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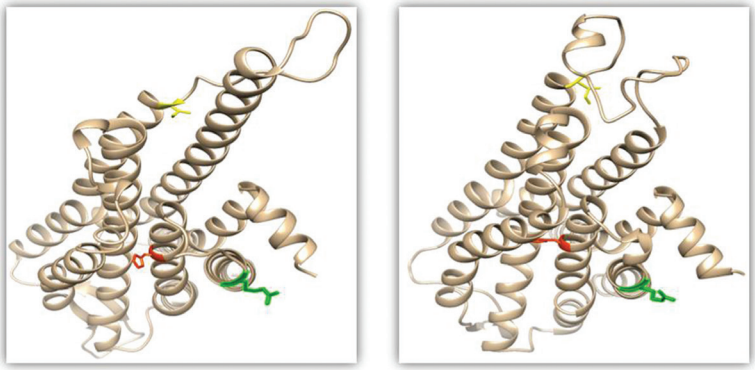

A Novel Mutation in the *AVPR2* Gene Causing Congenital Nephrogenic Diabetes Insipidus

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

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

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REVIEW

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Use of Vitamin D in Children and Adults: Frequently Asked Questions

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Abstract

In recent years, the increase in interest and use of vitamin D has been attributed mainly to the extra-skeletal effects of vitamin D and confusion about normal reference values for serum 25-hydroxy vitamin D (25-OHD). However, The Institute of Medicine, which determines daily intake of nutrients, vitamins and minerals in the United States, emphasizes that there is no additional benefit of having a 25-OHD level above 20 ng/mL in terms of parathyroid hormone suppression, calcium absorption and "fall risk". Taking into consideration that there has not been a significant increase in vitamin D deficiency and related conditions in Turkey over the past five years, it is not hard to suppose that this increased interest is due to doctors, using mass media platforms, who have made claims that vitamin D is a "panacea". This paper aims to answer some frequently asked questions such as the threshold values recommended for the evaluation of vitamin D status, the clinical indications for measuring 25-OHD and suggestions on the use of lifelong vitamin D starting from pregnancy.

Keywords: Vitamin D, deficiency, maintenance

Introduction

Over the last 10 years an increasing interest in vitamin D deficiency and its effect, not only on extra-skeletal tissues but also on general human health, has been observed not only in Turkey but all over the world.

Recently published research, based on data of 711,718 children in the UK, showed that the frequency of diagnosis of vitamin D deficiency increased from 3.14/100,000 in 2000 to 261/100,000 in 2014 and a 15-fold increase was reported after adjustment for population increase (1). However, reliable institutions and researchers have expressed the opinion that we are not confronting a vitamin D deficiency pandemic but that this rise is related to the change in diagnostic behaviour of physicians and other health care professionals, as well as to an increase in the demand for vitamin D examination during routine visits (1,2).

Severe vitamin D deficiency may result in hypocalcemic seizures and hypocalcemic cardiomyopathy in infancy. Therefore, we suggest that 25-hydoxy-vitamin D (25-OHD) levels in infant and mother should be a routine part of evaluation of infantile hypocalcemia. It should also be noted that the regulation and action of parathyroid hormone (PTH) may be disturbed by vitamin D deficiency, particularly in infancy. Elevated PTH levels associated with hypocalcemia and normal or high phosphate indicate an element of end-organ resistance to PTH, mimicking pseudo-hypoparathyroidism (3). Studies performed in the last decade detected severe vitamin D deficiency (< 10 ng/mL) in 46-80 % of pregnant women and nursing mothers in different regions of Turkey (4,5,6,7,8). Low socioeconomic status, covered clothing style, low educational level and spending less time outdoors because of cultural and lifestyle factors are associated with maternal and perinatal vitamin D deficiency (4,5,6,7,8). There is no doubt that the only way of preventing vitamin D deficiency and its complications is vitamin D supplementation.

In addition nationwide data demonstrating increase in frequency of vitamin D deficiency over time in Turkey is not available. However, the serum 25-OHD levels of 110,774 individuals, obtained between January 2011 to December 2016 and assessed in a single laboratory serving the whole country using the liquid chromatography-tandem mass spectrometry method revealed no significant difference over time (9). Surprisingly, data obtained from Intercontinental Marketing Services Health 'IMS Health' showed that in

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2012, 2,280,626 boxes of vitamin D (each box contains 300,000 units of vitamin D) were sold in Turkey, which rose to 8,376,319 in the first eight months of 2016. According to the same data, in 2015, only 925,734 of 8,754,753 boxes of vitamin D (less than one in ten) were prescribed.

Taking into consideration that there has been no observable increase in vitamin D deficiency-related conditions in Turkey during recent years, it is possible to assume that this increase is due to declarations of doctors, appearing via the mass media, who have made claims that vitamin D is a "panacea". The Turkish National Pediatric Endocrinology and Diabetes Society was concerned enough that it released a statement about the harm that these physicians might cause and has drawn attention to false information on vitamin D (10). This paper aims to answer some frequently asked questions, such as the threshold values recommended for the evaluation of vitamin D status and suggestions on the use of lifelong vitamin D starting from pregnancy.

What is the reason for the increasing interest in vitamin D in recent years? What are the normal threshold values for vitamin D?

In recent years, the increase in interest and use of vitamin D has been attributed mainly to the extra-skeletal effects of vitamin D and confusion about normal reference values for serum 25-OHD and in particular The American Endocrine Society's recommendation proposing at least 30 ng/mL for the lower limit of normal range for serum 25-OHD level (11).

The Institute of Medicine (IOM), which determines daily intake of nutrients, vitamins and minerals in the United States, emphasized that there is no additional benefit of having a 25-OHD level above 20 ng/mL in terms of PTH suppression, calcium absorption and "fall risk". In several reports it was stated that skeletal effects of vitamin D plateau when the 25-OHD level is between 12-16 ng/mL and 25-OHD levels below 20 ng/mL should not be accepted as 'deficiency' in all cases. The IOM remarks 'it is false to specify 25-OHD >30 ng/mL as the "desired" threshold and there is no need to supplement high doses of vitamin D for obese individuals' (12,13). Recently, members of the IOM D Vitamin Committee addressed supplementing vitamin D and recommend 400 units per day in the first year of life, 600 units in the first 70 years of life, and 800 units of vitamin D after the age of 70 years. They noted that it is possible to achieve serum vitamin D levels of 16-20 ng/mL in 97.5% of the general population.

In this report the authors highlighted the misinterpretation of a value of 20 ng/mL 25-OHD as a threshold value for bone health given that 97.5% of the general population actually have a 25-OHD level equal to or below 20 ng/mL (2). This misinterpretation may lead a shifting the entire population to a higher intake and could cause the upper intake limit (4000 units of vitamin D per day) will become normal practice, which would also bring risks.

It should not be forgotten that inadequate dietary calcium intake is as important as vitamin D to the development of rickets/osteomalacia. The IOM's recommendation for daily calcium intake is 700-1300 mg for children, 1000-1200 mg for adults (12).

How important are extra-skeletal effects of vitamin D? Is there a need to define a different threshold of 25-OHD level for these effects?

In addition to the intestine, which is the main site of active vitamin D (calcitriol) effect, several tissues such as breast, bone marrow, nerve cells and the immune system have vitamin D receptors and it has been suggested that calcitriol plays a role in the functions of 230 different genes (14). Recently, attention has focused on the extra-skeletal effects of calcitriol and numerous studies establishing a relationship between calcitriol and many diseases (especially cancer) have appeared widely in medical journals (15). The vast majority of this research is made up of correlational studies and fails to meet the cause and effect relation criteria.

Some researchers in The United States claim milder deficiency of vitamin D causes a predisposition to diseases of extra-skeletal tissues (15). Those publications have caused concern in the community by highlighting the supposed risks associated with 25-OHD levels below 30 ng/mL and they have encouraged healthy people to check vitamin D levels and to take high doses of vitamin D (15).

However, data from vitamin D receptor knock-out animal studies indicated the effects of calcitriol on extra-skeletal tissues were not significant, yet entirely confirmed its effects on calcium absorption and indirect effects through calcium supply on bone texture (16). Besides, a study carried out in human with hereditary vitamin D resistant rickets showed that calcium absorption was highly dependent on vitamin D from infancy until the end of puberty, however hereditary 1.25-dihydroxyvitamin-D-resistant rickets patients have normal plasma renin activity, without any indications of hypertension or gross heart abnormities, such as reduced contractility or hypertrophy, at least until the age of 37 years (17).

The IOM remarked that the outcomes of studies that relate the level of vitamin D to non-skeletal pathologies such as

cancer, cardiovascular disease, diabetes and auto-immune diseases are not consistent with each other and do not require establishing a different 25-OHD threshold or a higher intake of vitamin D to prevent these diseases (12,13).

From a clinical point of view, an increase in problems expected from extra-skeletal effects of vitamin D deficiency in countries/regions/groups where vitamin D deficiency is frequent has not been reported. For instance, there are no reports of a high frequency of type 1 diabetes among children who had rickets. The relationship between vitamin D deficiency and the occurrence of type 1 diabetes has almost been a "cliché" and this information is often regarded as correct because it has been repeated for many years. Recent research in Finland has shown that there is no association between type 1 diabetes antibody positivity, the development of clinical type 1 diabetes and serum 25-OHD levels (18).

In conclusion, studies on the effects of vitamin D and extra-skeletal effects do not provide coherent data and the recommendation for 25-OHD level to be at least 30 ng/mL to obtain protective effects is unproven.

Does total 25-OHD show the whole truth? Is it necessary to supplement a high dose of vitamin D in obese children and adolescents?

Approximately 80% of total 25-OHD is transported by vitamin D binding protein (VDBP), which has a half-life of 1-2 days. It is known that VDBP is a negative acute phase reactant and in cases such as sepsis, synthesis in the liver decreases and therefore total 25-OHD is found to be low (19). A study from the USA showed that total 25-OHD levels in African-American women were associated with low VDBP, so that African-American women and Caucasian women had similar "bioavailable" D vitamin levels (20). In another study, despite low levels of total 25-OHD in obese children, the bioavailable D vitamin level was shown to be normal and there was a negative correlation between insulin resistance and VDBP (21). Similarly, research from Turkey revealed no relationship between insulin resistance parameters and vitamin D levels in obese children (22).

It is well known that 25-OHD levels are generally low in obese people, however these recover with weight loss and vitamin D requirements are not different from non-obese people (13).

On the basis of this evidence, there is no need to routinely monitor serum vitamin D level in obese subjects and it is not necessary to prescribe vitamin D at doses higher than 400 IU per day to enhance the low levels of 25-OHD levels found.

Is routine vitamin D testing and/or intake of vitamin D ampoules necessary for healthy people?

Overall, when vitamin D deficiency is severe (serum 25-OHD level ≤12 ng/mL) bone metabolism deteriorates and diseases such as rickets in children and osteomalacia in adults result (23).

In Turkey, a nationwide 'vitamin D prophylaxis augmentation programme' was initiated in 2005 using a simple but effective method which included free distribution of vitamin D drops to all new-borns and infants (0-12 months) visiting primary healthcare stations throughout the country. This programme has reduced the number of clinical rickets cases and the incidence of severe vitamin D deficiencies dramatically in Turkey (24). There is absolutely no need to test vitamin D levels in routine follow-up and to prescribe high doses of vitamin D because of low vitamin D levels in infancy and childhood. Indeed, the Global Consensus clearly states that testing is not indicated in asymptomatic individuals (23). Instead, all infants from birth, all pregnant women and all ethnic/cultural risk groups require supplementation. Nevertheless, the frequency of serum 25-OHD testing has increased approximately 2.60 times in the 0-18 years old age group and 32% in the over 18s between 2011-2016 (4).

Adults with osteomalacia might suffer from widespread bone pain and muscle weakness, particularly in the vertebrae, when vitamin D concentrations drop to 12 ng/mL or less. Therefore, neither routinely testing vitamin D in healthy, aymptomatic subjects over 40 years of age, nor prescription/intake of high dose vitamin D for serum concentrations of vitamin D below 20 ng/mL is required. This is because serum concentrations of vitamin D are only a biochemical parameter and do not give the whole picture. It is necessary to test serum alkaline phosphatase (ALP) and PTH concentrations and make an assessment based on these findings and other clinical and radiological findings to diagnose the disease.

Has the definition of vitamin D deficiency changed? Who should have been treated with high dose vitamin D? Is testing only serum 25-OHD enough to make a decision?

Thresholds used for vitamin D deficiency differ in children and adults, but many physicians tend to interpret values below 20 ng/mL as deficiency regardless of patient age.

In children, the laboratory 25-OHD threshold for vitamin D deficiency is 12 ng/mL and 12-20 ng/mL for insufficiency. Serum 25-OHD values above 20 ng/mL are accepted as vitamin D sufficiency (Table 1) (23). However, in The Endocrine Society's 2011 guidelines, a 25-OHD level below 20 ng/mL is defined as deficiency and a level between 20-30 ng/mL is defined as insufficiency (11). This older recommendation has led physicians to administer high dose vitamin D treatment. It is also outdated in relation to the musculoskeletal effects of vitamin D. A similar recommendation exists in the guidelines of The Association of Adult Endocrinology and Metabolism in Turkey and administering "vitamin D stoss therapy" is recommended if the serum 25-OHD level is < 20 ng/mL (25). In contrast to The Endocrine Society's 2011 and The Association of Adult Endocrinology and Metabolism in Turkey's recommendations the IOM consider that an intake of 400 IU/day of vitamin D is adequate in order to ensure a serum vitamin D level between 16 and 20 ng/mL (12). In addition, in a CDC report analysing vitamin D status in the US, the threshold serum 25-OHD level was taken as 12 ng/mL as a definition of vitamin D deficiency and it was reported that levels above 50 ng/mL are 'possibly harmful' (26). Furthermore, it is essential to confirm an elevation of serum ALP and/or PTH before administration of vitamin D at the treatment dose. Treatment dose is determined as a peroral, single dose of 50,000 IU vitamin D for children aged 3-12 months, 150,000 IU for children aged 12 months to 12 years and 300,000 IU for those aged > 12 years in The European Society for Pediatric Endocrinology's Global Consensus Recommendations on Prevention and Management of Nutritional Rickets (23).

As mentioned above, vitamin D stoss therapy (a single high dose vitamin D) or 2,000-6,000 IU/day vitamin D administration, based only on serum vitamin D level, is not advisable.

Seasonal variations of 25-OHD level should also be taken into consideration when vitamin D status is being assessed.

Table 1. Classification of vitamin D status, based on serum 25-OHD levels (22)

Vitamin D status	Serum 25-OHD levels in nmol/L (ng/mL equivalent values)
Sufficiency	> 50 (> 20 ng/mL)
Insufficiency	30-50 (12-20 ng/mL)
Deficiency	< 30 (< 12 ng/mL)
Recommended upper limit	250 (100 ng/mL)
Toxicity	> 250 (> 100 ng/mL) + hypercalcemia, hypercalciuria and suppressed PTH

25-OHD: 25-hydoxy-vitamin, PTH: parathyroid hormone

A seasonal decline in serum 25-OHD levels has been well documented from summer to winter in two large scaled studies from different regions of Turkey (27,28).

What is the lifelong daily maintenance dose of vitamin D? Is vitamin D supplementation needed during pregnancy?

Lifelong daily vitamin D requirements are regularly updated by the IOM in the United States. These updates include the amount that meets at least 97.5 % of the healthy target population [Recommended Dietary Allowance (RDA)] and the maximum amount that can be taken per day without any risk [Upper Intake Level (UL)]. The last update from 2011 specified the RDA for vitamin D was 400 IU/day in the first year of life (UL was 1000 IU/day for infants > 6 month-old, 1500 IU/day for infants 6 months-1 year) and 600 IU/day for individuals between one and 71 years (UL was 2500 IU/day for children between 1 and 3 years, 3000 IU/day for children between 4 and 8 years and 4000 IU/day for individuals > 8 years) and 800 IU for individuals > 71 years old (7). A recent global consensus report has recommended that 400 IU of vitamin D be given orally to all infants until one year of age (23). The IOM's recommended dose for supplementing vitamin D in pregnancy is 600 IU/day (UL: 4000 IU). D-vitamin supplementation during pregnancy is primarily required for the prevention of late hypocalcemia in the new-born period. In countries where maternal vitamin D deficiency is common, such as Turkey, a dose of 1200 IU/day or more is recommended (29).

400 IU/day vitamin D for new-borns (from the first day of life) and 1200 IU/day vitamin D for women from the third month of pregnancy and during lactation is recommended through the national program for the prevention of vitamin D deficiency in Turkey (30,31).

It is considered that supplementation of vitamin D in the form of oral drops until at least the first year of life, and preferably up to the age of three years, is sufficient.

Sunlight exposure, 30 minutes per week with only diaper and at least two hours per week when they are fully clothed, is also sufficient for babies after six months of age to have a vitamin D level at 11 ng/mL, however the duration of sunlight exposure that is necessary for infants and children to maintain vitamin D levels at 50 nmol/L (20 ng/mL) in children remains to be determined. In the meantime, it is necessary to keep in mind that sunscreens and sunlight exposure through glass reduce the synthesis of vitamin D by more than 90 % (32,33).

What are the main incorrect attitudes regarding use of vitamin D in children?

In Turkey, vitamin D deficiency rickets was a common problem in the first two years of life in the past. Affected infants had signs such as delayed walking and teething so that some families, pharmacists and sometimes physicians had a tendency to make toddlers drink vitamin D ampoules with the idea of "earlier walking" and "earlier teething" due to this association.

However, supplementing a baby with higher doses of vitamin D than required has no effect on early walking and teething. Beyond that, it may result in permanent damage by causing "vitamin D intoxication" and renal calcifications.

Another misconception is the administration of high dose D vitamin to children with bowed legs without thorough examination. Bowed legs may be seen in vitamin D deficiency rickets but it is not the only etiology of leg bowing which include physiologic bowing and genetic skeletal disorders. Thus, children with bowed legs should not be randomly given high dose vitamin D and any such cases should definitely be examined by a pediatric endocrinologist.

Finally, some physicians in Turkey discontinue supplementing vitamin D in the first few months of life because of "small" fontanelles. This is another misconception because closure of the fontanelles is delayed in the case of vitamin D deficiency and normal or even high doses of vitamin D are not associated with early closure or smallness of the fontanelles.

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Concept: Gül Yeşiltepe Mutlu, Şükrü Hatun, Design: Gül Yeşiltepe Mutlu, Şükrü Hatun, Data Collection or Processing: Gül Yeşiltepe Mutlu, Şükrü Hatun, Analysis or Interpretation: Gül Yeşiltepe Mutlu, Şükrü Hatun, Literature Search: Gül Yeşiltepe Mutlu, Şükrü Hatun, Writing: Gül Yeşiltepe Mutlu, Şükrü Hatun.

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The Role of Irisin, Insulin and Leptin in Maternal and Fetal Interaction

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What is already known on this topic?

Irisin regulates physiological insulin resistance in pregnancy. The effects of irisin on fetal growth have not yet been completely elucidated. It is known that obesity, type 2 diabetes and the metabolic syndrome, insulin resistance and consequently increased risks of cardiovascular disease can be programmed in the intrauterine period.

What this study adds?

Cord blood levels of irisin, insulin, and leptin were associated with fetal growth. These findings may help to identify the risks that may arise in the later stages of life due to intrauterine growth problems.

Abstract

Objective: Insulin is an important hormone for intrauterine growth. Irisin is an effective myokine in the regulation of physiological insulin resistance in pregnancy. Leptin and insulin are associated with fetal growth and fetal adiposity. In this study, we aimed to investigate the relationships between irisin, insulin and leptin levels and maternal weight gain, as well as anthropometric measurements in the newborn.

Methods: Eighty-four mothers and newborns were included in the study. Irisin, leptin and insulin levels were measured in the mothers and in cord blood. Anthropometric measurements in the newborn, maternal weight at the beginning of the pregnancy and at delivery were recorded.

Results: Birth weight were classified as small for gestational age (SGA), appropriate for gestational age (AGA) and large for gestational age (LGA). There was no difference in irisin levels among the groups. Leptin and insulin levels were found to change significantly according to birth weight ($p = 0.013$, and $p = 0.012$, respectively). There was a negative correlation between the anthropometric measurements of the AGA newborns and irisin levels. This correlation was not observed in SGA and LGA babies. Leptin levels were associated with fetal adiposity.

Conclusion: While irisin levels are not affected by weight gain during pregnancy nor by birth weight, they show a relationship with anthropometric measurements in AGA infants. These results may lead to the understanding of metabolic disorders that will occur in later life.

Keywords: Irisin, insulin, leptin, fetal growth, maternal weight gain

Introduction

Boström et al (1) first discovered irisin, a myokine, in 2012 and they reported that irisin stimulates the transition of white fat tissue to brown fat tissue. Increased levels of irisin by exercise and exposure to cold leads to the production of fibronectin-type 3 domain-containing protein 5 (FNDC5), a membrane protein (1,2,3). Obese people with higher levels of basal irisin were shown to lose more weight with dieting and increased levels of irisin with early intervention in obesity have been reported to reduce insulin secretion and blood sugar level (4). The

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post acute exercise level was temporarily increased, while the level of prolonged exercise decreased and was higher in prediabetics than in controls. Moreover, it has been shown that the level of irisin can be heritable and that there is an irisin-related positive correlation between mother and son (5).

There are also various reports on associations of irisin with chronic disease processes such as type-2 diabetes, metabolic syndrome, cardiovascular diseases, osteoporosis, chronic kidney disease, proliferation of cancer cells and nonalcoholic fatty liver (6). It has been reported that irisin has an effect in increasing energy expenditure, weight loss, glucose and insulin resistance and that it exerts these effects by stimulating the transformation of white adipose tissue to brown-like adipose tissue (1,7).

Brown fat tissue is important in thermogenesis and energy metabolism. The amount of brown fat tissue is high in the neonatal period (8). Intrauterine growth restriction (IUGR) creates a predisposition to fetal fat tissue changes in fetal growth disorders leading to macrosomia, permanent hormonal changes and obesity and insulin resistance in future years (9,10). IUGR also causes a disproportion in fat mass compared to lean mass, and retardation in growth and skeletal muscle development (11,12). IUGR infants have high insulin sensitivity at birth and are predisposed to insulin resistance after rapid growth in the postnatal period (13). In addition, large for gestational age (LGA) infants are predisposed to obesity and cardiovascular disease later in life as they develop a high fat ratio and reduced insulin sensitivity (14).

Irisin has an important role in controlling maternal and fetal glucose hemostasis. It has been found that the growth of newborns with low irisin levels in their cord blood is delayed and that the proportion of their brown fat tissue is low. Thus, irisin may play an important role in the regulation of maternal-fetal glucose hemostasis (15).

The pregnancy period is a period of increased oxidative stress and it has been shown that irisin reduces oxidative stress (16,17,18). Accordingly, the level of irisin in pregnant women is higher than that of non-pregnant women (19). It is thought that irisin also contributes to the physiological insulin resistance found in pregnancy (19).

Babies of obese mothers have a higher incidence of LGA birth and are more at risk for obesity in the future due to intrauterine programming (14). Maternal leptin levels and newborn fat mass percentiles are interrelated (20). Maternal leptin level increases during pregnancy, as a consequence of placental production, and this increase is associated with fat mass gain (21,22). Leptin also has an effect on fetal adiposity (20).

The purpose of this study was to investigate the relationships between maternal weight at the beginning of pregnancy and weight gain during pregnancy, birth weight, anthropometric measurements; and the effects of these on irisin, leptin and insulin levels.

Methods

This study was conducted on mothers who delivered in the Perinatology Department of the Erciyes University Medical Faculty and their offspring. Signed consent was obtained from all women who volunteered to participate in the study. The study was approved by the University's Ethics Committee (date of approval: 04/04/2014, approval number: 234).

Mothers with diabetes, hypothyroidism, chronic kidney disease, epilepsy, chronic liver disease, hypertension, chronic drug use, asthma, smoking and those with placental and fetal problems were excluded. Premature births (<38 weeks) were excluded from the study. The mother's weight before and after pregnancy was obtained from the hospital files. A proportion of the births were elective cesarean sections. Blood samples were taken simultaneously from maternal venous fasting blood (8 hours fasting) and postnatal fetal umbilical artery under sterile conditions. These blood samples were centrifuged at 5,000 rpm for 5 minutes and stored at -80 °C, and all the samples were analyzed together. Anthropometric measurements were taken on all infants after delivery by the same physician using the same scale.

Ponderal index (PI) was calculated using the [birth weight (g) / birth height (cm^3) x 100] formula in all infants. Those with a PI <2.32 were classified as of low birth weight (SGA), those between 2.32 and 2.85 were classified as of normal birth weight (AGA), and those with >2.85 as overweight birth weight (LGA) (23).

Irisin levels were measured in serum using a commercial human enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Heidelberg, Germany). The measurement range of this assay was between 0.01 and 100 µg/mL.

Maternal and infant serum leptin levels were measured using a human ELISA kit (DIAsource ImmunoAssays S.A., Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium). Serum insulin levels were measured using the Dia Metra kit for immunoenzymatic determination.

Statistical Analysis

Statistical analysis of the data was performed with the IBM SPSS 22.0 program (IBM Inc., Chicago, Ill., USA). The normality distributions of the groups were determined

according to the Kolmogorov-Smirnov test because the number of cases was small. Since the distribution between groups was not normal, the data were given as median and range. Mann-Whitney U test was used for nonparametric tests in comparison of variables including continuous data. The p value was expected to be less than 0.05 for significance. Kruskal Wallis variance analysis was applied as a post-hoc test between the groups. Bonferroni correction was applied for post hoc analyzes and a p value of <0.0125 was taken as significant. Spearman correlation analysis was used to evaluate the relationship between the two continuous variables.

Results

Eighty four mothers with a mean age of 29.8 ± 5.2 years and their newborns were enrolled in the study. The mean duration of pregnancy was 38.7 ± 0.9 weeks. 53.6% of the births were cesarean delivery. At the beginning of the pregnancy, 32.1% of the mothers were overweight, while 15.5% were obese and 27.4% of them gained too much weight during pregnancy (>15 kg). Forty six (55%) of the newborns were male and 38 (45%) were female.

9.5% of the infants were classified as SGA, 73.8% as AGA and 16.7% as LGA. When the anthropometric and hormonal values of the mother and the baby were compared among these three groups, significant differences were found between all anthropometric parameters except for the birth length of the infants (Table 1). Hormonal levels for leptin and insulin differed among the infants in the 3 groups, but no differences were found in other parameters (Table 1).

Birth weight, PI values, head, neck, middle arm circumference and skin fold thicknesses of the infants were significantly higher in the infants who were the offspring of mothers who were overweight when the mothers were divided into two groups according to weight they gained in pregnancy (Table 2). However, maternal weight gain did not cause a difference in hormone levels in either mothers or infants (Table 2).

When the relationship between maternal and infant anthropometric measurements and hormonal changes were analyzed, while there was no relationship between pre-pregnancy body mass index (BMI) values and anthropometric values of the infants, a statistically significant positive correlation was found between these values and the PI,

Table 1. Anthropometric and hormonal parameters in small for gestational age, appropriate for gestational age and large for gestational age infants and their mothers (median values and ranges)

	SGA (n = 8)	AGA (n = 62)	LGA (n = 14)	p
Maternal age (years)	26 (21-37)	30 (21-42)	34 (23-38)	0.230
Weight gained during pregnancy (kg)	11.5 (8.0-14)	12 (7-51)	14 (8-28)	0.130
BMI at the beginning of pregnancy (kg/m^2)	23.9 (15.6-28.0)	24.8 (17.3-45.5)	24.60 (20.4-36.7)	0.519
BMI at the end of pregnancy (kg/m^2)	28.7 (19.1-32.9)	29.2 (21.3-58.5)	31.2 (26.04-42.2)	0.199
Gestational age at birth (weeks)	38.1 (37.4-40.1)	38.6 (37-41)	38.9 (38-42.4)	0.144
Length at birth (cm)	48.5 (44-51)	49.0 (45-53)	48.0 (45-51)	0.152
Weight at birth (g)	2505 (1920-3080)	3130 (2230-4200)	3475.0 (2800-4030)	**<0.001**
Neonatal ponderal index (g/cm^{3}x100)	2.28 (1.93-2.32)	2.58 (2.33-2.83)	3.00 (2.88-3.33)	**<0.001**
Head circumference (cm)	33.8 (31.5-35.5)	34.5 (31-38.5)	35.3 (32-38.5)	**0.017**
Thoracic perimeter (cm)	31.5 (28.5-34.0)	33.0 (29-39)	35.0 (30-36.5)	**0.002**
Neck circumference (cm)	17.5 (15.5-20)	19.4 (15.5-24)	20.5 (17-25)	**0.005**
Abdominal circumference (cm)	29.3 (27-34)	31.5 (27-38)	33.0 (30-36)	**0.001**
Left arm circumference (cm)	9.3 (7.5-10)	11.0 (8.5-14)	11.3 (9-13)	**<0.001**
Triceps skinfold thickness (mm)	5.0 (4-7.5)	7.0 (4-10.5)	9.0 (7-11)	**<0.001**
Subscapular skinfold thickness (mm)	4.5 (2-5.5)	7.0 (4.11.5)	9.0 (5-11)	**<0.001**
Mother irisin level (µg/mL)	2.53 (1.75-12.99)	2.54 (0.01-13.39)	2.94 (2.05-11.38)	0.230
Baby irisin level (µg/mL)	2.6 (1.91-13.06)	2.82(0.01-13.87)	2.45 (1.98-11.48)	0.881
Mother leptin level (ng/mL)	12.9 (1.89-33.19)	9.64 (1-45.75)	6.38 (1.09-27.41)	0.652
Baby leptin level (ng/mL)	1.15 (1.06-7.05)	3.31 (0.18-21.10)	6.88 (1.15-16.13)	**0.013**
Mother insulin level (µIU/mL)	4.36 (2.92-21.68)	4.59 (1.84-36.41)	4.01 (1.53-31.76)	0.838
Baby insulin level (µIU/mL)	2.6 (1.76-12.53)	5.9 (1.84-24.09)	10.33 (2.30-28.27)	**0.012**

BMI: body mass index, AGA: appropriate for gestational age, SGA: small for gestational age, LGA: large for gestational age

abdomen and arm circumference of the infants and post-pregnancy BMI score and between PI and the infants' head, chest, abdomen, left mid arm circumference and skinfold thicknesses (triceps and biceps) (Table 3).

When assessed in terms of the relationships between mother-infant hormone changes and anthropometric measurements, there was a strong positive correlation between maternal-infant irisin levels and a moderately strong positive correlation between mother-infant insulin and leptin levels (Figure 1). There was a weak negative correlation between maternal irisin levels and infant chest, neck, and left middle arm circumference values. There was no relationship between maternal insulin and leptin levels and anthropometric measurements of the infants. There was a positive correlation between the leptin levels of the infants and all anthropometric measurements and between insulin levels and PI, neck circumference and skinfold thickness. Only the neck circumference showed a weak negative correlation with irisin levels of the infants (Table 4).

When the mothers were classified according to weight gain in pregnancy and anthropometric measurements in the infants, hormonal interactions are observed; there was a

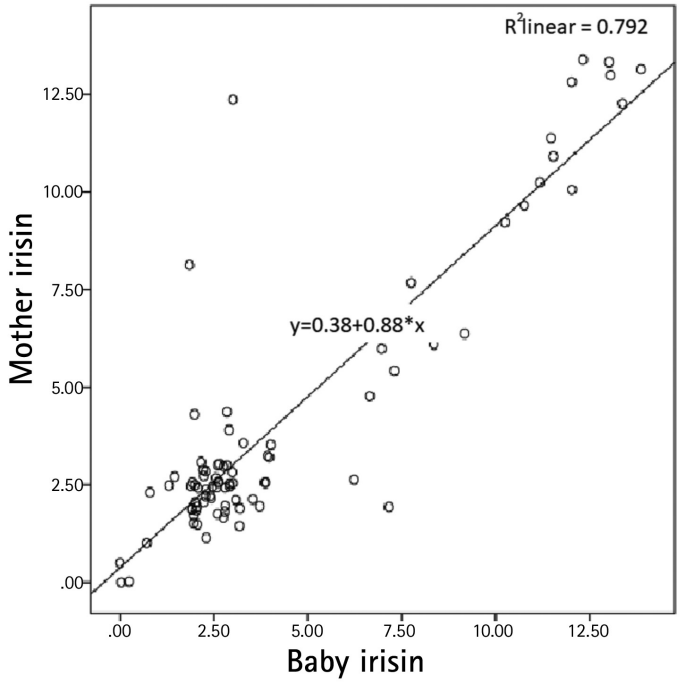

Figure 1. Correlation analysis between mother and baby irisin levels

Table 2. Maternal and infant anthropometric and hormonal parameters according to the weight gain of the mother during pregnancy (median values and ranges)

	Normal weight gain (n = 61)	Excess weight gain (n = 23)	p
Maternal age (years)	30 (21-42)	30 (21-38)	0.717
Weight gained during pregnancy (kg)	11.0 (7.0-15.0)	18.0 (16.0-51.0)	**< 0.001**
BMI at the beginning of pregnancy (kg/m^2)	24.77 (15.62-45.49)	24.84 (17.90-39.06)	0.483
BMI at the end of pregnancy (kg/m^2)	28.65 (19.05-49.10)	31.14 (24.56-58.48)	**0.040**
Gestational age at birth (weeks)	38.71 (37-42.42)	38.42 (37.14-40.14)	0.108
Length at birth (cm)	49.00 (44.00-53.00)	50.00 (45.00-53.00)	0.134
Weight at birth (g)	3040 (1920-4000)	3290 (2510-4200)	**0.016**
Neonatal ponderal index (g/cm^{3}x100)	2.56 (1.93-3.33)	2.68 (2.33-3.22)	**0.026**
Head circumference (cm)	34.5 (31.0-37.0)	35.0 (32.0-38.5)	**0.033**
Thoracic perimeter (cm)	33.0 (28.50-38.50)	33.5 (30.0-39.0)	0.107
Neck circumference (cm)	25.25 (25.50-25.00)	20.00 (16.50-23.50)	**0.018**
Abdominal circumference (cm)	31.50 (27.00-36.00)	32.00 (28.00-38.00)	0.232
Left arm circumference (cm)	10.50 (7.50-13.00)	11.50 (9.50-14.00)	**< 0.001**
Triceps skinfold thickness (mm)	7.00 (4.00-11.00)	8.00 (4.50-10.50)	**0.005**
Subscapular skinfold thickness (mm)	6.50 (2.00-10.50)	7.00 (4.00-11.50)	**0.036**
Maternal irisin level (µg/mL)	2.56 (0.001-13.39)	2.70 (0.50-12.82)	0.771
Infant irisin level (µg/mL)	2.79 (0.03-13.87)	2.92 (0.001-12.03)	0.916
Maternal leptin level (ng/mL)	7.32 (1.00-33.19)	12.54 (1.07-45.75)	0.053
Infant leptin level (ng/mL)	3.57 (0.18-16.13)	5.00 (1.00-21.10)	0.129
Maternal insulin level (µIU/mL)	4.16 (1.53-31.76)	5.71 (1.84-36.41)	0.165
Infant insulin level (µIU/mL)	6.26 (1.76-23.62)	5.79 (2.15-28.27)	0.108

BMI: body mass index

negative correlation between the irisin levels of the mothers of normal weight-gain during pregnancy and the baby's chest, neck, arm circumference, triceps thickness and a positive correlation between maternal insulin levels and the head circumference of the infants.

There was a negative relationship between irisin levels of the infant and neck circumference, a weak positive relationship between insulin levels and middle arm circumference; leptin levels were correlated with all anthropometric values showing a moderate to strong positive correlation. However, these relationships, including the mothers' hormone levels and the infants' anthropometric measurements, disappeared in the infants of mothers who gained excess weight during pregnancy (Figure 2).

