categorized into two groups: (a) patients with normal thyroid function after therapy withdrawal at three years of age were classified as transient CH; (b) patients with persistent hypothyroidism following cessation of therapy at three years of age were considered to have permanent CH. Both groups were compared for the time to achieve target hormone levels from onset of treatment.

(ii) Patients were categorized into three groups according to pretreatment plasma fT_4 concentrations: severe CH (fT_4 ≤0.31 ng/dL); moderate CH (0.31 < fT_4 ≤ 0.62 ng/dL) and mild CH (fT_4 > 0.62 < 0.93 ng/dL). Mild, moderate and severe CH groups were compared for the time to reach target hormone levels from onset of treatment.

(iii) Patients were divided into two groups with respect to initial dose of Na-LT_4. Those with an initial dose less than 10 µg/kg/day were defined as the low-dose group and those with an initial dose more than 10 µg/kg/day were defined as high-dose group. Both groups were compared for the time to achieve target hormone levels from onset of treatment.

Hormone Levels

Initial and follow-up thyroid hormone levels were extracted from medical records to analyze time to reach euthyroidism. Treatment targets were: (1) to achieve upper half of normal range for serum fT_4 (>1.32 ng/dL); and (2) serum TSH <10 mIU/L. In most patients, records included serum fT_4 and TSH levels every 1-2 weeks for the first two months of life, then every 1-3 months until 12 months of age, and thereafter every three months; and more often in those with problems with compliance during follow-up, though due to the retrospective nature of the study the time intervals between visits were not homogeneous. Blood samples were obtained in the morning before Na-LT_4 administration.

Patients who experienced a serum fT_4 level lower than 0.93 ng/dL at the first visit were considered to be hypothyroid.

Dose management of Na-LT_4 was made on an individual basis by the primary endocrinologist. However, the general approach in our department is to adjust the initial dose to pretreatment fT_4 levels.

This was a retrospective analysis of medical records. Serum fT_4 and TSH levels were measured by chemiluminescence method using IMMULITE 2000 System (Siemens, UK) during the studied period. Intra- and inter-assay variation coefficients for TSH were 5.3% and <6.4% and <7.1% and <7.8% for fT_4, respectively.

Iatrogenic Hyperthyroxinemia

Serum fT_4 levels higher than 1.94 ng/dL in the first visit among transient/permanent CH, mild/moderate/severe CH, and low-dose/ high-dose initial therapy groups were analyzed.

This study was approved by Hacettepe University Medical Faculty Non-Invasive Clinical Research Ethics Committee (GO 13/406-24).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 22.0 software (IBM Inc., Chicago, IL, USA). Numerical variables were summarized by mean ± standard deviation or median (minimum-maximum) as appropriate. Normality of the numerical variables was assessed with the Shapiro-Wilks test. As all numerical data sets had skewed distribution, nonparametric tests including Kruskal-Wallis and Mann-Whitney U test were used to compare independent groups (such as CH groups). Differences between groups in terms of categorical variables were examined by the chi-square test or Fisher's exact test. A p value less than 0.05 was considered statistically significant.

Results

Seventy-one patients with CH were extracted from hospital records within the time frame. Of these, seven patients were excluded since the age at diagnosis was later than 45 days and eight patients were excluded for non-compliance leading to treatment failure to reach target serum levels. Furthermore, four patients with subclinical hypothyroidism with unknown cause (etiology was unknown at diagnosis, and serum fT_4 were normal, however TSH levels were elevated after cessation of treatment at three years of age) were also excluded. Thus the final study group consisted of 52 children. Twenty-three (44.2%) were girls and 29 (55.5%) were boys. Mean age at diagnosis and treatment was 20.6 ± 9.9 days (7-43). Consanguinity was present in 28.8% of the parents.

Twenty-three (44.2%) patients had spot urinary iodine measurements, none of the results showed exposure to excessive iodine. Twenty-one (40.4%) mothers had spot urinary iodine measurements, which showed iodine sufficiency in four (19%) and deficiency in 17 (91%). Seven (41.2%) had mild (5-10 µg/dL), seven (41.2%) moderate (2-5 µg/dL) and three (17.6%) severe (<2 µg/dL) iodine deficiency. Babies of ten mothers with iodine deficiency (58.8%) were in the transient CH group, whereas the remaining seven (41.2%) were in the permanent CH group.

Mean serum levels of fT_4 and TSH at diagnosis were 0.75 ± 0.48 ng/dL and 70.6 ± 48.8 mIU/L, respectively. Mean initial Na-LT_4 dose was 8.4 ± 3.1 µg/kg/day. The mean time from initial treatment to first control visit was 14.8 ± 6 days (1-36).

The median time for fT_4 level to rise above 1.32 ng/dL was 16 (1-100) days, and the median time for TSH level to go below 10 mIU/L was 17 (1-88) days.

None of the patients experienced hypothyroidism at the first visit; 48 patients (92.3%) achieved target serum fT_4 levels and only four children (7.7%) had fT_4 levels in the lower half of the normal range (0.99-1.31 ng/dL). Three of these four had achieved target serum fT_4 levels by the second visit (12-31 days); one had initial low-dose Na-LT_4 (7.14 µg/kg/day) and the other two had initial high-dose Na-LT_4 (10.53 and 12.2 µg/kg/day). The remaining one achieved target serum fT_4 levels by the third visit (100th day), he had initial low-dose Na-LT_4 (7.35 µg/kg/day).

Disease Severity

Twenty-seven (51.9%) patients had mild, 11 (21.1%) moderate, and 14 (26.9%) severe CH. The mean initial Na-LT_4 dose given to mild, moderate and severe groups were 6.9 ± 3.3, 9.4 ± 2.2 and 10.2 ± 2 µg/kg/day, respectively. The initial treatment dose of the mild group was significantly lower than that of moderate and severe groups ($p < 0.001$). High-dose initial Na-LT_4 treatment was initiated in four (14.8%), six (56.5%) and eight (57.1%) patients in the mild, moderate and severe groups, respectively ($p = 0.006$).

The median time for patients to reach target serum levels of fT_4 (>1.32 ng/dL) was 16 (3-49), 13 (7-100) and 16 (1-36) days for mild, moderate and severe groups, respectively ($p = 0.478$). The median time for patients to achieve target serum TSH levels (<10 mIU/L) was 16 (5-31), 15 (8-27) and 30 (1-88) days in the mild, moderate and severe groups, respectively ($p = 0.003$).

Permanent versus Transient Congenital Hypothyroidism

Twenty-four (46.1%) patients had permanent CH, and 28 (53.9%) had transient CH. In the permanent CH group, 13 patients had dysgenesis and 11 had dyshormonogenesis. Of 13 patients with thyroid dysgenesis, three (23.1%) had

agenesis, eight (61.5%) had ectopy, with one case (7.7%) each of hypoplasia and hemiagenesis.

The mean initial Na-LT_4 dose was 9.8 ± 2.8 and 7.1 ± 2.9 µg/kg/day in patients with permanent and transient CH, respectively with the initial dose being significantly higher in patients with permanent CH (p = 0.003) (Table 1). Twelve patients (50%) in the permanent, and six patients (21.4%) in the transient CH group underwent high-dose initial Na-LT_4 therapy (p = 0.031).

Thirteen (54.2%), seven (29.2%) and four patients (16.7%) with severe, moderate and mild CH, respectively, were in the permanent CH group. In contrast in the transient group there was one (3.6%) child with severe CH, and four (14.3%) and 23 (82.1%) children with moderate and mild CH, respectively, based on pre-treatment fT_4 concentration. While the patients with severe CH were mainly in the permanent group, the patients with mild CH were principally in the transient group (p < 0.001).

The median time for serum levels of fT_4 to rise above 1.32 ng/dL was 15.5 (1-49) and 16 (3-100) days in patients with permanent and transient CH, respectively (p = 0.927). The median time for serum levels of TSH to decrease below 10 mIU/L was 18.5 (1-88) and 16 (5-31) days in patients with permanent and transient CH, respectively (p = 0.079). No statistically significant difference was found between the two groups.

Low versus High Initial Dose of Na-LT_4

The mean initial Na-LT_4 dose was 11.8 ± 1.4 µg/kg/day for the patients in the high dose group (n = 18, 34.6%) and 6.4 ± 2.1 µg/kg/day for the low dose group (n = 34, 65.4%) (Table 2).

The median time for fT_4 level to increase above 1.32 ng/dL was 12.5 (1-49) days for patients in the high-dose, and 16 (3-100) days for patients in the low-dose group (p = 0.081). The median time for TSH level to decrease below 10 mIU/L was 17 (1-83) days for patients in the high-dose, and 17 (5-88) days for patients in the low-dose group (p = 0.664). No statistically significant difference was found between the groups.

Overtreatment

Analysis of fT_4 levels revealed that 23 (44.2%) patients experienced serum levels of fT_4 > 1.94 ng/dL at the first visit. None of them showed any signs/symptoms of hyperthyroidism.

The effect of high initial doses on fT_4 at the first visit were compared. The mean time from initial treatment visit to first follow-up visit was 11.7 ± 5.1 and 16.4 ± 6 days in the high-dose, and low-dose treatment groups, respectively (p = 0.005). We compared high-dose, and low-dose initial treatment in terms of iatrogenic hyperthyroxinemia during the course of reaching treatment goals; 10 (55.5%) and 13 (38.2%) patients experienced serum levels of fT_4 > 1.94 ng/dL in the high-dose and low-dose treatment groups at the first visit, respectively (p = 0.36).

Mild, moderate and severe CH groups were also compared in terms of iatrogenic hyperthyroxinemia at the first visit. The mean time from initial treatment visit to first follow-up visit was 14.9 ± 7.7, 13.4 ± 5.8 and 15.3 ± 5.3 days in mild, moderate, and severe CH groups, respectively (p = 0.33). Seven (25.9%), eight (72.7%) and eight (57.1%) patients experienced serum levels of fT_4 > 1.94 ng/dL in mild, moderate, and severe CH groups at the first visit, respectively (p = 0.016)

Finally, permanent and transient CH groups were compared in terms of iatrogenic hyperthyroxinemia at the first visit. The mean time from initial treatment visit to first follow-up visit was 14.5 ± 6.6 and 15.1 ± 5.1 days in permanent and transient groups, respectively (p = 0.47). Fifteen (62.5%) and eight (28.6%) patients experienced serum levels of fT_4 > 1.94 ng/dL in the permanent and transient CH groups at the first visit, respectively (p = 0.03).

Table 1. Pretreatment serum fT_4 and thyroid stimulating hormone (TSH) levels, initial Na-LT_4 doses and days to achieve target serum fT_4 and TSH levels in permanent and transient congenital hypothyroidism groups

		Initial levels			Time to achieve target hormone levels (days)	
		fT_4*	TSH*	Dose*	fT_4**	TSH**
Etiology	N	(ng/dL)	(mIU/L)	(µg/kg/day)	> 1.32 ng/dL	< 10 mIU/L
Permanent	24	0.4 ± 0.29	95.1 ± 51.7	9.8 ± 2.8	15.5	18.5
Transient	28	1.05 ± 0.4	49.6 ± 35.1	7.1 ± 2.9	16	16
p		< 0.001	< 0.001	0.003	0.927	0.079

*Mean ± standard deviation, **median.
TSH: thyroid stimulating hormone, fT_4: free thyroxine, Na-LT_4: sodium levothyroxine

Table 2. Pretreatment serum fT_4 and thyroid stimulating hormone (TSH) levels, initial Na-LT_4 doses and days to achieve targeted serum fT_4 and TSH levels in high and low-dose groups

		Initial levels			Time to achieve target hormone levels (days)	
		fT_4*	TSH*	Dose*	fT_4**	TSH**
Dose	N	(ng/dL)	(mIU/L)	(µg/kg/day)	> 1.32 ng/dL	< 10 mIU/L
High	18	0.44 ± 0.27	102.1 ± 54.7	11.8 ± 1.4	12.5	17
Low	34	0.94 ± 0.49	53.9 ± 36.2	6.4 ± 2.1	16	17
p		0.001	< 0.001	< 0.001	0.081	0.664

*Mean ± standard deviation, **median.
TSH: thyroid stimulating hormone, fT_4: free thyroxine, Na-LT_4: sodium levothyroxine

Discussion

CH is one of the most common treatable causes of intellectual disability. Studies have shown that thyroid hormones have a crucial role in the appropriate formation of neuronal architecture as well as differentiation (13,14). High initial doses of Na-LT_4 (10-15 µg/kg/day) are recommended for rapid normalization of thyroid hormones and TSH for all infants, irrespective of severity and cause of CH. There are studies that have shown lower doses may also have similar success with less risk of overtreatment (6,9,15,16). Supraphysiological levels of fT_4 may result in premature craniosynostosis, behavioral problems, and attention impairment, and furthermore, may have a negative effect on IQ at adolescence, as one Dutch study has shown (15,16,17,18). Although the importance of early detection and effective treatment of CH is beyond dispute, there are some controversies in standard high dose Na-LT_4 (19,20).

Soliman et al (21) reported that around one quarter of 45 patients who received high dose Na-LT_4 (15 µg/kg/ day) as initial therapy experienced hyperthyroxinemia during follow-up. Craven and Frank (9) showed high initial Na-LT_4 (> 12.5 µg/kg/day) may lead to hyperthyroxinemia that required dose reduction in more than half of the patients during follow-up. They suggested a narrower range for dosing would avoid over-treatment. Furthermore, limited information is available about how a targeted dosing strategy compares to initial high dosing (10-15 µg/kg/day) to achieve target serum fT_4 and TSH levels. We aimed to provide some data on this issue, and we have evaluated patients diagnosed with primary CH and investigated the influence of different initial doses of Na-LT_4 on thyroid hormone levels as well as the time to achieve target levels. We also analyzed the time to achieve target hormone levels from onset of treatment in perspective of etiologies and disease severity. Furthermore, we analyzed patients who developed hyperthyroxinemia during initial hormone treatment, and its relation to initial doses, disease severity and whether CH was permanent or transient.

Mathai et al (22) has questioned single initial Na-LT_4 dose for all CH patients and reviewed variable initial dose strategy in permanent CH. In their study, Na-LT_4 treatment was given in 10, 12 and 15 µg/kg/day doses for dyshormonogenesis, ectopia and athyreosis, respectively. They showed these doses succeeded in normalizing serum fT_4 within 14 days. They also showed that lower doses (9.98 ± 3.19 µg/kg/day) of Na-LT_4 enabled target serum fT_4 levels in cases with permanent CH within a median of 16 days. Bakker et al (23) studied 30 CH neonates who were treated with initial daily T_4 dosages ranging from 4.8 to 11.1 µg/kg and found no correlation between the initial dose (whether high or low) and the time for normalization of plasma fT_4 levels. The mean initial Na-LT_4 dose was 8.4 ± 3.1 µg/kg/day in the current study, moreover both low (6.4 ± 2.1 µg/kg/day) and high (11.8 ± 1.4 µg/kg/day) dose groups achieved target serum fT_4 and TSH levels within similar time frames. Tuhan et al (24) studied the effect of three different initial Na-LT_4 doses (6-9.9 µg/kg/day, 10-11.9 µg/kg/day and 12-17 µg/kg/day) on serum TSH levels in the first month, and reported no statistical difference between groups.

In the current study, varying initial doses of Na-LT_4 (6.9 ± 3.3; 9.4 ± 2.2; 10.2 ± 2 µg/kg/day) in mild, moderate, and severe CH groups achieved target fT_4 serum concentrations within similar time-frames, suggesting titration of initial Na-LT_4 doses to initial hormone levels may be a valid strategy for effective treatment. We showed comparable efficacy of lower doses to achieve target serum fT_4 levels in the mildly and moderately affected subgroups, against severe hypothyroidism treated with high doses. However, it took longer for the severe CH group to achieve target TSH serum concentrations than the mild and moderate groups (30 vs 16,15 days respectively) in our study. Persistent high serum levels of TSH in early phase of treatment has been reported. Furthermore fT_4 concentration is considered more helpful in determining T_4 supplementation doses in this specific time span in some studies (23,25). Our results suggest impairment of hypothalamic-pituitary-thyroid axis negative feedback control may be more pronounced in the severe CH

subgroup, as has previously been suggested by Hanukoglu et al (26).

Our cohort has an increased percentage of transient CH (53.9%), similar to the recently published French study in which more than half of the study population had transient CH (27). The increased percentage of transient CH in these studies may be attributed to iodine deficiency in both countries (27,28). Spot urinary iodine measurements of the mothers in the current study also showed that iodine deficiency is still a problem in the maternal age group. A good number of patients in both our cohort and French study presented with mild hypothyroidism at the time of diagnosis and received lower initial doses of Na-LT_4 in comparison to guidelines. We consider this special subgroup of patients with mild, and usually transient, hypothyroidism may be related to iodine deficiency rather than primary congenital defects of the thyroid gland or hormonogenesis, and they may need lower initial Na-LT_4 replacement doses. Furthermore, they may have an increased risk of overtreatment with standard high dose therapy.

Hyperthyroidism is an issue in high-dose treatment. Tuhan et al (24) studied 71 children with CH, and showed 43.1% of patients were overtreated. Furthermore, five of them experienced clinical signs of hyperthyroidism but initial follow-up in this study was delayed. They suggested following-up thyroid functions earlier than 30 days. Mathai et al (22) monitored thyroid functions weekly and reported 28% of their patients had supraphysiological fT_4 levels within the first month of treatment. Their data suggests close monitoring during the early follow-up may decrease risk of overtreatment. The mean time from initial treatment to first control visit was 14.8 ± 6 days in our study, and 23 (44.2%) of the infants experienced iatrogenic hyperthyroxinemia due to overtreatment at the first visit.

Lower doses titrated to initial hormone levels were preferred in the current study, rather than the high-doses recommended in the guidelines. Yet some patients still experienced hyperthyroxinemia. Thus these results support closer follow-up, especially in patients with higher initial doses.

Study Limitations

The current study has some constraints, such as its retrospective nature and the small number of cases. In addition, neither the doses nor follow-up schedule were predetermined. Rather, they were decided by the primary endocrinologists, and time to target range of hormones depended on the follow-up schedule so that the exact timing is unknown. A limited number of babies and mothers had urinary spot urinary iodine measurements. Furthermore, anthropometric parameters and data regarding long-term neuropsychological outcome are not available.

Conclusion

Our data adds to the growing number of studies suggesting that standard high dose initial therapy needs to be reconsidered in CH. This may be especially true for areas where mild transient CH is endemic due to iodine deficiency or frequent follow-up in the first weeks of treatment is not practical. We suggest that basal serum fT_4 level may guide the initial dose of thyroxine replacement in neonates suspected of having CH, and, where possible, more frequent follow-up should be employed for dose adjustment in the first weeks, in order to prevent overtreatment. Long-term studies are necessary to determine the validity of such treatment, and whether it has any impact on the neurocognitive outcomes of children with CH.

Ethics

Ethics Committee Approval: This study was approved by Hacettepe University Medical Faculty Non-Invasive Clinical Research Ethics Committee (GO 13/406-24).

Informed Consent: Due to the retrospective nature of the study, patient consent was waived.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ceren Günbey, Alev Özön, E. Nazlı Gönç, Ayfer Alikaşifoğlu, Nurgün Kandemir, Concept: Ceren Günbey, Alev Özön, Data Collection or Processing: Ceren Günbey, Alev Özön, Analysis or Interpretation: Ceren Günbey, Alev Özön, E. Nazlı Gönç, Ayfer Alikaşifoğlu, Sevilay Karahan, Nurgün Kandemir, Literature Search: Ceren Günbey, Alev Özön, Writing: Ceren Günbey.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2020.2020.0213

J Clin Res Pediatr Endocrinol 2021;13(3):276-284

Evaluation of Children and Adolescents with Thyroid Nodules: A Single Center Experience

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What is already known on this topic?

Thyroid nodules in children have high potential for malignancy (22-26%). Sonographic findings, such as parenchymal heterogeneity, hypoechogenicity, irregular margins, increased intranodular blood flow, presence of microcalcifications and abnormal cervical lymph nodes increase the likelihood of malignancy. Even nodules < 1 cm diameter associated with risk factors require fine needle aspiration biopsy.

What this study adds?

The malignancy rate in this relatively small cohort of children and adolescents with thyroid nodules was 10%. Patients with atypia of undetermined significance/follicular lesion of undetermined significance on fine needle aspiration (FNA) had 9% potential for malignancy. In addition, patients with initially benign FNA result had later changes, giving a 5.3% false negative rate.

Abstract

Objective: We aimed to evaluate the clinical, radiological and pathological findings of children and adolescents with thyroid nodules.
Methods: Data of 121 children and adolescent with thyroid nodules and had fine needle aspiration (FNA) were examined retrospectively. Concomitant thyroid disease, ultrasonography (US) features of the nodule, FNA and histopathological results were recorded. FNA results were assessed according to The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC).
Results: Median (range) age of the cases was 14 (3-18) years and 81% were female. FNA results of patients were: insufficient in 1 (0.8%); benign in 68 (56.2%); indeterminate in 44 (36.4%); and malignant in 8 (6.6%) patients. Among 39 patients who underwent surgery, 10 (25.6%) had differentiated thyroid cancer (DTC) and the overall malignancy rate was 10.0% (10/100). Follow-up FNA results showed progress based on TBSRTC in 18.7% of benign results and 4/75 patients had DTC on surgical excision. Two of 22 patients with atypia of undetermined significance (AUS) who continued follow-up was diagnosed with DTC. Male gender, presence of Hashimoto thyroiditis and US findings of uninodularity, hypoechogenicity, increased blood flow, irregular margins, solid structure, microcalcification and presence of abnormal cervical lymph nodes were associated with malignancy.
Conclusion: In this study 10% of thyroid nodules were malignant in children and adolescents. Patients with AUS have a 9% potential for malignancy. Patients with initially benign FNA result may have changes on repeat FNA when assessed with TBSTRC indicating a 5.3% false negative rate.
Keywords: Adolescents, children, fine needle aspiration, thyroid nodule

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Conflict of interest: None declared
Received: 07.09.2020
Accepted: 20.12.2020

Introduction

Thyroid nodule is a lesion characterized by focal abnormal overgrowth of thyroid cells within thyroid tissue. They are usually detected and of clinical importance when noted by the patient, by a clinician during routine physical examination, or during radiologic procedures. Prevalence of thyroid nodules depends on many factors including age, sex, iodine sufficiency status and therapeutic and environmental radiation exposure. Autoimmune thyroiditis, which affects 2-15% of the population, is also associated with increased risk of nodule formation and thyroid malignancy (1). However, there is no identifiable risk factor present in the majority of patients with thyroid nodules (2).

Thyroid nodules are less common in children (1-1.5%) and adolescents (up to 13%) compared to adults (19-68%) (3,4). Although most thyroid nodules are benign, the thyroid gland is more susceptible to irradiation and carcinogenesis in children and the risk of malignancy in thyroid nodules is higher in childhood versus adulthood (22-26% versus 7-15%) (5,6,7). Therefore, thyroid nodules in children should be investigated carefully, regardless of whether the patient is symptomatic or asymptomatic.

Nodules that warrant fine needle aspiration (FNA) biopsy are identified based on characteristics determined by ultrasonography (US) and clinical context. The American Thyroid Association (ATA) recommends US-guided FNA for thyroid nodules over 1 cm or <1 cm with concerning ultrasonographic features that include hypoechogenicity, irregular margins, increased intranodular blood flow, microcalcifications and abnormal cervical lymph nodes although hyperfunctioning nodules should be excepted from FNA as they require surgery directly (8). Cyopathology findings are categorized by The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) as nondiagnostic, benign, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm/suspicious for follicular neoplasm (FN/SFN), suspicious for malignancy (SFM), and malignant (9). Further treatment plan is structured, based on TBRSTC category, as intermittent follow-up with US, repeat FNA or surgery.

The aim of this study was to evaluate the clinical, radiological and pathological findings of children and adolescents with thyroid nodules who were followed-up in our clinic.

Methods

Children and adolescents who were followed up with thyroid nodules and underwent FNA between January 2010 and June 2019 were included in the study and their files were examined retrospectively. Normal serum levels of laboratory tests were accepted as: thyroid stimulating hormone (TSH): 0.6-5.5 µIU/mL; free thyroxine (fT4): 0.8-1.9 ng/dL; free tri-iodothyronine (fT3): 2-6.5 pg/mL; thyroid peroxidase antibody (anti-TPO): 0-60 IU/mL; thyroglobulin antibody (anti-TG): 0-60 IU/mL; calcitonin: 2-11.5 pg/mL; and thyroglobulin: 0-60 ng/mL. Thyroid function tests (TFTs) at the time of diagnosis were grouped as euthyroid (normal TSH and fT4 levels), subclinical hypothyroidism (TSH: 5.6-9.9 µIU/mL and normal fT4 levels), hypothyroidism (TSH ≥10.0 µIU/mL with normal or subnormal fT4 levels), subclinical hyperthyroidism (TSH <0.6 with normal fT4 and fT3 levels) and hyperthyroidism (TSH <0.6 with fT4 >1.9 ng/dL or fT3 >6.5 pg/mL). The presence of congenital hypothyroidism, Hashimoto's thyroiditis (HT) or Graves' disease was also noted. Patients with sonographic changes in thyroid gland, such as decrease in parenchymal echogenicity, irregularity, heterogeneity or nodular appearance, in addition to being positive for anti-TPO anti-TG were considered to have HT (10). In patients with subclinical hyperthyroidism or hyperthyroidism, Graves thyroiditis was considered if TSH receptor antibody was positive (11).

US and FNA of all cases were performed by experienced radiologists. Thyroid gland parenchymal structure (homogeneous, heterogeneous); number of nodules (single, multiple); size (largest 3-dimensional measurement); structure (solid, semisolid, cystic); echogenicity (hyperechoic, isoechoic, hypoechoic); characteristics of the margin (regular, irregular, lobulated) and the presence of calcification were examined. FNA was performed with a 22-gauge needle on a 10-mL injector. Three or four samples were taken (from the largest nodule with the highest risk for malignancy, if there were multiple nodules) in each process. Patients were discharged after ensuring hemostasis by US.

FNA and post-thyroidectomy tissues were evaluated by experienced pathologists. FNA specimens were categorized according to TBSRTC (9). In all cases with nondiagnostic results, FNA was repeated. Benign results were followed every six months and FNA was repeated if there was any change in the nature and size of the nodule. The decision to perform surgery for further categories (AUS/FLUS, FN/SFN, SFM and malignant) was made by an expert multidisciplinary council, consisting of pediatric endocrinologists, a pediatric surgeon, a pediatric oncologist, a radiologist and a pathologist.

Statistical Analysis

Statistical Package for the Social Sciences, version 22 (IBM Inc., Chicago, IL, USA) program was used for statistical analysis. Results were expressed as mean ± standard

deviation for parametric data and median + range for nonparametric data. Chi-square test or Fisher Exact test was used for comparing non-numeric data according to minimum expected value. Independent samples median test was used for comparison of non-parametric numerical data medians. Significance level was accepted as $p < 0.05$.

Ethics

This study was approved by the Dr. Sami Ulus Children Training and Research Hospital Specialty and Training Review Board with the decision number 2019/12. The need to obtain informed consent from the study participants was waived due to the study's retrospective nature.

Results

One hundred and twenty-one cases with thyroid nodules were included in the study. Mean age of the patients was 13 ± 3 years (3.4-18 years) and 81 % of them were female (Figure 1). The thyroid US that detected the thyroid nodule(s) was performed because 78 (64.4 %) of the patients had enlarged thyroid gland by inspection or palpation. In addition patients with detected nodules were being followed up for HT (n = 23; 19 %), congenital hypothyroidism (n = 4; 3.3 %) and Graves' disease (n = 2; 1.7 %). Furthermore, 11 (9.1 %) patients had defects in TFTs, two (1.7 %) had a positive family history of thyroid malignancy and one patient (0.8 %) had a history of radiotherapy. Ninety-three (76.7 %) patients had normal TFTs at the time of diagnosis. In the remainder, TFT results were compatible with hypothyroidism in 12 (9.9 %), subclinical hypothyroidism in nine (7.4 %), hyperthyroidism in three (2.5 %) and subclinical hyperthyroidism in four (3.3 %). In the whole cohort the median TSH level was 1.8 µIU/mL but varied widely from completely surpressed to significantly elevated (0.06-100.0 µIU/mL). In 34 (28.1 %) of patients at least one of anti-TPO or anti-TG antibodies were positive. Serum calcitonin and thyroglobulin levels were increased in one (0.8 %) and 43 (35.5 %) patients, respectively.

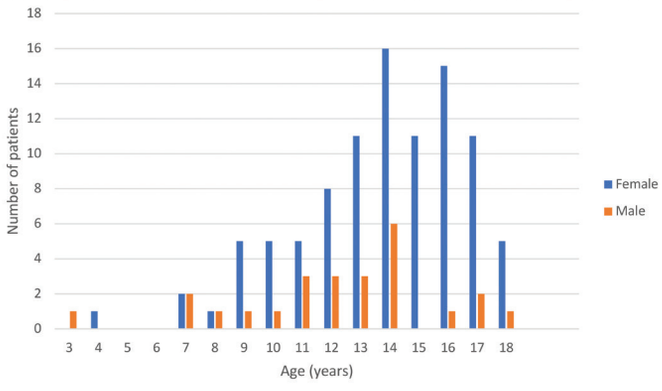

Figure 1. Age and sex distrubition of the patients

Median nodule size was 13 (5-55) mm. Less than half of patients (n = 54; 44.6 %) patients had a single nodule. Other US findings were as follows: nodule structure was solid in 63 (52.1 %), cystic in eight (6.6 %), solid/cystic in 47 (38.8 %) and there was a calcificated area without evident nodule formation in three (2.5 %) patients; the nodule was hypoechoic in 67 (55.4 %), isoechoic in 34 (28.1 %) and hyperechoic in 20 (16.5 %) patients; blood flow was increased in 30 (24.8 %) patients; nodule margins were irregular in 31 (25.6 %) patients; and microcalcification and parenchymal heterogeneity were present in 35 (28.9 %) and 68 (56.2 %) patients, respectively.

Fifty-three patients (43.8 %) had multiple FNAs and 192 FNA results were evaluated. Fifty-eight FNA results were non-diagnostic with an overall non-diagnostic rate of 30.2 %. Initial FNA results of patients (including the first biopsy results of the patients with multiple FNA) were as follows; nondiagnostic in 18 (14.9 %), benign in 62 (51.2 %), AUS/FLUS in 19 (15.7 %), FN/SFN in nine (7.4 %), SFM in seven (5.8 %) and malignant in six (5.0 %) patients. Final FNA results of patients (considering the most recent FNA results in patients with multiple FNA) were insufficient material in one (0.8 %), benign in 68 (56.2 %), AUS/FLUS in 22 (18.2 %), FN/SFN in 12 (9.9 %), SFM in 10 (8.3 %) and malignant in eight (6.6 %) patients.

The thyroid council decided that nodules of 55 patients required surgery and follow-up of 39 of them was continued in our center. Surgery undertaken consisted of nodulectomy (n = 2; nodulectomy only performed in the early period of the study), lobectomy (n = 10), subtotal thyroidectomy (n = 5) and total thyroidectomy (n = 22). Among 39 patients the cytological diagnosis of differentiated thyroid cancer (DTC) was made in one of 12 patients with AUS, 4 of 8 patients with FN/SFN, 2 of 4 patients with SFM and 3 of 3 patients with malignancy. Among the patients with DTC the types were papillary carcinoma (PTC) in seven and follicular carcinoma in three after histopathological examination and diagnosis. The total malignancy rate was 10 % (10/100) among the cases in whom thyroidectomies were performed in our center and whose histopathologic diagnosis were known (21 patients had surgical follow-up elsewhere). None of the ten patients with malignancy had a history of radiotherapy or history of thyroid malignancy in family. The median (range) largest thyroid nodule dimension in these cases with DTC was 12 (5-48) mm. Only two cases had a nodule size < 10 mm (both 5 mm) and FNA was planned due to the presence of microcalcification in these two patients. A 7 year old girl with congenital hypothyroidism

who had been on levothyroxine treatment since postnatal day 27 was found to have a 13 mm nodule in follow up and was diagnosed with PTC. Her average TSH level all through 7 year period was <2.5 ng/dL did not have gland enlargement in control US's till diagnosis. Individual clinical and ultrasonographic properties of patients with DTC is given in Table 1.

There were 35 patients with at least one non-diagnostic FNA result, either as an initial FNA result (n=18) or after repeat FNA's of initially benign or indeterminate results. Their subsequent FNA results were as follows: nondiagnostic in one (2.9%), benign in 22 (62.8%), AUS in seven (20%), SFN in four (11.4%) and malign in one (2.9%) patient.

When follow-up of 80 patients with at least one benign FNA result, either as an initial FNA result (n=62) or repeat FNA of an initially non-diagnostic FNA (n=14), AUB (n=2) or FN/SFN (n=2) results was evaluated, in 15 (18.7%) of the 80 patients' control biopsies there was evidence of progression, when evaluated by TBSRTC stage (AUS n=9, FN/SFN n=3, SFM n=2, malignant n=1), while 22 (27.5%) patients' control biopsies were benign and 43 (53.8%) patients' follow-up US did not require repeat FNA. Five patients with indeterminate (AUS, FN/SFN or SFM) repeat FNA results were lost to follow-up or refused surgery. Consequently, four patients with an initial benign FNA among 75 patients with at least one benign result (excluding five patients lost to follow-up) had DTC after surgical excision and histological examination giving a 5.3% false negative rate.

In the follow-up of 31 patients with AUS, FNA was repeated in 13 patients (benign n=4, AUS n=3, FN/SFN n=5, SFM n=1), nine patients had thyroidectomy and five patients were referred to adult endocrinology or were lost to follow-up. Two of the 15 patients operated in our hospital were diagnosed with PTC (Figure 2).

Risk factors for malignancy were evaluated among the 100 patients who were not referred on, did not quit follow-up or did not refuse surgery in whom DTC was present in 10 and the remainder (n=90) were benign (Table 2). TSH levels and nodule size were similar in both groups. Male gender, presence of HT, uninodularity, hypoechogenicity, increased blood flow, irregular margins, solid structure, microcalcification of the nodule and presence of abnormal cervical lymph nodes were found to be associated with malignancy. Parenchymal heterogeneity was found to be associated with benign nodules.

Discussion

In this study clinical, radiological and pathological findings of 121 children and adolescents with thyroid nodules were evaluated. The frequency of nodules increased and female dominance became evident with increasing age, especially after onset of puberty. Thyroid nodules are more common in women and their frequency increases with age. Female dominance can be explained by increased incidence of autoimmune thyroiditis, together with the influence of estrogen and progesterone on thyroid cells (12,13).

Dyshormonogenesis, HT, Graves disease, iodine deficiency, history of radiotherapy and some genetic disorders are known to increase nodule development (6,8). In this study, the vast majority of patients did not have any known thyroid disease or thyroid dysfunction, and only one patient had a history of radiotherapy. The fact that diagnostic US was requested according to the inspection and palpation

Table 1. Clinical and ultrasonographic findings of patients with differentiated thyroid cancer

Case	Age	G	TD	US findings of the nodule			FNA	Histopathological diagnosis
				L (mm)	Echogenicity	MC		
1	7	F	CH	13	Hypo	+	SFM	PTC, CS
2	8	M	-	21	Hypo	-	Malignant	PTC, CS
3	10	M	-	48	Iso	+	FN/SFN	FTC, invasive
4	13	M	HT	12	Hypo	+	FN/SFN	PTC, DSS
5	13	F	HT	12	Hypo	-	FN/SFN	FTC, WD
6	13	F	-	35	Hypo	+	Malignant	PTC, CS
7	14	F	HT	5	Iso	+	FN/SFN	PTC, FS
8	14	F	-	10	Hypo	-	AUS	PTC, FS
9	15	F	HT	5	Hyper	+	SFM	PTC, CS
10	16	M	HT	39	Iso	-	Malignant	FTC, MI

G: gender, F: female, M: male, TD: thyroid disease, HT: Hashimoto's thyroiditis, CH: congenital hypothyroidism, US: ultrasonography, L: length, MC: microcalcification, FNA: fine needle aspiration biopsy, FN/SFN: follicular neoplasm/suspicious for follicular neoplasm, SFM: suspicious for malignancy, AUS: atypia of undetermined significance, PTC: papillary thyroid carcinoma, FS: follicular subtype, DSS: diffuse sclerosing subtype, CS: classic subtype, FTC: follicular thyroid carcinoma, MI: minimal invasive, WD: well differentiated

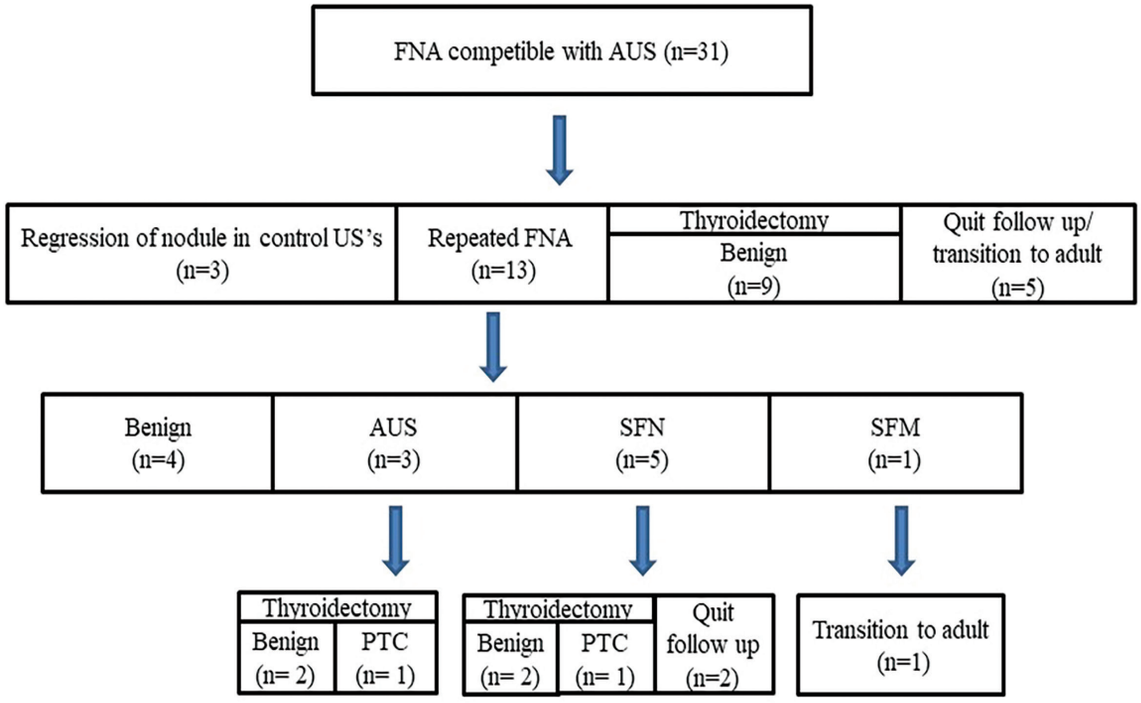

Figure 2. Follow up of patients with fine needle aspiration biopsies compatible with atypia of undetermined significance

FNA: fine needle aspiration biopsy, AUS: atypia of undetermined significance, US: ultrasonography, SFN: suspicious for follicular neoplasm, SFM: suspicious for malignancy, PTC: papillary thyroid carcinoma

Table 2. Risk factors for malignancy

	Total[1] (n = 100)		p
	Benign[2] (n = 90)	Malignant[3] (n = 10)	
TSH level (µIU/mL)	1.82 (0.2-100)	1.55 (0.7-5.3)	0.82
Gender (male/female)	18/72	4/6	< 0.001
Nodule size (mm)	12 (5-55)	12 (5-48)	0.74
Uninodularity n (%)	37 (41.1)	6 (60.0)	< 0.001
Solid structure n (%)	42 (46.6)	9 (90.0)	0.016
Hypoechogenicity n (%)	48 (53.3)	6 (60.0)	< 0.001
Increased blood flow n (%)	15 (16.6)	5 (50.0)	0.025
Irregular margin n (%)	19 (21.1)	3 (30.0)	< 0.001
Microcalcification n (%)	20 (22.2)	6 (60.0)	0.02
Parenchymal heterogeneity n (%)	49 (54.4)	4 (40.0)	< 0.001
Abnormal cervical lymph nodes n (%)	4 (4.4)	3 (70.0)	0.02
Hashimoto thyroiditis n (%)	23 (25.6)	5 (50.0)	< 0.001
Increased thyroglobulin n (%)	32 (35.9)4	4 (40.0)	> 0.99

[1]Patients who quit follow up or refuse surgery were excluded.
[2]Patients with cytopathologic or histopathologic benign results.
[3]Patients with histopathologic malignant results.
[4]Thyroglobulin result was missing in one patient.
TSH: thyroid stimulating hormone

findings in most of cases emphasizes the importance of holistic examination in pediatric practice.

