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Sample References

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

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

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

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

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CONGRESS CALENDAR

ESPE 2017 (10th International Meeting of Pediatric Endocrinology)
14-17 September 2017, Washington, DC, USA

ISPAD 2017 (43rd Annual Conference, International Society for Pediatric and Adolescent Diabetes) October 18-21, 2017, Innsbruck, Austria

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ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.4774

J Clin Res Pediatr Endocrinol 2018;10(1):1-7

Acute Effects of Blood Transfusion on Insulin Sensitivity and Pancreatic β-Cell Function in Children with β-Thalassemia/ Hemoglobin E Disease

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What is already known on this topic?

Chronic iron overload in transfusion-dependent thalassemia patients is a cause of insulin resistance and pancreatic β-cell dysfunction. In addition, severe anemia is also associated with insulin resistance. Essential regular blood transfusions will improve the anemic state in severe thalassemia but will also increase iron accumulation.

What this study adds?

This is the first study that reports the acute effects of blood transfusion on insulin sensitivity and β-cell function. We demonstrated that following blood transfusion in thalassemic patients, acute iron loading accompanied by partial correction of anemia, resulted in a rise in insulin secretion and a trend towards increasing insulin resistance.

Abstract

Objective: To assess the acute effects of blood transfusion on insulin sensitivity and pancreatic β-cell function in thalassemia patients.

Methods: Fifty children and adolescents with β-thalassemia/HbE disease were enrolled in a prospective cohort study. Hemoglobin, serum ferritin and oral glucose tolerance test (OGTT) were performed prior to, and one week after blood transfusion. Insulin sensitivity indices [homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR), whole body insulin sensitivity index (WBISI)] and β-cell function indices [HOMA of β-cell function (HOMA-β), insulinogenic index (IGI), and disposition index (DI)] were calculated from glucose and insulin levels obtained during the OGTT.

Results: Following blood transfusion, hemoglobin and serum ferritin increased significantly; 8.5 to 10.1 g/dL ($p < 0.001$) and 1764 to 2160 ng/mL ($p < 0.001$), respectively. β-Cell function indices also increased significantly [median HOMA-β: 74.3 vs. 82.7 ($p = 0.033$); median IGI: 59.6 vs. 79.3 ($p = 0.003$); median DI: 658 vs. 794 ($p = 0.01$)]. However, the insulin sensitivity index (WBISI) tended to decrease and the insulin resistance index (HOMA-IR) tended to increase although this did not reach significance. Multivariate analysis showed that pre-transfusion serum ferritin was the major factor negatively associated with WBISI and positively associated with HOMA-IR, but pre-transfusion hemoglobin had no significant association with insulin sensitivity indices post-transfusion.

Conclusion: This study demonstrated that acute increases in serum ferritin and hemoglobin following blood transfusion in patients with thalassemia might contribute to an increase in insulin secretion and to a trend towards increased insulin resistance.

Keywords: Insulin resistance, hemoglobinopathy, iron, hemochromatosis, ferritin

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Introduction

Thalassemia is a hereditary hemolytic disease caused by hemoglobinopathy. Among patients with moderate to severe disease, regular blood transfusion is a vital modality of treatment. However, chronic blood transfusions result in an increase in total body iron, thus iron overload, which in turn leads to iron deposition in multiple organs, including the pancreas. In animal studies, intravenous iron loading was shown to lead to pancreatic necrosis by free radical oxygen species through a Fenton reaction (1,2). Glucose dysregulation, secondary to increased insulin resistance and pancreatic β-cell dysfunction, has been widely reported in thalassemia major patients with iron overload (3,4). Furthermore, adverse effects of iron overload on insulin sensitivity and insulin secretion varied, depending on the degree of iron excess and may be reversible after reduction of tissue iron accumulation. In patients with hereditary hemochromatosis, there was a high prevalence of diabetes mellitus and impaired glucose tolerance (IGT) (5). Besides, normalization of serum ferritin by phlebotomy improved insulin secretory capacity (6). In addition, we recently demonstrated that there was a trend towards improvement of insulin sensitivity and β-cell function following six months of iron chelation therapy in adolescents with non-transfusion-dependent thalassemia (7). Phlebotomy in diabetic patients and blood donation in normal individuals were also shown to improve insulin sensitivity, and thus decrease insulin secretion (8,9).

In addition to increasing the serum iron level, blood transfusions immediately improve the anemic state. Anemia has been shown to be associated with increased insulin resistance in chronic renal failure. Previous studies of chronic renal failure patients treated with multiple episodes of hemodialysis reported that low hematocrit (Hct) may induce tissue hypoxia and cause insulin resistance. Erythropoietin treatment improves the anemic state and reduces insulin resistance in these patients (10,11,12).

Blood transfusion has also been shown to acutely raise serum ferritin level while simultaneously improving the anemic state. These effects can be detected within a week following blood transfusion (13,14). However, the acute effects of blood transfusion on insulin sensitivity and β-cell function in patients with thalassemia remain unknown. We therefore hypothesized that iron loading, along with improvement of the anemic state following blood transfusion might have negative effects on insulin sensitivity and might adversely increase β-cell function.

Methods

This prospective cohort study was conducted at the Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Bangkok, Thailand during the period from April 2015 to March 2016. Children and adolescents aged 5-20 years and diagnosed with β-thalassemia/hemoglobin (Hb) E disease, were enrolled into the study. All patients required regular, packed red cell transfusions every four weeks. Patients with other systemic illness, such as diabetes mellitus, chronic renal disease, cardiomyopathy and those taking medications affecting insulin sensitivity and β-cell function or who had had bone marrow transplantation performed were excluded. Anthropometric data including weight, height, body mass index (BMI) and Tanner's pubertal stage were recorded. Standard deviation (SD) scores of weight and height were calculated using the National Standard Growth Curve of the Ministry of Public Health, Thailand (15). Duration of the disease and median serum ferritin level during the past 3 years were recorded.

After an overnight fast, an oral glucose tolerance test (OGTT) was performed and blood samples were taken for measurement of basal Hb, Hct and serum ferritin. The patients then proceeded to receive packed red cell transfusions at a standardized dose of 10 mL/kg. One week following the transfusion, all patients underwent a second OGTT as well as measurement of Hb, Hct and serum ferritin.

Hb was measured using an Abbott Cell Dyne Ruby hematology analyzer (Abbott Diagnostics, Lake Forest, Illinois, USA) within four hours after blood collection. Vitros ferritin assay (Ortho Clinical Diagnostics, Johnson & Johnson, UK) was used for serum ferritin measurement. For glucose and insulin level assessments, Abbott ARCHITECT c16000 Clinical Chemistry Analyzer (Abbott Diagnostics, Lake Forest, Illinois, USA) and IMMULITE chemiluminescent immunoassay (Siemens Medical Solutions Inc., Malvern, Pennsylvania, USA) were used, respectively.

OGTT was performed in the morning, following an eight-hour overnight fast, using 1.75 g glucose/kg body weight (maximum 75 g). Plasma glucose and serum insulin concentrations were determined before and at 30, 60, 90 and 120 minutes following the ingestion of the glucose solution. Each sample was immediately sent to the laboratory right after blood drawing for analytic procedure. The result of the OGTT was interpreted according to American Diabetes Association criteria (16).

Homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR) (17), and whole body insulin sensitivity index (WBISI) (18) were calculated to determine insulin sensitivity. For β-cell function, HOMA of β-cell function (HOMA-β) (19), insulinogenic index (IGI) (20) and disposition index (DI) (21) were calculated for assessment of β-cell function. areas under the curve (AUC) of glucose and insulin were calculated

using the trapezoidal method. The above-mentioned indices were calculated using the previously published formulas as shown below.

$$\text{HOMA-IR} = \frac{\text{Glucose 0 min (mmol/L) x Insulin 0 min (μIU/mL)}}{22.5}$$

$$\text{WBISI} = \frac{10000}{\sqrt{\text{[Glucose 0 min (mg/dL) x Insulin 0 min (μIU/mL) x Mean glucose (mg/dL) x Mean insulin (μIU/mL)]}}}$$

$$\text{HOMA-}\beta = \frac{20 \times \text{Insulin 0 min (μIU/mL)}}{\text{Glucose 0 min (mmol/L)-3.5}}$$

$$\text{IGI} = \frac{\text{Insulin 30 min-Insulin 0 min (pmol/L)}}{\text{Glucose 30 min-Glucose 0 min (mmol/L)}}$$

DI = WBISI x IGI

This study was approved by the Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval number: 04-58-07; date: 22.04.2015). A written informed consent was obtained from all patients and their legal guardians before the enrollment.

Statistical Analysis

The data were analyzed using IBM SPSS Statistics version 24.0 (SPSS Inc., Illinois, USA). Data are presented as mean ± SD for parametric data and median (interquartile range, IQR) for non-parametric data. Paired-samples t-test and Wilcoxon signed-rank test were performed to compare differences between pre- and post-transfusion parameters for parametric and non-parametric parameters, respectively. A chi-square test was used to compare OGTT results between pre-transfusion and post-transfusion samples. Spearman rank test was used for analysis of correlation among parameters. Linear log regression was used for multivariate analysis. A p value of less than 0.05 was considered statistically significant.