When the babies were classified according to PI and the relationship between hormones and anthropometric measurements was analyzed, a more significant relationship was observed in the analysis of babies born with normal weight, a finding similar to the analysis according to maternal weight gain in pregnancy. Interestingly, in SGA infants, a positive correlation was observed between maternal irisin level and middle arm circumference while in the normal weight group this correlation, as well as correlations between all anthropometric measurements and skinfolds, were negative. Again, the relationship between baby irisin levels and some of the baby anthropometric measurements was negative. In LGA infants positive correlations were found between maternal leptin and infant PI, and also between maternal insulin levels and infant neck circumference. Maternal and infant hormone levels correlated with each other in all three groups (Figure 2).

Discussion

It is known that there is a relationship between weight gain of the mother during pregnany and the birth weight of the baby (24). In this study, the interaction of maternal and infant irisin, leptin and insulin levels and factors affecting these values were investigated. It has been shown that normal weight gain in pregnancy and a AGA are influenced by these three hormones, but the effect of these hormones on the infant's development and fat distribution is not so clear when the mother is overweight or the baby deviates from the norm to SGA or LGA.

Irisin is a hormone associated with brown fat tissue and is affected by nutrition, exercise and heat. Elevated fasting plasma glucose and increased levels of irisin have been suggested to play a protective role for insulin resistance (25,26). Physical exercise leads to an increase in peroxisome

Table 3. Relationships between maternal weight during pregnancy and anthropometric measurements of the infant

	BMI at the beginning of pregnancy (kg/m^2)	BMI at the end of pregnancy (kg/m^2)	Weight gain during pregnancy (kg)
Gestational age at birth (weeks)	0.177	0.144	-0.019
	0.107	0.191	0.866
Neonatal ponderal index (g/cm^{3}x100)	0.202	**0.248***	**0.231***
	0.066	**0.023***	**0.035***
Head circumference (cm)	0.029	0.121	**0.271***
	0.793	0.273	**0.013***
Thoracic perimeter (cm)	0.129	0.207	**0.244***
	0.242	0.059	**0.025***
Neck circumference (cm)	0.090	0.174	**0.242***
	0.414	0.112	**0.026***
Abdominal circumference (cm)	0.182	**0.232***	0.173
	0.097	**0.034***	0.115
Left arm circumference (cm)	0.123	**0.274***	**0.434****
	0.264	**0.012***	**<0.01****
Triceps skinfold thickness (mm)	0.001	0.105	**0.264***
	0.990	0.344	**0.015***
Subscapular skinfold thickness (mm)	-0.006	0.088	**0.260***
	0.958	0.424	**0.017***

The statistical values in the first row are r, and the values in the second row are p values.

Significant results *$p < 0.05$, **$p < 0.01$ in star, BMI: body mass index

proliferator-activated receptor-γ coactivator-1 alpha (a transcriptional coactivator) in the muscle tissue as well as increases in FNDC5 and irisin secretion (5).

Increase in irisin increases glucose transport. In addition, by increasing the brown fat tissue and by thermogenesis, glycolysis increases the consumption of glucose and lipids as energy by increasing oxidative phosphorylation (27). Zhang et al (28) found a significant reduction in irisin levels in type 2 diabetic patients.

Infants born as IUGR or LGA are known to develop obesity and subsequent susceptibility to insulin resistance and metabolic syndrome in later life (10,11). However, there may be signs predictive of obesity in later life in the intrauterine period (15). The mechanism of physiological insulin resistance in pregnancy is not fully understood (29). Parallel to the growth of the fetoplacental unit during the gestational period, insulin sensitivity decreases, and progressive weight gain continues (30). Maternal insulin resistance is an important mechanism for fetal growth (30). However, further increases in insulin resistance in the gestational period may cause abnormal fetal growth, fetal macrosomia and IUGR (31,32). Irisin plays a role in the regulation of this physiological insulin resistance in pregnancy and thus in intrauterine growth (33). Leptin is also effective in the development of fetal adiposity (20).

It is known that irisin has an effect on the normal growth of the baby in the fetal period. However, there are conflicting results among the studies in this area. In a study by Briana et al (33), no association was found between maternal irisin levels, BMI and insulin levels in SGA, LGA, and AGA newborns. In contrast Keleş and Turan (34) reported lower irisin levels in SGA infants than those in AGA infants.

In our study, we found no differences in relationships between birth weight and maternal and baby irisin levels in SGA, AGA and LGA infants. Similarly, irisin levels were similar when evaluated according to weight gain during pregnancy. A strong linear correlation was found between maternal and infant irisin levels consistent with previous studies. There was a weak negative relationship between maternal irisin levels and the infant's chest, neck and arm circumference. This relationship disappeared in mothers who gained excessive weight during pregnancy. While the negative relationship between maternal and infant irisin levels and anthropometric measurements became more obvious in normal weight mothers and infants, interestingly, strong positive correlations were found between maternal irisin and baby arm circumference values in SGA infants, and PI values in LGA infants. With these associations, it can be concluded that irisin can control the baby's adiposity in normal pregnancy weight gain in women of normal weight, but that this control is

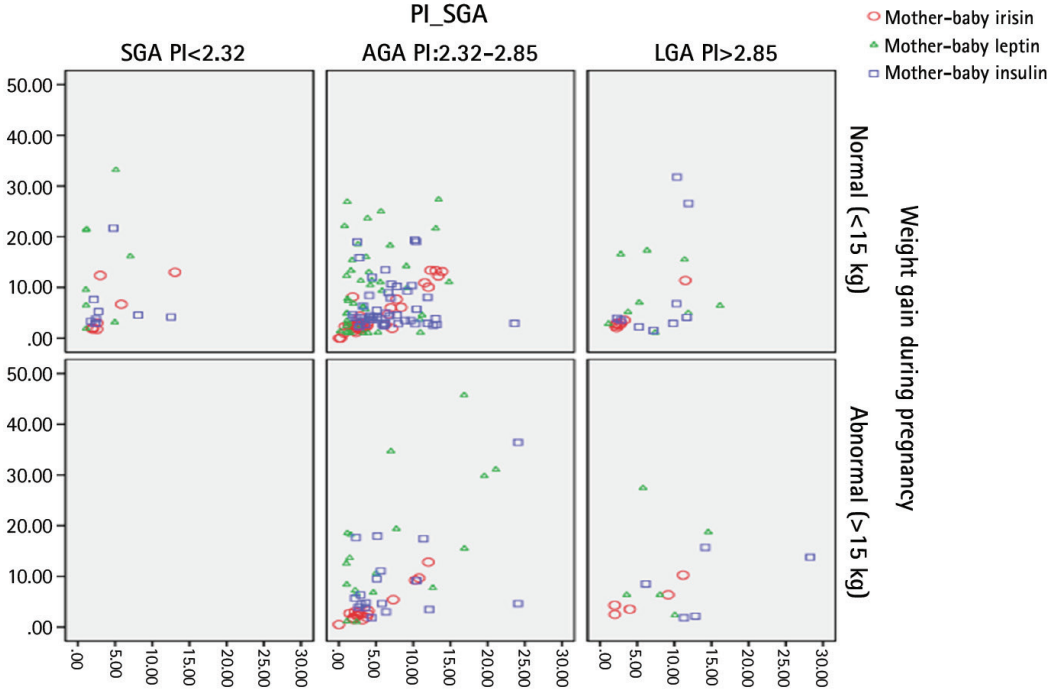

Figure 2. Correlation analysis of hormonal changes in the classification according to the weight gain of the mother during pregnancy and the birth weight of the baby

SGA: low birth weight, AGA: normal birth weight

lost in infants who deviate from the norm (such as SGA and LGA) and this may have pathological effects on fat content and fat distribution.

Leptin is a hormone that correlates with the amount of white fat tissue. Castro et al (35) found no association between maternal leptin levels and fetal adiposity. In our study, although maternal leptin levels correlated with leptin and insulin levels in infants, maternal leptin did not have any effect on the fat distribution parameters of the baby. However, leptin levels differed according to PI, mostly in LGA-born infants. In addition, leptin was found to be the only hormone correlated with all anthropometric parameters of the infant. This correlation was not found to be related to any measurement parameters of either the maternal leptin levels in mothers who gained more weight nor to leptin levels in the infants; while it continued to correlate in the mothers with normal weight gain and AGA infants. Leptin levels were found to correlate with the baby's arm circumference in SGA infants and PI in the LGA babies. This may be related to the development of a kind of resistance which occurs with disappearance of leptin due either to the pathologic weight gain of the mother or to SGA or high birth weight.

Table 4. Relationships between maternal and infant hormone levels and anthropometric measurements

	Mother			Baby		
	Irisin	**Leptin**	**Insulin**	**Irisin**	**Leptin**	**Insulin**
Maternal irisin (µg/mL)	1	0.090	0.091	**0.890****	-0.113	0.111
		0.415	0.410	**< 0.01****	0.305	0.313
Infant irisin (µg/mL)	**0.890****	0.120	0.144	1	-0.066	0.113
	< 0.01**	0.276	0.192		0.552	0.305
Maternal leptin (ng/mL)	0.090	1	**0.293****	0.120	**0.374****	0.102
	0.415		**0.007****	0.276	**< 0.01****	0.354
Infant leptin (ng/mL)	-0.113	**0.374****	**0.310****	-0.066	1	**0.337****
	0.305	**< 0.01****	**0.004****	0.552		**0.002****
Maternal insulin (µIU/mL)	0.091	**0.293****	1	0.144	**0.310****	**0.255***
	0.410	**0.007****		0.192	**0.004****	**0.019***
Infant insulin (µIU/mL)	0.111	0.102	**0.255***	0.113	**0.337****	1
	0.313	0.354	**0.019***	0.305	**0.002****	
Gestational age at birth (weeks)	-0.120	-0.032	0.107	-0.128	**0.303****	0.040
	0.278	0.772	0.334	0.248	**0.005****	0.717
Neonatal ponderal index (g/cm^{3}x100)	-0.092	-0.065	0.202	-0.013	**0.329****	**0.233***
	0.406	0.554	0.065	0.904	**0.002****	**0.033***
Head circumference (cm)	-0.199	-0.001	0.117	-0.126	**0.293****	0.104
	0.070	0.995	0.291	0.254	**0.007****	0.348
Thoracic perimeter (cm)	**-0.240***	0.021	0.082	-0.150	**0.344****	0.188
	0.028*	0.847	0.458	0.173	**< 0.01****	0.086
Neck circumference (cm)	**-0.240***	0.045	0.143	**-0.223***	**0.324****	**0.262***
	0.028*	0.684	0.195	**0.042***	**0.003****	**0.016***
Abdominal circumference (cm)	-0.140	0.031	0.127	-0.109	**0.353****	0.201
	0.204	0.783	0.249	0.323	**< 0.01****	0.066
Left arm circumference (cm)	**-0.244***	0.027	0.169	-0.153	**0.408****	**0.218***
	0.025*	0.804	0.124	0.163	**< 0.01***	**0.046***
Triceps skinfold thickness (mm)	-0.205	-0.118	0.017	-0.142	**0.339****	**0.250***
	0.062	0.284	0.879	0.199	**0.002****	**0.022***
Subscapular skinfold thickness (mm)	-0.148	-0.111	0.153	-0.092	**0.367****	**0.280****
	0.178	0.317	0.164	0.406	**< 0.01****	**< 0.01****

The statistical values in the first row are r, and the values in the second row are p values.

Significant results *p < 0.05, **p < 0.01 in star

The positive effect of insulin on growth in the fetal period is well documented (10,13). Consistent with this, in our study, insulin levels of LGA-born babies were significantly higher. However, in general, we did not find a significant association between insulin levels and irisin levels. This result is consistent with the existing information in the literature (33).

Study Limitations

An important limitation of the study was the low numbers of SGA and LGA cases. This was due to the exclusion of infants with chronic illness from the study.

Conclusion

In conclusion, a strong correlation between mother and baby irisin levels, as expected, was found in this study. There was a strong correlation also between maternal insulin and infant irisin levels in SGA infants, while in LGA infants a strong correlation between infant insulin and leptin levels were found. In SGA infants, elevation of irisin levels can be considered as a protective mechanism for high insulin levels in the mother. In LGA babies, the correlation between leptin and irisin suggests that increased fat tissue increases the effect of both hormones.

When the infants were evaluated from the developmental point of view, it can be concluded that the homeostasis system works well, especially in mothers who gained normal weight and in babies born of normal weight and especially when irisin correlates negatively with all measurements.

The effects of irisin on fetal growth have not yet been elucidated precisely. It is known that obesity, type 2 diabetes and the metabolic syndrome, insulin resistance and consequently increased risks of cardiovascular disease can be programmed in the intrauterine period. Disturbances in intrauterine growth are an important factor in this programming. In this context, further studies are needed to identify and prevent possible predisposing factors to abnormal growth in fetal life and to better understand the mechanism of action of irisin.

Ethics

Ethics Committee Approval: This study was performed at the Erciyes University Medical Faculty perinatology department on patients followed by the clinic who gave birth at the same clinic. Signed consent was obtained from all the volunteers who participated in the study. The study was approved by the University's Ethics Committee (date of approval: 04/04/2014, approval number: 234).

Informed Consent: Informed form received.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Deniz Ökdemir, Nihal Hatipoğlu, Ülkü Gül Siraz, Selim Kurtoğlu, Sabahattin Muhtaroğlu, Mehmet Serdar Kütük, Himmet Haluk Akar, Concept: Deniz Ökdemir, Selim Kurtoğlu, Nihal Hatipoğlu, Design: Deniz Ökdemir, Selim Kurtoğlu, Nihal Hatipoğlu, Data Collection or Processing: Deniz Ökdemir, Nihal Hatipoğlu, Ülkü Gül Siraz, Selim Kurtoğlu, Sabahattin Muhtaroğlu, Mehmet Serdar Kütük, Himmet Haluk Akar, Analysis or Interpretation: Deniz Ökdemir, Nihal Hatipoğlu, Literature Search: Deniz Ökdemir, Nihal Hatipoğlu, Ülkü Gül Siraz, Selim Kurtoğlu, Sabahattin Muhtaroğlu, Mehmet Serdar Kütük, Himmet Haluk Akar, Writing: Deniz Ökdemir, Nihal Hatipoğlu, Ülkü Gül Siraz, Selim Kurtoğlu, Sabahattin Muhtaroğlu, Mehmet Serdar Kütük, Himmet Haluk Akar.

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Efficacy and Safety of Continuous Subcutaneous Insulin Infusion vs. Multiple Daily Injections on Type 1 Diabetes Children: A Meta-Analysis of Randomized Control Trials

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What is already known on this topic?

A previous meta-analysis on children with type 1 diabetes indicated the advantages of continuous subcutaneous insulin infusion in blood glucose control. However, bias caused by age may exist.

What this study adds?

A better control of glycemia can be accomplished by continuous subcutaneous insulin infusion (CSII) compared with multiple daily injections (MDI) in children with type 1 diabetes aged ≤18 years old. The significantly reduced insulin requirement can be obtained after long term CSII treatment (12 months), compared with MDI. Age, treatment duration and study design are factors impacting the efficacy of CSII and MDI in children with type 1 diabetes.

Abstract

Objective: This meta-analysis was performed to evaluate the efficacy and safety of continuous subcutaneous insulin infusion (CSII) vs. multiple daily injections (MDI) in children with type 1 diabetes.

Methods: A literature search was conducted on databases including PubMed and Embase up to June 2017. The pooled weighted mean difference or risk ratio as well as 95% confidence intervals were calculated using RevMan 5.3 software.

Results: Eight studies involving 310 children with type 1 diabetes were included. Results showed that HbA1c (%) was significantly lower ($p = 0.007$) after CSII compared with MDI in children with type 1 diabetes. In addition, there was no significant difference between groups in HbA1c (%) change, total daily insulin doses, change of total daily insulin doses and incidence of ketoacidosis and severe hypoglycemia. However, subgroup analyses indicated that age, treatment duration and study design were influenced the efficacy of CSII and MDI in children with type 1 diabetes.

Conclusion: CSII is associated with lower HbA1c levels in children with type 1 diabetes but appears to have no effect on insulin requirement or incidence of ketoacidosis and severe hypoglycemia.

Keywords: Continuous subcutaneous insulin infusion, multiple daily injections, children, type 1 diabetes, meta-analysis

Introduction

Type 1 diabetes is caused by the immune system attacking and destroying the beta cells in the pancreas that produce insulin and commonly occurs in childhood with increasing incidence continuing in recent years (1). Multiple daily injection (MDI) treatment is the most widely used method of insulin administration for treating diabetes, which requires at least three or more injections a day. In recent years, to reduce the complications and to improve blood glucose control, continuous subcutaneous insulin infusion (CSII) has been used as a popular option for diabetes management, especially in preschool-aged children (2,3).

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Recently, many meta-analyses were performed to compare MDI and CSII in adult patients with type 1 diabetes (4,5). In these studies, CSII was shown to have many advantages including improvement of blood glucose control, reduction of daily insulin requirement and increase of treatment satisfaction. In addition, a previous meta-analysis (6) of studies involving children with type 1 diabetes also indicated the advantages of CSII in blood glucose control. However, a study investigating patients older than 18 years (7) was included in that meta-analysis, so bias caused by age may have had an impact on the results. Thus, it is necessary to compare the efficacy and safety of CSII and MDI with studies comprising only children aged ≤18 years. In this present study, we also investigated the influence of treatment duration, age and study design on efficacy of CSII as compared to MDI.

Materials and Methods

The methods used for this meta-analysis and generation of inclusion criteria were based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses recommendations. Approval by a research ethics committee to conduct this meta-analysis was not required.

Literature Search Strategy

Databases including PubMed and Embase were used for literature search up to June 2017, using the following keywords: [(insulin infusion) OR (insulin pump)] AND (children) AND [(diabetes) OR (diabetic)]. In addition, the references of relevant reviews were searched for additional studies.

Inclusion and Exclusion Criteria

The following criteria were met for all included studies: (1) the study type was a randomized study; (2) subjects were children with type 1 diabetes aged ≤18 years old; (2) CSII was used for glucose control (experimental group) compared with conventional MDI (control group); (3) clinical outcomes included at least one of the following: HbA1c (%), insulin dose and some adverse events.

The studies were excluded if they were (1) duplicate publications, or (2) reviews, letters or comments.

Data Extraction and Quality Assessment

The following data were recorded in a predesigned form: first author name, country, year, enrolled time, duration of diabetes, treatment duration, sample size, age, sex, treatment target, and outcomes. Data extraction was performed independently by two investigators. The quality of included studies was assessed by the Cochrane Collaboration's tool for assessing risk of bias as described previously (8). For data extraction and quality assessment, differences were resolved by discussion to ensure consistency of evaluation.

Statistical Analysis

The RevMan 5.3 software (RevMan 5.3, The Cochrane Collaboration, Oxford, UK) was used to perform this meta-analysis. The I-squared and Cochrane Q tests were used to assess the heterogeneity using $p < 0.1$ or $I^2 > 50\%$ indicating significant heterogeneity. An appropriate statistical model (fixed effect model or random effects model) was applied to pool the weighted mean difference (WMD) or risk ratio (RR) as well as the corresponding 95% confidence intervals (CIs), based on the results of heterogeneity test. The subgroup analysis was performed based on the age, treatment duration and study type. Publication bias was assessed using Egger's and Begg's Tests. For all these analyses, $p < 0.05$ indicated statistical significance.

Results

Characteristics of Included Studies

After initial literature search, a total of 312 articles (PubMed: n = 175, Embase: n = 137) were identified. After excluding duplicates, 88 potentially relevant articles remained. Of these, 56 articles were excluded including 15 obvious irrelevant studies, 25 non-randomised controlled trials (non-RCTs) and 16 reviews. Then the remaining 32 articles were assessed by reading the full-text. Among them, 26 articles were excluded (10 were non-RCTs, four articles did not report available data, six articles did not use the insulin injection and four more studies enrolled some participants aged over 18 years). Finally, eight studies (9,10,11,12,13,14,15,16) were included in this analysis (Figure 1).

The characteristics of these studies are shown in Table 1. A total of 310 children with type 1 diabetes were included and

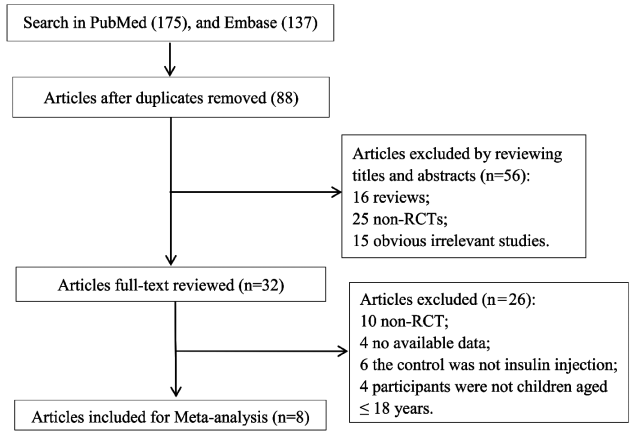

Figure 1. Flow diagram of the study selection process
RCT: randomised controlled trials

Table 1. The characteristics of included studies

Author year	Country	Enrolled time	Study type	Patients	Group	Duration of diabetes		Treatment duration	Target	Age (years)	Sex (F/M)	Duration of diabetes
Weintrob et al (13)	Israel	NA	RCOT	8 to 14 years children	CSII	5.3 ± 1.9 years	Total mean ± SD: 5.8 ± 2.3 years; Range: 2.8 to 11.9 years	3.5 months	The target range for glycemia was 80 to 150 mg/dL (4.4-8.3 mmol/L) before meals and at midnight and 120 to 180 mg/dL (6.7-10.0 mmol/L) at 2 hours after meals	9.25 ± 13.75	10/13	2.5 to 11 years (median, 6.0 years)
					MDI	6.3 ± 2.6 years						
Opipari-Arrigan et al (11)	USA	2002.09-2003.02	RCT	Preschool-age children	CSII	≥1 year		6 months	Blood glucose value 70-180 mg/dL	52.5 ± 9.4 months	4/4	At least a 1-year history of T1DM
					MDI				53.1 ± 7.7 months	3/5		
Dimeglio et al (9)	India	1999.11-2003.04	RCT	Preschool-aged children	CSII	1.8 ± 0.6 years		6 months	100 to 220 mg/dL during the day, with bedtime blood sugars > 150 mg/dL	3.8 ± 0.8	11/10	1.8 ± 0.6 years
					MDI	1.8 ± 0.6 years				3.7 ± 0.7	14/7	1.8 ± 0.6 years
Wilson et al (14)	USA	2001.05-2002.05	RCT	Preschool-aged children	CSII	Total mean ± SD: 1.4 ± 0.6 years; Range: 0.6-2.6 years		12 months	The goals for these young children were a preprandial glucose target of 8.3 mmol/L (150 mg/dL) and a bedtime target of 9.7 mmol/L (175 mg/dL), with an overall average glucose target between 5.6 and 11.1 mmol/L (100-200 mg/dL). HbA1c target range was 7.5-8.5%	3.6 ± 1.0	12/7	1.4 ± 0.7 years
					MDI							
Fox et al (10)	USA	2001.01-2003.09	RCT	Preschool-aged children	CSII	15.3 ± 3.4 years		6 months	NA	47.5 ± 4.8 months	4/7	15.3 ± 3.4 months
					MDI	19.7 ± 4.1 years				45.3 ± 4.3 months	5/6	19.7 ± 4.1 months

Table 1. Continued

Author year	Country	Enrolled time	Study type	Patients	Group	Duration of diabetes	Treatment duration	Target	Age (years)	Sex (F/M)	Duration of diabetes
Weintrob et al (12)	Israel	NA	RCOT	8 to 14 years children	CSII MDI	Median (range): 6 (2.5-11) years	3.5 months	The target range for glycemia was 4.4 to 8.3 mmol/L (80-150 mg/dL) before meals and at midnight, and 6.6 to 10 mmol/L (120-180 mg/dL) at 2 hours after meals	11.8 ± 1.4	10/13	5.8 ± 2.3 years
Skogsberg et al (16)	Sweden	2001.12-2004.04	RCT	7 to 17 years children	CSII MDI	NA	24 months	NA 12.3 ± 4.5	11.8 ± 4.9	30/42	NA
Abusaad (15)	Egypt	NA	RCT	12 to 17 years children	CSII MDI	≥1 year	6 months	NA	12-17 years 14/11	12/13	At least a 1-year history of T1DM

RCT: randomized control trial, RCOT: randomized crossover trial, CSII: continuous subcutaneous insulin infusion, MDI: multiple daily injections, NA: not reported, F/M: female/male, T1DM: type 1 diabetes mellitus

reanalyzed in this meta-analysis. The duration of diabetes was longer than one year in all these patients. The publication year ranged from 2003 to 2014. There were six randomized control trials and two randomized crossover trials. The treatment durations ranged from 3.5 to 24 months. The bias risk assessment is shown in Table 2. No study applied or reported the blind method. Performance bias was avoided by crossover design only in the studies by Weintrob et al (12,13).

Meta-analysis

All eight studies included in this analysis reported glucose control as the main outcome. As shown in Figure 2A, in children with type 1 diabetes, HbA1c (%) was significantly lower (WMD = -0.25, 95% CI = -0.43 to -0.07, p = 0.007) after treatment by CSII as compared with MDI. However, the significant difference disappeared in the subgroup analyses (Table 3) by studies with crossover design (p = 0.53) or in comparing prepubertal and pubertal patients of school age (p = 0.05). Moreover, no significant difference was found in mean change of HbA1c (%) (mean difference from baseline to end of study) between the children treated with CSII and MDI in the overall analysis (WMD = -0.02, 95% CI = -0.18 to 0.15, p = 0.84, Figure 2B) and in the subgroup analyses (p > 0.05, Table 3).

As shown in Figure 2C, the total daily insulin doses were similar in diabetic children after treatment by CSII and MDI (WMD = -0.14, 95% CI = -0.34 to 0.06, p = 0.16). The mean change of total daily insulin dose from baseline to the end of the study (mean difference from baseline to end of study) was also similar between CSII and MDI groups (WMD = -0.11, 95% CI = -0.25 to 0.03, p = 0.13, Figure 2D). In the subgroup analyses, the results indicated that children with type 1 diabetes needed significantly less daily insulin doses after 12 months of CSII treatment as compared with MDI (WMD = -0.21, 95% CI = -0.36 to -0.05, p = 0.009, Table 3).

As for adverse events, there was no significant difference in the incidence of ketoacidosis (RR = 2.22, 95% CI = 0.75-6.59, p = 0.15, Figure 2E) and severe hypoglycemia (RR = 0.77, 95% CI = 0.45-1.32, p = 0.34, Figure 2F) between the children treated with CSII and MDI. No inconsistent results for analysis of incidence of severe hypoglycemia were found in subgroup analysis (p > 0.05, Table 3).

Heterogeneity Results

In overall analyses, significant heterogeneity (p < 0.1 or I^2 > 50%) among studies was found in analyses for HbA1c (%), total daily insulin doses and change in total daily insulin doses. Therefore, the randomized effects model was applied to pool the data. Fixed effect model was used for other analyses (Figure 2). However, these significant heterogeneities were still absent (p > 0.1 or I^2 = 0%, Table 3) among studies in some

subgroup analyses for HbA1c (%) (treatment duration, 3 or 3.5 months; study design, crossover design; age, prepubertal school aged and pubertal patients) and change of total daily insulin doses (treatment duration, 6 months). Thus, beside age, treatment duration and study design, there were other sources of heterogeneity.

Publication Bias

No significant publication bias was found by Egger's and Begg's tests in this study ($p > 0.05$).

Discussion

In this study, significantly lower HbA1c (%) values were reported in the CSII group as compared with the MDI group. Moreover, subgroup analysis showed a significant difference between the groups after both three to three-and-a-half months and six months treatment. Indeed, many retrospectively or prospectively observational studies on the long term outcomes using HbA1c as the outcome measure in type 1 diabetic children (17,18), reported that CSII may have a significant better efficacy on glucose control after long term treatment. More studies should be performed to investigate the efficacy difference between long-term and short-term treatment. Our subgroup analysis also showed that study design may be a factor affecting the results, based on the subgroup analysis by study design for HbA1c (%). Lack of effect in RCTs suggests training in diabetes management may be main cause explaining CSII effects. In addition, the mean change of HbA1c (%) was similar among groups. The different baseline level or low number of studies may be the factors leading to the similar results between CSII and MDI groups. Furthermore, the effect of CSII on HbA1c (%) may be related to more diabetic education in children with diabetes and their families. The family or children treated by CSII may receive more diabetic education due to more opportunity to contact new treatment information and good economic incomes. More studies

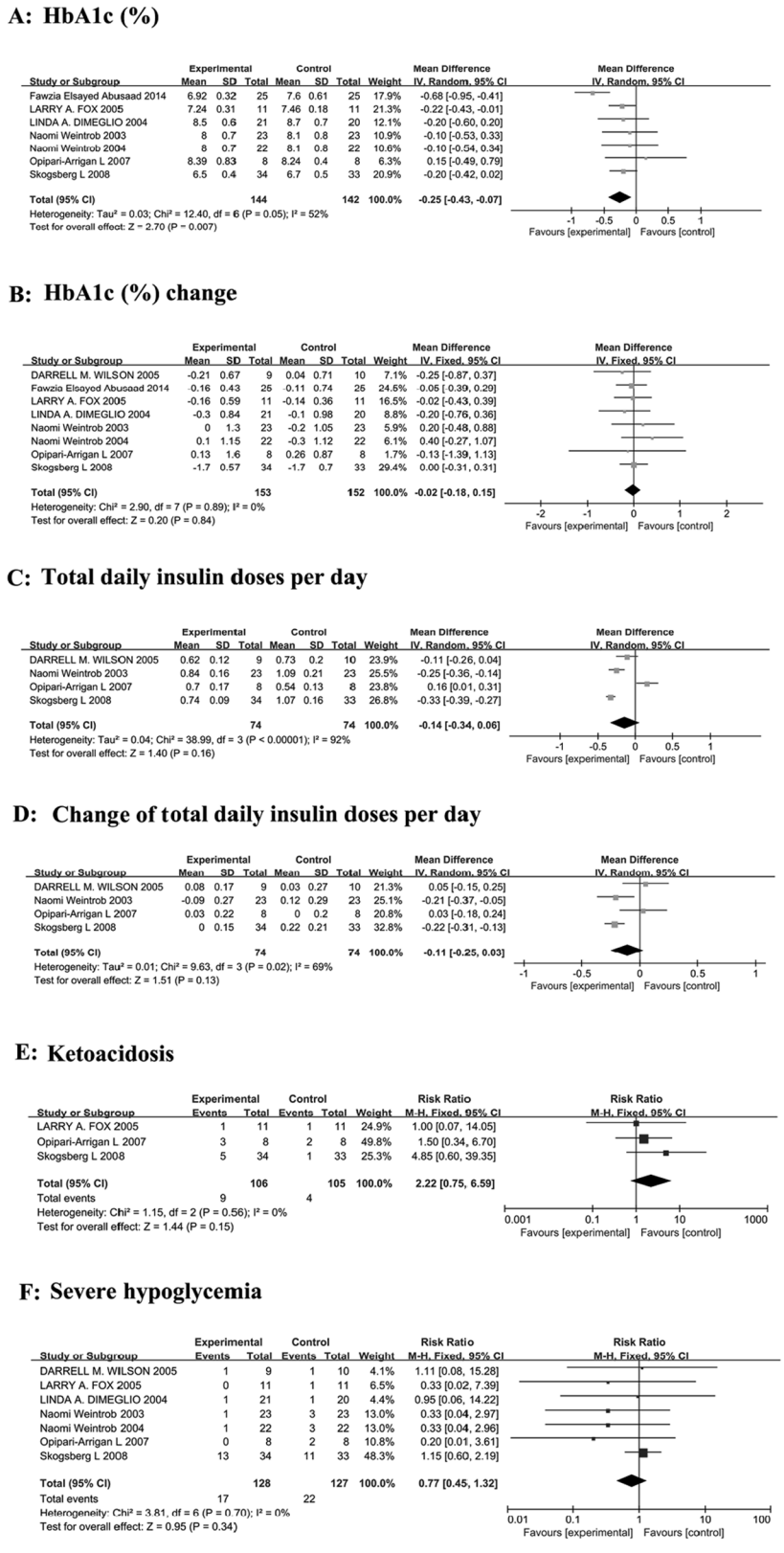

Figure 2. Forest plots for meta-analysis on HbA1c (%) (A), HbA1c (%) change (B), total daily insulin doses per day (C), change of total daily insulin doses per day (D) and incidence of ketoacidosis (E) and severe hypoglycemia (F)

Table 2. The assessment of bias risk of included studies

Author year	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Weintrob et al (13)	Low	Unclear	Low	Unclear	Low	Unclear	Unclear
Opipari-Arrigan et al (11)	Low	Unclear	High	Unclear	Low	Low	Unclear
Dimeglio et al (9)	Low	Unclear	High	Low	Unclear	Unclear	Unclear
Wilson (14)	Low	Unclear	High	Unclear	Low	Low	Unclear
Fox et al (10)	Low	Unclear	High	Unclear	Low	Unclear	Unclear
Weintrob et al (12)	Low	Unclear	Low	Unclear	Low	Unclear	Unclear
Skogsberg et al (16)	Low	Unclear	High	Unclear	Low	Unclear	Unclear
Abusaad (15)	Low	Unclear	High	Unclear	Low	Unclear	Unclear

Table 3. The results of subgroup analyses

Parameters	Subgroups		Number of studies	WMD/RR (95% CI), p-value	I^2, p-value
HbA1c (%)	Treatment duration	3/3.5 months	4	-0.24 (-0.40, -0.0) p = 0.003	0%, 0.68
		6 months	3	-0.28 (-0.48, -0.08) p = 0.0007	67%, 0.02
	Study design	RCT	5	-0.28 (-0.51, -0.06) p = 0.01	64%, 0.03
		RCOT	2	-0.10 (-0.41, 0.21) p = 0.53	0%, 1.00
	Age	Pre-school aged children	3	-0.19 (-0.37, -0.01) p = 0.04	0%, 0.56
		Prepubertal school aged and pubertal patients	4	-0.30 (-0.59, -0.00) p = 0.05	70%, 0.02
HbA1c (%) change	Treatment duration	3/3.5 months	4	-0.03 (-0.30, 0.24) p = 0.83	22%, 0.28
		6 months	3	-0.04 (-0.20, 0.13) p = 0.67	0%, 0.98
		12 months	2	0.13 (-0.12, 0.38) p = 0.31	41%, 0.19
	Study design	RCT	6	-0.06 (-0.24, 0.12) p = 0.50	0%, 0.98
		RCOT	2	0.30 (-0.18, 0.78) p = 0.22	0%, 0.68
	Age	Pre-school aged children	4	-0.12 (-0.40, 0.16) p = 0.41	0%, 0.92
		Prepubertal school aged and pubertal patients	4	0.04 (-0.17, 0.24) p = 0.73	0%, 0.65
Total daily insulin doses per day	Treatment duration	6 months	2	0.01 (-0.26, 0.28) p = 0.94	92%, 0.0003
		12 months	2	-0.21 (-0.36, -0.05) p = 0.009	76%, 0.04
Change of total daily insulin doses per day	Treatment duration	6 months	2	-0.01 (-0.07, 0.06) p = 0.88	0%, 0.72
		12 months	2	-0.08 (-0.28, 0.13) p = 0.46	72%, 0.06
Severe hypoglycemia	Treatment duration	3.5 months	2	0.30 (0.06, 1.59) p = 0.16	0%, 1.00
		6 months	3	0.39 (0.08, 1.92) p = 0.25	0%, 0.73
	Study design	RCT	5	0.92 (0.52, 1.65) p = 0.79	0%, 0.75
		RCOT	2	0.30 (0.06, 1.59) p = 0.16	0%, 1.00
	Age	Pre-school aged children	4	0.51 (0.13, 1.91) p = 0.32	0%, 0.80
		Prepubertal school aged and pubertal patients	3	0.86 (0.48, 1.55) p = 0.62	9%, 0.33

WMD: weighted mean difference, RR: risk radio, CI: confidence interval, RCT: randomized control trial, RCOT: randomized crossover trial

should be performed to investigate the impact of diabetic education level on CSII or MDI treatment efficacy.