A wide range of malignancy rate (6.6-56%) has been reported for childhood thyroid nodules (14,15,16). There may be overestimation in series of tertiary centres where patients with indeterminate and malignant FNA results are referred (16). Discordant results can also be explained by small sample sizes and the variation in inclusion criteria of the previous studies. Malignancy risk is underestimated in pediatric series which include cases up to the age of 21 and overestimated in series which limit inclusion to operated nodules (15,17,18). The overall incidence of thyroid carcinoma among operated children with nodules was given as 26.2% in a review summarizing 16 studies including 1164 patients since 1960 (6). When we evaluated the malignancy rate among the operated cases only, the malignancy rate in our study (10/36, 25.6%) was compatible with the literature.

FNA is a reliable method to assess the possibility of malignancy of a thyroid nodule and necessity for surgery. Its accuracy is 95% with 83% sensitivity, 92% specificity, 5% false negativity and 3% false positivity (19). Although FNA is a safe method and complications are very rare, it is an interventional process and patient selection should be made carefully. Which nodules should undergo FNA should be decided according to US findings. Adult guidelines, which primarily consider the size of the nodule as an indication for FNA and which do not recommend FNA for a <1 cm nodule unless the patient is considered high risk with a history of ionizing radiation exposure or pathologic regional lymph nodes, have been applied to children and adolescents for a long time (20). However, children and adolescent demonstrate differences in pathophysiology and clinical presentation and the 2015 ATA guideline for children with thyroid nodules and DTC recommends using US features and clinical context rather than size alone to identify nodules that require FNA (7). It is notable that extended indications for FNA, considering US features and clinical context primarily, had already been in use in most pediatric endocrinology clinics, including ours. Thus, two patients with nodule sizes of 5 mm were directed to FNA because of microcalcifications within the nodule and were diagnosed with PTC.

Categorizing FNA results according to TBSRTC in children has equal accuracy, sensitivity and specificity as in adults (5,21). Risk of malignancy in nondiagnostic samples in adults is very low, however it is not known if this holds true for children (22). Repeat of FNA at least three months after, is given as an option in the ATA guideline (8). In this study, FNA was repeated in all cases with nondiagnostic results and a malign result was found in one patient. However, this can not be given as malignancy rate of nondiagnostic results because nearly one third of the repeated FNA were compatible with AUS or SFN which have potential for malignancy.

Patients with benign FNA are followed-up with US after 6-12 months. Repeat FNA and/or lobectomy plus isthmusectomy is required if the nodule is growing or there are suspicious US findings (8). The probability of having a benign nodule on surgical excision is 90% in patients with one benign FNA and 98% in patients with at least two benign FNAs. Patients can be safely monitored without going to surgery with repetitive biopsies, unless clinical changes develop (23). There is a small but significant false negative rate with FNA (20). False negativity is increased in larger nodules and lobectomy is an option in patients with nodules over >4 cm (8,24). In this study, 17.5% of the 80 patients with initial benign FNA, had indeterminate cytology and one patient with a 12 mm nodule had malignant cytology on repeat FNA. The mean nodule size was 17 ± 8 mm in patients with indeterminate cytology. False negativity in FNA is not specific to very large nodules. FNA should be repeated if there is increase in nodule size or there are specific US features, such as microcalcification.

Repeat FNA in indeterminate results is recommended as an option in adult guidelines that have also been used for children (20). Risk of malignancy in indeterminate nodules is higher in children (28% in AUS/FLUS and 58% in FN/SFN) than in adults (5-15% in AUS/FLUS and 15-30% in FN/SFN) (25,26). Hence, the 2015 ATA Guideline for children recommends definitive surgery for indeterminate results (8). All follow up decisions for indeterminate nodules were made by the thyroid council directing FN/SFN and SFM results to surgery. AUS/FLUS results tended to be directed to surgery after 2015 and total the malignancy rate was 9% (2 among 22 patients who weren't lost to follow up or refused surgery). Recently, Cherella et al (27) reported that 28% of nodules with AUS on initial FNA were benign on repeat FNA, while this rate was 31% in this study. Based on this data, repeat FNA may still be considered for AUS/FLUS cytology, however small number of cases in these studies suggest further investigation is warranted (2).

TSH has a major role on the proliferation and functioning of thyroid cell and persistently elevated TSH levels increase the risk of DTC formation (28). Even patients with a nodule and TSH levels in upper tertiles of reference range may have increased risk for malignancy (29). Mussa et al (30) showed that TSH levels of children with DTC were higher than children with benign nodules after excluding the ones already on levothyroxine treatment or the ones with hypo/hyperthyroidism. However, its hard to documentate how

long the patients' TSH levels had been over or within the upper tertile of the normal range. So that, in this study, none of the 10 patients with DTC had subclinical or overt hypothyroidism (six of them were already on levothyroxine) and TSH levels were similar in both groups even after excluding the patients with abnormal TSH levels from the benign group.

Primary congenital hypothyroidism due to dyshormonogenesis may have increased risk of developing goiter, thyroid nodules and malignancy. European Society for Pediatric Endocrinology recommends periodical neck US every 2 to 3 years in patients with goitrous dyshormonogenesis. Although poor compliance to treatment leading to persistently high TSH levels and presence of goiter are thought to be the possible causes, malignancy can develop despite adequate levothyroxine treatment in patients with dyshormonogenesis (31). Our patient with congenital hypothyroidism who developed PTC neither had high TSH levels nor goiter. Drut and Moreno (32) also reported a case of PTC in a five-year-old girl with congenital nongoitrous dyshormonogenetic hypothyroidism. Even if there is no goiter in patients with congenital hypothyroidism, thyroid nodules should be checked periodically with US.

HT is the most common inflammatory thyroid disease and is characterized by a wide range of morphological changes in the gland. Co-existence of DTC and HT has been reported in many publications (33,34). However, it is not clear whether lymphocytic infiltration due to HT facilitates DTC formation or the immune response against the tumour initiates lymphocytic infiltration (28). Adult studies that have investigated the prevalence of HT among patients with DTC have reported a variable prevalence of 5-85% (35). Our findings were compatible with the study of Hacıhamdioğlu et al (36) which reported HT prevalence as 45% (all with PTC) among 20 children with DTC. Older ages at diagnosis and smaller tumour sizes were also reported by them, and our findings support this. In a study focusing on malignancy risk among children with HT, risk of malignancy among nodules that required FNA was 25% while this was 17.9% (5/28) in our study, both of which indicate a higher malignancy prevalence than in nodules in the absence of HT (37).

US findings, such as hypoechogenicity, increased blood flow, irregular margins, solid structure, microcalcification of the nodule and presence of abnormal cervical lymph nodes, which were identified as malignancy risk factors in our cohort, are in keeping with previous studies which have reported these characteristics to be more common in malignant nodules (38,39,40,41). Although hypoechogenicity and increased blood flow have high negative predictive value and high sensitivity, microcalcifications and presence of abnormal cervical lymph nodes have the highest specificity and positive predictive value and because of this, FNA is recommended for nodules with microcalcifications and abnormal lymph nodes, independent of nodule size (21). The two subcentimeter malignant nodules, both with microcalcifications and one with abnormal lymph nodes but without hypoechogenicity or increased blood flow in this study, supports the importance of this recommendation.

Study Limitations

The main limitations of this study were the small sample size and retrospective design of the study. Additionally, about 1/6 of the patients' progress was unknown due to them being lost to follow-up, refusing surgery, or having surgery elsewhere, mostly because they had reached an age (16-18 years) and had applied to adult clinics.

Conclusion

There is a considerable malignancy risk of 10% in childhood thyroid nodules. Nodules ≥1 cm or < 1 cm with additional high-risk US findings, such as microcalcification or abnormal lymph nodes, should be directed to FNA. However, due to the 5.3% false negative rate in FNA, patients with benign FNA result should continue to be followed regularly and FNA should be repeated if their findings progress. Although the malignancy rate was not different in AUS/FLUS cases compared to the general sample of this study, due to the low number of cases, routinely repeating FNA before committing to surgery cannot be recommended in patients with AUS.

Ethics

Ethics Committee Approval: This study was approved by the University of Health Sciences Turkey, Ankara Dr. Sami Ulus Children Training and Research Hospital Specialty and Training Review Board with the decision number 2019/12.

Informed Consent: The need to obtain informed consent from the study participants was waived due to the study's retrospective nature.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Selin Elmaoğulları, Şervan Özalkak, Semra Çetinkaya, İbrahim Karaman, Çiğdem Üner, Nilüfer Arda, Şenay Savaş-Erdeve, Zehra Aycan, Concept: Selin Elmaoğulları, Semra Çetinkaya, Şenay Savaş-Erdeve, Zehra Aycan, Design: Selin Elmaoğulları, Semra Çetinkaya, Şenay Savaş-Erdeve, Zehra Aycan, Data Collection or Processing: Selin Elmaoğulları, Şervan Özalkak, Şenay Savaş-Erdeve, Zehra Aycan, Analysis or Interpretation:

Selin Elmaoğulları, Şervan Özalkak, Semra Çetinkaya, Şenay Savaş Erdeve, Zehra Aycan, Literature Search: Selin Elmaoğulları, Şervan Özalkak, Writing: Selin Elmaoğulları, Şervan Özalkak.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0191

J Clin Res Pediatr Endocrinol 2021;13(3):285-292

An Evaluation of Glucagon Injection Anxiety and Its Association with the Fear of Hypoglycemia among the Parents of Children with Type 1 Diabetes

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What is already known on this topic?

Previous research has shown that parental anxiety and prior experiences of hypoglycemia can impair the management of diabetes and make parents more fearful about hypoglycemia. Research on parental attitudes and beliefs towards glucagon administration is lacking.

What this study adds?

The current study shows a positive association between parental fear of hypoglycemia and parental anxiety concerning glucagon administration. Practical training should be carried out to improve the self-confidence of caregivers.

Abstract

Objective: Hypoglycemia is a common acute complication of type 1 diabetes (T1D), which may cause seizure, loss of consciousness, and temporary motor or sensory impairment. Glucagon administration is an effective way of treating severe hypoglycemia, especially in a free-living setting. Nonetheless, families have difficulties in managing severe hypoglycemia due to their anxiety and challenges with current glucagon administration techniques. The aim of the current study was to explore the associations between parental fear of hypoglycemia (FoH) and their general anxiety level, and in particular, their attitudes towards and thoughts on glucagon administration.
Methods: Parents of children with T1D completed questionnaires assessing background and clinical information, FoH, generalized anxiety disorder (GAD) and parental anxiety for glucagon administration (PAGA).
Results: Sixty-eight parents participated. Positive correlations were found between parental GAD-7 score and both FoH and the number of night-time blood glucose measurements and there was a negative correlation with the child's age. Parents mean self-evaluation score of their competence in glucagon administration was 6 (standard deviation ± 2.9) on a scale of 0 to 10. Unsurprisingly, this score was negatively correlated with the PAGA scores. There was no significant difference between children using continuous glucose monitoring system and self-monitoring of blood glucose in terms of parental FoH, anxiety and misconceptions about glucagon administration.
Conclusion: The results showed that parents of children with T1D had anxiety and fear connected with hypoglycemia and glucagon administration. Structured and practical training should be implemented to increase parents' self-confidence including annual refresher training for home glucagon administration.
Keywords: Hypoglycemia, glucagon, anxiety, diabetes mellitus type 1

Introduction

Hypoglycemia is a common complication in type 1 diabetes mellitus (T1D) treatment and refers to conditions where blood glucose levels are ≤70 mg/dL (1). The symptoms of hypoglycemia include tremors, sweating, palpitations, increased feeling of hunger, anxiety, nausea, headache, sleepiness, excessive fatigue, and attention difficulties. Hypoglycemia may also have more serious consequences, such as coma, seizure, and temporary motor or sensory

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Conflict of interest: None declared
Received: 17.09.2020
Accepted: 02.01.2021

impairment (2). The symptoms of hypoglycemia, which are acute, occasionally dramatic, and may be accompanied by convulsions, cause fear of hypoglycemia (FoH) in families, especially at night (3). This fear is more evident in the families of young children (<6 years) who are unable to recognize the symptoms of hypoglycemia quickly enough to counter it, often causing these families to spend the night without sleeping (4). The feelings of families, and especially the mothers, regarding hypoglycemia vary according to local cultures (5). Although coma due to severe hypoglycemia is very rare and the prevalence of hypoglycemia-related death is unclear, many families fear that they might find "their children dead in bed", even if their children have only had severe hypoglycemic convulsions once (1). This false notion may change their priorities in diabetes treatment. Although diabetes technologies, especially continuous glucose monitoring (CGM) systems with alarms, have played an important role in reducing the fears of families in recent years, it is still known that FoH could be a significant barrier to achieving improvement in glycemic targets (4).

Moreover, with regard to the treatment of severe hypoglycemia, for many years, the only available form of glucagon has involved preparation by injecting a diluent into the powdered drug (6). Recently launched intranasal glucagon and premixed glucagon are inaccessible to most people with T1D outside of the USA. The most commonly used glucagon form is administered with the injector perpendicular to the front side of the arm or leg. Studies show that families have difficulty in managing the process of severe hypoglycemia, especially when it is accompanied by impaired consciousness (7). Our experience has suggested that families tend to go to the hospital as a matter of urgency rather than using glucagon itself due to their anxieties involving glucagon administration. Consequently, this anxiety related to glucagon administration, in addition to the FoH may have reduced the observed rates of parental/patient administered glucagon and consequently the value of glucagon. Given that it is assumed that caregivers can easily administer glucagon in the treatment of severe hypoglycaemia, parental concerns about this process may not have been taken into account sufficiently in standard diabetes education by healthcare professionals.

While previous research has shown that anxiety of the parent and prior experiences of hypoglycemia can impair the management of diabetes (7,8), research on attitudes towards glucagon administration is lacking. The current study aimed to explore the associations between parental FoH and their general anxiety level, in particular, their attitudes towards and thoughts about glucagon administration.

Methods

Study Sample

The parents of children aged between 2 and 18 years who had been diagnosed with T1D at least six months previously and followed by our pediatric endocrinology department were invited to participate in the study. At the beginning of the survey, the informed consent was obtained from the participants. There was no ethical committee application for the current study. Nonetheless, the Koç University Ethics Committee has concluded that the research had not violated the bioethical principles. The relevant letter from the committee is included in the supplementary file.

Measures

All forms were sent in a format suitable for online completion by the participants.

The Demographic form consisted of information about the child's gender, date of birth, date of diagnosis of diabetes, frequency of daytime and nighttime glucose measurement, diabetes technology usage, frequency of hypoglycemia, and information related to glucagon usage, such as injection sites and deciding accurate doses. The last HbA1c results of the children given in percentages were gathered from our hospital's laboratory data. The parents were also asked to rate their competence in glucagon administration on a scale of 0 to 10 points.

The Fear of Hypoglycemia Questionnaire-Parent Form was developed by Gonder-Frederick et al (8). The reliability and validity of the scale in Turkish was conducted by Şen Celasin et al (9). It measures parental FoH with a total of 25 items divided into 15 items for the anxiety subscale and 10 items for the behavior subscale. Increasing scores represent increasing severity of FoH.

The Generalized Anxiety Disorder-7 Scale (GAD-7) was developed according to the Diagnostic and Statistical Manual of Mental Disorders-IV-TR criteria (10) and its Turkish validity and reliability were confirmed (11). It is a short, self-reported 7-item scale evaluating GAD. Increasing scores represent the increasing severity of anxiety.

The Questionnaire Evaluating Parental Anxiety for Glucagon Administration (PAGA) is a 10-item questionnaire that aims to investigate parental misconceptions and perceptions of barriers to administration of glucagon. It is a 5-point Likert scale response eliciting how concerned they are about each statement. The questionnaire consists of statements including "the belief that the needle will hurt because of the length", "the belief that if I administer the glucagon, I will be too late to take him to the emergency room", "the

belief that my child will not get better, even if I administer the glucagon". In many years of experience, our pediatric endocrinology team observed common misconceptions and barriers reported by families about glucagon usage. Due to the lack of any existing questionnaire regarding this matter, the questions were developed based on the clinical observations of the team. The internal consistency evaluated with Cronbach's alpha was found to be 0.89. Increasing scores represent increasing misconceptions about glucagon administration. The questionnaire is shown in Appendix 1.

Statistical Analysis

Analyses were conducted using Statistical Package for the Social Sciences version 23.0. (IBM Corp., Armonk, N.Y., USA). Descriptive statistics (means, standard deviations, frequencies, and percent) were used to summarize demographic and clinical variables. T-tests were used to assess the associations between categorical variables and continuous variables. The group differences were analyzed using Mann-Whitney U test. Bivariate and partial correlations were used to determine the relation between continuous variables.

Results

A total of 153 parents of children aged between 2 and 18 years who had been diagnosed with T1D at least six months previously and followed by our pediatric endocrinology department were invited to participate in the study. Of these, 96 accepted, and 68 parents completed all of the surveys. The participation flow chart is shown in Figure 1. The mean age of the children was 9.5 ± 4.1 years and the mean diabetes duration was 2.9 ± 2.2 years. Of the participants' children, 14 (21%) were on insulin infusion pump (IIP) therapy and all the pumps were Medtronic® 640G. The rest of the participants were on multiple dose injection therapy (MDI). The descriptive information is shown in Table 1. The analysis of the survey data showed that the education levels of the participants were as follows: two (2.9%) parents were primary school graduates, two (2.9%) were secondary school graduates, 20 (29.4%) of the

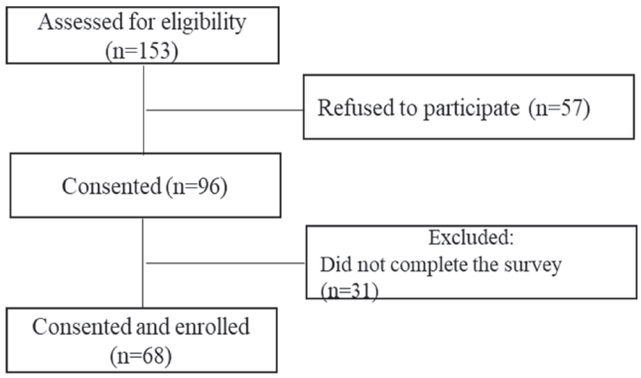

Figure 1. Study flow diagram

Table 1. Demographic and clinical characteristics of the children with type 1 diabetes whose parents participated (n = 68)

% or mean ± SD		Range
Sex (female %)	51.5	
Child's age (years)	9.5 ± 4.1	2.7-17.5
Diabetes duration (months)	2.9 ± 2.2	0.5-9.6
HbA1c (%)	7.6 ± 1.4	5.5-14
Insulin regimen (pump %)	21	
CGM usage (%)	57.4	
Number of the times blood glucose < 50 mg/dL in the past 3 months	5 ± 5.9	0-20
Glucagon administration or emergency room visit in the past 12 months	0.1 ± 0.5	0-3
Number of times experiencing unconsciousness/seizures ever	0.3 ± 0.6	0-4
Number of day-time glucose measurement		
< 3 times/day	12	
4-6 times/day	35	
≥7 times/day	53	
Number of night-time glucose measurement:		
< 1 time/week	28	
1 time/week	12	
2-4 times/week	23	
≥7 times/week	36	

SD: standard deviation, CGM: continuous glucose monitoring

parents were high school graduates, 36 (52.9%) parents had university degrees and eight (11.8%) had masters-degrees. Of the parents, 23 (34%) were unemployed. Ninety-three percent of the parents (n = 63) stated that they were trained in glucagon administration and 13 reported administering glucagon to their child in a severe hypoglycemia emergency. Glucagon was available in 65 of the houses and 29 of the schools of the children. On average, the parents evaluated their competence in glucagon administration as 6 ± 2.9 out of 10. Of the participants, 85% (n = 58) reported that they would administer glucagon immediately if there was a loss of consciousness.

The mean GAD-7 score of the participants was 6.6 ± 5.4 (0-21). According to the cut off points in the GAD-7 (11), 30 (44.1%) of the parents had no anxiety, 20 (29.4%) of them had mild anxiety, 11 (16.2%) of them had moderate levels of anxiety and 7 (10.3%) of them had severe anxiety.

A positive correlation was found between the FoH and the GAD-7 scores of the parents. This relationship was also significant when the education level of the parents, the time of diagnosis and the age of the child were controlled for ($r = 0.35$; $p < 0.005$). There was a positive correlation between the number of night time blood glucose measurements and the GAD-7 scores and a negative correlation with the age of the child. The perceived proficiency in glucagon administration of parents was negatively correlated with the PAGA scores. Table 2 shows the correlation between the diabetes related variables and the scores in the questionnaires.

In further analysis, the parents were grouped into two sub-groups: those whose children had experienced loss of consciousness or seizures; and those whose children had not. The two groups did not differ in terms of FoH, anxiety, misconceptions about glucagon administration, HbA1c levels, and day and night time measures (Table 3). There was no significant difference between the children using CGM and self-monitoring of blood glucose (SMBG) in terms of parental FoH, anxiety and misconceptions about glucagon administration (Table 4). Furthermore, there was no significant difference between MDI and IIP users in terms of GAD-7 ($p = 0.38$) and FoH scores ($p = 0.84$).

Discussion

Mostly, the parents of children with T1D have concerns about hypoglycemia and its unfavorable short-term and long-term effects. Severe hypoglycemia can be safely treated with glucagon administration by the caregivers in a free setting. Nonetheless, families have difficulties in preparing, drawing the correct dose and administering it during a severe hypoglycemia episode, which, in turn, raises anxiety (7,12). In the current study, parents evaluated their competence in glucagon administration as 6 out of 10 and 85% of them reported that they would administer glucagon immediately if there was a loss of consciousness.

Table 2. Correlation between diabetes-related variables and scores in the questionnaires

	Age in months	Diabetes duration	HbA1c	Day time glucose measurement	Nighttime glucose measurement	Perceived proficiency in glucagon administration	PAGA Score	FoH Score	GAD-7 Score
Age in months	1								
Diabetes duration	0.450**	1							
HbA1c	0.257*	0.432**	1						
Day time glucose measurement	-0.217	0.054	0.087	1					
Nighttime glucose measurement	-0.375**	-0.029	-0.182	0.306*	1				
Perceived proficiency in glucagon administration	0.016	0.08	0.031	-0.012	-0.016	1			
PAGA score	-0.186	-0.067	0.159	0.077	0.068	-0.541**	1		
FoH score	-0.254*	0.045	0.121	0.140	0.088	-0.042	0.211	1	
GAD-7 score	-0.243*	-0.134	0.099	0.169	0.241*	-0.052	0.246*	0.395**	1

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).
PAGA: parental anxiety for glucagon administration, FoH: fear of hypoglycemia, GAD-7: generalized anxiety disorder-7

Table 3. The comparison of parents of children who had loss of consciousness/seizures and those whose children have not lost consciousness or had seizures

	Loss of consciousness/ seizures (n = 16)	No loss of consciousness/ seizures (n = 52)	p value
PAGA score	12.9 (0-25)	13.4 (0-30)	0.644
FoH score	49.8 (27-81)	34.9 (21-91)	0.449
GAD-7 score	8.1 (0-21)	6.1 (0-19)	0.303
HbA1c (%)	7.7 (5.7-12.3)	7.6 (5.5-14)	0.569
Day-time glucose measurement (≥7 times/day) (%)	37.5	58	0.327
Night-time glucose measurement (≥7 times/week) (%)	44	35	0.816

Mean and range values are given.
PAGA: parental anxiety for glucagon administration, FoH: fear of hypoglycemia, GAD-7: generalized anxiety disorder-7

Table 4. Continuous glucose monitoring and self-monitoring of blood glucose groups

	CGM group (n = 39)	SMBG group (n = 29)	p value
PAGA score	12.4 (0-30)	14.5 (2-30)	0.911
FoH score	46.9 (20-85)	47.6 (21-91)	0.961
GAD-7 score	6.5 (0-20)	6.8 (0-21)	0.413
Child's age (years)	8.7 (2.7-17.2)	10.5 (2.8-17.5)	0.598
Diabetes duration (months)	32.8 (6-116)	38.4 (6-107)	0.799

PAGA: parental anxiety for glucagon administration, FoH: fear of hypoglycemia, GAD-7: generalized anxiety disorder-7, CGM: continuous glucose monitoring, SMBG: self-monitoring of blood glucose

These results indicate some parents have hesitations and regard themselves as incompetent when it comes to glucagon administration. Diabetes teams should emphasize the importance of glucagon administration in their training about managing severe hypoglycemia. Moreover, structured and practical education should be given to caregivers in order to lessen their anxiety and increase self-confidence. In our experience, as most families rarely need to use glucagon for severe hypoglycemia in any given year, it is likely they may forget the details of the administration in an acute emergency situation. Therefore, practical education about glucagon administration should be repeated annually (12).

Recently, nasal glucagon has been approved for treatment of severe hypoglycemia and has subsequently gained some popularity. It is ready to use, needle-free and involves a one-step administration compared to the other form. Pharmacodynamic studies support that intranasal glucagon had similar efficacy compared to intramuscular glucagon in the treatment of hypoglycemia in children and adolescents with T1D (13). Research reveals that caregivers and acquaintances administering intranasal glucagon have been able to administer it faster, more confidently and in accurate doses (14). Whereas intramuscular glucagon creates fear and anxiety (15), nasal glucagon seems an effective, user friendly and well tolerated method of treating hypoglycemia for caregivers in the home and school setting (16,17). In the current study, some of the PAGA questions included physical difficulties concerning intramuscular glucagon administration such as "the needle will hurt because of the size". The parents stated that they had difficulty with these issues. They also reported a lack of confidence in glucagon administration. These results show that practical and simple administrations are needed for the correct use of glucagon. Expanding intranasal glucagon use could ease and strengthen the administration process before it becomes more serious (18) and would address an important unmet medical need (16). In turn, it may help to reduce parental concerns and pave the way for more effective use of glucagon. Although research into the psychosocial impact of intranasal glucagon is rare, caregivers stated it was less stressful to use compared to intramuscular glucagon (14). Therefore, in order to use nasal glucagon in all countries, an international initiative should be advanced under the leadership of the International Society for Pediatric and Adolescent Diabetes. Moreover, health systems of countries should reimburse nasal glucagon as it constitutes an emergency medicine.

Concerns about hypoglycemia may lead children with T1D and their parents to inject lower doses of insulin, over-eat or feed, limit their daily exercises and generally follow frequent blood glucose monitoring (8,9). These interventions may cause undesirable elevations in blood glucose levels (19). Due to the fact that 75% of hypoglycemia in children is seen at night (20), some parents often wake up at night

and measure blood glucose. This may cause anxiety in the children and parents (21). With increasing levels of anxiety, a decrease in quality of life and metabolic control and diabetes-related burnout can be seen in both parents and children (8,20,22). It is also known that the prevalence of hypoglycemia and loss of consciousness or seizure as a result of hypoglycemia are associated with FoH among the parents (8,19,23,24). The current study supports the literature in these aspects. In the validity and reliability study of the Turkish adaptation of GAD-7 scale, patients who were diagnosed with GAD-7 scored an average of 12.03 [standard deviation (SD) ± 5.07] and the healthy control group scored 6.11 (SD ± 4.35) (11). The mean GAD-7 score of the participants (6.6 ± 5.4) suggested that the study group did not differ from the general population. It was found that the anxiety level of the parent was positively correlated with the frequency of glucose measurements during night time, FoH and misconceptions about glucagon administration. However, there was no association between HbA1c levels of the children and their parents' anxiety levels and FoH. Some research also reports that there was no direct association between FoH and HbA1c but an indirect association with parenting stress was reported (25).

The literature reveals both negative and positive psychological effects of CGM technologies in T1D treatment. Some research demonstrated that parental FoH and distress levels diminished with CGM use (26,27). On the other hand, there are studies indicating no alleviation in FoH in CGM use compared to the control groups (28,29). In the current study, we compared parental FoH and their anxiety levels when their children were CGM users compared to the parents of SMBG users. There was no significant difference between their anxiety level, FoH, and frequency of daily and nightly glucose measurements.

Study Limitations

There are certain limitations to the current study. First of all, the number of cases in the study was small. In further research, the number of participants could be increased and grouped according to CGM and, or only IIP and SMBG use. Another limitation was the lack of reliability and validity of the PAGA questionnaire used in the study. The PAGA questionnaire was generated by the study team because of the lack of a standardized measure regarding this issue. Further research may be needed to demonstrate that the PAGA questionnaire is a reliable measure to understand the misconceptions regarding glucagon administration of parents with T1D children. There is no doubt that when nasal glucagon is available in Turkey, it would be of value to include parental experiences regarding the use of nasal glucagon in a future study. Despite the limitations, we feel that this pilot study is valuable as it is the first study that has investigated the attitudes and misconceptions of the parents of children with T1D regarding intramuscular glucagon administration.

Conclusion

In conclusion, parents of children with T1D state their anxiety and fear associated with hypoglycemia and glucagon administration even without presence of prior experiences with severe hypoglycemia. Structured and practical training should be carried out to increase parents' self-confidence including annual refresher training for home glucagon administration. Moreover, the availability and widespread use of intranasal glucagon should be encouraged, as it will lead to a reduction in glucagon administration failure and parental anxiety, emergency department visits and hospital time for the parents.

Acknowledgement

The authors would like to thank all the participants and Alan J. Newson for the English language editing.

Ethics

Ethics Committee Approval: There was no ethical committee application for the current study. This work is a survey study.

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept – Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

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

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Appendix 1. Questionnaire evaluating parental anxiety for glucagon administration

Below are some concerns about glucagon use. Please mark how much you are concerned about each item.

	Not at all	Slightly	Moderately	Very	Extremely
1. The belief that my knowledge/experience is inadequate	0	1	2	3	4
2. The belief that because glucagon should only be injected into the muscle, I cannot accomplish it	0	1	2	3	4
3. The belief that the needle will hurt because of its length	0	1	2	3	4
4. The belief that blood sugar will increase too much	0	1	2	3	4
5. The belief that glucagon will harm my child as a medicine	0	1	2	3	4
6. The belief that my child won't get better, even if I administer the glucagon	0	1	2	3	4
7. The belief that I may panic and make a mistake that may harm my child	0	1	2	3	4
8. The fear of permanently damaging nerves/veins while administering glucagon	0	1	2	3	4
9. The concern that after administering glucagon side effects (nausea, vomiting, etc.) may occur	0	1	2	3	4
10. The belief that if I administer the glucagon, I will be too late to take him to the emergency room	0	1	2	3	4

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0303

J Clin Res Pediatr Endocrinol 2021;13(3):293-299

Midkine: Utility as a Predictor of Early Diabetic Nephropathy in Children with Type 1 Diabetes Mellitus

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What is already known on this topic?

Microalbuminuria is the gold standard for the detection and prediction of diabetic nephropathy (DN). However several studies have indicated that microalbuminuria lacks specificity for accurate prediction of DN.

What this study adds?

Serum midkine is a useful, novel, practical marker for the evaluation of renal involvement in children with type 1 diabetes mellitus, especially in normoalbuminuric children.

Abstract

Objective: This study aimed to assess the role of serum midkine (MK) as a biomarker for early detection of diabetic nephropathy in children with type 1 diabetes mellitus (T1DM) before microalbuminuria emerges.
Methods: A total of 120 children with T1DM, comprising 60 microalbuminuric patients (Group 1), 60 normoalbuminuric patients (Group 2), and 60 healthy participants as a control group (Group 3) were included. Detailed medical history, clinical examination, and laboratory assessment of high-sensitivity C-reactive protein (hs-CRP), hemoglobin A1c percentage (HbA1c%), lipid profile, urinary albumin to creatinine ratio (ACR), serum MK and estimated glomerular filtration rate based on serum creatinine were performed in all participants.
Results: Both Group 1 and Group 2 had significantly higher serum MK compared to controls ($p < 0.001$). Additionally, significantly higher MK concentrations were present in Group 1 compared with Group 2 ($p < 0.001$). Receiver operating characteristic curve analysis revealed that the MK concentration cutoff value of 1512 pg/mL was able to predict microalbuminuria with a sensitivity of 96% and specificity of 92%. Stepwise regression analysis revealed that HbA1c%, hs-CRP, and ACR were independently related to MK levels ($p < 0.001$ for each).
Conclusion: The results of this study suggest that serum MK is a useful, novel, practical marker for the evaluation of renal involvement in children with T1DM, especially in normoalbuminuric children.
Keywords: Midkine, type 1 diabetes mellitus, diabetic nephropathy, urinary albumin creatinine ratio

Introduction

Midkine (MK) is a multifunctional heparin-binding growth factor that was primarily identified as the retinoic acid–response gene product (1). It has pleiotropic activities including enhancement of cell proliferation, differentiation, survival, and migration and is also involved in angiogenesis and oncogenesis (2,3). Furthermore, functional evidence has suggested a possible role for MK in modifying inflammatory responses (4). MK is implicated in the pathogenesis of multiple disease processes, including cancer development, neuronal survival, tissue inflammation, and acute and chronic kidney disease (CKD) (5,6,7). In the kidney, MK is expressed in both proximal tubular cells and distal tubular epithelial cells (7) and to a lesser extent in endothelial cells (8). MK is induced by oxidative stress via the activation of

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Conflict of interest: None declared
Received: 14.12.2020
Accepted: 29.01.2021

hypoxia-inducible factor-1-alpha (7). The pathological roles of MK in renal disease are broad, ranging from progression of CKD (8), to hypertension (9), diabetes mellitus (DM) associated kidney damage (10) and drug toxicity (11).

Diabetic nephropathy (DN) is a grave complication that may occur in both type 1 and 2 DM and, unless arrested, leads to end-stage kidney disease (12). Pathologically, DN is a diffuse process affecting glomerular endothelial cells, tubular epithelial cells, and interstitium (13). The natural history of DN may be divided into several stages beginning with glomerular hyperfiltration and progressing to the silent phase (normoalbuminuria), incipient nephropathy (microalbuminuria), overt nephropathy (macroalbuminuria), and finally established renal failure (14,15). The occurrence of glomerular basement membrane and tubular basement membrane thickening on histopathology in type 1 diabetes mellitus (T1DM) kidney tissues suggests that tubular injury is not independent of glomerular injury, as both can occur after a same duration of disease (16). As with other kidney diseases, the outcome of diabetic kidney disease is better determined by tubulointerstitial changes than glomerular changes (17). Classically, microalbuminuria is the gold standard for the detection and prediction of DN (12). However, studies have shown that histopathologic changes associated with DN may occur in normoalbuminuric diabetic patients (18). Furthermore, microalbuminuria appears once significant renal damage has actually occurred (19). Moreover, there are several confounding factors with which microalbuminuria is associated, such as urinary tract infection, or following exercise or acute illness, thus indicating the non-specificity of the presence of microalbuminuria for accurate prediction of DN (20). Consequently, there is a urgent need for a more specific and sensitive biomarker for earlier diagnosis of DN during the "tubular stage" of renal damage, before microalbuminuria appears. To the best of our knowledge, there are no data available regarding the association between serum MK and DN in children with T1DM. The current study aimed to assess the diagnostic value of serum MK as a novel biomarker in the prediction of microalbuminuria, thus allowing for early recognition of DN in children with T1DM before the manifestation of microalbuminuria was evident.

Methods

Patients

This is a case-control study included sixty children and adolescents with T1DM having microalbuminuria, defined as urinary albumin excretion 30-299 mg/g creatinine, (the microalbuminuric group; Group 1) and sixty children and adolescents with T1DM who were normoalbuminuric, defined as urinary albumin excretion < 30 mg/g creatinine, (the normoalbuminuric group; Group 2) (21). Exclusion criteria were: the presence of any clinical or laboratory evidence of chronic infection, immunosuppression, liver diseases, heparin therapy, connective tissue disease, or other autoimmune disorders. Patients on antiplatelet drugs, lipid-lowering medication, or anti-hypertensive therapy including angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) were also excluded, as the protective role of both ACEIs and ARBs on glomerular and tubulointerstitial compartments have been proven in human studies (22). Patients were recruited over a period of two years, from April 2017 to March 2019, from the outpatient pediatric diabetes clinic of the Children's Hospital, Assiut University, Assiut, Egypt. This study also included sixty healthy children and adolescents who were recruited from the general population and matched for age, sex, pubertal stage, body mass index (BMI) standard deviation (SD) score (SDS), and socioeconomic status; these healthy children were designated Group 3. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Assiut Children's University Hospital, Assiut, Egypt (21/2017). Informed consent was obtained from each patient or control subject or their legal guardians before enrollment into the study.

Methodology

A detailed medical history was obtained from the studied patients were also subjected to a thorough clinical examination, with special emphasis on the age of onset of diabetes, disease duration, and insulin dose. Anthropometric measurements including weight, height, and waist circumference were obtained by a trained nurse according to standardized techniques. BMI was calculated as weight in kilograms divided by squared height in metres (kg/m^2). These BMI values were then converted to SDS using reference data for Egyptian children and adolescents (23). Puberty was assessed using the standardized method of Tanner stages (24). Systolic and diastolic blood pressures (SBP and DBP, respectively) were measured by standard technique. SDS for mean BP were calculated according to the report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents (25). Hypertension was defined as mean systolic or mean diastolic BP > 1,645 SDS as 1,645 SDS corresponds to the 95th percentile in a standard normal distribution).

Laboratory Investigations

Fasting lipid profile and high sensitivity C-reactive protein (hs-CRP) were measured using Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany). Serum total cholesterol, high-density lipoprotein (HDL), and triglyceride concentrations were measured by standard enzymatic methods using commercial reagents (Boehringer Mannheim GmbH, Germany). Calculation of low-density lipoprotein (LDL) concentration was performed using Friedewald's equation (26). Assessment of mean hemoglobin A1c percentage (HbA1c%) in the year preceding the study was performed using high-performance liquid chromatography on a Variant Analyzer (Bio-Rad Inc., Cairo, Egypt). Urinary albumin excretion (as an indicator of nephropathy) was measured in an early morning urine sample as an albumin-to-creatinine ratio (ACR) by an immuno-nephelometric method on a prime photometer (BCP BioSed, Rome, Italy). Microalbuminuria is present if urinary albumin excretion in at least two out of three consecutive urine samples, two months apart was 30-299 mg/g creatinine and patients were defined as normoalbuminuric if urinary albumin excretion was <30 mg/g creatinine (21). Potential factors affecting urinary albumin excretion, such as exercise, fever, and posture were excluded. Serum creatinine (Cr, mg/dL) was measured on a Dimension Xpand plus chemistry analyzer using commmercial methods (Siemens Technology, Illinois, USA). Serum creatinine-based estimated glomerular filtration rate (eGFR-Cr) was calculated using the updated Schwartz formula. eGFR-Cr = 0.413*height (cm)/serum Cr (mg/dL) (27). Determination of serum MK was performed using an enzyme-linked immunosorbent method (Human Midkine ELISA Kit, Boster Biological Technology Co., Pleasanton, CA, USA).

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS), version 19.0 (SPSS Inc., Chicago, IL, USA). Qualitative variables are presented as number and percent [n (%)] and compared by the chi-square test. Quantitative variables were tested for normality using the Kolmogorov-Smirnov test. Normally distributed quantitative variables were expressed as mean and SD (mean ± SD) and the one-way analysis of variance (ANOVA) test was used to compare the three studied groups with Bonferroni post-hoc test used to detect pair-wise comparison. Spearman correlation was used for non-parametric correlation between quantitative variables and Pearson correlation was used for parametric correlation. Multiple linear regression analysis was employed to assess the relationship between MK and clinical and laboratory variables. Logistic regression was used to examine the relationship between MK and ACR, after adjustment for other variables. Receiver operating characteristic (ROC) curve was used to determine the best cutoff value of MK in the prediction of microalbuminuria in children with T1DM. The area under the curve (AUC), specificity and sensitivity were computed based on the ROC. A p value <0.05 was considered significant in all analyses.

Results

The demographic data and laboratory finding of the patient groups and the control group are shown in Table 1. Children with T1DM, both Group 1 and Group 2, had significantly higher SBP SDS, DBP SDS, serum total cholesterol, LDL cholesterol, triglycerides, HbA1c%, hs-CRP, ACR, and MK compared with control subjects ($p<0.05$ for all). Children in Group 1 were older with longer disease duration. They had significantly higher blood pressure SDS, HbA1c, hs-CRP, urinary ACR, serum lipids (except HDL cholesterol), and insulin dose compared with Group 2 ($p<0.05$). ROC curve analysis revealed that the MK concentration cutoff value of 1512 pg/mL was able to distinguish microalbuminuria with a sensitivity of 96% and specificity of 92% [AUC, 0.94; confidence interval (CI), 0.87-1.00; $p<0.001$] (Figure 1).