Results

Fifty children and adolescents with β-thalassemia/HbE disease were enrolled in this study. Clinical characteristics of the patients are shown in Table 1. 64% (32/50) were pubertal and 76% (38/50) had been receiving only one kind of iron chelator, deferiprone. Short stature and underweight were common. None of them was obese (median Z-score of BMI -0.40, IQR -1.35 to 0.09). Only eight patients (16%) had a previous history of splenectomy. Serum ferritin levels during the three years prior to enrollment were high (median 1725, range 362-5740 ng/mL). Acute illness was not observed in our patients during the one-week post-transfusion period.

Results of OGTT between pre- and post-transfusion were compared (Table 2). Forty-three (86%) had normal glucose tolerance (NGT) at both pre- and post-transfusion assessments. Two patients had IGT at both tests. Two patients with NGT before transfusion developed IGT at post-transfusion. One patient with NGT at pre-transfusion had impaired fasting glucose (IFG) at post-transfusion. Conversely, one patient with IGT and another with IFG at pre-transfusion had normal OGTT result at post-transfusion. There was no significant difference between pre- and post-transfusion groups.

Following blood transfusion, there were significant increases in mean Hb (8.5 to 10.1 g/dL), mean Hct (26.6 to 31.4%) and median serum ferritin level (1764 to 2160 ng/mL)

Table 1. Clinical characteristics of all enrolled patients (n = 50)

Characteristics	
Age (years)*	14 (10-16)
Sex (male/female) (n)	27/23
Pubertal stage (n)	
- Pre-pubertal	18
- Pubertal	32
Z-score of weight*	-0.57 (-1.40 to 0.58)
Z-score of height*	-0.75 (-1.75 to 0.11)
Z-score of BMI*	-0.40 (-1.35 to 0.09)
Iron chelator used (n)	
- None	1
- Single	38
- Combined	11
Splenectomy (n)	8
Past 3-year ferritin level (ng/mL)*	1725 (362-5740)
Duration of transfusions (years)*	9 (1-18)

*Data are presented as median (interquartile range)

BMI: body mass index

Table 2. Pre-transfusion and post-transfusion oral glucose tolerance test results of all enrolled patients (n = 50)

	Number of patients		
	NGT	IFG	IGT
Pre-transfusion	46	1	3
Post-transfusion	45	1	4

$\chi^2 = 0.154$, $p = 0.93$

NGT: normal glucose tolerance, IFG: impaired fasting glucose, IGT: impaired glucose tolerance

(Table 3). Insulin sensitivity tended to decrease following the transfusion as defined by a reduction in WBISI and an increase in HOMA-IR, but these were not statistically significant. However, β-cell function indices, including HOMA-β, IGI and DI were all significantly increased following transfusion. Additionally, AUC of plasma glucose obtained during the OGTT pre- and post-transfusion was not significantly different. However, AUC of serum insulin at pre-transfusion was significantly lower than that of the post-transfusion point.

Correlation analysis of pre-transfused Hb and serum ferritin with insulin sensitivity and β-cell function indices showed that only pre-transfused ferritin had a negative correlation with percentage change of WBISI ($r = -0.32$, $p = 0.031$), (data not shown).

Differences in total body iron and anemic state potentially influence the changes in insulin sensitivity and β-cell function. We therefore performed subgroup analysis according to pre-transfused Hb and pre-transfused serum ferritin (Table 4). Since most of our patients were sub-optimally transfused [mean (SD) pre-transfused Hb 8.5 (1.1) g/dL] and sub-optimally iron-chelated [median (IQR) pre-transfused serum ferritin 1764 (799-2662) ng/mL], Hb of 8.5 g/dL and serum ferritin of 1500 ng/mL were used as the cut off points for subgroup analysis. In the low pre-transfused Hb group (< 8.5 g/dL), there were significant increases in all β-cell function indices (HOMA-β, IGI, DI), but no significant changes in insulin sensitivity indices (HOMA-IR, WBISI) following the transfusion. In comparison, no significant changes in either β-cell function or insulin sensitivity indices were observed in the high pre-transfused Hb group (≥8.5 g/dL). In the high pre-transfused serum ferritin group (> 1500 ng/mL), there was a significant decrease in insulin sensitivity (decreasing WBISI and increasing HOMA-IR) and significant increases in β-cell function indices (HOMA-β, IGI) following transfusion. In contrast, in the low pre-transfused serum ferritin group, there were no changes of insulin sensitivity indices (WBISI, HOMA-IR) and β-cell function indices (HOMA-β, IGI), but slightly and significantly increased DI following the transfusion. Using multivariate analysis, pre-transfused serum ferritin was the only factor associated with changes of HOMA-IR ($F = 4.080$, $p = 0.049$) and WBISI ($F = 6.799$, $p = 0.012$).

Discussion

Studies in patients with thalassemia and hereditary hemochromatosis who had chronic iron overload showed that glucose dysregulation occurred as a result of insulin resistance followed by β-cell dysfunction (5,22,23,24). Excessive iron causes insulin resistance and subsequently, pancreatic β-cell apoptosis and insulin deficiency (25,26). Most of our patients had NGT at both pre-transfusion and post-transfusion assessments. Their AUC of plasma glucose remained unchanged following the transfusion, while AUC of serum insulin was increased significantly at post-transfusion, reflecting a rise in β-cell function. In fact, in the early phase of glucose dysregulation, change of insulin secretion is reciprocal to that of insulin sensitivity. In parallel with AUC of serum insulin, all β-cell function indices were also elevated following blood transfusion. Meanwhile, there was a trend towards decreasing insulin sensitivity. Therefore, we speculate that acute iron loading, concomitant with partial correction of anemia following a single dose of packed erythrocytes, resulted in a trend towards reduction in insulin sensitivity and thus caused a rise in insulin secretion.

Table 3. Pre- and post-transfusion values for hemoglobin, hematocrit (mean ± standard deviation), serum ferritin, insulin sensitivity and β-cell function indices (median, interquartile range) and area under the curve of glucose and insulin (mean ± standard deviation) in 50 thalassemic children and adolescents

Parameters	Pre-transfusion	Post-transfusion	p
Hemoglobin (g/dL)	8.5 ± 1.1	10.1 ± 1.2	< 0.001
Hematocrit (%)	26.6 ± 3.6	31.4 ± 3.5	< 0.001
Ferritin (ng/mL)	1764 (799-2662)	2160 (1095-3033)	< 0.001
HOMA-IR	0.76 (0.45-1.26)	0.82 (0.48-1.27)	0.14
WBISI	11.8 (7.6-17.8)	10.1 (7.0-17.1)	0.27
HOMA-β	74.3 (45.4-109.9)	82.7 (56.2-123.4)	0.033
IGI	59.6 (36.2-99.2)	79.3 (41.3-135.3)	0.003
DI	658 (374-1135)	794 (458-1492)	0.01
AUC-G (mmoL•h/L)	13.7 ± 2.1	12.7 ± 2.3	0.15
AUC-I (pmol•h/L)	394 ± 284	463 ± 358	0.026

HOMA-IR: homeostatic model assessment of insulin resistance, WBISI: whole body insulin sensitivity index, HOMA-β: homeostatic model assessment of β-cell function, IGI: insulinogenic index, DI: disposition index, AUC-G: area under the curve of plasma glucose, AUC-I: area under the curve of serum insulin

Table 4. Insulin sensitivity and β-cell function indices at pre- and post-transfusion according to pre-transfused hemoglobin and pre-transfused serum ferritin

Median (IQR)	Pre-transfused hemoglobin (g/dL)						Pre-transfused serum ferritin (ng/mL)					
	<8.5 (n=25)		p	≥8.5 (n=25)		p	<1500 (n=23)		p	≥1500 (n=27)		p
Parameters	Pre-t	Post-t		Pre-t	Post-t		Pre-t	Post-t		Pre-t	Post-t	
HOMA-IR	0.68 (0.47-0.99)	0.79 (0.42-1.21)	0.07	0.90 (0.45-1.51)	0.89 (0.60-1.30)	0.71	0.85 (0.58-1.27)	0.81 (0.44-1.16)	0.32	0.71 (0.44-1.26)	0.91 (0.59-1.43)	**0.004**
WBISI	13.62 (8.75-20.30)	14.03 (7.69-22.1)	0.82	10.38 (7.54-12.70)	8.53 (5.61-15.0)	0.16	11.11 (7.67-16.70)	11.66 (7.70-17.70)	0.13	12.17 (7.28-22.0)	8.52 (5.55-17.0)	**0.002**
HOMA-β	51.4 (43.1-103.0)	86.8 (66.2-121)	**0.009**	83.4 (57.2-133)	82.0 (48.8-131.0)	0.60	70.4 (44.5-134)	82.0 (72.0-121.0)	0.58	76.7 (45.5-106.0)	95.3 (51.8-134)	**0.015**
IGI	55.1 (25.3-95.8)	71.3 (38.1-146)	**0.02**	65.5 (44.9-105)	83.1 (47.5-164.0)	0.07	44.6 (26.5-78.4)	71.3 (34.0-89.9)	0.07	66.0 (46.8-111.0)	93.5 (48.4-211)	**0.011**
DI	555 (368-942)	789 (448-1750)	**0.028**	669 (368-1252)	798 (468-1314)	0.12	466 (334-738)	780 (411-1129)	**0.031**	873 (492-1533)	972 (459-1741)	0.12

IQR: interquartile range, Pre-t: pre-transfusion, Post-t: post-transfusion, HOMA-IR: homeostatic model assessment of insulin resistance, WBISI: whole body insulin sensitivity index, HOMA-β: homeostatic model assessment of β-cell function, IGI: insulinogenic index, DI: disposition index

Among patients with chronic renal failure treated with hemodialysis, moderate to severe anemia was also a risk factor for insulin resistance (10,11,12,27). Partial correction of anemia with erythropoietin was shown to reduce insulin resistance as well as reduce insulin secretion (11,12). In chronic renal failure, erythropoietin treatment improved the anemic state with no change of iron status (11,12). In contrast, blood transfusions in thalassemic patients improve the anemic state, but lead to increased iron load.