However, based on the results of subgroup analyses, the advantage [as measured by reduction in HbA1c (%)] of CSII compared with MDI was just absent in prepubertal school aged and pubertal patients in this study ($p = 0.05$). Thus, age may be a factor affecting the efficacy of CSII and MDI treatment for type 1 diabetes. The pathogenesis of type 1 diabetes is mainly related to immune system mediated cell injury in the pancreas. Significant heterogeneity ($I^2 = 70\%$, $p = 0.02$) existed among the included studies on prepubertal school aged and pubertal patients. Compliance with therapy may be a factor influencing the results, which is notoriously poor among pubertal aged patients but may be improved using CSII whereas prepubertal and preschool children age more under the control of their patients. Thus, the results are conflicting. More studies should be performed to confirm the impact of age on efficacy of CSII and MDI.

In addition, the insulin requirement was reported to be significantly reduced after long-term (12 months) CSII treatment compared with MDI, but not after short-term treatment (six months), which is inconsistent with the previous meta-analysis (6). This previous meta-analysis included a study on type 1 diabetes patients aged 8-21 years old (7). The findings on adult patients (of ages over 18 years) in this series with type 1 diabetes may have led to a result in bias risk affecting the results on children. Thus, we only included studies with children aged ≤18 years old in this meta-analysis Moreover, we included more studies in this meta-analysis, such as Opipari-Arrigan et al (11), Abusaad (15) and Skogsberg et al (16). In addition, we performed the subgroup analyses by study design. The heterogeneity changes and inconsistent results between subgroup analyses and overall analyses indicated that age, study design and treatment duration may be sources of heterogeneity and factors impacting the efficacy of CSII and MDI in children with type 1 diabetes.

In addition, no significantly different incidence of complications, in particular ketoacidosis and/or severe hypoglycemia, were found in this meta-analysis. However, some previous observational studies indicated that the CSII could significantly reduce the incidence of severe hypoglycemic episodes compared with MDI after long term treatment (five years) (19). Thus, more studies with longer follow-up periods need to be performed to further compare the complications after CSII and MDI in children with type 1 diabetes and explore the factors influencing the safety of CSII and MDI in these children.

Study Limitations

Firstly, the number of included studies and sample size were small. Secondly, significant heterogeneity was found among the results of the studies. Although the subgroup analyses were performed, the significant heterogeneity still existed in some subgroup analyses. In addition to differences in study design, age and treatment duration, some other confounding factors (such as sex, duration of diabetes, country and treatment target) may also be sources of this heterogeneity. With increase in duration of diabetes, there is more and more risk of ''burn-out'' and noncompliance of patients, which will affect the efficacy of treatment for glycemic control. However, the data for duration of diabetes is inadequate in the studies analyzed to perform the subgroup analyses in this meta-analysis. Therefore, this factor (duration of diabetes) needs to be investigated in further studies. Thirdly, in addition to HbA1c (%), duration of blood glucose value at the target range is also a key index evaluating the efficacy of blood glucose control. However, there were not sufficient data to perform a subgroup analysis in this meta-analysis.

Conclusions

In conclusion, CSII is associated with lower HbA1C levels in children with type 1 diabetes but may have no effect on insulin requirement and in reducing incidence of ketoacidosis and severe hypoglycemia. Age, treatment duration and study design may be the factors influencing the comparison results. Diabetic education level may be one of the important factors influencing treatment efficacy. More studies should be performed to investigate the impact of diabetic education level on CSII or MDI treatment efficacy.

Ethics

Ethics Committee Approval: Approval by a research ethics committee to conduct this meta-analysis was not required.

Informed Consent: Not required.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Concept: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Design: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Data Collection or Processing: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Analysis or Interpretation: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Literature Search: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y., Writing: Y.Q., L.H.Y., X.Li.H., X.H.C., H.Y.

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Under-recognized Hypoparathyroidism in Thalassemia

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What is already known on this topic?

Symptomatic hypoparathyroidism in patients with transfusion-dependent thalassemia is relatively rare. Data on prevalence of asymptomatic hypoparathyroidism are also scanty.

What this study adds?

Hypoparathyroidism in patients with thalassemia is not uncommon. In comparison with patients with normoparathyroidism, plasma fibroblast growth factor 23 was lower in patients with hypoparathyroidism. Screening for asymptomatic mild hypocalcemia without elevation of parathyroid hormone should be considered in transfusion-dependent thalassemia for early detection and proper treatment.

Abstract

Objective: Symptomatic hypoparathyroidism [symptomatic hypocalcemia without elevated serum parathyroid hormone (PTH)] in patients with thalassemia is relatively rare. Asymptomatic mild hypocalcemia without elevated PTH, which is considered hypoparathyroidism, may be more common but under-recognized.

Methods: Sixty-six transfusion-dependent thalassemic patients and 28 healthy controls were enrolled. Serum calcium (Ca), phosphate (P), creatinine (Cr), albumin, intact PTH, 25-hydroxyvitamin D (25-OHD), plasma intact fibroblast growth factor-23 (FGF-23), urinary Ca, P and Cr were measured. Tubular reabsorption of P was calculated.

Results: Thalassemic patients had significantly lower median serum Ca levels than the controls [8.7 (7.8-9.7) vs 9.6 (8.7-10.1) mg/dL, $p < 0.001$]. Hypoparathyroidism was found in 25 of 66 (38%) patients. Symptomatic hypoparathyroidism was not encountered. Thalassemic patients also had significantly lower median plasma FGF-23 levels than the controls [35.7 (2.1-242.8) vs 53.2 (13.3-218.6) pg/mL, $p = 0.01$]. In patients with hypoparathyroidism, median plasma FGF-23 level was significantly lower than that of normoparathyroid patients [34.8 (2.1-120.0) vs 43.1 (3.2-242.8) pg/mL, $p = 0.048$]. However, serum P, Cr, intact PTH and 25-OHD levels were not significantly different in the two groups.

Conclusion: Hypoparathyroidism was not uncommon in patients with transfusion-dependent thalassemia treated with suboptimal iron chelation. Plasma intact FGF-23 level in hypoparathyroid patients was lower than that of normoparathyroid patients.

Keywords: Thalassemia, hypoparathyroidism, hypocalcemia, iron overload, fibroblast growth factor-23

Introduction

Thalassemia is an inherited disease caused by abnormal hemoglobins. It leads to ineffective erythropoiesis and increased peripheral hemolysis. Regular blood transfusion is inevitable in patients with moderate to severe thalassemia. Overt hypoparathyroidism in thalassemia is relatively rare (1). However, asymptomatic hypoparathyroidism has been rarely reported, although its incidence was as high as 42% in one study (2). Previous studies have shown that hypoparathyroidism was primarily associated with iron overload (3,4,5,6).

Relatively high serum phosphate (P) levels in children and adults with thalassemia were reported in several studies (7,8,9,10). Our previous study also demonstrated that serum P levels in transfusion-dependent thalassemia had a trend to be higher than those in non-transfusion

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dependent thalassemia cases, but not significantly so ($p = 0.081$) (11). In transfusion-dependent thalassemia, high P loading due to regular blood transfusions, hemolysis and hypoparathyroidism contribute to elevated serum P levels (12).

Fibroblast growth factor-23 (FGF-23), a phosphaturic hormone, is mainly synthesized and secreted by osteoblasts and osteocytes in response to hyperphosphatemia and elevated 1.25-dihydroxyvitamin D ($1.25\text{-}(OH)_2D$) concentration (13). FGF-23 acts at the renal tubular cell level to reduce P reabsorption. In addition, FGF-23 inhibits 1α-hydroxylase, leading to a reduction in formation of $1.25\text{-}(OH)_2D$ (14). FGF-23 also reduces parathyroid hormone (PTH) secretion from the parathyroid glands, thereby attenuating the PTH-mediated phosphaturic effect (15). However, the mode of FGF-23- P axis control in thalassemia has not been elucidated.

Our previous histomorphometric study demonstrated that iron deposits in thalassemic bones impaired bone mineralization and reduced bone formation (16). *In vitro* studies demonstrated that excessive iron inhibited osteoblast proliferation and differentiation (17,18). Therefore, iron accumulation in thalassemic bones may compromise FGF-23 production by osteoblasts and osteocytes.

We therefore hypothesized that asymptomatic hypoparathyroidism might be common but under-recognized in patients with thalassemia. In addition, impaired FGF-23 production, secondary to iron deposits in bones, might partly contribute to elevated serum P in thalassemia. Our study aimed to determine serum calcium (Ca), P and 25-hydroxyvitamin D (25-OHD), PTH and plasma FGF-23 levels in transfusion-dependent thalassemic patients.

Methods

Children and adolescents with transfusion-dependent thalassemia attending the Hematology Clinic at the Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand were enrolled in the study. Most of these transfusion-dependent thalassemic patients had received a standard regular blood transfusion therapy every 3-4 weeks to maintain their hemoglobin levels at 9-10.5 g/dL. Desferrioxamine was the only iron chelating agent used in patients who had a serum ferritin level greater than 1000 ng/mL. Additional oral iron chelators such as deferiprone and deferasirox have been used in the past five years. All patients received daily folic acid and multivitamin supplementation. Each tablet of multivitamin contains 400 IU of vitamin D_2. Patients with known underlying conditions including hypoparathyroidism, renal disease and acute hemolysis, and patients who had been taking other medications affecting Ca, P and vitamin D metabolism, were excluded. The controls were healthy children who attended the day camp regularly arranged by our hospital during the end of each school semester. All these controls were the offspring of hospital personnel. None of them had been taking medications known to affect Ca, P and vitamin D metabolism.

Anthropometric measurements were performed at the time of enrollment. Measurements included weight to the nearest 0.1 kg measured using a digital weighing scales; height to the nearest 1 mm, measured using a Harpenden stadiometer (Holtain Ltd, Crymych, Dyfed, Wales). Z-scores of height and weight were calculated using the National Standard Growth Curve of the Ministry of Public Health, Thailand. The Z-score of body mass index (BMI) was calculated using the World Health Organization BMI for age and sex. Median serum ferritin was determined using serum ferritin levels periodically obtained during routine clinic visits. Cumulative iron loading was estimated from cumulative volume of packed red cell (PRC) transfusion as follows:

Cumulative iron loading (mg) = volume (mL) of PRC given x hematocrit of PRC x 1.16 (19).

Fasting blood samples were obtained in thalassemic patients and controls for determination of serum Ca, P, Cr, albumin, intact PTH, 25-OHD and plasma intact FGF-23 levels. In thalassemic patients, fasting blood samples were obtained on the day of transfusion just before the scheduled transfusion. Simultaneous spot morning urine samples for Ca, P and Cr in thalassemic patients were obtained. Serum PTH and 25-OHD were measured by chemiluminescence assay (Roche Diagnostics GmbH, Mannheim, Germany). Corrected serum Ca, tubular reabsorption of P (TRP) and ratio of tubular P (TP) reabsorption to the glomerular filtration rate (TP/GFR) were calculated using the following formulas:

Corrected serum Ca (mg/dL) = Total serum Ca (mg/dL) + 0.8 x [4 – serum albumin (g/dL)]

TRP (%) = $\{1\text{- [(urine P / serum P) / (urine Cr / serum Cr)]}\}$ x 100

TP/GFR (mg/dL) = Serum P – (urine P x serum Cr / urine Cr) (20)

Definitions used in this study: Hypocalcemia = corrected serum Ca < 8.5 mg/dL; normocalcemia = corrected serum Ca 8.5-10.4 mg/dL; hypoparathyroidism = hypocalcemia without elevated intact PTH (reference range: 20-75 pg/mL).

Plasma intact FGF-23 was measured in duplicate by a commercial the enzyme-linked immunosorbent assay (ELISA) kit (2nd generation human intact FGF-23 ELISA kit, (Immutopics Inc, San Clemente, CA). The intra-assay coefficient of variation (CV) was 2.0-4.1% and the inter-assay CV was 3.5-9.1%. The lower limit of detection was 1.5 pg/mL.

The study was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (ethics approval no. MURA2013/24 Np1). Informed consent was obtained from the patients and their legal guardians.

Statistical Analysis

Statistical analyses were performed using the software package SPSS 15.0 (SPSS, IBM Inc., Chicago, USA). All parameters were not normally distributed determined by Kolmogorov-Smirnov test and therefore, they were presented as median (range). Comparison between the patient and control groups was performed using Mann-Whitney U test. Pearson's correlation was used to determine the correlation between two variables. A p value of less than 0.05 was considered statistically significant.

Results

Sixty-six transfusion-dependent thalassemic patients, with a median (range) age of 13.5 (5.1-23.2) years, and 28 healthy controls, median (range) age of 8.9 (4.8-17.2) years participated in the study. Sixty of these patients were β-thalassemia/hemoglobin E (β-thal/E) cases and six were patients with β-thalassemia homozygote (β-major) disease. Twelve patients were splenectomized. All patients had been receiving monthly PRC transfusions and iron chelation therapy, including desferrioxamine in combination with either deferiprone or deferasirox. In comparison with the controls, thalassemic patients were significantly older, but their Z-scores for weight, height and BMI were lower. Thalassemic patients had significantly lower corrected serum Ca and plasma intact FGF-23 levels than those of the controls. No significant differences in serum P, PTH and 25-OHD levels were found between the two groups (Table 1). There were no significant differences in corrected serum Ca, P, 25-OHD, PTH and plasma intact FGF-23 levels between patients with β-thal/E and β-major. Hypoparathyroidism (corrected serum Ca 7.5-8.4 mg/dL and no elevation of PTH) was found in 25 of 66 (38%) thalassemic patients. None of the controls had hypocalcemia. Most thalassemic patients (94%) had normal vitamin D status (25-OHD ≥20 ng/mL). Only 4 of 66 (6%) patients (3 β-thal/E, 1 β-major) had mild vitamin D insufficiency (25-OHD, range 18.6-19.7 ng/mL). All but one of the controls (27 of 28) had normal vitamin D status (one child had mild vitamin D insufficiency, 25-OHD 16.8 ng/mL).

Twenty-five thalassemic patients had asymptomatic mild hypocalcemia. All these patients who had either an inappropriately low or normal serum PTH were considered to have hypoparathyroidism. The

Table 1. Characteristics and blood chemistry of thalassemic patients and controls

Parameters Median (range)	Thalassemic (n = 66)	Control (n = 28)	p value
Age (years)	13.5 (5.1-23.2)	8.9 (4.8-17.2)	0.004
Male, n (%)	35 (53)	10 (36)	NS
Z-score of weight	-0.8 (-3.2 to 2.6)	+0.8 (-1.5 to 5.4)	<0.001
Z-score of height	-0.7 (-4.0 to 2.0)	+0.2 (-1.4 to 0.8)	<0.001
Z-score of BMI	-0.6 (-3.6 to 2.3)	+0.2 (-0.8 to 3.3)	0.016
Puberty, n (%)	39 (59)	11 (39)	<0.001
Corrected serum calcium (mg/dL)	8.7 (7.8-9.7)	9.6 (8.7-10.1)	<0.001
Serum phosphate (mg/dL)	4.9 (3.8-6.2)	4.9 (4.1-6.0)	NS
Serum creatinine (mg/dL)	0.4 (0.2-0.8)	0.4 (0.2-0.8)	NS
Serum PTH (pg/mL)	31.5 (12.5-74.6)	29.6 (20.5-65.8)	NS
Serum 25-OHD (ng/mL)	27.6 (18.6-57.3)	26.6 (16.8-38.7)	NS
Plasma FGF-23 (pg/mL)	35.7 (2.1-242.8)	53.2 (13.3-218.6)	0.010
Hemoglobin (g/dL)	8.6 (5.9-11.0)	-	-
Serum ferritin (ng/mL)	1,333 (372-6,752)	-	-

Data are presented as median (range), otherwise as indicated.

BMI: body mass index, PTH: parathyroid hormone, 25-OHD: 25-hydroxyvitamin D, FGF-23: fibroblast growth factor-23, NS: not significantly different

remaining 41 patients had normal serum Ca and PTH levels. There were no significant differences in gender, age, Z-scores of weight, height and BMI and ages of onset of transfusion and chelation therapy between patients with hypoparathyroidism and those with normoparathyroidism. In the hypoparathyroid group, the lowest level of 25-OHD was 18.6 ng/mL; almost all patients had vitamin D sufficiency (median 27.6 ng/mL). In comparison with normoparathyroid patients, median plasma intact FGF-23 was slightly but significantly lower in the hypoparathyroid group (43.1 vs 34.8 pg/mL, $p = 0.048$). There were no significant differences in the serum P levels of these two groups. Cumulative iron loading was greater, while serum ferritin was lower in the hypoparathyroid group (Table 2). Serum Ca level had a negative correlation with duration of transfusions in hypoparathyroid patients ($r = -0.45$, $p = 0.022$), but had no correlation in the normoparathyroid group.

Discussion

The present study demonstrates asymptomatic hypoparathyroidism in transfusion dependent thalassemia. Previous studies reported low prevalences of hypoparathyroidism, ranging from 0.5 to 7.6% (5,12,21). Those prevalences primarily represented overt or symptomatic hypoparathyroidism. This cross-sectional study looked at Ca-P metabolism in patients with transfusion-dependent thalassemia. No patients had symptoms of hypocalcemia or a history of fractures. The prevalence of hypoparathyroidism was 38% in this study, which suggests a high prevalence of unrecognized, asymptomatic hypoparathyroidism. This is in line with previous studies of Mostafavi et al (22) and Adil et al (23) that reported hypoparathyroidism in 22.7% and 35.3% of thalassemic patients, respectively. Previous studies demonstrated that hypoparathyroidism was associated with high serum ferritin levels (3,4,5,6). A serum ferritin level higher than 2,500-3,000 ng/mL has been demonstrated to be associated with higher frequency of hypoparathyroidism (5,6). In addition, Belhoul et al (6) also reported that patients with a serum ferritin >2,500-3,000 ng/mL were 3.27 times more likely to develop hypoparathyroidism. However, no relationship between hypoparathyroidism and serum ferritin has been reported (24,25). In the present study, the patients had modestly elevated, median serum ferritin level of 1333 ng/mL and the median serum ferritin level in

Table 2. Anthropometric and biochemical parameters in hypoparathyroid and normoparathyroid thalassemic patients

Parameters Median (range)	Hypoparathyroid* (n = 25)	Normoparathyroid** (n = 41)	p value
Male, n (%)	15 (60)	20 (49)	NS
Age (years)	14.2 (7.3 -18.9)	11.8 (5.1-23.2)	NS
Z-score of weight	-0.8 (-1.9 to 2.3)	-0.7 (-3.2 to 2.6)	NS
Z-score of height	-0.9 (-2.4 to 1.6)	-0.5 (-4.0 to 2.0)	NS
Z-score of BMI	-0.8 (-2.9 to 2.3)	-0.5 (-3.6 to 1.4)	NS
Puberty, n (%)	18 (72)	20 (49)	0.030
Corrected serum calcium (mg/dL)	8.2 (7.8-8.4)	8.9 (8.5-9.7)	<0.001
Serum phosphate (mg/dL)	4.9 (4.0-5.8)	5.1 (3.8-6.1)	NS
Serum creatinine (mg/dL)	0.4 (0.2-0.8)	0.4 (0.2-0.7)	NS
Serum PTH (pg/mL)	31.8 (15.3-74.6)	31.3 (12.5-57.5)	NS
Serum 25-OHD (ng/mL)	28.9 (18.6-57.3)	27.4 (19.4-43.9)	NS
Plasma FGF-23 (pg/mL)	34.8 (2.1-120.0)	43.1 (3.2-242.8)	0.048
Urine Ca/Cr (mg/mg)	0.08 (0.01-0.28)	0.06 (0.01-0.51)	NS
TRP (%)	95.6 (88.6-99.1)	96.2 (87.8-98.6)	NS
TP/GFR (mg/dL)	4.7 (3.8-5.6)	4.9 (3.7-5.9)	NS
Serum ferritin (ng/mL)	1,087 (372-6.197)	1,957 (449-6.752)	0.001
Cumulative iron loading (g)	26.8 (3.7-60.8)	17.3 (3.6-62.9)	0.046

Data are presented as median (range), otherwise as indicated.

*Corrected serum calcium <8.5 mg/dL; **Corrected serum calcium 8.5-10.4 mg/dL.

PTH: parathyroid hormone, 25-OHD: 25-hydroxyvitamin D, FGF-23: fibroblast growth factor-23, Ca/Cr: calcium to creatinine ratio, TRP: tubular reabsorption of phosphate, TP/GFR: ratio of tubular phosphate reabsorption to glomerular filtration rate, NS: not significantly different

patients with hypoparathyroidism was lower than that of normoparathyroid patients. This finding can be explained by recent additional iron chelation treatment with oral deferiprone and deferasirox. Iron chelation improved strikingly with this treatment, resulting in rapid reduction of serum ferritin. However, tissue iron accumulation may still persist to a degree. In fact, serum ferritin levels were greater than 3,000 ng/mL in our transfusion-dependent thalassemics during the past 5-10 years when only desferrioxamine injection had been used (11).

Previous studies also demonstrated that serum ferritin may not be a reliable indicator of tissue iron overload (24,26). In patients with suboptimal iron chelation therapy, the amount of iron from PRC transfusion may better reflect tissue iron accumulation (27). Hence, our patients with hypoparathyroidism had higher cumulative iron loading than the normoparathyroid patients, despite lower serum ferritin levels. The cause of hypoparathyroidism is likely due to iron deposition in parathyroid glands, as previously reported (1,24,28,29).

FGF-23 is a phosphaturic hormone secreted by osteoblasts and osteocytes in response to elevated serum P. Elevated serum FGF-23 levels have been demonstrated in hypoparathyroid patients secondary to other causes such as parathyroidectomy, thyroidectomy or accidental parathyroidectomy and transient hypoparathyroidism in the offspring of hyperparathyroid mothers (30,31,32). In contrast, our hypoparathyroid thalassemic patients did not have elevated FGF-23 levels. A previous study reported that excessive iron disturbed the metabolism of mouse osteoblastic cells (17). In addition, ferric iron was shown to inhibit osteoblast proliferation, differentiation and mineralization. Moreover, the inhibition of human osteoblast activity was concentration-dependent (18). Iron overload inevitably occurs in transfusion-dependent thalassemic patients and iron accumulation in thalassemic bones has also been demonstrated (16,33). Thus, FGF-23 production by osteoblasts and osteocytes could be compromised in thalassemic patients. Our study showed that plasma intact FGF-23 level was significantly higher in the controls as compared with the thalassemic group. This finding could reflect an impaired FGF-23 production among thalassemic patients. Median plasma FGF-23 level in patients with hypoparathyroidism was significantly lower than that of normoparathyroid patients (34.8 vs 43.1 pg/mL, $p = 0.048$) although serum P levels and serum PTH levels were comparable. These findings suggest that FGF-23 response in patients with hypoparathyroidism might be impaired. One would expect to see elevated serum P levels in these hypoparathyroid patients because of impairment of both phosphaturic hormones, PTH and FGF-23. The reason for an absence of elevated serum P in these patients is unclear and merits further investigation.

Iron deficiency, an opposite condition to iron overload state, has been reported to be associated with elevated serum FGF-23 levels in patients with autosomal dominant hypophosphatemic rickets (ADHR), in the elderly population and in undernourished Gambian children (34,35,36). In addition, iron deficiency upregulated *Fgf23* mRNA in bones of *Fgf23* knock-in mice and consequently led to an ADHR phenotype (37). Improvement of iron status following iron supplementation was associated with a decrease in serum FGF-23 level in undernourished Gambian children and patients with ADHR (36,38). Moreover, the latter had a complete loss of biochemical ADHR phenotype following iron supplementation (38). The mechanism of iron status in influencing FGF-23 concentration remains to be elucidated. However, to our knowledge, the impact of an iron overload state on FGF-23 level, secondary to thalassemia or hereditary hemochromatosis, has not been reported. One might speculate that an iron overload leads to a decrease in FGF-23 production in an opposite direction to the effect of iron deficiency. Our previous study demonstrated "iron-associated focal osteomalacia" in bone histology of patients with thalassemia (16). Osteoblasts and osteocytes could be disturbed by iron accumulation in bones and thus lead to impaired FGF-23 production. Further studies are required to assess the effects of iron overload on the synthesis, secretion and metabolism of FGF-23.

Study Limitations

There were some limitations of this study. First, the sample size was relatively small. Second, the control and thalassemic groups were not matched for age and pubertal maturation level. However, previous studies reported no age- and puberty-associated changes in FGF-23 levels (39,40). Third, serum 1.25-$(OH)_2$ vitamin D was not measured. Thus, pathophysiological changes of serum 1.25-$(OH)_2$ vitamin D related to plasma FGF-23 during hypoparathyroidism or normoparathyroidism could not be assessed.

Conclusion

Hypoparathyroidism was not uncommon in patients with transfusion-dependent thalassemia treated with suboptimal iron chelation. Plasma FGF-23 level in patients with hypoparathyroidism was lower than that of patients with normoparathyroidism.

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Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (ethics approval no. MURA2013/24 Np1).

Informed Consent: Informed consent was obtained from the patients and their legal guardians.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Pat Mahachoklertwattana, Hataitip Tangngam, Preamrudee Poomthavorn, Ampaiwan Chuansumrit, Data Collection or Processing: Pat Mahachoklertwattana, Hataitip Tangngam, Preamrudee Poomthavorn, Ampaiwan Chuansumrit, Nongnuch Sirachainan, La-or Chailurkit, Patcharin Khlairit, Analysis or Interpretation: Pat Mahachoklertwattana, Hataitip Tangngam, Preamrudee Poomthavorn, Ampaiwan Chuansumrit, Nongnuch Sirachainan, La-or Chailurkit, Patcharin Khlairit, Literature Search: Pat Mahachoklertwattana, Hataitip Tangngam, Preamrudee Poomthavorn, Ampaiwan Chuansumrit, Nongnuch Sirachainan, La-or Chailurkit, Patcharin Khlairit, Writing: Pat Mahachoklertwattana, Hataitip Tangngam, Preamrudee Poomthavorn, Ampaiwan Chuansumrit.

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ORIGINAL ARTICLE

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Evaluation of the Ovarian Reserve in Adolescents with Hashimoto's Thyroiditis Using Serum Anti-Müllerian Hormone Levels

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What is already known on this topic?

Hashimoto's thyroiditis (HT) is the most common disease accompanying premature ovarian failure in adult women. In adolescents, there are only two studies examining ovarian reserve of HT patients. Anti-Müllerian hormone levels of adolescent girls with HT were significantly higher than controls in both studies.

What this study adds?

There were no statistically significant differences between the Hashimoto's thyroiditis (HT) and the control group in serum anti-Müllerian hormone (AMH) concentrations. This study contributes to the limited existing literature on this topic and highlights two important research questions via secondary findings: association of AMH levels and menarche age and determination of AMH levels according to puberty stage.

Abstract

Objective: This study aims to evaluate ovarian reserve in adolescent girls with Hashimoto's thyroiditis (HT) by assessment of serum anti-Müllerian hormone (AMH) levels. It was hypothesized that HT decreases ovarian reserve and AMH levels are lower in the HT group.
Methods: Thirty HT patients, aged between 10-18 years, and 30 healthy girls as the control group were enrolled in this cross-sectional study. The mean serum AMH levels of the groups were compared using the Mann-Whitney U test.
Results: There was no statistically significant difference between the patient and the control groups in terms of serum AMH levels. There was a negative correlation between serum AMH and thyroid stimulating hormone (TSH) levels and no correlation between serum AMH and anti-thyroid peroxidase (anti-TPO) or anti-thyroglobulin (anti-Tg) antibody levels.
Conclusion: Our results show that ovarian reserve of adolescent girls, as measured by serum AMH levels, is not affected by HT. Autoimmune damage to the ovaries may take time and the adolescent period may be too early to see these effects. Follow up of the patients for reproductive abnormalities and initiation of prospective studies is recommended.
Keywords: Hashimoto's thyroiditis, ovarian reserve, anti-Müllerian hormone, adolescents

Introduction

Hashimoto's thyroiditis (HT) is an autoimmune disease of the thyroid gland characterized by the lymphocytic infiltration of the thyroid gland and is the most common thyroid disorder in children and adolescents (1). Susceptible individuals who have the combination of abnormalities in cellular immune responsiveness, presence of anti-thyroid auto antibodies, immune susceptibility genes and environmental triggers may develop the disease (1,2).

Anti-Müllerian hormone (AMH) is produced by the granulosa cells of the primary follicles, from fetal life to menopause. Serum AMH levels are correlated with a low antral follicle count (3). Due to its level remaining relatively stable during the menstrual cycle and it not being affected by hormonal feedback mechanisms (3,4), AMH is established as a reliable marker for the quantitative evaluation of ovarian reserve (3,4,5,6). Thyroid hormones are involved in control of the menstrual cycle. Oocytes possess cell surface receptors for triiodothyronine and thyroid hormones affect

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the actions of follicle-stimulating hormone and luteinizing hormone through steroid biosynthesis. Thyroid dysfunction is associated with menstrual irregularities, anovulation and infertility (7). Premature ovarian failure (POF) describes gonadal failure before the age of 40, defined by clinical and laboratory findings. Abnormalities of cellular immunity and autoimmune processes have a role in the autoimmune etiology of POF. Eighty percent of females with idiopathic POF were reported to have a personal or family history of autoimmune disease, 50% to have high titers of anti-thyroid antibodies and 20% anti-ovary antibodies (8). HT is the most common disease accompanying POF in adult women (8,9,10). Even in women with euthyroid HT, the presence of thyroid autoantibodies is related to female infertility (11,12,13).

In adolescents there are only two studies examining ovarian reserve of HT patients. Results of these two recent studies showed that serum AMH levels of adolescent girls with HT were significantly higher than controls (14,15). In the current study it is hypothesized that HT decreases ovarian reserve and AMH levels are lower in the HT group.

Methods

Thirty adolescent HT patients aged between 10-18 years were recruited to the study. Thirty euthyroid and autoantibody-negative age-matched adolescents were included in the control group. The patients were diagnosed and followed as HT in Dr. Sami Ulus Children's Health and Diseases Training and Research Hospital's Pediatric Endocrinology Outpatient Clinic. Diagnoses of HT were based on clinical evidence, autoantibodies [presence of anti-thyroid peroxidase (anti-TPO) or anti-thyroglobulin (anti-Tg) or both required for the diagnosis], hormone levels and ultrasonography findings. At the time of study all patients had either normal thyroid function or hypothyroidism. Patients with Graves' disease, hyperthyroidism or irregular menstruation cycles were not included in the study. The control group was composed of adolescent girls who were admitted to our hospital or who had presented to our pediatric outpatient clinic for minor acute illnesses such as upper respiratory tract infections. No patient had a history of chronic disease, chronic drug use or irregular menstruations. An appointment was made for each to assess thyroid function, anti-TPO and anti-Tg autoantibody concentrations.

The study protocol was approved by the Clinical Research Ethics Committee of Zekai Tahir Burak Women's Health, Training and Research Hospital (with the approval number: 75). Informed consent was obtained from all the subjects and controls prior to enrollment.

All participants were evaluated for pubertal stage, according to Tanner staging (16). Venous blood samples of the patient and control groups were collected for AMH, thyroid stimulating hormone (TSH), free thyroxine (fT4) and anti-TPO and anti-Tg antibody levels. For AMH, blood samples were centrifuged and stored at -20 degrees Celsius and assessed using the AMH Gen II enzyme-linked immunosorbent assay (ELISA; Eastbiopharm, Zhejiang, China) kit. According to the manufacturer, the lowest amount of AMH in a sample that can be detected with a 95% probability is 0.08 ng/mL. The patient group was compared with the control group in terms of serum AMH levels.

The study group was evaluated using thyroid ultrasonography for presence or absence of goiter, thyroid heterogeneity, nodules or any other abnormality by experienced pediatric radiologists. Thyroid volume was measured by the following formula using sonographic measurement of three dimensions in centimeters (a, b, c) of each lobe of the thyroid gland:

Thyroid volume = (a*b*c*0.52) + (a*b*c*0.52)

Statistical Analysis

All the statistical analyses were conducted using the IBM SPSS for Windows Version 21.0 program (IBM Inc., Chicago, Ill., USA). Results were presented as mean ± standard deviation or median (minimum-maximum). Categorical variables were shown using numbers and percentages. Mann-Whitney U test was performed to confirm the difference between the two groups; Kruskal-Wallis test was used for more than two groups as non-parametric tests. A chi-square test was used to confirm the relationship of the categorical variables. Spearman's correlation coefficient was used to determine the relationship between the numeric variables. P values <0.05 were considered statistically significant.

Results

The patient and the control groups had the same mean [± standard deviation (SD)] age which was 14.4 (± 1.85) years. The mean (± SD) follow up time for the patient group was 8.5 (± 4.5) months. The minimum Tanner stage of puberty was 2 and median stage was 5 in both groups. There were no significant differences between the groups in terms of pubertal stage and age. Ten of the 60 subjects (five from the patient group, five from the control group) had not reached menarche at the time of study. Excluding these 10 subjects, the mean menarche age of the patient group was significantly earlier than the control group, (11.4 ± 0.86 versus 12.4 ± 1.04, $p=0.001$).

Median serum AMH level of the patient group was 1.7 ng/mL (minimum 0.5 ng/mL, maximum 5.1 ng/mL), lower than in the control group which was 1.8 ng/mL (minimum 0.29 ng/mL, maximum 5.5 ng/mL). However this was not statistically significant (p = 0.784)

There was no statistically significant difference between groups in terms of TSH and fT4 levels, probably because 86% of the patient group was on levothyroxine treatment. Four newly diagnosed cases had subclinical hypothyroidism. Twenty-six cases were euthyroid. There was no subject with overt hypothyroidism. There was a significant negative correlation between serum AMH and TSH levels in the total sample: r = -0.29, p = 0.02 and in the study group: r = -0.29, p = 0.02. When the control group alone was assessed this relationship was very close to significance: r = -0.36, p = 0.05. There was no correlation between serum AMH and anti-TPO or anti-Tg levels.

Forty-three of 60 subjects (71%) were at stage 5 puberty and their median serum AMH level was higher than the median AMH levels of girls at puberty stages 3 and 4 (Table 1), but the difference was not statistically significant.

Forty percent of the patient group had goiter, 93.3% had thyroid tissue heterogeneity and 43.3% had septation on the thyroid ultrasonography imaging. Goiter, heterogeneity or septation of the thyroid gland were not found to be associated with serum AMH levels.

Discussion

This study showed that no statistically significant differences were present in terms of serum AMH levels between the HT adolescents and the control group. Serum AMH levels were negatively correlated with serum TSH levels for patients and for the subject plus control groups.

An unexpected result reported in a recent study on adults, conducted to evaluate the ovarian reserve of 32 women with HT as compared to 49 healthy females was that serum AMH levels were higher in women with HT (17). The authors suggested that this finding may be due to polycystic ovary syndrome, which may share a common etiologic linkage with autoimmunity and HT. Another aspect of this study was the lack of a statistically significant difference between the study and control patients in terms of antral follicle count.