MK was positively correlated with disease duration, SBP and DBP SDS, HbA1c, hs-CRP, and urinary ACR ($p<0.05$), while no correlation was found between MK and age, serum lipids, BMI SDS, or insulin dose ($p>0.05$) (Table 2). Stepwise regression analysis (Table 3) revealed that HbA1c, hs-CRP, and urinary ACR were independently related to MK levels ($p<0.001$). Moreover, logistic regression revealed that MK was a significant independent factor for DN, after adjustment of other variables, including age, gender, disease duration, BMI SDS, BP, HbA1c, ACR, and fasting lipids (Odds ratio: 2.05, 95% CI: 1.16-5.26; $p<0.001$).

Discussion

In this study, MK levels were found to be significantly higher in diabetic children with microalbuminuria compared to those with normoalbuminuria and controls so that in Group 1 the mean MK level was 1.6-fold higher than in Group 2 and 2.8-fold higher than in healthy children. Most importantly, the normoalbuminuric children had significantly higher levels of MK compared with controls (mean value 1.76-fold higher). This suggests that serum MK levels are related to subclinical tubular impairment and may be used as an earlier, measurable marker of renal involvement before the onset of microalbuminuria. Furthermore, MK correlated positively and significantly with urinary ACR ($p<0.001$), suggesting that MK may influence the severity of renal involvement and thus MK may be used as a marker to

Table 1. Clinical and laboratory variables of diabetic patients (normoalbuminuria and microalbuminuria), and healthy control groups

	Microalbuminuric (Group 1) (n = 60)	Normalbuminuric (Group 2) (n = 60)	Healthy controls (Group 3) (n = 60)	p value
Age	16.5 ± 2.7	13.8 ± 3.4	14.3 ± 3.9	0.011
Male	32 (53.3)	29 (48.3)	28 (46.6)	0.865
Diabetic duration (yrs)	9.3 ± 2.6$^{\#}$	6.2 ± 1.4	-	**< 0.001**
Insulin dose (IU/kg/day)	0.99 ± 0.26	0.89 ± 0.27	-	0.910
BMI SDS	0.68 ± 0.8	0.64 ± 0.4	0.63 ± 0.5	0.172
SBP SDS	1.77 ± 0.4$^{*\#}$	1.3 ± 0.2*	0.63 ± 0.2	**< 0.001**
DBP SDS	0.99 ± 0.3$^{*\#}$	0.4 ± 0.1*	0.32 ± 0.05	**< 0.001**
Triglycerides (mg/dL)	183.9 ± 35.8$^{*\#}$	126.7 ± 16.9*	107.1 ± 11.2	**< 0.001**
Total cholesterol (mg/dL)	198.7 ± 37.3$^{*\#}$	165.3 ± 25.1*	127.3 ± 15.3	**< 0.001**
LDL cholesterol (mg/dL)	136 ± 35.4$^{*\#}$	94.7 ± 22.5*	87.7 ± 12.2	**< 0.001**
HDL cholesterol (mg/dL)	41.2 ± 15.2$^{*\#}$	61.7 ± 15.9	69.8 ± 13.2	**< 0.001**
ACR (mg/g creatinine)	218.6 ± 35.6$^{*\#}$	22.2 ± 2.7*	10.3 ± 2.7	**< 0.001**
Hb1Ac (%)	9.2 ± 1.3$^{*\#}$	7.8 ± 0.4*	4.7 ± 0.3	**< 0.001**
hs-CRP (mg/L)	6.55 ± 1.3$^{*\#}$	2.81 ± 0.78*	0.39 ± 0.11	**< 0.001**
Serum creatinine (mg/dL)	0.71 ± 0.11	0.67 ± 0.12	0.65 ± 0.13	0.148
eGFR-Cr (mL/min/1.73 m^2)	100.71 ± 27.88	103.09 ± 33.22	113.93 ± 30.21	0.208
Serum Midkine (pg/mL)	1847.2 ± 266.4$^{*\#}$	1158.4 ± 157.6*	658.3 ± 79.3	**< 0.001**

Data are shown as mean ± standard deviation, or n (%).
*Indicates significance versus control subjects; $^{\#}$indicates significance between Group 1 and Group 2.
yrs: years, BMI: body mass index, SDS: standard deviation score, LDL: low-density lipoprotein, HDL: high-density lipoprotein, ACR: urinary albumin creatinine ratio, HbA1c: hemoglobin A1c, hs-CRP: high-sensitivity C-reactive protein, eGFR: estimated glomerular filtration rate, SBP: systonic blood pressure, DBP: diastonik blood pressure

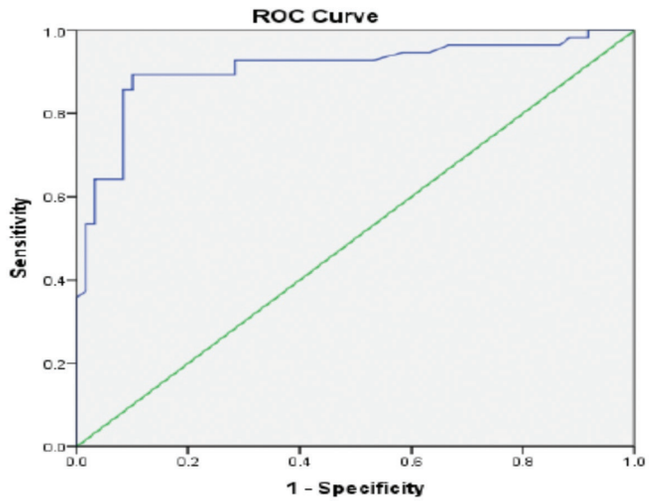

Figure 1. Receiver operating characteristic curve of fasting C-peptide to predict microalbuminuria with a sensitivity of 96% and specificity of 92% (area under the curve was 0.94)

stratify DN into different stages. In a previous study, Kosugi et al (10) reported that kidney biopsy tissue from eight adult patients with DN revealed that marked tubular atrophy, interstitial fibrosis and interstitial cell infiltration were evident in the specimens of DN, in which MK induction was detected in the glomeruli, tubules and interstitium. In addition, MK expression was detected in all the examined cases, despite the patients exhibiting different degrees of DN. These data were in agreement with the MK expression pattern in a mouse model induced by streptozotocin (10). Although glomerular dysfunction is thought to be a major factor in the development and progression of DN, tubulointerstitial damage may also play an important role in the pathogenesis of DN. MK is up-regulated in damaged tubular epithelial cells during the extremely early phase, both *in vivo* and *in vitro*, when only ischemia and hypoxia are evident (28). Experimental studies have shown that MK antisense oligodeoxynucleotides (anti-MK ODN) can improve ischemic reperfusion-induced renal damage, arterial restenosis and cisplatin-induced nephropathy (11). In line with our results, we suggest that MK inhibitors may be useful in treating DN, which may offer new avenues for the development of therapy for DN (29).

The International Society of Nephrology recommend annual screening for albuminuria and measurement of eGFR to detect and monitor DN in patients with DM (30). In the current study, no significant difference in eGFR-Cr among

Table 2. Correlations between the levels of midkine and demographic, clinical, and laboratory variables

Variable	r	p
Age (yrs)	0.298	0.071
Disease duration (yrs)	0.411	**0.006**
BMI SDS	– 0.086	0.514
SBP SDS	0.465	**0.002**
DBP SDS	0.311	**0.002**
HbA1c (%)	0.373	**0.003**
hs-CRP (mg/L)	0.324	**0.041**
Total cholesterol (mg/dL)	0.298	0.071
Triglycerides (mg/dL)	0.254	0.052
HDL (mg/dL)	– 0.145	0.320
LDL (mg/dL)	0.221	0.089
eGFR-Cr (mL/min/1.73 m^2)	0.105	0.415
ACR (mg/g creatinine)	0.754	**0.001**

yrs: years, BMI: body mass index, SDS: standard deviation score, BP: blood pressure, LDL: low-density lipoprotein, HDL: high-density lipoprotein, ACR: urinary albumin creatinine ratio, HbA1c: hemoglobin A1c, hs-CRP: high-sensitivity C-reactive protein, eGFR: estimated glomerular filtration rate

Table 3. Multiple regression analysis of the relationship of midkine levels to clinical and laboratory variables

Variable	Standardized coefficients	p
Age (yrs)	0.24	0.48
Disease duration (yrs)	0.38	0.09
SBP SDS	0.22	0.86
DBP SDS	0.54	0.08
hs-CRP (mg/L)	0.61	**< 0.001**
HbA1c (%)	0.66	**< 0.001**
ACR (mg/g creatinine)	0.49	**< 0.001**

yrs: years, BP: blood pressure, ACR: urinary albumin creatinine ratio, HbA1c: hemoglobin A1c, hs-CRP: high-sensitivity C-reactive protein

the studied groups was detected. Moreover, serum MK was not correlated with eGFR-Cr. These data indicate that serum MK is a better predictor of eGFR when compared to the classical eGFR-Cr method in children with T1DM, as eGFR-Cr was not able to detect the early renal effect in the present study.

In this study, HbA1c % was significantly higher in Group 1 compared with Group 2 and Group 3. Moreover, there was also a significant correlation between HbA1c and MK. Kosugi et al (10) reported that MK expression was up-regulated by high glucose in primary cultured tubular epithelial cells. They also identify MK as a key molecule in patients with DN and suggested that MK accelerates the intracellular signaling network evoked by hyperglycemia in DN. High glucose levels and diabetic substrates, such as glycation end products, affect mostly proximal renal tubular cells, leading to tubular cell hypertrophy and the interstitial deposition of chemokines and cytokines like MK, which in turn cause inflammation and fibrosis of the tubules (31). In line with these data, Brito et al (32) have shown that the proximal tubular basement membrane is already thickened in normoalbuminuric patients with diabetes.

In the current study, we observed that the circulating levels of hs-CRP were significantly higher in Group 1 the other two groups and also in Group 2 compared with Group 3. Furthermore, the hsCRP levels were positively correlated with MK levels ($r = 0.323$, $p = 0.01$). These findings support the hypothesis that MK, which is expressed in the proximal tubular epithelial cells of the kidney, plays a role in the pathophysiology of inflammation-related renal diseases (7). MK is an endogenous inflammatory marker and a key molecule in the development of DN. It enhances both neutrophil and macrophage migration into the tubulointerstitial regions, which is detrimental to kidney health (7). Studies involving human biopsy specimens and animal models have reported that macrophage infiltration is a characteristic of DN, confirming the concept that inflammation plays a crucial role in the pathogenesis of DN (33).

In the current study, we showed that SBP SDS and DBP SDS were significantly higher in Group 1 compared to Group 2 and Group 3. Besides, DBP SDS was significantly higher in Group 2 than in Group 3. Hobo et al (34) showed that SBP and mean BP were significantly higher in the Mdk + / + mice than in the Mdk – / – mice in the remnant kidney model as a model of advanced renal injury. They also reported that MK up-regulated pulmonary ACE in the 5/6 renal ablation model of CKD, leading to increase BP. These data suggest that MK plays an important role in the hypertensive response (29). MK was induced in the lung endothelium by oxidative stress and subsequently up-regulated by ACE, which hydrolyzes Ang II to induce more oxidative stress, thus accelerating MK generation. This leads to a vicious cycle of positive feedback in the MK-Ang II pathway (34). Kidney-lung interactions involving positive feedback between the renin-angiotensin system and MK might partly account for the pathogenesis of hypertension and kidney damage (35).

In our study, ROC curve analysis showed that an MK concentration cutoff value of 1512 pg/mL was able to predict microalbuminuria in children with T1DM with a sensitivity of 96 % and specificity of 92 % . To the best of our knowledge, this is the first study to evaluate MK levels in children with DN and define a cut-off value. Therefore, further prospective studies are needed to validate this threshold.

Study Limitations

The small sample size is probably related to strict inclusion criteria of the studied cases.

Single-center study limits generalizability.

Due to the cross-sectional nature of the work, it is difficult to conclude whether higher MK levels are directly involved in the pathogenesis of DN complications or this is simply an association

We were also unable to compare the examined parameters based on a histological diagnosis. It should be taken into consideration that the increased levels of MK might not only reflect renal tubular cell damage. There may also be an extra-renal source.

Microalbuminuria was determined in spot urine samples rather than with a 24-hour urine sample, which is considered the standard method in determining microalbuminuria.

Conclusion

The results of this study suggest that serum MK is a useful, novel, practical marker for the evaluation of renal involvement in children with T1DM, especially in normoalbuminuric diabetic children. However, further research with a larger sample size and a prospective design are requied to clarify the predictive and pathophysiological role and significance of MK in the early phase and also in the progression of DN.

Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Assiut Children University Hospital, Assiut, Egypt (approval number: 21/2017, date: 09.06.2021).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Kotb Abbass Metwalley, Hekma Saad Farghaly, Safwat Mohamed Abdel-Aziz, Asmaa Mohamed Ismail, Islam Fathy Elnakeeb, Concept: Kotb Abbass Metwalley, Hekma Saad Farghaly, Magda Farghali Gabri, Asmaa Mohamed Ismail, Duaa Mohamed Raafat, Islam Fathy Elnakeeb, Design: Kotb Abbass Metwalley, Hekma Saad Farghaly, Magda Farghali Gabri, Safwat Mohamed Abdel-Aziz, Islam Fathy Elnakeeb, Data Collection or Processing: Kotb Abbass Metwalley, Hekma Saad Farghaly, Asmaa Mohamed Ismail, Duaa Mohamed Raafat, Analysis or Interpretation: Kotb Abbass Metwalley, Hekma Saad Farghaly, Magda Farghali Gabri, Safwat Mohamed Abdel-Aziz, Asmaa Mohamed Ismail, Duaa Mohamed Raafat, Islam Fathy Elnakeeb, Literature Search: Kotb Abbass Metwalley, Hekma Saad Farghaly, Magda Farghali Gabri, Safwat Mohamed Abdel-Aziz, Duaa Mohamed Raafat, Asmaa Mohamed Ismail, Writing: Kotb Abbass Metwalley, Hekma Saad Farghaly.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0192

J Clin Res Pediatr Endocrinol 2021;13(3):300-307

Long-term Clinical Follow-up of Patients with Familial Hypomagnesemia with Secondary Hypocalcemia

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What is already known on this topic?

Hypomagnesemia with secondary hypocalcemia (HSH) is a rare autosomal recessive disease which is characterized by selective magnesium malabsorption related to a mutation on the transient receptor potential melastatin 6 (*TRPM6*) gene. Affected cases are usually diagnosed when seizures occur, due to severe hypocalcemia and hypomagnesemia, during infancy. Early diagnosis and treatment play a crucial role in the prevention of sudden deaths, which although rare, occur due to irreversible neurological deficits and arrhythmias.

What this study adds?

Long-term follow-up data and treatment responses in six cases of HSH is presented. Of the four mutations identified in the *TRPM6* gene, three were novel. Controversial topics in HSH are discussed, including short stature and testicular hypofunction. In addition, the genetic and clinical features of all Turkish patients previously reported are reviewed.

Abstract

Objective: Familial hypomagnesemia with secondary hypocalcemia (HSH) is an autosomal recessive disease caused by a mutation in the transient receptor potential melastatin 6 (*TRPM6*) gene and is characterized by selective magnesium malabsorption. Affected cases are usually diagnosed during infancy and usually present with seizures due to hypocalcemia and hypomagnesemia. Irreversible neurological deficits and arrhythmias can be observed without appropriate treatment. The aim was to evaluate the long-term follow-up of patients with genetically confirmed HSH.

Methods: A total of six patients with HSH, two of whom were siblings, were included. Age at diagnosis, clinical, laboratory and follow-up data on admission were recorded. All 39 exons of the *TRPM6* gene and flanking exon-intron junctions from genomic DNA were amplified and sequenced in all cases.

Results: The median (range) follow-up duration was 12.1 (7.6-21.7) years. All cases were diagnosed in infancy. Four different mutations, three of which had not been previously reported, were detected in the *TRPM6* gene. Treatment compliance was good and there were no severe complications in the long-term follow-up of cases. However, mental retardation, specific learning difficulty and attention deficit/hyperactive disorder were observed as comorbidities.

Conclusion: Of the four different *TRPM6* mutations in this small cohort, three had not been previously reported. The long-term prognosis of HSH appears to be good, given early diagnosis and good treatment compliance. This long-term follow-up and prognostic data and the three novel mutations will contribute to the published evidence concerning this rare condition, HSH, and it is hoped will prevent negative outcomes.

Keywords: Hypomagnesemia, hypocalsemia, *TRPM6* mutation

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Conflict of interest: None declared
Received: 14.08.2020
Accepted: 03.02.2021

Introduction

Familial hypomagnesemia with secondary hypocalcemia (HSH) is a rare, autosomal recessively inherited disease, due to transient receptor potential melastatin 6 (*TRPM6*) gene mutation. HSH is seen in the early infancy period and may be characterized by hypocalcemia secondary to hypomagnesemia and symptoms of neuromuscular excitability, such as generalized seizures, muscle cramps and agitation (1). TRPM6 functions as a cation channel with high permeability to magnesium (Mg) ions (Mg^{+2}) and the activity of the TRPM6 protein is regulated by intracellular Mg^{+2} levels (2). It is expressed in intestine and renal distal convoluted tubules. *TRPM6* mutations cause hypomagnesemia by intestinal and renal Mg wasting. Hypocalcemia in HSH is secondary to parathyroid hormone (PTH) resistance and decreased PTH release because of hypomagnesemia (3,4). Mg plays essential roles in normal cell physiology throughout the body. It is difficult to distinguish the clinical manifestations of HSH from other causes of hypocalcemia such as hypoparathyroidism. If hypomagnesemia cannot be detected and treated rapidly, fatal convulsions, irreversible neurodevelopmental deficits and life-threating arrhythmias may develop. No genotype-phenotype correlation between mutations in *TRPM6* and the severity of HSH have been identified.

In this study, clinical features and long-term follow-up data of six patients with HSH who had *TRPM6* mutation are presented.

Methods

Patients

The clinical files of six HSH patients from five different families and who were followed-up in our clinic since 1998 were evaluated retrospectively. Primary hypomagnesemia was diagnosed biochemically and the low serum Mg^{+2} levels present a requirement for high dose Mg^{+2} treatment. Exclusion criteria were secondary hypomagnesemia, such as being an infant of diabetic mother, intestinal malabsorption, and clinical conditions such as short bowel syndrome and exposure to various drugs, such as proton pump inhibitors, antibiotics, diuretics, and chemo-therapeutic agents. The levels of serum electrolytes, serum creatinine (Cr), alkaline phosphatase, PTH, 25 (OH) vitamin D and urinary Mg^{+2}, calcium (Ca), phosphate (P) and Cr were evaluated in all cases.

The ultra-filtrated fraction of serum Mg^{+2} was calculated as UFMg = 0.7xSMg. Renal Mg^{+2} handling was assessed by calculating fractional Mg^{+2} excretion (normal range 3 to 5 % for normomagnesemic individuals) with FeMg = (UMgxSCr)/(UFMgxUCr) x100 where Fe is fractional excretion, SMg is serum Mg^{+2}, UMg is urinary Mg^{+2}, SCr is serum Cr and UCr is urine creatinine. Hypercalciuria was defined as urine Ca/Cr ratio (UCa/UCr) higher than 0.21 mg/mg. Renal ultrasound was performed to rule out nephrocalcinosis. Clinical and laboratory findings of the patients undergoing Mg^{+2} treatment were evaluated regularly at outpatient clinic visits. Diarrhea is the main side effect of high oral Mg administration and was defined as three or more loose or watery bowel movements per day. Diagnoses of all cases were confirmed by genetic analysis. Neurodevelopmental status was assessed by Revised Wechsler Intelligence Scale for children, which was evaluated by the department of child and adolescent psychiatry.

The clinical and laboratory findings including serum Ca, serum Mg^{+}, alkaline phosphatase, PTH, 25 (OH) vitamin D and urinary Mg^{+2}, Ca, P, Cr, of the parents were also evaluated.

All participants and their parents received oral and written information concerning the study before providing signed consent. All procedures performed in this study were in accordance with ethical standards. This study was approved by the Sultangazi Haseki Training and Research Hospital Local Ethical Committee (no: 2020-58, date: 14.05.2020).

Mutational Analysis

Extraction of DNA from leukocytes was performed using standard protocols. *TRPM6* mutational screening was performed by Single-Strand Conformation Polymorphism analysis. For that purpose, an overlapping set of polymerase chain reaction primers, based on the sequence of the human *TRPM6* gene (genomic contig GenBank accession number AL354795), was used to amplify the complete coding sequence and the intron/exon boundaries from genomic DNA (primer sequences available upon request). Amplified products were separated on polyacrylamide gels by electrophoresis (Multiphor II; Pharmacia Biotech®, Sweden). Subsequently, exons with conformational variants were directly sequenced from both strands using an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, USA).

Results

Five male cases and one female case from five different families, diagnosed with primary hypomagnesemia, were included in the study. All patients presented with afebrile convulsions from between one and nine months of age at first admission. Consanguineous marriage was present in three families, one of which contained two affected siblings.

The clinical and laboratory findings of the subjects at the time of diagnosis and follow-up are presented in Table 1. The follow-up period of the subjects ranged from 7.5 to 21.6 years.

All cases were diagnosed with hypomagnesemia on admission, except one case (F4). This patient was admitted with a seizure at 3.5 months of age and the initial diagnosis was hypoparathyroidism because serum Mg was not assesed. Anticonvulsant treatment (phenytoin, phenobarbital) was started due to persistence of seizures despite calcitriol treatment. Hypomagnesemia was eventually diagnosed at seven months of age with the

Table 1. Laboratory, genetic and follow-up features of cases with familial hypomagnesemia with secondary hypocalcemia

	F1.1	F1.2	F2	F3	F4	F5
Age on admission (months)	9	1	1.5	3	3.5	5
Age on diagnosis (months)	9	1	1.5	3	7	5
Gender	Male	Male	Male	Female	Male	Male
Symptoms on admission	Seizures	Seizures	Seizures	Seizures	Seizures	Seizures
Serum Ca on admission (2.1-2.55 mmol/L)	1.68	1.82	1.92	1.62	1.42	1.55
PTH on admission (pg/mL) (n = 9-67)	4.9	3.8	5.5	6.5	9.1	8.7
Serum Mg on admission (0.66-1.07 mmol/L)	0.23	0.16	0.21	0.18	0.13	0.17
Age at last control (years)	22	15.3	8.4	7.8	9.4	22
Height SDS at last control	-0.93	-0.92	0.8	2.32	-0.22	-0.43
Follow-up period (years)	21.2	15.2	8.3	7.6	8.9	21.7
Magnesium doses at last control (mg/kg/day)	12.5	13.6	13.5	31.5	24	13
Neurodevelopmental status	Mild-moderate MR	SLD	Normal	Normal	Normal	ADHD
Serum Mg at final control (0.66-1.07 mmol/L)	0.62	0.59	0.51	0.71	0.72	0.59
Serum Ca at final control (2.1-2.55 mmol/L)	2.25	2.52	2.37	2.52	2.42	2.52
FeMg (%) (n = 3-5%)	2.1	1.9	1.2	5.1	5.9	1.8
Urinary Ca/Cr	0.1	0.06	0.19	0.04	0.06	0.036
Nefrocalsinosis	No	No	No	No	No	No
Parental consanguinity	Yes	Yes	Yes	Yes	No	No
Affected gene	TRPM6	TRPM6	TRPM6	TRPM6	TRPM6	TRPM6
Mutation	c.3158A > G (p.Tyr1053Cy)	c.3158A > G (p.Tyr1053Cys)	*c.841(+1) G > A	*c.1751A > G (p.His584Arg)	*c.841(+1) G > A	*c.3514C > T (p.Arg1172*)
Localization	Exon 23	Exon 23	IVS7 donorsplice site	Exon 16	IVS7 donorsplice site	Exon 25
Mutation/mother	c.3158A > G (p.Tyr1053Cy) (het)	c.3158A > G (p.Tyr1053Cy) (het)	*c.841(+1) G > A (het)	*c.1751A > G (p.His584Arg) (het)	*c.841(+1) G > A (het)	*c.3514C > T (p.Arg1172*) (het)
Mutation/father	c.3158A > G (p.Tyr1053Cy) (het)	c.3158A > G (p.Tyr1053Cy) (het)	*c.841(+1) G > A (het)	*c.1751A > G (p.His584Arg) (het)	*c.841(+1) G > A (het)	*c.3514C > T (p.Arg1172*) (het)

*No association with the disease has been reported before.
Ca: calcium, Mg: magnesium, PTH: parathormone, SDS: standard deviation score, Cr: creatinine, FeMg: fractional magnesium excretions, MR: mental retardation, SLD: specific learning difficulty, ADHD: attention deficit/hyperactive disorder

observation of hypomagnesemia and recurrent resistant seizures despite calcitriol and anticonvulsant therapy.

On admission the range of serum Mg^{+2} levels was 0.13-0.23 mmol/L, serum Ca levels were 1.42-1.92 mmol/L, and PTH levels were 3.8-9.1 pg/mL. Following Mg^{+2} treatment, serum Ca and PTH levels returned to normal levels during follow-up of all patients.

Oral Mg^{+2} treatment doses were adjusted to sustain normocalcemia, according to the tolerance of the cases. Dose was not increased in patients with diarrhea. Treatment doses ranged between 12.5 and 31.5 mg/kg/day. In two cases (F3, F4), serum Mg^{+2} concentrations were maintained at the reference intervals with oral treatment and were subnormal in four cases (0.51-0.59 mmol/l). However, no symptoms were observed. Fractional Mg^{+2} excretion was between 1.2% and 5.9%. Hypercalciuria and nephrocalcinosis were not detected in any case.

None of the patients had short stature at the last follow-up. Height standard deviation (SD) scores ranged from -0.93 to 2.36 SD. In one case, who was diagnosed at nine months of age (F1.1), mild-to-moderate mental retardation was observed while his brother had a specific learning difficulty (F1.2). In another case (F5) attention deficit and attention deficit/hyperactivity disorder (ADHD) was diagnosed. Neurodevelopmental status of all patients, except F1.1 and F1.2, were consistent with their ages.

Mutation Analysis

Four different mutations were detected in the *TRPM6* gene in six cases out of five families (Table 1). A homozygous missense mutation, which has been identified previously in a Turkish family on Exon 23 was detected in F1.1 and F1.2. [c.3158A > G, (p.Tyr1053Cys)]. Both of the parents were heterozygous carriers of this mutation. Two patients from non-consanguineous families (F2 and F4) had the same splice-site mutation, which had not been previously described (IVS7 splice-site, c.841 (+ 1)G > A). This mutation affected an essential splice site. The parents of these patients were heterozygous for the same mutation. A missense mutation [c.1751A > G, (p.His584Arg)] on Exon 16 was detected in patient F3. This variant was reported in the ExAC database (exac.broadinstitute.org) as a rare variant at a frequency of 29/120000 and classified as a variant of unknown significance. However, this variant was predicted to be disease causing by *in silico* analysis in Mutation Taster. Furthermore, the parents of these patients were heterozygous for the same variant. In F5, a nonsense mutation on exon 25 [c.3514C > T, (p.Arg1172)], previously reported in the ExAC database as a rare variant with a frequency of 1/120000, was detected. This mutation is predicted to cause a premature stop codon to be formed; the parents were heterozygous for this mutation. Therefore, this mutation, which was not previously reported in any patient, was accepted as a pathogenic variant (Figure 1).

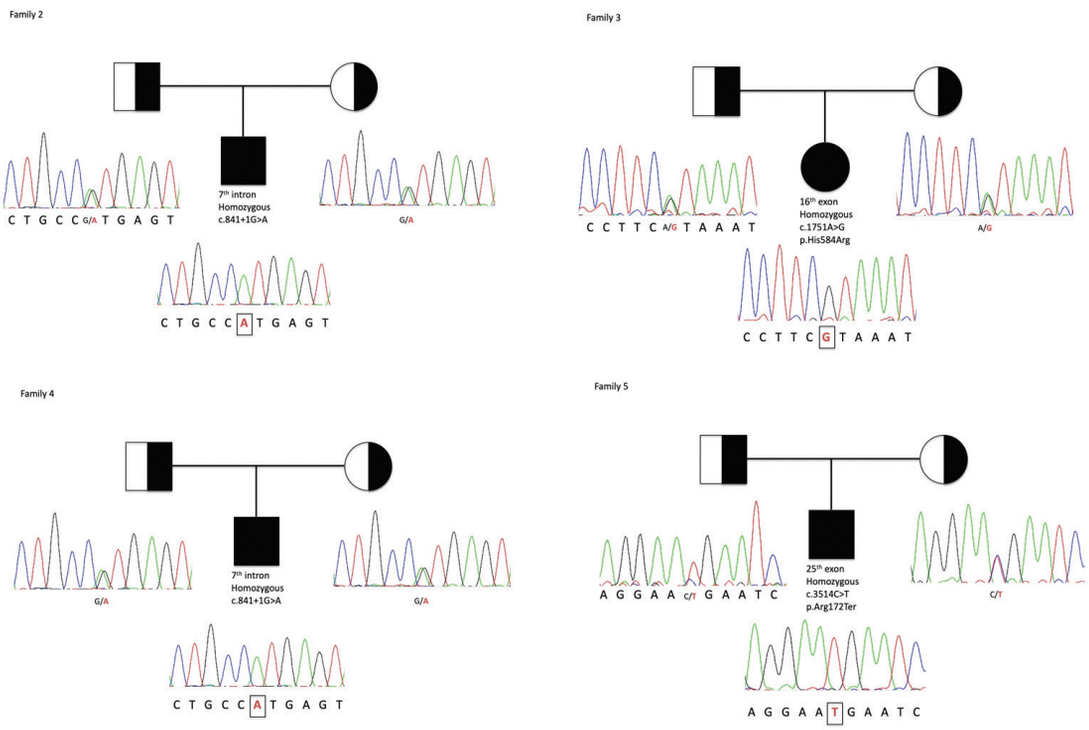

Figure 1. Pedigree and mutational analysis of the patients with novel *TRPM6* variant

The clinical and laboratory findings, including serum Ca, serum Mg^{+}, alkaline phosphatase, PTH, 25 (OH) vitamin D and urinary Mg^{+2}, Ca, P, and Cr, of the heterozygous parents were evaluated and were found to be unremarkable.

Discussion

In this study, the long-term follow-up results of six genetically confirmed HSH patients are presented. Three novel mutations in the *TRPM6* gene were identified, in addition to one known pathogenic mutation. The first presentation of all cases was afebrile seizures in the first year of life. Mild mental retardation, specific learning difficulty and ADHD were found as comorbidities. On long-term follow-up, growth was normal with Mg supplementation and maintenance of normal serum Ca levels.

Mg^{+2} is a cofactor for many enzymes and transporters, including phosphatases and phosphokinases. It is required for energy storage and use, and plays an important role in the synthesis of nucleic acids and proteins (5). Therefore, insufficient cellular Mg^{+2} concentrations affect many systems. Mg is strictly regulated by intestinal absorption and renal excretion and/or reabsorption. Intestinal absorption of Mg^{+2} occurs in the jejunum and the ileum. Most of the renal-filtered Mg^{+2} is absorbed by passive paracellular transport from the proximal tubule and the thick ascending loop of Henle. In the distal convoluted tubule, the fine tuning of Mg^{+2} equilibrium is made by active transcellular transport (6).

Hypomagnesemia in children may develop secondary to clinical conditions, such as intestinal malabsorption, short bowel syndrome, being the infant of a diabetic mother and the use of various drugs (proton pump inhibitors, antibiotics, diuretics, chemotherapeutic agents) and may also develop as a result of primary familial hypomagnesemia disorders (7). HSH is usually characterized by clinical findings, such as restlessness, tremor, muscle cramps, tetany, perioral cyanosis and generalized convulsions, in neonates or in the early infancy period. Mg levels are normal at delivery due to free passage of Mg^{+2} across the placenta. Mg levels progressively decrease within weeks or months and clinical findings begin (8). The age at presentation varies between two weeks and nine months, and in 96% of patients, generalized seizures have been reported as a presenting symptom, which was the finding in all of our cases (9,10).

Hypocalcemia and hypoparathyroidism may cause misdiagnosis of primary hypomagnesemia if the serum Mg level was not assessed (11), as in our F4 case. Improper or delayed diagnosis and treatment may cause recurrent, convulsions and irreversible neurological damage (9). The mechanism by which hypomagnesemia causes neurological damage is not known; a defect of voltage-dependent Mg passage in the N-methyl-D aspartate receptor is thought to trigger convulsions (12). Abnormal development and neural tube defects have been reported in *TRPM6* knock-out mice. In addition, mental retardation, paranoid delusions and death due to recurrent and eventually fatal seizures were reported in cases with delayed diagnosis (8,9,13). Mental retardation was diagnosed in one of our cases (F1.1), who was diagnosed relatively late, at 9 months of age. In contrast, his brother had specific learning difficulty even though (F1.2) he had an early diagnosis at 1 month of age and was treated apropriately. Interestingly, Lainez et al (14) also described a case with mental retardation and the same genetic mutation decribed in the siblings in our series. Lastly, in one patient who was diagnosed at 5 months of age, we observed ADHD (F5), and another patient with recurrent seizures had normal neurological development (F4).

Short stature has been reported rarely in HSH patients and the underlying mechanism is not completely explained (9,12,15). Short stature may be the result of late diagnosis and/or non-compliance with treatment, but it is also seen in cases diagnosed in early infancy and treated appropriately. So, it has been suggested that short stature may be a clinical feature of the disease (15). There was no short stature in any of our patients.

TRPM6 has been shown to be expressed in testicles, but the effect of mutations on male fertility is unknown (16). In two 21-years-old male patients, puberty was consistent with Tanner stage 5 and the sperm number, motility and morphology were normal in the spermiogram. In our 15-years-old male, puberty was consistent with stage 5, but a spermiogram could not be performed. None of the patients had any children.

The standard treatment in HSH is high dose Mg^{+2}. On diagnosis, intravenous or intramuscular administration can be preferred and maintenance therapy is high dose oral Mg^{+2}. A significant variation of mean oral Mg dose (0.41-3.9 mmol/kg) has been reported between patients and centers (9,17). In the literature, it has been shown that serum Mg levels do not reach normal values in patients with HSH, with the exception of only three cases undergoing high dose Mg treatment (9,14). In line with the litherature, in our study, oral Mg doses ranged between 0.51-1.28 mmol/kg (12.5-31.5 mg/kg). Normal serum Mg levels near the lower limit of the reference intervals were obtained in only two patients under Mg treatment.

Physiological fractional renal Mg excretion is 3-5%, but this falls below 0.5-1% in order to maintain serum Mg levels

in the presence of hypomagnesemia (18,19). In the current study fractional renal Mg excretion was measured over 5% in two patients whose serum Mg levels were normal but close to the lower limit of the reference intervals. In patients with subnormal course of serum Mg levels, renal Mg excretion was over 1% (1.2-2.1%). Increased renal Mg excretion has a clear role in the pathogenesis of the disease and prevents the achievement of physiological serum Mg values, despite adequate treatment. In other words, the treatment should not provide normomagnesemia, but should provide normocalcemia, and if serum Ca is normal, Mg doses should not be increased.

It has been shown that the mutations previously identified in patients with HSH are not localized in a specific region and may be distributed across many areas of the *TRPM6* protein (3,9,14). To date, 11 different mutations have been identified in 17 Turkish patients (Table 2) (9,14,15,20,21,22). The most common *TRPM6* mutations in Turkish patients were c.5775A>G (in five cases from three non-consanguineous families), c.469G>T (in three cases from three family with), and c.3158A>G (in three cases from two non-consanguineous families). In our study, missense mutation (F1.1 and 1.2) was found in the twenty third exon, previously described in a Turkish case by Lainez et al (14). In addition, we demonstrated three novel mutations. The first mutation, c.841(+1)G>A, was found in the IVS7 splice site and was present in two unrelated patients (F2 and F4). The second novel mutation was c.3514C>T (p.Arg1172) in the twenty fifth exon. It was reported as a rare variant in the database, however it causes the formation of a premature stop codon and both parents were heterozygous carriers for the same mutation; therefore this mutation is accepted as pathogenic. The missense variant found in F3 was c.1751A>G (p.His584Arg) and both parents were heterozygous carriers for the same mutation. This variant was reported in ClinVar database as a variant of unknown significance. It may be pathogenic when evaluated together

Table 2. Clinical data and results of the *TRPM6* mutational analyses of Turkish patient with familial hypomagnesemia with secondary hypocalcemia

Turkish patients (references)	Gender	Age at diagnosis	Symptoms at manifestation	Initial serum Mg^{+2} (mmol)	Initial serum Ca^{+2} (mmol)	Oral/ IMMg^{+2} (mmol/ kg/d)	Mg^{+2} under therapy	FeMg (%)	Additional finding	Mutation
P1 (9)	F	2 mo	Seizures	0.21	1.63	1.03 (o)	0.59	2.6	-	c.1769G>G
P2 (9)	M	6 yr	Seizures	ND	1.29	0.62 (o)	0.57	2.8	MR	c.2667+G>A
P3 (9)[a]	M	3 mo	Seizures	0.09	1.6	0.54 (o)	0.33	ND	-	c.5775A>G
P4 (9)[a]	M	4 mo	Asymptomatic	0.16	1.75	0.94 (o)	0.53	ND	-	c.5775A>G
P5 (9)	M	4 mo	Seizures	0.1	1.45	2.0 (o)	0.50	3.7	-	c.469G>T
P6 (9)	F	3 wk	Seizures	0.2	1.72	0.93 (o)	0.52	ND	-	c.2667+G>A
P7 (14)	F	Infancy	Seizures	0.05	1.78	0.97 (o)	0.50	ND	MR	c.3158A>G
P8 (14)	F	8 mo	Seizures	0.2	1.6	0.5 (o)	0.53	ND	-	c.469G>T + 5261G>A
P9 (21)	F	2 mo	Seizures	<0.24	1.5	1.6 (o)	0.57	3.9	-	c.3447delT>p.F1149fs
P10 (15)[b]	M	3 mo	Seizures	0.16	1.8	0.2 (o)/ 0.8 (im)	0.38	0.1	-	c.3556C>T
P11 (15)[b]	F	3 mo	Seizures	0.08	1.0	0.4 (o)/ 0.9 (im)	0.45	0.1	Short stature	c.3556C>T
P12 (15)	M	1 mo	Seizures	0.2	2.4	0.9 (im)	0.41	0.8	-	c.5775A>G
P13 (15)	M	1 yr	Seizures	0.14	2.6	0.8 (o)	0.75	2.7	-	c.1444-1 G>T
P14 (15)[c]	M	1 mo	Seizures	0.5	1.7	0.6 (o)/3.7 (im)	0.58	2.3	Short stature	c.5775A>G
P15 (15)[c]	F	3 mo	Seizures	0.5	1.7	0.5 (o)/7 (im)	0.66	1.9	-	c.5775A>G
P16 (22)	M	1 mo	Seizures	0.16	1.42	0.7 (o)	0.69	ND	-	469G>T +
P17 (23)	M	8 mo	Seizures + hypotonia	0.19	1.67	1.72 (o)	ND	0.18	-	3178A>T

[a, b, c]: siblings, F: female, M: male, yr: year, mo: month, wk: week, o: oral, im: intramusculer, ND: not defined, MR: mental retardation, Mg: magnesium, FeMg: fractional magnesium excretions, Ca: calcium

with the clinical features and segregation analysis of the patient, however functional analysis is required.

In a study, in which 28 HSH cases were evaluated regarding genotype-phenotype relationship, normal serum Mg levels were obtained by Mg treatment in two cases with a deletion in exon 32 or 33 (9). Since mutations in these exons only affect a small portion of the *TRPM6* protein, it has been suggested that the channel function may be partially protected. However, it was reported, after functional analysis, that all mutations resulted in complete loss of function in the *TRPM6* ion channel and no genotype-phenotype correlation was reported (9). In another study, the age of admission, serum Mg and Ca levels and oral Mg doses have been compared in 30 cases previously described in the literature. No relation was found between genotype and these clinical and laboratory parameters (10). In our study, clinical and laboratory findings of all patients with different mutations were similar. In addition, in one patient, normal serum Mg levels were obtained with Mg treatment, while Mg levels were subnormal in another patient with the same mutation, suggesting phenotypic variability despite genotypic homogeneity.

Mg treatment and close follow-up are essential to prevent clinical symptoms and to obtain normal Ca metabolism in HSH patients. In our case series, we observed that with an early diagnosis, appropriate treatment and good treatment compliance the long-term (about 15 years) prognosis was good and no serious complications developed in HSH patients, similar to the study of Astor et al (16) which reported long-term (about 40 years) follow-up data.

Study Limitations

The main limitation of our study was the small number of patients. Additionally, functional analysis could not be performed although we identified new pathogenic mutations.

Conclusion

Evaluation of our cases with HSH revealed a homogenous clinical picture at manifestation with onset in the first year of life with generalized seizures. However there were heterogenous molecular findings, including four different *TRPM6* mutations, of which three were novel. Early diagnosis, appropriate treatment and good treatment compliance are crucial for the prognosis. Enlightment of the genetic etiology of autosomal recessive disorders like HSH is important, and can reveal mutations especially in populations where consanginous marriages are prevalent. Molecular studies in cases with HSH and their families will contribute to increase our knowledge about Mg homeostasis. Determination of the genetic mutation is also useful to know the prognosis and associated comorbidities.