Considering the present study, acute iron loading from blood transfusion with an increase in serum ferritin about 400 ng/mL concomitant with an increase in Hb of about 1.5 g/dL caused increased insulin secretion and may have had a detrimental effect on insulin sensitivity. In addition, the acute change in total body iron within only a week may cause increased insulin resistance in chronically transfused patients, particularly in those with relatively high pre-transfused serum ferritin. As shown in the multivariate analysis, pre-transfused serum ferritin was the only factor associated with insulin sensitivity indices.

To the best of our knowledge, acute effects of blood transfusion on insulin sensitivity and β-cell function have not been reported in thalassemia patients. Early changes in insulin sensitivity and β-cell function may help in understanding the sequences of pathophysiology underlying glucose dysregulation in thalassemia patients.

Study Limitations

There were some limitations in this study. Firstly, the majority of the patients were sub-optimally transfused and sub-optimally iron-chelated. A high iron store may mask the actual effect of correction of anemia on insulin sensitivity and β-cell function. Secondly, since β-thalassemia/HbE disease has a spectrum of severity, that is varied degrees of hemolysis and transfusion requirement, these may be important factors determining insulin sensitivity and β-cell function. Thirdly, a larger sample size is required for the subgroup analysis to distinguish the effects of anemia from iron overload on insulin sensitivity and β-cell function. Understanding the relationship between Hb and glucose homeostasis, independently of iron status would be beneficial in identifying optimal target Hb for maintaining near-normal insulin sensitivity and β-cell function.

Conclusion

Our study has demonstrated that acute increases in serum ferritin and Hb following blood transfusion in patients with β-thalassemia may contribute to an increase in insulin secretion and thus to a trend towards increased insulin resistance.

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Ethics

Ethics Committee Approval: This study was approved by the Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval number: 04-58-07; date: 22.04.2015).

Informed consent: A written informed consent was obtained from all patients and their legal guardians before the enrollment.

Peer-review: Internally peer-reviewed by all authors.

Authorship Contributions

Concept: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana, Design: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana, Data Collection and Processing: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana, Patcharin Khlairit, Sarunyu Pongratanakul, Analysis and Interpretation: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana, Literature Search: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana, Writing: Somboon Wankanit, Ampaiwan Chuansumrit, Preamrudee Poomthavorn, Pat Mahachoklertwattana.

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ORIGINAL ARTICLE

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Serum Nesfatin-1 Levels in Girls with Idiopathic Central Precocious Puberty

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What is already known on this topic?

Nesfatin-1, a recently discovered anorexigenic neuropeptide, seems to play an important role in hypothalamic pathways regulating food intake and energy homeostasis. There are a few reports suggesting the possible role of nesfatin-1 in metabolic regulation of reproductive function and the gonadotropic axis.

What this study adds?

This is the first study investigating the role of nesfatin-1 in human puberty. Although the sample size of our study is too limited to make precise comments, we found no evidence to support the role of nesfatin-1 in the regulation of human puberty.

Abstract

Objective: Nesfatin-1, an anorexigenic neuropeptide, is expressed mainly in the central nervous system and in some peripheral tissues. The role of nesfatin-1 in energy balance has been investigated. Despite the suggestion of a role for nesfatin-1 in reproductive function, data are limited on the role of nesfatin-1 in human puberty.

Methods: The aim of this study was to investigate the following: i) the role of nesfatin-1 in puberty, and ii) relationship between nesfatin-1 and anthropometric measurements and gonadotropin levels in girls with idiopathic central precocious puberty (CPP). Twenty-four girls with CPP (7.68 ± 1.02 years) and 20 female, prepubertal, healthy controls (7.48 ± 0.88 years) were enrolled in the study. All patients with CPP were treated by the intramuscular administration of leuprolide acetate at a daily dose of 3.75 mg for 28 days. Nesfatin-1 was measured before and during treatment.

Results: There was no difference in serum nesfatin-1 levels in girls with CPP and healthy controls [5.67 (2.5-20.6) mmol/L and 5.75 (2.51-9.64) mmol/L], respectively. There was a negative correlation between nesfatin-1 levels and body weight and body mass index-standard deviation score ($p = 0.01$, $r = -0.83$; $p = 0.025$, $r = -0.81$, respectively). No correlation was found between nesfatin-1 and gonadotropin, estradiol levels, uterine length or endometrial thickness.

Conclusion: The results of this study suggest that there are no differences between girls with CPP and healthy, prepubertal girls regarding nesfatin-1 levels.

Keywords: Leuprolide acetate, nesfatin-1, nucleobindin-2, precocious puberty

Introduction

Nesfatin-1, an 82-amino acid product of the post-translational processing of nucleobindin-2 (NUCB2), was initially described as an anorexigenic neuropeptide (1). Widespread expression of NUCB2 mRNA has been demonstrated in the central nervous system, especially in several hypothalamic nuclei and in forebrain-hindbrain areas that integrate both energy balance and reproduction (2). Several studies reported the co-localization of nesfatin-1 immunoreactive neurons with other neurotransmitters (including pro-opiomelanocortin, α-melanocyte-stimulating hormone and neuropeptide Y) that regulate food intake and with pituitary hormones (thyrotropine-releasing, growth hormone-releasing and corticotropin-releasing hormones). Immunohistochemical studies indicate that nesfatin-1/NUCB2 protein is expressed

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in peripheral organs including the stomach, pancreas, testes and the pituitary gland (3,4,5). The role of nesfatin-1 at the onset of puberty has been investigated in a few experimental studies. These studies have revealed data demonsrating the link between the neuroendocrine control of puberty and energy reservoirs and also the closer distribution of nesfatin-1 neurons to the key areas which control reproduction (6,7,8,9,10,11). These studies also showed that NUCB2 mRNA expression in hypothalamus, pituitary and testis changed during the pubertal transition (9,10). Furthermore, intracerebroventricular injection of nesfatin-1 in rats induced a significant increase in the serum levels of gonadotropins, mainly luteinizing hormone (LH) (6,9). In summary, evidence from rodent studies suggested that nesfatin-1; i) plays a role in the onset of puberty and in maturation and ii) affects the gonadotropic axis, mainly via increased secretion of LH (6,7,8,9,10,11). There is a close relationship between the adequacy of energy stores, adipose tissue and onset of puberty in children. The hypothalamo-pituitary-gonadal (HPG) axis has a capacity to respond to metabolic cues from energy stores. The most studied adipocytokine regarding the relationship between adipose tissue and puberty is leptin, which is an anorexigenic adipokine. Leptin plays an important role in the maturation of the HPG axis, and in onset and regulation of puberty, particularly in girls. However, there is no study investigating the role of nesfatin-1 in human puberty. In light of these findings, we hypothesised higher serum nesfatin-1 levels in girls with central precocious puberty (CPP). In this study, we aimed to investigate; i) serum nesfatin-1 levels in girls with CPP and ii) the relationship of nesfatin-1 with anthropometric parameters and gonadotropin levels.

Methods

Girls who received a diagnosis of idiopathic CPP were enrolled in the study. The main criteria for a CPP diagnosis were; i) onset of breast development (Tanner breast stage 2) before the age of 8, ii) progression of pubertal development during follow-up for at least six months, iii) accelerated linear growth and advancement of bone age (BA), and iv) a basal LH level of ≥1 IU/L or a peak LH >5 IU/L measured during an intravenous gonadotropin-releasing hormone (GnRH) stimulation test. The control group consisted of healthy, prepubertal girls (Tanner stage 1) of similar age and body mass index (BMI) to the CPP patients. None of the girls in the control group had acute or chronic illnesses nor used any kind of medication. Physical examination and anthropometric measurements of the patients were performed by the same clinician (A.A.). Pubertal staging of each subject was recorded according to the Tanner classification (12). Height was measured by a stadiometer and weight was measured by a calibrated scale. The BMI was calculated as weight divided by height squared (kg/m^2) and the BMI-standard deviation score (SDS) was calculated by using Centers for Disease Control and Prevention charts (13). All patients with CPP were treated with an intramuscular injection of leuprolide acetate (LA) at a daily dose of 3.75 mg for 28 days. A single X-ray of the left hand and wrist was performed to evaluate BA in the CPP group and the assessment of BA was performed by a single clinician, according to Greulich and Pyle (14). Pituitary magnetic resonance investigation (MRI) was performed on all patients. Pelvic ultrasonography for the evaluation of ovarian and uterine measurements was performed in all required patients.

Early morning blood samples were taken after 12 hours of overnight fasting and were immediately centrifuged. Serum samples were collected in Eppendorf tubes and stored at -80 °C until the day of analysis for nesfatin-1 levels. Early-morning basal serum levels of LH, follicle-stimulating hormone (FSH) and estradiol were measured in all patients.

The GnRH stimulation test with gonadorelin acetate (LHRH Ferring®, Ferring Pharmaceuticals Inc., Tarrytown, New York) was performed between 8 a.m. and 8.30 a.m. on subjects with basal LH <1 IU/L (15). GnRH (0.1 mg/m^2) was administered intravenously and samples for measuring FSH and LH were drawn at 20, 40, 60 and 90 minutes after the injection. A peak LH level of >5 IU/L was considered to be indicative of puberty (16). For the assessment of hormonal suppression, a repeat GnRH test (retest) was performed during the course of treatment. Retests were done three weeks after the third dose of LA. Peak LH levels of <2 IU/L were considered to be an adequate suppression of puberty (17).