Table 1. Serum anti-Müllerian hormone levels of the sample according to Tanner puberty stages

Puberty stage	n (%)	AMH level (ng/mL)		
		Median	Minimum	Maximum
Stage 2	5 (8.3)	1.9	0.7	2.7
Stage 3	4 (6.6)	1.1	1.0	4.1
Stage 4	8 (13)	1.3	0.5	4.2
Stage 5	43 (71)	1.9	0.3	8.8

AMH: anti-Müllerian hormone

Only two studies on ovarian reserve of HT adolescents have been reported to date. Results of these two recent studies showed that serum AMH levels of adolescent girls with HT were significantly higher than controls. In one of these studies (14), 30 newly diagnosed HT adolescents were enrolled as the study group and compared to healthy adolescents. In both our study and in Pirgon et al's (14) study the sample had no menstural irregularities. Our study is different in terms of follow up time of the patient group (mean 8.5 ± 4.5 months), from Pirgon et al's (14) study in which the cases were just diagnosed. So there was a longer time for the autoimmune process to affect the ovaries. Higher serum AMH levels and lower serum anti-oxidant levels in euthyroid HT subjects were reported in the second study on this topic (15). The discrepancy of serum AMH levels between our study and these former reports may be due to differences in the thyroid status of the HT groups and the duration of autoimmune thyroiditis. However all three studies support the finding that ovarian reserve of HT patients is not decreased in adolescence when assessed by serum AMH concentrations.

Serum AMH concentration varies during a woman's lifetime. Hormone expression from the ovaries starts with fetal life, reaching the maximum level at puberty, starts to decrease in adulthood and disappears following the menopause. This hormone is expressed by the granulosa cells of the primary follicles, specifically in the preantral and small antral follicles. The expression is decreased in the large antral follicles (18,19). In our study the difference between the serum AMH concentrations at different pubertal stages were not statistically significant but the higher AMH levels at puberty stage 5 and lower levels in earlier stages were noteworthy findings. The number of cases in our study group at puberty stages 2 (n = 5), 3 (n = 4) and 4 (n = 8) was small whereas 43 of the 60 cases were at stage 5 puberty. A larger group of subjects in earlier stages of puberty are needed to examine the relationship between puberty stages and AMH concentration.

The variation of serum AMH concentrations during puberty have been evaluated in a previous study (20). In this well-planned study, serum AMH levels of 381 girls, aged eight years, were recorded. Thirty-nine of these girls had telarche and their serum AMH concentrations were significantly lower than those who had not attained thelarche (p = 0.001). In the longitudinal part of this study, 32 girls were followed and their AMH concentration was recorded at seven,

nine and 11 years of age. Between seven and nine years, the concentration of serum AMH was increased. This was explained by the transition of AMH-silent primordial follicles into AMH-secreting, small antral follicles. Between the ages of nine and 11 years, serum AMH concentration decreased, which was explained by the transition of the small antral follicles into large antral follicles which secrete less AMH than small antral follicles. These findings support the concept that puberty is a special stage of life which may require AMH reference ranges to be set for puberty stage rather than for chronological age.

Serum AMH concentrations were negatively correlated with serum TSH concentrations, but not correlated with anti-TPO and anti-Tg autoantibodies in the present study. Independent of autoimmunity, subclinical hypothyroidism may affect both ovarian function and reserve. Tuten et al (17) reported that serum AMH and both anti-TPO and anti-Tg autoantibody concentrations were positively correlated, while serum AMH and TSH levels were not in adult HT patients. In a large cross sectional study from Belgium, women were divided into low, middle and high ovarian reserve categories according to serum AMH levels (21). There was no significant difference in the prevalence of positive anti-TPO antibodies between the different ovarian reserve categories, a finding compatible with the present study.

In our study, mean menarche age of the patient group was significantly lower than the control group. It is possible that early menarche may be related to early menopause or POF. The relationship between autoimmunity and early menarche is unclear. In a prospective study, 46 children and adolescents with HT were followed for six years and the mean age of menarche was not found to be different from normal children (22). Another autoimmune disorder, celiac disease, is associated with late age of menarche and this has been attributed to autoimmunity and micro- and macro-nutrient deficiencies (23). In a retrospective study, anti-nuclear antibody prevalence in postmenopausal women was found to be associated with late menarche age (24). Early menarche is associated with cardiovascular risk factors, obesity and breast cancer. Therefore these patients should be followed for these entities.

Our study is one of the firsts conducted on AMH levels of children with HT. Including our report, currently there are only three studies investigating AMH concentrations of adolescent with HT, and none of them showed ovarian reserve impairment. However, follow up time and thyroid status differ between these studies. More comprehensive, prospective studies with higher sample sizes are required to investigate the relationship between HT and ovarian reserve in adolescents. Our study expands the limited existing literature on ovarian reserve in HT adolescents and highlights two other important research areas. These are the association of AMH concentrations and menarche age and AMH concentrations according to pubertal stage.

Study Limitations

The major limitations of our research is the small sample size and the cross-sectional design. Long-term follow up is needed to see if there will be any ovarian reserve impairments due to autoimmunity in time.

Conclusion

In conclusion, our study showed that the ovarian reserve of adolescents, evaluated by serum AMH concentration, was not affected by HT. It is possible that autoimmune damage to the ovaries takes time and adolescence may be too early in the process to see the effects. Follow up of adolescents with HT for reproductive abnormalities and prospective studies starting from childhood will be enlightening.

Ethics

Ethics Committee Approval: The Clinical Research Ethics Committee of Zekai Tahir Burak Women's Health, Training and Research Hospital (13/12/2012, with the approval number: 75).

Informed Consent: Informed consent was taken from all subjects.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ezgi Özalp Akın, Concept: Ezgi Özalp Akın, Zehra Aycan, Design: Ezgi Özalp Akın, Zehra Aycan, Data Collection or Processing: Ezgi Özalp Akın, Analysis or Interpretation: Ezgi Özalp Akın, Zehra Aycan, Literature Search: Ezgi Özalp Akın, Zehra Aycan, Writing: Ezgi Özalp Akın, Zehra Aycan.

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Incidence of Type 1 Diabetes in Children Aged Below 18 Years during 2013-2015 in Northwest Turkey

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What is already known on this topic?

Incidence of type 1 diabetes mellitus (T1DM) peaked in the age groups 5-9 and 10-14 years. Diagnosis of T1DM showed a seasonal pattern peaking in autumn-winter.

What this study adds?

The incidence of type 1 diabetes mellitus in children and adolescents aged 0-17 years was 8.99/100,000 during 2013-2015 in the Northwestern region of Turkey and constant over the course of these 3 years.

Abstract

Objective: To assess the incidence of type 1 diabetes mellitus (T1DM) in children under 18 years of age in the northwest region of Turkey during 2013-2015.

Methods: All newly diagnosed T1DM cases were recorded prospectively during 2013-2015. Total, as well as gender and age group specific (0-4, 5-9, 10-14 and 15-17 age) mean incidences per 100,000 per year were calculated.

Results: There were 1,773 patients diagnosed during 2013-2015 (588 cases in 2013, 592 cases in 2014, 593 cases in 2015). Of these, 862 (48.6%) were girls and 911 (51.4%) were boys. The mean age at diagnosis was 9.2 ± 4.2 years and it was not significantly different between girls (9.0 ± 4.1 years) and boys (9.4 ± 4.4 years) (p = 0.052). The crude mean incidence was 8.99/100.000 confidence interval (CI) (95% CI: 8.58-9.42). Although mean incidence was similar between boys [8.98/100.000 (CI: 8.40 to 9.58)] and girls [9.01/100.000 (CI: 8.42 to 9.63)], there was male predominance in all groups except for 5-9 year age group. The standardized mean incidence was 9.02/100.000 according to the World Health Organization standard population. The mean incidence for the 0-4, 5-9, 10-14 and 15-17 age groups was 6.13, 11.68, 11.7 and 5.04/100.000 respectively. The incidence of T1DM was similar over the course of three years (p = 0.95). A significant increase in the proportion of cases diagnosed was observed in the autumn-winter seasons.

Conclusion: The northwest region of Turkey experienced an intermediate incidence of T1DM over the period of the study.

Keywords: Type 1 diabetes mellitus, childhood, incidence

Introduction

Type 1 diabetes mellitus (T1DM) is a common, chronic disease in children and adolescents. In many populations, an increase in the incidence of T1DM in children has been observed (1,2,3). Studies have shown that the incidence of T1DM varies widely between and within countries (1,2,3,4). Seasonal variations in the presentation and gender differences in incidence of T1DM have been reported (5,6,7,8).

In Turkey, data on incidence and incidence trends of childhood T1DM are limited. Our aim was to determine the incidence of T1DM in children and adolescents (aged under 18 years) during the years 2013-2015 in the northwestern region of Turkey and to analyze the seasonal presentation pattern of T1DM in these children.

Methods

Turkey is divided into seven geographical regions determined by topography and climate and defined by central government. The northwestern region, where this prospective study was conducted, is one of these regions. All children younger than 18 years of age, diagnosed as T1DM in pediatric endocrinology units in this region during 2013-2015 were included in the study. The pediatric endocrinology units' locations were 11 university hospitals, 15 state hospitals and one private hospital.

The diagnosis of T1DM was made by the pediatric endocrinologist who took care of the child, according to the accepted criteria of the International Society for Pediatric and Adolescent Diabetes (9). The date of diagnosis of diabetes was accepted as the day of the first insulin injection.

In the Turkish health care system, all children aged 0-17 years with T1DM are referred to a pediatric endocrinology department for treatment. Over the three year period (2013-2015), data on all hospitalized or referred new cases in the institutions in the Northwestern region of the country were reported to our team on a special form containing information about the patient's personal identification number, sex, date of birth, date of diagnosis and some clinical and laboratory data. All forms were sent monthly to one investigator (SP) for data collection and verification. We excluded children with type 2 diabetes mellitus, neonatal diabetes, maturity onset diabetes of youth, transient hyperglycemia, and diabetes caused by other conditions (chemotherapy, cystic fibrosis, etc).

Statistical Analysis

Incidence of T1DM was calculated using the numbers of patients reported for each year by age (0-4, 5-9, 10-14

and 15-17 years aged) and gender groups (girls and boys). Annual numbers for the age groups in the geographical area were used as denominators, and incidence (per 100,000 per year) was calculated with 95% CIs, assuming a Poisson distribution. The annual population sizes were obtained from the Turkish census data of 2013-2015 from the address-based population registration system of the Turkish Statistical Institute. For comparison with data from other countries, the incidence was standardized by the direct method according to the age distribution of the world population (10).

The percentage of patients diagnosed during each calendar month was calculated in age groups for both sexes and then compared, to identify any seasonal variation in diagnosis of T1DM.

In order to assess the significance of the differences between the groups, normality of variables was tested by Kolmogorov Smirnov test; Mann-Whitney U and chi-square tests were used. Results are reported as means ± SD. Two-tailed p values were calculated. Statistical significance was accepted as $p < 0.05$.

Results

A total of 1773 cases were identified over the three year period (588 cases in 2013, 592 cases in 2014, 593 cases in 2015). Of these, 862 (48.6%) were girls and 911 (51.4%) were boys, giving a male to female ratio of 1.05:1. The mean age at diagnosis was 9.2 ± 4.2 years and showed no sex difference (9.0 ± 4.1 years in the girls and 9.4 ± 4.4 years in the boys, $p = 0.052$). Table 1 shows mean ages and distribution of the patients by age groups over the three year period. The proportion of newly diagnosed T1DM cases was highest among children aged 5-9 years (35.9%), followed by the age groups 10-14 years (35.3%), 0-4 years (19.1%) and 15-17 years (9.6%).

The crude mean annual incidence in children aged 0-17 years over this period was 8.99 per 100,000 [95% confidence interval (CI): 8.58 to 9.42]. The standardized mean incidence was 9.02 per 100,000 according to the World Health Organization (WHO) standard population.

There was no significant difference between the mean annual incidence figures for boys [8.98/100.000 (CI: 8.40 to 9.58)] and girls [9.01/100.000 (CI: 8.42 to 9.63)] during the study period ($p = 0.95$) (Table 2). The mean annual incidence for the 0-4 year age group was 6.13/100.000. Incidence increased significantly with age, reaching a peak in the age groups 5-9 and 10-14 years. It was 11.68/100.000 for the 5-9 year age group and 11.7/100.000 for the 10-14 year age group and subsequently the incidence declined at age 15-17 years. The lowest incidence was seen in the age group 15-17 years (5.04/100,000) (Table 2). The incidence of the age group 0-14 years was 9.82/100,000 (95% CI: 9.34 to 10.31). Male predominance was seen in all groups except for the 5-9 years age group (Table 2). The incidence of T1DM was similar over the course of the three years (Table 2, $p = 0.95$).

A significant increase in proportion of diagnosis of T1DM was observed in the autumn-winter seasons (Figure 1). It was similar over the three year period in all age and gender groups.

Discussion

In this study we investigated the incidence of T1DM in children residing in the Northwestern region of Turkey, our results demonstrate that the incidence of T1DM here is

Table 1. Mean ages at diagnosis and distribution by age groups of newly diagnosed type 1 diabetes mellitus cases over the three year period

	2013	2014	2015	2013-2015
Total number of cases	588	592	593	1773
Boys	279	309	323	911
Girls	309	283	270	862
Mean age at diagnosis (years)				
Total group	9.2 ± 4.3	8.9 ± 4.3	9.5 ± 4.1	9.2 ± 4.2
Boys	9.6 ± 4.5	9.1 ± 4.4	9.5 ± 4.3	9.4 ± 4.4
Girls	8.9 ± 4.1	8.7 ± 4.2	9.4 ± 3.9	9.0 ± 4.1
Proportion by age groups (%)				
0-4 years	20	20.9	16	19.1
5-9 years	32.3	38.2	37	35.9
10-14 years	39.1	31.4	35.4	35.3
15-17 years	8.3	9.5	11.1	9.6

intermediate (8.99/100,000) in the pediatric age group (4). In Turkey, there are only a few reports on the epidemiology of T1DM in children and most of them focused on children below 15 years of age (11,12,13,14). Our study is one of the few population-based reports presenting the incidence of T1DM among children in Turkey.

Considerable differences in incidence rates for T1DM have been reported from different countries, and even within the same country (1,2,3,4). Recently, in 2013, a nationwide incidence of T1DM among Turkish children was reported and this study covered T1DM incidence in Turkey divided 5 geographic regions (14) with notable differences in incidence across the five regions. The northwestern region is a developing part of Turkey and there have been big changes in the economy, urbanization and lifestyles in recent decades in this region. A slightly higher incidence (10.1/100,000 per year) was reported in the all western part of Turkey in this nationwide study and the results were consistent with our data (9.01/100,000 per year). However, the methodology for case ascertainment used in our study is different from the nationwide study. The nationwide study used data from the universal health insurance system about prescriptions for essential medicines for diabetics for the calculation of incidence. In our study the data were collected prospectively from T1DM patient data from pediatric endocrinology units in the region.

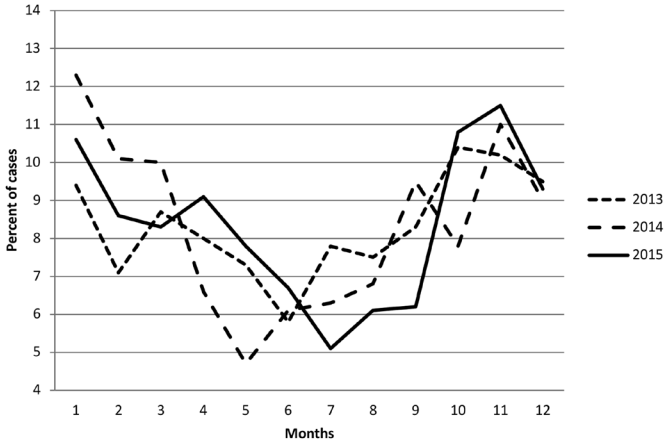

Figure 1. Distribution of age of onset of type 1 diabetes according to months of the year

We cannot detect the incidence trends from our study due to the short period covered and the lack of epidemiological data before our study in children younger than 18 years in Turkey. Our neighbour countries reported intermediate rates for incidence, similar to our results (2,3,15) and reported an increase in incidence of T1DM over time. Although our observation period was very short, incidence was quite stable over the three year period. Although the global increase in the incidence of T1DM is widely recognized in recent decades, some studies in populations with a higher incidence of T1DM have demonstrated that the increase in the incidence slowed down in the last decade (16,17,18). In the EuroDiab study, it was reported that between 2001 and 2009 the

Table 2. Incidence of type 1 diabetes mellitus over the three year period

Incidence (95% CI)	2013-2015	2013	2014	2015	p
Total group	8.99	9.01	8.99	8.98	0.95
Boys	8.98	8.31	9.11	9.51	0.07
Girls	9.01	9.76	8.85	8.43	0.1
By age groups (total group)					
0-4 years	6.13	6.6	6.74	5.09	0.92
5-9 years	11.68	10.56	12.37	12.10	0.85
10-14 years	11.7	12.78	10.42	11.9	0.48
15-17 years	5.04	4.37	4.93	5.82	0.60
Boys					
0-4 years	6.39	6.26	6.98	5.94	0.78
5-9 years	10.26	8.43	11.08	11.25	0.06
10-14 years	12.73	12.29	12.39	13.51	0.41
15-17 years	5.19	5.01	4.26	6.31	0.32
Girls					
0-4 years	5.86	6.96	6.48	4.18	0.07
5-9 years	13.18	12.8	13.74	13.01	0.91
10-14 years	10.6	13.31	8.32	10.18	0.06
15-17 years	4.88	3.69	5.65	5.28	0.23

CI: confidence interval

increase in T1DM incidence was significantly different in regions within Europe and the highest increase occurred in Central Eastern European countries while Finland, other Nordic countries and the Czech Republic showed a much lower increase or a stabilization in the incidence of T1DM (16,17,19,20,21). An average relative increase of 3-4% per year has been reported worldwide (22). Environmental factors are thought as the most likely reason for this increase in incidence (3,20,23). For this reason, evaluation of incidence in different regions is important and warranted.

In our cohort, although the mean annual incidence for boys and girls was similar, a male predominance was seen in all age groups except for the age group 5-9 years. The female predominance in the 5-9 years age group could be due to the earlier onset of puberty in girls than in boys. Gender differences in T1DM have been identified in many studies (4,6,24,25,26,27). Overall, high incidence countries tend to have a slight male predominance and low incidence countries a female predominance (4,24,25,26,27). Karvonen et al (4) found that 88% of low incidence populations were predominantly girls and patients in high incidence populations were more likely to be predominantly boys. In Sardinia, a very high incidence area, a male predominance is reported in the 0-14 year age group (26). The Danish Study Group of Diabetes in Childhood reported male predominance in their population (24). However, no significant difference in T1DM incidence between boys and girls was observed in Shanghai or in Kuwait (25,27).

Age differences in T1DM incidence have also been observed in previous studies (2,15,27,28,29). The incidence in our cohort increased with age in both sexes and was highest in the 5-14 year age group. This was followed by a decrease in the 15-17 year age group. Incidence was similar in children aged 5-9 and 10-14 years in our study. The youngest age group (0-4 years) had a lower incidence as compared with older children (5-14 years). This difference in incidence by age groups has also been shown in other counties. The WHO analyzed standardized incidence data on T1DM in the Multinational Project (DIAMOND) in 112 centres from 57 countries during 1990-1999. The DIAMOND study showed that 5-9 years old children had a higher risk of developing T1DM compared with 0-4 year old children (2). Some countries reported a high incidence in the 5-9 years old group, but others found the highest incidence in children aged 10-14 years (2,15,27,28,29).

Our cohort showed a significant seasonal variation in diagnosis of T1DM. More cases were diagnosed during autumn and winter months, which are the cooler seasons in the Northwest region. This seasonality of diagnosis of T1DM was identified in both sexes and in all age groups and thus seems to be a robust finding. Although Turkey is situated in the Mediterranean geographical location, the diverse regions have different climates because of irregular topography. To evaluate the impact of weather on incidence of TIDM in Turkey, each region should be evaluated separately. Similar to our results some countries show significant seasonality in diagnosis for all age groups, with higher incidence rates in the winter period (5,7,8). Some countries reported no seasonality in all age groups or absent in some age groups (5,8,30,31,32). Different interpretations have been suggested for this seasonal pattern in diagnosis of T1DM, including physical activity, stress, viral infections and vitamin D synthesis during different seasons (5,7,8,33). The DIAMOND group reported seasonality in T1DM incidence with winter or summer peaks in 40% of all participating centers depending on the geographic position of the country (2). It was shown that in Japan there was a bimodal pattern in the diagnosis of T1DM, that is common in April/May and in December with no seasonal pattern of incidence among preschool children (6).

Study Limitation

The limitation of our study was the short duration of the registry. It would be important to continue monitoring incidence of T1DM in the same region and evaluate trends.

Conclusion

To conclude, this is the first paper that analyzes the time-related trends in the incidence of T1DM in Turkish children aged from 0 to 17 years in the Northwest region of Turkey. The results showed an intermediate incidence of T1DM and a similar mean annual incidence between boys and girls. Considering the increasing incidence of T1DM worldwide, we suggest that it would be important to follow trends in incidence in the next few years in this same region to determine the possible triggering factors and also to develop preventive strategies.

Ethics

Ethics Committee Approval: This study was approved by the Local Ethical Committee of İstanbul Faculty of Medicine.

Informed Consent: Informed consent was obtained from the families and children.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Şükran Poyrazoğlu, Rüveyde Bundak, Design: Şükran Poyrazoğlu, Rüveyde Bundak, Data Collection and Processing: All authors, Analysis and Interpretations: Şükran Poyrazoğlu, Rüveyde Bundak, Halim İşsever, Writing: Şükran Poyrazoğlu, Rüveyde Bundak, Feyza Darendeliler.

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SLC34A3 Intronic Deletion in an Iranian Kindred with Hereditary Hypophosphatemic Rickets with Hypercalciuria

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What is already known on this topic?

Hereditary hypophosphatemic rickets with hypercalciuria is a very rare inheritable hypophosphatemic rickets/osteomalacia. Biallelic mutations in the *SLC34A3/NPT2c* gene are responsible for the disease.

What this study adds?

In this paper, we describe clinical findings, biochemical profile and gene analysis of Iranian kindred with a 101bp deletion in the *SLC34A3* gene. Genetic counseling and screening for *SLC34A3* mutations can be helpful in adult onset phenotype with unexplained osteoporosis, bone deformities and recurrent renal calculi.

Abstract

Objective: To describe clinical findings, biochemical profile and genetic analysis in an Iranian kindred with hereditary hypophosphatemic rickets with hypercalciuria (HHRH).

Methods: Clinical examination and biochemical profile results and gene analysis of 12 members of a family of a patient previously diagnosed with HHRH due to *SLC34A3* mutation. Ten healthy controls were also evaluated.

Results: Of the twelve family members three were homozygote and seven heterozygote for the same *SLC34A3* variant found in the proband while two others were unaffected. All patients had significantly increased risk of kidney stone formation, bone deformities and short stature compared with unrelated healthy controls. The heterozygous patients displayed milder clinical symptoms compared with homozygous patients. In particular they had mild or no hypophosphatemia and they did not develop skeletal deformities. Recurrent renal stones and hypercalciuria were the main presentations of the heterozygous patients which may be confused with familial hypercalciuria. In addition, biochemical analysis showed significantly low serum sodium and elevated alkaline phosphatase levels in these patients.

Conclusion: Genetic counseling and screening for *SLC34A3* mutations can be helpful in adult onset phenotype with unexplained osteoporosis, bone deformities and especial recurrent renal stones. In subjects with vitamin D deficiency the results should be interpreted cautiously.

Keywords: Hereditary hypophosphatemic rickets with hypercalciuria, *SLC34A3* gene, hypophosphatemia, hypercalciuria

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Introduction

Loss of function in the third member of the sodium phosphate cotransporter family type II (NaPi-IIc/*NPT2c*) which is encoded by the *SLC34A3* gene causes hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (1). HHRH is a rare metabolic disorder (OMIM #241530) with an autosomal recessive mode of inheritance that was first described in a large, consanguineous Bedouin kindred (1,2,3). The candidate gene, which is located on chromosome 9q34, codes for NaPi-IIc/*NPT2c* which is expressed at the apical domain of renal proximal tubule cells and plays a fundamental role in the maintenance of phosphate homeostasis (2,4). *NPT2c* contributes to renal phosphate reabsorption from glomerular filtrate under the hormonal control of parathyroid hormone (PTH) and fibroblast growth factor 23 (2). Phosphate participates in a remarkably wide array of cellular processes, intracellular signaling, pH buffering, bone mineralization, phospholipid structures and nucleic acids synthesis (4). Clinically, HHRH patients, who carry homozygous or compound heterozygous *SLC34A3/NPT2c* mutations, often show hypophosphatemia following decreased renal phosphate reabsorption, rickets and/or osteomalacia and frequently kidney stones or nephrocalcinosis. Hypophosphatemia is followed by upregulation of renal 1-α-hydroxylase and increased serum level of 1.25-dihydroxy vitamin D ($1.25(OH)_2D$) resulting in elevated intestinal absorption of calcium and urinary calcium excretion despite suppressed parathyroid function (1,5,6). High serum $1.25(OH)_2D$ concentrations and hypercalciuria distinguish HHRH from other hypophosphatemic disorders (7). Other features include slow growth, limb deformities, muscle weakness, bone pain, bowing and short stature (5,8,9). Since the initial description a few families and sporadic cases have been reported in Turkey, Holland, Morocco, North America, Japan, Africa, Caucasus, Germany and Iran (2,9,10,11). This study reports a case series in a kindred describing clinical features, biochemical profile and subsequent candidate gene analysis of a family with a 101-bp intronic deletion within the *SLC34A3* gene.

Methods

Twelve members of a family of a previously reported patient (11) with HHRH (Figure 1) were evaluated in the endocrine unit of the Shariati Hospital, Tehran University of Medical Sciences. Analysis of the extended family's medical history disclosed other members with a history of nephrolithiasis. Ten unrelated healthy subjects (no deformity, no history of renal calculi or calcium and bone disease) were included in this study as the control group. The study was approved by the Ethical Committee of Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences (No: IR.TUMS.EMRI.REC.1390). Written informed consent was obtained from the family.

A detailed clinical examination was conducted to identify any physical signs and symptoms of rickets including; skeletal deformities, leg pain, difficulty in walking, and leg

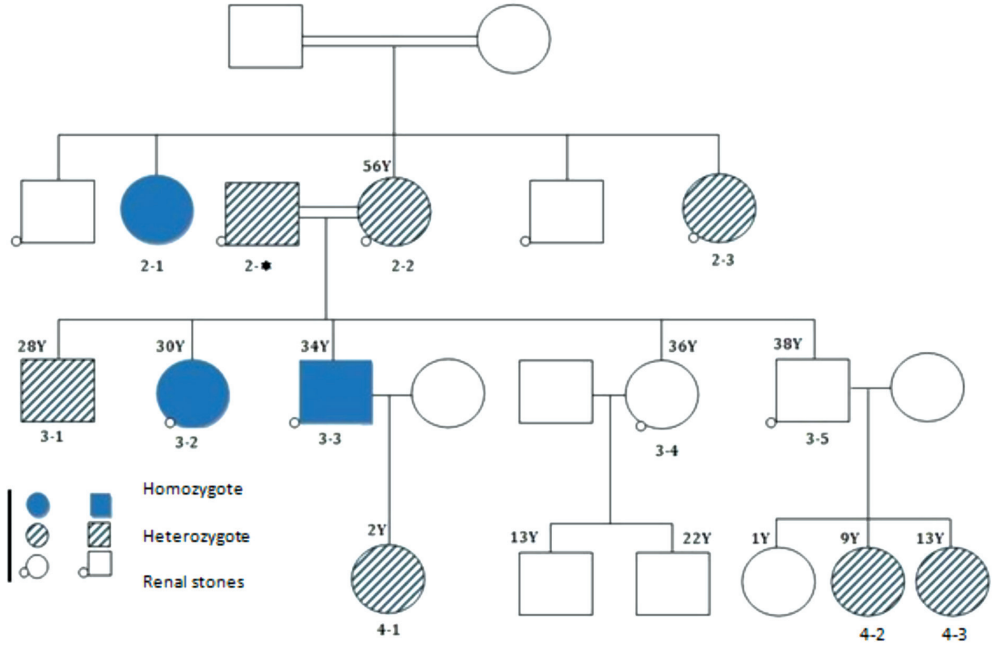

Figure 1. Genetic relationship of patients with hereditary hypophosphatemic rickets with hypercalciuria. Genetic analysis was not done for subject 3-5

bowing. Height and weight were measured in all patients. Skeletal X-rays were taken and renal ultrasonography and radiological examination were performed. Bone mineral density (BMD) of the spine, right hip and forearm was assessed for two of the homozygous patients using dual-energy X-ray absorptiometry.

Twelve hour overnight fasting blood and 24 hour urine samples were collected to measure calcium, creatinine and phosphorus. Serum levels of intact PTH, serum 25-hydroxyvitamin D [25(OH)D] and total alkaline phosphatase were assessed. Fasting tubular reabsorption of phosphate (TRP) and maximal renal phosphate reabsorption per glomerular filtration rate (TMP/GFR) were calculated using the following formula: 1 - (urine phosphorus × serum creatinine/serum phosphorus × urine creatinine).

Genetic Analysis

Genomic DNA was isolated from peripheral blood leukocytes. The previously detected 101bp deletion in intron 9 of the *SLC34A3* gene was screened for after direct sequencing of the entire *SLC34A3* gene on ABI 3130 genetic analyzer (Applied Biosystems, Thermo Fisher Scientific corporation, USA) as described by Bergwitz et al (8).

Statistical Analysis

All statistical analysis was performed using SPSS software version 16 (IBM Inc., Chicago, Ill., USA). Normal distribution of continuous variables was assessed using Kolmogorov-Smirnov test. Continuous variables with normal distribution are presented as mean (standard deviation). Comparison of continuous variables between groups was done using ANOVA test. P values < 0.05 were considered as statistically significant.

Results

A total of 12 individual members of an HHRH kindred were included in this study, three of whom were homozygote and seven heterozygote whilst the remaining two were healthy. Genetic analysis revealed the presence of a previously detected 101-bp deletion in intron 9 (Figure 1). BMD of the spine, right hip and forearm in two homozygous patients was measured (Table 1) and very low BMD and osteoporosis was found (one of homozygous patient did not consent to do the BMD). The T score of the spine, right hip and forearm were -1.6 and -3.4, -2.7 and -2.3, -3.9 and -3.7 in patients 3-3 and 3-2, respectively. Table 2 shows clinical examination and biochemistry results carried out on the homozygous and heterozygous individuals of the investigated kindred. Seven members of the kindred had a history of kidney stone. Serum creatinine was high in two members (3-1 and 3-3) and serum calcium was high in another two members (4-1 and 4-3). In six of the eight adults tested alkaline phosphatase level was found to be above the reference range. Comparison of biochemical examinations between mutant homozygous and heterozygous individuals in the kindred and healthy controls was shown in Table 3. Serum concentrations of sodium and alkaline phosphatase and mean corpuscular hemoglobin concentration were significantly different among the three groups ($p < 0.05$). Serum concentrations of sodium, potassium and calcium were lower in homozygous individuals compared to normal individuals. Serum alkaline phosphatase was higher in homozygous and heterozygous individuals in comparison with healthy controls ($p = 0.008$). Elevated hematocrit and low serum phosphate levels were not significantly different. Twenty four hour urine volume and urine calcium were higher in homozygous patients.

Homozygote and heterozygote mutations in the *SLC34A3* gene were found to lead to a significantly increased risk of kidney stone formation and bone deformities which in turn led to short stature and growth delay.

Discussion

In this case series study, we describe and discuss the clinical examination, biochemical profile and gene analysis results of the family members (affected and unaffected) of an HHRH patient. A total of 12 individuals of an Iranian

Table 1. Comparison of bone mineral densitometry values (g/cm^2) of spine, right hip and forearm in two of the homozygous patients

			Patient 3-2	Patient 3-2
		BMD	0.910	0.672
Spine	Total	T-score	-1.6	-3.4
		Z-score	-1.6	-3.4
		BMD	0.238	0.538
	Neck	T-score	-5.1	-2.8
		Z-score	-4.8	-2.7
		BMD	0.264	0.525
Right hip	Troch	T-score	-4.1	-1.8
		Z-score	-3.9	-1.8
		BMD	0.629	0.657
	Total	T-score	-2.7	-2.3
		Z-score	-2.6	-2.3
		BMD	0.485	0.378
Forearm	Total	T-score	-3.9	-3.7
		Z-score	-3.8	-3.6

BMD: bone mineral density

Table 2. Clinical examination and biochemical findings of the mutant homozygous and heterozygous individuals

	Reference range (adult)	2-1 (Homo)	2-* (Hetero)	2-2 (Hetero)	2-3 (Hetero)	3-1 (Hetero)	3-2 (Homo)	3-3 (Homo)	3-4	3-5	4-1 (Hetero)	4-2 (Hetero)	4-3 (Hetero)
Age (years)		50	60	56	53	26	30	33	36	38	3	9	13
Sex (F or M)		Female	Male	Female	Female	Male	Female	Male	Female	Male	Female	Female	Female
Height (cm)		147	173	150	153	178	135	150					158
Weight (kg)		68	63	55	72	95	47	55		75		22	40
Kidney stone (Y/N)		No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No	No	No
Deformity (Y/N)		Yes	No	No		No	Yes	Yes	No	Yes		No	No
Serum creatinine (mg/dL)	0.3-1.40	0.87	0.89	1	0.83	1.97	0.78	2.26		1.20	0.53	0.63	0.73
Serum calcium (mg/dL)	8.9-10.1 (1-14 years: 9.5-10.6)	9.4	8.9	9.5	9.3	9.8	8.6	9.6		9.7	10.6	9.7	10.6
Serum phosphorus (mg/dL)	2.7-4.5 (1-12 years: 3-6)	3.0	3.2	3.1	3.0	3.9	5.1	3.0		2.3	4.7	4.4	4.8
Alkaline phosphatase (U/L)	Female: 64-306 Male: 80-306 (1-15 years: 180-1200)	537	255	359	245	319	357	407		578	512	715	267
Parathyroid hormone (pg/mL)	10-62	18	29	30	196	100	36	66		29	37	38	35
Serum 25-hydroxy vitamin D (ng/mL)	20-100	62	99.1	118	12	25	12.8	19.3		22	59	19	16
Urine creatinine (mg/ 24 h)	600-1800	611	1071	585	933	1817	693	966		1272		376	864
Urine calcium (mg/ 24 h)	Up to 300 (Up to 4 mg/kg)	188	289	221	50	48	209	172		29.7		56	92
Urine phosphorus (mg/ 24 h)	-	266	799	312	518	667	385	907		468		252	328
FEp		0.10	0.20	0.17	0.15	0.18	0.08	0.70		0.19		0. 09	0.05
TRP	0.85-0.98	0.89	0.79	0.82	0.84	0.81	0.91	0.29		0.81		0.90	0.92
GFR		97.7	77	64.1	104	76.4	92	36.2		86		63.5	95.9
TMP/GFR (mg/dL)	2.5-4.2	3.0	2.7	2.7	2.3	3.2	5.0	0.9		1.8		4.4	4.8

Three subjects (4-1, 4-2, 4-3) were younger than 13 years. Interpretation for biochemical tests should be considered in the reference range of this group. 2-1, 3-2 and 3-3 are homozygote and 2-*, 2-2, 2-3, 3-1, 4-1, 4-2 and 4-3 are heterozygote.

Y: yes, N: no, F: female, M: male, FEp: free erythrocyte protoporphyrin, TRP: tubular reabsorption of phosphate, GFR: glomerular filtration rate, TMP: maximal renal phosphate reabsorption

kindred were included in this study. Of these, three were homozygote, seven heterozygote and two were healthy.