Acknowledgement

We thank Professor Karl Peter Schlingmann, University Children's Hospital, Department of General Pediatrics in Münster/Germany and Naz Güleray, University of Health Sciences Turkey, Ankara Dr. Sami Ulus Obstetrics and Gynecology and Child Health and Diseases Training and Research Hospital, Department of Medical Genetic in Ankara, Turkey for genetic analyses and sequencing data.

Ethics

Ethics Committee Approval: This study was approved by the Sultangazi Haseki Training and Research Hospital Local Ethical Committee (no: 2020-58, date: 14.05.2020).

Informed Consent: All participants and their parents received oral and written information concerning the study before providing signed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Elvan Bayramoğlu, Melikşah Keskin, Zehra Aycan, Şenay Savaş-Erdeve, Semra Çetinkaya, Concept: Elvan Bayramoğlu, Semra Çetinkaya, Design: Elvan Bayramoğlu, Semra Çetinkaya, Data Collection or Processing: Elvan Bayramoğlu, Melikşah Keskin, Zehra Aycan, Şenay Savaş-Erdeve ve Semra Çetinkaya, Analysis or Interpretation: Elvan Bayramoğlu, Zehra Aycan, Semra Çetinkaya, Literature Search: Elvan Bayramoğlu, Melikşah Keskin, Zehra Aycan, Şenay Savaş-Erdeve ve Semra Çetinkaya, Writing: Elvan Bayramoğlu, Semra Çetinkaya.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0228

J Clin Res Pediatr Endocrinol 2021;13(3):308-319

Clinical Characteristics and Growth Hormone Treatment in Patients with Prader-Willi Syndrome

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What is already known on this topic?

Prader-Willi syndrome (PWS) is a genetic disorder characterized by short stature, low lean body mass (LBM), muscular hypotonia, mental retardation, behavioral abnormalities, dysmorphic features, and excessive appetite with progressive obesity. Growth hormone (GH) treatment is beneficial for children with PWS. It improves linear growth, increases LBM, basal energy expenditure, muscle strength and reduces fat mass.

What this study adds?

Although clinical and genetic characteristics of PWS are well defined, national Turkish data regarding patients with PWS is lacking. This study reports clinical and genetic characteristics, the rate and timing of GH treatment initiation, and response to GH treatment in Turkish PWS patients. Additionally, by increasing pediatricians' awareness of PWS, it is hoped that earlier diagnosis and therefore earlier treatment may occur.

Abstract

Objective: To investigate clinical characteristics and response to growth hormone (GH) treatment in patients with Prader-Willi syndrome (PWS) in Turkey.

Methods: The data of 52 PWS patients from ten centers was retrospectively analyzed. A nation-wide, web-based data system was used for data collection. Demographic, clinical, genetic, and laboratory data and follow-up information of the patients were evaluated.

Results: The median age of patients at presentation was 1.5 years, and 50% were females. Genetic analysis showed microdeletion in 69.2%, uniparental disomy in 11.5%, imprinting defect in 1.9% and methylation abnormality in 17.3%. Hypotonia (55.7%), feeding difficulties (36.5%) and obesity (30.7%) were the most common complaints. Cryptorchidism and micropenis were present in 69.2% and 15.3% of males, respectively. At presentation, 25% had short stature, 44.2% were obese, 9.6% were overweight and 17.3% were underweight. Median age of obese patients was significantly higher than underweight patients. Central hypothyroidism and adrenal

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Conflict of interest: None declared
Received: 23.09.2020
Accepted: 03.02.2021

insufficiency were present in 30.7% and 4.7%, respectively. Hypogonadism was present in 75% at normal age of puberty. GH treatment was started in 40% at a mean age of 4.7 ± 2.7 years. After two years of GH treatment, a significant increase in height SDS was observed. However, body mass index (BMI) standard deviation (SDS) remained unchanged.

Conclusion: The most frequent complaints were hypotonia and feeding difficulty at first presentation. Obesity was the initial finding in 44.2%. GH treatment was started in less than half of the patients. While GH treatment significantly increased height SDS, BMI SDS remained unchanged, possibly due to the relatively older age at GH start.

Keywords: Prader-Willi syndrome, endocrine dysfunction, growth hormone treatment, body composition

Introduction

Prader-Willi syndrome (PWS) is a genetic disorder resulting from lack of paternally inherited imprinted genes on chromosome 15 in the 15q11-q13 region, either due to deletions from the paternal chromosome, maternal uniparental disomy (UPD) or, rarely, defects in the imprinting center (1). The estimated incidence of PWS is around 1 in every 15,000-30,000 births. Both sexes are affected equally (2).

PWS is a complex disorder with different phenotypic features developing at different ages. It is characterized by severe hypotonia with poor sucking and feeding difficulties in early infancy, followed by excessive eating and gradual development of obesity in later infancy or early childhood, if access to food is unrestricted (3,4). Hypothalamic dysfunction is characteristic of PWS and this is hypothesized to underlie many of the syndrome's cardinal features, such as hyperphagia, temperature instability, sleep-disordered breathing, and multiple endocrine abnormalities that include growth hormone (GH) deficiency, central adrenal insufficiency (CAI), hypogonadism and hypothyroidism (5). Global developmental delay, cognitive dysfunction and neurobehavioral problems are other features of the syndrome (1).

GH deficiency is very common in PWS (6). Recombinant human GH (rGH) is indicated in the treatment of growth failure in PWS and provocation testing to demonstrate GH deficiency is unnecessary for patients with genetically confirmed PWS (7). In addition, treatment with GH can improve body composition and physical strength, as well as motor and mental development (8).

In Turkey, the data regarding the purpose of GH treatment in patients with PWS are not clear. In addition to describing the prevailing current situation, an aim of this study was to determine the clinical, demographic and accompanying endocrine and non-endocrine co-morbid conditions of pediatric Turkish patients with PWS.

Methods

In this study, the data of 52 patients with PWS who were being followed in 10 centers in Turkey were retrospectively analyzed. PWS patients aged between 0 and 18 years were enrolled in the study. A nation-wide, web-based, CEDD-NET Data System (http://cedd.saglik-network.org/) was used for data collection between March 2016 and February 2018. A case recording form, including demographic, clinical, genetic, and laboratory findings and follow-up information was uploaded to the website and completed by the patient's managing physician. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Gülhane Training and Research Hospital Institutional Ethical Review Board (approval number: 2016-16, date: 02.19.2016).

Short stature was defined as a height that was two standard deviation (SD) score (SDS) or more below the mean height for age and sex (9). Overweight and obesity were defined as body mass index (BMI) that was between $>85^{th}$ and $<95^{th}$ percentile and $\geq 95^{th}$ percentile for age and sex, respectively. Underweight was defined as BMI that was $<5^{th}$ percentile for age and sex (10). SDS and percentiles of height, weight and BMI were calculated according to national data (11).

Preterm delivery was defined as a gestational age of <37 weeks. LBW was defined as birth weight below 2500 gr. Small for gestational age (SGA) was defined as birth weight below the 10^{th} percentile for gestational age (12).

GH deficiency was diagnosed after two stimulation tests (L-dopa, clonidine, glucagon or insulin, depending on each center's normal practice). Complete GH deficiency was diagnosed after a stimulation test with a GH peak <5 µg/L and partial GH deficiency with a GH peak between 5 and 10 µg/L. Diagnosis of central hypothyroidism (CH) was made with low free T4 (fT4) concentrations associated with low/normal serum thyroid stimulating hormone (TSH) levels. Primary hypothyroidism was diagnosed with low fT4 associated with elevated TSH levels (13). Presence of CAI was investigated by estimation of serum adrenocorticotrophic hormone (ACTH) and cortisol levels

in blood samples obtained in the early morning and by low-dose ACTH stimulation test, when needed. The cut-off level for appropriate cortisol response was accepted as 18 mcg/dL (14). Hypogonadism was investigated when puberty was delayed. Low sex steroid levels, along with high gonadotropin levels suggested primary hypogonadism. Diagnosis of secondary hypogonadism was made with low sex steroid levels and negative luteinizing hormone-releasing hormone stimulation test in patients above pubertal age (15). Micropenis was defined as penile length smaller than 2.5 SD below the mean; SDS of penile length was calculated according to national data (16). Osteoporosis was considered present when the child had sustained: (a) one or more low-traumatic vertebral fractures in the absence of local disease or high-impact injury; or (b) two or more low impact fractures of the long bones if less than ten years of age or three or more low impact fractures before 19 years of age, together with bone mineral density as assessed by dual-energy X-ray absorptiometry that was <-2 SDS below the mean for sex, chronological age, and height/height age (17).

Clinical and laboratory characteristics of the patients were evaluated by each center at presentation and during follow-up. Clinical characteristics including birth weight, gestational age at delivery, history of developmental milestones, feeding difficulties in infancy, complaints, anthropometric measurements (height, weight, BMI) and pubertal status were recorded. A detailed PWS-specific questionnaire regarding cardiac, renal, endocrine, otorhinolaryngological and skeletal systems, as well as neuromotor, psychosocial and sleep problems relevant to PWS, were completed by managing physicians. Cardiac findings were based on echographic examinations. If available, sella magnetic resonance imaging (MRI) and polysomnography (PSG) results were requested. Confirmatory genetic test results were recorded. In addition, any other clinical features present in individual patients but not specifically queried in the questionnaire were also requested and recorded. Patient's anthropometric measurements were recorded yearly over two years follow up. Annual laboratory assessments were collected which included serum insulin-like growth factor 1 (IGF-1), glycated hemoglobin, fasting glucose and insulin, and lipid profile consisting of total triglyceride, total cholesterol, high density lipoprotein (HDL), and low density lipoprotein (LDL) concentrations. SDS values were calculated for IGF-1, according to age- and sex-matched reference values for the Turkish population (18). Dyslipidemia was defined according to the guidelines of the National Heart, Lung, and Blood Institute as total cholesterol ≥200 mg/dL, LDL ≥130 mg/dL, HDL <40 mg/dL, triglyceride ≥100 mg/dL for younger than 10 years old and ≥130 mg/dL for those older than 10 years (19). Homeostasis model of assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = [fasting insulin (μIU/mL) × fasting glucose (mg/dL)]/405 (20).

The researchers were asked to record the GH dose, if treated, and whether the GH treatment had been interuprted or discontinued completely, and if so, why.

Entering additional information not included in the questionnaire form was optional.

Genetic Tests

DNA methylation analysis was performed as first line test to confirm the diagnosis of PWS. Polymerase chain reaction along with Southern blotting of the small nuclear ribonucleoprotein polypeptide N probe for the 15q11-q13 region or Methylation-Specific Multiplex Ligation Dependent Probe Amplification (MS-MLPA) were used to determine methylation status, depending on each centers' preference. If the DNA methylation patterns were consistent with PWS, further tests were performed to identify the exact genetic etiology of PWS (deletion, UPD or imprinting defect). Fluorescence in situ hybridization with high resolution karyotype, chromosomal microarray analysis with single nucleotide polymorphism (SNP) and copy number variant probes or MS-MLPA methods were used to determine the deletion status of the 15q11-q13 region, again depending on each centers' preference. If no deletion was detected, DNA polymorphism analysis was performed to distinguish between maternal UPD and imprinting defects. The diagnosis of imprinting defect was made after exclusion of UPD. In some centers, the diagnosis of PWS was made only with DNA methylation analysis without further genetic tests.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences, version 21.0 (IBM Inc., Chicago, Ill., USA). Descriptive statistics for categorical variables are presented as frequencies and percentages. Normality was tested using the Shapiro-Wilk test. Depending on the distribution of the data set, data are presented as mean ± SD or median (25^{th} to 75^{th} percentile). Wilcoxon signed-ranks and Friedman tests were used to compare baseline values between first year and second year values in the group receiving GH treatment. A p value <0.05 was assumed to indicate statistical significance.

Results

Baseline Characteristics

Data of a total of 52 patients with PWS (26 males, 26 females) were collected. At first presentation, the median

age of patients was 1.5 (0.08-15.4) years and 96.1% (n = 50) of the patients were prepubertal. The most frequent complaints were hypotonia in 55.7% (n = 29) patients, feeding difficulties in 36.5% (n = 19) and obesity in 30.7% (n = 16). Cryptorchidism and micropenis had been detected in 69.2% (n = 18) and 15.3% (n = 4) of male patients, respectively (Table 1). Demographic and anthropometric data of the patients at presentation is shown in Table 2. Mean height SDS and BMI SDS were -1.25 ± 1.23 and 0.96 ± 2.56, respectively. Short stature was detected in 25% (n = 13) of the patients. Height SDS was between -2 SDS and -3 SDS in 17.3% (n = 9) and less than -3 SDS in 7.7% (n = 4) of the patients. Among the patients 44.2% (n = 23) were obese, 9.6% (n = 5) were overweight and 17.3% (n = 9) were underweight. The median age of the obese patients was 4.1 (range 0.9-15.4) years, whereas the median age of the underweight patients was 0.12 (range 0.08-1.4) years. Median age of the obese patients was significantly higher than the underweight patients ($p < 0.001$).

Table 1. Clinical features of the patients at presentation

Characteristics	n (%)
Hypotonia	29 (55.7)
Feeding problems	19 (36.5)
Cryptorchidism*	18 (69.2)
Obesity	16 (30.7)
Developmental delay	14 (26.9)
Short stature	13 (25)
Typical dysmorphic facies	7 (13.4)
Mental retardation	5 (9.6)
Micropenis*	4 (15.3)
Failure to thrive	4 (7.6)
Sleep disorders	4 (7.6)
Small hands and feet	3 (5.7)

*In male patients.

Birth Characteristics

The mean gestational age and birth weight of the patients were 38 ± 1.8 weeks and 2550 ± 450 g, respectively. Median birth weight SDS was -1.25 SD (-3.9; 1.4). Birth weight SDS was lower than -2 SD in 29% (n = 16) of the patients. Preterm delivery was present in 17.3% (n = 9) of the patients. Of the patients 40.3% (n = 21) were SGA and 42.3% (n = 22) had low birth weight (LBW).

Neuromotor Development

The mean time for onset of developmental milestones were as follows: independent sitting at 17.9 ± 8.9 months (n = 30/52); walking at 33 ± 13 months (n = 27/52); and first spoken words at 31.3 ± 16.1 months (n = 20/52).

Nutritional Characteristics

In infancy, need for assisted feeding with nasogastric tube, spoon and nursing bottle were recorded in 15.3% (n = 8) of the patients.

Genetic Evalution

Genetic analysis results of patients are shown in Figure 1. PWS was diagnosed, based solely on methylation abnormality, in 17.3% (n = 9) of the patients. In these patients, microdeletion, UPD and imprinting center mutation could not be examined to define genetic etiology. In the remaining patients, further genetic tests revealed microdeletion, maternal UPD, and imprinting center defects in 69.2% (n = 36), 11.5% (n = 6), and 1.9% (n = 1) of the patients, respectively

Table 2. Clinical characteristics of the patients at presentation

	Mean ± SD/n (%)	Median	Min-Max
Age (years)	2.7 ± 3.2	1.5	0.08-15.4
Gender, female	26 (50)		
Height SDS	-1.25 ± 1.23	-1.25	-4.9-0.9
Target height SDS	-0.66 ± 0.73	-0.69	-2.5-1.2
Weight SDS	0.25 ± 2.16	0.05	-4.7-4.85
BMI (kg/m^2)	19.8 ± 7.01	19.2	8.8-41.6
BMI SDS	0.96 ± 2.56	1.64	-4.7-4.7
BMI percentile			
< 5th	9 (17.3)		
≥5th to < 85th	15 (28.8)		
≥85th to < 95th	5 (9.6)		
≥95th	23 (44.2)		

Min-Max: minimum-maximum, SDS: standard deviation (SD) score, BMI: body mass index

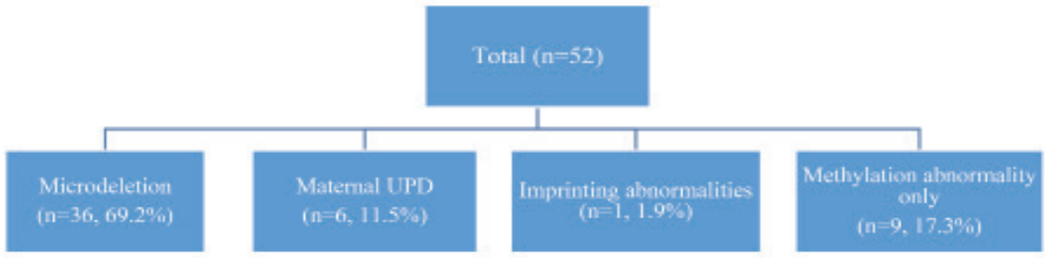

Figure 1. The results of genetic analysis of patients

UPD: uniparental disomy

Endocrinologic Evaluation

CH and acquired primary hypothyroidism were observed in 30.7% (n = 16) and 1.9% (n = 1) of the patients, respectively. Etiology of acquired primary hypothyroidism remained undetermined, thus autoimmune thyroid disease was excluded in the patient. Adrenal function was evaluated in 80.7% (n = 42) and CAI was detected in 4.7% (n = 2) of them. These two patients had no clinical signs of adrenal insufficiency; CAI was detected with screening, and hydrocortisone replacement was provided in case of adrenal stress, for example, because of infection.

In 48% of the patients (n = 25) GH stimulation tests were performed and 23/25 (92%) had a deficient GH response. Sella MRI was normal in all of the patients with GH deficiency.

Among patients at pubertal age (n = 4), two had hypogonadotropic hypogonadism, and one had hypergonadotropic hypogonadism. The patient with hypergonadotropic hypogonadism was diagnosed due to arrest of pubertal development. One of the patients with hypogonadotropic hypogonadism presented with secondary osteoporosis. Overall, two patients had osteoporosis; one with normal pubertal development, thus the etiology of osteoporosis remained undetermined. Cryptorchidism and micropenis were present in 69.2% (n = 18) and 15.3% (n = 4) of the male patients, respectively. Orchidopexy was performed in 57.6% (n = 15) of male patients. In one patient, orchiectomy was performed due to atrophic testis.

Skeletal Assessment

Skeletal problems were present in 30.7% (n = 16). The most common problem was scoliosis, observed in 23% (n = 12). Lower extremity abnormalities, including developmental dysplasia of hip, pes equino varus, pes cavus, and x-bain and o-bain deformities were present in 15.3% (n = 8).

Otorhinolaryngological Assessment

Seventy-one percent of patients (n = 37) were formally evaluated by an otorhinolaryngologist. Pathologic findings, including adenoid vegetation and/or tonsillar hypertrophy, were reported in 43.2% (n = 16) and surgical interventions (adenoidectomy and/or tonsillectomy) were performed in 50% (n = 8) of these. One patient had conductive hearing loss.

Sleep Apnea and Polysomnography Findings

PSG was performed in 57.6% (n = 30) and pathologic findings, including obstructive/central/mixed apnea and hypopnea, were detected in 70% (n = 21). Narcolepsy was reported in one patient (1.9%).

Other Chronic Diseases

Epilepsy was reported in 9.6% (n = 5). Three patients were operated for strabismus. Echocardiographic evalution was performed in 53.8% (n = 28) and pathologic findings, including atrial septal defect, ventricular septal defect, subaortic ventricular septal hypertrophy, patent foramen ovale, pulmonary stenosis, pulmonary hypertension, minimal aortic insufficiency and tricuspid insufficiency, were present in 28.5% (n = 8). One patient was operated due to atrial septal defect. One patient had a pacemaker due to arrhythmia. One patient died at the age of nine months due to lower respiratory tract infection. One patient had tracheostomy and she also had severe mental motor retardation.

Growth Hormone Treatment

Twenty-one (40.3%) patients were treated with rGH (mean dose: 25 ± 5 μg/kg/day) of whom 19 had GH deficiency. GH was started in one patient without GH stimulation testing and one patient without GH deficiency (Figure 2). The mean age at onset of GH treatment was 4.7 ± 2.7 (range 1.6-9.4) years. At the beginning of treatment 28.5% of the patients had short stature, 52.3% were obese, 14.2% were overweight and 4.7% were underweight. Treatment was continued for one year in 21 patients and for two years in 11 patients. The mean growth velocity was 9.9 ± 2.5 cm/year

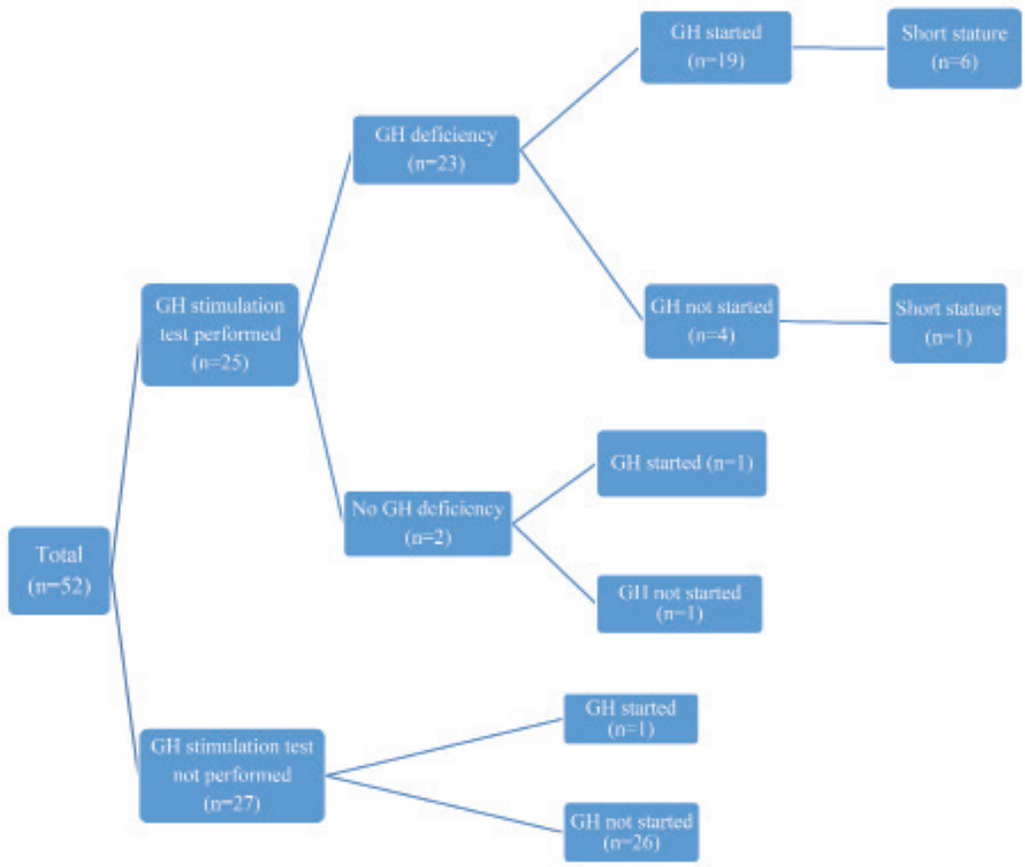

Figure 2. Flow chart of the patients whom growth hormone treatment was started

GH: growth hormone

for the first year and 8.1 ± 3.1 cm/year for the second year. Data showing the anthropometric and laboratory changes after the first and second year of GH treatment are shown in Table 3 and Table 4, respectively. After one year of GH treatment, a significant increase in height SDS parallel to the increase in serum IGF-1 SDS was observed. However, there was slight but significant increase in weight SDS (p = 0.035) and BMI SDS remained unchanged. Serum glucose levels did not change in the first year of treatment, but insulin levels increased slightly (p = 0.047). Fasting glucose levels were normal in all patients before GH treatment, while impaired fasting glucose was detected in only one patient after the first year of treatment. IR was evaluated by HOMA-IR in fifteen patients before treatment and 2/15 (13.3%) had high pre-rGH treatment HOMA-IR values. At the end of the first year of rGH, high HOMA-IR values were detected in 5/15 patients (33.3%); thus three patients progressed from normal to abnormal HOMA-IR on GH treatment. Before GH treatment, elevated total cholesterol, LDL and triglyceride levels, and decreased HDL levels were detected in 22.2% (n = 4/18), 27.7% (n = 5/18), 31.2% (n = 5/16) and 16.6% (n = 3/18), respectively. After the first year of GH treatment, elevated total cholesterol, LDL and triglyceride levels were detected in 43.75% (n = 7/16), 46.6% (n = 7/15) and 23% (n = 3/13), respectively. When compared to baseline, there was no change in triglyceride levels, but total cholesterol, HDL and LDL levels increased after the first year of GH treatment (Table 3). None of the patients had low HDL levels after the first year of GH treatment.

In the eleven patients completing two years of GH treatment, a significant increase in height and IGF-1 SDS was observed, compared to baseline, but there was no difference in terms of weight and BMI SDS. After two years of treatment, despite no change in insulin and HOMA-IR levels, there was a slight increase in glucose levels compared to baseline. Since there was not enough data, the effect of GH on lipid profile was not evaluated in the second year.

In all but two patients receiving GH treatment, PSG was performed before treatment (19/21). Sleep apnea was

Table 3. Clinical and laboratory evaluation of patients before and after one year of growth hormone treatment

	Baseline	First year	p
Height SDS (n = 21)	-1.4 (-2.0; -0.6)	-0.9 (-1.3; -0.4)	< 0.001
Weight SDS (n = 21)	0.3 (-0.8; 2.5)	1.2 (-0.2; 2.6)	0.035
BMI SDS (n = 21)	1.8 (0.6; 3.2)	2.0 (1; 3.3)	> 0.05
Glucose (mg/dL) (n = 21)	81.5 (67.7; 86.2)	85.5 (79.2; 91)	> 0.05
Insulin (µIU/mL) (n = 15)	7.6 (5.2; 9.6)	10 (8.1; 12.4)	0.047
HOMA-IR (n = 15)	1.5 (1.0; 2.0)	2.15 (1.6; 2.6)	0.016
Total cholesterol (mg/dL) (n = 16)	153.5 (126.7; 198.7)	197.5 (155.7-234)	0.004
LDL cholesterol (mg/dL) (n = 15)	101 (78; 143)	120 (83; 174)	0.047
HDL cholesterol (mg/dL) (n = 15)	44 (37; 63)	52 (45; 64)	0.021
Triglyceride (mg/dL) (n = 13)	92 (59; 116)	82 (51; 107)	0.1
IGF-1 SDS (n = 17)	-2.5 (-2.7; -2)	-0.6 (-1.0; 0.8)	< 0.001

Results are given as median (25; 75p).
SDS: standard deviation (SD) score, BMI: body mass index, HDL: high density lipoprotein, LDL: low density lipoprotein, HOMA-IR: homeostasis model of assessment of insulin resistance, IGF-1: insulin-like growth factor 1

Table 4. Clinical and laboratory evaluation of patients before and after the first and second years of growth hormone treatment

	Baseline	First year	Second year	p
Height SDS (n = 11)	-1.6 (-2.5; -1.2)	-1.0 (-1.4; -0.8)	-0.9 (-1.3; -0.2)	< 0.001*
Weight SDS (n = 11)	0.2 (-0.8; 1.4)	1.1 (-0.5; 1.4)	0.8 (0.4; 1.9)	> 0.05
BMI SDS (n = 11)	1.6 (0.8; 2.8)	1.5 (0.3; 2.7)	1.7 (1.4; 2.2)	> 0.05
Glucose (mg/dL) (n = 11)	80 (71; 85)	83 (77; 90)	90 (78; 93)	0.023µ
Insulin (µIU/mL) (n = 9)	7.7 (4.9; 8.8)	8.9 (4.7; 10.8)	8.8 (5.5; 15.2)	> 0.05
HOMA-IR (n = 9)	1.5 (1; 1.8)	2.1 (0.9; 2.3)	1.7 (1.1; 3.5)	> 0.05
IGF-1 SDS (n = 10)	-2.4 (-2.7; -2)	-0.6 (-1.2; 0.9)	0.9 (-0.6; 3.1)	0.002¶

Results are given as median (25; 75p).
*The difference was due to the baseline and second year comparison (p = 0.001).
µThe difference was due to the baseline and second year comparison (p = 0.023).
¶The difference was due to the baseline and second year comparison (p = 0.001).
SDS: standard deviation (SD) score, BMI: body mass index, HOMA-IR: homeostasis model of assessment of insulin resistance, IGF-1: insulin-like growth factor 1

observed in nine patients before treatment and in one patient during GH treatment. In three of them, GH treatment was started after adenotonsilectomy. In three patients, GH treatment was started under continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BiPAP) support. Due to exacerbation of apnea, two patients underwent adenotonsilectomy, after which GH treatment was continued. In one patient treatment was discontinued in the second year due to worsening of sleep apnea. In one patient who did not have sleep apnea before rGH treatment, sleep apnea was observed in the first year of treatment and treatment was continued under BiPAP support. In the remaining nine patients whose pre-treatment PSG was normal and in two patients who did not undergo PSG, no complication was reported during GH treatment. GH treatment was not started in 11 further patients with sleep apnea.

Adrenal insufficiency was not reported in patients receiving GH. In seven patients, CH was associated with GH deficiency. Hypothyroidism did not develop under GH treatment.

Discussion

A total of 52 patients (26 males, 26 females) with PWS who had been registered to the CEDD-NET Data System were involved in this study. Of the cohort, 55.7% and 36.5% of the patients had presented with hypotonia and feeding difficulties, respectively. It is known that clinical signs of PWS vary by age. In infants, the most prominent findings are hypotonia and feeding difficulties. The characteristic findings, such as hyperphagia, obesity, and intellectual disability develop later in childhood (21,22). In a multicenter study investigating maternal and neonatal outcomes in patients with PWS, all neonates were hypotonic, and 99%

had feeding difficulties (23). In our series, frequency of hypotonia and feeding difficulty were lower than in the literature. Clinical diagnosis of PWS is difficult during infancy because hypotonia is a non-specific feature and the typical clinical features of the later period are not yet present. Beside this, hypotonia is not an indication for admission to endocrine clinics. Thus, in our cohort, later age at presentation makes hypotonia a relatively less frequent symptom. It is recommended that PWS should be considered in any infant with significant hypotonia, particularly in the setting of poor feeding and genital hypoplasia (cryptorchidism, small penis, or small clitoris). Tuysuz et al (24) reported PWS in 11% of the patients referred for hypotonia. Infantile history should be actively sought during evaluation of older children (25).

Excessive weight gain follows the period of failure-to-thrive in early infancy in PWS (21). It is reported that obesity usually begins between the ages of one and six years, with an average age of onset of two years (3). In our series, 44.2% of the patients presented with obesity. Median age at presentation of obese patients was 4.1 years. In the 17.3% of patients presenting with underweight, median age at presentation was 0.12 years. However, data regarding the age at onset of obesity was not present. Given the expected natural history of PWS, patients presenting with undernutrition were unsurprisingly younger than those who were obese at presentation.

Toddlers with PWS have delayed motor and language development, with milestones achieved at about double the normal age (3). In our series, the average age at independent sitting, walking and speaking first words were at 18, 33 and 31 months, respectively. Developmental delay was a presenting feature in 26.9% of the patients.

The prevalance of preterm birth, SGA and LBW in our cohort was 17.3%, 40.3% and 42.3%, respectively, which was in concordance with the increased incidence of preterm birth, LBW and intrauterine growth retardation reported in PWS (26,27,28).

In our series, 17.3% were diagnosed by methylation analysis only. Unfortunately, further genetic tests were not performed in these patients. In the patients in whom further genetic analysis was performed the frequencies of microdeletion, UPD and genomic imprinting center defect were 69.2%, 11.5% and 1.9%, respectively. In the literature, paternal 15q11.2-q13 deletion is responsible for 65-75% of cases, maternal UPD is responsible for 20-30% of cases, and 1-3% of cases are sporadic or due to genomic imprinting center defect (1,29). Thus in this group of Turkish PWS patients the incidence of microdeletion and imprinting is in line with previous reports but the incidence of UPD is around half that expected. Nevertheless, if further genetic tests could have been performed in the group with only methylation analysis, these incidence rates may be different.

Hypothalamic dysfunction is thought to be responsible for some endocrinopathies in PWS, including CH (30). In our series, the prevalence of CH was 30.7%, and one patient had acquired primary hypothyroidism due to unknown etiology. There are conflicting data in the literature regarding the prevalence of CH in PWS. In some studies, a prevalence of 2-4%, which is similar to that of the general population was reported, while others reported a prevalence of 20-30% (31,32) or even of 72% in a study conducted in patients with PWS during the first 2 years of life (33).

Children with PWS are at risk for CAI, also thought to be due to hypothalamic dysfunction (30). However, the frequency of CAI in patients with PWS is not clear and frequencies have varied widely between studies. In a cross-sectional study, CAI was detected in 60% of cases after metirapone testing (34). Subsequent studies, conducted with other methods, did not confirm the reported high frequency. Corrias et al (35) found CAI in 14.3% of the cases. Beauloye et al (36) reported CAI with a prevalance of 5% in children with PWS, similiar to the frequency of 4.7% found in our series. By conrast, some studies showed normal hypothalamic pituitary adrenal axis in PWS patients (37,38).

Hypogonadism is a common clinical feature of the syndrome and both hypothalamic and gonadal abnormalities can cause hypogonadism (39,40). In both genders, hypogonadism manifests as genital hypoplasia, incomplete pubertal development, and infertility in the majority (1). Unilateral or bilateral cryptorchidism is a common finding, ranging from 66% to 100% of males (39,41). However, genital hypoplasia is often overlooked in females (1). Frequency of cryptorchidism and micropenis in our cases were 69.2% and 15.3%. Genital hypoplasia was not reported in females. In our series, 92.3% of the patients were in the prepubertal period and thus gonadal function was not evaluated. Among four patients with ages consistent with the normal pubertal period, two had hypogonadotropic hypogonadism and one had hypergonadotropic hypogonadism (75%). In a cohort of 115 adult patients with PWS, all males and 93% of females had hypogonadism (42). In the French national PWS pediatric database of 142 patients, the frequency of hypogonadism was 49% (32). The number of patients whose gonadal function was evaluated was limited in our cohort. Therefore, it is unreliable to attempt to draw definite conclusion for frequency of hypogonadism in this cohort.

The body composition of patients with PWS, characterized by reduced lean body mass (LBM) and increased fat mass,

resembles that of individuals with GH deficiency (43). Diminished GH secretion in PWS is well documented and it has been reported to be present in 40% to 100% of the patients (6,7,32,44).

In our series, the GH/IGF axis was evaluated in 48% (n = 25) and GH stimulation tests revealed GH deficiency in 23 patients. GH treatment was started in 40.3% of the patients. Short stature is a common finding in PWS patients and occurs because of linear growth retardation and lack of a pubertal growth spurt (3). In our series, 25% of the patients had short stature at presentation. Among the patients who received GH treatment 28.5% had short stature at the initiation of the treatment. The rationale for treating PWS children with GH is to not only to enhance linear growth but also to improve body composition, energy expenditure and muscle strength (45). In our study, height velocity increased with treatment and significant improvement in height SDS was observed at the end of the first year. However, there was no change in BMI SDS. Data for the second year of GH treatment was suboptimal due to a low number of patients (n = 11). Compared to baseline, there was an improvement in height SDS, however there was no change in BMI SDS. Several studies conducted on PWS children treated with GH and followed longitudinally have shown that prolonged treatment with GH improves but does not normalize body composition (45,46,47). Although there is no consensus on this issue, it is recommended to start GH treatment before the onset of obesity, which often begins by two years of age (7). Carrel et al (48) conducted a study with 21 patients with PWS, in whom GH treatment was started prior to two years of age and continued for six years. At the end of treatment, the anthropometric measurements of these children were compared with 27 PWS patients of similar age, who did not receive GH treatment. These authors reported that GH therapy begun early in life favorably altered the natural history of PWS by reducing body fat mass. In our series, the median age at onset of GH treatment was four years (range 1.6-9.4). One reason for the lack of improvement in BMI SDS in our patients may be the relatively late initiation of treatment; 66.5% were already obese or overweight. Bakker et al (49) conducted a study in 60 PWS patients who started GH therapy between 3-7 years of age and continued for eight years. In this study, LBM significantly increased, and percent fat mass and BMI significantly decreased in the first year. However, in the subsequent seven years, LBM and BMI remained stable, but percent fat mass gradually increased. At the end of eight years, LBM SDS was higher than baseline, but percent fat mass and BMI SDS were not significantly different from baseline. In this study, BMI of patients was compared with reference values of untreated age and sex matched children with PWS and BMI_{PWS} SDS was calculated according to these reference values. BMI_{PWS} SDS decreased significantly in the first year of treatment and this effect persisted during the entire study period and at the end BMI_{PWS} SDS was significantly lower than at baseline. The authors concluded that GH treatment is a potent force for counteracting the clinical course of obesity in patients with PWS. In the present study, only change in BMI was evaluated while body composition was not. Thus, even though no change in BMI was observed, fat and lean mass ratio may have changed. Nevertheless, there was no improvement in mean BMI SDS. However, stabilization of BMI SDS may be an acceptable outcome of the treatment compared to increasing worsening of BMI. We do not have enough data about the untreated PWS group to compare the anthropometric changes with the GH treated group. However, tendency to deterioration of auxological and body composition parameters over time in untreated patients is widely accepted. Nutritional management is the mainstay of treatment in PWS, even during GH therapy (7). The lack of nutrional data was another limitation of our study. In our cohort, the dose of GH treatment was not uniform so that the variation in dosages may have confounded the anthropometric results.

We found an increase in fasting insulin and HOMA-IR levels, with no change in fasting glucose levels after the first year of GH treatment. Carrel et al (50) also reported similar results; although there was no change in glucose level, there was a statistically insignificant increase in insulin level. Bakker et al (49) showed an increase in fasting glucose and insulin levels after one year of treatment. However, some studies reported that GH treatment was not associated with adverse effects on glucose and insulin parameters (48,51).

Previous studies have shown improvements in patients' lipid profiles with GH treatment (48,49,50). However, in our study, despite a significant increase in HDL levels, there was also a significant increase in LDL and total cholesterol levels. In our series, the change in serum lipid levels may not be due to GH therapy and may be part of the natural course of the disease.

Children with PWS have a high incidence of both central and obstructive apnea (52). In the patients who underwent PSG, abnormalities were detected in 70%. This finding shows the importance of PSG in the follow up of PWS, especially in the patients in whom GH treatment is planned, because GH treatment can hypothetically lead to expansion of airways-associated lymphoid tissue in PWS children, due to increased IGF-1 effects, and thus exacerbate obstructive apnea (53). Severe sleep apnea is a contraindication for GH treatment (7). In our cohort, GH treatment was started in

three patients with sleep apnea who also received BİPAP/CPAP support and in three patients after adenotonsilectomy. Due to exacerbation of apnea, two patients underwent adenotonsilectomy and treatment was discontinued in one patient after the second year. In one patient, sleep apnea was detected during GH treatment and treatment was continued under BiPAP support. Deaths have been reported associated with GH treatment, especially in the early phase of GH treatment (52,53,54). In our cohort, no death was reported during GH treatment. However, GH treatment increased the severity of apnea in four patients. Therefore, during GH treatment in PWS, close follow-up of patients with ENT and/or PSG is recommended.

Study Limitations

The most important limitation of this study is its retrospective design. Data was collected from different centers with a web-based national data system and the clinical follow up protocols were heterogenous. We did not have data on neuromotor development involving all patients. Additionally, data was collected only from pediatric endocrinology clinics and thus patient characteristics could be different in those who were admitted to genetic or other clinics. As noted earlier, data regarding body composition and nutritional status are incomplete. Also, there is insufficient anthropometric data in the untreated PWS group to compare the changes with those observed in the rGH treated group. Here, we report short-term results of GH treatment in a small group. Prospective studies in larger populations with long-term follow-up are needed to assess the effect of GH treatment and to draw definite conclusions.

Conclusion

The present study provides data on the demographic characteristics and frequency of associated problems in PWS during childhood, based on the experience of pediatric endocrinology centers in Turkey. This study has highlighted the lack of a national protocol for follow up and GH treatment in pediatric patients with PWS. The most frequent complaint was hypotonia followed by feeding difficulties. Obesity was the initial finding in 44.2% of the patients suggesting relatively late diagnosis. GH treatment was started in less than half of the patients. While GH treatment significantly increased the height SDS, BMI SDS remained unchanged, which might be due the relatively late onset of GH treatment. National programs to increase awareness of PWS to improve diagnosis and guidelines for standardized follow-up to improve clinical care should be instituted.