Serum nesfatin-1 measurement was performed using a human nesfatin-1, enzyme-linked immunosorbent assay commercial kit (Sunred Biological Technology, catalog 32 No. 201-12-4341, limit of determination 0.2-35 mmol/L) as recommended by the manufacturer's protocol [Sensitivity: 0.113 mmol/L; Intra-Assay: coefficient of variability (CV) <10%; Inter-Assay: CV <12%]. The study design complied with the Declaration of Helsinki. Patient enrollment was started after the approval of the Ethics Committee of Pamukkale University (approval number: 60116787-020/25027). Informed written consent was obtained from the parents.

Statistical Analysis

Data were analyzed using SPSS 17.0 computer software (SPSS, Chicago, Illinois, USA). Variables were given as the median (range). A Wilcoxon (two-related sample) test was used to compare medians of pretreatment and post treatment nesfatin levels of girls with CPP. The Mann-

Whitney U test was used to compare the medians of the study and control groups. Univariate correlation analysis was performed using the Spearman test. A p value of < 0.05 was considered to be statistically significant.

Results

The study group consisted of 24 girls with CPP and 20 healthy prepubertal controls. The clinical and laboratory characteristics of patients are summarized in Tables 1 and 2. The median age interquartile range (IQR) at the time of treatment was 8.24 (6.6-10) years. Five patients (20.8%) were at Tanner stage 2, 12 (50%) at stage 3 and seven (29.2%) at stage 4 of pubertal development at the time of treatment. Eight patients (33.3%) underwent menarche before the age of 9.5 years. Median IQR BA at the time of treatment was 11 (8.8-12) years. The nesfatin-1 level of the CPP group was slightly higher than that of the healthy control group; however, the difference was not significant [5.67 (2.5-20.6) and 5.75 (2.51-9.64) mmol/L, respectively, p = 0.32]. Also, there was no difference in nesfatin-1 levels prior to or during treatment (Table 1). There was a negative correlation between nesfatin-1 and body weight, BMI-SDS (p = 0.01, r = -0.83; p = 0.025, r = -0.81, respectively). Nesfatin-1 was not correlated with basal LH, basal FSH, basal estradiol, stimulated peak LH, retest peak LH, uterine length, endometrial thickness or pubertal stage in the CPP group.

The pituitary MRI of all patients was normal.

Discussion

There are only a few studies on the role of nesfatin-1 in the HPG axis. Increased expression of hypothalamic NUCB2/nesfatin-1 during pubertal transition has been reported in female rats (9). Additionally, the central administration of nesfatin-1 induced elevation of circulating gonadotropins, especially LH in rodents (9). García-Galiano et al (8) reported that the expression of nesfatin-1 in mature Leydig cells was under the control of pituitary LH secretion. In contrast to these studies, a suppressive effect of nesfatin-1 on the hypothalamo-pituitary-ovarian axis of goldfish and rats has been demonstrated (10,18). Intracerebroventricular injection of nesfatin-1 was reported to significantly decrease the expression of the hypothalamic genes for *GnRH,* kisspeptin (*Kiss-1*), and pituitary genes for *FSH*β, *LH*β (18). However, these experimental studies were performed on non-mammalian vertebrates. To date, only a few reports exist on the role of nesfatin-1 in the human HPG axis. Çatlı et al (19) reported that serum nesfatin-1 levels were higher in girls with premature thelarche compared to those in healthy prepubertal controls. However, these researchers did

Table 2. Biochemical and radiological characteristics of patients with central precocious puberty

	Mean ± SD	Median (range)
Basal LH (IU/L) (n = 24)	2.22 ± 2.31	1.35 (0.05-12.60)
Basal FSH (IU/L) (n = 24)	3.36 ± 1.6	2.86 (1.82-4.96)
Peak LH (IU/L) (n = 9)	10.31 ± 5.51	8.45 (6.10-18.25)
Peak FSH (IU/L) (IU/L) (n = 9)	9.47 ± 1.59	9.12 (8.08-11.59)
Re-test peak LH (IU/L) (n = 24)	1.38 ± 0.79	1.14 (0.74-2.90)
Uterine length (mm) (n = 15)	43.16 ± 5.77	40.5 (38-51)
Endometrial echo (mm) (n = 10)	4.16 ± 1.72	4 (2-7)

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

Table 1. The clinical characteristics of patients with central precocious puberty and the control group

	CPP (n = 24)		Controls (n = 20)	p
Age at presentation (years)				
Median (range)	7.61 (6.0-9.5)		7.56 (5.7-9.0)	0.63
Height SDS				
Median (range)	1.25 (-0.70-2.10)		0.06 (-1.2-1.68)	< 0.05
Weight SDS				
Median (range)	1.12 (-0.52-2.19)		-0.07 (-0.2-1.8)	0.06
BMI-SDS				
Median (range)	1.0 (-0.5-1.2)		0.8 (-0.4-1.7)	0.07
Serum nesfatin level (mmol/L)	**Pre-treatment**	**During treatment**		
Median (range)	5.67 (2.5-20.6)	5.59 (3.10-17.13)	5.75 (2.51-9.64)	0.52*/0.32**

*Wilcoxon test between pre-treatment and under treatment nesfatin-1 levels, **Mann-Whitney U test, between pre-treatment nesfatin-1 and healthy control
BMI: body mass index, SDS: standard deviation score, CPP: central precocious puberty

not find a correlation between nesfatin-1 and gonadotropin levels. In a study by Abaci et al (20), there was no difference in nesfatin-1 levels between pubertal and prepubertal obese children (1.2 ± 1.2 ng/mL and 1.3 ± 2.0 ng/mL, respectively). In contrast to this study, Anwar et al (21) reported that there was a rise in the nesfatin-1 level as the pubertal stage advanced in both obese and healthy children. In this study, we did not find a difference in nesfatin-1 levels in girls with CPP and healthy controls. Also, there was no correlation between nesfatin-1 and gonadotropin levels, estradiol and pubertal stage. Intra-individual comparison of nesfatin-1 levels (pretreatment and during treatment) was not different. This finding suggested that circulating levels of nesfatin-1 may not have a role in the maturation of the HPG axis in the human. It is not possible to measure the central concentration of nesfatin-1 in humans. Thus it is not possible to investigate in humans its reported central role suggested by animal studies. There is a controversy in the literature about the relationship between nesfatin-1 and body weight and BMI-SDS. A positive correlation was reported between serum nesfatin-1 and BMI-SDS in obese children in previous studies (21). Serum nesfatin-1 was found to be significantly higher in obese children than in control groups. Additionally, these same authors reported a positive correlation between serum nesfatin-1 with serum insulin, BMI-SDS, body fat % and fat mass. In addition Ustabaş Kahraman et al (22) have reported lower nesfatin-1 levels in underweight children compared to healthy controls. In contrast to these findings, Abaci et al (20) found a negative correlation between nesfatin-1 and BMI-SDS in obese children, and nesfatin-1 levels were lower in obese children than in healthy controls. It was speculated that lower levels of nesfatin-1 might be the reason for uncontrolled appetite in obese children. In the present study, we found a negative correlation between nesfatin-1 and BMI-SDS. However, obese children were excluded from our study group and median BMI-SDS was within the normal range in this group.

Study Limitations

Our study has limitations. Firstly, we have a relatively small number of participants, so this study could be considered as a preliminary report. Secondly, indices of adiposity such as body composition, fat distribution or waist circumference measurements were not investigated in our study.

Conclusion

In conclusion, the results of our study suggest that there are no differences in nesfatin-1 concentrations between girls with CPP and prepubertal girls. Further, larger scale studies, including those on the expression of nesfatin-1/NUCB2 in the pituitary and in gonads in humans with CPP, are needed to clarify this matter.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Pamukkale University (approval number: 60116787-020/25027).

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

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ayça Altıncık, Concept: Ayça Altıncık, Design: Ayça Altıncık, Data Collection or Processing: Ayça Altıncık, Oya Sayın, Analysis or Interpretation: Ayça Altıncık, Oya Sayın, Literature Search: Ayça Altıncık, Writing: Ayça Altıncık, Oya Sayın.

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An Assessment of Retinal Nerve Fiber Layer Thickness in Non-Diabetic Obese Children and Adolescents

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What is already known on this topic?

Obesity affects almost all systems in the body and can also cause injury to the retinal nerve fibers due to a chronic inflammatory process. The literature concerning this issue is scarce. Optical coherence tomography may show early retinal damage.

What this study adds?

Retinal nerve fiber layer thickness was found to be decreased in both obese and non-obese children as body mass index standard deviation score values increased.