Homozygote and heterozygote mutations in the *SLC34A3* gene lead to a significantly increased risk of kidney stone formation and bone deformities, compared with healthy controls. A hallmark feature of familial hypophosphatemic rickets is short stature resulting from deformity and growth retardation, which is observed in both homozygous and heterozygous individuals. Hypophosphatemia in HHRH leading to elevation in the serum level of $1.25(OH)_2D$ which results in hypercalciuria. Episodes of hypercalciuria may cause development of recurrent renal stones. Based on biochemical follow-up data that were available for the homozygote and heterozygote members in this kindred, significantly low serum sodium levels and elevated alkaline phosphatase levels were observed.

The causative gene, *SLC34A3*, which is mapped to chromosome 9q34 encodes a member of the SLC34A transporter family of proteins which is involved in transporting phosphate into cells mediated by sodium-

Table 3. Comparison of biochemical findings between mutant homozygote and heterozygote and normal controls

Variables	Normal	Homozygote	Heterozygote	p-value
Weight (kg)	61.3 (8.1)	56.7 (10.6)	57.8 (25.4)	0.88
WBC	5.8 (1.0)	6.5 (2.5)	7.2 (1.9)	0.24
RBC	5.0 (0.4)	5.6 (0.5)	5.2 (0.5)	0.22
Hemoglobin	14.5 (1.4)	16.0 (2.0)	14.5 (1.9)	0.38
Hematocrit	42.9 (3.2)	48.7 (5.4)	45.0 (4.8)	0.14
MCV	85.6 (8.1)	86.5 (2.5)	86.3 (4.0)	0.96
MCH	29.0 (3.0)	28.5 (1.0)	28.9 (2.3)	0.71
MCHC	33.8 (0.9)	33.0 (0.5)	32.3 (1.5)	0.04
Platelets	263.8 (41.2)	259.0 (38.7)	276.4 (110.2)	0.92
FBS	100.4 (5.9)	79.3 (3.51)	109.3 (60.8)	0.52
Urea	32.3 (7.5)	30.0 (13.07)	34.1 (11.0)	0.82
Creatinine	1.0 (0.1)	1.3 (0.83)	0.9 (0.5)	0.43
Triglyceride	110.6 (58.1)	142.0 (87.1)	113.1 (55.8)	0.74
Total cholesterol	208.7 (55.5)	227.0 (39.0)	206.3 (44.0)	0.82
HDL cholesterol	56.9 (8.8)	56.3 (7.2)	57.0 (7.4)	0.99
LDL cholesterol	117 (36.7)	123.3 (24.4)	111.6 (34.0)	0.88
Sodium	142.1 (2.0)	138.4 (0.6)	138.6 (1.4)	0.001
Potassium	4.3 (0.3)	3.9 (0.47)	4.1 (0.1)	0.12
Calcium	9.8 (0.4)	9.2 (0.53)	9.8 (0.6)	0.22
FEp	0.15 (0.05)	0.29 (0.2)	0.14 (0.04)	0.24
TRP	0.84 (0.05)	0.69 (0.19)	0.85 (0.17)	0.45
GFR	89.9 (30.2)	75.3 (0.2)	80.1 (21.5)	0.35
Alkaline phosphatase	198.0 (63.9)	433.6 (92.9)	381.7 (173.4)	0.008
Phosphorus	3.8 (0.4)	3.7 (1.21)	3.9 (0.77)	0.94
PTH	58.7 (44.8)	40.0 (24.2)	66.4 (62.3)	0.75
25-OH vitamin D	39.0 (20.8)	31.4 (26.7)	49.7 (43.4)	0.67
Urine volume (24 hrs)	1333 (582.5)	2200 (1735)	1250 (677)	0.28
Urine creatinine (24 hrs)	990 (328)	757 (186)	941 (497)	0.66
Urine calcium (24 hrs)	152 (110)	189 (18.5)	126 (103)	0.67
Urine phosphorus (24 h)	657.7 (313)	506 (356)	479 (220)	0.48

Variables are presented as mean (standard deviation).

Three heterozygous individuals were younger than 13 years and were not included in the analysis.

WBC: white blood cells, RBC: red blood cells, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, FBS: fasting blood sugar, FEp: free erythrocyte protoporphyrin, TRP: tubular reabsorption of phosphate, GFR: glomerular filtration rate, PTH: parathyroid hormone, HDL: high-density lipoprotein, LDL: low-density lipoprotein

phosphate cotransporters in the renal brush border membrane and plays a key role in phosphate homeostasis, despite its low expression levels (4,8). It has been demonstrated that mice homozygous for the disrupted *NPT2* gene show many of the features of HHRH and that the *SLC34A1/NaPi7* gene plays a key role in phosphate homeostasis and in normal skeletal development (12). Not many cases of biallelic *SLC34A3/NPT2c* mutations resulting in HHRH syndrome, have been reported worldwide (5). Different mutations in *SLC34A3/NPT2c* with different phenotypes have been reported in these patients (1,2,5, 6,8,9,10,13,14,15,16,17). This present study reports the first kindred of HHRH in Iran and describes a previously described mutation, a 101bp deletion, within the *SLC34A3* gene, which affects transcription or splicing of pre-mRNA, causes aberrant RNA splicing, between exons 9 and 10 (11).

Since HHRH is an autosomal recessive disease, biallelic mutations are required for full-scale disease manifestations; loss of one *SLC34A3* allele does not always lead to laboratory abnormalities. However, clinical phenotypes are sometimes seen in carriers of single *SLC34A3* mutations (2,6). Similarly, several heterozygous members of the Bedouin kindred for the c.228delC mutation, displayed mild hypophosphatemia, reduced TMP/GFR, and elevations in $1.25(OH)_2D$ concentrations in addition to increased urinary calcium excretion (8). In the present study, the heterozygous patients displayed milder clinical symptoms compared with homozygous patients. These patients displayed mild or no hypophosphatemia and they did not develop skeletal deformities. Recurrent renal stones and hypercalciuria were the main presenting features of these patients, which could be confused with familial hypercalciuria. HHRH diagnosis can be missed in this situation and appropriate interpretation of the clinical symptoms is important. Absence of clinical symptoms and of biochemical alterations have also been reported in previous heterozygous HHRH families (2,5,6,8,17). Therefore, genetic screening for *SLC34A3* mutations can be helpful in patients with suspicious clinical findings.

Hypophosphatemia and renal stones are common in homozygous patients. In addition bone deformities may not always develop in these patients and may create difficulties in diagnosing this disease. Further investigation and genetic evaluation are needed in this situation.

Poor vitamin D status is highly prevalent among Iranian adults with vitamin D deficiency and insufficiency reported in 90.7% of the adult population (18). Moreover, hypercalciuria and renal stones are prevalent in Iran from childhood, as patient 3-4 also had kidney stones without any mutation (19,20). Thus, evaluation of vitamin D level and/or increase in serum creatinine should also be measured when assessing serum and urine levels of phosphorous and calcium. As the findings of patients 3-1 and 3-3 demonstrate, chronic kidney diseases and vitamin D deficiency are two important issues for interpretation of biochemical findings.

Study Strengths and Study Limitations

The large number of individuals included in the study is the strength of this study. However, we realize that the sample size of the control cohort is small. One of the limitations of the study was the lack of clinical and genetic testing for all members of the family. Moreover, the concentration of $1.25(OH)_2D_3$ which is very important in differentiating HHRH patients, was not measured in this study. It should be noted that the 24 hour urine samples are influenced by dietary phosphate and three hour, fasting, spot urines were not determined in order to calculate TRP%. In addition, genes encoding other phosphate transporters were not sequenced.

Conclusion

In conclusion, as the clinical phenotype of HHRH can be quite variable with different penetrance even in the same family with identical mutations, it is not possible to be certain about a genotype-phenotype effect, and a proper diagnosis requires molecular genetic analysis. Screening for *SLC34A3* mutations to evaluate the importance of treatment and close follow-up to avoid complications can be helpful.

Ethics

Ethics Committee Approval: Ethical Committee of Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences (No: IR.TUMS.EMRI.REC.1390).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Mahsa M. Amoli, Fatemeh Bitarafan, Concept: Shirin Hasani-Ranjbar, Design: Shirin Hasani-Ranjbar, Akbar Soltani, Data Collection or Processing: Mahsa M. Amoli, Fatemeh Bitarafan, Bahareh Yarjoo, Analysis or Interpretation: Shirin Hasani-Ranjbar, Hanieh-Sadat Ejtahed, Mahsa M. Amoli, Mostafa Qorbani, Akbar Soltani, Literature Search: Hanieh-Sadat Ejtahed, Bahareh Yarjoo, Writing: Shirin Hasani-Ranjbar, Hanieh-Sadat Ejtahed.

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A Novel Mutation in the *AVPR2* Gene Causing Congenital Nephrogenic Diabetes Insipidus

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What is already known on this topic?

About 90 percent of all cases of hereditary nephrogenic diabetes insipidus result from mutations in the *AVPR2* gene. To date, more than 250 mutations have been identified comprising missense, nonsense, small insertions and deletions, large deletions and complex rearrangements in *AVPR2* gene.

What this study adds?

In this study, we found a novel hemizygous missense mutation in the *AVPR2* gene at the position 80th in exon 2 causing congenital nephrogenic diabetes insipidus in a 6-year-old boy presenting with growth failure and dull normal cognitive functions.

Abstract

Objective: Congenital nephrogenic diabetes insipidus (CNDI) is a rare inherited disorder characterized by a renal insensitivity to arginine vasopressin (AVP). In the majority of the cases, CNDI is caused by mutations in the arginine vasopressin receptor 2 *(AVPR2)* gene. Our objective is to report a novel mutation in the *AVPR2* gene causing CNDI in a 6-year-old boy, presenting with growth failure and dull normal cognitive functions.

Methods: The proband was the third off-spring of non-consanguineous parents and had polyuria (4.3 L/day), polydipsia (5 L/day). The diagnosis of CNDI was established by a water-deprivation test and a desmopressin challenge test. Genetic studies were also carried out in the mother, siblings and affected family members, since excessive fluid intake and diuresis were also reported in these individuals. All exons of the *AVPR2* gene for all participants were amplified and sequenced. Bioinformatics analysis for wild-type and mutant AVPR2 were obtained with Swiss-Model and UCSF Chimera 1.10.2.

Results: A novel, hemizygous, missense mutation was identified at the position 80th in exon 2 (p.H80Y) of *AVPR2* in the proband. The proband's mother, maternal aunt and grandmother were heterozygous and his maternal uncle was hemizygous for this mutation. Bioinformatic analysis indicates this mutation would cause significant conformational changes in protein structure.

Conclusion: p.H80Y mutation will cause inappropriate folding of the protein compromising water homeostasis via AVPR2 and AVP and leading to diabetes insipidus. We suggest that future functional investigations of the H80Y mutation may provide a basis for understanding the pathophysiology of the NDI in patients with this variant.

Keywords: *AVPR2*, congenital nephrogenic diabetes insipidus, mutation

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Introduction

Water is vital and hydration is important for physical and mental performance. Water balance of the body is controlled through fluid intake, which is stimulated by thirst and renal excretion of water as urine (1,2,3).

Increase in plasma osmolality or decrease in blood volume leads to secretion of arginine vasopressin (AVP), also called antidiuretic hormone (ADH), from the posterior pituitary gland (4,5). AVP increases water permeability in the renal collecting ducts by activating arginine vasopressin receptor 2 (AVPR2), one of the family of G protein-coupled receptor, located on the basolateral membrane of the kidney collecting duct cells (6,7). Binding of AVP to AVPR2 activates Gs/adenylate cyclase and leads to a series of intracellular events resulting in exocytic insertion of the water channel aquaporin-2 (AQP2) from intracellular storage compartments into the luminal membrane. This results in water reabsorption from the pro-urine by the kidney tubule after an osmotic gradient (1,8,9). Any impairment in this pathway can lead to a metabolic disease called diabetes insipidus (DI) (10). DI has two major types; a deficiency of AVP causes central DI, whereas inadequate sensitivity to AVP in the kidney leads to nephrogenic DI (NDI) (11,12).

Congenital nephrogenic DI (CNDI) is an inherited form of NDI and this disorder occurs as a result of loss-of-function mutations of the *AVPR2* or *AQP2* genes. *AQP2* gene defects cause autosomal recessive or dominant NDI and are responsible for a small percentage of NDI cases. However, loss-of-function mutations in *AVPR2* lead to X-linked recessive NDI and this accounts for 90% of cases with CNDI. In addition, X-linked NDI occurs with a frequency of 4-8/1,000,000 male live births (13,14,15,16). The well-known clinical symptoms of CNDI are polydipsia, polyuria, hypernatremia and hyperchloraemia (17,18). The cause of these symptoms is the inability of the kidney to concentrate urine. This defect may be due to loss-of-function mutations of *AVPR2* (19).

In this study, we described a novel, hemizygous, missense mutation causing a conversion of the histidine residue to tyrosine in the protein sequence, at position 80th in exon 2 of *AVPR2* in a 6-year-old proband with symptoms of CNDI and an X-linked recessive family pedigree. The pedigree became evident after investigating affected and unaffected family members.

Methods

A 6-year-old boy was referred to the Pediatric Nephrology Department of Keçiören Training and Research Hospital for abnormal fluid intake (5 L/day) and excessive urine output (4.3 L/day), reported to have started in the early phases of the patient's life. The parents indicated that it was possible to make him stop crying only if he drank water in addition to breast milk. His height and weight were less than his peers in preschool and he succeeded moderately well in preschool activities. He was the third child of non-consanguineous parents. His maternal grandmother, aunt and uncle were also reported to have excessive fluid intake and high urine output. This was the patient's first admission to a medical center for his symptoms of polyuria and polydipsia. The values of his height, weight, blood pressure, skin turgor, serum sodium (Na), potassium, chloride, calcium, phosphorus, albumin, urea, creatinine, urine specific gravity, plasma vasopressin were obtained from physical and laboratory examinations. His serum and urinary osmolality were calculated using the ffollowing formulas, 2 x [serum Na^+] + [blood urea nitrogen (BUN) ÷ 2.8] + (serum glucose ÷ 18) and 1.86 x [urine Na^+] + [urea nitrogen (UN) ÷ 2.8] + (urine glucose ÷ 18) + 9. The water deprivation test and desmopressin (DDAVP) challenge test was also performed. The patient's blood pressure and heart rate were measured. In addition, his urinary ultrasonography, pituitary magnetic resonance imaging, verbal and performance tests of the Wechsler Intelligence Scale for Children fourth edition (WISC-IV) were performed.

The collection of blood samples from the proband and his family members was approved by the Ethics Committee of the Faculty of Medicine of Hacettepe University (approval no: 2607). Written informed consent was obtained from all participants and/or their parents.

DNA Isolation

Genomic DNA was extracted from peripheral blood leukocytes following a standard protocol using QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extracted genomic DNA was quantified spectrophotometrically (Quawell Technology, Inc., United States) and stored in aliquots at -20°C until use.

PCR Amplification and Direct Sequencing of *AVPR2* Gene

The entire coding regions and flanking intronic sequences of the *AVPR2* gene were amplified from genomic DNA using polymerase chain reaction (PCR). The sequences of the primers that were used in PCR are as follows: Exon 1, forward 5'-GTC TGACCA TCC CTC TCA ATC TTC-3'and reverse 5'-GGA GTC GGG AAG AGG GCC TGG TTA-3'; Exon 2a, forward 5'-ATA ACA TGG CTT CCT GGA GTC CC-3' and reverse 5'-TGC GCT GGG CGA AGA TGA AGA GCTG-3'; Exon 2b, forward 5'-TGG AAG TGG GGC TCACTG GAA CCG GC-3'

and reverse 5'-GCT GTT GAG GCT GGC CAG CAA ACA TG-3'; Exon 3, forward 5'-TGT AGC CGT GGC TAG GGC TGA CGG G-3' and reverse 5' CCT GCC CCA GGA AGG CAG CTG AGC-3'. All PCR amplifications were performed under the following conditions: 45 seconds at 95 °C, 45 seconds at 64 °C and 45 seconds at 72 °C for 32 cycles. After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel. These products were purified enzymatically and sequenced using the Big Dye kit (Applied Biosystems, Foster City, CA, USA).

Analyses of Bioinformatics

Three-dimensional protein structures for wild-type and mutant AVPR2 proteins, comparing amino acid sequence properties and predictions of binding sites of these proteins, were obtained with computational tools including Swiss-Model and UCSF Chimera 1.10.2 servers.

To predict the functional effect of mutation on the resulting mutant AVPR2, it was also analysed using Phenotyping Polymorphism v2 (PolyPhen-2) software.

Results

At physical examination, the patient's height [104 cm, standard deviation scores (SDS): -2.66] and weight (14 kg, SDS: -3.92) were below the 3rd percentile, his blood pressure and skin turgor were normal. Laboratory examination disclosed the following values: serum Na 137 mmol/L (range: 136-146), potassium 4.86 mmol/L (range: 3.5-5.1), chloride 102 mmol/L (range: 101-109), calcium 9.52 mg/dL (range: 8.8-10.6), phosphorus 5.67 mg/dL (range: 4-7), albumin 4.07 g/dL, urea 23 mg/dL and creatinine 0.59 mg/dL. Urine specific gravity was 1.000. The patient's calculated serum osmolality was high 281.5 mOsm/kg, his calculated urinary osmolality was low 67.7 mOsm/kg and he had hyponatriuria (urine Na^+: 15 mmol/L). The water deprivation test was stopped due to a weight loss exceeding 3% at 3.5th hour of the test. Plasma vasopressin (16.75 pmol/l, range: 0-13) at the conclusion of the dehydration test was high and the urine parameters showed insignificant changes. The administration of DDAVP, 20 μg intranasally, also did not have any effect on urine parameters (Table 1). A high urine output, low urine osmolality and impaired ability to increase urine osmolality to normal levels were noted after ADH administration. The patient's blood pressure and heart rate were stable at 112/75 mmHg and 82/min during the test. His urinary ultrasonography and pituitary magnetic resonance imaging showed no abnormality. On the verbal and performance tests of the WISC-IV, the child obtained a score of 81 and 82, respectively. Both verbal and performance tests indicated a "dull normal" intellectual function. The patient was treated with thiazide diuretics (2 mg/kg/day), indomethazine (2 mg/kg/day), low-protein diet, low Na diet and unlimited amounts of fluid. He responded well to the treatment during the first year; fluid intake (3-3.5 L/day) and diuresis (2.7-3 L/day) were both diminished. At no time during follow-up did the patient develop hydronephrosis nor did he experience dehydratation and/or hypernatremia. The family pedigree was compatible with a presumed X-linked recessive CNDI due to similar symptoms associated with NDI in some of his family members (Figure 1).

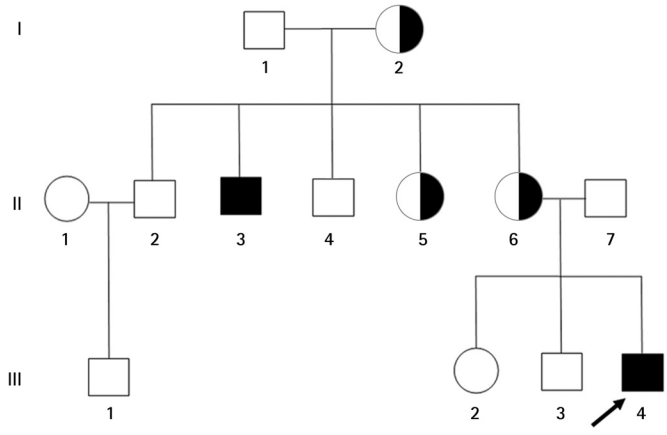

Figure 1. Pedigree of the family. The individuals marked with numbers are those who were available for mutation screening of the *AVPR2* gene. Black and white symbols represent clinically affected and unaffected individuals, respectively. Arrow represents proband.

Table 1. Results of the water-deprivation and desmopressin challenge tests

	Weight (kg)	Urine density	Urine sodium (mmol/L)	Urine osmolarity (mOsm/kg)	Serum sodium (mmol/L)	Serum osmolarity (mOsm/kg)
Before the tests	14	1.000	15	67.7	137	281.5
1st hour	13.9	1.005	13	63.5	ND	ND
2nd hour	13.7	1.005	12	60.5	138	285.3
3.5th hour- desmopressin given	13.5	1.005	13	70.1	141	291.4
5th hour	ND	1.005	13	65.9	ND	ND

ND: not done

All of the coding regions for the *AVPR2* gene of the proband were screened by DNA sequence analysis. The analysis revealed the presence of a novel hemizygous missense mutation at coding position 238 (c.C238T) in exon 2 (Figure 2). This mutation leads to a conversion of the histidine residue to tyrosine in the protein sequence, at position 80. After the screening of the novel mutation, we also performed sequence analysis in the proband's close family members. The results showed that his mother (II-6), maternal aunt (II-5) and grandmother (I-2) were heterozygous and maternal uncle (II-3) was hemizygous for this mutation (Figure 2). No mutation in *AVPR2* gene was detected in other family members (II-2, II-4, III-1, III-2, III-3).

According to bioinformatic analysis, based on DNA sequence, we made a prediction on mRNA structures of the wild-type and mutated proteins. H80Y mutation is located in the second transmembrane domain (amino acid positions 78-98) of the protein (20). Using the Swiss-Model and UCSF Chimera 1.10.2 servers, we found some differences between alpha-helix and beta-sheet structures of wild-type and mutant AVPR2 proteins (Figure 3). Comparisons of amino acid sequences revealed that

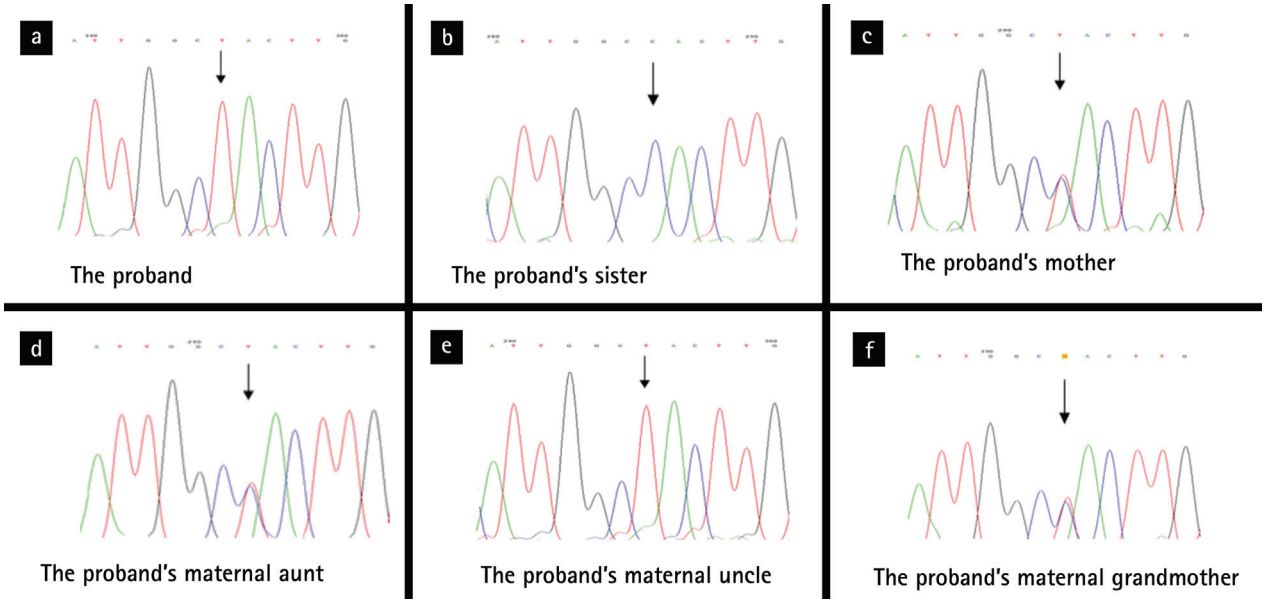

Figure 2. (a, b, c, d, e, f) DNA sequencing results from a part of exon 2 of the *AVPR2* gene of proband and his family members. Arrows represent the mutation site.

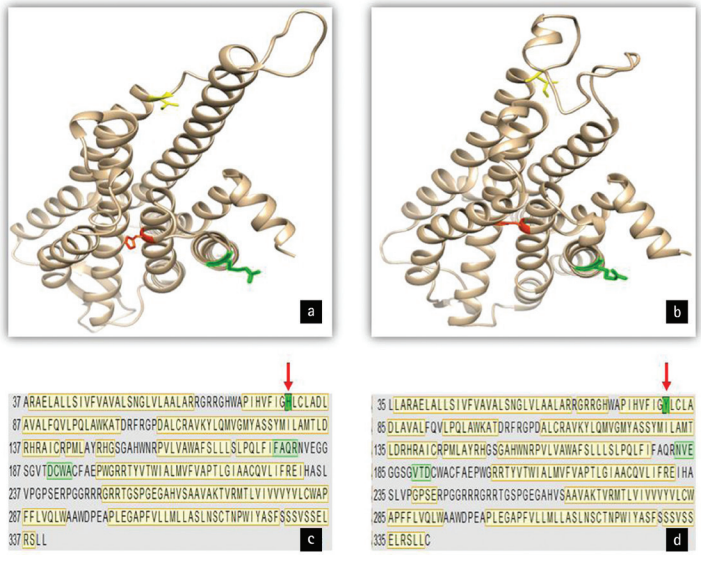

Figure 3. Three-dimensional protein structure predictions of wild-type (a) and mutant (b) AVPR2. Primary structures of wild-type (c) and mutant (d) AVPR2 proteins. Yellow boxes represent α-helixs structures and light green boxes represent β-sheet structures. Arrows represent mutation site (processed by UCSF Chimera 1.10.2.).

theoretical pI values of wild type and mutant protein are 9.49 and 9.47, respectively and their theoretical molecular weights are 40,279.09 and 40,305.12, respectively. In addition, PolyPhen-2 analyses predicted the effect of the mutation with a score of 0.999 (out of 1), sensitivity of 0.14 and specificity of 0.99, therefore p.H80Y mutation was identified as a probable damaging mutation.

Discussion

CNDI is a rare disease, most commonly caused by mutations in the AVPR2 in the collecting duct epithelial cells, which is encoded by the *AVPR2* gene (Xq28). The gene encodes a 371-amino acid, G protein-coupled receptor with seven transmembrane, four extracellular and four cytoplasmic domains (7,14). *AVPR2* mutations causing CNDI can vary in their functional severity; clinical symptoms and the response to DDAVP can be diverse.

CNDI is a severe form of DI and is difficult to treat. It is commonly due to inherited defects (14). The urine output in patients with CNDI can be lowered with a low-salt, low-protein diet, thiazide diuretics and/or potassium-sparing diuretic (amiloride) and non-steroidal anti-inflammatory drugs (21). Diuretics in NDI patients reduce theurine output by promoting the reabsorption of Na and water in the proximal tubule, thus delivering less water to the collecting ducts (22). The inhibitory effect of indomethacin on urine volume is thought to be mediated by an AVP-independent water reabsorption resulting from an increase in solute reabsorption and consequent medullary hypertonicity (23). Nevertheless, many patients still experience significant polyuria and polydipsia while receiving these therapeutic measures. The investigational therapeutic strategies for CNDI include the rescue of AVPR2 mutants by chemical chaperones and by passing defective AVPR2 signaling (24). In infants, early recognition is very important as the proper treatment can avert the physical and mental retardation that results from repeated episodes of dehydration and hypernatremia. Patients with CNDI should be monitored for growth, serum Na concentration and hydration status, and also for the development of hydronephrosis. Genetic analyses for CNDI can also be a key aid in obtaining diagnosis at an early age and should be performed in all patients with a family history of the disorder (14,21,25). Therefore, the definitive diagnosis of CNDI will provide appropriate genetic counseling and development of specific treatment strategies.

To date, more than 250 putative disease-causing *AVPR2* mutations have been found comprising missense, nonsense, small insertions and deletions, large deletions and complex rearrangements in the *AVPR2* gene (14,26). The most common category of *AVPR2* mutations causing NDI are missense mutations. Many disease-causing mutations occur in the transmembrane domains compared to extracellular or intracellular domains. *AVPR2* missense mutations are likely to impair folding and lead to rapid degradation of the misfolded polypeptide (14,25,26,27).

We report here a male child with repeated episodes of dehydration, polyuria and polydipsia in early infancy. The water deprivation test and the DDAVP challenge test confirmed the diagnosis of CNDI. The genetic analysis revealed a novel, X-linked recessive, missense mutation (p.H80Y) causing replacement of histidine residue with tyrosine in the protein sequence of AVPR2. Histidine is a basic, polar, positively charged amino acid and tyrosine is an aromatic, non-polar amino acid. The function of the altered AVPR2 structure is presumably significantly impaired as tyrosine joins in beta-strand conformations in proteins (26) in addition to the physicochemical differences between histidine and tyrosine amino acids. Therewith, an altered protein conformation might impair the intracellular trafficking of AVPR2 and affect proper localization of the receptor into plasma membrane or decrease AVP binding characteristics (20). The novel mutation reported in this study is located in the second transmembrane domain of the protein. This mutation point (codon 80) is a conserved residue among rat V1 and V2 vasopressin receptors and the human oxytocin receptor (28,29). A different missense mutation at codon 80 (p.H80R) was previously reported by Yuasa et al (20) and the authors emphasized the sequence conservation and functional importance of this codon.

The functional significance of this mutation was analysed by utilizing the PolyPhen-2 software. This software can be used to predict the consequence of an amino acid change on the structure and function of a protein using physical and evolutionary comparative considerations (18,30). As a result of the analysis, the H80Y mutation was identified as a probable pathogenic mutation. In addition, according to the bioinformatic analysis of this mutation, protein conformation is predicted to be impaired which may also lead to abnormal protein function. However, definitive functional analyses of this mutation are needed to determine the structure-function relationship in patients with CNDI.

Study Limitation

The limitation of this study is the lack of mutation function analysis data.

Conclusion

In conclusion, this study reports the clinical and molecular characterization of a novel mutation in *AVPR2* resulting in CNDI and emphasizes the importance of definitive diagnosis in CNDI patients.

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Ethics

Ethics Committee Approval: The collection of blood samples from the proband and his family members were approved by the Ethics Committee of the Faculty of Medicine of Hacettepe University (approval no: 2607)

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Hatice Mergen, Aslı Çelebi Tayfur, Design: Hatice Mergen, Aslı Çelebi Tayfur, Data Collection or Processing: Aslı Çelebi Tayfur, Tuğçe Karaduman, Merve Özcan Türkmen, Analysis or Interpretation: Tuğçe Karaduman, Merve Özcan Türkmen, Dilara Şahin, Literature Search: Tuğçe Karaduman, Merve Özcan Türkmen, Dilara Şahin, Ayşe Derya Buluş, Aysun Çaltık Yılmaz, Bahar Büyükkaragöz, Writing: Aslı Çelebi Tayfur, Tuğçe Karaduman, Merve Özcan Türkmen.

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ORIGINAL ARTICLE

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Perceived Expressed Emotion, Emotional and Behavioral Problems and Self-Esteem in Obese Adolescents: A Case-Control Study

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What is already known on this topic?

Family climate is important for the mental health of youths. Expressed emotion (EE) is a measure of the family climate. Studies have shown that family climate and thus EE is important in prevention and treatment of obesity. Although family attitude and psychopathology is an important issue in obesity treatment, there is only one published study on children and one on adults in this field.

What this study adds?

In this study, the relationship between perceived expressed emotion (EE) and emotional and behavioural problems (BP) and self-esteem in obese adolescents was investigated. A higher rate of perceived EE, emotional and BP were observed in the obese group than in the control group. According to our findings, the obese adolescents had significantly lower levels of self-esteem than the control group. To the best of our knowledge, no study concerning EE and psychopathology and self-esteem of obese adolescents has been conducted in Turkey.

Abstract

Objective: Obesity is a chronic disease which leads to medical and psychiatric complications. Family climate is a critical factor in the treatment of obesity and comorbid psychiatric disorders. In our study, perceived expressed emotion (EE), psychopathology, self-esteem and emotional and behavioural problems (BP) among obese adolescents were investigated and compared with their non-obese peers.
Methods: The subjects were 49 obese adolescents and 47 non-obese adolescents served as the control group. All participants were requested to fill out the Socio-demographic Data Form, Shortened Level of Expressed Emotion Scale, Rosenberg Self-Esteem Scale, Strength and Difficulties Questionnaire-Adolescent Form.
Results: In our study, obese adolescents showed a significant difference in perceived EE ($p < 0.001$). Subscales of EE, such as Lack of Emotional Support ($p < 0.001$), intrusiveness ($p < 0.001$), irritability ($p < 0.001$), self-esteem ($p < 0.001$), emotional and BP ($p < 0.001$), attention deficit-hyperactivity disorder ($p < 0.001$), problems in peer relationships ($p < 0.001$) and social skills ($p < 0.001$) were significantly worse when compared with the control group. There was a strong relationship between EE and emotional and BP and self-esteem.
Conclusion: The higher rate of perceived EE, psychopathology and low self-esteem among obese adolescents showed that obesity prevention and treatment are also crucial for good mental health in adolescents. The important role of the family in mental health of obese adolescents was emphasized. It was shown that identification of risk factors in childhood that promote obesity should be done so that targeted intervention and prevention programs can be developed.
Keywords: Obesity, self-esteem, expressed emotion, psychopathology

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Introduction

The prevalence of obesity among young people has recently increased dramatically in both developed and developing countries (1). Obesity affects all physiological systems of humans at every age. Obese adolescents exhibit psychiatric problems such as body dissatisfaction, lower self-esteem, depression and attention deficit-hyperactivity disorder (ADHD) (2,3,4). Behavioral problems, neurocognitive function deficits and ADHD symptoms in childhood have been found to affect weight gain over time (5). Pathological family environment factors, such as mental illness and inadequate care, are also found to be strongly associated with the psychopathology of obesity in children (5). In this context, family-based programs have been shown to be effective in targeting childhood obesity (6).

Expressed emotion (EE) is a measure of the family climate in the home which is characterized by communication styles of family members, such as emotional support, irritability and intrusiveness. This concept was formulated because of the strong relationship between environmental changes in the family system and the mental health of family members (7). In considering the association between obesity and mental health, EE becomes an important factor. Although the relationship between EE and mental-physical illnesses is well understood, researchers generally focus on the relationship between EE and eating disorders such as anorexia nervosa, bulimia, binge eating and emotional eating (8,9).

However, EE is an under-investigated issue in obesity. While the importance of the effect of the family on obesity has been shown (10,11), there is still little information regarding the difference between perceptions of EE of obese adolescents and those of their healthy peers. To the best of our knowledge, only one study regarding EE and obesity in children has been conducted to date (7). These researchers concluded that childhood obesity intervention programs may benefit from targeting maternal psychopathology, EE and coping skills. Also there is only one poster report of a study in the adult literature regarding obesity and EE (12). This study concluded that levels of EE should be considered when planning treatment interventions to enhance compliance in obese patients.

The goal of the current study was to investigate the perception of the family climate, as well as the emotional and behavioral problems of obese adolescents, by comparing obese adolescents with a non-obese control group. A second objective was to examine the association between perceived EE and psychopathology and self-esteem in obese adolescents. We also aimed to investigate the role of the family in the mental health of obese adolescents, as well as in their treatment and to identify the risk factors in childhood that promote obesity. Identifying these risk factors will help to develop targeted intervention and prevention programs.