Ethics

Ethics Committee Approval: The study was conducted according to the principles of the Declaration of Helsinki and approved by the Gülhane Training and Research Hospital Institutional Ethical Review Board (approval number: 2016-16, date: 02.19.2016).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Aydilek Dağdeviren Çakır, Aslı Derya Kardelen, Elvan Bayramoğlu, Şükran Poyrazoğlu, Concept: Aydilek Dağdeviren Çakır, Elvan Bayramoğlu, Design: Aydilek Dağdeviren Çakır, Elvan Bayramoğlu, Data Collection or Processing: Aydilek Dağdeviren Çakır, Firdevs Baş, Onur Akın, Zeynep Şıklar, Bahar Özcabı, Merih Berberoğlu, Aslı Derya Kardelen, Zehra Aycan, Semih Bolu, Damla Gökşen, Ayça Törel Ergür, Murat Aydın, Beyhan Tüysüz, Oya Ercan, Olcay Evliyaoğlu, Analysis or Interpretation: Aydilek Dağdeviren Çakır, Şükran Poyrazoğlu, Literature Search: Aydilek Dağdeviren Çakır, Şükran Poyrazoğlu, Writing: Aydilek Dağdeviren Çakır, Şükran Poyrazoğlu.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0285

J Clin Res Pediatr Endocrinol 2021;13(3):320-331

The Application of Next Generation Sequencing Maturity Onset Diabetes of the Young Gene Panel in Turkish Patients from Trakya Region

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What is already known on this topic?

Maturity-onset diabetes of the young (MODY) cases represent 1-2% of all diabetes cases. Since the clinical findings of MODY patients are similar to type 1 and type 2 diabetes, the majority of these patients are mistakenly diagnosed with type 1 or type 2 diabetes mellitus. Despite all the advances of molecular tests, mutations in any known gene may still not be detected in some of the individuals clinically diagnosed with MODY. This indicates that monogenic diabetes is a subject that needs to be investigated more in terms of new genes and new criterias.

What this study adds?

This is the first study of MODY variants in the Trakya region of Turkey. There was a relatively high case frequency for the population in a cohort of 61 patients, 47.5% had variants which were pathogenic/likely pathogenic and a further 18% had variants of uncertain clinical significance. In addition 12 novel variants were detected and are firstly reported here.

Abstract

Objective: The aim of this study was to investigate the molecular basis of maturity-onset diabetes of the young (MODY) by targeted-gene sequencing of 20 genes related to monogenic diabetes, estimate the frequency and describe the clinical characteristics of monogenic diabetes and MODY in the Trakya Region of Turkey.

Methods: A panel of 20 monogenic diabetes related genes were screened in 61 cases. Illumina NextSeq550 system was used for sequencing. Pathogenicity of the variants were assessed by bioinformatics prediction software programs and segregation analyses.

Results: In 29 (47.5%) cases, 31 pathogenic/likely pathogenic variants in the *GCK, ABCC8, KCNJ11, HNF1A, HNF4A* genes and in 11 (18%) cases, 14 variants of uncertain significance (VUS) in the *GCK, RFX6, CEL, PDX1, KCNJ11, HNF1A, G6PC2, GLIS3* and *KLF11* genes were identified. There were six different pathogenic/likely pathogenic variants and six different VUS which were novel.

Conclusion: This is the first study including molecular studies of twenty monogenic diabetes genes in Turkish cases in the Trakya Region. The results showed that pathogenic variants in the *GCK* gene are the leading cause of MODY in our population. A high frequency of novel variants (32.4%-12/37) in the current study, suggests that multiple gene analysis provides accurate genetic diagnosis in MODY.

Keywords: Monogenic diabetes, MODY, NGS, pathogenic variant, novel variant

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Conflict of interest: None declared
Received: 30.11.2020
Accepted: 09.02.2021

Introduction

The types of diabetes associated with single gene defects, including neonatal diabetes, syndromic diabetes and maturity onset diabetes of the young (MODY), are called monogenic diabetes (1). MODY is caused by pathogenic variants in genes responsible for the embryonic development or function of beta cells of the pancreas (2). When it was first defined, autosomal dominant inheritance, a history of diabetes in at least three generations, positive clinical findings before the age of 25, no need for insulin or needing low dose (< 0.5 U/kg), and good metabolic control were determined as diagnostic criteria for MODY (3). However, with the current understanding that MODY is a heterogeneous group and the clinical findings and treatment differ due to the underlying genetic defect, clinical suspicions for MODY pre-diagnosis have been expanded. In addition, due to the fact that some patients who are followed as type 1 or type 2 diabetes are diagnosed with MODY, diabetes autoantibody negativity and absence of insulin resistance findings in obese diabetics are also included in the criteria for MODY.

MODY is responsible for an estimated 1% of all diabetes cases in children and adolescents (2). However, it is very difficult to find the true prevalence as individuals with MODY are mistakenly classified as type 1 or type 2 diabetes. In some studies, while the rate of diagnosis of MODY clinically is 10-20% among all diabetics, pathogenic variants in the known genes are not detected in approximately 20% of patients when genetic testing is performed (2).

There are MODY subtypes related to many different gene defects. The most common pathogenic variants are in the *HNF4A, GCK* and *HNF1A* genes, and these three genes account for about 95% of all MODY cases (4). The clinical spectrum of individuals with MODY can vary considerably according to the underlying genetic problem. For example, GCK-MODY causes mild fasting hyperglycemia, which does not progress and does not require treatment, while HNF1A-MODY or HNF4A-MODY leads to diabetes with progressive beta cell destruction and microvascular complications. Some types of MODY, such as HNF1B-MODY or CEL-MODY, can also be classified as syndromic diabetes, as they are associated with kidney and pancreatic malformations or exocrine pancreatic insufficiency (5).

MODY due to *HNF1A, HNF4A, HNF1B* and *GCK* mutations accounts for most of all known cases (6). The next most common gene variants found in MODY are *ABCC8* and *KCNJ11* mutations, and more rarely, *INS, PDX1, NEUROD1* and *CEL* mutations. While MODY cases due to heterozygous *INS, PDX1, NEUROD1* mutations create isolated clinical diabetes in a few pedigrees, a decrease in fecal elastase and other exocrine pancreatic functions have also been reported in a small number of MODY individuals due to heterozygous *CEL* mutation (7).

Until recently, the diagnosis of MODY was made by scanning the intended candidate gene for point mutations or small insertions/deletions by Sanger sequencing. This method could result in both high cost and a limited number of genes being sequenced. In addition, this method could miss large insertions and deletions. To date, with the advent of the next generation sequencing (NGS) method, many genes can be analyzed together at the same time and at lower cost. Thus, many gene panels have been created and 7-29 genes are analysed simultaneously (8). However, accurate evaluation of the results is very important. In the current study, we aimed to analyse 20 different genes (*ABCC8, BLK, CEL, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11, KLF11, NEUROD1, NKX2-2, PAX4, PDX1, RFX6, ZFP57, GLIS3, FOXP3, NEUROG3, G6PC2*) in cases with a pre-diagnosis of monogenic diabetes using NGS method.

Methods

The patient files of 61 cases with a clinical diagnosis of monogenic diabetes, mostly MODY, who were examined in Pediatric Endocrinology clinic or Medical Genetics clinic were included. For the clinical diagnosis of MODY, the following criterias were considered: diabetes mellitus in a parent and a history of diabetes mellitus in first-degree relatives for at least two generations; negativity for two or more type 1 diabetes mellitus associated autoantibodies [anti-glutamic acid decarboxylase (GAD) antibody, insulin antibody and/or islet cell antibody (ICA)]; low insulin requirement (< 0.5 U/kg/day) and measurable C-peptide level. Written informed consent was obtained from the parents of the patients.

EDTA-blood samples were collected from participants. Genomic DNA was isolated from peripheral blood mononuclear cells using EZ1 DNA Investigator Kit (Qiagen, Hilden, Germany). Primary quality control of the isolated DNA samples was performed using NanoDrop (Thermo Fisher Scientific, Waltham, MA), and samples having A260/280 values between 1.8-2.0 were included in the study.

QIAseq Targeted DNA Panel (Qiagen, Hilden, Germany) was used to analyse 20 genes (*ABCC8, BLK, CEL, GCK, HNF1A, HNF1B, HNF4A, INS, KCNJ11, KLF11, NEUROD1, NKX2-2, PAX4, PDX1, RFX6, ZFP57, GLIS3, FOXP3, NEUROG3, G6PC2*). Libraries were prepared according to manufacturer's instructions. Quality control of the prepared libraries was done with Qubit dsDNA BR Assay system (Invitrogen, Carlsbad, CA, USA). Fastq generation was performed on

Illumina NextSeq550 (Illumina Inc., San Diego, CA, USA). Libraries covering the target genes were prepared according to the QIAseq Targeted DNA Panel protocol (Qiagen, Hilden, Germany). Following the target enrichment process, libraries were sequenced on the Illumina NextSeq550 (Illumina Inc., San Diego, CA, USA). QCI analysis (Qiagen, Hilden, Germany) was used for Quality control and ordering Variant Call Format file. Variant analysis was performed with Ingenuity software (Qiagen, Hilden, Germany).

For segregation analysis, primers were designed for all needed regions and Sanger sequencing was performed using an ABI 3130 (Applied Biosystems, USA) capillary electrophoresis system.

The American College of Medical Genetics 2015 (9) guidelines were followed for the classification of all the variants, recommendations of the Human Genome Variation Society (10) were followed to describe the novel variants. ClinVar (11), HGMD-Professionel 2020 database and literature information were considered for collecting the information about known variants.

Descriptive analysis was done with patient numbers and percentages in the current study.

This study was approved by the Ethical Committee of the Trakya University (approval number: 2020/263, date: 10.08.2020) and performed in consonance with the principles of the Declaration of Helsinki.

Results

Twenty different genes were analysed in 61 unrelated cases with a pre-diagnosis of MODY. The cases had a mean age of 14.93 years and included 33 female cases (mean age 15.1 years) and 28 male cases (mean age 14.7 years). Thirty-two of the patients (52.4%) had an affected family member.

Thirty-one pathogenic/likely pathogenic variants in 29 (47.5%) cases and 14 variants of uncertain significance (VUS) in 11 (18%) cases were detected. Pathogenic/likely pathogenic variants were distributed among the target genes as follows: *GCK* n=24; *ABCC8* n=3; *KCNJ11* n=2; *HNF1A* n=1; and *HNF4A* n=1. Similarly, VUS were identified in: *GCK* n=2; *RFX6* n=3; *CEL* n=2; *PDX1* n=2; *KCNJ11* n=1; *HNF1A* n=1; *G6PC2* n=1: *GLIS3* n=1; and *KLF11* n=1 (Table 1). In total 37 different variants were identified in this study (Table 2).

Six novel pathogenic/likely pathogenic variants, five in the *GCK* gene and one in the *ABCC8* gene were found. In addition, six novel VUS (*RFX6* n=3, *HNF1A* n=1, *CEL* n=1, and *GLIS3* n=1) were detected. Thus, 12 of 37 (32.4%) variants found were novel (Table 2). All variants in this study had been inherited in heterozygously.

Family members of cases 2, 3, 4, 10, 12, 15, 17, and 18 who also had the same variants with the reported cases also had clinical findings consistent with MODY (Table 1). They were all diagnosed as diabetes mellitus 1 or 2 previously. However, due to these moleular results, the diagnoses of these family members could be reconsidered.

Cases 4 and 5 each had two different likely pathogenic variants. Case 4 had *GCK* and *KCNJ11* likely pathogenic variants, which were maternally inherited and *de novo*, respectively. He had been diagnosed with type 1 diabetes mellitus initially. After revealing the maternal history, he had been tested for MODY, and had the correct diagnosis. It is interesting that he had two likely pathogenic variants for MODY type 2 and type 13. However, during follow-up, neither he nor his mother had a complication related with diabetes. Case 5 had *ABCC8* and *GCK* likely pathogenic variants, related with the phenotypes of MODY type 2 and diabetes mellitus, noninsulin dependent. She was diagnosed with incidentally detected high serum glucose level and she had been taking unnecessary insulin because of the diabetes mellitus diagnosis. A MODY test was planned due to the strong family history of diabetes with her mother, sister and grandmother all affected. During follow-up, case 5 also never exhibited complication associated with diabetes.

Discussion

MODY is a rare form of diabetes that has genetic, metabolic and clinical differences and is autosomal dominantly inherited (12). MODY should be considered in the differential diagnosis of patients diagnosed with type 1 or type 2 diabetes but who have clinically atypical findings. MODY diagnosis should be kept in mind in patients diagnosed with type 1 diabetes but with negative pancreatic autoantibodies and/or measurable C-peptide level at the time of diagnosis and/or good glycemic control with low-dose insulin therapy. In addition, the diagnosis of MODY should be considered in patients without obesity and acanthosis nigricans, followed by a diagnosis of type 2 diabetes in the laboratory without signs of insulin resistance. Diagnosis of diabetes in three generations or similar findings in the family is also suggestive for MODY.

Many MODY-related genes have been identified. The most common of these genes are *HNF4A* (MODY1), *GCK* (MODY2), *HNF1A* (MODY3), *IPF1* (MODY4), *HNF1B* (MODY5), *NEUROD1* (MODY6), and *CEL* (MODY8). Glucokinase, the protein product of the *GCK* gene (also called pancreatic β-cell glucose sensor) acts as the key regulatory enzyme in glucose-

induced insulin release. The heterozygous loss-of-function mutation in the *GCK* gene, which may be diagnosed at any age, leads to a slight increase in the glucose threshold value in the glucose-insulin release curve. This results in fasting glucose levels of between 100-153 mg/dL and HbA1c levels do not exceed 7.5% while the increase in glucose level at 0 and 120 min on oral glucose tolerance test (OGTT) does not exceed 90 mg/dL in 95% of cases (13).

In our study, twenty-four cases had a *GCK* pathogenic variant; all were diagnosed due to incidental hyperglycemia. The youngest patient was 9 months old (case 14). Only one patient (case 2) was obese [body mass index (BMI) > 95 P] and the other cases had normal weight. All these cases were negative for ICA although two of them were positive for GAD antibody (cases 18 and 20). At diagnosis, the lowest HbA1c was 6.1% and the highest was 6.7%, (normal range 3.6-5.8). The same mutation was detected in one of the parents of six patients with *GCK* pathogenic variants. Three of the parents (mothers of cases 2, 17 and 18) were diagnosed with gestational diabetes and had to use insulin. All our patients have been managed with a controlled carbohydrate diet. None of them needed medical treatment.

Haliloglu et al (14) selected cases for *GCK* analysis with a pre-diagnosis of MODY in a Turkish population. In 11 cases of 21 probands (52%) a pathogenic/likely pathogenic variant was identified. In our study, *GCK* pathogenic/likely pathogenic variants were detected 39.3% of cases. Pathogenic variants in the *GCK* gene are the most frequent in the literature (15). Since hyperglycemia is mild in these patients, microvascular complications are not observed. Therefore, confirming molecular diagnosis in these patients will prevent unnecessary insulin treatment. Although there is no long-term data on macrovascular complications, it is thought that cardiovascular risk does not increase in these patients (16). In a study, it was reported that GCK-MODY patients' BMI and blood glucose level increased and insulin sensitivity decreased during follow-up (17). Therefore, HbA1c is recommended to be observed once a year for GCK-MODY patients.

The presence of GCK-MODY in pregnant women may also be a clinical concern. A genetically unaffected baby of a mother with GCK-MODY will be born macrosomically. In cases where both mother and baby are GCK-MODY, starting treatment for the mother may lead to the birth of the baby at low weight (18). For this reason, identifying GCK-MODY during pregnancy is important for the problems which may occur for either the mother or the baby.

Another common cause of MODY is heterozygous mutations in the *HNF1A* gene. The *HNF1A* gene is a transcription factor that plays a role in the development, proliferation and death of beta cells, as well as regulating insulin secretion (19). For this reason, individuals with HNF1A-MODY are born normoglycemic, but with advancing age (usually puberty and after) the clinical spectrum progresses due to the onset of beta cell destruction and obvious diabetes develops. With a high penetration, 63% of those carrying the mutation develop diabetes before the age of 25 years and 96% before the age of 55 years. The type and location of the mutation affects the age of onset of diabetes (20). A mutation in the dimerization or binding region of *HNF1A* leads to the development of diabetes 10 years earlier than a mutation in the transactivation region. Although the clinical features of cases varies according to the mutation, there can be phenotypic variability in the same family with genotypic homogeneity. Although ketoacidosis is rare, approximately 25% of patients can present with a type 1 diabetes-like clinical picture. Most present type 2 diabetes-like clinical findings, but patients are generally not obese and there are no signs of insulin resistance (21).

We identified *HNF1A* pathogenic variant in one case (case 12), and *HNF1A* variant of unknown significance in a further case (case 31). Case 31 was 12 years old, at the beginning of the pubertal period, and case 12 was 16 years and 11 months old at the end of the pubertal period. Case 31 had no positive family history of diabetes. In the OGTT test, this patient, had a blood glucose level in the normal range and the HbA1c level was 6%. Case 31 is managed with a controlled carbohydrate diet. Case 12 had a typical positive three-generation family history of diabetes mellitus. These two cases were negative for ICA, GAD antibodies and insulin auto-antibody. At diagnosis, case 12 had an HbA1c level of 8.5% and high blood sugar (fasting blood sugar > 100 mg/dL, postprandial blood sugar > 200 mg/dL), and she is managed with sulfonylurea treatment.

HNF4A-MODY is rarer than HNF1A-MODY and is responsible for approximately 5-10% of all MODY cases. *HNF4A* is also a transcription factor, abnormality of which leads to progressive beta cell destruction, similar to a dysfunctional heterozygous mutation in the *HNF1A* gene. Clinical findings are the same as HNF1A-MODY, and 50% of individuals with *HNF4A* heterozygous mutation have a history of macrosomic delivery, and about 15% have neonatal hyperinsulinemic hypoglycaemia that is diazoxide responsive. Extra-pancreatic laboratory findings in HNF4A-MODY are low high-density lipoprotein, low triglyceride and high low-density lipoprotein (22). We identified a pathogenic variant of *HNF4A* in one case (case 23) with HbA1c of 12% and fasting blood sugar of 237 mg/dL at diagnosis. He was diagnosed as type 1 diabetes and treated with insulin before molecular analysis by MODY panel.

Table 1. Genotypes and phenotypes of the cases and segregation results in the study

Case	Age/Gender	Gene	Variant
1	16/F	*GCK*	ENST00000403799.3:c.387C > A
2	13/F	*GCK*	NM_033507.3:c.1256 + 1G > T (c.1253 + 1G > T)
3	5/F	*GCK*	ENST00000403799.3:c.387C > A
4	16/M	*GCK*	NM_000162.5:c.943C > T
		KCNJ11	NM_000525.3:c.668C > T
5	21/F	*ABCC8*	NM_000352.6:c.3517G > A
		GCK	NM_000162.5:c.943C > T
6	11/F	*GCK*	ENST00000403799.3:c.398T > A
7	7/F	*GCK*	NM_033507.3:c.133G > A
8	3/F	*GCK*	NM_033507.3:c.537delG
9	18/F	*GCK*	NM_000162.5:c.943C > T
10	13/F	*GCK*	ENST00000403799.3:c.387C > A
11	11/F	*GCK*	NM_033507.3:c.867-1G > A
12	16/F	*HNF1A*	NM_000545.8:c.864delGinsCC (c.872dupC)
13	5/F	*ABCC8*	NM_000352.6:c.4014G > A
14	9 months/M	*GCK*	NM_000162.5:c.943C > T
15	3/F	*GCK*	NM_000162.5:c.880G > C
16	8/F	*GCK*	NM_000162.5:c.1248C > G
17	14/M	*GCK*	NM_000162.5:c.746G > A
18	1/F	*GCK*	NM_000162.5:c.667G > A
19	21/M	*GCK*	NM_000162.5:c.506A > G
20	12/F	*GCK*	NM_000162.5:c.565A > G
21	14/M	*GCK*	NM_000162.5:c.565A > G
22	1/M	*GCK*	NM_000162.5:c.617C > T
23	14/M	*HNF4A*	NM_000457.4:c.844G > A
24	33/F	*GCK*	NM_000162.5:c.943C > T
25	28/M	*GCK*	NM_000162.5:c.1222G > T
26	16/M	*ABCC8*	NM_000352.6:c.2768T > G
27	12/F	*KCNJ11*	NM_000525.3:c.481G > A
28	1/M	*GCK*	NM_000162.5:c.115_117delAAG
29	12/F	*GCK*	NM_000162.5:c.214G > A
30	29/M	*KCNJ11*	NM_000525.3:c.1117G > A
31	12/F	*HNF1A*	NM_000545.8:c.1769-3C > T
32	33/M	*G6PC2*	NM_021176.3:c.89C > T
33	13/F	*CEL*	NM_001807.6:c.2184_2216del
		PDX1	NM_000209.4:c.226G > A
34	13/M	*RFX6*	NM_173560.4:c.428G > A
		GCK	NM_000162.5:c.863 + 3A > G
35	33/F	*GCK*	NM_000162.5:c.863 + 3A > G
36	8/M	*CEL*	NM_001807.6:c.2049_2082del
		RFX6	NM_173560.4:c.1072G > A
37	12/F	*RFX6*	NM_173560.4:c.246C > G
38	14/F	*PDX1*	NM_000209.4:c.97C > A
39	18/F	*GLIS3*	NM_001042413.2:c.589G > T
40	2/M	*KLF11*	NM_003597.5:c.1447C > T

ACMG: American College of Medical Genetics, dbSNP: The Single Nucleotide Polymorphism Database, VUS: variants of unknown significance, NA: not applicable, M: male, F: female

Protein	Pathogenicity (ACMG-2015)	Segregation analysis	Phenotype
p.(Cys129Ter)	Pathogenic	NA	MODY type 2
	Pathogenic	Inherited from the affected mother	MODY type 2
p.(Cys129Ter)	Pathogenic	Affected sister has the same variant	MODY type 2
p.(Leu315Phe)	Likely pathogenic	Inherited from the affected mother	MODY type 2
p.(Thr223Ile)	Likely pathogenic	*de novo*	MODY type 13
p.(Val1173Met)	Likely pathogenic	NA	Diabetes mellitus, noninsulin dependent
p.(Leu315Phe)	Likely pathogenic	NA	MODY type 2
p.(Phe133Tyr)	Likely pathogenic	NA	MODY type 2
p.(Gly45Ser)	Likely pathogenic	NA	MODY type 2
p.(Asn180ThrfsTer25)	Pathogenic	NA	MODY type 2
p.(Leu315Phe)	Likely pathogenic	NA	MODY type 2
p.(Cys129Ter)	Pathogenic	Inherited from the affected mother	MODY type 2
	Pathogenic	NA	MODY type 2
p.(Gly292ArgfsTer25)	Pathogenic	Inherited from the affected mother	MODY type 3
p.(Trp1338Ter)	Pathogenic	NA	Diabetes mellitus, noninsulin dependent
p.(Leu315Phe)	Likely pathogenic	NA	MODY type 2
p.(Gly294Arg)	Likely pathogenic	Inherited from the affected mother	MODY type 2
p.(His416Gln)	Likely pathogenic	NA	MODY type 2
p.(Gly249Asp)	Likely pathogenic	Inherited from the affected mother	MODY type 2
p.(Gly223Ser)	Pathogenic	Inherited from the affected mother	MODY type 2
p.(Lys169Arg)	Likely pathogenic	NA	MODY type 2
p.(Ile189Val)	Likely pathogenic	NA	MODY type 2
p.(Ile189Val)	Likely pathogenic	NA	MODY type 2
p.(Thr206Met)	Likely pathogenic	NA	MODY type 2
p.(Asp282Asn)	Likely pathogenic	NA	
p.(Leu315Phe)	Likely pathogenic	NA	MODY type 2
p.(Val408Leu)	Likely pathogenic	NA	MODY type 2
p.(Leu923Arg)	Likely pathogenic	NA	Diabetes mellitus, noninsulin dependent
p.(Ala161Thr)	Likely pathogenic	NA	MODY type 13
p.(Lys39del)	Likely pathogenic	NA	MODY type 2
p.(Gly72Arg)	Pathogenic	NA	MODY type 2
p.(Val373Met)	VUS	NA	MODY type 13
	VUS	NA	MODY type 3
p.(Ser30Phe)	VUS	NA	NA
p.(Gly729_Thr739del)	VUS	NA	MODY type 8
p.(Asp76Asn)	VUS	NA	MODY type 4
p.(Cys143Tyr)	VUS	NA	Mitchell-Riley syndrome
	VUS	NA	MODY type 2
	VUS	NA	MODY type 2
p.(Thr684ArgfsTer9)	VUS	NA	MODY type 8
p.(Val358Ile)	VUS	NA	Mitchell-Riley syndrome
p.(Asn82Lys)	VUS	NA	Mitchell-Riley syndrome
p.(Pro33Thr)	VUS	NA	MODY type 4
p.(Asp197Tyr)	VUS	NA	Diabetes mellitus, neonatal, with congenital hypothyroidism
p.(Pro483Ser)	VUS	NA	MODY type 7

Table 2. ***In silico*** **predictions and previous database access number information of each variant identified in this study**

Gene (transcript ID)	Variant	Variant type	Chr position (hg19)	dbSNP
GCK	NM_000162.5:c.115_117delAAG p.(Lys39del)	In frame	7:44192991	NA
	NM_033507.3:c.133G > A p.(Gly45Ser)	Missense	7:44192978	rs267601516
	NM_000162.5:c.214G > A p.(Gly72Arg)	Missense	7:44192019	rs193922289
	ENST00000403799.3:c.387C > A p.(Cys129Ter)	Nonsense	7:44190651	rs1583601365
	ENST00000403799.3:c.398T > A p.(Phe133Tyr)	Missense	7:44190640	NA
	NM_000162.5:c.506A > G p.(Lys169Arg)	Missense	7:44189641	NA
	NM_033507.3:c.537delG p.(Asn180ThrfsTer25)	Frameshift	7:44189613	NA
	NM_000162.5:c.565A > G p.(Ile189Val)	Missense	7:44189582	rs757978639
	NM_000162.5:c.617C > T p.(Thr206Met)	Missense	7:44189421	rs1441649062
	NM_000162.5:c.667G > A p.(Gly223Ser)	Missense	7:44189371	rs1360415315
	NM_000162.5:c.746G > A p.(Gly249Asp)	Missense	7:44187366	NA
	NM_000162.5:c.863 + 3A > G	Splicing	7:44187246	rs193922334
	NM_033507.3:c.867-1G > A	Splicing	7:44186218	rs1167675604
	NM_000162.5:c.880G > C p.(Gly294Arg)	Missense	7:44186201	NA
	NM_000162.5:c.943C > T p.(Leu315Phe)	Missense	7:44186138	rs1583594350
	NM_000162.5:c.1222G > T p.(Val408Leu)	Missense	7:44185127	NA
	NM_000162.5:c.1248C > G p.(His416Gln)	Missense	7:44185101	NA
	NM_033507.3:c.1256 + 1G > T	Splicing	7:44185095	NA
ABCC8 **(NM_000352.6)**	c.2768T > G p.(Leu923Arg)	Missense	11:17429991	NA
	c.3517G > A p.(Val1173Met)	Missense	11:17426099	rs141322087
	c.4014G > A p.(Trp1338Ter)	Nonsense	11:17418568	NA
HNF1A **(NM_000545.8)**	c.864delGinsCC p.(Gly292ArgfsTer25)	Frameshift	12:121432117	rs1593058932
	c.1769-3C > T	Splicing	12:121438865	NA
KCNJ11 **(NM_000525.3)**	c.481G > A p.(Ala161Thr)	Missense	11:17409158	rs1363707190
	c.668C > T p.(Thr223Ile)	Missense	11:17408971	rs561086953
	c.1117G > A p.(Val373Met)	Missense	11:17408522	rs770375846

ClinVar variation ID	HGMD	SIFT	DANN	GERP	Mutation taster	Classification (ACMG-2015)
NA	CD191970	NA	NA	5.07	NA	Likely pathogenic (PM1, PM2, PM4, PP3)
76898	CM013265	Damaging	0.9989	5.07	Disease causing	Pathogenic (PS1, PM1, PM2, PM5, PP2, PP3, PP5)
36209	CM023383	Damaging	0.9983	4.67	Disease causing	Pathogenic (PS1, PM1, PM2, PP2, PP3, PP5)
804846	CM012111	NA	0.9939	4.94	NA	Pathogenic (PVS1, PM2, PP3, PP5)
Novel	Novel	Damaging	0.9906	5.05	Disease causing	Likely pathogenic (PM1, PM2, PM5, PP2, PP3)
NA	CM141531	Damaging	0.9991	5.69	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3, PP5)
Novel	Novel	NA	NA	5.79	NA	Pathogenic (PVS1, PM2, PP3)
NA	NA	Tolerated	0.9986	5.82	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
NA	CM012122	Damaging	0.9993	5.96	Disease causing	Pathogenic (PM1, PM2, PM5, PP2, PP3, PP5)
435306	CM012123	Damaging	0.9992	6.17	Disease causing	Pathogenic (PS1, PM1, PM2, PP2, PP3, PP5)
Novel	Novel	Tolerated	0.9986	5.23	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
36261	NA	NA	0.9673	5.5	NA	VUS (PM2, BP4)
804861	NA	NA	0.9943	4.59	Disease causing	Pathogenic (PVS1, PM2, PP3, PP5)
Novel	Novel	NA	0.9991	4.76	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
804863	CM064013	Damaging	0.9985	4.59	Disease causing	Likely pathogenic (PM1, PM2, PM5, PP2, PP3)
NA	CM171422	Damaging	0.9955	5.57	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
Novel	Novel	Damaging	0.994	5.57	Disease causing	Likely pathogenic (PM1, PM2, PM5, PP2, PP3)
NA	CS032698	NA	0.9949	5.57	Disease causing	Pathogenic (PVS1, PM2, PP3)
Novel	Novel	Damaging	0.9981	6.03	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
35609	NA	Tolerated	0.9944	5.32	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP5)
NA	CM994652	NA	0.9945	4.76	Disease causing	Pathogenic (PVS1, PM2, PP3)
817605	CX1310026	NA	NA	4.15	NA	Pathogenic (PVS1, PM2, PP5)
Novel	Novel	NA	0.9708	6.05	NA	VUS (PM2)
NA	NA	Damaging	0.9993	4.92	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
NA	NA	Damaging	0.9988	5.28	Disease causing	Likely pathogenic (PS2, PM2, PP2, PP3)
RCV001280332.1	NA	Tolerated	0.9367	5.42	Disease causing	VUS (PM2, PP2, BP4)

Table 2. Continued

Gene (transcript ID)	Variant	Variant type	Chr position (hg19)	dbSNP
HNF4A **(NM_000457.4)**	c.844G > A p.(Asp282Asn)	Missense	20:43048468	rs1236613475
CEL **(NM_001807.6)**	c.2049_2082del p.(Thr684ArgfsTer9)	Frameshift	9:135946938	NA
	c.2184_2216del p.(Gly729_Thr739del)	In frame	9:135947057	rs756606428
PDX1 **(NM_000209.4)**	c.97C > A p.(Pro33Thr)	Missense	13:28494372	rs192902098
	c.226G > A p.(Asp76Asn)	Missense	13:28494501	rs137852783
RFX6 **(NM_173560.4)**	c.246C > G p.(Asn82Lys)	Missense	6:117198981	NA
	c.428G > A p.(Cys143Tyr)	Missense	6:117201754	NA
	c.1072G > A p.(Val358Ile)	Missense	6:117240349	NA
G6PC2 **(NM_021176.3)**	c.89C > T p.(Ser30Phe)	Missense	2:169757930	rs142189264
GLIS3 **(NM_001042413.2)**	c.589G > T p.(Asp197Tyr)	Missense	9:4125741	NA
KLF11 **(NM_003597.5)**	c.1447C > T p.(Pro483Ser)	Missense	2:10192542	rs761563032

VUS: variants of uncertain significance, NA: not applicable, dbSNP: The Single Nucleotide Polymorphism Database

Gain of function mutations in the *ABCC8* and *KCNJ11* gene are known to cause temporary or permanent neonatal diabetes. In addition, dysfunctional mutations lead to congenital hyperinsulinism. However, some children have been shown to develop diabetes years after remission of neonatal hyperinsulinism. A mutation in the *ABCC8* or *KCNJ11* gene, which results in a serious clinical situation in the neonatal period, may exhibit a more moderate clinical picture, such as type 2 diabetes, gestational diabetes, or impaired glucose tolerance in other family members carrying the same mutation (23). Moreover, patients with mild hyperglycemia due to *ABCC8* pathogenic variants and no family history or neonatal diabetes history were also identified. The mechanism underlying these two gene-dependent variable clinical situations is still unknown. Case 13 presented with abdominal pain, nausea, and vomiting. Hyperglycemia was detected at 214 mg/dL. She had also a high postprandial blood sugar and a diabetes mellitus family history. Case 26 had clinical findings indicative of diabetes mellitus, but there was a very strong family history with his mother, grandmother, mother's uncles and aunts all having a diagnosis of diabetes mellitus.

A diagnosis of MODY has many clinical benefits for both the family and the patient. Diagnosing GCK-MODY will eliminate unnecessary treatment (insulin or oral antidiabetic) due to accidental classification of type 1 diabetes, or mostly type 2 diabetes in adults, and will affect the patient's quality of life. In addition, the absence of complications in GCK-MODY will prevent unnecessary visits by providing more comfortable follow-up for the patient's diabetes.

Individuals with *HNF1A* or HNF4A-MODY can stop unnecessary insulin treatment once the diagnosis is confirmed and achieve better metabolic control with oral sulfanylurea. In addition, genetic diagnosis with MODY enables early diagnosis of affected individuals in the family and all patients may be closely followed up in terms of complications. Again, individuals with HNF1A-MODY have a 5-10% risk of hepatic adenomatosis (24). Therefore, genetic diagnosis enables the patient to be followed in this respect.

If the pathogenic variants of individuals with neonatal hyperinsulinemic hypoglycemia is found in *HNF4A* or *ABCC8/KCNJ11* genes, it also allows genetic counseling to be given in terms of MODY screening of the family and the risk

ClinVar variation ID	HGMD	SIFT	DANN	GERP	Mutation taster	Classification (ACMG-2015)
NA	NA	Damaging	0.9993	4.98	Disease causing	Likely pathogenic (PM1, PM2, PP2, PP3)
Novel	Novel	NA	NA	1.34	NA	VUS (PVS1)
NA	NA	NA	NA	2.67	NA	VUS (PM2)
36414	CM056344	Damaging	0.9977	5.46	Disease causing	VUS (PM2, PP2, PP3)
8859	CM992901	Damaging	0.9982	4.96	Disease causing	VUS (PM2, PP2)
Novel	Novel	Tolerated	0.9171	5.36	Polymorphism	VUS (PM2)
Novel	Novel	Damaging	0.9981	5.61	Disease causing	VUS (PM1, PM2, PP3, BP1)
Novel	Novel	Tolerated	0.9951	6.07	Disease causing	VUS (PM2, BP1)
NA	NA	Damaging	0.9982	5.42	Disease causing	VUS (PP3, BS1)
Novel	Novel	Damaging	0.9885	5.26	Disease causing	VUS (PM2, PP3, BP1)
NA	NA	Tolerated	0.9953	5.78	Disease causing	VUS (PM2)

for future pregnancies (25). In our study there were three pathogenic/likely pathogenic variants in *ABCC8* gene and two pathogenic/likely pathogenic variants in *KCNJ11* gene. Case 4 had both *GCK* and *KCNJ11* and case 5 had both *GCK* and *ABCC8* pathogenic/likely pathogenic variants. Notably, cases 4 and 5 had both received a diagnosis of type 1 diabetes mellitus previously. The mother of case 4 and the mother, sister and grandmother of case 5 also had a diagnosis of diabetes mellitus. Both case 4 and case 5 had shown mild clinical signs while being followed-up for diabetes.

A diagnosis of HNF1B-MODY in an individual who was being followed-up with the diagnosis of diabetes suggests that care should be taken when assessing for extrapancreatic findings that accompany and/or may occur and requires multidisciplinary planning of their follow-up (26). There were no *HNF1B* pathogenic variants detected in our cohort.

Six of our cases were found to harbor novel pathogenic variants in *GCK* and *HNF1A* genes. If only known variants are analyzed in suspicious cases, many novel variants are at risk of being missed. In our study, analyzing all the exons of the genes increased the rate of diagnosis.

There are 14 known genes for MODY (*HNF4A, GCK, HNF1A, PDX1, HNF1B, NEUROD1, KLF11, CEL, PAX4, INS, BLK, ABCC8, KCNJ11, APPL1*) (27). We analysed these genes, with the exception of *APPL1*, and included extra seven genes (*NKX2-2, RFX6, ZFP57, GLIS3, FOXP3, NEUROG3, G6PC2*) to exclude similar phenotypes.

After determining the molecular diagnosis in the index case, first and second degree relatives should be informed that they are at risk for monogenic diabetes, this type of diabetes can affect their general health, and that their treatment is different depending on the subtype. Molecular analysis can also be planned in asymptomatic cases with a family history, depending on consent. Genetic counselling was given to all cases and their families in this study.

Study Limitations

A number of limitations of the present study should be noted. Firstly, the number of cases should be higher to provide more robust results. This was a cross-sectional study, which was performed in a region of our country with a low density population. Secondly, MODY type 14 could

not be investigated due to the *APPL1* gene was not included in the targeted gene panel in the current study. However, variants in the *APPL1* gene cause less than 1 % of all MODY types.

Conclusion

As new genes are identified through developing more widespread and more comprehensive molecular testing, the category of monogenic diabetes is expected to expand gradually. Suspicion of monogenic diabetes in patients diagnosed with type 1 or type 2 diabetes mellitus but showing atypical course, and confirming the diagnosis with appropriate molecular tests plays a key role in providing appropriate treatment for their condition. Molecular diagnosis is of great importance in terms of identifying the most appropriate treatment, and this will affect the prognosis of the patient, allow provision of genetic counseling and prompt screening of individuals at risk.

Acknowledgments

We would like to thank the subjects who participated in the study.

Ethics

Ethics Committee Approval: The study were approved by the Trakya University of Local Ethics Committee (approval number: 2020/263, date: 10.08.2020).

Informed Consent: The written informed consent forms were obtained from the parents of the patients.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sinem Yalçıntepe, Fatma Özgüç Çömlek, Hakan Gürkan, Filiz Tütüncüler Kökenli, Concept: Sinem Yalçıntepe, Fatma Özgüç Çömlek, Hakan Gürkan, Design: Sinem Yalçıntepe, Hakan Gürkan, Filiz Tütüncüler Kökenli, Data Collection or Processing: Sinem Yalçıntepe, Fatma Özgüç Çömlek, Selma Demir, Emine İkbal Atlı, Engin Atlı, Damla Eker, Filiz Tütüncüler Kökenli, Analysis or Interpretation: Sinem Yalçıntepe, Hakan Gürkan, Selma Demir, Emine İkbal Atlı, Engin Atlı, Damla Eker, Literature Search: Sinem Yalçıntepe, Writing: Sinem Yalçıntepe, Fatma Özgüç Çömlek.

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

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2021.2020.0238

J Clin Res Pediatr Endocrinol 2021;13(3):332-341

Evaluation of Growth Hormone Results in Different Diagnosis and Trend Over 10 Year of Follow-up: A Single Center Experience

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What is already known on this topic?

Growth hormone (GH) treatment has long been used in rare diseases such as isolated GH deficiency (IGHD), multiple pituitary hormone deficiency (MPHD), small for gestational age (SGA), and Turner syndrome (TS). Early diagnosis and early initiation of GH treatment are important to optimize the effects of treatment.

What this study adds?

Although there are larger series in the literature, our study is one of the largest single-center patient series performed after the The Pfizer International Growth Study database was terminated. GH treatment onset age was late in our cohort and no differences have been observed in the last 10 years. The improvement in the height standard deviation score was seen most in the IGHD and MPHD groups, the least in the TS and SGA groups, the patients' treatment compliance was high (92%) and the incidence of side effects was low (2.7%).

Abstract

Objective: The aim was to evaluate the results of diagnosis, follow-up and treatment of the patients who recieved growth hormone (GH) treatment for the last 10 years and to determine the differences in the process and results over the years.

Methods: Anthropometric, clinical, laboratory data, treatment adherence and side effects were evaluated retrospectively in 767 patients who recieved GH treatment between 2009-2018. Patients were grouped as isolated GH deficiency (IGHD), multiple pituitary hormone deficiency (MPHD), small for gestational age (SGA), and Turner syndrome (TS) depending on diagnosis.

Results: GH treatment was started in 689 cases (89.8%) with IGHD, 24 (3.1%) with MPHD, 26 (3.4%) with SGA and 28 (3.7%) with TS. Median age of GH treatment onset was the earliest in SGA (8.4 years) and the latest in the IGHD group (12.0 years). At the time of treatment cessation, height standard deviation score (SDS) in IGHD and MPHD was significantly higher than treatment initiation time, whereas there was no significant difference in TS and SGA. One hundred eighty-nine cases reached the final height. Final heights for girls/boys were: IGHD 154/164.9 cm; MPHD 156.2/163.5 cm; TS 146.7 cm; and SGA 145.7/-cm, respectively. Target height SDS-final height SDS median values were IGHD: 0.1, MPHD: 0.6, SGA: 0.5, TS: 2.4 respectively. The patients' treatment compliance was high (92%) and the incidence of side effects was low (2.7%).