Abstract

Objective: Obesity affects almost all systems in the body. This includes the retinal nerve fibers which may be damaged due to a chronic inflammatory process. To determine changes in retinal nerve fiber layer (RNFL) thickness in non-diabetic children and adolescents using optical coherence tomography (OCT) and to evaluate the relationship between this change, metabolic risk factors and pubertal stage.
Methods: Thirty-eight obese and 40 healthy children and adolescents aged 10-18 years were included in the study. RNFL measurements from the optic disk and all surrounding quadrants were obtained using OCT from both eyes of the individuals in the study groups. Correlations between RNFL thickness and age, auxological measurements, pubertal stage, systolic and diastolic blood pressure, homeostasis model assessment-insulin resistance (HOMA-IR) index and lipid values were investigated.
Results: A general decrease was observed in RNFL thickness in obese subjects compared to the controls, the decrease being highest in the inferior quadrant, although these differences were not statistically significant ($p > 0.05$). RNFL thickness was negatively correlated with body mass index (BMI) standard deviation score (SDS) in both groups (control group $r = -0.345$, $p = 0.029$; obese group $r = -0.355$, $p = 0.022$). Significant negative correlations were determined between diastolic blood pressure, HOMA-IR, low density lipoprotein cholesterol level and RNFL thickness ($r = -0.366$, $p = 0.024$; $r = -0.394$, $p = 0.016$; and $r = -0.374$, $p = 0.022$, respectively) in the obese group, while there was no association between these parameters and RNFL thickness in the control group.
Conclusion: In this cross-sectional study, no statistically significant difference in RNFL thicknesses between the obese and control groups was determined. However, RNFL thickness was found to decrease in both healthy and obese children as BMI-SDS values increased. Further prospective studies may be of benefit to determine whether the decrease in RNFL values might become more pronounced on long-term follow-up.
Keywords: Obesity, optical coherence tomography, children, retinal nerve fiber layer

Introduction

The optic nerve carries signals originating from the retina to the visual cortex. Progressive loss of vision may occur when the transmission of these signals is impaired (1,2). The measurement of retinal nerve fiber layer (RNFL) thickness is a valuable tool for demonstrating early retinal damage. Optical coherence tomography (OCT) measures the delay in the reflection of laser light reflected from the retina. The RNFL can thus be visualized in a painless and non-invasive manner (3). Since it is rapid and simple, the technique is widely used in several diseases of the optic nerve and

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retina (optic neuropathies, retinal damage due to a range of causes, retinal vascular changes, glaucoma and so on). It is particularly useful in pediatric ophthalmology (4). Obesity is currently increasing globally. It affects almost all systems in the body and is a significant risk factor for vascular diseases (5,6). There are few studies in children and adolescents in this regard and they are controversial. The purpose of this study was to evaluate changes in RNFL thickness using OCT imaging in non-diabetic obese children and adolescents and to assess the association between any changes found and metabolic risk factors and pubertal stage.

Methods

This prospective observational study was performed with the approval of the University of Health Sciences Tepecik Training and Research Hospital Medical Research Ethical Committee (approval number: 29/12/2014-20) and in line with the ethical principles of the Declaration of Helsinki.

Inclusion criteria for study and control subjects were;

1. Aged between 10-18 years old,

2. Not having neurological diseases,

3. Not having a history of ocular disease and/or surgery,

4. Children and their parents being compatible with the examinations,

5. Subjects with spheric values between -0.50 diopter (D) and +0.50 D.

Exclusion criteria for study and control subjects were;

1. Having diabetes mellitus or any systemic disease,

2. Being on continuous medication,

3. Not being sufficiently cooperative for OCT measurement.

An ophthalmological assessment of both eyes was conducted on 38 obese children and adolescents aged 10.1-17.2 years who presented to the University of Health Sciences Tepecik Training and Research Hospital Clinic of Pediatric Endocrinology, İzmir, Turkey, between January 2015 and May 2016, and on 40 healthy children and adolescents aged 10.2-18.0 years who acted as the control group. Informed consent was obtained from patients and their families. Demographic data for the obese and control groups were obtained from their medical files. The control group was randomly selected from volunteers aged 10-18 years who were within normal limits for body mass index (BMI) standard deviation score (SDS) and in whom ophthalmological examination revealed no pathological findings. Anthropometric parameters, blood pressure values and pubertal stages were assessed by an experienced pediatric endocrinologist. Pubertal stages were classified according to Tanner and Whitehouse (7). Height measurements, accurate to the nearest centimeter, were performed using a rigid stadiometer. Weight measurement was accurate to the nearest 0.1 kg using a calibrated balance scale with the subject unclothed. Obesity was diagnosed according to World Health Organization criteria (8). BMI was determined using the formula weight (kg)/height (m^2). Reference values established for Turkish children were employed to calculate SDS for weight, height and BMI (9). Blood pressure was measured in all cases after a period of resting and were repeated at least three times at 10 minute intervals. Subjects with systolic and/or diastolic blood pressure values greater than the 95th percentile were regarded as hypertensive (10). Blood glucose, insulin and serum lipids in obese cases were measured using an automatic analyzer from fasting venous specimens taken on the same day. Insulin resistance using the homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as fasting insulin (μIU/mL) × fasting glucose (mg/dL)/405 (11). All cases underwent comprehensive eye examinations by the same ophthalmologist. This included best corrected visual acuity, ocular motility examination and intraocular pressure using a Goldmann applanation tonometer, a detailed anterior segment assessment using a slit-lamp biomicroscope and optic nerve and retina examination using a 90 D lens. For pupil dilation, 1% cyclopentolate hydrochloride drops (Cycloplegin R; Abdi İbrahim İlaç Sanayi, İstanbul-Turkey) were used twice at 5 minute intervals, and the mean value was taken of three measurements performed 30 minute after the last drop using an auto-rephractometer device (Canon RK-F1). Subjects with spheric values between -0.50 D and +0.50 D were enrolled. RNFL thickness was measured using the OCT method (Spectralis HRA + OCT, 870 nm; Heidelberg Engineering, Heidelberg, Germany). In all cases, scans were carried out with pupillary dilatation under the identical level of dim room lighting by the same experienced technician. To avoid diurnal fluctuations, all OCT scans were performed at the same time in the morning. Internal fixation targets were employed in all tests together with a real-time eye tracking system in order to adjust for eye movements. RNFL thickness was determined around the disc with consecutive circular B scans (3.5 mm diameter). The thickness (between the interior margin of the internal limiting membrane and the exterior margin of the RNFL layer) was automatically segmented using the Spectralis version 6.3.2.0 software. Mean RNFL thicknesses were used in analyses. RNFL thickness measurements were taken from the optic disk

and all surrounding quadrants of both eyes. Statistical analysis was performed with mean right and left eye RNFL values. Control and obese group RNFL values were then compared. Correlations between RNFL thickness and age, body measurements, pubertal stages, systolic and diastolic blood pressure, HOMA-IR and lipid values were investigated.

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS 20.0; IBM, USA) software. The Kolmogorov-Smirnov test was used to assess the normality of the sample distribution. Mean and standard deviation values were given for all parameters. Partial correlations were used in relationship analysis for variables with normal distribution. Simple correlation analysis was applied to the variables for which normality was not provided. A p value <0.05 was considered statistically significant.

Results

No differences were found between the study and control groups in terms of age, gender distribution, pubertal stages or systolic and diastolic blood pressure values ($p>0.05$). BMI-SDS values were 0.5 ± 0.4 in the control group and 3.0 ± 0.4 in the obese group ($p<0.001$). No difference was observed between the groups in terms of fasting blood glucose values (control group: 82.1 ± 8.8 mg/dL, obese group: 85.3 ± 9.9 mg/dL, $p=0.65$). Fasting insulin and HOMA-IR values were significantly higher in the obese subjects compared to the controls (fasting insulin: 19.6 ± 9.8 vs. 8.3 ± 3.1 mIU/mL, respectively, $p=0.02$; HOMA-IR: 4.7 ± 2.7 vs. 1.9 ± 0.7, respectively, $p=0.01$). There was no difference between the groups in terms of serum lipid levels ($p>0.05$). Clinical and laboratory characteristics of the groups are shown in Table 1.

No difference between the sexes or between the two eyes was found at RNFL thickness evaluations using OCT imaging ($p>0.05$). RNFL thickness was lowest in both the control and obese groups in the nasal quadrant, followed by the temporal, superior and inferior quadrants in respective order. A general decrease in RNFL thickness was observed in obese subjects compared to controls, ranging from 2 % to 7 % in mean values, the greatest change occurring in the inferior quadrant, although these differences were not statistically significant. Obese and control group RNFL thickness measurements are shown in Table 2. Correlation analyses of RNFL with age, pubertal stage, BMI-SDS, blood pressure and metabolic parameters were performed. No correlation between RNFL thickness and age, pubertal stage, systolic blood pressure, fasting glucose, fasting insulin, triglyceride or high-density lipoprotein cholesterol were determined in the groups ($p>0.05$). RNFL thickness was negatively correlated with BMI-SDS in both groups (control group $r=-0.345$, $p=0.029$; obese group $r=-0.355$, $p=0.022$). Significant negative correlations were determined between diastolic blood pressure, HOMA-IR, low density lipoprotein-cholesterol level and RNFL thickness ($r=-0.366$, $p=0.024$; $r=-0.394$, $p=0.016$; and $r=-0.374$, $p=0.022$, respectively) in the obese group, while there was no association between these parameters in the control group. Correlations between clinical and laboratory values and RNFL thickness in the study and control groups are shown in Table 3.