Methods

Subjects and controls were recruited at the Paediatric Endocrinology Outpatient Clinic of Uludağ University Medical School between January 1 and July 31, 2015. The control group of adolescents was matched to the patient group for age and gender. The necessary legal permission and approval were obtained from the Uludağ University Ethics Committee (Date: 14.11.2014, No: 2014-19/5) before proceeding to the data collection stage. All participants in the study and their parents gave informed consent after being informed of the methods and objectives of this study. A child and adolescent psychiatrist evaluated all participants using a clinical interview, based on Diagnostic and Statistical Manual of Mental Disorders (13). The exclusion criteria were the presence of a neurological disorder, clinical mental retardation, autism spectrum disorder, physical disability with significant functional impairment, alcohol and/or substance abuse and any clinical conditions that cause obesity, such as steroid use or antipsychotic drug use. The weight and heights of adolescents were measured by the same nurse working in our outpatient clinic and body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. BMI-standard deviation (SD) score (SDS) and BMI percentiles were calculated using age and gender-specific norms published by the Centers for Disease Control and Prevention (14). To compare BMI across different ages, BMI Z-scores were also calculated. The Z-score represents the number of SD above or below the considered population mean value based on standardized tables for children. Obesity was defined as a BMI Z-score value above 2 SD for age and gender. Adolescents who had a BMI above the 95th percentile for age and gender or a BMI SDS above +2.0 SD were defined as obese and taken into the study group (15). All participants were requested to fill out a Strength and Difficulties Questionnaire (SDQ)-Adolescent Form and the socio-demographic form prepared by the researchers. EE was assessed using the Shortened Level of Expressed Emotion Scale (S-LEES), the Rosenberg Self-Esteem Scale (RSES) assessed self-esteem and SDQ.

Data Collection Materials

Socio-demographic Form

The "Socio-demographic Form" was created by the researchers. Data items collected included age, gender,

educational level of the parents, employment status of the parents, number of siblings, birth order and economic status. Income levels were determined based on the official starvation and poverty limits of 2015.

S-LEES in Adolescents

This was developed by Nelis et al (16) and was translated into Turkish by Vural et al (17) in 2013. S-LEES consists of 33 items measuring the EE of the person perceived to be most important in the participant's life over the previous three months. The three subscales of the S-LEES include Lack of Emotional Support (LES), irritability and intrusiveness. Higher scores indicate higher levels of EE. Nelis et al (16) reported Cronbach's alpha coefficients, a measure of reliability, of 0.88, 0.82 and 0.70 for the S-LEES, irritability and intrusiveness subscales, respectively. When the reliability scale was evaluated by Vural et al (17), Cronbach's alpha coefficient was found to be as high as 0.90. The forms were completed by the adolescents themselves. Items were answered on a Likert scale with four units ranging from "1 (not true)" to "4 (true)".

SDQ - Adolescent Form

SDQ was translated into Turkish by Güvenir et al (18). It was developed in 1997 by British psychiatrist Goodman (19) for screening behavioral and emotional problems in children and adolescents. The SDQ has 25 items evaluating both positive and negative emotions and behavioral features. These items are grouped into five sub-scales, each containing five items, according to both appropriate diagnostic measures and the results of factor analysis: ADHD, behavioral problems, emotional problems, peer relationship problems and social behaviors. In Turkey, the Cronbach's alpha for the SDQ was reported to be 0.73 by Güvenir et al (18).

RSES

The RSES was adopted to evaluate self-esteem in children and adolescents (20). This scale consists of 12 sub-tests, with the first ten articles aiming to evaluate self-esteem. The numerical levels of self-esteem are evaluated as: 0 to 1 points - high, 2 to 4 points- medium and 5 to 6 points-low. In our study, we used the first subtest to evaluate self-esteem. In Turkey, the validity and reliability of the scale was tested by Çuhadaroğlu (21).

Statistical Analysis

Data were evaluated using IBM Statistical Package for the Social Sciences Statistics version 22 program (22). The median and range were used for descriptive statistics of the groups. Comparison of the non-normally distributed variables and non-parametric parameters was performed using the Mann-Whitney U test. Comparison of the categorical variables of the groups was performed using the chi-square tests. The majority of data were not normally distributed. Therefore, data were analyzed using nonparametric analysis. The correlations were performed using Spearman's rho testing. A p-value of <0.05 was considered statistically significant.

Results

A total of 108 participants were initially recruited. However 12 subjects were excluded for the following reasons: two subjects with clinical intellectual disabilities, two with epilepsy, two who had had treatment with exogenous steroids and six who did not complete all of the questionnaires. Thus the study was conducted on 49 obese subjects and 47 healthy adolescents formed the control group.

The overall median age was 14 years. The median age of the obese group was 14 (minimum: 12-maximum: 17). 52.0% (n = 26) of the obese group were girls. The median age of the control group was 15 (minimum: 12-maximum: 17) and 56.0% (n = 28) were girls. The groups were similar in terms of age ($p = 0.175$) and gender ($p = 0.841$).

To compare socio-economic status (SES), the poverty line defined as an income of 250 dollars/month and the hunger line of 400 dollars/month, as defined by the Turkish Trade Union Confederation, were used (23). The groups were divided into high and low SES subgroups. In terms of family income, the difference was not statistically significant between the patient and control groups. The groups were also similar in terms of cohabitation of parents, mothers' and fathers' educational levels and living conditions ($p > 0.05$).

The perceived EE scores and the S-LEES scores of the patient and control groups are shown in Tables 1 and 2. There was a significant difference in the total perceived EE scores and also a significant difference in the subscales concerning irritability and intrusiveness between the two groups.

All cases were evaluated using the RSES. There was a statistically significant difference between the obese and control groups ($p < 0.001$) (Table 1).

The presence of psychopathology in the two groups was evaluated using SDQ. There was a statistically significant difference between the obese group and the control

group in the subscales of SDQ as follows: "emotional problems" ($p < 0.001$), "behavioral problems" ($p = 0.001$), "attention deficiency and hyperactivity" ($p < 0.001$) and "peer relationship problems" ($p < 0.001$). However, there was no significant difference in prosocial behaviors when comparing the two groups ($p = 0.077$) (Table 2).

Correlations between EE scores and RSES scores and SDQ scores in the obese group are shown in Table 3. A strong correlation was found between EE emotional problems ($p = 0.012$, $r = 0.36$), behavioural problems (BP) ($p = 0.107$, $r = 0.23$), and self-esteem ($p < 0.001$, $r = 0.56$) in the obese group (Table 3).

Table 1. Perceived expressed emotion scores in the obese and control groups

			Mean (IQR)	p value
Shortened Level of Expressed Emotion Scale	Total score	Obese group	110 (31)	< 0.001
		Control group	50 (17)	
	Lack of Emotional Support	Obese group	49 (15)	< 0.001
		Control group	23 (14)	
	Irritability	Obese group	41 (21)	< 0.001
		Control group	16 (5)	
	Intrusiveness	Obese group	21 (7.5)	< 0.001
		Control group	13 (6)	

IQR: interquartile range

Table 2. Rosenberg Self-Esteem scores and Strengths and Difficulties Questionnaire scores in the obese and the control groups

			Median (IQR)	p value
Rosenberg Self-Esteem Scale		Obese group	3.41 (2.08)	< 0.001
		Control group	0.75 (0.58)	
Strength and difficulties questionnaire	Emotional symptoms	Obese group	6 (3)	< 0.001
		Control group	2 (2)	
	Behavioral problems	Obese group	4 (3)	< 0.001
		Control group	2 (2)	
	Attention deficit-hyperactivity	Obese group	6 (2)	0.001
		Control group	5 (3)	
	Peer relationship problems	Obese group	6 (2)	< 0.001
		Control group	2 (2)	
	Prosocial behaviors	Obese group	8 (3)	0.77
		Control group	9 (3)	

IQR: interquartile range

Table 3. Correlation (Spearman rho) between expressed emotion and Rosenberg Self-Esteem or Strengths and Difficulties Questionnaire scores in the obese group

		EE total score	LES	Irritability	Intrusiveness
EP	p (r)	0.012 (0.36)	0.001 (0.47)	0.094 (0.24)	0.058 (0.27)
BP	p (r)	0.107 (0.23)	0.013 (0.35)	0.226 (0.18)	0.645 (0.07)
ADHD	p (r)	0.067 (0.26)	0.161 (0.20)	0.267 (0.16)	0.330 (0.14)
PRP	p (r)	0.930 (0.01)	0.832 (0.31)	0.185 (-0.19)	0.328 (0.14)
PB	p (r)	0.562 (-0.085)	0.063 (-0.27)	0.887 (0.021)	0.998 (< 0.001)
SDQ total score	p (r)	0.015 (0.345)	0.003 (0.42)	0.239 (0.17)	0.080 (0.25)
RSE total score	p (r)	< 0.001 (0.56)	< 0.001 (0.53)	0.079 (0.25)	0.001 (0.46)

EP: emotional problems, BP: behavioural problems, ADHD: attention deficit-hyperactivity disorder, PRP: peer relationship problems, PB: prosocial behaviours, SDQ: Strengths and Difficulties Questionaire, RSES: Rosenberg Self-Esteem Scale, EE: expressed emotion, LES: Lack of Emotional Support

Discussion

Although obesity is a common disorder, its social determinants and psychosocial consequences are still inadequately understood. Our results emphasize that family climate, as evaluated by EE in obese patients, is essential. Prevention and treatment of obesity are also necessary for the mental well-being of these adolescents. When the vulnerability and tendency to psychiatric disorders of obese adolescents is considered, it can be seen that there is a need for further studies regarding obese adolescents and their families in regard of the psycho-social interactions which occur in families. The findings of our study, showing significant differences in the intra-family psychosocial interactions when comparing obese and non-obese adolescents, suggests that this aspect merits further investigation and may help to improve management of interventions in the future.

In this study, we investigated perceived EE, psychopathology and self-esteem of obese youths by comparing them with their non-obese peers. A higher rate of perceived EE was observed in the obese group than in the control group. Also a higher rate of emotional and BP were observed in obese adolescents. According to our findings, the obese adolescents had significantly lower levels of self-esteem than the control group. Similarity of the study and control groups in terms of age and gender strengthens the validity of our results.

Studies have been conducted on interactions in the families of obese children (24). The families of obese children have been reported to be more angry and critical towards to their obese children (10). In studies of family functioning and obesity, it has been reported that families of obese children are more dysfunctional than those of their non-obese peers (11). In addition, inappropriate parental attitudes were found to be associated with increased risk for abnormal eating behaviour and obesity (25). EE is an empirical concept that was developed for evaluating family climate. When we examined the perceptions of adolescents regarding their families' EE, the obese adolescents perceived significantly lower levels of emotional support, higher irritability and greater intrusiveness. The studies investigating the parents of obese adolescents and EE also recommended taking EE into account while providing treatment for obesity (12).

In the literature dealing with obesity and psychopathology a strong association between emotional and BP, peer problems and obesity has been shown (26,27,28,29). A strong relationship between ADHD and obesity was also reported which is in keeping with our findings. There are studies underlining the role of abnormal eating pattern, possible genetic factors and sedentary life style of patients with ADHD and obesity (26,29). There are also studies reporting a causal role of ADHD in contributing to weight gain (29). According to a longitudinal study, anxiety and depression are more common in children and adolescents with obesity (27). Our results again support these findings in adolescents.

Self-esteem in obese adolescents was another issue investigated in our study. Lower self-esteem in obese adolescents has been reported in several studies (2,30,31). A strong relationship between body shame and vulnerability to eating problems has been reported and this increases the risk of low self-esteem and eating disorders among both obese and non-obese youngsters (32). A 4-year follow-up study by Strauss (30) with 1520 participants found that self-esteem was significantly lower in obese adolescents compared to non-obese adolescents, again in line with our findings. Our results are also consistent with previously published reports which show a higher ratio of psychiatric problems, such as depression, BP and low esteem, among obese adolescents when compared to non-obese adolescents.

In our study a strong association was observed between EE and emotional behavioral problems and self-esteem of obese adolescents. This has been previously reported. When we investigate the subscales of EE, the relationship between LES seems to be critical in the mental health of obese adolescents. Adolescents who perceived their parents as less emotionally supportive had more psychiatric problems and lower self-esteem. In addition intrusiveness of the parents was found to be associated with low self-esteem. In a six-month follow up study, it was reported that the decrease of BMI during follow-up was affected by the emotional response of the caregivers (7).

Study Limitations

The limitations of our study were the low number of the clinically obese patients who had consulted our outpatient clinic and failure to evaluate psychopathology in the parents. Also lack of follow-up can be considered a limitation of this study. It is debatable if the high EE is a risk factor for exhibiting obesity and mental problems in adolescents or a result of them. More follow-up studies are necessary to enhance our understanding of this field. The strengths of our study were evaluating EE using a self-report scale specially designed for adolescents and comparing the obese subjects with their healthy peers. Additionally, this study is the first controlled study to evaluate the relationship between perceived EE and obesity in adolescents.

Conclusion

To conclude, we can recommend that EE should be considered when planning treatment interventions to enhance compliance in obese adolescents.

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Ethics

Ethics Committee Approval: The study were approved by the Uludağ University Ethics Committee (date: 14.11.2014, No: 2014-19/5).

Informed Consent: All participants in the study and their parents gave informed consent after being informed of the methods and objectives of this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Merve Çolpan, Concept: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray, Design: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray, Data Collection or Processing: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray, Analysis or Interpretation: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray, Literature Search: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray, Writing: Merve Çolpan, Ayşe Pınar Vural, Erdal Eren, Şafak Eray.

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ORIGINAL ARTICLE

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Insulin Resistance as Related to Psychiatric Disorders in Obese Children

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What is already known on this topic?

Childhood adiposity has been reported to be related to mental health conditions such as depression, behavioral and emotional disorder, anxiety and mood disorder. BeLue and colleagues reported that adolescents who were obese were 1.6 times more likely to have depression or anxiety. Other research has demonstrated obese children are 3.1 times more likely to have anxiety symptoms and 3.6 times more likely to have depressive symptoms compared to their same-age peers.

What this study adds?

Our findings suggest that insulin resistance, rather than obesity-related metabolic comorbidities, is more predictive of psychiatric illness. The results of our study underline the importance of assessing psychiatric functioning in obese children, particularly those with insulin resistance. We recommend routine screening of these children for the identification of psychiatric disorders.

Abstract

Objective: The current study aimed to investigate psychiatric consequences of obesity and the relationship between componenets of the metabolic syndrome and psychiatric disorders in children. Our second aim was to elucidate which of the anthropometric parameters or metabolic components were most strongly associated with psychiatric disorders.

Methods: The study included 88 obese and overweight children with a body mass index (BMI) greater than 85th percentile. The patients were evaluated for psychiatric disorders by a single child and adolescent psychiatrist. Forty patients diagnosed with psychiatric disorders and 48 patients with normal psychiatric evaluation were compared in terms of anthropometric and metabolic parameters. BMI, BMI-standard deviation score and BMI percentile, waist circumference, waist to hip ratio, blood pressure and pubertal stage of all patients were recorded. Fasting serum glucose, insulin, lipid profile and homeostatic model assessments of insulin resistance (HOMA-IR) were measured to evaluate the metabolic parameters. Serum and 24 hour urine cortisol levels were measured.

Results: HOMA-IR in the group with psychiatric disorders was found to be significantly higher than in the group without psychiatric disorders (6.59 ± 3.36 vs 5.21 ± 2.67; $p = 0.035$). Other anthropometric measurements and metabolic parameters were not significantly different between the two groups.

Conclusion: An understanding of the relationships between obesity related medical comorbidities and psychiatric pathologies is important to encourage patients and their families to make successful healthy lifestyle changes and for weight management in terms of appropriate treatment.

Keywords: Child, obesity, insulin resistance, mental disorder

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Introduction

Childhood obesity is an important public health problem worldwide. The prevalence of obesity in children has risen dramatically in recent years. Childhood obesity has various and considerable adverse consequences for health outcomes (1).

There is an increasing recognition of the relationship between mental illness and obesity. Childhood overweight and obesity are reported to be more strongly associated with psychiatric comorbidities as compared to their healthy-weight peers, including those with a lower health-related quality of life, lower self-esteem and body image concerns (2,3). It is known that overweight and obese children are exposed to some difficulties in social life, such as pervasive peer victimization, weight-related teasing, weight stigma and bullying (4,5). These may complicate their physical and medical health outcomes (1,6).

Obesity related psychiatric comorbidities include a variety of psychiatric illness (2). In a study by Britz et al (7), more than 40% of the obese adolescents in their sample met Diagnostic and Statistical Manual of Mental Disorders, 4th edition DSM-4 criteria for a psychiatric illness. Increased lifetime rates for low mood (42.6%), anxiety (40.4%), substance use (36.2%), somatoform (14.9%) and eating (17.0%) disorders were reported in the obese group as compared with the general population. In a large population study sample from the United States, 43297 children aged between 10-17 years were evaluated; 15% of them were overweight and 16% were obese. In this study obese children compared with children classified as of normal weight were more likely to have internalizing and externalizing problems. Attention deficit and hyperactivity disorder (ADHD), conduct disorder, depression, learning disability and developmental delay were found to be more common in obese children (8).

It is still not clear whether psychiatric disorders and psychological problems are causes or consequences of childhood obesity or whether common factors promote both obesity and psychiatric disturbances in children and adolescents. The first aim of this study was to investigate psychiatric consequences of obesity and to evaluate the associations between childhood obesity related comorbidities and psychiatric disorders in children. The second aim was to identify which of the anthropometric or metabolic parameters related to obesity had an effect on mental health. To this end, we compared obese children with and without mental disorders to reveal differences in anthropometric and metabolic parameters.

Methods

This study was conducted in Manisa Celal Bayar University Pediatric Endocrinology and Child Psychiatry Clinics. A total of 88 obese and overweight children with a body mass index (BMI) value greater than the 85th percentile for age and sex, according to growth charts from the Center for Disease Control and Prevention (CDC-2000), aged nine to 17 years, who attended or were referred to our pediatric endocrinology outpatient clinic for evaluation of obesity and related comorbidities, were included in the study. We excluded individuals with developmental delays, chronic diseases, a history of drug use, a previous diagnosis of psychiatric disorders, those with any disease affecting the endocrine system (for example hypothyroidism or Cushing's disease) or suspected syndromes associated with obesity such as Prader-Willi and Laurence-Moon-Biedl syndromes.

The study was approved by the Local Ethics Committee of Celal Bayar University, Faculty of Medicine in Manisa (number/date: 20478486-382/11.11.2015), and written informed consent were taken from the primary caregiver and patient, before the study.

All overweight and obese patients underwent a thorough physical examination, laboratory evaluation and psychiatric assessment. The assessments were all performed by specially trained clinical research staff.

Height was measured in all subjects by a wall-mounted stadiometer and weight by a calibrated scale. All subjects were measured with no clothing other than undergarments. BMI was calculated as weight (in kilograms) divided by square of height (in metres). BMI-standard deviation (SD) score and BMI percentiles were calculated using age and gender specific norms published by the CDC (9). Obesity was defined as a BMI ≥95th percentile and overweight as a BMI ≥85th for age and sex (10).

Waist circumference (WC) was measured with a non-stretchable tape to the nearest 0.1 cm, midway between the lowest rib and the highest point of the iliac crest parallel to the floor, without clothing and during expiration in a standing and relaxed position (as recommended by the World Health Organization Expert Committee, 1995). Hip circumference (HC) was measured in centimetres around the widest portion of the buttocks. Waist-to-hip ratio was calculated by dividing the WC by the HC.

Findings for pubertal development were recorded according to the classification of Tanner and Whitehouse (11). A testicular volume of ≥4 mL in males, and a breast development of stage 2 and over in females, were considered to be findings consistent with puberty.

Blood samples were taken in the morning after 10 to 12 hours of night-time fasting (water permitted) for glucose, insulin and lipids including triglycerides (TG), total cholesterol and high-density lipoprotein (HDL), low-density lipoprotein cholesterol and serum cortisol measurements. A 24 hour urine sample was collected for cortisol measurement.

Insulin resistance was evaluated according to the homeostasis model assessment-insulin resistance index. Different cut-off values for prepubertal and pubertal stages were used to determine the status of insulin resistance (prepubertal >2.5, pubertal >4.0) (12).

Oral glucose tolerance test was performed after overnight fasting for 12-14 h. After glucose drink containing 1.75 g/kg glucose to a maximum of 75 g, blood samples were obtained every 30 min for 120 min for measurement of plasma glucose and insulin.

Blood pressure was taken with the appropriate cuff, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice in the supine position, after a ten minute rest, using the right arm and a calibrated sphygmomanometer and the mean of these two BP values were calculated.

According to the International Diabetes Federation (IDF), metabolic syndrome (MetS) can be diagnosed in children ten to 16 years old when the following criteria are fulfilled: a WC ≥90th percentile, together with two more risk factors defined as a fasting blood glucose level ≥100 mg/dL (5.6 mmol/L), a serum TG level ≥150 mg/dL (1.7 mmol/L) or being under treatment for elevated TG, HDL cholesterol <40 mg/dL (1.03 mmol/L) or being under treatment for low HDL and with a SBP ≥130 or DBP ≥85, or being under treatment for hypertension. For children 16 years and older, adult criteria can be used. Ethnic-specific WC percentiles for the Turkish population are ≥94 cm for men, ≥80 cm for women and a sex-specific cut off level for HDL are <40 mg/dL (1.03 mmol/L) in men or <50 mg/dL (1.29 mmol/L) in women. For children younger than ten years of age, MetS cannot be diagnosed, but vigilance is recommended if WC is ≥90th percentile (13).

Psychiatric measurements included the following:

1. Kiddie Schedule for Affective Disorders and Schizophrenia for School Age Children-Present and Lifetime Version was used (14). This is a semi-structured interview developed by Kaufman et al (14), to evaluate present and lifetime psychopathology in children and adolescents according to DSM-3-R and DSM-4 criteria. The reliability and validity study of the Turkish translation was conducted by Gökler et al (15). Psychiatric evaluation of obese patients was performed by the same clinician. The individuals were classified into two groups as follows: (i) obese group with normal psychiatric evaluation; (ii) obese group with psychiatric disorder.

2. A sociodemographic form was developed by the study coordinators, and included questions on parental education and vocation, physical/mental illnesses in the family and information about the patient.

Statistical Analysis

Statistical analysis of the study was performed using the Statistical Package for Social Sciences 15.0 program (IBM Inc., Chicago, Ill., USA). Descriptive data were presented as number ± SD, frequency and percentage values. Sociodemographic data, medical, anthropometric and physical measurements for cases with and without a psychiatric disorder were analysed using the chi-square test for categorical variables, t-test for those that were normally distributed and the Mann-Whitney U test for data that were not normally distributed.

Results

In this study, 88 obese children and adolescents were evaluated. The mean age of the participants was 13.20 ± 2.44 (range 9-17) years. Fifty-nine (67%) were female and 29 (33%) were male. The mean weight and height of the subjects were 73.94 ± 16.93 kg and 155.93 ± 11.56 cm, respectively. The number of subjects attending school was 84 (95.5%).

Psychiatric disorder was found in 40 (45.5%) of the children and five of them had multiple psychopathologies. These disorders consisted of anxiety disorders in 31 subjects (35.2%), depressive disorders in two (2.3%), oppositional defiant disorders in two (2.3%) and comorbid anxiety and depressive disorders in five subjects (5.7%).

Demographic, clinical and metabolic parameters were compared in children with and without mental disorder and the results are presented in Tables 1, 2 and 3. The group with mental disorders was not statistically different from the group without mental disorder in terms of age, sex, family history of psychiatric and chronic disorders, parents' employment status, pubertal status, degree of obesity and MetS components. Insulin resistance was significantly higher in children diagnosed with psychiatric disorder and school attendance was found to be significantly lower ($p = 0.035$).

Discussion

The worldwide rates for overweight and obesity in children has increased rapidly among all age groups and

Table 1. Demographic and clinical characteristics of obese patients with and without psychiatric disorders

Sociodemographic characteristics	Obese patients with psychiatric disorders (n = 40)	Obese patients without psychiatric disorders (n = 48)	p
Age (years)	13.7 ± 2.48	12.38 ± 2.36	0.083
Gender			
Male	11	18	0.368
Female	29	30	
Family history of psychiatric disorder			
Positive	15	10	0.100
Negative	25	38	
Family history of chronic disorder			
Positive	18	28	0.284
Negative	22	20	
Mother's occupation status			
Working	8	15	0.334
Unemployed	30	33	
Father's occupation status			
Working	31	46	0.073
Unemployed	6	2	
School attendance			
Positive	36	48	0.039
Negative	4	0	
Clinical characteristics			
Tanner stage			
Prepubertal (1)	3	1	0.326
Pubertal (2-5)	37	47	
Weight status			
Overweight	8	9	
Obesity	26	26	0.382
Morbid obesity	6	13	

Data about the occupation status of the parents is missing in some families (2 related to the mother/3 related to the father)

Table 2. Metabolic characteristics of obese patients with and without psychiatric disorders

Metabolic syndrome components	Obese patients with psychiatric disorders (n = 40)	Obese patients without psychiatric disorders (n = 48)	p
Waist circumference (cm)	99.10 ± 11.20	94.67 ± 10.20	0.056
Increased WC - n (%)	40 (100%)	47 (97.9%)	0.361
Fasting blood glucose (mg/dL) (mean ± SD)	84.07 ± 7.68	86.75 ± 7.52	0.104
High fasting blood glucose (≥100 mg/dL) - n (%)	1 (2.5%)	3 (6.3%)	0.623
Triglycerides (mg/dL) (mean ± SD)	135.37 ± 70.61	139.34 ± 118.80	0.853
High triglycerides (≥150 mg/dL) - n (%)	11 (27.5%)	11 (22.9%)	0.631
HDL cholesterol (mg/dL) (mean ± SD)	47.12 ± 7.86	46.77 ± 8.64	0.843
Low HDL cholesterol (< 40 mg/dL) - n (%)	6 (15%)	12 (25%)	0.296
SBP (mmHg) (mean ± SD)	120.66 ± 11.17	115.77 ± 13.30	0.070
High SBP ≥130 - n (%)	10 (25.0%)	8 (16.7%)	0.425
Diastolic blood pressure (mmHg) (mean ± SD)	77.89 ± 10.95	75.20 ± 11.52	0.272
High diastolic blood pressure - (DBP ≥85) - n (%)	9 (22.5%)	7 (14.6%)	0.406

WC: waist circumference, SD: standard deviation, HDL: high-density lipoprotein, SBP: systolic blood pressure, DBP: diastolic blood pressure

Table 3. Metabolic syndrome related components in obese patients with and without psychiatric disorders (mean ± standard deviation values are given)

Metabolic syndrome related components	Obese patients with psychiatric disorders (n = 40)	Obese patients without psychiatric disorders (n = 48)	p
BMI (kg/m^2)	30.74 ± 4. 51	29.41 ± 4.50	0.173
BMI SDS	2.02 ± 0.37	2.00 ± 0.38	0.849
Hip circumference (cm)	108.63 ± 11.38	105.67 ± 10.29	0.225
Waist/hip ratio	0.91 ± 0.062	0.89 ± 0.051	0.186
Total cholesterol (mg/dL)	162.40 ± 30.29	161.20 ± 31.75	0.858
LDL cholesterol (mg/dL)	86.09 ± 28.45	41.74 ± 22.72	0.303
Two-hour 75-g OGTT glucose (mg/dL)	123.35 ± 23.21	123.45 ± 24.15	0.983
Insulin (mU/L)	29.46 ± 13.23	24.31 ± 11.79	0.057
HOMA-IR	**6.59 ± 3.36**	**5.21 ± 2.67**	**0.035**
Cortisol	11.34 ± 5.01	11.98 ± 5.30	0.569
24-hour urine cortisol (µg/24 h)	119.07 ± 76.08	104.63 ± 49.53	0.290

BMI: body mass index, SDS: standard deviation score, OGTT: oral glucose tolerance test, LDL: low-density lipoprotein, HOMA-IR: homeostatic model assessments of insulin resistance

both sexes over the past few decades (16). Childhood obesity is associated with several short- and long-term consequences (cardiovascular diseases, hypertension, hypercholesterolemia, insulin-resistance, type 2 diabetes, pulmonary and liver disease in addition to mental disorders) (6,17,18,19,20).

In this study, we evaluated psychiatric disorders in children who were obese and overweight, and compared the anthropometric and biochemical data in individuals with and without psychiatric impairment. We also assessed whether there is a metabolic or anthropometric difference that may be related to psychopathology among obese children and investigated the association of psychiatric disorders with the MetS, diagnosed according to the criteria of the IDF.

In our study, a psychiatric disorder was detected in 40 out of 88 obese and overweight patients (45.45%). We consider that this is a very high percentage of patients who are diagnosed during screening. Anxiety disorder was found as the most common psychiatric disorder among our study sample. According to the data obtained from a review of nine previous studies about the relationship between childhood adiposity and mental health (1), our study findings were consistent in relating childhood adiposity with mental health conditions such as depression, behavioral and emotional disorder, anxiety and mood disorder. BeLue et al (21) reported that adolescents who were obese were 1.6 times more likely to have depression or anxiety. Pervanidou et al (22) demonstrated that obese children are 3.1 times more likely to have anxiety symptoms and 3.6 times more likely to have depressive symptoms compared to same-age peers. In a study by Fox et al (23), 102 adolescents were evaluated and in the overall sample, 34% had symptoms consistent with depression and 32% symptoms of anxiety. Similar results have been found in studies from Turkey. In the study by Taner et al (24), 54 obese children were evaluated and psychopathology was detected in 50% of these children. In the study of Topçu et al (25), there were significant differences among obese and control groups in terms of the total score of state-trait anxiety inventory-C and child depression inventory. Our study results are consistent with studies conducted in both Turkey and other countries.

There are many articles evaluating the relationship between ADHD and obesity in the literature. In the study of Erermis et al (26) obese cases admitted to the endocrine clinic were evaluated and 13.3% of them were diagnosed with ADHD. Cortese et al (27) analyzed 42 studies including a total of 48,161 ADHD and 679,975 control subjects and found a significant relationship between obesity and ADHD in children. In another review, Cortese and Tessari (28) presented seven studies evaluating the prevalence of ADHD in individuals referred for obesity treatment. All these studies, except one, have confirmed significantly higher rates of ADHD in individuals with obesity compared to normal weight controls. Conversely, there were no cases diagnosed with ADHD in our study. In children, ADHD affects academic achievement and social adjustment negatively in the school setting, and is mostly recognized and diagnosed during the primary school period. In this present study, a known mental illness or drug use were among the exclusion criteria and the mean age of the children was 13.2. Thus most of them were of the secondary school age. In this age

group, children have high rates of previous ADHD diagnosis and were therefore presumably excluded from this study which explains why we did not find any ADHD diagnosis in the study group.

It has been reported that when obesity is accompanied by a psychiatric disorder, the children become disoriented to obesity treatment along with a decrease in their school performance. Also, their body sense becomes more negative and their quality of life more distorted (24,29). With the diagnosis and treatment of the existing psychiatric disorder and improved self-esteem or improvement in other factors associated with mental health, obese individuals may be more successful in increasing their motivation (30). The present study emphasizes the importance of mental health assessment prior to treatment in order not to miss diagnoses that may affect the outcome of the treatment. Establishment of multidisciplinary teams and psychiatric evaluation are important in the effective treatment of obesity.

The relationship between obesity and mental disorder has not yet been clearly elucidated and questions such as which one triggers the other or *vice versa* or whether they co-occur remain to be clarified. The association of psychiatric disorders such as depression and anxiety disorders with MetS are relatively well-documented in adults (31). There are only limited data on the nature of the association between obesity related MetS and other comorbidities and psychiatric disorders in children.

A number of studies have documented the association of depressive symptoms or disorders with the MetS (32,33,34,35). It was reported that the MetS in childhood predicted higher levels of depressive symptoms in adulthood (36). The association of the MetS with anxiety disorder has received significantly less attention and the results of the studies on this issue are controversial. Some authors have reported more severe anxiety symptoms and more frequent anxiety disorders in MetS patients, while other researchers have not confirmed this association (37,38,39,40).

Phillips and Perry (31) compared depression and anxiety symptoms among metabolically healthy and unhealthy obese and non-obese individuals. The risk of anxiety and depressive symptoms were found to be greater among the metabolically unhealthy, obese subjects than the metabolically healthy, non-obese individuals. Increased risk for these conditions was not observed among the metabolically healthy obese subjects. Hamer et al (41), investigated whether the association between obesity and depressive symptoms is dependent on an individual's metabolic health and report that the metabolically unhealthy obese had increased risk of depressive symptoms after a two year follow-up but that this relationship was not found in metabolically healthy, obese individuals. Furthermore, the data obtained from a recent analysis of eight studies by Jokela et al (42), demonstrate that obese individuals with a favorable metabolic profile have a slightly increased risk of depressive symptoms compared with non-obese, but the risk is greater when obesity is combined with an adverse metabolic profile. Phillips and Perry (31) investigated associations between the metabolic risk factors and depressive symptoms and anxiety among metabolic unhealty, obese subjects. Insulin resistance and abdominal obesity were associated with depressive symptoms, only insulin resistance remained significant in adjusted models for both depressive symptoms and anxiety.

We expected that accompanying obesity-related comorbidities or MetS components, rather than obesity alone, would relate to impaired psychiatric functioning and greater psychiatric distress. In view of the MetS and related components, we found that in our sample children with psychiatric disorders had higher measures of insulin resistance. We know that, obesity related cardiometabolic comorbidities are less common in children and tend to occur later in life. Insulin resistance, which is the most common early onset obesity-related comorbidity, is significantly associated with an increase in the frequency of mental disorders, even when other metabolic changes have not yet begun. We believe that this is an important outcome for this study.

Obesity and mental disorders share some behavioral factors and adverse dietary habits but are also related to different stress systems such as disturbances in the hypothalamic-pituitary-adrenal axis and dysregulation of the central serotonin, norepinephrine and dopamine neurotransmitter systems which may contribute to changes in body composition and metabolic parameters (43,44,45,46,47). Endocrinologic abnormalities may play a role in the association between psychiatric disorders and insulin resistance. It has been shown that fluoxetine, improves insulin-mediated glucose utilization independent of its effect on body weight (48). These findings indicate that the serotonergic system plays a role in the pathogenesis of both mental disorders and insulin resistance and may have a role linking these two pathogeneses. In the current study, our findings suggest that psychiatric disorders may affect peripheral insulin sensitivity, possibly via behavioral and/or neuroendocrinologic pathways.

Comorbid psychiatric disorders and related lifestyle factors affect the development of insulin resistance in obese patients. It may be predicted that adequate treatment of psychiatric disorders resulting in improvement of

psychopathology related factors, such as an increase in daily physical activity, improvement in sleep disturbances, or changes in eating behavior will improve the insulin resistance. Thus, early diagnosis and adequate treatment of an underlying psychiatric disorder in obese children are very important for the improvement of impaired insulin sensitivity and may serve to decrease the risk of developing diabetes, hypertension and cardio-vascular disease in these subjects.

Study Limitations

This study has some limitations. Firstly, it is unclear from this study whether obesity worsens psychosocial factors or psychosocial factors worsen obesity. There may also be other factors affecting these relationships, such as personal characteristics of the patient, duration of the psychiatric illness and family stress factors which have not been analyzed in the present study. Secondly, the severity of the mental disorders was not assessed in this study. Longitudinal data are needed to understand the nature of the relationship between obesity and mental disorders, as well as to document any changes in psychosocial functioning with reduction in BMI.

Conclusion

In conclusion, our findings suggest that insulin resistance, rather than obesity-related metabolic comorbidities, is more predictive of psychiatric illness in younger obese patients. The results of our study underline the importance of assessing psychiatric functioning among obese children, particularly those with insulin resistance. Routine screening of these children is recommended for the identification of psychiatric disorders and the identification of patients who require clinical intervention. In the absence of such information, it is unlikely that lifestyle recommendations will be successful in weight management of obese patients. Also, screening for presence of the MetS in those with psychiatric disorder may help to reduce the risk of developing cardiovascular disease and type 2 diabetes mellitus.

Ethics

Ethics Committee Approval: The study was approved by the Local Ethics Committee of Celal Bayar University, Faculty of Medicine in Manisa (number/date: 20478486-382/11.11.2015).