Conclusion: In our cohort, GH treatment start age was late and no difference in this was observed in the last 10 years. The improvement in the height SDS was most marked in the IGHD and MPHD groups, the least in the TS and SGA groups.

Keywords: Rare disease, growth hormone treatment, follow-up

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Conflict of interest: None declared

Received: 13.10.2020

Accepted: 25.02.2021

Introduction

The introduction of recombinant human growth hormone (GH) in 1985 ended the phase of pituitary-derived human GH and its associated limitations and risks, opening the possibility of widespread clinical use (1). GH treatment has long been used in rare diseases, such as isolated GH deficiency (IGHD), multiple pitutiary hormone deficiency, (MPHD), small for gestational age (SGA), and Turner syndrome (TS). Today, it is also used in other indications such as chronic renal failure, SHOX deficiency, Prader-Willi syndrome and idiopathic short stature, besides GH deficiency (GHD) (2).

The foremost aims of GH treatment in children are the normalization of height during childhood, attainment of a timely and normal pubertal growth and the achievement of an adult height that is normal for the population and genetic target, in conjunction with normalization of other aspects, such as body composition, metabolism and quality of life (1). In all pediatric indications, early diagnosis and early initiation of GH treatment are important to optimize the effects of treatment.

The Pfizer International Growth Study (KIGS) (3), the National Cooperative Growth Study (4) and the NordiNet International Outcome Study (5), are multicenter, international databases created to monitor the efficacy and safety of GH treatment. The advantage of these databases is to create a standardized, common platform for uniform documentation of data on GH treatment in centers participating in the database, potentially reveal differences between clinics, and offer the possibility of reliable observation of potentially rare results due to the large number of participants (6). The KIGS database, in which data entries were made from many centers in our country and where we evaluate the treatment results of patients with GH treatment was terminated approximately 10 years ago. However, there are no new outputs regarding the diagnosis and treatment processes of these diseases in our country in recent years. In this study, we aimed to determine the follow-up, and treatment results and final heights of patients with rare diseases who had been treated with GH treatment in the last 10 years, and to determine the differences in the process and results over the years.

Methods

In the present study, 767 patients who had received GH treatment in Ankara Dr. Sami Ulus Obstetrics and Gynecology, Children's Health and Disease Training and Research Hospital between 2009 and 2018 were recruited. The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by a Ankara Keçiören Training and Research Hospital Local Ethics Committee (no: 1686, date: 23.05.2018). Anthropometric, clinical, laboratory findings, treatment adherence and side effects of patients during, admission, GH treatment initiation time, follow-up, and GH treatment cessation time were evaluated retrospectively. Patients were grouped by diagnosis as IGHD, MPHD, TS, and SGA.

After systemic disease screening, at least two different GH stimulation tests were performed in patients with pathological short stature whose growth rate $<25^{th}$ percentile and height standard deviation score (SDS) <-2.5. Apart from these, height SDS >-2.5 but with a growth rate below -2 SDS in the last year or below -1.5 SDS in the last two years, cases thought to have GHD, and patients with a regression in growth rate for more than six months clinically and genetically confirmed TS were also evaluated (7,8). Before the last change in the social security institution regulation regarding TS and SGA patients, two different GH tests were required from all patients in order to pay for GH treatment in our country. Since TS and SGA patients included in the study were diagnosed before these changes related to these patients, all patient groups, including TS and SGA cases, were administered a GH stimulation test.

GHD was defined as <10 ng/mL serum peak GH concentration (7). It was required that the bone age should be at least 2 years retarded compared to chronologic age in the prepubertal period, and the epiphyseal plates were open in puberty. In addition, male and female subjects were primed with sex steroids prior to provocative GH testing, particularly in the patients with delayed puberty. For both boys and girls, 2 mg β-oestradiol (1 mg for body weight <20 kg) (not ethinyl oestradiol) was administered orally on each of the two evenings preceding the test, while boys were also given intramuscular testosterone (50-100 mg of a depot formulation administered one week before the test). Puberty was defined as breast development ≥2 Tanner stage in girls and testicular volume ≥4 mL in boys (9).

IGHD was defined as a condition of GHD not associated with other pituitary hormone deficiencies. MPHD was defined as a deficiency of at least two pituitary hormones, with one being GH. SGA was defined as birth weight less than -2 SDS for gestational age. TS was defined as females who have partial or complete absence of the second sex chromosome with a variety of phenotypic features.

All measurements were calculated with the reference developed for Turkish children and expressed as SDS (10,11). Target (mid-parental) height was calculated by adding 6.5 cm to the mean of the parents' heights for boys

or by subtracting 6.5 cm from the mean of the parents' heights for girls (12). If those who reached the final height were within range of ±5 cm of the target height, they were considered as having reached target height.

After GH tests were evaluated, organic pathology that may accompany cases with GH deficiency was evaluated by performing pituitary magnetic resonance imaging (MRI). GH was administered subcutaneously at a dose range of 0.2-0.4 mg/kg/week, six days per week. According to the rules of the social security institution in our country, the GH treatment is discontinued when height reaches 155 cm in girls and 165 cm in boys. In addition, GH treatment was discontinued if the annual growth rate was <2 cm and/or bone age was ≥16 in boys and ≥14 in girls (7).

GH product, type of injection device, dosage, GH storage conditions, number of missed injections, reasons for missed injections, person administering daily GH injections and problems in follow-up were recorded at each visit and patients' compliance was evaluated. Adherence categories were established following the criteria of Smith et al (13), and patients were categorized into one of four compliance groups, based on the percent of doses omitted at each evaluation period: excellent if 0%, good if 5%, fair if 5 to 10%, and poor if >10%. Patients in the poor category were considered to be incompatible with treatment.

Statistical Analysis

The Predictive Analytics Software 18, (2009) program was used for statistical analysis. The conditions where the type-1 error level was below 5% were interpreted statistically. Kolmogorov-Smirnov and Shapiro-Wilk tests were used for assessment of normality of distribution of the data. In descriptive statistics, categorical variables are expressed as number and percentage, and numerical variables are presented as median, and minimum and maximum values. Student's t-test was used to compare two groups when the parametric test prerequisites were met and Mann-Whitney U test was used otherwise. The Friedman test was used to examine the change in the age, bone age, height SDS, body mass index (BMI) SDS, puberty, and follow-up time, GH treatment initiation time, and GH treatment cessation time separately in all patients and groups. The Wilcoxon signed-ranks test was used in post-hoc analysis. Bonferroni correction was used in post-hoc analysis whenever appropriate. In all patients and in the IGHD group, the Kruskal-Wallis test was used for comparison analysis of numerical values between date groups. To evaluate the relationship between final height-SDS and target height SDS, first year growth velocity, treatment duration, age at GH treatment initiation, bone age, height SDS, puberty, gender, and concentrations of insulin-like growth factor-1 (IGF-1), IGF binding protein 3 (IGFBP3), multiple linear regression analysis was performed with the backward method. A p value <0.05 was considered statistically significant.

Results

The median age of patients (63% male) who were admitted to the clinic due to short stature was 10.4 years at first visit. GH treatment was started in 689 cases (89.8%) with IGHD, 24 (3.1%) with MPHD, 26 (3.4%) with SGA, and 28 (3.7%) with TS. The median age of GH treatment start was 12.0 years, the earliest was in SGA (8.4 years) and the latest was in IGHD (12.0 years). When the age of first admission to the hospital and the GH treatment onset age were compared by year, it was found that there was no difference between them at the beginning or at the end of the 10 years study period ($p>0.05$) (Table 1).

The height SDS at the GH treatment initiation time was below <-2.5 in the entire group and subgroups. The lowest height SDS was in the MPHD group, and the height SDS values of TS, and SGA groups were lower than the IGHD group. The lowest peak response to GH tests was in the MPHD, IGHD, and TS groups, respectively. The lowest serum IGF-1 and IGFBP3 values were in the MPHD group. The serum IGF-1 level was <-2 SDS in 330 (43.9%) patients, between -2 SDS and -1 SDS in 380 (50.5%) patients, and >-1 SDS in 42 (5.6%) patients (Table 2).

The pituitary MRI was pathological in 27.1% of the patients, and the most common accompanying pathology was pituitary hypoplasia (60%). Various pathologies, including pituitary hypoplasia, ectopic neurohypophysis, microadenoma/suspected microadenoma, empty sella, partial empty sella, Rathke cleft cyst, and arachnoid cyst, were detected in 25.9% of patients with IGHD and 78.3% of patients with MPHD. Patients with suspected microadenoma and microadenoma underwent neurosurgery consultation before GH treatment. In none of the cases, organic pathology that could interfere with GH treatment was found on MRI.

The median follow-up time without treatment was 11 months and the median follow-up time with treatment was 2.1 years. The longest duration of treatment was in the MPHD group at 3.8 (range, 0.3-9) years, and the shortest duration of treatment was in the IGHD group at 2 (range, 0.3-10.8) years. The median treatment dose was 0.2 (range, 0.2-0.4) mg/kg/week in the entire group and subgroups, while it was 0.3 mg/kg/week in the TS group. During the treatment, the changes in patients' GH dose were minimal (7.0%) and the

doses of GH were adjusted in relation to weight, elevation of IGF-1 concentration or changes in glucose metabolism.

Growth velocity was highest in the first year of treatment in the entire group and subgroups, and gradually decreased in the following years. The median value of the first-year growth velocity was 8.2 cm/year in entire group, while it was 9.8 cm/year in MPHD, 8.3 cm/year in IGHD, 7.8 cm/year in TS and 7.1 cm/year in SGA.

Table 1. Age at presentation and growth hormone therapy initiation age by year

year	Age at admission-entire group		Age at start of treatment-entire group		Age at presentation IGHD group		Age at start of treatment IGHD group	
	n	Median (min-max)	n	Median (min-max)	n	Median (min-max)	n	Median (min-max)
2009	23	9.9 (3.6-17)	23	11.5 (4-17.3)	18	10.1 (4-17)	18	11.5 (4.2-17.3)
2010	84	9.7 (0.1-15.4)	84	11.7 (4.6-16.3)	68	10.2 (2.9-15.4)	68	12 (4.6-16.3)
2011	98	10.9 (1.8-16.6)	98	12 (5.1-17)	92	10.8 (1.8-16.6)	92	12 (5.1-17)
2012	86	10.7 (0.2-15.6)	86	1.8 (0.8-16.4)	77	10.8 (0.6-15.6)	77	11.8 (0.8-16.4)
2013	104	11 (0-16.8)	104	12 (1.4-16.9)	95	11.1 (1-16.8)	95	12.1 (1.4-16.9)
2014	93	11.1 (0-15.6)	93	12.2 (2.1-17)	88	11.2 (0.5-15.6)	88	12.2 (2.1-16)
2015	83	10.5 (0-15.8)	83	12 (3.1-16.5)	78	10.7 (2-15.8)	78	12.2 (3.1-16.5)
2016	70	9.1 (0.3-15.1)	70	11.8 (2-16.4)	60	9.2 (0.8-15.1)	60	12 (2.5-16.4)
2017	87	9.3 (0-16)	87	11.7 (3.1-16.6)	80	9.8 (0-16)	80	11.7 (3.1-16.6)
2018	39	11.3 (2.7-15.7)	39	12.8 (3.7-16)	33	11.6 (3.3-15.7)	33	12.8 (5.1-16)
p	0.091		0.232		0.294		0.472	

IGHD: isolated growth hormone deficiency, min-max: minimum-maximum

Table 2. Anthropometric and laboratory features of patients at start of growth hormone therapy

	Entire group (n = 767)	IGHD (n = 689)	MPHD (n = 24)	SGA (n = 26)	TS (n = 28)
Chronologic age (years)	12 (0.83-17.3)	12.0 (0.83-17.3)	9.3 (1.8-17)	8.4 (3.0-14.4)	10.6 (2.4-15.4)
Bone age (years)	9 (0.5-15)	10 (0.5-15)	5 (0.5-13.5)	5.3 (1.1-13.5)	8.1 (2-13)
Sex n/% (female) (male)	289 (37.7)	240 (34.8)	6 (25)	15 (57.7)	28(100)
	478 (62.3)	449 (65.2)	18 (75)	11 (42.3)	0 (0)
Birth weights SDS	0.09 (-3.30-3.19)	0.11 (-1.97-3.19)	0.09 (-1.96-1.42)	-2.45 (-3.30- -2.02)	-0.47 (-1.72-1.17)
Height SDS	-3 (-8.5 - -1.0)	-2.9 (-8.5 - -1.7)	-3.8 (-7.8 - -1)	-3.4 (-5.9 - -2.5)	-3.4 (-6.9 - -1.82)
BMI SDS	-0.8 (-6.3-3.6)	-0.9 (-6.3-3.6)	0.2 (-4-3.2)	-1.21 (-2.8-1.7)	0.7 (-2.8-1.9)
Tanner stage	1 (1-5)	1 (1-5)	1 (1-2)	1 (1-5)	1 (1-2)
L-dopa-peak GH (ng/mL)	3.86 (0.01-18.9)	3.86 (0.01-9.88)	0.48 (0.07-9.6)	9.5 (0.46-18.9)	3.66 (0.55-11.4)
Clonidine peak GH (ng/mL)	5.05 (0-25.1)	5.05 (0.02-9.74)	0.52 (0.13-9.11)	11.36 (0-25.1)	5.19 (0.73-10.1)
ITT peak GH (ng/mL)	1.8 (0-10.4)	1.8 (0.04-9.67)	0.3 (0-1.7)	6.39 (1.1-10.4)	2.46 (0.28-7.66)
Serum IGF-1 (ng/mL)	146.3 (11.5-555)	148 (11.5-555)	50.1 (16.9-231)	117 (37.3-222)	147 (43.3-375)
IGF-1 SD <-2 (n/%)	330 (43.9%)	303 (44.6%)	16 (80%)	7 (26.9%)	4 (15.4)
IGF-1 SD -1 to -2 (%) (n/%)	380 (50.5%)	340 (50%)	4 (20%)	17 (65.4%)	19 (73.1)
IGF-1 SD >-1 (%) (n/%)	42 (5.6)	37 (5.4%)	0 (0%)	2 (7.7%)	3 (11.5)
Serum IGFBP3 (ng/mL)	3840 (49.4-8800)	3890 (49.4-8800)	1470 (500-5570)	3245.5 (1800-6120)	3759 (1340-6780)
IGFBP3 SD <-2 (n/%)	83 (11.1%)	72 (10.5%)	11 (55%)	0 (0%)	1 (3.9)
IGFBP3 SD -1 to -2 (n/%)	489 (65.5%)	454 (67.3%)	7 (35%)	14 (53.8%)	14 (53.8)
IGFBP3 SD >-1 (n/%)	175 (23.4%)	150 (22.2%)	2 (10%)	12 (46.2%)	11 (42.3)

IGHD: isolated growth hormone (GH) deficiency, MPHD: multiple pituitary hormone deficiency, SGA: small for gestational age, TS: Turner syndrome, SDS: standard deviation (SD) score, BMI: body mass index, ITT: insulin tolerance test, IGF-1: insulin-like growth factor-1, IGFBP3: insulin-like growth factor-binding protein 3, median (minimum-maximum)

Considering GH treatment cessation time, height SDSs in IGHD and MPHD groups were significantly higher than at treatment start ($p < 0.001$), whereas there was no significant difference in TS ($p = 0.225$) and SGA groups ($p = 0.191$). In the same period, no statistically significant difference was found in terms of the BMI SDS in the subgroups, except for the IGHD group (Table 3).

In total 189 patients reached final height; by diagnosis subgroup this was IGHD n = 166, TS n = 11, MPHD n = 8, SGA n = 4. Except for the TS and SGA groups, the percentage of patients reaching final height was higher in boys. In groups outside TS and SGA, final height SDSs were above -2 SDS. Final height for girls/boys were as follows: IGHD: 154/164.9 cm, MPHD: 156.2/163.5 cm, TS:146.7 (range, 133-156.4) cm, and SGA:145.7 (range, 136.7-150.3) cm. Of the 166 IGHD patients who reached final height, 104 (67.5%) were found to reach their target height. Target height SDS-final height SDS was the greatest in the TS group and the proportion reaching final height was the lowest in the TS

Table 3. Anthropometric and clinical findings of patients at first presentation, growth hormone therapy start and growth hormone therapy cessation

		n	Admission median (min-max)	GH treatment start time median (min-max)	GH treatment offset time median (min-max)	p
Entire Group A groups	Age (years)	499	11.2 (0-17)[bc]	12.2 (0.8-17.3)[ac]	15.1 (2.9-21)[ab]	< 0.001*
	Bone age (years)	449	8.1 (0-15)[bc]	10 (0.8-15)[ac]	14 (1-17)[ab]	< 0.001*
	Height SDS	499	-2.9 (-8.5-1.8)[bc]	-3 (-8.5--1.7)[ac]	-2 (-7.2-1)[ab]	< 0.001*
	BMI SDS	498	-0.9 (-5.5-4)[c]	-1 (-6.3-3.6)	-0.8 (-10.2-4.2)[b]	0.005*
	Puberty	495	1 (1-5)[c]	1 (1-5)[c]	4 (1-5)[ab]	< 0.001*
	Follow-up (year)	767	-	0.9 (0-12.5)	2.1 (0.3-10.8)	< 0.001*
IGHD	Age (years)	453	11.4 (0.6-17)[bc]	12.3 (0.8-17.3)[ac]	15.1 (2.9-19)[ab]	< 0.001*
	Bone age (years)	407	8.1 (0-15)[bc]	10 (0.8-15)[ac]	14 (1-17)[ab]	< 0.001*
	Height SDS	453	-2.8 (-8.5--0.2)[bc]	-3 (-8.5--1.7)[ac]	-1.9 (-7.1-1)[ab]	< 0.001*
	BMI SDS	452	-0.9 (-4.3-3.5)	-1 (-6.3-3.6)[c]	-0.8 (-10.2-4.2)[b]	0.011*
	Puberty	449	1 (1-5)[c]	1 (1-5)[c]	4 (1-5)[ab]	< 0.001*
	Follow-up (year)	689	-	0.9 (0-12.5)	2.1 (0.3-10.8)	< 0.001*
MPHD	Age (years)	15	7.2 (0-14.9)[bc]	9.9 (1.8-17)[ac]	16.3 (3.8-21)[ab]	< 0.001*
	Bone age (years)	13	5 (1-11)[c]	6 (2.9-13.5)[c]	14 (7-17)[ab]	< 0.001*
	Height SDS	15	-3.5 (-5.98- -0.97)[bc]	-3.84 (-6.08- -2.18)[ac]	-1.69 (-6.3- -0.24)[ab ab]	< 0.001*
	BMI SDS	15	-0.2 (-2.4-1.9)	-0.52 (-2.39-1.91)	-0.16 (-3.38-1.54)	0.207
	Puberty	15	1 (1-2)[c]	1 (1-2)[c]	3 (1-5)[ab]	0.007*
	Follow-up (year)	24	-	1.5 (0-6.8)	3.8 (0.3-9)	0.023*
SGA	Age (years)	14	9.01 (0.19-14.01)[bc]	10.35 (3.01-14.4)[ac]	13.91 (5.1-17.3)[ab]	< 0.001*
	Bone age (years)	13	8.1 (1.6-13.6)[c]	9 (1.06-13.5)[c]	14 (3-16)[ab]	< 0.001*
	Height SDS	14	-3.87 (-5.81- -2.6)	-3.84 (-5.87- -2.49)	-3.11 (-7.2- -1.9)	0.191
	BMI SDS	14	-1.2 (-3.7-4)	-1.67 (-2.64-0.97)	-1.21 (-3.08-1.82)	0.257
	Puberty	17	1 (1-3)[c]	1 (1-3)[c]	2 (1-5)[ab]	0.040*
	Follow-up (year)	26	-	1.5 (0.1-5.3)	2.4 (0.5-8.3)	0.038*
TS	Age (years)	17	9.4 (0-13.6)[bc]	11.11 (7-13.8)[ac]	14.6 (7.3-17)[ab]	< 0.001*
	Bone age (years)	16	7.6 (0.5-13)[c]	8.1 (5-13)[c]	13.5 (10-15)[ab]	< 0.001*
	Height SDS	17	-3.42 (-4.33-1.79)	-3.5 (-4.33 - -1.95)	-2.85 (-5.2- -1.1)	0.225
	BMI SDS	17	0.7 (-5.5-1.7)	0.75 (-1.22-1.9)	0.4 (-1.16-2.41)	0.814
	Puberty	17	1 (1-2)[c]	1 (1-2)[c]	4 (1-5)[ab]	0.001*
	Follow-up (year)	28	-	0.5 (0-11)	2.8 (0.3-6.3)	0.008*

[a]: different from admission time, [b]: different from GH treatment start-time, [c]: different from GH treatment cessation-time, *: $p < 0.05$.
SDS: standard deviation (SD) score, BMI: body mass index, IGHD: isolated growth hormone (GH) deficiency, MPHD: multiple pituitary hormone deficiency, SGA: small for gestational age, TS: Turner syndrome, min-max: minimum-maximum

group (Table 4). The change in height SDS of the patients from the beginning of treatment to the final height is given in Figure 1.

Of our IGHD patients who reached their final height, 93 (56.0%) were prepubertal and 73 (44%) were pubertal at the beginning of GH treatment. At the time of initiation of GH treatment, the age and bone age of pubertal IGHD patients were significantly higher than in prepubertal IGHD patients ($p < 0.001$). The duration of treatment was longer in prepubertal IGHD patients than in pubertal patients ($p < 0.001$) (Table 5). There was no statistically significant difference between prepubertal and pubertal IGHD patients in terms of height SDS, BMI SDS, final height SDS, target height-SDS, first year growth velocity and treatment dose.

In multiple linear regression analysis, GH treatment start time height SDS, target height SDS, first year growth velocity and puberty status were predictive factors for final height SDS (Table 6).

Patients' compliance with treatment was high (92%), and treatment was interrupted in 16% of patients due to problems in compliance with treatment during treatment,

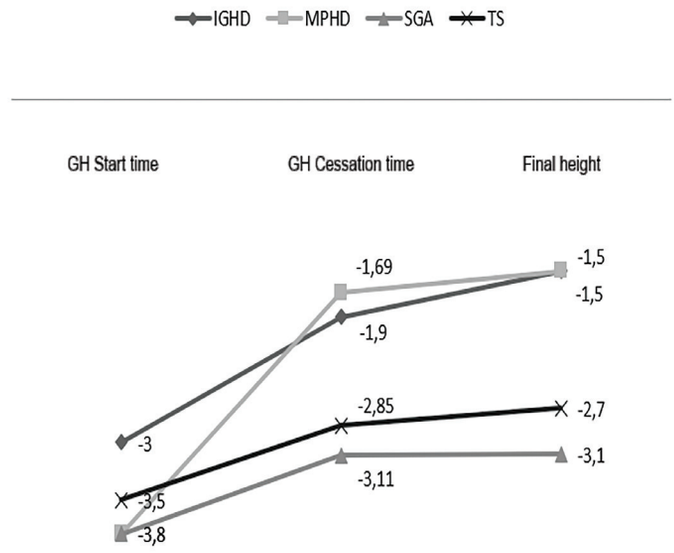

Figure 1. Graph of changes in height standard deviation score values of the groups

IGHD: isolated growth hormone deficiency, GH: growth hormone, SGA: small for gestational age, MPHD: multiple pituitary hormone deficiency, TS: Turner syndrome

Table 4. Descriptive analysis of patients reaching final height

	Entire group (n = 189)	IGHD (n = 166)	MPHD (n = 8)	SGA (n = 4)	TS (n = 11)
Age (years)	17 (12.7-23)	17 (12.7-23)	17.8 (14.6-19.6)	16.3 (14.9-18)	16.4 (15-18)
Bone age (years)	16 (12.5-17.4)	16 (13.6-17.4)	16 (12.5-16)	16	-
Sex					
Female	95 (50.3)	77 (46.4)	3 (37.5)	4 (100)	11 (100)
Male	94 (49.7)	89 (53.6)	5 (62.5)	0 (0)	0 (0)
FH (girl)	153.3 (133-170.8)	154 (137.5-170.8)	156.2 (150-160.6)	145.7 (136.7-150.3)	146.7 (133-156.4)
FH (boy)	164.9 (146.7-173.2)	164.9 (152-173.2)	163.5 (146.7-171.8)	-	-
FH SDS	-1.6 (-4.6-0.7)	-1.5 (-3.5-0.7)	-1.5 (-2.5--0.4)	-3.1 (-4.5--2.1)	-2.7 (-4.6--1.2)
TH SDS	-1.5 (-3.4-0.5)	-1.5 (-3.4-0.5)	-1.1 (-1.8--0.3)	-2 (-3.4--1.06)	-1.2 (-1.7-0.1)
TH-FH SDS	0.2 (-2.3-3.4)	0.1 (-2.3-3)	0.6 (-1.2-1.8)	0.5 (-0.1-2.8)	2.4 (0.4-3.4)
BMI	20.4 (15-37.6)	20.1 (15-33)	23.4 (16.7-26.6)	19.3 (18.9-20.7)	24.2 (19.2-37.6)
BMI SDS	-0.6 (-4.2-4.3)	-0.7 (-4.2-3.8)	0.5 (-3.1-1.6)	-0.6 (-1.5-1.5)	0.7 (-1.1-4.3)
Puberty	5 (2-5)	5 (3-5)	4 (2-5)	5 (4-5)	5 (3-5)
Age at start of treatment	12.4 (4-17.3)	12.5 (5.4-17.3)	9 (4-17)	12.3 (12.1-13.9)	11.1 (8.4-13.2)
Duration of treatment (year)	2.9 (0.2-12)	2.8 (0.2-12)	6 (1.5-9)	1.9 (1-4)	4.5 (1.3-6.3)
Treatment dose (mg/kg/wk)	0.2 (0.2-0.4)	0.2 (0.2-0.3)	0.2 (0.2-0.2)	0.2 (0.2-0.3)	0.3 (0.2-0.4)
Not reaching target height	62 (35.8)	50 (32.5)	3 (42.9)	2 (50)	7 (87.5)
Reaching target height	111 (64.2)	104 (67.5)	4 (57.1)	2 (50)	1 (12.5)

SDS: standard deviation score, BMI: body mass index, IGHD: isolated growth hormone deficiency, MPHD: multiple pituitary hormone deficiency, SGA: small for gestational age, TS: Turner syndrome, TH: target height, FH: final height, wk: week, FH: final height, TH: target height

low growth rate, and high IGF-1. Treatment incompatibility was lowest in the IGHD group and highest in the SGA group. Adverse effects were seen in 2.7% (n = 21) of our patients. These side effects were; significant creatinine kinase elevation (n = 8), scoliosis (n = 5), cardiac causes (n = 2 with one each of subaortic segmental hypertrophy and left ventricular hypertrophy), orthopedic causes including slipped capital femoral epiphysis (n = 1) and Osgood-Schlatter's disease (n = 1), non-injection site rash (n = 2), disorders of glucose metabolism (n = 1, impaired fasting glucose) and malignancy (n = 1, osteochondroma). Both patients with cardiac side effects were in the IGHD group and neither had syndromic features. Scoliosis, slipped capital femoral epiphysis and impaired fasting glucose were thought to be related to GH treatment. Scoliosis was newly developed in four cases and an increase in existing scoliosis in one case. Our patient with malignancy was followed up because of TS, the total treatment duration was 2.92 years, and the treatment dose was 0.3 mg/kg/week. It was found that the patient, whose treatment was discontinued after malignancy was detected, did not continue with her subsequent follow-ups.

Discussion

Our study, which is the second largest of pediatric patients receiving GH from Turkey, after the Turkey KIGS Database analysis published in 2004 with 1008 patients, evaluated etiology and treatment outcomes (14). In our study, in keeping with earlier reports, the highest proportion of patients were in the IGHD group and patients were mostly male (14,15).

It has been shown that the age at initiation of GH treatment is correlated negatively with the response to treatment, which emphasizes the need for early diagnosis and treatment (7). In a recently study by Sävendahl et al (16) data from the American Norditropin Studies: Web-Enabled Research Program (ANSWER-USA) and the NordiNet International Outcome Study (NordiNet IOS-Europe) were compared. Growth hormone initiation age in GHD, TS, and SGA patients were 11.09, 8.92 and 9.0 years in the ANSWER trial, respectively, while it was 9.12, 8.72 and 7.92 years in the NORDINET-IOS trial, respectively. The authors concluded that starting age of GH therapy was higher in all indications in the USA. Pfäffle et al (17) reported that the age of initiation of treatment was similar between the USA and Germany, but higher in the indications in France. Data

Table 5. Comparison of isolated growth hormone deficiency patients reaching final height according to their puberty status at the beginning of treatment

	Prepubertal (n = 93) median (min-max)	Pubertal (n = 73) median (min-max)	p
Age (years)	12 (5.4-14.4)	14 (11.11-17.3)	< 0.001
Bone age (years)	8.1 (3-10)	12 (10.50-15.00)	< 0.001
Height SDS	-3.00 (-5.54--2.39)	-2.81 (-5.10--1.70)	0.140
BMI SDS	-1.16 (-3.91-1.98)	-1.14 (-5.03-1.70)	0.912
TH SDS	-1.63 (-3.38-0.13)	-1.23 (-3.03-0.49)	0.052
FH SDS	-1.59 (-3.10--0.13)	-1.42 (-3.5-0.70)	0.444
First year growth velocity (cm)	8.5 (3.9-11.8)	8.7 (2.0-11.6)	0.867
Duration of treatment (year)	3.5 (1.4-9)	2.00 (0.3-4.8)	< 0.001
Treatment dose (mg/kg/wk)	0.2 (0.2-0.3)	0.2 (0.2-0.3)	0.335

SDS: standard deviation score, BMI: body mass index, TH: target height, FH: final height, wk: week, min-max: minimum-maximum

Table 6. Multiple linear regression analysis on final height standard deviation score

$R^2 = 0.377$ $p < 0.001$

Variable	B	SE	Beta	t	p
TH SDS	0.161	0.080	0.139	2.019	0.045
Bone age (year)	-0.078	0.042	-0.195	-1.847	0.067
Height SDS at GH treatment start	0.482	0.093	0.358	5.199	< 0.001
Puberty	-0.168	0.076	-0.161	-2.209	0.029
First year growt velocity (cm/year)	0.144	0.035	0.278	4.105	< 0.001

SDS: standard deviation score, TH: target height, GH: growth hormone, SE: standard error

from these different analyses show that the average age at the start of GH treatment is higher than desired worldwide.

In the study in which patients were registered in the KIGS database in Turkey and were treated with GH, the age at onset of GH treatment was 11.3 years (14), and 11.2 ± 2.67 years in the study performed by Soyöz and Dündar (18). In our study, the median age at onset of treatment was 12.0 years; the age at onset of treatment was oldest in the IGHD group and youngest in the SGA group, and there was no difference in the ages at first presentation and at initiation of treatment in the last 10 years. Our findings show that despite the increase in health awareness and easier access to health services in recent years, age at onset of GH treatment is still late in our cohort.

In our study, the highest growth velocity in the first year of treatment was in the MPHD and IGHD groups, besides height SDS was -3.0 and -3.84 in patients in the IGHD and MPHD groups at the GH treatment onset time, while the final height SDS was -1.5 in both of these groups. In previous studies from Turkey final height SDSs in IGHD and MPHD were found to be -1.8 and -1.6 by Kurnaz et al (19) and -1.4 and -1.1 by Darendeliler et al (20). The final height SDSs in the IGHD and MPHD groups in our study, with a similar dose range but shorter median treatment time, were similar to other studies from our country. It was thought that the better response in our patients in the MPHD group was associated with lower IGF-1 and peak GH values in the GH stimulation tests, as well as lower chronological age and bone age at the beginning of treatment compared with patients with IGHD.

The effect of GH treatment on final height in TS is variable and many factors, such as polymorphisms associated with the GH receptor and/or *IGFBP3* gene, age at the beginning of treatment, dose of GH, duration of treatment, bone age retardation, maternal X chromosome origin, first year response to target height, and oxandrolone treatment affect the treatment response (21,22,23). The *IGFBP3* gene promoter region contains several single nucleotide polymorphisms (SNPs). The 202 A/C SNP which located 202 bp upstream of the transcription start site consists of an A to C nucleotide change and is correlated with serum IGFBP-3 concentrations in healthy adults. Serum IGFBP-3 levels are highest in patients with the AA genotype, followed by the AC and CC genotypes (24). An association of the A allele in the IGFBP-3 promoter region with increased IGFBP-3 concentration and growth velocity after GH therapy has been observed in prepubertal children with GHD and TS (25,26).

Recently Ahn et al (27), in a study of 73 patients with TS, reported that the height SDS at the beginning was correlated with final height SDS, and that early treatment was very important. Evaluation of the data of 70 TS patients registered from 11 centers in Turkey in the KIGS database who received GH at a dose of 33 µg/kg/d subcutaneously, 6-7 times per week, with onset of therapy at age 12.5 (7.1-15.6) years revealed a non-significant increase in growth velocity 6.3 cm/year in the first year and 5.9 cm/year in the second year (28). In another study in which 842 patients with TS were evaluated with the participation of 35 centers from our country, it was reported that the average age to diagnosis with TS was 10.5 ± 4.8 years and that treatment was initiated at the age of 10.7 ± 3.5 year (29). In our study, the age at onset of treatment, the dose of treatment, and the first year response to treatment in patients with TS were consistent with earlier studies from our country, and although there was no significant difference in terms of height SDS between GH treatment initiation and cessation times, the rate of reaching the target height was the lowest in the TS group. We hypothesize that this was due to the age at onset of treatment being late in our patients and that the height SDS at the beginning of treatment were significantly lower.

GH treatment in infants with SGA is effective in the correction of body composition and improvement of metabolic complications, in addition to its contribution to stature in adulthood (30). The dose recommended by the Pediatric Endocrinology and Growth Hormone Research Society in children with SGA is 35-70 mg/kg/day, and higher doses are recommended for patients with severe growth retardation. Treatment dose, age at initiation, height at initiation of treatment, and mid-parental height are among the factors affecting the response of GH in children with SGA (31). The multidisciplinary follow-up of many of the SGA cases by other departments in our hospital has caused these patients to be referred to our clinic earlier and to start treatment earlier because of earlier diagnosis of growth disorders. However, there was no significant difference between GH treatment initiation and cessation in terms of height SDSs in the SGA group and the final height SDS was the lowest in the SGA group. These findings were thought to be due to the fact that the doses used in the SGA group were at the lower limit of the recommended dose and were associated with a treatment mismatch in this group.

In this study, although the chronological age and bone age were higher in the pubertal IGHD patients and the duration of GH treatment was longer in the prepubertal IGHD patients, there was no statistically significant difference between the two groups in terms of final height SDS. Similarly, Kurnaz et al's (19) study did not show a difference in final height SDS

of prepubertal and pubertal patients, but it was reported that delta height SDS was higher in pubertal patients (20). These results suggest that even if the GH treatment is initiated at pubertal age, it may be beneficial in achieving a final height compatible with the genetic potential together with the pubertal growth spurt.

Finally, our results justify the incorporation of height SDS at the beginning of treatment, target height SDS, and first-year response to treatment as major parameters in all predictive models of final height in all GH-treated children (21,32,33).

Study Limitations

The main limitations of this study are that it was designed retrospectively and the number of patients who could be evaluated in terms of final height was low.

Conclusion

This study has shown that GH treatment was started late in the entire group and there was no improvement in the 10 year study period. It was observed that patients who were admitted with short stature received GH treatment approximately 1.5 years later and this is likely to have negatively affected treatment responses. We suggest that efforts should be made to reduce the period between first presentation at the pediatric endocrinology clinic and initiation of GH therapy, if indicated. As a result of late start of GH treatment, improvement in the height SDSs of SGA and TS groups was minimal. In the IGHD group, it was seen that approximately 68% of those who reached final height also achieved the target height. Treatment compliance of patients receiving GH treatment was high.

Although our results cannot be generalized for the whole country, we believe that GH treatment probably does not show significant regional difference, the data obtained from large patient series are important, and in this context, our study may reflect the current situation in GH treatment in our country. Therefore, the results of this study suggest that clinical awareness of causes of short stature should be improved, diagnosis in patients with pathological short stature should be more rapid, the period between first presentation at pediatric endocrinology and initiation of GH therapy should be shortened and that in all children who would benefit from GH therapy, treatment should be started at earlier ages.

Ethics

Ethics Committee Approval: The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by a Ankara Keçiören Training and Research Hospital Local Ethics Committee (no: 1686, date: 23.05.2018).

Informed Consent: The study was retrospective and no interventions were used. Therefore we did not obtain informed consent from the patients or their parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Medical Practices: Aslıhan Araslı Yılmaz, Servet Yel, Zehra Aycan, Concept: Zehra Aycan, Şenay Savaş-Erdeve, Semra Çetinkaya, Design: Zehra Aycan, Şenay Savaş-Erdeve, Semra Çetinkaya, Data Collection or Processing: Aslıhan Araslı Yılmaz, Servet Yel, Zehra Aycan, Analysis or Interpretation: Aslıhan Araslı Yılmaz, Servet Yel, Zehra Aycan, Şenay Savaş-Erdeve, Semra Çetinkaya, Literature Search: Aslıhan Araslı Yılmaz, Zehra Aycan, Writing: Aslıhan Araslı Yılmaz, Zehra Aycan.

Financial Disclosure: This study was unrequitedly funded by Pfizer Turkey.

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0051

J Clin Res Pediatr Endocrinol 2021;13(3):342-346

Vandetanib in a Child Affected by Neurofibromatosis Type 1 and Medullary Thyroid Carcinoma with Both *NF1* and Homozygous *RET* Proto-oncogen Germ-line Mutations

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What is already known on this topic?

Medullary thyroid carcinoma or C-cell hyperplasia are usually associated with other endocrine tumors or a patients with multiple endocrine neoplasia type 2 clinical findings. Germline mutations in both *NF1* and *RET* proto-oncogene have been reported only in a patient with thyroid C-cell hyperplasia. Although vandetanib is frequently used in thyroid medullary carcinoma in the adult age group, there is little data regarding its use in the childhood age group.

What this study adds?

This is the first report the presence of a double germline mutation involving both *NF1* and *RET* genes and treated with vandetanib.

Abstract

Cases of neurofibromatosis type 1 (NF1)-associated medullary thyroid carcinoma (MTC) or C-cell hyperplasia are rarely associated with other endocrine tumors or cases with a multiple endocrine neoplasia type 2. In these patients, mutations were detected in the *NF1* gene but no mutations were detected in the *RET* gene. Although vandetanib has been shown to improve progression-free survival in adults with advanced MTC, data in pediatric patients are limited. Herein, we report the use and outcome of vandetanib in a pediatric MTC case in which *NF1* gene and *RET* proto-oncogen mutation were identified together.
Keywords: Medullary thyroid carcinoma, vandetanib, *RET* proto-oncogene, *NF1* gene, children

Introduction

Neurofibromatosis type 1 (NF1) is a common, autosomal dominant, multi-systemic neurocutaneous disorder. An increased frequency of various endocrine pathologies, such as central precocious puberty, short stature, diencephalic syndrome, growth hormone deficiency or growth hormone hypersecretion has been reported in children. In addition, pheochromocytoma, parathyroid carcinoma, parathyroid adenoma, somatostatin producing neuroendocrine tumor, duodenal carcinoid tumor producing somatostatin, thyroid papillary carcinoma with pheochromocytoma have been described in patients with NF1 (1,2,3).

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumour arising from the calcitonin producing parafollicular C-cells of the thyroid. It accounts for approximately 1-2% of all thyroid cancers. Clinically, 70-80% of MTCs are sporadic, while 20-30% are inherited in an autosomal dominant

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Conflict of interest: None declared
Received: 24.03.2020
Accepted: 01.07.2020

fashion (4). Hereditary MTCs may be part of multiple endocrine neoplasia type 2 (MEN) and occur in three different clinical forms as MEN2A, MEN2B and familial MTC. Mutated "REarrangement during Transfection" (*RET*) proto-oncogene plays a very significant role in the development of human neuroendocrine tumors and tumor syndromes. *RET* proto-oncogene mutation has been reported in both sporadic and familial cases (4).

Cases of NF1-associated MTC or C-cell hyperplasia are rarely associated with other endocrine tumors or with MEN2 clinical findings. In these patients, mutations were detected in the *NF1* gene but no mutations were detected in the *RET* gene (5,6,7,8,9,10,11,12).

To our knowledge, germline mutations in both *NF1* and *RET* proto-oncogene have been reported only in one patient with thyroid C-cell hyperplasia, but no simultaneous mutation of these two genes in MTC has been reported (12).