Table 1. Clinical and laboratory characteristics of the study and control groups

Clinical/laboratory characteristics	Controls (n = 40)	Obese subjects (n = 38)	p[a]
Gender (male/female)	21/19	18/20	0.81[b]
Age (years)	12.9 ± 2.4	12.8 ± 2.1	0.99
Puberty stage (pre-pubertal/pubertal)	11/29	10/28	0.98[b]
BMI-SDS	0.5 ± 0.4	3.0 ± 0.4	< 0.001
Systolic BP (mmHg)	106.1 ± 9.1	111.8 ± 9.4	0.19
Diastolic BP (mmHg)	66.3 ± 6.7	69.2 ± 9.3	0.28
Fasting glucose (mg/dL)	82.1 ± 8.8	85.3 ± 9.9	0.65
Fasting insulin (mIU/mL)	8.3 ± 3.1	19.6 ± 9.8	0.02
HOMA-IR	1.9 ± 0.7	4.7 ± 2.7	0.01
Triglycerides (mg/dL)	125.3 ± 62.0	138.5 ± 72.9	0.07
LDL-cholesterol (mg/dL)	89.3 ± 18.6	96.9 ± 25.7	0.06
HDL-cholesterol (mg/dL)	46.1 ± 11.3	43.2 ± 10.3	0.72

[a]Student's t test, [b]chi-square test

BMI-SDS: body mass index-standard deviation score, BP: blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low density lipoprotein, HDL: high density lipoprotein

Table 2. Mean ± standard deviation retinal nerve fiber layer thickness in controls and obese children

Retinal nerve fibre layer thickness	Controls (n = 40)	Obese subjects (n = 38)	p
Central (μm)	100.3 ± 8.9	98.4 ± 10.3	0.949
Superior (μm)	124.6 ± 19.7	120.1 ± 21.3	0.687
Nasal (μm)	73.1 ± 14.1	71.3 ± 14.5	0.772
Inferior (μm)	129.7 ± 23.0	121.2 ± 25.2	0.072
Temporal (μm)	76.0 ± 10.7	74.6 ± 19.1	0.449

Table 3. Correlation analysis of retinal nerve fiber layer with the clinical and laboratory parameters of the obese and control groups

	Controls		Obese subjects	
Parameters	**r**	**p**	**r**	**p**
Age	0.261	0.087	0.291	0.091
Puberty stage	-0.232	0.065	-0.312	0.056
BMI-SDS	-0.345	0.029	-0.355	0.022
Sistolic BP	0.021	0.872	0.051	0.760
Diastolic BP	0.056	0.503	-0.366	0.024
Fasting glucose	0.057	0.612	-0.175	0.211
Fasting insulin	0.061	0.456	-0.256	0.072
HOMA-IR	0.011	0.919	-0.394	0.016
Triglycerides	0.034	0.657	0.146	0.401
LDL-cholesterol	0.081	0.552	-0.374	0.022
HDL-cholesterol	0.078	0.671	0.024	0.921

BMI-SDS: body mass index-standard deviation score, BP: blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low density lipoprotein, HDL: high density lipoprotein

Discussion

Obesity and severe obesity have become a growing problem in children in recent years (5,6). The effect of obesity has been extensively investigated. However the effect of obesity on visual health, including RNFL, is one area in which there is a scarcity of data. RNFL thickness values in the children and adolescents with normal BMI SDS measured using OCT in this study were similar to those reported in the literature (12,13,14,15). Pehlivanoğlu et al (15) investigated RNFL thicknesses in healthy children and reported the thinnest values in the nasal and temporal quadrant and the thickest values in the inferior and superior quadrants. In agreement with that study, we also observed that the RNFL in both obese and control groups was thinnest in the nasal quadrant, followed by the temporal, superior and inferior quadrants in respective order. Pehlivanoğlu et al (15) reported the mean RNFL thickness values of the right and left eyes in normal healthy Turkish children with a mean age of 10.7 years. However, measurements by age groups were not given separately in this study. The authors reported that the RNFL thickness did not change with age. In our study, RNFL thickness in 16 of the 38 cases (42.1%) in the obese group was below the normal values reported by Pehlivanoğlu et al (15). In the control group, mean RNFL values in 7 out of 40 (17.5%) patients were below the normal reference value. Clinical findings were not observed in any of the cases in which the RNFL thickness was lower than normal. Various studies have investigated the relationship between RNFL thickness values measured using OCT and variables such as age, sex and race. Budenz et al (16) reported a significant relationship between age and RNFL thickness, with a 2.2-μ decrease in RNFL thickness for every 10-year increase in age. El-Dairi et al (17) reported that RNFL thickness measurements in the under-18 years old population were not age-dependent. In our study in children and adolescents, RNFL thickness values were not correlated with age. RNFL thickness values in the pediatric and adolescent age group can exhibit ethnic variation. Studies performed in the Turkish population show that mean RNFL thickness values are compatible with general values reported for Caucasians (18). The mean values in our study were also consistent with this. Although there was no statistically significant difference between the obese and control group RNFL values, a decrease was observed in all quadrants in the obese group compared to the controls, this change being greatest in the inferior quadrant. Similar studies have reported inconsistent results previously. In their study of obese children aged between 5 and 14, Pacheco-Cervera et al (2) reported a significant decrease in RNFL values in the severely obese group (BMI-SDS > 4). A negative correlation was also determined in that study between RNFL values and serum leptin and interleukin (IL)-6 levels. In a study in adults, no correlation was reported between BMI and RNFL thickness in women, but a decrease was found in RNFL in men as BMI increased (19). Elía et al (20) found no relationship between BMI and RNFL thickness measured using OCT in healthy children. Karti et al (21) investigated 55 obese and 33 healthy children and reported a negative correlation between BMI-SDS and RNFL values. The presence of a refraction defect can cause inaccurate measurement of OCT and RNFL values (22,23). The study of Karti et al (21), included patients with high refractive status (up to 5 D), a factor which may have affected RNFL values by OCT. In our study, the refraction values of all the cases that were recruited were between -0.5 and +0.5 D. We thus excluded any error caused by refraction defect from this study. There was no statistical difference in our study between the controls and the obese group in RNFL values despite a generalized reduction in RNFL in the obese subjects. However, correlation analysis revealed that RNFL thickness in both groups decreased as BMI-SDS values increased. The reason for the decrease in RNFL thickness in obese subjects is unclear. Pacheco-Cervera et al (2) suggested that RNFL values decreased with an increase in inflammatory mediators. Obesity is known to involve low levels of systemic inflammation (24,25,26). This long-term state of chronic inflammation may result in a decrease in RNFL values (27). It has been hypothesized that neuronal cell damage may occur in obesity due to changes in levels

of hormones such as leptin and adipokines, and due to oxidative stress. Retinal ganglion cell (RGC) death occurs via apoptosis following axonal injury. The production of reactive oxygen species (ROS) is an important factor in RGC necrosis and apoptosis (21). Mac Nair et al (28) suggested that ROS can initiate RGC loss following axonal injury. This suggestion is supported by animal studies (29,30). Long-term chronic inflammation associated with obesity may cause a decrease in RNFL thickness through oxidative stress. Further studies are needed to clarify this hypothesis. Also there was a negative correlation between BMI-SDS and RNFL thickness in our healthy control group with normal BMI. In children with normal BMI, the increase in body fat may affect RNFL thickness although, again, the mechanism is unclear. Detailed longitudinal follow-up studies are needed on this issue.

Study Limitations

As a limitation in this study, plasma levels of inflammatory mediators such as adiponectin, leptin and IL-6 were not measured. However, the effects of these adipokines in metabolic pathways were indirectly demonstrated by measuring insulin, glucose and lipid levels. The negative correlation between RNFL thickness and insulin resistance parameters supported the metabolic pathogenesis of retinal changes in obesity. No studies have shown whether changes may occur in RNFL values due to weight loss in obese subjects. Prospective observational studies involving weight control are needed to reveal the effect of obesity, and therefore of the chronic inflammatory process, on RNFL.

Conclusion

In conclusion, in this study we observed a decrease in RNFL thickness in both healthy and obese children and adolescents as BMI-SDS values increased. No statistically significant differences in RNFL thickness between the obese and control groups were found. The decrease in RNFL values may have been revealed more clearly if the patients had been monitored prospectively. The clinical significance of the decrease in RNFL thickness is as yet unclear.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Tepecik Training and Research Hospital Medical Research Ethical Committee (approval number: 29/12/2014-20).

Informed Consent: Consent form was obtained from patients and their families.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Bediz Özen, Hakan Öztürk, Design: Bediz Özen, Gönül Çatlı, Data Collection or Processing: Bediz Özen, Hakan Öztürk, Gönül Çatlı, Analysis or Interpretation: Bediz Özen, Hakan Öztürk, Literature Search: Bediz Özen, Gönül Çatlı, Bumin Dündar, Writing: Bediz Özen, Hakan Öztürk.

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ORIGINAL ARTICLE

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Could Alerting Physicians for Low Alkaline Phosphatase Levels Be Helpful in Early Diagnosis of Hypophosphatasia?

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What is already known on this topic?

Hypophosphatasia is a rare disorder with significant morbidity and mortality. A high level of alkaline phosphatase is commonly highlighted by biochemistry labs. Asfotase alpha is a new and effective medication for hypophosphatasia treatment.

What this study adds?

Unlike high alkaline phosphatase, low alkaline phosphatase is not always highlighted by biochemistry labs.
Identification of children presenting with non-specific clinical features and who have more than one reading of low alkaline phosphatase could help diagnose children with hypophosphatasia. Devising lab specific reference ranges for alkaline phosphatase is important to avoid missing abnormally low levels.

Abstract

Objective: Hypophosphatasia (HPP) is an inborn error of metabolism with significant morbidity and mortality. Its presentation is nonspecific leading to delayed or missed diagnosis. Low alkaline phosphatase (ALP) is a diagnostic test. Unlike high ALP, low level is commonly not flagged by laboratories as abnormal. A new treatment was shown to be effective in HPP. In this study we aimed to establish the frequency of low ALP levels requiring notification to physicians by the laboratory and also to describe the clinical manifestations of patients presenting with low ALP for a possible diagnosis of HPP.