Informed Consent: Written informed consent were taken from the primary caregiver and patient, before the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Deniz Özalp Kızılay, Betül Ersoy, Şermin Yalın Sapmaz, Concept: Deniz Özalp Kızılay, Semra Şen, Şermin Yalın Sapmaz, Design: Deniz Özalp Kızılay, Semra Şen, Şermin Yalın Sapmaz, Data Collection or Processing: Deniz Özalp Kızılay, Semra Şen, Yekta Özkan, Şermin Yalın Sapmaz, Analysis or Interpretation: Deniz Özalp Kızılay, Şermin Yalın Sapmaz, Literature Search: Deniz Özalp Kızılay, Şermin Yalın Sapmaz, Writing: Deniz Özalp Kızılay, Şermin Yalın Sapmaz.

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SHORT COMMUNICATION

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Evaluation of Efficacy of Long-term Growth Hormone Therapy in Patients with Hypochondroplasia

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What is already known on this topic?

Children with hypochondroplasia were given recombinant human growth hormone (GH) treatment in a limited number of studies. In these studies, treatment doses of GH and height gain were not uniform.

What this study adds?

This study presents the results of long term follow up of patients with hypochondroplasia by clinical, radiological and genetic examination and evaluates their response to growth hormone (GH) treatment. The study shows that high-dose GH therapy may be required for patients with skeletal dysplasia.

Abstract

Hypochondroplasia is a cause of disproportionate short stature and characterized by minor clinical manifestations. The aim of this study was to evaluate the efficacy of long-term growth hormone (GH) therapy in hypochondroplastic cases with inadequate response to GH stimulation tests. In this study, six patients who had a height standard deviation score of -3.43 before the treatment and a mean age of 7.42 years and who had received GH treatment at a dose of 0.2 mg/kg/week for a mean period of 4.45 years were evaluated. A good response was found in the first year of treatment, but this increase was not found to be sufficient for the patients to achieve an adequate final height.

Keywords: Hypochondroplasia, growth hormone therapy, skeletal dysplasia

Introduction

Hypochondroplasia is a common skeletal dysplasia that has an autosomal dominant inheritance and clinical manifestations of which can become evident over time. The patients may present with disproportionate short stature, macrocephaly, lumbar lordosis, rhizomelic and mesomelic shortness and brachydactyly (1,2). The patients may appear as normal or almost normal in early childhood and the clinical manifestations may not be evident until puberty. Final height has been reported to be 146 ± 4.9 cm for males and 137.6 ± 6.3 cm for females (3). Diagnostic radiological findings include decreased interpedicular distance between L1 and L5 and short lumbar pedicles (4). Hypochondroplasia is frequently due to the *FGFR3* mutation located at 4p16,3. However, mutations may not be observed in all hypochondroplasia patients. Children with hypochondroplasia were given recombinant human growth hormone (rhGH) treatment in a limited number of studies (5). In these studies, treatment doses of GH and height gain were not uniform. Very few data pertaining to the GH-insulin-like growth factor-1 (IGF-1) axis has been mentioned in the reported studies and very few studies have reported final height results following GH treatment.

In this study, we aimed to evaluate response to GH treatment in patients who had a definite diagnosis of hypochondroplasia by clinical, radiological and genetic examination, who also met the criteria for growth hormone deficiency and who received rhGH replacement therapy.

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Methods

In this study, we evaluated the growth of patients with hypochondroplasia who were followed in our clinic between the years 2000 and 2017 and who showed an inadequate response to growth hormone stimulation tests. A Harpenden stadiometer (Holtain Ltd, Crymych, Dyfed) was used in height measurements. Pubertal staging was done according to Tanner/Marshall criteria and bone ages were evaluated using the Greulich and Pyle (6) bone age atlas (7,8). The diagnosis of hypochondroplasia was made by clinical anthropometric evaluation (height, sitting height, upper/lower segment ratio etc.), presence of specific radiological findings (decreased interpedicular distance in the vertebrae, a short square iliac bone, extension in distal fibula) and demonstration of *FGFR3* gene mutation when possible. Presence of any affected parents in the family history supported the diagnosis. In all cases rhGH treatment was given at a dose of 0.2 mg/kg/week. Serum IGF-1 and IGF binding protein-3 concentrations, pubertal progression, change in upper/lower segment ratio, bone age progression rate and the achieved final height were assessed in the follow-up of the cases. All participants' families gave informed consent and the study protocol was approwed by Ankara University Ethic Committe (approval number: 15-638-15).

Results

Six cases (1 male/6 females) with an initial mean age of 7.08 ± 3.2 years and one in puberty were included in the study. Before the treatment, mean annual height velocity of the patients was 3.96 ± 0.83 cm, height standard deviation (SD) score (SDS) was -3.86 ± 0.75, bone age was 4.84 ± 2.97 years, upper segment/lower segment ratio was 1.34 ± 0.2, and specific radiographic findings were present in all cases (Table 1). In the first year of growth hormone therapy, height velocity ranged between 6.9 and 10 cm (mean 8.4 ± 1.29 cm) and it decreased to a mean of 5.5 ± 1.76 cm in the third year and to 5.3 ± 1.8 cm in the fourth year. In the first year of treatment, mean height SDS was -3.35 ± 0.78, and Δ height SDS of + 0.5 was found to decrease gradually in the following years. In the last follow-up of the cases when they had been on growth hormone therapy for a mean period of 4.45 ± 1.3 years; their mean age was 12.48 ± 3.19 years, mean height SDS -3.2 ± 1.2 Δ height SDS 0.66 ± 1.2 bone age 12.9 ± 3.5 years and IGF-1 SD 1.34. Four of the patients were pubertal, with a mean puberty onset age of 10.66 ± 1 years and achieved their final height. In these four patients, final height SDS was -3.57 ± 1, Δ height SDS was + 0.26 ± 1.19. The upper/lower segment ratio did not change significantly and there was no increase in disproportionality. During treatment, IGF-1 levels did not exceed + 2 SDS and remained within the confidence interval (Table 2).

Table 1. Features of the patients at presentation

Features	Findings
Gender	5 females / 1 male
Chronological age in years, mean (range) ± SD	7.8 (5.1-10.44) ± 3.2
Height SDS mean (range) ± SD	-3.86 (-2.66-4.67) ± 0.75
BA (years) mean (range) ± SD	4.84 (2-8.8) ± 2.97
Upper/lower segment ratio, mean (range) ± SD	1.34 (1.2-1.6) ± 0.2
Affected parent	Mother - 1 patient Father - 4 patients No affectionà 1 patient
FGFR3 mutation	1 patient 1612 A > G (p.IIE538Val) heterozygous Mutation
Pretreatment height velocity (cm) mean ± SD	3.96 cm ± 0.83
THSDS mean (range) ± SD	-2.39 (between -0.25 and -3.79) ± 1.09
IGF-1 SD mean (range) ± SD	-1.49 (-0.32 and -2.73) ± 0.74
IGFBP3 SD mean (range) ± SD	-2.09 (1.08 and -5.7) ± 2.1
GH deficiency - partial/complete	Partial GH deficiency 3 patients Complete GH deficiency 3 patients

SD: standard deviation, GH: growth hormone, SDS: standard deviation score, IGF-1: insulin-like growth factor-1

Table 2. Characteristics of patients at cessation of treatment

Gender	**4 females**
Chronological age in years, median (range) ± SD	13.71 (12.62-14.93)
Bone age in years median (range)	14.00 (14-15.5)
Upper/lower segment ratio, median (range)	1.36 (1.56-1.28)
GH therapy duration (years) median (range)	4.50 (3.25-6)
Total increase in height (cm) median (range)	26.5 (20.4-42)
Height SDS median (range)	-3.49 (-2.4 and -4.6)
Delta height SDS median (range)	0.21 (-0.91 and 1.77)
IGF-1 SD median (range)	0.36 (-0.67 and 5.34)
Height median (range)	136.47 (129.5-144)
Predicted adult height/final height	1. patient: -/129.5 cm 2. patient: 140.5 cm/144 cm 3. patient: 146.5 cm/142 cm 4. patient: 126.7 cm/132.6 cm

SD: standard deviation, GH: growth hormone, SDS: standard deviation score, IGF-1: insulin-like growth factor-1

Discussion

The literature data on the use of growth hormone in skeletal dysplasias are scarce except for in achondroplasia, and usually short-term treatment results have been reported (9). Due to the genetic heterogeneity and phenotypic diversity of the diagnostic features, different conclusions regarding treatment efficacy were reported. Activating mutations of *FGFR3*, which have an effect on the negative regulation of cartilage growth are encountered in hypochondroplasia and achondroplasia (10). The N540K mutation is also seen in hypochondroplasia and is associated with more severe shortness and disproportion. However, mutations are not observed in all hypochondroplasia patients (11,12). In one of our cases, 1612 A > G (p.Ile538Val) heterozygous mutation in the *FGFR3* gene was detected.

In children with achondroplasia, with growth hormone treatment, a transient increase in height velocity without any effects on final height is observed, and consequently, the use of routine recombinant growth hormone in achondroplasia is not recommended (13). In children with hypochondroplasia, there is no placebo-controlled study on the effects of growth hormone treatment on adult height. Previous studies have shown that the use of growth hormone improves the increase in rate of height velocity in children with hypochondroplasia, especially at puberty (14,15). In a study by Pinto et al (16) in 2014, 40 patients were followed up without treatment until they achieved their final height, and these patients were compared with 19 patients with hypochondroplasia who were given 0.057 mg/kg/day growth hormone. In the growth hormone treated group the height increase rate of 5.1 ± 0.3 cm/year at the beginning of treatment, reached 8.1 ± 1.9 cm/year in the first year, 6.2 ± 1.7 cm/year in the second and 4.8 ± 2.2 cm/year in the third year. Similar to other reports, the best response was noted in the first year of treatment and decreased in the following years.

In a meta-analysis published in 2015, effects of growth hormone therapy was evaluated in hypochondroplastic cases. A total of seven publications and 113 patients with rhGH treatment were included in this meta-analysis. Among these patients, 59.7% were male and most of them were pre-pubertal at the beginning of the treatment. The average growth hormone dose was 0.25 mg/kg/week. In all studies, adult height which was predicted at the beginning of the treatment was below the normal level and an increase in adult height after rhGH treatment over predicted final height was reported. In the meta-analysis of these seven studies, in the median post-treatment predicted adult height SDS, there was an increase of 0.414 for the first year, 0.530 for the second year and 0.609 for the third year. This increase was especially notable in the first year of treatment and continued to decline thereafter. It was observed that the highest rate of height increase under treatment was the first year, suggesting that the effect of treatment on adult height was related to the height achieved for the first year. In our study, at the end of the first year of growth hormone treatment, delta height SDS was + 0.51, suggesting that the response to growth hormone treatment was good. However, growth rates in subsequent years of follow-up decreased. IGF-1 levels remained within the confidence interval during treatment. In total, Δ height SDS remained at 0.21 and was interpreted as an insufficient response.

During the follow-up, we found that puberty started in the expected normal age range and that pubertal findings did not accelerate with GH treatment. Progression of bone age with GH therapy, which was reported in some skeletal dysplasia cases, is a finding that is important because of the effect on final height (17). In our cases, Δ bone age was found to be 6.6 years at the end of the treatment and the fact that there was not a significant increase was attributed to the accompanying GH deficiency.

An average of 28.5 cm height increase due to the treatment was observed in the four patients achieving their final height. After treatment, Δ height SDS was found to be +0.2. Upper/lower segment ratios during growth hormone therapy did not significantly worsen and no side effects were observed. The lack of change in disproportionality in patients with hypochondroplasia after GH therapy is a finding that suggests that the treatment does not have side effects. In patients with hypochondroplasia and GH deficiency, although rhGH therapy appeared to be effective, no improvement in final height was noted with the dose of 0.2 mg/kg/week.

In conclusion, this study showed that in GH deficient patients with hypochondroplasia, a standard dose of GH treatment was found to be sufficient to reach an increase in height just above 0.5 SD in the first year, which is acceptable, but not sufficient for the patients to achieve a sufficient increment in their final height. This finding supports the idea that high-dose GH therapy may be required for skeletal dysplasia, especially after the first year of rhGH therapy. Normal progression of puberty with a standard dose of GH therapy, lack of acceleration of bone age and skeletal disproportion and IGF-1 level remaining within the normal range are consistent with the idea that standard dose GH therapy is safe.

Ethics

Ethics Committee Approval: The study protocol was approwed by Ankara University Ethic Committe (approval number: 15-638-15).

Informed Consent: All participants provided a informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Merih Berberoğlu, Design: Merih Berberoğlu, Zeynep Şıklar, Data Collection or Processing: Tuğba Çetin, Zeynep Şıklar, Pınar Kocaay, Merih Berberoğlu, Analysis or Interpretation: Merih Berberoğlu, Zeynep Şıklar, Tuğba Çetin, Literature Search: Tuğba Çetin, Zeynep Şıklar, Merih Berberoğlu, Writing: Tuğba Çetin, Zeynep Şıklar, Merih Berberoğlu.

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CASE REPORT

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Aromatase Deficiency due to a Novel Mutation in *CYP19A1* Gene

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What is already known on this topic?

Aromatase deficiency is an autosomal recessive genetic disorder that is rarely reported in the literature. Aromatase enzyme converts androgens into estrogen in many tissues. Aromatase deficiency causes ambiguous genitalia in the female fetus and maternal virilization during the pregnancy due to increased concentration of androgens. Ovaries are usually large and polycystic in girls with aromatase deficiency.

What this study adds?

We identified a novel mutation in the *CYP19A1* gene in a patient who presented with ambiguous genitalia and maternal virilization during pregnancy. In our patient, the ovaries were hypoplasic despite increased gonadotropin levels

Abstract

Aromatase deficiency is a rare autosomal recessive genetic disorder with an unknown incidence. Aromatase converts androgens into estrogen in the gonadal and extra-gonadal tissues. Aromatase deficiency causes ambiguous genitalia in the female fetus and maternal virilization (hirsutism, acne, cliteromegaly, deep voice) during pregnancy due to increased concentration of androgens. A 19 months old girl patient was assessed due to presence of ambiguous genitalia. There were findings of maternal virilization during pregnancy. The karyotype was 46,XX. Congenital adrenal hyperplasia was not considered since adrenocorticotropic hormone, cortisol, and 17-hydroxyprogesterone levels were within normal ranges. At age two months, follicle-stimulating hormone and total testosterone levels were elevated and estradiol level was low. Based on these findings, aromatase deficiency was suspected. A novel homozygous mutation IVS7-2A > G (c.744-2A > G) was identified in the *CYP19A1* gene. Pelvic ultrasound showed hypoplasic ovaries rather than large and cystic ovaries. We identified a novel mutation in the *CYP19A1* gene in a patient who presented with ambiguous genitalia and maternal virilization during pregnancy. Presence of large and cystic ovaries is not essential in aromatase deficiency.
Keywords: Aromatase deficiency, *CYP19A1* gene, maternal virilization, ambiguous genitalia

Introduction

Aromatase is a member of the cytochrome P450 superfamily and encoded by the *CYP19A1* gene located on chromosome 15q21.1 (1). It is the key enzyme for estrogen biosynthesis in all vertebrates. *CYP19A1* gene and aromatase are expressed in numerous tissues including ovaries, testes, placenta, adipose tissue, skin and brain. Aromatase catalyzes the three precursors including androstenedione, testosterone and 16-α-hydroxy dehydroepiandrosterone sulfate (after conversion to 16-α-hydroxyandrostenedione) into estrone, estradiol and estriol, respectively (1,2,3). Aromatase deficiency leads to increased androgen levels both in the mother and the fetus. Aromatase deficiency causes specific signs of maternal virilization including cystic acne, hirsutism, cliteromegaly and deep voice while resulting in significant masculinization in the external genitalia of the female fetus (4).

In this study, we present a case with a novel homozygous IVS7-2A > G (c.744-2A > G) mutation in the *CYP19A1* gene

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causing significant virilization both in the mother and the female fetus.

Case Report

The patient was born at term at another centre via spontaneous vaginal delivery with a birth weight of 3500 g. The parents were first-degree cousins. Ambiguous genitalia were recognized at birth. Signs of maternal virilization (hirsutism, acne, cliteromgaly, deep voice) were noted at approximately 20 weeks of gestation. The patient represented at age fifteen days. Congenital adrenal hyperplasia was not considered since adrenocorticotropic hormone, cortisol, and 17-hydroxyprogesterone levels were within normal ranges. The other parameters were as follows: follicle-stimulating hormone (FSH) 66 mIU/mL (0.24-14.2), luteinising hormone (LH) 9.7 mIU/mL (0.02-7.0), total testosterone 0.9 ng/mL (0.2-0.64), estradiol 5 pg/mL (< 15). The karyotype was 46,XX and pelvic ultrasonography revealed the uterus dimensions as 5x8x13 mm (normal range 33.1 ± 4.1 mm for uterus long axis), those of the right ovary as 5x3x3 mm (0.02 mL; normal range 0.2-0.9 mL), and those of the left ovary as 5x3x3 mm (0.02 mL; normal range 0.2-0.9 mL).

At presentation to our centre at 19 months of age she had ambiguous genitalia. The patient had a body weight of 11 kg [standard deviation (SD) score + 0.07] and a length of 82 cm (SD score + 0.48). Genital examination showed bilateral impalpable gonads, a penis-like phallus of 1.5 cm, single penoscrotal urethral opening, and labioscrotal fusion defect (Prader stage 4). Hormonal analyses were unremarkable except for a significantly elevated FSH level (Table 1). Aromatase deficiency was considered due to the presence of maternal virilization, detection of hypergonadotropic hypogonadism during mini-puberty and low estradiol levels despite elevated total testosterone levels. *CYP19A1* gene mutation analysis was performed by sequencing the coding exons and the exon-intron boundaries of the genes. Genomic DNA was isolated from peripheral blood cells with QIAGEN DNA Blood Midi Kit according to the manufacturer's protocol. To amplify the exons of the *CYP19A1* gene, primers were used as listed in Table 2. Sequencing was performed with MiSeq V2 chemistry on a MiSeq instrument (Illumina California, USA) and the analysis was performed with IGV software. A novel homozygous [IVS7-2A > G (c.744-2A > G)] mutation was found in the

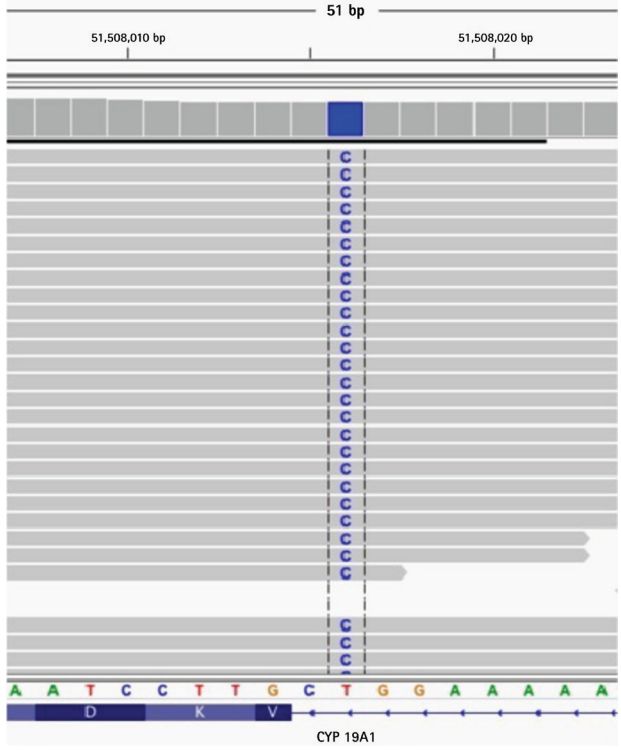

Figure 1. Homozygous mutation IVS7-2A > G (c.744-2A > G) in intron 7 of the *CYP19A1* gene

Table 1. Hormone levels of the patient at different age time points

	15 days	70 days	19 months
FSH (mIU/mL)	66 (0.24-14.2)	52.4 (0.24-14.2)	110 (1.0-4.2)
LH (mIU/mL)	9.7 (0.02-7.0)	4.9 (0.02-7.0)	13.7 (0.02-0.3)
Testosterone (ng/mL)	0.9 (0.2-0.64)	0.39 (< 0.1)	0.03 (< 0.03-0.01)
Estradiol (pg/mL)	< 5 (< 15)	< 5 (5-50)	< 5 (5-20)
Androstenedione (ng/mL)			0.2 (0.08-0.5)
17-OH progesterone (ng/mL)		1.67 (0.4-2.0)	0.73 (0.03-0.9)
Cortisol (mcg/dL)		22.3 (2.8-23.0)	18.0 (3.0-21.0)
ACTH (pg/mL)		28.7 (10.0-60.0)	42.0 (10.0-60.0)
DHEA-S (ug/dL)		61 (5-111)	
Progesterone (ng/mL)		0.27 (0.07-0.52)	0.2 (0.07-0.52)

FSH: follicle-stimulating hormone, LH: luteinizing hormone, 17-OH: 17-hydroxy, ACTH: adrenocorticotropic hormone, DHEA-S: sulfated dehydroepiandrosterone

CYP19A1 gene (Figure 1). To our knowledge, this mutation has not been previously reported. The mutation was interpreted as a "disease-causing" mutation by the MutationTaster and Splice Site Finder modeling programs. The parents were heterozygous carriers for the same mutation (Figure 2).

Informed consent was obtained from the parents for publication of the case.

Discussion

Aromatase deficiency is a rare disease caused by *CYP19A1* gene mutation and characterized by a decrease in estrogen synthesis. Aromatase deficiency is an autosomal recessive disorder and was first described by Shozu et al (5). To date, a total of 36 cases from various ethnic origins have been reported in the literature (1,2,6,7,8,9,10,11,12,13,14). In patients with aromatase deficiency, more than 30 distinct mutations have been identified in the *CYP19A1* gene, including missense, nonsense, small deletions and insertions, splice-site mutations, and one large intragenic deletion (1,2,6,7,8,9,10,11,12,13,14,15,16). Most of these mutations have been found to be located in exon 9 and 10 (9). The mutations identified in cases from Turkey have been reported in different exons (exon 5, 10, 11) (16,17,18). In our patient, the mutation was located in intron 7 of the *CYP19A1* gene.

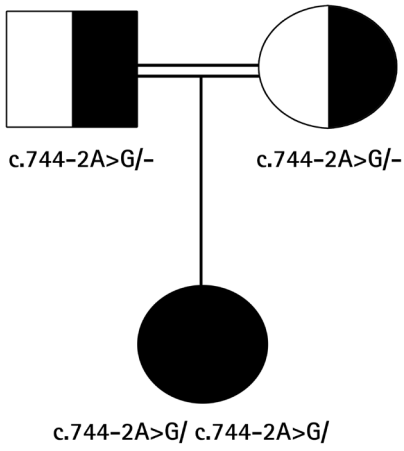

Figure 2. Pedigree of the patient's family. Solid black symbols depict affected individuals; half-filled symbols represent heterozygous carriers

Table 2. Primers used for sequencing the coding region of the *CYP19A1* gene

CYP19A1-2F	TCTGAAGCAACAGGAGCTAT
CYP19A1-2R	CAGAGATCTTTCCAGGTTTG
CYP19A1-3F	GAAGTGAAGAGCCTCATGTT
CYP19A1-3R	TGTTAGATTTCTGGGGATTG
CYP19A1-4F	CAACATGCATTTGCTAAGAG
CYP19A1-4R	CTGGGTGATAGAGTCAGAGC
CYP19A1-5F	TTAGGAGACCACAGAAAAGC
CYP19A1-5R	GCAGAAACACTAGGGAAAAA
CYP19A1-6F	GAAGATGGAATCTTGCTGAG
CYP19A1-6R	TTAATCAACAGCTCCCTTGT
CYP19A1-7F	CACTTACTCATAAGCACCAAT
CYP19A1-7R	TTGGATTGGGATTACAGAAC
CYP19A1-8F	TCAATCACAGAGACATGTGG
CYP19A1-8R	TCTTTTCCGTCTATCTGGTG
CYP19A1-9F	GCTGGTGTGCATTAGAATTA
CYP19A1-9R	GCACAGGGAATGAGTAAGAA
CYP19A1-10F	AGGGCATTGTAGCTGATAAC
CYP19A1-10R	TGTTCACTGTGAGGATGACA

Clinical characteristics of patients with aromatase deficiency vary depending on gender, age and enzymatic activity (1). Aromatase deficiency leads to an increase in intrauterine androgen concentration, thereby result in varying degrees of postnatal virilization in the external genitalia in girls and no change in the external genitalia in boys at birth. Our patient had a karyotype of 46,XX and was born with ambiguous genitalia (Prader stage 4). During infancy and childhood there are usually no symptoms of aromatase deficiency (particularly in boys) while some girl patients may present with abdominal symptoms of ovarian cysts because of mild changes in the hypothalamic-pituitary-gonadal axis due to lack of feedback regulation (3). Aromatase deficiency may lead to a number of clinical conditions in adolescent girls such as delayed puberty, hypergonadotropic hypogonadism, multicystic ovaries and primary amenorrhea in accordance with estrogen deficiency. Signs of virilization such as acne, hirsutism, and cliteromegaly in keeping with androgen excess may also be present (1,2,19,20). Estrogen deficiency, on the other hand, causes delayed epiphyseal closure, eunuchoid body habitus, osteopenia and osteoporosis that develop in both genders (21). A previous study reported a 27-year-old patient with bone pain and recurrent bone fractures secondary to minor trauma. The patient had open epiphyses and also developed lumbar osteoporosis. Aromatase deficiency was detected and the study concluded that estrogen has a key role in maintaining bone mineral density (17).

In most of the fetuses with aromatase deficiency, early (12 weeks) or late onset (up to 30 weeks) maternal virilization can be noted (20,22). The non-aromatized fetoplacental and maternal androgen precursors are converted to testosterone in the placenta and also in peripheral maternal tissues, thereby resulting in maternal virilization. After giving birth, the signs of virilization disappear gradually and the androgen levels return to normal (1). In our

patient, the signs of maternal virilization (hirsutism, acne and deep voice) developed at approximately 20 weeks of gestation. Although hirsutism and acne resolved after birth, interestingly the deep voice persisted, which was consistent with the literature (13).

Both basal and GnRH-stimulated FSH levels have been shown to be higher in girls with aromatase deficiency during the first two years of life compared to normal subjects (50-75 and 200-255 mIU/mL, respectively). However, the estradiol and estrone levels tend to be remarkably low during this same period (22,23). Moreover, basal LH is often within normal limits or slightly elevated during infancy (5-10 mIU/mL). A previous study showed that in a girl with aromatase deficiency, the FSH and LH levels persistently increased and multicystic ovaries developed between the ages of three and four years (22). However, Belgorosky et al (23,24) reported that the basal FSH and LH levels in a girl with aromatase deficiency were found to be increased during mini-puberty and to show a dramatic decrease between two and five months. In our patient, gonadotropin (FSH, LH) levels were found to be elevated since birth.

In girls with aromatase deficiency, the ovaries are usually large and polycystic in every stage of life (newborn, childhood and puberty) due to the chronic stimulation by gonadotropins that cannot be suppressed owing to estrogen deficiency or androgen excess (1,2). In our patient, no cystic formation was observed in the ovaries despite high gonadotropin levels and also the ovarian volumes were below the age-matched limits. To date, hypoplasic ovaries have been reported in a total of five cases from three studies, the characteristics of which were similar to those of our patient (9,16,18).

Literature reviews indicate that there is little documentation on the effects of estrogen replacement to prevent estrogen deficiency in women with aromatase deficiency. Moreover, there is no consensus on the dosage and age of initiation of estrogen replacement therapy. On the other hand, data regarding early initiation of the treatment and the long-term follow-up of the patients are extremely rare. To our knowledge, there has been only one study investigating the effects of estrogen replacement therapy on longitudinal growth, bone age maturation, multicystic ovaries, bone density and regulation of the pituitary gonadotropin feedback in a girl with aromatase deficiency who was started on low-dose estrogen therapy at the age of 3.5 years and continued the therapy until the age of 15 years. The study revealed that estrogen is required for normal growth, pituitary-gonadal development and bone maturation not only in puberty but also in early childhood (3). In a review of treatment of aromatase deficiency, it was reported that estrogen replacement therapy can be initiated at as early as two years of age. The study also noted that this treatment should be initiated and sustained with the lowest dose of estrogen possible to prevent the development of ovarian cysts and to avoid early development of breasts and acceleration of bone age. The study suggested that oral conjugated estrogens (0.15 mg/day or every other day) or micronized estradiol (0.25 mg/day or every other day) can be used and the dose may be titrated to maintain the suppression of FSH and LH (4). In view of these findings, low-dose estrogen replacement therapy was planned for our patient at age two years for enhancement of the development of uterus and ovaries, normal growth, bone maturation and normalization of bone mineral density.

In conclusion, the case reported here presented with ambiguous genitalia and exisiting aromatase deficiency, findings which were due to a novel mutation in the *CYP19A1* gene. Presence of large and cystic ovaries is not essential in aromatase deficiency. On the contrary, the ovaries may be hypoplastic as in this case and a number of other previous reports (9,16,18). Aromatase deficiency should be kept in mind in patients with 46,XX karyotype presenting with ambiguous genitalia along with the signs of maternal virilization.

Ethics

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Edip Unal, Ruken Yıldırım, Funda Feryal Taş, Yusuf Kenan Haspolat, Design: Edip Unal, Vasfiye Demir, Yusuf Kenan Haspolat, Data Collection or Processing: Edip Unal, Hüseyin Onay, Ruken Yıldırım, Funda Feryal Taş, Analysis or Interpretation: Edip Unal, Vasfiye Demir, Hüseyin Onay, Yusuf Kenan Haspolat, Literature Search: Edip Unal, Ruken Yıldırım, Vasfiye Demir, Funda Feryal Taş, Writing: Edip Unal, Hüseyin Onay.

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CASE REPORT

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ROHHAD Syndrome, a Rare Cause of Hypothalamic Obesity: Report of Two Cases

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What is already known on this topic?

Rapid-onset obesity with hypoventilation, hypothalamic dysfunction and autonomic dysregulation syndrome is a rare disease. There are around 80 reported patients.

What this study adds?

Rapid-onset obesity with hypoventilation, hypothalamic dysfunction and autonomic dysregulation (ROHHAD) syndrome should be considered in the differential diagnosis of obese patients with hypothalamic dysfunction and autonomic alterations. We aimed to increase awareness about ROHHAD syndrome which does not have dysmorphological features with obesity.

Abstract

Rapid-onset obesity with hypoventilation, hypothalamic dysfunction and autonomic dysregulation (ROHHAD) syndrome is a rare disease that is difficult to diagnosis and distinguish from genetic obesity syndromes. The underlying causes of the disease have not been fully explained. Hypothalamic dysfunction causes endocrine problems, respiratory dysfunction and autonomic alterations. Currently there are around 80 reported patients although this is likely due to underdiagnosis due to lack of recognition. We present two female patients suspected of ROHHAD due to weight gain starting in early childhood. Clinical and biochemical findings such as respiratory and circulatory dysfunction, hypothalamic hypernatremia, central hypothyrodism, hyperprolactinemia and central early puberty in these patients matched the criteria for ROHHAD syndrome. ROHHAD syndrome should be considered in the differential diagnosis of monogenic obesity.

Keywords: ROHHAD syndrome, hypothalamic dysfunction, endocrinological disorders

Introduction

Rapid-onset obesity with hypoventilation, hypothalamic dysfunction and autonomic dysregulation (ROHHAD) syndrome is a rare cause of obesity, characterized by early and rapid onset of obesity, hypoventilation, hypothalamic dysfunction and autonomic dysfunction. To date, 80 patients with ROHHAD have been reported (1,2,3). Obesity and endocrine abnormalities comprise major components of the syndrome. It should therefore be kept in mind in the differential diagnosis, particularly of pediatric endocrinology patients.

The syndrome begins with an uncontrollable eating impulse and rapid weight gain starting in early childhood, usually around 2-4 years of age. In this period, episodes of apnea and cyanotic attacks, due to respiratory distress caused by weight gain and hypothalamic dysfunction, are clinically apparent. Hyponatremia and/or adipsic hypernatremia may also be seen due to involvement of the thirst center. Abnormalities of hypothalamo-hypophyseal hormones may lead to presentation with diabetes insipidus, hyperprolactinemia, growth hormone (GH) deficiency, central hypothyroidism, secondary adrenal insufficiency and/or early or delayed puberty (2,4,5).

There may be findings of autonomic dysfunction including thermal dysregulation (hypothermia/hyperthermia), cold, pale hands and feet (Raynaud's phenomenon), excessive sweating, decreased pain sensitivity, impaired

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pupillary response to light, bradycardia, hypotension and gastrointestinal dysmotility (2,6,7). Moreover, patients may present with psychiatric problems such as anxiety, aggressive behavior and personality disorder (8). The majority of patients were reported to have an underlying neural crest tumor (5). Thus, this rare syndrome can manifest with various clinical and endocrine findings.

In this paper, we discuss two cases with distinct clinical symptoms who presented for evaluation of obesity.

Case Reports

Case 1

A 7 years 4 months old girl presented with excessive weight gain. She was born at term with a birth weight of 2900 g [-0.96 standard deviation (SD)]. The parents reported that she began to gain weight rapidly at 1.5 years of age. There was no consanguinity between the parents. The patient had two healthy siblings (of ages 11 years and 9 months). Body weight was 61 kg (±3.9 SD) and height was 130 cm (±1.8 SD) with a body mass index (BMI) of 36 kg/m^2 (±3.3 SD). She had a plethoric facial appearance, axillary acanthosis nigricans, pale/blue fingers and toes and stage 2 thelarche, bilaterally. The patient was admitted to hospital for further evaluation. During follow-up, it was observed that she had episodes of excessive sweating and a body temperature as low as 35.4 °C. Blood pressure was 95/60 mmHg (95 percentile: 120/80 mm/Hg). Laboratory evaluation showed the following results: sodium (Na), 156 mmol/L [normal range (NR): 135-145 mmol/L]; aspartate transaminase, 87 U/L (NR: 8-45 U/L); alanine aminotransferase, 57 U/L (NR: 7-55 U/L); urine density, 1024. The remaining liver function tests, serum electrolytes, lipids kidney function tests and complete blood count were normal. The patient had no polydipsia; thus, hypernatremia was considered to be due to insufficient intake. Oral fluid replacement was given and the hypernatremia was corrected (Na: 141 mmol/L). Impaired glucose tolerance (141 mg/dL at two hours) was detected in the oral glucose tolerance test performed due to morbid obesity and acanthosis nigricans; and the patient was started on metformin. An abdominal ultrasound was performed, due to the elevated transaminase levels, which revealed grade 3 hepatic steatosis.

In terms of hormonal problems that may present in the patient's follow-up hormonal evaluation revealed the following results: free T4, 0.7 ng/mL (NR: 0.98-1.6 ng/mL; thyroid stimulating hormone (TSH), 4.8 µIU/mL (NR: 0.5-4.3 µIU/mL); adrenocorticotropic hormone (ACTH), 24 pg/mL (NR: 10-60 pg/mL); cortisol, 1.5 µg/mL (NR: 3-21 µg/mL); LH, 1.3 mIU/mL (prepubertal NR: <0.3 mIU/mL); estradiol, 12.9 pg/mL (prepubertal NR: <12 pg/mL); prolactin (PRL), 33 ng/mL (NR: 4.7-23.3 ng/mL). Insulin like growth factor-1 (IGF-1) was <25 ng/mL and IGF binding protein 3 (IGFBP3) 1870 ng/mL. Bone age was advanced at 10 years. Peak cortisol response to low dose ACTH stimulation test was low (9.9 µg/mL).

A diagnosis of secondary adrenal insufficiency, central hypothyroidism and central precocious puberty was made and treatment was initiated with hydrocortisone, thyroxine and leuprolide acetate. No further evaluation or treatment was considered for GH deficiency, due to the patient's normal height. However, a predisposition to neural crest tumor was considered despite the low IGF-1 and IGFBP3 levels of the patient. Brain and pituitary magnetic resonance (MR) imaging studies were found to be normal. The IQ score was 65. The pale appearance of the fingers was considered to be due to Raynaud's phenomenon (Figure 1). Alterations in body temperature and Raynaud's phenomenon were attributed to autonomic dysfunction. Pulmonary hypertension was detected on echocardiography and nifedipine was prescribed.