In this article, we present a 15-year-old male patient diagnosed with both NF1 and MTC, and also had mutations in both *NF1* and *RET* genes, and will discuss the effectiveness of vandetanib therapy in MTC.

Case Report

A 15-year-old boy was admitted to a hospital with progressively increasing midline neck swelling for two months. Physical examination revealed a firm and mobile 3×2 cm swelling on the left side of the neck, multiple lymph nodes, multiple café-au-lait macules, and inguinal and axillary freckling. Lisch nodule was detected in the eye examination. Cranial magnetic resonance imaging showed focal areas of signal intensity and bilateral optic glioma. Family history revealed that his brother and mother had similar findings and also that his mother had a diagnosis of neurofibromas. Cervical ultrasonography and computed tomography showed a heterogeneous mass lesion in the left thyroid lobe and multiple lymphadenopathy. Total blood count and biochemical analysis were within normal range. Thyroid-stimulating hormone, free triiodothyronine and free thyroxine levels were 21.038 µIU/mL (0.35-5.5 µIU/mL), 6.28 pmol/L (8-22 pmol/L) and 14.76 pmol/L (0.83-1.43 ng/dL), respectively. After the cervical nodes were resected, pathological investigation demonstrated metastasis of MTC, and the patient was referred to the department of pediatric surgery. Preoperative calcitonin and carcinoembryonic antigen (CEA) levels were 2000 pg/mL (0-8.4 pg/mL) and 116.52 ng/mL (0-2.5 ng/mL), respectively. The patient was admitted to our department after total thyroidectomy and radical neck dissection. He was investigated for MEN syndrome. Serum parathyroid hormone, serum gastrin, 24-hour urinary catecolamine and metanephrine levels were within normal range.

Histopathologic examination showed MTC with presence of perineural and lymphovascular invasion (Figure 1A-1F). Due to the residual thyroid tissue and bilateral pathological lymph nodes detected on Tc99m-pertechnetate thyroid scintigraphy and positron emission tomography, the patient was re-operated but excision was incomplete. The patient's stage was T2N1aM0 (stage 3) according to the tumor node metastasis system proposed by the "American Joint Committee on Cancer" (13). Molecular testing revealed a heterozygous mutation in *NF1* gene [IVS38-2A>G (c.5610-2A>G) both in our patient and his brother. There was no NF1-related mutation in his mother. In addition, homozygous *RET* proto-oncogene mutation [c.2671T>G (p.S891A) (p.Ser891Ala)] was found in the patient with a heterozygous mutation in his mother, father and brother (Figure 2). As a result of incomplete removal of lymph nodes and remaining thyroid tissue, serum calcitonin level was 1563 pg/mL and serum CEA level was 57.28 ng/mL. Vandetanib treatment was initiated at a dose of 300 mg/day. Serum calcitonin levels at the sixth, twelfth and twenty-fourth months of treatment were 34.7 pg/mL, 4.4 pg/mL and 1.2 pg/mL, while CEA levels were 12 ng/mL, 3.2 ng/

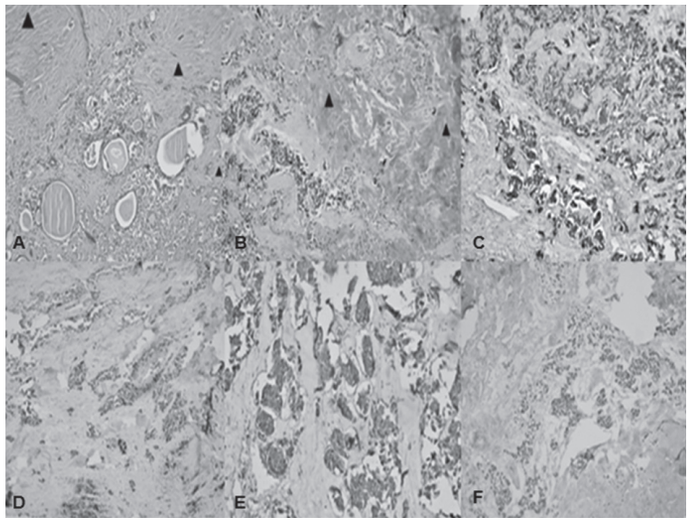

Figure 1. (A) Large areas of amyloid deposits (black triangle) can be seen around and between tumor cells and thyroid follicules (Hematoxylin and eosin x100), **(B)** Large deposits of amyloid (black triangle) in thyroid parenchyma (Congo Red x100), **(C)** Tumor cells showed positive immunoreactivity against monoclonal carcinoembryonic antigen (CEA) antibodies (CEA x200), **(D)** Tumor cells showed positive immunoreactivity against monoclonal calcitonin antibodies (Calcitonine x100), **(E)** Tumor cells showed positive immunoreactivity against monoclonal chromogranin antibodies (Chromogranin x200), **(F)** Tumor cells showed positive immunoreactivity against monoclonal TTF-1 antibodies (TTF-1 x100)

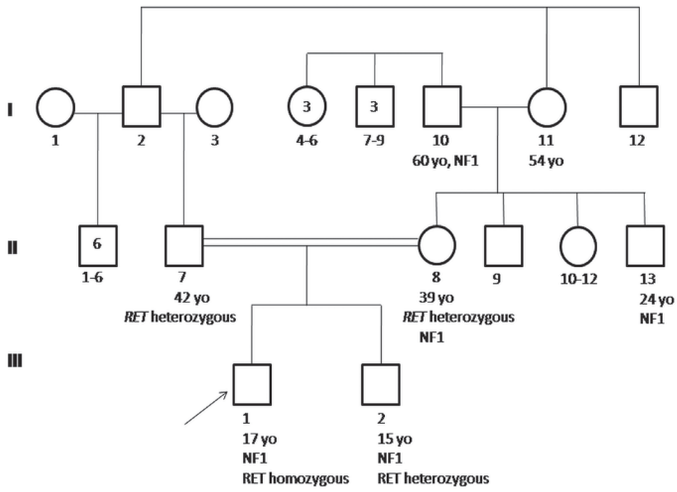

Figure 2. The pedigree of the family with *NF1* and *RET* mutations; the arrow (III-1) indicates the proband

mL and 1.1 ng/mL, respectively. The patient has been on vandetanib treatment for 32 months and no residual tissue and lymphadenopathy were detected in the neck tomography taken at the 30th month of the treatment. No side effects were observed during vandetanib treatment in our patient in this period.

Discussion

Besides leukemia, somatic *NF1* mutations have been reported in various cancers occurring in many different regions such as breast, colorectum, urothelium, lung, ovary, skin and nerve tissues (14). In addition, pheochromocytoma, parathyroid carcinoma, somatostatin producing neuroendocrine tumors, duodenal carcinoid tumors, and thyroid papillary carcinoma are reported endocrine neoplasms in patients with NF1. In these patients, it can be assumed that *NF1* mutations predispose to the development of endocrine tumors by affecting the growth and differentiation of parafollicular C cells, parathyroid cells and other cells from which different endocrine tumors develop.

To our knowledge, MTC has not been reported in patients with NF1 mutation. In these patients, the cause of this association is unclear because no germ-line mutation in the *RET* gene could be demonstrated. Mutations in both the *NF1* and *RET* genes have been described to date only in one case with thyroid C cell hyperplasia (12). Our case is remarkable since it is the first case of MTC in which both *NF1* gene and *RET* proto-oncogen mutation were identified simultaneously.

MTC is a rare tumor originating from the parafollicular or C-cells of the thyroid gland. MTC is sporadic in 75% of patients and usually occurs in the fourth to sixth decade of life. Less commonly, hereditary MTCs are found in MEN2A or MEN2B or as a part of familial MTC (15). *RET* proto-oncogene mutation is detected in almost all hereditary cases and in more than 40% of sporadic cases (15).

In our patient, homozygous mutation was detected in codon 891 in the *RET* gene. The p.S891A mutation was first described by Hofstra et al (16) in 1997 and associated with MEN 2A and MTC. Less than 5% of all MTC patients reported to date have RET mutationorrected as p.S891A and this mutation is reported to be heterozygous because *RET* oncogene, acts dominantly as is usual in oncogenes (17). Giacché et al (18) analyzed 251 relatives of individuals with 28 p.S891A mutations and reported that 108 had asymptomatic carriage and 64 had undergone thyroidectomy. As a result of histological examination, they reported that the mean age of patients with C-cell hyperplasia, micro-MTC and MTC was 30.2 ± 13.7, 37.9 ± 10.3 and 55.0 ± 14.7, respectively, and that malignancy development increased with age in individuals carrying the p.S891A mutation. As a result of the ItaMEN study, in which the germline RET mutations of 250 families with hereditary MTC were evaluated, the p.S891A mutation was present in 9.2% and was lower than other European studies (19). Also Schulte et al (20) stated that they found p.S891A mutation in 5% of patients followed up for MEN 2A. According to our knowledge there is only one study about the frequency of p.S891A mutation in Turkish patients (21). In this study 12 different RET oncogen mutations were detected in 32 of 155 patients who were diagnosed with isolated MTC or as part of MEN2, and p.S891A mutation was reported in two patients (6%). In this case the mutation was homozygous and, to our knowledge, it was not reported previously. The p.S891A mutation poses a moderate risk for MTC development according to the American Thyroid Association (ATA). The recommended ATA approach in individuals with moderate-risk *RET* mutations is to follow annual calcitonin and perform a total thyroidectomy when high values are detected (22). The mothers, fathers and siblings of our patient with heterozygous p.S891A mutations did not have any symptoms, pathological examination or laboratory findings in favor of cancer. Since the mother and father carry a moderately risky mutation according to ATA criteria and MTC risk will increase in later years, prophylactic thyroidectomy was recommended but they did not accept. Similarly, the family preferred long-term follow-up instead of total thyroidectomy for the other child with a heterozygous p.S891A mutation.

The *NF1* gene encodes neurofibromin, a GTPase-activating protein that negatively regulates the Ras/mitogen-activated protein kinase (MAPK) signalling pathway (23). Loss-of-

function mutations in *NF1* lead to uncontrollable activation of kinase and tumorigenesis. Also, the *RET* protooncogene encodes a receptor tyrosine kinase that mediates extracellular neurotropic signaling to intracellular transduction pathways including the MAPK/ERK pathway (24). We think that these two diseases occurred coincidentally, because MTC in our patient does not have a common etiological pathway with the NF1, according to the evidence concerning both the *NF1* gene and the *RET* oncogene.

Our patient was investigated for MEN2 due to *RET* proto oncogene mutation and MTC. In the family history, we learned that there were no patients with thyroid disease and therefore operated. *RET* proto-oncogene mutation analysis of the parents revealed that they were carriers of germ-line S891A mutation. On three-generation pedigree analysis no family member with cancer, including MTC, was reported. Although we could not perform the molecular testing of the *RET* gene for the rest of the family, since they lived in different cities, we believe the maternal grandmother and paternal grandfather to be carriers because they were siblings and the case had a homozygous mutation. The case was thought to be familial MTC although there was no clinical or laboratory finding in any of the heterozygotes, despite the mutation in the family; and currently follow-up was performed without prophylactic thyroidectomy.

In the follow-up of our patient, it was thought that vandetanib treatment would be appropriate, since residual tissue was still present after the second operation. Vandetanib is an orally available tyrosine kinase inhibitor that targets vascular endothelial growth factor dependent tumor angiogenesis and epidermal growth factor receptor, *RET* and *RET* dependent tumor cell proliferation (25). Several studies have evaluated the efficacy of vandetanib in the treatment of advanced MTC. In the ZETA trial, 331 patients with 5% local advanced stage and 95% metastatic MTC were randomized to vandetanib and placebo. At the end of the study, it was determined that the median survival of 19.3 months in the placebo group and the median 30.5 months in the vandetanib group were progression-free survival and a significant difference was found between the two groups (26). In a meta-analysis, 300 mg of vandetanib treatment was demonstrated to have a better objective response than 150 mg of vandetanib treatment (27). When compared to 150 mg and 300 mg vandetanib treatments, Hu et al (28) showed that administration of 300 mg increased overall response rate.

The efficacy of vandetanib in childhood and adolescence was investigated in 16 patients aged 5-18 years with locally advanced or metastatic MEN2B-associated MTC. In this study, the dose of vandetanib was 100 mg/m^2. M918T *RET* germline mutation was present in 15 patients and 7 of them (47%) had a partial response (29). Kraft et al (30) reported that the duration of vandetanib therapy was 6.1 (0.1-9.7+) years in children treated with vandetanib, which lasted a median of 7.4 years and that progression-free survival was 6.7 years. Our patient has been receiving vandetanib for two years and serum calcitonin and CEA levels gradually decreased and reached the normal reference range. Since the dose we administer is higher than the dose in other pediatric studies and our patient is 15 years old, we think that the 300 mg/day dose stated in adult studies may have contributed to our good response. Also, considering that vandetanib suppresses *RET* oncogene and *RET* oncogene dependent cell proliferation, we hypothesize that the high dose we applied may be more effective due to the homozygous mutation in our patient.

Conclusion

In conclusion, it should be kept in mind that different endocrinological tumors may rarely develop with NF1 and the patients should be carefully evaluated in this regard. Furthermore, we believe that vandetanib dose for children, especially for older or adolescent children, with MTC may be the same as in adults but this needs to be supported by further pediatric studies with larger sample size.

Ethics

Informed Consent: Written informed consent was obtained from the patient's parents for publication of this case report and the accompanying images.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices- Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: Begümhan Demir Gündoğan, Fatih Sağcan, Sevcan Tuğ Bozdoğan, Yüksel Balcı, Ferah Tuncel Daloğlu, Elvan Çağlar Çıtak.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0064

J Clin Res Pediatr Endocrinol 2021;13(3):347-352

Unusual Presentation of Denys-Drash Syndrome in a Girl with Undisclosed Consumption of Biotin

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What is already known on this topic?

WT1 gene mutations are associated with Denys-Drash syndrome (DDS), characterized by steroid-resistant nephrotic syndrome, Wilms tumor, disorder of sex development with dysgenetic gonads and gonadoblastoma risk in males. The renal manifestations are generally the only pathological condition in females. Hormone assays support endocrinological assessment and the suspicion of gonadal dysgenesis.

What this study adds?

We describe a girl with an unusual presentation of DDS, with end stage renal failure, severe genital abnormalities, signs of hyperandrogenism, and suspected dysgenetic gonads. Recent clinical history revealed that the patient consumed biotin, and the high levels of testosterone were due to analytical interference of the laboratory immunoassay. Enquiring about biotin supplementation should be conducted, since patients may not consider biotin as a medication and therefore may not mention it in their medication list.

Abstract

We describe a 46,XX girl with Denys-Drash syndrome, showing both kidney disease and genital abnormalities, in whom a misdiagnosis of hyperandrogenism was made. A 15 year-old girl was affected by neonatal nephrotic syndrome, progressing to end stage kidney failure. Hair loss and voice deepening were noted during puberty. Pelvic ultrasound and magnetic resonance imaging showed utero-tubaric agenesis, vaginal atresia and urogenital sinus, with inguinal gonads. Gonadotrophin and estradiol levels were normal, but testosterone was increased up to 285 ng/dL at Tanner stage 3. She underwent prophylactic gonadectomy. Histopathology reported fibrotic ovarian cortex containing numerous follicles in different maturation stages and rudimental remnants of Fallopian tubes. No features of gonadoblastoma were detected. Unexpectedly, testosterone levels were elevated four months after gonadectomy (157 ng/dL). Recent medical history revealed chronic daily comsumption of high dose biotin, as a therapeutic support for hair loss. Laboratory immunoassay instruments used streptavidin-biotin interaction to detect hormones and, in competitive immunoassays, high concentrations of biotin can result in false high results. Total testosterone, measured using liquid chromatography tandem mass spectrometry, was within reference intervals. Similar testosterone levels were detected on repeat immunoassay two weeks after biotin uptake interruption. Discordance between clinical presentation and biochemical results in patients taking biotin, should raise the suspicion of erroneous results. Improved communication among patients, health care providers, and laboratory professionals is required concerning the likelihood of biotin interference with immunoassays.

Keywords: Denys-Drash syndrome, testosterone, biotin, disorder of sex development

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Conflict of interest: None declared
Received: 03.04.2020
Accepted: 06.07.2020

Introduction

Wilms' tumor suppressor gene 1 (*WT1*, OMIM *607102) is essential for kidney and gonadal development (1,2). Mutations in the *WT1* gene are associated with Denys-Drash syndrome (DDS). In 46,XY subjects, *WT1* mutations are associated with steroid-resistant nephrotic syndrome, Wilms tumor, disorder of sex development (DSD) with dysgenetic gonads and gonadoblastoma risk. In contrast, the impact of *WT1* gene on the genital development of 46,XX subjects is not clear and most affected subjects only show the renal manifestations of the condition (1,2).

We describe a girl with end stage renal failure, severe genital abnormalities, signs of hyperandrogenism, and suspected dysgenetic gonads. Recent clinical history revealed that the patient consumed biotin, and the erroneous high levels of testosterone were due to an analytical interference of laboratory immunoassay.

Case Report

A 15 year-old Caucasian Italian girl had exhibited steroid-resistant nephrotic syndrome in the first month of life. Kidney biopsy at onset showed mesangial proliferative glomerulonephritis with focal segmental glomerulosclerosis. End-stage renal failure was reached by two years of age. Cytogenetic analysis showed a normal 46,XX female karyotype. Sanger sequencing of the *WT1* gene, performed at five years of age at another center, showed the missense mutation c.1097G>A in exon 8, causing the amino acid change Arg366His affecting the zinc finger 2 region. The mutation was *de novo* and present in the heterozygous state.

She underwent left nephrectomy at one year of age. Right nephrectomy and a first renal transplantation were performed at 3.3 years of age. Chronic primary Epstein-Barr virus infection was diagnosed early after transplantation and did not respond to reduction in immunosuppression therapy and rituximab. In the following years progressive chronic allograft nephropathy developed and renal function worsened. At the age of 12 years hemodialysis was restarted and 10 months later the transplanted kidney was removed.

Puberty started at 13 years. A few months later, hair loss and voice deepening were observed. Repeated hormone assays, measured by chemiluminescence on an ADVIA Centaur XPT Immunoassay System (Siemens Healtineers Diagnostics, Erlangen, Germany) showed normally increasing pubertal female levels of gonadotrophins and estradiol, but testosterone level progressively increased up to abnormally high concentrations (285 ng/dL) when the girl reached Tanner stage 3 of breast and pubic hair development. The levels of adrenal androgens and precursors including delta4 androstendione, dehydroepiandrosterone sulfate and 17-hydroxyprogesterone, were in the normal range for a pubertal female, excluding the adrenal origin of hyperandrogenism. These data suggested the presence of dysgenetic gonads. Pelvic ultrasound and magnetic resonance imaging showed absence of uterus and Fallopian tubes, vaginal atresia and urogenital sinus. Both gonads were located at the internal inguinal ring. The right gonad appeared small, with a relatively homogeneous, streak-like, structure and rare anechoic areolas. The left gonad was larger and showed an anechoic area consistent with a dominant follicle. As a second step, targeted next generation sequencing was performed using a customized panel for DSD, including all coding exons and flanking introns of the following genes: *AR, FOXL2, FST, HSD3B2, NR5A1, RSPO1, SOX3, SOX9, SRY, WNT4, WT1*. Sequence enrichment was performed using the NimbleGen SeqCap Target Enrichment kit (Nimblegen Roche, Basel, Switzerland) and sequenced on the Illumina NextSeq550 platform (Illumina, San Diego, California). The *WT1* Arg366His mutation was confirmed, while no others mutations were found (Figure 1).

The neoplastic risk associated with *WT1* mutations, the need to plan a second renal transplantation with the related long-term immunosuppressive therapy, the absence of Mullerian structures with potentially dysgenetic gonads producing testosterone led to consideration of prophylactic gonadectomy. She underwent gonadectomy at the age of 14 years. Gross examination revealed small multicystic ovaries. Microscopy showed fibrotic ovarian cortex containing numerous follicles in different maturation stages, from primordial to secondary follicles (Figure 2); some follicles were cystic and scattered corpora lutea were observed. No features of gonadoblastoma were detected. Rudimental remnants of Fallopian tubes were present.

Four months after gonadectomy, hair loss appeared improved but hormonal tests unexpectedly showed elevated testosterone levels (157 ng/dL). In-depth interview on recent medical history revealed daily consumption of high dose biotin, started eight months before, as a therapeutic support for hair loss. Plasma level of biotin was higher than 800 mg/L.

The patient's total testosterone levels, collected during biotin intake and after its cessation, were measured using liquid chromatography tandem mass spectrometry and were both found within reference intervals, at 4 and 5 ng/dL, respectively. Similar testosterone levels were confirmed using immunoassay testing two weeks after biotin uptake interruption (Table 1).

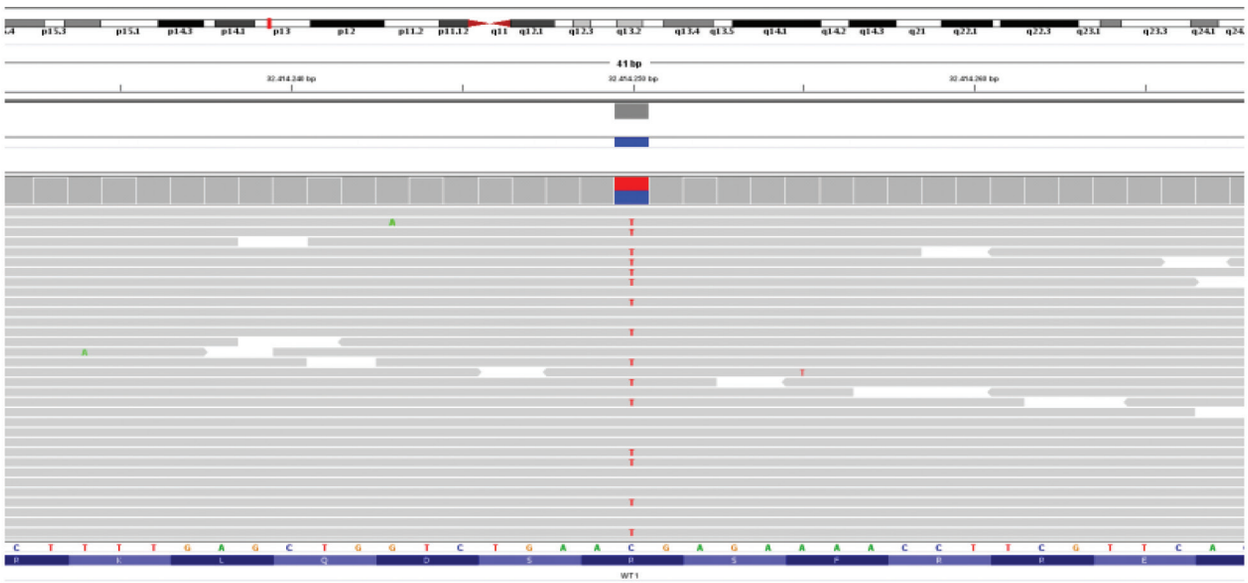

Figure 1. Next generation sequencing analysis WT1: variant visualization on integrative genome viewer (IGV). Patient DNA was sequenced using a custom panel including genes involved in 46,XX disorder of sex development. Sequence enrichment was performed using the NimbleGen SeqCap Target Enrichment kit (Roche) and sequenced on the Illumina NextSeq550 platform (Illumina, San Diego, California). VariantStudio software (Illumina, http://variantstudio.software.illumina.com/) was used for variants annotation. Each single variant has been evaluated for the coverage and the Qscore, and visualized via IGV software. The variant was analyzed *in silico* using prediction pathogenicity software (Scale-Invariant Feature Transform-SIFT and Polymorphism Phenotyping v2 -PolyPhen2) and database of variants frequency

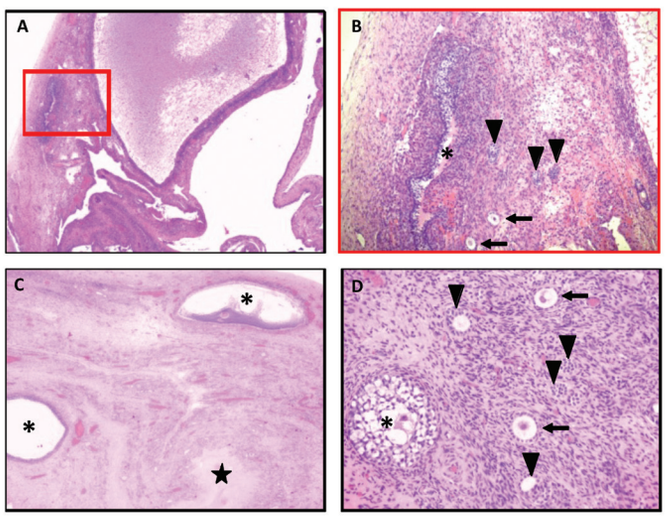

Figure 2. Gonadal histology. **(A)** Right ovary: multiple cystic follicles in the fibrotic cortex (hematoxylin and eosin x2.5). **(B)** Higher magnification of the red insert in A) Follicles in different maturation stages: primordial (arrows), primary (arrowheads) and late stage secondary (asterisk) follicles (hematoxylin and eosin x10). **(C)** Left ovary: fibrotic cortex containing some dilated follicles (asterisks) and a small *corpus luteum* (star) (hematoxylin and eosin x2.5). **(D)** Left ovary: Follicles in different maturation stages: primordial (arrows), primary (arrowheads) and early stage secondary (asterisk) follicles (hematoxylin and eosin x20)

Discussion

WT1 encodes a DNA-binding protein, containing four zinc finger structures, which is essential for normal mammalian urogenital development (3). *WT1* knockout mice lack gonads in both sexes, suggesting a role of this gene during the formation of the genital ridge, an early stage of genital development when the gonad is still undifferentiated (4). Classically, its pathogenic variants are associated with abnormal testis development, leading to 46,XY DSD, while 46,XX subjects generally show normal female genitalia (5,6,7).

WT1 mutations have been described in two 46,XX patients with premature ovarian insufficiency (8). Minor genital abnormalities, such as streak ovaries or bicornuate uterus have been reported sporadically (2). Steroid-resistant nephrotic syndrome, associated with absence of both ovaries, has been described in a single case (9). A 46,XX woman showing adult onset of both focal segmental glomerulosclerosis and hypergonadotropic hypogonadism has been reported recently (10). Laparoscopy showed myomas of uterus and cervix, and streak gonads. Both tubes were lying face up with absent fimbrian funnel. None of the reported 46,XX patients showed abnormalities of the external genitalia.

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2021;13(3):347-352

Table 1. Laboratory measurements before and after gonadectomy and with or without assumption of biotin

	Before gonadectomy	After gonadectomy	
	With biotin	With biotin	Without biotin
Testosterone (ng/dL)	285	157	13.4
LH (mU/mL)	7.4	191.2	143.4
FSH (mU/mL)	5.3	328.7	273.5
Estradiol (ng/dL)	136.3	44.1	47.7

LH: luteinizing hormone, FSH: follicle-stimulating hormone

Recently, a novel frameshift *WT1* variant (c.1453_1456del; p.Arg485Glyfs*14) has been reported in a SRY-negative 46,XX girl with clitoridomegaly, single perineal opening, and short blind-ending vagina. At 10 years of age, basal gonadotrophins were low, but gonadotrophin releasing hormone analog stimulation test showed a significant elevation of testosterone levels, without an increase in estradiol levels. She underwent bilateral gonadectomy, confirming bilateral testes with seminiferous tubules containing predominantly Sertoli cells and rare germ cells. An immature right uterine tube was also identified (11).

The Arg366His mutation found in our patient, was first described in a 46,XY subject with early onset renal disease, female external genitalia with right dysgenetic testis, left streak-gonad and absence of both Mullerian and Wolffian structures. Histopathology of the removed gonads showed a gonadoblastoma (2). The Arg366His mutation has been subsequently described in several 46,XY subjects with DDS, while 46,XX patients with this mutation generally show normal female genitalia and normal pubertal development (5,6,7). An exception are the two identical twins described by Dharnidharka et al (12). Both were phenotypically females and died a few weeks after birth due to multiorgan failure. At autopsy, the gonads were normal sized ovaries in both twins. Twin A had a complete duplication of uterus and vagina. Mesonephric remnants were prominent in the mesovarium of both twins. Twin B had a microscopic cluster of tubules within the mesovarium consisting of germ cells and supporting cells, reminiscent of testicular architecture. Fluorescent in situ hybridization analysis for detection of the Y chromosome was negative in both twins.

Biotin (also known as vitamin H, vitamin B7, and coenzyme R) is a water-soluble vitamin, naturally present in some foods, with plasma levels between 100-250 ng/L and undergoes urinary excretion. In Western populations, dietary biotin intake is estimated to be 35 to 70 µg daily, a level in line with the recommended dietary allowance. In recent years, high-dose supplementation (doses greater than 1 mg/d) has played a role in the treatment of several diseases, including biotinidase deficiency, mitochondrial metabolic disorders, and multiple sclerosis. Furthermore, advised doses up to 10 mg/day are frequently encountered in nutritional supplements taken to improve hair, skin, and nail health. Many common blood tests employ a biotin-streptavidin reaction as part of the test procedure. While the expected amount of dietary biotin intake is not expected to be high enough to affect these tests, biotin supplementation at doses greater than 1 mg per day can cause either falsely low or falsely high test results, depending on the analyte and platform used for testing (13). Briefly, excess biotin in blood competes with biotinylated antibody of the assay, which produces falsely decreased hormone concentrations in sandwich or non-competitive immunoassays and falsely increased hormone concentrations in competitive immunoassays. Several reports have shown analytical biotin interference, especially in thyroid function tests, but only one included total testosterone measurement (14). Our analytical platform measured testosterone by a competitive immunoassay: elevated concentrations of biotin compete with biotin-antibody-(labeled) analyte complexes for binding to the streptavidin-coated well, leading to the detection of a diminished signal causing a falsely high analyte result.

Our case confirms that the clinical phenotype of subjects with *WT1* mutation and 46,XX karyotype may include a severe DSD. The clinical signs of hyperandrogenism, partially improved after gonadectomy, suggest that the girl had hypersecretion of ovarian androgens during puberty, but the "male" levels of testosterone mostly resulted from the analytical interference. Unfortunately, the "true" testosterone levels before gonadectomy are unknown, because there were no testosterone measurements made without in periods when there was no biotin consumption. Several different factors primarily impacted the decision-making process leading to prophylactic gonadectomy: the potential risk of gonadoblastoma associated with *WT1* mutations; the need for a second renal transplantion with long-term immunosuppressive therapy; and the absence of Mullerian structures associated with abnormally located

and potentially dysgenetic gonads. However, the spuriously elevated testosterone levels apparently confirmed clinical hyperandrogenism, and supported the suspicion of gonadal dysgenesis. Surprisingly, histology showed normal appearance of ovarian tissue, but we cannot exclude the presence of abnormal clusters of androgen secreting cells. Experimental studies on mouse models demonstrated that *WT1* gene expression controls the differentiation of genital ridge somatic cells into granulosa or Sertoli cells in genetically female and male gonads, respectively. When *WT1* is deleted, these somatic cells turn into steroidogenic cells, hyper-expressing enzymes involved in androgen synthesis, without any sex dimorphism (15).

During patient history taking, enquiring about biotin supplementation should be conducted, since patients may not consider biotin as a medication and therefore may not mention it in their medication list. In the presence of discordance between clinical presentation and biochemical results in patients taking biotin-containing medications, considering biotin half-life of 15 hours (16), we recommend to repeat specimen collection after at least 48 hours of interruption.

Acknowledgments

We thank Simona Pancotti for technical support.

Ethics

Informed Consent: Informed consent was obtained from all individual participants included in the study

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Carla Bizzarri, Luca Dello Strologo, Isabella Guzzo, Francesco Emma, Marco Cappa, Concept: Carla Bizzarri, Marco Cappa, Ottavia Porzio, Luca Dello Strologo, Isabella Guzzo, Francesco Emma, Design: Carla Bizzarri, Marco Cappa, Ottavia Porzio, Data Collection or Processing: Carla Bizzarri, Germana Antonella Giannone, Jacopo Gervasoni, Sabina Benedetti, Federica Albanese, Francesca Diomedi Camassei, Analysis or Interpretation: Carla Bizzarri, Germana Antonella Giannone, Jacopo Gervasoni, Sabina Benedetti, Federica Albanese, Luca Dello Strologo, Isabella Guzzo, Mafalda Mucciolo, Ottavia Porzio, Literature Search: Carla Bizzarri, Ottavia Porzio, Writing: Carla Bizzarri, Ottavia Porzio.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0149

J Clin Res Pediatr Endocrinol 2021;13(3):353-357

A Case of Congenital Central Hypothyroidism Caused by a Novel Variant (Gln1255Ter) in *IGSF1* Gene

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What is already known on this topic?

Mutations in the *immunoglobulin superfamily, member 1 (IGSF1)* gene that mainly regulates pituitary thyrotrope function lead to X-linked hypothyroidism characterized by congenital hypothyroidism of central origin and testicular enlargement. The clinical features associated with IGSF1 mutations are variable, but prolactin and/or growth hormone deficiency, and discordance between timing of testicular growth and rise of serum testosterone levels could be seen.

What this study adds?

Genetic analysis revealed a novel c.3763C > T variant in the *IGSF1* gene. To our knowledge, this is the first reported case of *IGSF1* deficiency from Turkey. Additionally, as in our case, early testicular enlargement but delayed testosterone rise should be evaluated in all boys with central hypothyroidism, as macro-orchidism is usually seen in adulthood.

Abstract

Loss-of-function mutations in the *immunoglobulin superfamily, member 1* (*IGSF1*) gene cause X-linked central hypothyroidism, and therefore its mutation affects mainly males. Central hypothyroidism in males is the hallmark of the disorder, however some patients additionally present with hypoprolactinemia, transient and partial growth hormone deficiency, early/normal timing of testicular enlargement but delayed testosterone rise in puberty, and adult macro-orchidism. Here, we report a boy with congenital central hypothyroidism caused by a novel variant in the *IGSF1* gene. In our patient, early testicular enlargement but delayed testosterone rise with central hypothyroidism and hypoprolactinemia were the most important clues for diagnosis. In genetic analysis, we identified a novel, hemizygous nonsense c.3763 C > T (G1n1255Ter) variant in *IGSF1* gene. To our knowledge, this is the first reported case of *IGSF1* deficiency from Turkey.
Keywords: Central hypothyroidism, hypoprolactinemia, IGSF1

Introduction

Congenital central hypothyroidism (CCH) is a rare disease characterized by impaired thyrotropin secretion with a normal thyroid gland. The pathogenic mechanism of CCH is heterogeneous and dysfunction of thyrotroph-specific genes such as the *thyroid-stimulating hormone β-subunit* and *thyrotrophin releasing hormone (TRH) receptor (TRHR)* can result in isolated central hypothyroidism (1,2). Many CCH patients, however, have additional pituitary hormone deficiencies (3). Some patients with combined pituitary hormone deficiencies were reported to carry mutations in transcription factors genes involved in pituitary development, including *POU1F, PROP1, HESX1*, and *LHX3* (4). Recently, loss-of-function mutations in the *immunoglobulin*

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Conflict of interest: None declared
Received: 02.07.2020
Accepted: 04.08.2020

superfamily, member 1 (IGSF1) gene have been described as an X-linked cause of CCH with an estimated prevalence of 1/100,000 (5,6). Central hypothyroidism in males is the hallmark of the disorder. However, some patients additionally present with hypoprolactinemia, transient and partial growth hormone deficiency (GHD), early/normal timing of testicular enlargement but delayed testosterone rise in puberty resulting in delayed adolescent growth spurt, and adult macro-orchidism (7). The *IGSF1* gene resides on the X-chromosome (Xq 26.2) and therefore its mutation affects mainly males, although some female heterozygous carriers may present with central hypothyroidism (7). *IGSF1* encodes a plasma membrane immunoglobulin superfamily glycoprotein (8). After proteolytic cleavage, the C-terminal portion traffics to the plasma membrane where it is expressed as a large extracellular domain, suggesting a possible function in cell-cell adhesion or signaling (9). *IGSF1* is mainly expressed in Rathke's pouch, adult pituitary gland, and the hypothalamus (5,10).

Here we report a boy with CCH caused by a novel variant in the *IGSF1* gene. In our patient, early testicular enlargement but delayed testosterone rise with central hypothyroidism and hypoprolactinemia were the most important clues for the diagnosis. Additionally, to our knowledge, this is the first reported case of *IGSF1* deficiency from Turkey.

Case Report

A 10.1 year-old boy was referred to our pediatric endocrinology out-patient clinic for hypothyroidism. He has been followed up in another hospital due to congenital hypothyroidism, and using levothyroxine (1.7 mcg/kgper day). His medical history revealed that he was born at term with 3240 g weight without perinatal hypoxia. His mental-motor development was normal. He had used short-term growth hormone therapy two years earlier. His parents were not consanguineous, and had no history of hypothyroidism.

In physical examination, height was 135 cm [-0.55 standard deviation score (SDS)] and weight was 37 kg (+0.62 SDS). Thyroid gland was not palpable, bilateral testicular volumes were 6 mL, and penis stretched length was 4 cm without pubarche. Target height was 170 cm (-0.92 SDS). Laboratory findings were as follows; fT4: 0.59 ng/dL (0.61-1.68); ree triiodothyronine (fT3): 3.46 ng/dL (2.9-6.1); thyroid stimulating hormone (TSH): 0.02 uIU/mL (0.37-5.1); thyroglobulin: 10.7 µg/L (3.5-41); prolactin 0.76 µg/L (2.64-13.13); and thyroid auto-antibodies were negative. Thyroid ultrasonography revealed a hypoplastic thyroid gland with a total volume of 0.9 mL. Levothyroxine dosage was increased until euthyroidism was achieved. Bone age was nine years. There was a 38 mm arachnoid cyst in the right temporal pole on brain magnetic resonance imaging, and pituitary gland was normal in structure. Growth hormone deficiency was excluded on follow-ups. His growth rate and insulin-like growth factor-1 (IGF-1) level (120.3 µg/L) were normal for his age. Total testosterone was low (0.01 µg/L), and other laboratory tests (morning basal values) were as follows; luteinizing hormone (LH): 0.01 U/L; follicle-stimulating hormone (FSH): 0.65 U/L; adrenocorticotropic hormone (ACTH): 8.9 ng/L (4.7-48.8); and cortisol: 7 µg/dL (6.7-22.6). Low dose ACTH stimulation test was performed, and peak cortisol level was found to be normal (22.1 µg/dL). Additionally, on TRH stimulation test, peak TSH response was very low (0.01 uIU/mL) confirming the pituitary central hypothyroidism. Laboratory findings of the mother were normal: free thyroxine (fT4): 1.1 ng/dL; fT3: 4.2 ng/dL; TSH: 3.3 uIU/mL; and prolactin 16 µg/L. fT4, TSH and prolactin levels of the father and the two other siblings (one sister and one brother) were also normal.

At the age of 11.9 years, his bilateral testicular volumes were 6-8 mL without pubarche. Laboratory tests were as follows: total testosterone: 0.07 ng/mL (0.21-0.82); LH: 0.13 U/L; FSH: 1.85 U/L; dehydroepiandrosterone sulfate (DHEAS): 48 µg/dL (20-550); androstenedione: 0.3 ng/mL (0.3-0.6); 11-deoxycortisol 0.41 ng/mL (0.2-1.5); 17-OH progesterone: 1.5 ng/mL (0.5-1.5); and anti-Mullerian hormone: 24.2 ng/mL (28.4-113.8). As a result, *IGSF1* gene mutation was considered in the patient because of central hypothyroidism, hypoprolactinemia, and low testosterone level incompatible with testicular volume.

After written informed consent was provided from the parents, genetic analysis with next gene sequencing (Illumina, NovaSeq, 6000, San Diego, California, United States) was performed. A novel hemizygous nonsense c.3763C>T (G1n1255Ter) variant in the *IGSF1* gene was identified (Figure 1). We considered this variant as *pathogenic* by using American College of Medical Genetics (ACMG) criteria (11). According to the ACMG criteria *IGSF* c.3763C>T variant met the criteria for PVS1, PM2 and PP4. The explanations of these criteria are as follows; its nonsense nature and loss of function is a known mechanism for central hypothyroidism (PVS1), absence in population databases (PM2), and compatible clinical findings with *IGSF1* gene mutations (PP4). Sanger sequencing was performed in the patient's mother, and she was found as an obligate carrier (Figure 2). Unfortunately, other relatives of the mother did not consent for genetic analysis.