Methods: Patients under age 18 years with low ALP levels were identified from biochemistry records over a period of 6 months. Reference ranges were used as per the Associated Regional and University Pathologists Reference Laboratory (Utah, USA). Electronic results for patients with low levels were checked for flagging as abnormal/low ALP results. Charts of identified patients were reviewed. Presenting features were categorized under groups of disorders.

Results: ALP levels were tested in 2890 patients. 702 had values less than 160 U/L. Of these patients, 226 (32%) had age/gender specific low ALP. None of the low ALP results was flagged as low. Twenty-one had more than one low reading and their charts were reviewed. Four patients in the neuromuscular and four in the miscellaneous group presented with features consistent with HPP despite these patients having no specific diagnoses.

Conclusion: Laboratories do not alert physicians in cases with low ALP levels. A persistently low level in patients with unspecified diagnoses could be a key to diagnose HPP. Implementing lab-specific ranges and alerting for low levels could prompt physicians to investigate for undiagnosed HPP.

Keywords: Alkaline phosphatase, hypophosphatasia, inborn error, laboratory, biochemistry

Introduction

Hypophosphatasia (HPP) is an inborn error of metabolism characterized by a low serum alkaline phosphatase (ALP) level due to a defect in the gene encoding the tissue-nonspecific isozyme of ALP (*TNSALP*) (1). Inheritance can be autosomal recessive or dominant. As many as 260 genetic mutations in the *TNSALP* gene have been associated with HPP (2). Penetrance is variable which results in a wide range of clinical features, with the spectrum ranging from

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stillbirth with no bone mineralization to early loss of teeth without bone symptoms. Clinically, there are six forms of HPP based mainly on age at presentation: perinatal (lethal); perinatal (benign); infantile; childhood; adult and odontohypophosphatasia (1,3). Severe forms of HPP (perinatal and infantile) are inherited as autosomal recessive traits and in milder forms (adult and odontohypophosphatasia), autosomal recessive and autosomal dominant inheritance coexist (4). Genotype is known to be associated with specific outcomes in the perinatal lethal type, whereas genotype/phenotype correlation is less pronounced in other, less severe forms (5). HPP causes major morbidity in patients with substantial bone disease, myopathy and weakness. Hypercalcemia associated with nephrocalcinosis is a known feature of HPP (1). Craniosynostosis and skull dysmorphology occur in around 40% of infants (6). HPP is almost always fatal early in life when severe skeletal disease is obvious at birth (1,7). Skeletal deterioration typically results in death from respiratory insufficiency (7). Bone fragility and recurrent fractures can be presenting features of HPP in childhood (8). The perinatal form might present with intractable seizures caused by secondary pyridoxine-deficiency encephalopathy. This is due to deficiency of ALP that is required for the metabolism of pyridoxal-5'-phosphate neurotransmitters (9). Accordingly, HPP should be considered in neonates presenting with convulsions responding to pyridoxine. Although some of the above features might point to the diagnosis of HPP, other presenting features of HPP can be less specific and include various symptoms and signs encountered in more common diseases (10). Accordingly, clinical diagnostic criteria for HPP are unspecific and confirming the diagnosis requires biochemical, radiological and possibly genetic testing. This fact has been a major reason for the disease to be both underdiagnosed and misdiagnosed (11). A low ALP is a key for differentiating the diagnosis of HPP from many other more common paediatric disorders (2). Alerts by biochemistry laboratories on abnormal levels of ALP are useful to draw attention to specific diagnoses. Although a high level of ALP is usually highlighted by biochemistry labs, low levels are not usually flagged. Alerting for low ALP level could be an opportunity for the early diagnosis of HPP patients presenting with nonspecific manifestations. Early detection of HPP will offer these patients the opportunity to benefit from a new enzyme replacement treatment that has recently been shown to be an effective modality to treat this potentially fatal disease (12,13). This study was designed to check if biochemistry laboratories alert physicians to low ALP levels and also on the necessity of examining clinical features in those patients who have persistently low ALP levels.

Methods

Using a cut-off level of 160 U/L, the electronic records of the Biochemistry Laboratory at Mafraq Hospital, covering a study period of six months, from July 2014 to Dec 2014, were screened for patients aged 18 years or under who had low ALP readings (phase I). As the study was based on charts review, no consent was deemed necessary as per the local research and ethics committee who approved the study. The cut-off value of 160 U/L was selected based on the Associated Regional and University Pathologists (ARUP) Reference Laboratory (Utah, USA) online test directory, being the highest level of the low range of ALP (14) (www.aruplab.com). The list of patients with low ALP was filtered by age and gender in accordance with the ARUP lab reference ranges (phase II). The biochemistry laboratory records were also evaluated for highlighting abnormally. Patients who had at least two readings of ALP lower than normal value per age and gender together with no other normal values had their charts reviewed (phase III).

Three groups of patients were excluded:

- Patients who had two low values of ALP but had one or more normal value detected on subsequent testing.

- Those with a single low value of ALP with a normal value after or before.

- Only one borderline normal value at one presentation of acute illness with no further history of illness.

In the remaining records, a list of the main diagnoses of the patients was made and stratified into subcategories (phase IV). Details of the patients' presentations, working diagnoses and features suspicious of HPP were noted. The categories of diseases linked with a possible diagnosis of HPP included musculo-skeletal, rheumatological, neurological, renal, respiratory-related diseases and fractures. The approximate number of patients with suspicion of HPP was estimated and their presenting features reported for each disease category was noted for use in further studies. The study was approved by the Research and Ethics Committee at Mafraq Hospital (approval number: MAF-REC-12/2015_06). The ALP level was measured on a fully automated Roche Cobas® 8000 modular analyzer series c701 system (Roche Diagnostics GmbH, Mannheim, Germany, 2010). The assay is a basic, standardized, colorimetric assay traceable to the International Federation of Clinical Chemistry Reference Gen2 method as an optimized assay. ALP is measured in a reaction whereby ALP catalyzes the cleavage of phosphate from 4-nitrophenyl phosphate to form 4-nitrophenoxide (benzenoid form), which undergoes spontaneous rearrangement at alkaline pH to the quinonoid form (yellow

color). The reaction is followed by measuring absorbance of the reactant color at 405 nm on the automated analyzer detection system. The ALP assay performance specifications include an analytical measuring range of 5-1200 U/L, with a lower detection limit (analytical sensitivity ie the lowest measurable analyte level that can be distinguished from zero) of 5.00 U/L. The assay has a clinically reportable range of 5.00-6000 U/L. The ALP assay has a within-run precision coefficient of variation (CV%) of 0.7% at an ALP mean of 84.3 U/L and of 2.4% at a mean of 92.8 U/L, while the assay demonstrates a CV of 0.5% at an ALP mean of 222 U/L and a CV% of 1.7% at a mean of 224 U/L. The inter-individual CV is 6.7%, with an intra-individual CV of 25.4% and a critical significant difference of 37%.

Results

During the six-month study period, there were 2890 tests for ALP performed in subjects 18 years of age or younger. In phase I of the study, this number was reduced to 702 patients who had readings below 160 U/L. None of the low levels of ALP was flagged as abnormal by the biochemistry lab. Age stratification for normal reference values was performed, resulting in 349 patients being selected (phase II). Further filtering was done to this group to match reference range with gender. This reduced the number of patients to 226 at this stage (phase III). Of those, 1 patient (male) was in the age bracket of 16-18 years, 24 (20 males) between 14-15 years, 24 (14 males) between 12-13, 19 between 10-11 years (NB from this age and younger, there is no gender difference in the quoted lower normal value for ALP), 35 between 7-9 years, 74 between 4-6 years, 48 between 1-3 years and 1 between 1-11 months (Table 1). Charts for all patients identified in phase III were reviewed. Two hundred and five patients were excluded as per the exclusion criteria and 21 patients were studied further (Figure 1). The 21 patients were classified under disease categories based on presentation and working diagnoses. These were; rheumatologic disorders (five patients), fractures (five patients), neuromuscular diseases (four patients), immobility and repeated fractures (three patients) and a miscellaneous group (four patients) (Table 2). The five patients in the rheumatology category had a confirmed diagnosis of systemic lupus erythematosus (three patients) and juvenile rheumatoid arthritis (two patients). Five patients had a single fracture of a long bone. Of those, one had a dislocated shoulder with a fracture of the humerus and another had orthodontic treatment for teeth malposition and crowding. Three patients were diagnosed with cerebral palsy and