Case 2

A five year old girl with suspected epileptic seizures was referred for evaluation of obesity. The patient was reported to have sleep apnea and aggressive behavior. She was born at term, with a birth weight of 2800 g (-1.2 SD) and she had uncontrollable eating, starting at two years of age, with rapid weight gain. There was no consanguinity between parents and she had two healthy siblings. Body weight was 11 kg (±3.7 SD) and height was 101 cm (±1.7 SD) with a BMI of 30.4 kg/m^2 (±5.7 SD) (Figure 2). The patient had central cyanosis. Blood pressure was 90/60 mm/Hg (95 percentile: 115/75 mm/Hg). Axillary body temperature measurements varied from 35.6 to 39.5 °C.

Laboratory evaluation revealed the following results: Na, 164 mmol/L (NR: 135-145 mmol/L); urine density, 1018.

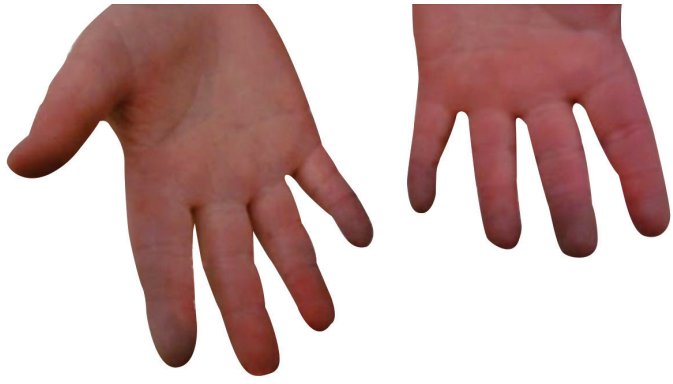

Figure 1. Raynaud's phenomenon in case 1

The remaining biochemical parameters including liver enzyme levels and lipid profile were normal. The patient was considered as a case of adipsic hypernatremia. Oral fluid replacement was given, which normalized the Na value (140 mmol/L). Pituitary evaluation revealed the following results: free T4: 0.8 ng/mL (NR: 0.98-1.6 ng/mL); TSH: 1.8 µIU/mL (NR: 0.5-4.3 µIU/mL); PRL: 56 ng/mL (NR: 4.7-23.3 ng/mL). Remaining pituitary hormone levels were within normal limits.

Treatment was started for central hypothyroidism. The mild hyperprolactinemia persisted but no treatment was needed. Brain and pituitary MR imaging studies revealed normal results. Genetic tests for the Prader-Willi syndrome revealed no abnormality in the 15q11-q13 (*SNRPN* gene). In addition, the genetic analysis did not identify any abnormality in the gene associated with congenital hypoventilation syndrome, *PHOX2B*. IQ was compatible with a chronological age of 3 years. Imaging studies for neural crest tumor gave normal results. However, sleep apnea persisted and the central cyanosis progressed. The patient was transferred to the intensive care unit due to development of carbon dioxide retention (pH: 7.27, pCO_2: 55 mm/Hg) and mechanical ventilation was initiated. Since the patient needed continuous ventilator support, she was discharged with a home ventilator after tracheostomy.

The clinical and laboratory characteristics of the two patients are summarized in Table 1.

Figure 2. Appearance of case 2

Discussion

ROHHAD syndrome, characterized by high morbidity and hypothalamic dysfunction is a rare cause of obesity with unknown etiology (7). It has been suggested that the syndrome is associated with underlying genetic, autoimmune and paraneoplastic factors and studies aiming to detect such factors are ongoing (4). However, in one such study investigating candidate autonomic system genes no genetic defect was found (9).

In the Smiths-Magenis syndrome (SMS), presenting in late childhood/adolescence with hyperphagia and dysmorphism, a point mutation in *RAI1*, which is a transcription factor involved in craniofacial and neural development, is implicated (10). In 2015, a novel mutation was detected in the *RAI1* gene in a boy aged 11 years who presented with the clinical findings of ROHHAD syndrome and the authors suggested that the patient had an overlap syndrome with SMS (11).

Since patients with ROHHAD syndrome resemble congenital hypoventilation syndrome patients, *PHOX2B*, a gene encoding a transcription factor critical for hypothalamic embryogenesis, was investigated but no abnormalities were found (9). Congenital hypoventilation syndrome manifests with autonomic dysfunction, respiratory problems and gastrointestinal motility disorders in the neonatal period in most instances. In both our patients, onset of obesity was after 18 months of age and autonomic dysfunction developed later in the course of the disease.

Prader-Willi syndrome is also included in the differential diagnosis of these cases, due to early onset of obesity. Mental retardation, hypotonia, small hands and feet, ocular findings and hypogonadism may also be present (2,10,12). Prader-Willi syndrome was not considered in the differential diagnosis of the first case due to the presence of precocious puberty. A genetic investigation was performed in the second case, which proved to be negative.

Leptin deficiency or resistance can be distinguished from ROHHAD syndrome by earlier onset of weight gain. This condition is associated with immune deficiency, but also causes hyperphagia, obesity and deficiency in pituitary hormones. MRC4 receptor resistance - proopiomelanocortin (POMC) deficiency is also a rare cause of monogenic obesity but in these patients obesity begins in the first year of life and no autonomic dysfunction is described. In addition, there is usually reddish hair and extremely light skin color in POMC deficiency (13,14).

Cushing syndrome should be kept in mind in the differential diagnosis of patients with ROHHAD syndrome. No

Table 1. Clinical and laboratory features in the two patients

	Case 1	Case 2
Age at presentation (years)	7.3	5
Age at onset of hyperphagia (years)	1.5	2
Birth weight (g) (SD)	2900 (-0.96 SD)	2800 (-1.2 SD)
Weight (kg) (SD)	61 (3.9)	31 (3.7)
Height (cm) (SD)	130 (1.8)	101 (-1.7)
BMI (kg/m²) (SD)	36 (3.3)	30.4 (5.7)
Endocrine pathology	Central hypothyroidism Secondary adrenal insufficiency Central precocious puberty Hyperprolactinemia GH deficiency Adipsic hypernatremia	Central hypothyroidism Hyperprolactinemia Adipsic hypernatremia
Autonomic dysfunction	Hypothermia Sweating Raynaud's phenomenon	Hypothermia/hyperthermia Sleep apnea
Mental state	IQ 65	Compatible with age 3 years
Respiration	-	Sleep apnea Carbon dioxide retention Ventilator support required
Cardiac	Pulmonary hypertension	-
Neural crest tumor	-	-
Genetic abnormalities	-	15q11-q13 (*SNRPN* gene) (-) *PHOX2B* no variation

SD: standard deviation, BMI: body mass index

hypercortisolism was detected in either of our patients; indeed secondary adrenal insufficiency was present in the first case.

Autonomic dysfunction is one of the common findings in patients with ROHHAD syndrome. Tachycardia/bradycardia, cardiac arrest, constipation, hypothermia/hyperthermia, sleep apnea and narcolepsy may be present. Dysfunction of hypocretin-1, involved in acetylcholine release in the autonomic nervous system, has been implicated in autonomic dysfunction (7). Hypothermia, excessive sweating and Raynaud's phenomenon were present in the first case, while there were hypothermia-hyperthermia episodes and sleep apnea in the second case. Respiratory problems and obstructive sleep apnea can be observed in other monogenic obesity cases but there are no additional clinical findings such as thermal dysfunction, excessive sweating and circulatory problems such as Raynaud's phenomenon. In addition adipsic hypernatremia has not been reported in other monogenic obesity cases.

The presence of lymphocytic infiltration on postmortem hypothalamic examination is suggestive of autoimmunity in ROHHAD syndrome and a partial response has been reported to intravenous immunoglobulin and immunosuppressive therapies (5,12). In addition, detection of an oligoclonal band in the cerebrospinal fluid analysis supports intrathecal immunoglobulin synthesis (12).

Ganglioneuroma and neuroblastoma have been reported in some ROHHAD cases, suggesting paraneoplastic involvement of the hypothalamus (2). Thus, there is a group of patients comprising 40% of all patients, the term "ROHHAD-neuroendocrine tumors" has been coined in recent years (15). We screened both cases for the presence of such tumors but no mass lesion was detected.

ROHHAD syndrome is a rare cause of hypothalamic obesity and is accompanied by autonomic dysfunction and pituitary hormone abnormalities. It is a multi-systemic disease with unclear etiology, requiring a multidisciplinary palliative approach. It is thought that the diagnosis is missed in many of these cases, most of whom die due to respiratory or cardiac problems or to an underlying neoplasm. ROHHAD syndrome should be kept in mind in the differential diagnosis of monogenic obesity.

Ethics

Informed Consent: Written consent was obtained from the patient's parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ülkü Gül Şiraz, Deniz Ökdemir, Gül Direk, Concept: Ülkü Gül Şiraz, Design: Ülkü Gül, Şiraz, Nihal Hatipoğlu, Data Collection or Processing: Ülkü Gül Şiraz, Deniz Ökdemir, Analysis or Interpretation: Leyla Akın, Mustafa Kendirci, Selim Kurtoğlu, Literature Search: Ülkü Gül Şiraz, Nihal Hatipoğlu, Writing: Ülkü Gül Şiraz, Nihal Hatipoğlu.

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CASE REPORT

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A Novel Mutation of *AMHR2* in Two Siblings with Persistent Müllerian Duct Syndrome

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What is already known on this topic?

Persistent Müllerian Duct syndrome develops due to deficiency of anti-Müllerian hormone (AMH) or AMH receptor resistance in individuals with 46,XY karyotype. The condition is characterized by a penis of normal length in association with unilateral or bilateral undescended testes and persistence of müllerian structures in individuals with 46,XY karyotype.

What this study adds?

A novel homozygous mutation in the *AMHR2* gene was found in two siblings. These siblings were phenotypically different, suggesting that this mutation may present with a variable clinical picture.

Abstract

Persistent Müllerian Duct syndrome (PMDS) develops due to deficiency of anti-Müllerian hormone (AMH) or insensitivity of target organs to AMH in individuals with 46,XY karyotype. PMDS is characterized by normal male phenotype of external genitals, associated with persistence of Müllerian structures. This report includes the presentation of a 2.5 year old male patient due to bilateral undescended testis. His karyotype was 46,XY. The increase in testosterone following human chorionic gonadotropin stimulation test was normal. The patient was referred to our clinic after uterine, fallopian tube and vaginal remnants were recognized during the orchiopexy surgery. The family reported that the eight year old elder brother of the patient was operated on for right inguinal hernia and left undescended testis at the age of one year. A right transverse testicular ectopia was found in the elder brother. Both cases had normal AMH levels. *AMHR2* gene was analyzed and a homozygous NM_020547.3:c.233-1G > A mutation was found that was not identified previously. In conclusion, we determined a novel mutation in the *AMHR2* gene that was identified for the first time. This presented with different phenotypes in two siblings.

Keywords: Undescended testis, anti-Müllerian hormone, persistent Müllerian Duct syndrome

Introduction

Persistent Müllerian Duct syndrome (PMDS) is a rare disorder of 46,XY sex development. The condition is characterized by a penis of normal length in association with unilateral or bilateral undescended testis and persistence of Müllerian structures in individuals with 46,XY karyotype. PMDS develops mostly due to deficiency of anti-Müllerian hormone (AMH) or insensitivity of target organs to AMH. Mutations of either the *AMH* or *AMHR2* gene have been detected in 88% of cases (1). PMDS shows an autosomal recessive inheritance and its incidence is not clearly known. However, published numbers of cases have increased, due to cryptorchidism being investigated at earlier stages of life, laparoscopic examination being included in routine clinical work-up and surgeons being more aware of this condition in comparison to the past (1).

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In this study, we present two siblings with homozygous NM_020547.3:c.233-1G > A mutation that is identified for the first time in the *AMHR2* gene. One sibling presented with bilateral undescended testis, while the other presented with transverse testicular ectopia.

Case Reports

Case 1

This 2.5 years old male patient presented to our outpatient clinic with bilateral undescended testis. Family history is notable for 3rd degree consanguineous marriage in the parents. It is understood that bilateral, undescended testes were recognized by the family immediately after the birth, but the family did not visit a doctor. At presentation the patient had a weight of 13.5 kg [standard deviation score (SDS): -0.18] and a height of 94.0 cm (SDS score: 0.36). Stretched penile length was 4 cm. The testes could not be palpated bilaterally. A pelvic ultrasound detected a formation, suggestive of testis in the proximal segment of the inguinal canal, bilaterally. These structures were 7x5x7 mm in size on the right side and 7x5x9 mm in size on the left side. A uterus, Fallopian tubes or ovaries could not be visualized. Laboratory tests revealed a follicle stimulating hormone (FSH) concentration of 1.2 mIU/mL, luteinizing hormone (LH) concentration of 0.1 mIU/mL and total testosterone of 0.03 ng/mL. Concentration of 17-hydroxyprogesterone was 0.48 ng/mL and AMH: 35.1 ng/mL (normal range: 5-265 ng/mL). As the patient had bilateral undescended testes and the hormone profile was prepubertal, a human chorionic gonadotropin stimulation test was performed and testosterone response was normal. The patient was referred to the pediatric surgery clinic for orchiopexy. Rudimentary uterine, fallopian tube and vaginal remnants were seen at the orchiopexy operation. It was also reported that bilateral gonads resembling testes were found and biopsies were taken. The patient was referred to our outpatient clinic again due to the presence of these Müllerian structures. The biopsy specimens were consistent with bilateral testicular tissue. In the light of these findings, a diagnosis of PMDS was considered. Since AMH level was normal, *AMHR2* gene mutation was considered.

AMHR2 gene mutation analysis was performed by sequencing of the coding exons and the exon-intron boundaries of the genes. Genomic DNA was isolated from peripheral blood cells with QIAGEN (Maryland, USA) DNA Blood Mini Kit according to the protocol provided with the kit. Sequencing was performed with Miseq V2 chemistry on MiSeq instrument (Illumina California, USA). Analysis was performed with Integrative Genomics Viewer software. Genetic analysis revealed a homozygous NM_020547.3:c.233-1G > A mutation in the *AMHR2* gene (Figure 1). This mutation has not been identified in databases previously. Analyses made with MutationTaster, and splicing modeling software (NNSplice, GeneSplicer and SpliceSiteFinder) showed that the mutation can cause the condition. According to the American College of Medical Genetics and Genomics 2015 criteria, this mutation was classified as "Pathogenic" (2).

Case 2

After case 1 was diagnosed with PMDS, the elder sibling (eight years old) presented due to left undescended testis. From the patient history it was learned that the family had noted a swelling in the right groin when the child was two months old and that the child was operated on by a pediatric surgeon at age one year. Right inguinal hernia and presence of both testes in the right scrotum were reported after the surgery. Right inguinal hernia was repaired, but Müllerian structures were not mentioned in the operative note. At presentation the patient weighed 23.5 kg (SDS: -0.65) had a height of 124 cm (SDS: -0.54). The left testis could not be palpated, but both testes were palpated in the right scrotum. The stretched penile length was 5 cm. The scrotal ultrasound was consistent with the presence of two testes in the right scrotal cavity, measuring 15x7 mm and 17x7 mm respectively. A structure consistent with a uterus, measuring 8x4 mm in size, was found posterior to the urinary bladder. FSH level was 0.72 mIU/mL, LH: 0.1 mIU/mL, total testosterone: 0.03 ng/mL, AMH: 35.1 ng/mL (normal range: 3.2-182.4). Since AMH level was normal, *AMHR2* gene mutation was considered and a homozygous NM_020547.3:c.233-1G > A mutation was found in the *AMHR2* gene in the genetic analysis.

Written informed consent was obtained from the patient's family for publication of this report.

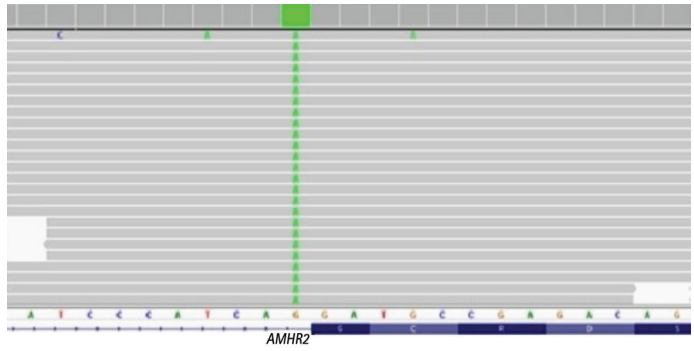

Figure 1. Homozygous mutation (NM_020547.3:c.233-1G > A) in intron 2 of the *AMHR2* gene

Discussion

AMH is synthesized by immature Sertoli cells in men and ovarian granulosa cells in women. It is responsible for total regression of Müllerian structures in week 10 of fetal development in the male fetus. External genitalia are completely normal in men with AMH deficiency. However, AMH deficiency causes persistence of Müllerian structures along with testes and male excretory ducts (1,3). PMDS usually originates from gene mutations in *AMH* or *AMHR2* (1,4). It is recognized during conventional surgery or laparoscopic examination of undescended testis alone or in combination with inguinal hernia (1). PMDS has three main clinical presentations:

1. Bilateral cryptorchidism. This presentation accounts for approximately 55% of AMH pathway mutations and 86% of idiopathic cases;

2. Unilateral cryptorchidism. A testis and the accompanying fallopian tube and uterus cause an inguinal hernia. This presentation is known as "hernia uteri inguinalis". This presentation accounts for approximately 20% of AMH pathway mutations and 14% of idiopathic cases;

3. Transverse testicular ectopia. This term refers to unilateral herniation of both testes and a part of the Müllerian structures through the processus vaginalis. This condition is the most specific anatomic situation of PMDS and it is found in 25% of cases with *AMH* or *AMHR2* gene mutation. However, it is never seen in idiopathic cases (5). Our first case was diagnosed with this condition, after Müllerian structures were recognized during surgical treatment of bilateral undescended testis.

AMH is a member of the transforming growth factor-β family. It contains fixed exons and it is 2.8 kb long (6). Cohen-Haguenauer et al (7) determined that the *AMH* gene is located in the short arm of chromosome 19 (p13.3). It was reported that the *AMHR2* gene is located on the long arm of chromosome 12 and that it contains 11 exons (8). Picard et al (1) conducted a study of 157 families with PMDS from 1990 to 2016 and they found mutations of *AMH* or *AMHR2* genes in 88% of the cases. The same study demonstrated 64 different mutations in the *AMH* gene in 80 families and the authors found that mutations are more commonly located in Exon 1, 2 and 5. Similarly, *AMHR2* gene mutations were discovered in 75 families in 58 different alleles. No mutation was found in the AMH or *AMHR2* genes in 12% of the cases and these are referred to as idiopathic PMDS (1). Serum *AMH* levels are undetectable or low in *AMH* gene mutations, while normal or high when *AMHR2* mutations are present (4,9). Since serum *AMH* levels were normal in our patient, *AMHR2* gene mutation was considered and this diagnosis was made. There is no significant anatomic difference between patients with *AMH* or *AMHR2* gene mutations. Previous studies demonstrated that the position of testes and that of Müllerian structures may vary between siblings with PMDS and with the same mutation (10). Our study showed that a mutation causes bilateral undescended testis in one patient and transverse testicular ectopia in the sibling.

Recently, early orchiopexy has been recommended, if and whenever possible, in order to prevent damage to the germ cells in patients with cryptorchidism. Previously, it was estimated that the incidence of testicular cancer in PMDS was not higher than that in cases with cryptorchidism and that the incidence was around 18% (11). However, Picard et al (1) showed that unilateral or bilateral malignant testicular degeneration develops in 33% of patients with PMDS at ages 18 or above and stated that the most common malignant degeneration is seminoma. Malignant degeneration of Müllerian derivatives is less common. Farikullah et al (12) have detected degeneration of Müllerian structures releated to PMDS with only in 3 cases in their study.

The most common complication of PMDS is infertility. Although fertility is rare in PMDS, it is possible if at least one testis is present and if the excretory ducts are intact (1). A comprehensive literature review showed that 19% of adult patients have one or more children (1). Farag (13) reported the rate of fertile patients as 11%. On the other hand, there are many reported cases of infertility and azoospermia. Late orchidopexy, damage of testis and vas deferens during surgery and abnormal anatomic connection of testes to the excretory ducts are some of the causes of infertility (13,14,15). The testes are usually not properly connected to the male excretory ducts due to aplasia at the upper part of the vas deferens and epididymis or absence of a connection between the testis and the epididymis (16).

In conclusion, PMDS is a rare condition that is usually seen in men who present with cryptorchidism and/or inguinal hernia. It should be diagnosed early for both protection of fertility and for prevention of potential malignant degeneration. Considering the possibility of damage to the vas deferense and testis during surgical procedures, the patient should always be referred to experienced surgeons.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Edip Unal, Ruken Yıldırım, Suat Tekin, Yusuf Kenan Haspolat, Design: Edip Unal, Vasfiye Demir, Yusuf Kenan Haspolat, Data Collection or Processing: Edip Unal, Hüseyin Onay, Ruken Yıldırım, Suat Tekin, Analysis or Interpretation: Edip Unal, Vasfiye Demir, Hüseyin Onay, Yusuf Kenan Haspolat, Literature Search: Edip Unal, Ruken Yıldırım, Vasfiye Demir, Suat Tekin, Writing: Edip Unal, Hüseyin Onay.

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CASE REPORT

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Acquired Chiari I Malformation Secondary to Spontaneous Intracranial Hypotension Syndrome and Persistent Hypoglycemia: A Case Report

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What is already known on this topic?

Spontaneous intracranial hypotension (SIH) is a rare and potentially serious pathological syndrome in childhood. Concomitant presentation of Chiari I malformation with SIH has rarely been reported. Diagnostic criteria wide-ranging due to variable clinical manifestations. Myelography computerized tomography and epidural blood patch are reliable diagnostic and treatment modalities.

What this study adds?

Chiari I malformation may mimic spontaneous intracranial hypotension (SIH) and to provide ideal therapy requires recognition of SIH. Persistent hypoglycemia was an early central feature of our patient which is an unusual finding in SIH. Some possible causes of hypoglycemia in SIH are discussed and we present vagotomy as a new treatment modality.

Abstract

Spontaneous intracranial hypotension (SIH) is a rare and potentially serious condition in childhood. Cerebrospinal fluid (CSF) volume depletion is thought to be the main causative feature for intracranial hypotension and results from a spontaneous CSF leak, often at the spine level. SIH is increasingly diagnosed in clinical practice, although it manifests a varied symptomatology. The downward displacement of the brain, sometimes mimicking a Chiari I malformation, has rarely been reported. We present a case of a SIH with Chiari I malformation accompanied by an unusual clinical presentation of persistent hypoglycemia.

Keywords: Intracranial hypotension, hypoglycemia, vagotomy

Introduction

Spontaneous intracranial hypotension (SIH) is a rare condition with an estimated prevalence of only one in 50.000 individuals (1). The clinical spectrum of SIH is quite variable and includes headache, neck stiffness, cranial nerve dysfunction, radicular arm pain and symptoms of diencephalic or hindbrain herniation (1,2). Intracranial hypotension is a well-recognized sequel of a spontaneous cerebrospinal fluid (CSF) leak, particularly in cases in which the leak involves the thoracic spine (3). The cause for these CSF leaks remains unclear, but authors have postulated minor trauma, weakness of the dural sac or a combination of both (4,5). More cases are being diagnosed due to advances in imaging, but the diagnosis is still challenging because of the number of atypical, unconfirmed and doubtful cases. The current diagnostic criteria have a wide spectrum due to very variable clinical manifestations. The diagnosis of

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SIH is mainly based on presence of an orthostatic headache together with at least one of the following: low CSF pressure, sustained improvement of symptoms after epidural blood patching, demonstration of an active spinal CSF leak and cranial magnetic resonance imaging (MRI) changes demonstrating intracranial hypotension (6). Myelography computerized tomography is the most reliable method for the accurate localization of the CSF leak (7). In most cases, epidural blood patch is the main treatment modality (8).

Chiari I malformation is defined radiographically as a simple displacement of the cerebellar tonsils 5 mm or greater below the foramen magnum and is distinguished from Chiari II and Chiari III malformations occurring with myelodysplasia and cervical encephalocele, respectively (9). Spontaneous CSF leakage with development of SIH and acquired Chiari I malformation due to lumbar spinal CSF diversion procedures have both been well described. However, concomitant presentation of both syndromes has rarely been reported. Not to be confused with idiopathic Chiari I malformation, ideal therapy requires recognition of the syndrome and treatment directed to the site of the spinal CSF leak (10).

This is a case of a 13-year-old girl with acquired Chiari I malformation secondary to SIH with a unique coexistence of persistent hypoglycemia.

Case Report

A 13-year-old girl was admitted to the pediatric emergency unit of Kanuni Sultan Süleyman Training and Research Hospital suffering from hypoglycemia, syncope and convulsive seizures. She had no notable health problem in her past medical history up to 11.5 years of age. Thereafter, she had six subsequent hospital admissions in the previous 1.5 years, mostly at emergency services for hypoglycemic convulsions and syncope attacks. She was born at term, weighing 3100 g from non-consanguineous parents after an uncomplicated delivery. On evaluation of her records, it was found that she had undergone hypoglycemic periods 2-3 times per day, but syncope attacks were independent from hypoglycemic episodes. She was diagnosed with hyperinsulinemia with a serum glucose level of 29 mg/dL with a concomitant serum insulin level of 25 IU/L. Positron emission tomography and abdominal ultrasonography were performed to determine the etiology of hyperinsulinemia, but revealed normal anatomic findings. At neurological counseling electroencephalography showed bilateral delta waves with spikes and cranial MRI revealed a 7 mm herniation of the cerebellar tonsils from the foramen magnum (Figure 1). Further work-up with brainstem auditory evoked potentials and somatosensory evoked potentials, cardiac evaluation with echocardiography and holter monitoring revealed normal findings. Hypoglycemic episodes resolved in the following weeks but, although reduced in number, syncope episodes persisted. Pediatric psychiatry counseling results during her previous admission were not contributory.

At her present admission, the patient's body weight was 55 kg (90th percentile) and height was 145 cm (25th percentile). Laboratory investigations revealed hypoglycemia (serum glucose level: 30 mg/dL) with a high insulin level of 50 IU/L. Serum C-peptide level was 5 pmol/mL (N: 0.5-1.30 pmol/mL) and cortisol 23 µg/dL (N: 6.2-19.4 µg/dL). After intravenous glucose, intramuscular glucagon and methylprednisolone treatment, glucagon infusion was initiated. Glucose levels were 40 to 65 mg/dL during the first 24 hours, but surprisingly, glucose levels of 80-90 mg/dL were detected in the course of 24 hours after stopping the infusion. Oral glucose tolerance test (OGTT) showed hypoglycemia at the 30th minute (glucose: 25 mg/dL) with an insulin level of 300 IU/L. We monitored our patient's daily glucose levels by continuous glucose monitoring system [CGMS System Gold® (Medtronic Minimed, Northridge, CA)] and 14 hypoglycemia episodes

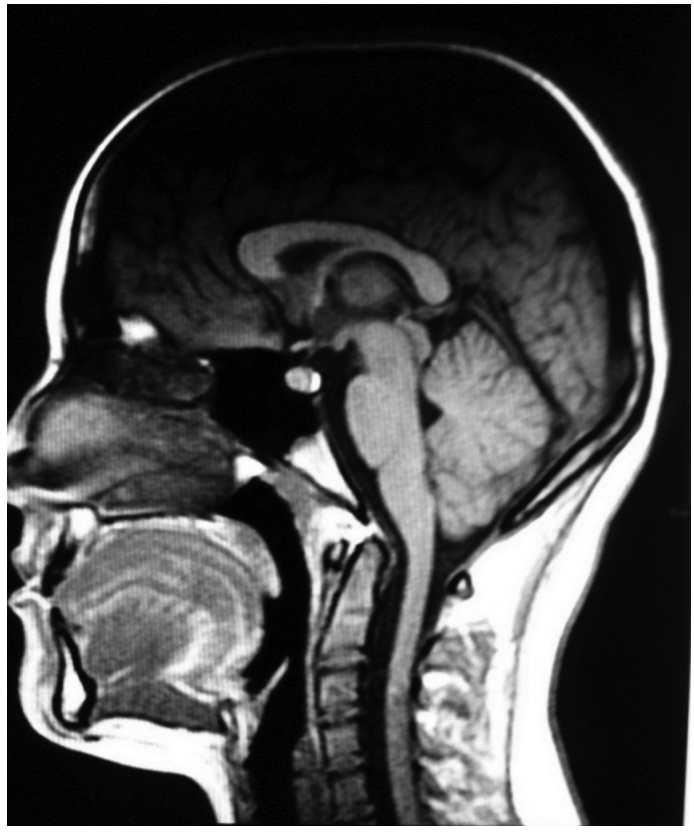

Figure 1. A simple displacement of the cerebellar tonsils 7 mm below the foramen magnum compatible with Chiari I malformation

were noted, most occurring during sleep or defecation or in the postprandial period with a maximum glucose level of 70 mg/dL in three days of follow-up. We measured glucose levels before and after defecation; results were 85 mg/dL and 29 mg/dL respectively. No adrenergic symptoms were observed during hypoglycemic episodes. Diazoxide (40 mg/kg) and octreotide (40 µg/kg) treatment had no effect. Dysarthria was noted in the first month of hospitalization with frequent hypoglycemia episodes. During this period, syncope attacks were observed four times, independent of hypoglycemia. Additionally, the patient had severe biparietal headache episodes in the morning lasting for two hours. These were unrelated to hypoglycemia and were followed by anisocoria. Myelography was performed for SIH and verified with two CSF leaks originating at the lumbar 2 level. The patient underwent a procedure of autologous epidural blood patch at the CSF leak site (Figure 2), with good clinical results including complete control of her episodes of syncope, headache and hypoglycemia.

However, hypoglycemia recurred with dysarthria after two months and was attributed to displacement of the cerebellar tonsils, due to an epidural patch failure. Although the patient remains in good clinical condition after two subsequent epidural patch surgery interventions, neurologic problems and hypoglycemia persisted. Truncal vagotomy and partial pancreatectomy were planned for persistent hypoglycemia, because glucose levels were continuously under 29 mg/dL. At the initiation of this surgical intervention serum glucose was 25 mg/dL. Glucose level spontaneously normalized and was about 100 mg/dL during anesthesia. Truncal vagotomy was performed firstly. In order to test the efficiency of vagotomy, dextrose 10% solution infusion was initiated instead of 0.9% sodium chloride and serum glucose level increased to 180 mg/dL. Distal, partial pancreatectomy was performed additionally, to prevent another surgery risk.

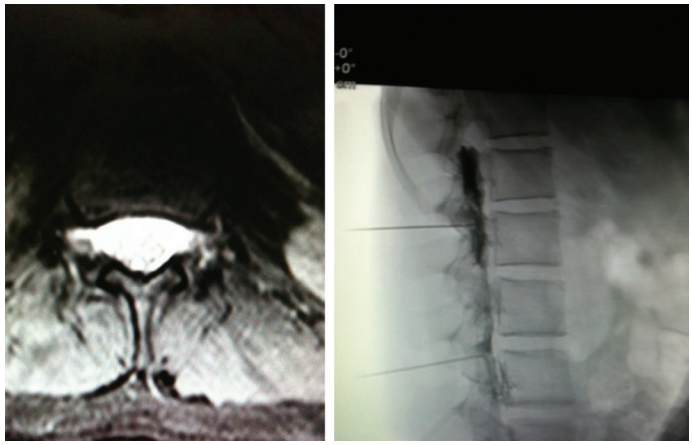

Figure 2. Two cerebrospinal fluid leaks at the lumbar 2 level and the procedure of autologous epidural blood patch

Post operatively diabetes mellitus developed. The patient was discharged with single-dose insulin glargine treatment and her follow-up has been successful for four years.

Discussion

In clinical practice, SIH may manifest itself as a loss of the prepontine cistern due to leak of CSF, with flattening of the brainstem and downward herniation of the cerebellar tonsils, which may mistakenly lead to a diagnosis of Chiari I malformation (10,11). Our patient was diagnosed initially as a Chiari I malformation, but thereafter, with evaluation of symptoms and laboratory results, a diagnosis of SIH was reached.

SIH is initially suspected on the basis of presenting signs and symptoms such as headache, syncope and some neurological problems. However, in our patient, hypoglycemia was the leading clinical feature and this symptom is not a usual finding in SIH patients.

It has been suggested that hyperinsulinism is responsible for the hypoglycemia. Rekate et al (12) reported four cases diagnosed with Chiari malformation who were suffering from intermittent hyperinsulinemic hypoglycemia and proposed that vagal hypertonia, caused by variation in intracranial pressure, affected the pancreas leading to hypoglycemia in their patients. Tarani et al (13) proposed that the brainstem compression due to hindbrain herniation leads to dysfunction of the normal homeostatic mechanisms to correct hypoglycemia and direct stimulation of the vagal nuclei stimulates pancreatic islet cells to secrete insulin. In our patient, hypoglycemia mostly occurred during parasympathetic activities such as when in a postprandial state, defecation or sleep. Moreover, adrenergic activities had never been observed even with severe hypoglycemic episodes, thus this condition may be attributed to autonomic failure related to parasympathetic dominancy due to vagal stimulus. Vagal efferent activity starts with stimulus of oropharyngeal receptors by oral intake and increases with gastrointestinal peristaltic activity, consequently leading to insulin release, inhibition of norepinephrine from splanchnic nerves, gluconeogenesis and activation of glycogen synthesis (14). Vagal stimulus also produces an early phase of insulin response with postprandial insulin release (15,16). The occurrence of hypoglycemia at the 30th minute of the OGTT with a high insulin level is noteworthy and supports a vagal effect. Moreover, in our opinion the syncope episode in our patient may be related to unbalanced reflexes of the sympathetic system (17).

SIH in childhood is rare (18). Occurrence of orthostatic headaches in the morning suggested a diagnosis of SIH

and the myelogram showed two dural puncture areas in the lumbar region (Figure 2). Treatment with the patch procedure improved hypoglycemia and other symptoms with resolving caudal displacement of the cerebellar tonsils. Rekate et al (12) used continuous-drip feeding for hypoglycemia in their cases with Arnold Chiari syndrome. Unfortunately, persistent hypoglycemia relapsed after epidural patch procedure failures in our patient. In concordance with our estimation of parasympathetic dominancy, truncal vagotomy and partial pancreatectomy were planned as a radical therapy for her persistent hypoglycemia. Surgery was started with a serum glucose level of 25 mg/dL, and surprisingly, serum glucose level normalized with anesthesia induction. This outcome may be related to a possible effect of anesthesia on sympathetic-parasympathetic balance (19). Additionally, glucose response after the vagotomy procedure during surgery verified the role of a vagal effect on hypoglycemia. Diabetes mellitus presented after the surgery due to partial pancreatectomy and our patient continues to take single-dose glargine insulin treatment.

In clinical practice, intracranial hypotension syndrome may manifest with a variety of symptoms, one of which may be persistent hypoglycemia. This paper reports the findings in a patient with this syndrome, along with some pathophysiological considerations, diagnostic processes and possible treatment modalities in SIH patients with persistent hypoglycemia.

Ethics

Informed Consent: A written informed consent was obtained from the parent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Hasan Önal, Concept: Erdal Adal, Design: Zerrin Önal, Data Collection or Processing: Serdar Sander, Analysis or Interpretation: Sait Albayram, Literature Search: Atilla Ersen, Hakan Gemici, Writing: Atilla Ersen.

Financial Disclosure: There is no financial or other relationship.

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