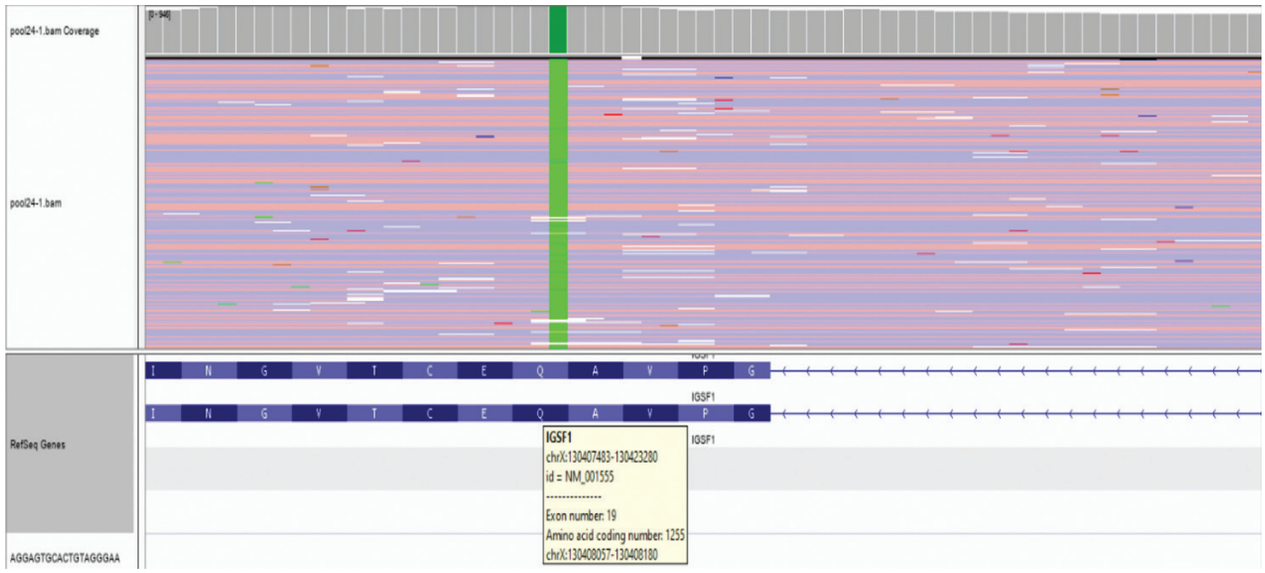

Figure 1. Next gene sequencing image of the novel hemizygous c.3763C > T change in *IGSF1* gene of the patient

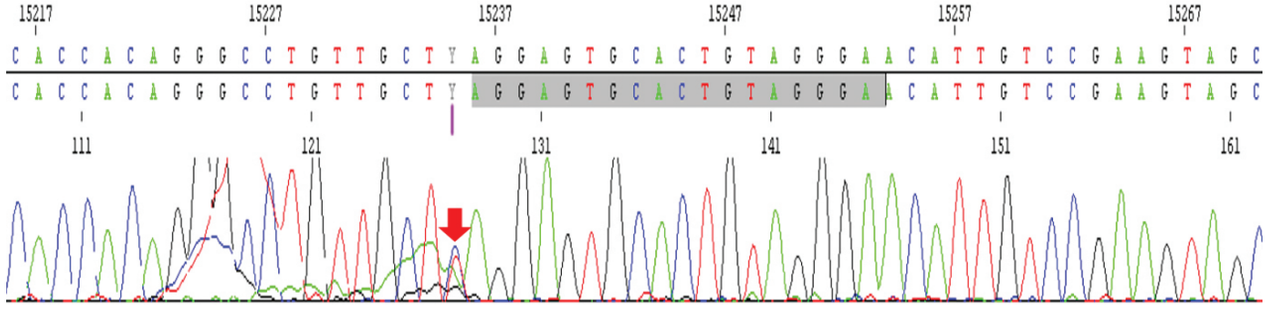

Figure 2. Sanger sequencing image of heterozygous c.3763C > T change in *IGSF1* gene of the patient's mother

Discussion

The IGSF1 protein contains 12 immunoglobulin-like domains in two clusters, which are separated by a linker segment and followed by a transmembrane and a cytoplasmic region (12). Lack of IGSF1 protein impairs glycosylation and trafficking of the protein to the cell surface. Therefore, mutations in *IGSF1* gene that mainly regulates pituitary thyrotrope function lead to X-linked hypothyroidism characterized by congenital hypothyroidism of pituitary origin. The clinical features associated with *IGSF1* mutations are variable, but prolactin and/or growth hormone deficiency, discordance between timing of testicular growth and rise of serum testosterone levels, and abnormal weight gain may be seen (5). In a recent study, pituitary central hypothyroidism was found as the cardinal finding of the disease (100 % in the cases), however only 62 % had prolactin deficiency, and the remaining patients had normal prolactin secretion. Interestingly, prolactin deficiency was not consistent in patients from the same family who carry the same mutations. The reason for this variability is unknown but might be caused by interplay of several gene polymorphisms (13). In the same study, the rate of transient GH deficiency was 11 %. Surprisingly, all children with GH deficiency had normal IGF-1 levels, whereas adults had higher than normal IGF-1 levels. Many of the children with GH deficiency were retested in adulthood and found to have normal results on GH stimulation tests. Additionally, delayed pubertal testosterone rise, and early/normal timing of testicular growth was found in 75 % of the patients in this cohort. In our case, pubertal testicular volume without pubarche and with low testosterone level prompted us to consider IGSF1 deficiency.

Another important issue in these patients is adrenal function. Hypocortisolism was diagnosed in 21 % of newborns. However, this proved to be transient within a few years in all cases (13). The late adrenarche in patients with prolactin deficiency is another clue for adrenal dysfunction.

However, this is considered to occur as a result of prolactin deficiency. Prolactin receptors are highly expressed in the adrenal gland and are stimulated by ACTH to increase adrenal androgen secretion (14). Furthermore, DHEAS levels are usually found elevated in hyperprolactinemia, and lowering of prolactin concentration decreases DHEAS level. In our case, the patient has delayed pubarche with low DHEAS and androstenedione levels for his age without adrenal insufficiency.

A small proportion of heterozygous females may also show central hypothyroidism, prolactin deficiency and delayed menarche. Heterozygous females carrying *IGSF1* mutations generally exhibit fT4 levels in the lower tertile of the normal range with nearly 20% fulfilling the criteria for central hypothyroidism. Up to 20% of the cases demonstrate hypoprolactinemia, and to date four females have been reported to have surgery for benign ovarian cysts (13). In our case, the mother was an obligate carrier, and had neither hypothyroidism nor hypoprolactinemia.

Conclusion

In conclusion, in the present case, genetic analysis revealed a novel c.3763C>T variant in the *IGSF1* gene. To our knowledge, this is the first reported case of *IGSF1* deficiency from Turkey. Early testicular enlargement but delayed testosterone rise with central hypothyroidism and hypoprolactinemia were the most important clues for the diagnosis. Importantly, as in our case, early testicular enlargement but delayed testosterone rise should be evaluated in all boys with central hypothyroidism, as macro-orchidism is only usually seen late in adulthood. Additionally, although the current clinical findings of the patient are sufficient for the diagnosis and treatment of central hypothyroidism, genetic diagnosis played a key role in management and follow-up of the patient.

Ethics

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

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Doğa Türkkahraman, Design: Doğa Türkkahraman, Data Collection or Processing: Doğa Türkkahraman, Nadide Cemre Randa, Analysis or Interpretation: Doğa Türkkahraman, Nadide Cemre Randa, Literature Search: Doğa Türkkahraman, Nimet Karataş Torun, Writing: Doğa Türkkahraman, Nimet Karataş Torun, Nadide Cemre Randa.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0039

J Clin Res Pediatr Endocrinol 2021;13(3):358-361

Brain Abscess in a Patient with Osteopetrosis: A Rare Complication

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What is already known on this topic?

The most common infectious complication of osteopetrosis is osteomyelitis; in particular, mandibular osteomyelitis may be seen due to bone sclerosis and reduced vascular supply. Otolaryngological complications, such as recurrent otitis media, are also seen frequently.

What this study adds?

To our knowledge, this report is only the second published description of brain abscess in association with osteopetrosis. Therefore, routine otologic examination should be an integral component of management and plays an important role in preventing more severe complications, such as brain abscess.

Abstract

Brain abscess formation is extremely rare in patients with osteopetrosis. Herein, we report a case of viridans streptococci brain abscess in an immunocompromised child diagnosed with osteopetrosis. The patient presented with a sudden change in mental status and convulsions. Radiological evaluation revealed a temporal lobe brain abscess, and intravenous antibiotherapy was started immediately. The patient underwent abscess drainage, and laboratory investigation of pus material revealed viridans streptococci.
Keywords: Osteopetrosis, chronic otitis media, brain abscess, viridans streptococci

Introduction

Osteopetrosis describes a group of rare genetic skeletal disorders characterized by reduced osteoclast activity, which results in defective bone resorption and increased bone mass and density (1). Osteopetrosis is normally classified descriptively by its clinical severity and inheritance pattern (2,3). The adult (autosomal dominant) type is a mild form of the disease characterized by normal life expectancy while the infantile (autosomal recessive) type is characterized by diagnosis in early life and fatal prognosis. The intermediate type of osteopetrosis, which is a subgroup of the autosomal recessive type, is seen less frequently; diagnosis is made during early childhood, with patients being clinically normal at birth (2,3,4). Currently, with the improvement in molecular and genetic techniques, forms of osteopetrosis can also be classified according to their genetic basis (2,3). In autosomal recessive osteopetrosis, loss of bone marrow causes anemia, thrombocytopenia, and leukopenia, in turn resulting in extramedullary hematopoiesis, hepatosplenomegaly, and recurrent infection. Bone fractures with minor trauma and osteomyelitis of especially the long bones and mandible can be seen (1). Differential diagnosis of osteopetrosis including dysosteosclerosis, which is more rare, and is distinguished from osteopetrosis by the presence of early acquired

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Conflict of interest: None declared
Received: 21.02.2020
Accepted: 10.08.2020

sclerotic platyspondyly and metaphyseal expansion. Another differential diagnostic tool is mutation analysis (5).

The factors associated most strongly with the appearance of infectious complications in osteopetrosis are impaired resistance to infection due to neutropenia and reduced vascular supply to the bone, which limits the availability of antibiotics in the infected area. Otolaryngological complications, such as recurrent otitis media, are also seen frequently, but brain abscess formation due to recurrent otitis media is extremely rare. Herein, we discuss a case of viridans streptococci brain abscess in an immunocompromised child with osteopetrosis. To our knowledge, this report is only the second published description of brain abscess in association with osteopetrosis (6).

Case Report

A 14-year-old boy with a previous diagnosis of autosomal recessive osteopetrosis type 7 (OPTB7) presented to our pediatric emergency care unit with complaints of confusion, sudden abnormal involuntary muscle contractions, and temporary cessation of breathing and cyanosis. He had experienced nausea, vomiting, and diarrhea for the past two weeks. There was no history of trauma or fever. The patient had taken oral antibiotics, primarily amoxicillin/clavulanic acid, on numerous occasions in the past year for recurrent suppurative otitis media. He had a history of frequent purulent otorrhea, which had never fully resolved. In his medical history, abnormal eye movement was first observed by his family when he was two months old and then, at age eight months, a bone fracture was seen. During follow-up, the patient was diagnosed with blindness caused by optic nerve compression and hypogammaglobulinemia in addition to multiple arm and leg fractures. He was diagnosed with osteopetrosis in the infancy period and a homozygous tumor necrosis factor receptor superfamily member 11A (*TNFRSF11A*) mutation [c.838G > T (p.G280X)] was detected in an another tertiary hospital (7). There is parental consanguinity and both parents were heterozygous for the mutation. He was receiving intravenous immunoglobulin replacement therapy alone, with no other treatment.

On physical examination, the patient showed a change in mental status and abnormal involuntary contractions of the left arm. The Glasgow Coma Scale (GCS) score was 8 (E 2, V 2, M 4). Intravenous midazolam was started immediately to control seizures. Laboratory examination revealed a white blood cell count of 17,500/mm³ (80 % neutrophils, 20 % lymphocytes), a hemoglobin level of 8.5 g/dL, platelet count of 456,000/mm³, and C-reactive protein level of 9.2 mg/dL. Serum electrolytes, renal function, and liver function were all normal. Cranial computed tomography revealed a brain abscess in the right temporal lobe. The patient was prescribed intravenous ceftriaxone (100 mg/kg/day in two doses), vancomycin (60 mg/kg/day in four doses) and metronidazole (30 mg/kg/day in three doses). Dexamethasone was also started for brain edema, and levetiracetam was started to control the seizures.

On the fourth day of treatment, the patient was referred to our clinic for evaluation by pediatric infectious disease specialists. On physical examination, he was conscious with symmetrical and equally reactive pupils and no meningeal sign or respiratory problem. He was agitated. There were accompanying exophthalmos, left facial paralysis, and purulent otorrhea. He had locomotor difficulties and could speak a few simple words with hearing loss. Hepatosplenomegaly was also detected. His height was 96 centimeters [-8.89 standard deviation (SD)], while his weight was 15 kilograms (-6.65 SD), respectively. Contrast-enhanced magnetic resonance imaging of the brain showed a multiloculated lesion with perilesional edema and slight contrast enhancement in the right temporal lobe (approximately 6 × 5 × 4.5 cm in diameter); subfalcine herniation and uncal herniation were also seen (Figure 1). Post-intubation plain chest radiography showed diffuse

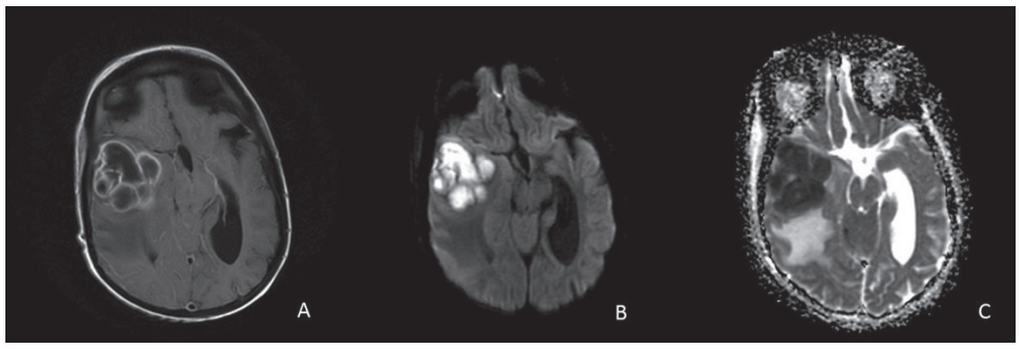

Figure 1. (A) Brain abscess with peripheral contrast enhancement in the right temporal lobe. **(B, C)** Reduced signal intensity on diffusion magnetic resonance images

increased bone density and callus formation due to fracture healing (Figure 2).

The patient underwent abscess drainage via temporal-lobe burr-hole craniotomy under general anesthesia. Yellowish-brown pus was aspirated from the affected region and the abscess was excised completely. The abscess was grey-brownish, rigid, and filled with a necrotic material ($7 \times 5.8 \times 0.9$ cm in diameter). The abscess material was sent to the laboratory for microbiological and histopathological investigations. Gram staining showed gram-positive cocci and surgical drainage culture showed the growth of viridans streptococci, sensitive to β-lactam antibiotics. The histopathology report revealed active chronic inflammation, including abscess formation, proliferating blood vessels, congestion, and fibrosis, suggestive of pyogenic, intracerebral abscess formation. After surgery, the patient was followed in our pediatric intensive care unit (PICU) with respiratory support. He had an unfavorable clinical outcome, dying within 24 hours of admission to the PICU.

Discussion

The classification of osteopetrosis is challenging, due to variability in the severity of clinical manifestations, genetic factors and associated complications. Disease severity ranges from the occurrence of life-threatening complications in neonates to incidental findings of osteopetrotic features on plain radiography in adults with no complaint (8). Mutations in *TNFRSF11A* are associated with osteoclast-poor forms of autosomal recessive osteopetrosis because of impaired interaction between RANK (encoded by *TNFRSF11A*) with RANK ligand (encoded by *TNFSF11*) which is important not only in osteoclast differentiation but also for immune system function (2,3,7,9). Our patient had blindness caused by optic nerve compression, multiple fractures of the extremities and hypogammaglobulinemia. He was being followed by physicians from another center under a diagnosis of osteoclast-poor with immunoglobulin deficiency, autosomal recessive, infantile form OPTB7.

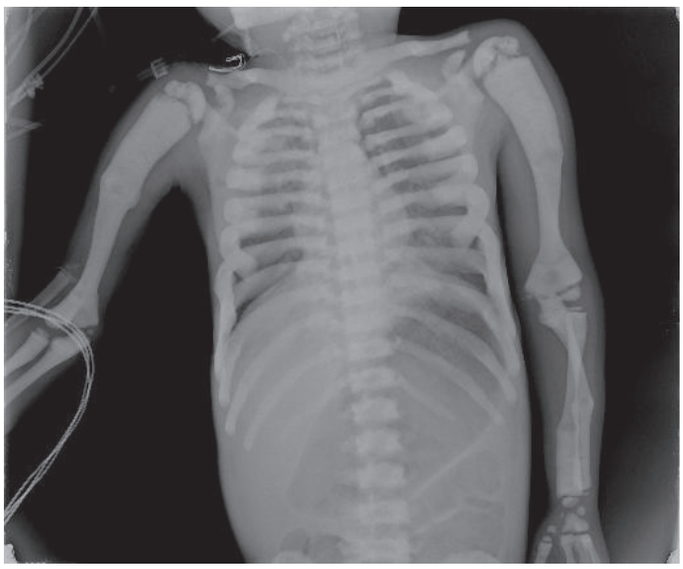

Figure 2. Diffuse increased bone density and formation of multiple calluses due to fracture healing

The most common infectious complication of osteopetrosis is osteomyelitis; in particular, mandibular osteomyelitis may be seen due to bone sclerosis and reduced vascular supply. Dental abscess formation and tooth decay are the main predisposing factors for infection (8,10,11). Recurrent otitis media is another important and commonly seen entity (10,11). Abnormal temporal bone anatomy, such as poor mastoid pneumatization and Eustachian tube narrowing, increases the risk of otologic infection (11).

Hypogammaglobulinemia due to bone marrow failure can also occur in patients with osteopetrosis and is an important risk factor for recurrent infection (1,12,13). In one study, 15 of 32 patients with autosomal recessive osteopetrosis experienced multiple episodes of otitis media (11). Our patient had a history of recurrent, suppurative otitis media episodes in the past year, and physical examination revealed purulent otorrhea. The most common infections preceding brain abscess formation in children were sinusitis (36.3%), periorbital/orbital cellulitis (16.1%), otitis media (13.5%) and meningitis (11.9%) (14). Brain abscess formation should be suspected in patients with osteopetrosis, although only one case has been reported in the literature (6). This rare clinical entity can occur after antibiotherapy for otitis media episodes.

Brain abscess is aggressive and life-threatening infection with a high fatality rate, especially among immunocompromised children. Furthermore, a low GCS score at presentation is associated with a poor outcome (14). Our patient was immunodeficient and had a low GCS score at presentation. The clinical picture of brain abscess can be confusing and uncertain in the early phase of the disease. The initial symptoms are typically nonspecific, and few patients show the classic triad of headache, fever, and focal neurological deficits. In particular, brain abscess should be included in the differential diagnosis of immunocompromised patients with headache, altered mental status, vomiting, seizures, focal neurological deficits, and speech and visual disturbances (14).

The only effective treatment known for osteopetrosis is allogeneic bone marrow transplantation. Historically, the best outcomes have been achieved by using bone marrow from a genotypically human leukocyte antigen-identical

donors (2,3,15). Acute or chronic otitis media episodes are seen in half of all patients with osteopetrosis, the majority of whom require the insertion of tympanostomy tubes (10,11). In our case, the patient had a history of chronic otitis media in the past year without appropriate treatment. The etiology of osteopetrosis is thought to be related to a contiguous focus of infection.

Conclusion

Therefore, a routine otologic examination should be an integral component of management and plays an important role in preventing more severe complications, such as brain abscess.

Ethics

Informed Consent: Informed consent was obtained from the patient for the publication of this case report and any accompanying images.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Merve İşeri Nepesov, Eylem Kıral, Gürkan Bozan, Concept: Ömer Kılıç, Kürşat Bora Çarman, Coşkun Yarar, Ener Çağrı Dinleyici, Design: Merve İşeri Nepesov, Ömer Kılıç, Data Collection or Processing: Merve İşeri Nepesov, Ömer Kılıç, Suzan Şaylısoy, Analysis or Interpretation: Ömer Kılıç, Kürşat Bora Çarman, Coşkun Yarar, Suzan Şaylısoy, Ener Çağrı Dinleyici, Literature Search: Merve İşeri Nepesov, Ömer Kılıç, Writing: Merve İşeri Nepesov, Ömer Kılıç.

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

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CASE REPORT

DOI: 10.4274/jcrpe.galenos.2020.2020.0219

J Clin Res Pediatr Endocrinol 2021;13(3):362-366

Co-existence of Congenital Adrenal Hyperplasia and Familial Hypokalemic Periodic Paralysis due to *CYP21A2* and *SCN4A* Pathogenic Variants

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What is already known on this topic?

Pathogenic variants in the *CYP21A2* gene are the most common cause of congenital adrenal hyperplasia. Familial hypokalemic periodic paralysis (FHPP) is a rare disorder in which affected individuals may experience paralytic episodes associated with hypokalemia caused by pathogenic variants in *SCN4A* and *CACNA1S*. It is important to obtain a careful and detailed medical history from the patient and family and to identify family members at risk by segregation analyses.

What this study adds?

This is the first case report of 21-hydroxylase enzyme deficiency (21-OHD) and FHPP in the literature. Elevated adrenocorticotropic hormone and androgens may trigger a hypokalemia attack in FHPP. It is necessary to reconsider routine fludrocortisone treatment in children with 21-OHD based on additional, newly available scientific evidence.

Abstract

Steroid 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia (CAH), usually due to biallelic variants in *CYP21A2*. Classical 21-hydroxylase deficiency is characterised by virilisation of the external genitalia in females and hypocortisolism. Hyponatremia and hyperkalemia are among the common biochemical findings. Familial hypokalemic periodic paralysis (FHPP) is a rare disorder in which affected individuals may experience paralytic episodes associated with hypokalemia, caused by pathogenic variants in *SCN4A* or *CACNA1S*. A 14-year-old female, who had been diagnosed with classical 21-hydroxylase deficiency and treated with hydrocortisone and fludrocortisone since early infancy, presented with acute onset weakness. The laboratory results revealed a remarkably low serum potassium level. The family history revealed that both her father and uncle had the same hypokalemic symptoms, which suggested an FHPP diagnosis. We found two previously reported homozygous variants in the *CYP21A2* (p.Ile173Asn) and *SCN4A* (p.Arg672His) genes in the patient. Therefore, diagnoses of simple virilising 21-hydroxylase deficiency and FHPP were genetically confirmed. Here, FPHH and chronic overtreatment with fludrocortisone may explain the presentation of our patient with severe hypokalemia. The family's medical history, which is always a valuable clue, should be investigated in detail since rare inherited conditions may co-occur in geographies where consanguineous marriages are common and the genetic pool is diverse. In patients with CAH, care should be taken to avoid overtreatment with fludrocortisone. Androgens may have triggered the hypokalemic attack in FHPP, as supported in a previous study.

Keywords: *CYP21A2*, familial hypokalemic periodic paralysis, *SCN4A*, congenital adrenal hyperplasia

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Conflict of interest: None declared
Received: 18.09.2020
Accepted: 17.12.2020

Introduction

Autosomal recessive pathogenic variants in the gene encoding *CYP21A2* cause 21-hydroxylase enzyme deficiency (21-OHD), which is the most common reason for pediatric adrenal insufficiency, resulting in hyponatremia and hyperkalemia. The incidence ranges from 1:13,000 to 1:15,000 births, and the prevalence varies according to ethnicity and geographic area (1). Familial hypokalemic periodic paralysis (FHPP) is associated with pathogenic variants in genes encoding ion channel subunits. These include heterozygous pathogenic variants which may occur in the *CACNA1S* gene or the *SCN4A* gene. About 40-60% of FHPP cases are attributed to *CACNA1S* pathogenic variants, whereas in 7-10% of the cases, *SCN4A* pathogenic variants are responsible. FHPP is an uncommon cause of transient episodes of painless muscle weakness due to low serum potassium levels, with an estimated prevalence of 1 in 100,000. The disorder is less common in women. Paralytic attacks can be triggered by high-carbohydrate meals, exercise, stress, and certain medications. The mechanism that leads to episodic potassium transfer into the cells and causes weakness due to calcium channel defect is not precisely understood. The initial symptoms occur during the first or second decades of life, and these attacks vary in frequency and duration (2). To our knowledge, this is the first case report of the co-existence of FHPP with 21-OHD, which causes a reversed pathology in blood potassium levels.

Case Report

A 14-year-old female presented with acute onset weakness in both upper and lower extremities, which had progressed over a period of one day. The medical history revealed that she was diagnosed with 21-OHD as a new-born and was given oral hydrocortisone and fludrocortisone treatment. The patient was born at term in another primary care centre. There was a consanguineous marriage between her parents. Unfortunately, we could not retrieve detailed data on the relevant laboratory parameters before her referral to our clinic.

She presented with clitoromegaly, electrolyte imbalance, elevated adrenocorticotropin hormone (ACTH) and 17-OH progesterone levels. To the best of our knowledge ACTH stimulation tests were not performed. She had undergone clitoroplasty in infancy. Six months before a hypokalemic attack, while she was taking hydrocortisone (17 mg/m^2/day-irregularly) and fludrocortisone (0.1 mg/day) treatment, her total testosterone (1.03 ng/mL), 17-OH progesterone (162 ng/mL) and ACTH (190 pg/mL) levels were elevated, reflecting poor control. The patient had developed central precocious puberty with advanced bone age and received gonadotrophin releasing hormone analogue treatment until 11 years of age.

She presented to the pediatric emergency clinic with severe generalised weakness in both arms and legs, which was our first evaluation of her. There was no history of fever, acute gastroenteritis, substance abuse or carbohydrate-rich meal or exercise before the paralysis episodes. She had no history of recent upper respiratory tract infection or vomiting, and she did not experience any neurological symptoms. She did not report similar episodes in the past, but her father and uncle had a history of paralytic episodes accompanied by hypokalemia without a genetic diagnosis. The muscle strength in both arms and legs was at grade two, and reflexes were absent. Blood pressure was 100/70 mmHg. The rest of the physical examination was unremarkable. Complete blood count, blood gas, blood urea nitrogen, creatinine, creatine phosphokinase, and serum electrolytes (calcium, phosphate, magnesium) were in the normal ranges, while serum potassium and sodium levels were 1.9 mEq/L and 146 mmol/L, respectively. Hyperthyroidism was excluded.

She was admitted to the hospital, fludrocortisone treatment was terminated, intravenous potassium replacement was initiated. When the lower limit of the normal serum potassium level was achieved, intravenous potassium treatment was stopped, and oral potassium (citrate and bicarbonate) administration was initiated. Oral potassium was preferred to intravenous treatment to reduce the risk of rebound hyperkalemia and discontinuation of the normal serum potassium level. Cardiac activity and serum electrolyte levels were monitored during and after the treatment. Potassium levels were closely checked for potential rebound hyperkalemia for 24 hours. In her first endocrinology evaluation, 15 days after the hypokalemic attack and without fludrocortisone treatment, her laboratory examinations showed normal plasma aldosterone concentration of 36 ng/dL (4-58 ng/dL) and normal plasma renin activity (PRA) of 2.38 ng/mL/hr (2.3-37.0 ng/mL/hr) with normal blood pressure. The levels of biochemical parameters of the patient are shown in Table 1.

The patient's clinic and laboratory findings and family history were consistent with FHPP. Although the patient's father had the same diagnosis, FHPP, genetic analysis had not been performed before. Blood samples for genetic analyses were obtained after informed consent was received from the patient and her parents. Sanger sequencing in the proband revealed a homozygous p.Ile173Asn (c.518T > A) pathogenic variant in *CYP21A2* (NM_000500.9). The patient's mother and father were heterozygous for this variant. No pathogenic variant was detected in *CACNA1S*. The sequencing of *SCN4A*

Table 1. The levels of biochemical parameters of the patient

Time according to hypokalemic attack	Na mmol/L (136-146)	K mEq/L (3.5-5.5)	Total testosterone ng/dL (2.3-13.9)	17-OH progesterone ng/mL (0.4-12)	ACTH pg/mL (10-60)	Aldosterone ng/dL (4-58)	PRA ng/mL/hr (2.3-37.0)	Blood pressure mm/Hg
Six months before	141	4.2	133	53	67	NA	NA	Normotensive*
Three months before	142	4.5	103	162	190	NA	NA	Normotensive*
Days of attack	146	1.9	NA	NA	NA	NA	NA	100/70
15 days' after	143	4.6	NA	NA	NA	36.8	2.38	90/60

*: Blood pressure value unspecified, reported normotensive in data, numbers in parentheses refer to normal value.
PRA: plasma renin activity, NA: not available, ACTH: adrenocorticotropin hormone

(NM_000334.4) in the proband identified the previously reported heterozygous p.Arg672His (c.2015G > A) variant. Her affected father and uncle were heterozygous for this variant. Two variants were considered to be 'pathogenic' variants based on the 2015 American College of Medical Genetics/Association for Molecular Pathology (ACMG/AMP) guideline. Pedigree and segregation analyses are shown in Figure 1.

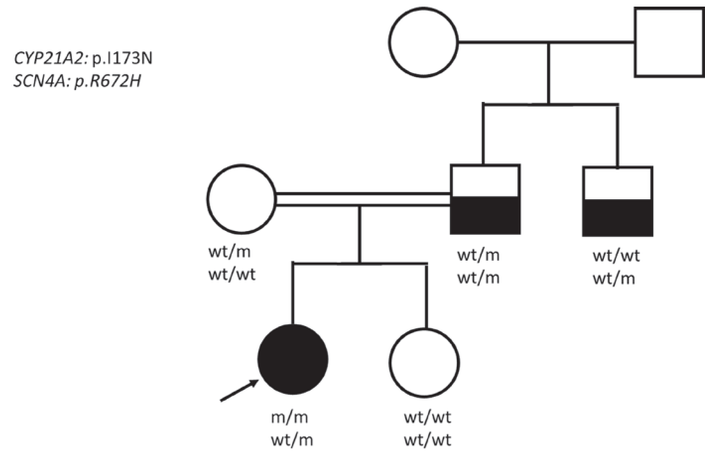

Figure 1. Affected males (Familial Hypokalemic Periodic Paralysis) are represented by black and white squares, affected, females (two diseases) are represented by black circles, and index individuals are indicated by arrows. White square symbols indicate unaffected male family members, White circle symbols represent unaffected female family members, and the double line indicates consanguinity. Under each symbol are the genotypes with WT and M denoting wildtype and mutant, respectively. The first line, and the second line indicates CYP21A2:p.I173N, and SCN4A:p.R672H, respectively

Discussion

Here, we present the first patient with co-existence of congenital adrenal hyperplasia (CAH) and hypokalemic periodic paralysis. This is a rare case that presented with hypokalemia and a tendency to hyperkalemia due to 21-OHD.

In *CYP21A2*, a T-to-A change at nucleotide 518 was predicted to be a substitution of the isoleucine at residue 173 for asparagine, p.Ile173Asn. This pathogenic variant has been widely reported in 21-OHD, and functional analysis has been performed (3,4). The p.Arg672His was located at a hot-spot region in *SCN4A*. The missense variant was not found in gnomAD. Alternative variants Arg672Ser, Cys, Gly and Ser have been reported previously (5). Jurkat-Rott et al (6) reported a family with hypokalemic periodic paralysis type 2, in which the family member had p.Arg672His in *SCN4A*. Lastly, this variant was considered to be a 'pathogenic' variant based on the 2015 ACMG/AMP guideline. These findings led us to conclude that this variant is the disease-causing variant.

Since enzyme activity is 1-5% in patients with p.Ile173Asn in *CYP21A2*, most patients do not need fludrocortisone treatment. The presence of homozygous p.Ile173Asn genotype, classified in group c, predicts simple virilising 21-OHD. However, these patients tend to have hyperkalemia (3,4). Fludrocortisone treatment is initiated before the definite diagnosis, as suggested in CAH guidelines because of its life-threatening nature, causing 25% of patients to receive unnecessary fludrocortisone treatment (1). It is necessary to measure PRA and aldosterone levels, blood pressure and serum electrolytes of each patient with 21-OHD to avoid overtreatment or mistreatment. We observed that this management was insufficient in the patient's history.

Additionally, genetic analysis is guiding in these patients (3,7). Prior to deciding on such treatment, it is vital to review all possibilities, take a careful medical history and family history and perform a physical examination. Here, *CYP21A2* genetic analysis is also highly useful in deciding upon fludrocortisone treatment (3). It was unusual to detect hypokalemia with a sign of hyperaldosteronism in

a case where we expected a possible lack of aldosterone production. However, the patient's family history was suspicious for hypokalemic periodic paralysis. We determined that genetic analysis had not been performed for any family members before, although molecular genetic confirmation of the possible diagnosis is extremely helpful in such complex situations. In this patient, genetic testing confirmed an autosomal dominant *SCN4A*-related disorder and a simple virilising form of 21-OHD. Fludrocortisone treatment was discontinued gradually, with the molecular genetic prediction, as it could exacerbate possible episodes of hypokalemia. As research in upcoming fields, such as molecular genetics and metabolomics, increases, more evidence-based approaches may be created rather than empirical treatments. This provides a better medical approach to orphan cases. In our patient, when the serum potassium level was 1.9 serum mEq/L, the serum sodium level was 146 mmol/L, at the upper end of the range. Additionally, 15 days after the hypokalemic attack, PRA seemed to be suppressed at the lower end of the range without fludrocortisone treatment. This finding led us to infer that mistreatment with fludrocortisone may have aggravated hypokalemia.

When patients are evaluated after or between attacks, the diagnosis of FHPP may be challenging. Molecular genetic testing is recommended when the diagnosis of FHPP is suspected (2). Other diagnostic options include provocative testing and electromyography. Provocative testing with oral glucose, insulin, exercise and ACTH can induce FHPP (8). The father and uncle of the case, carrying the same variant in *SCNN4*, had experienced their first hypokalemia attack in their 20s. Although there is an opinion that penetrance is lower in women, our case had her first attack at the age of 14 years. Hypokalemia that occurs earlier than we expected could be due to two reasons; first, based on the ACTH provocation test, the elevated ACTH, due to poor metabolic disease control, may trigger the attack or second, elevated androgens due to 21-OHD may trigger an attack in FHPP, as shown in the study of Ke et al (9).

Our patient had undergone clitoroplasty in infancy, without any complications. The association between malignant hyperthermia and hypokalemia is uncertain, but has been described previously (10). If surgery with general anaesthesia is required, patients should be monitored for signs of malignant hyperthermia. However, in a future surgical operation, the surgeon, anaesthesiologist and endocrinologist should evaluate possible risks and take the necessary precautions. Simultaneously, the patient was warned about drugs and general anaesthesia that could trigger an attack. In this patient's clinical management, with two co-existing conditions, we advised her to have a low-carbohydrate diet and to refrain from strenuous exercise to prevent hypokalemic attacks. However, potassium-sparing treatment is not preferred for the risk of hyperkalemia. It can, paradoxically, worsen hypokalemia. As soon as symptoms begin, we recommend that hypokalemia must be confirmed with blood tests before potassium therapy. The patient was educated about the regular use of glucocorticoid therapy to avoid poor metabolic control with CAH.

In medical evaluation, it is essential to take a careful and detailed patient medical history and obtaine the same from family members. The pedigree is critical. Many patients are not diagnosed, and their diseases are not defined due to insufficient examination time and clinical attention, as well as a lack of medical information, especially in orphan patients. This is the group of patients who suffer most from the difficulties in getting appropriate support. According to our clinical experience from these cases, identifying individuals at risk with segregation analysis after taking a complete family history will keep the case safer in terms of future risky situations.

Conclusion

In our case, a disease predisposing to hypokalemia, 21-OHD, was suspected at the first evaluation, and molecular genetic tests supported the diagnosis. Periodic paralysis is a rare disease, and the possibility of concomitant 21-OHD is extremely low. Rare inherited conditions may co-occur in geographies where consanguineous marriage is common and the genetic pool is diverse. Due to the heavy patient load, insufficient number of doctors and the presence of widespread routine algorithms, such rare cases can be missed. In this case, our experiences highlighted once again, the importance of obtaining a careful and detailed family medical history, which allows the clinician to reduce the possible risks. Family members at risk should be identified by segregation analysis in inherited genetic diseases and disorders. Androgens may have triggered the hypokalemic attack in FHPP, as previously suggested, and overtreatment of fludrocortisone may have aggravated hypokalemia.

Ethics

Informed Consent: The subject and her parents have given their written informed consent to publish their case in accordance with the Declaration of Helsinki.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices- Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: Tuğba Kontbay, İhsan Turan.

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

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LETTER TO THE EDITOR

DOI: 10.4274/jcrpe.galenos.2020.2020.0299

J Clin Res Pediatr Endocrinol 2021;13(3):367-368

Analysis of the Performance of Neck Circumference to Identify Overweight and Obese Children

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Dear Editor,

I have read with interest the article by Asif et al (1) on the potential usefulness of neck circumference to identify overweight and obese children. It is indeed a potential tool applicable to any context. However, there are two specific aspects about this article that I find interesting to point out.

In the statistical analysis section, they mention that a diagnostic test was considered highly accurate if the area under the curve (AUC) was between 0.65 and 1.00, while it was moderately accurate if the AUC was between 0.5 and 0.65 (1). AUC values close to 0.5 are not useful in clinical practice so perhaps it would be better to refer to AUC values between 0.5 and 0.65 as "not accurate" rather than "moderately" accurate. That said, one could calculate the sample size required to find an AUC equal to the minimum considered "highly accurate", that is, 0.65. The pROC package (2) for R uses the formula published by Obuchowski et al (3) to perform this calculation. When using this package (using a power of 0.80 and a group ratio of 1:1) it is obtained that the required sample size is 110 (55 controls and 55 cases) for each AUC that is estimated to be at least 0.65. In the study, four AUC values were calculated for each age group (overweight boys, overweight girls, obese boys and obese girls) (1). Therefore, for each age group, a minimum of 440 subjects would be required. One of the strengths of the study is that it far exceeds this number of subjects in each age subgroup (1). With the availability of such a large number of study subjects, it could have been considered to perform cross-validation, with which an internal validation of the AUCs could have been carried out. However, for this, approximately 200 subjects would have been required as a minimum ideal number for each AUC to be validated (4). That is, a total of 800 subjects would have been required for each age group, which exceeds the number of subjects available to the researchers for several age groups (1). Despite this, similar to the way they grouped those aged 5 to 9 and 10 to 14 years (1), they could have made groups aged 5 to 6, 7 to 8, 9 to 10, 11 to 12 and 13 to 14 years in order to have a sufficient number of subjects per group, not only to calculate cut-off points but also to be able to carry out an internal validation of the discriminative ability of neck circumference.

On the other hand, the group of 7-year-old boys had a very low AUC (0.555) unlike other groups analyzed (1). It would be important to propose possible explanations for this in order to know in which situations the neck circumference usefulness could be diminished.

Ethics

Peer-review: Internally peer-reviewed.

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

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Received: 08.12.2020
Accepted: 17.12.2020

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DOI: 10.4274/jcrpe.galenos.2021.2021.0222

J Clin Res Pediatr Endocrinol 2021;13(3):369

In reply Asif M et al.

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Dear Editor,

Firstly, we are very thankful to the reader who took really a very keen interest in our research work. In our study, we checked the diagnostic performance and determined the best cut-off points of the neck circumference (NC) for identification of overweight and obese Pakistani children (1). The diagnostic ability of NC to discriminate children with or without overweight and obesity was assessed using area under the curve (AUC).

1) The reader raised the point that it would be better to refer to AUC values between 0.5 and 0.65 as "not accurate" rather than "moderately" accurate. We (the authors) want to explain that we used the AUC cut-off points that were suggested by the Perkins and Schisterman (2) and the same cut-points for AUC in determining the diagnostic ability of NC were also used by Kelishadi et al. (3).

2) The reader also reported that one could calculate the sample size required to find an AUC equal to the minimum considered "highly accurate", that is, 0.65. The pROC package (4) for R uses the formula published by Obuchowski et al (3) to perform this calculation. For getting AUC at least 0.65 in pROC package, minimum sample size for each age-group should be 110 (number of cases = number of controls). He mentioned that a total of 800 subjects would have been required for each age group and these numbers were greater in our study. In our study, the number of cases were not equal to number of controls in each age-group and we used the software; "Statistical Package for Social Sciences (SPSS)" version 21.0 for ROC analyses which doesn't require such type of sample size conditions. That's why, one who used the pROC package in R could follow the required sample size conditions.

Ethics

Peer-review: Internally peer-reviewed.

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

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Conflict of interest: None declared
Received: 11.08.2021
Accepted: 15.08.2021