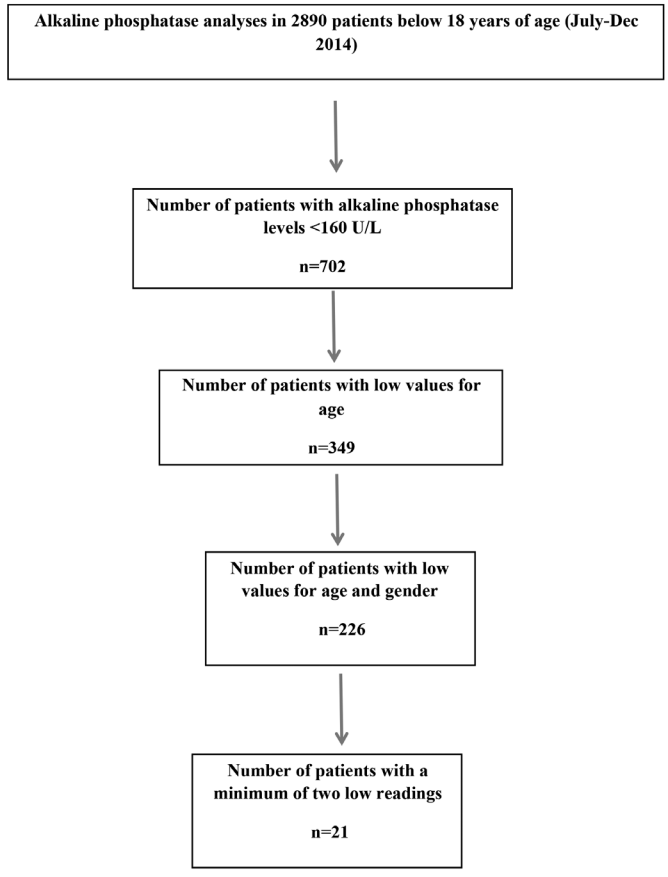

Figure 1. Flow chart showing of number subjects with low alkaline phosphatase levels by age and gender

Table 1. Numbers of patients with low alkaline phosphatase concentrations for age and gender (total 226)

Age range	16-19 (years)		14-15 (years)		12-13 (years)		10-11 (years)	7-9 (years)	4-6 (years)	1-3 (years)	1-11 (months)
Gender	F	M	F	M	F	M	No gender difference in reference range				
Lowest normal value U/L	40	60	55	130	110	160	150			125	70
Number of patients	0	1	4	20	10	14	19	35	74	48	1

F: female, M: male

Table 2. System involvement in 21 patients with persistent low alkaline phosphatase and repeated medical presentations

Category	Neuromuscular diseases	Rheumatological diseases	Repeated fractures and immobility	Single fractures	Other conditions/ associations
Number of patients	4	5	3	5	4
Clinical description					
Arthrogry-posis	SLE, lupus nephritis	Cerebral palsy, single fracture	One fracture (wrist)	Nemaline myopathy and kidney stones	
Multiple skeletal deformities	Juvenile rheumatoid arthritis	Cerebral palsy, repeated fractures	One fracture (radius and ulna)	Demyelinating sensory and motor neuropathy	
Arthrogry-posis and repeated fractures	SLE	Cerebral palsy, single fracture	One fracture, distal ulna	Recurrent infections	
Neuromuscular deformities and fractures	Juvenile rheumatoid arthritis		One humerus fracture and shoulder dislocation	Down syndrome, repeated ICU admission for respiratory infections. Short femur/humerus	
Juvenile rheumatoid arthritis					
One forearm fracture and teeth crowding					

SLE: systemic lupus erythematosus, ICU: intensive care unit

were immobile with repeated fractures. The neuromuscular category included four children who did not have definite diagnoses. Two had arthrogryposis, one of whom also had repeated fractures. One presented with multiple skeletal deformities and the fourth patient had neuromuscular deformities with fractures. The miscellaneous group included a child with Down syndrome who was diagnosed with short limbs antenatally and admitted to the intensive care unit repeatedly with recurrent chest infections. One child was diagnosed with nemaline myopathy and had kidney stones and another was diagnosed with severe demyelinating sensory and motor neuropathy. The fourth patient had repeatedly low ALP readings and suffered from recurrent infections. The selected patients could potentially have HPP as their presentation is quite unspecific and their ALP is persistently low. Further diagnostic testing (particularly genetic testing) is recommended in these scenarios. This is mentioned below as a limitation of our study.

Discussion

HPP is a disease that is associated with major co-morbidity and poor prognosis. The wide range of presenting features which are non-specific constitutes a complicating factor in its diagnosis (11). In the past, various treatment approaches have been tried to treat the severe form of the disease with poor results. Treatment modalities included transplantation therapy using bone fragments and cultured osteoblasts (7), infusion of enriched plasma with ALP from patients with Paget disease (15), bone marrow transplant (16) and conservative treatment using low calcium milk and pamidronates (17). Calcitonin and chlorothiazide have been used to reduce calcium level, which can reach very high levels (18). Bisphosphanates are pyrophosphate analogs and can precipitate the disease progression. Patients with undiagnosed HPP presenting with fractures and osteoporosis and treated with bisphosphanate are reported to progress into renal failure (19) and using bisphosphanate to treat HPP is currently contraindicated. Asfotase Alfa is a recombinant, fusion protein comprising the *TNSALP* ectodomain and a terminal deca-aspartate motif for bone targeting (20). It has been used in clinical trials and was shown to enhance healing of skeletal abnormalities and improve respiratory and motor dysfunction (12). Asfotase alfa has now been approved by the European Medicines Agency for use in patients with HPP (13).

In our cohort, we detected a group of patients who had low ALP levels, but no specific diagnosis (Table 2). Despite the low ALP level in more than one occasion of testing, there was no alert by the biochemistry laboratory drawing attention to the low value. In two groups of patients, those with rheumatic diseases and those with fractures causing immobility, the ALP abnormality could be possibly attributed to the underlying disease. In a third group of patients, those with single fractures, the patients were healthy otherwise and unlikely to have an undiagnosed HPP. However,

some patients in the neuromuscular disorder group (four patients) and the miscellaneous group (four patients) are worth examining further to rule out the possibility of HPP. Two patients in particular in the miscellaneous group had repeated episodes of chest infection and intensive care unit admissions and one had a kidney stone. The four patients in the neuromuscular group had skeletal deformities and fractures and they did not have a specific diagnosis. They, too, qualify for further investigations to exclude HPP. High levels of ALP can be encountered in a variety of bone disorders, but low levels are not as frequently seen in clinical practice. A high ALP level is routinely flagged up by the biochemistry lab but this is not the case for low ALP levels. Biochemistry labs need to have reference ranges for ALP levels to highlight possible abnormalities particularly in case of associated hypercalcemia. The ALP assay is widely available and is a fairly inexpensive test. It is a key for diagnosing HPP and makes a good screening test to diagnose HPP for which an effective treatment is now available.

Study Limitations

The main limitation of the study is that we did not confirm the diagnosis of HPP in those suspected cases where a definite cause for the ALP was not reached. Further plans for genetic testing on such suspected cases will facilitate the diagnosis.

Conclusion

We conclude that persistent low ALP levels in patients presenting with non-specific signs and symptoms can be used as a guide to further investigate and exclude HPP. This is particularly important because medication is now available for HPP and has been shown to be effective in ameliorating morbidity and improving quality of life in this disease. Accordingly, the alerting of physicians to low levels of ALP by biochemistry labs can be very useful. We highlight the importance of having age and gender adjusted ALP reference ranges, specific for local laboratories or populations, to avoid missing the diagnosis of HPP. A clear plan of action needs to be drawn on how to proceed with patients with low ALP levels and non-specific presentation.

Ethics

Ethics Committee Approval: The study was approved by the Research and Ethics Committee of Mafraq Hospital (approval number: MAF-REC_12/2015_06).

Informed Consent: As the study involved patients chart reviews rather patients interview or samples taking, it was deemed by the ethics committee that informed consent is not necessary to undertake the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Asma Deeb, Concept: Asma Deeb, Abubaker Elfatih, Design: Asma Deeb, Abubaker Elfatih, Data Collection or Processing: Abubaker Elfatih, Analysis or Interpretation: Asma Deeb, Abubaker Elfatih, Literature Search: Asma Deeb, Abubaker Elfatih, Writing: Asma Deeb.

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High Prenatal Exposure to Bisphenol A Reduces Anogenital Distance in Healthy Male Newborns

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What is already known on this topic?

Bisphenol A (BPA) is suspected to alter genital development. Several animal studies have shown significant results regarding this issue. At the same time, several human studies have reported significant results on the relationship between BPA and genital development.

What this study adds?

To our knowledge, this is the first study investigating the relationship between cord blood BPA levels and anogenital measurements in healthy newborns.

Abstract

Objective: To estimate the relationship between cord blood bisphenol A (BPA) levels and anogenital measurements in healthy newborns.
Methods: Pregnancy and birth history, together with body mass and length data, anogenital measurements, penile measurements and cord blood samples were obtained from healthy newborns. Cord blood concentration of BPA was analyzed by sandwich enzyme-linked immunosorbent assays kit.
Results: Among 130 healthy newborns (72 boys, 58 girls), mean anopenile distance was 45.2 ± 6 mm and anoscrotal distance was 21.9 ± 5.4 mm in boys; mean anoclitoral distance was 33.8 ± 6.6 mm and mean anofourchette distance was 12.2 ± 4.9 mm in girls. Mean cord blood BPA level was 4.75 ± 2.18 ng/mL. 90th percentile value for cord blood BPA was 8.26 ng/mL and the analysis showed a statistically significant correlation between anoscrotal distance and cord blood BPA levels above the 90th percentile ($p = 0.047$) in boys. The changes in anogenital distance in girls were not statistically significant.
Conclusion: We showed a significant association between high cord blood BPA levels and shortened anoscrotal distance in male newborns. However, this result should be interpreted with caution since there were no significant external genital abnormalities in our study group.
Keywords: Anogenital distance, bisphenol A, cord blood, newborn

Introduction

Being first produced as a synthetic estrogen, bisphenol A (BPA) was later widely used in the plastic and resin production industry as a plasticizer due to its cross-linking qualities. Exposure of products containing BPA to increased temperatures was shown to cause leakage of this molecule into food and beverages (1,2). BPA is detectable in many body fluids including blood, urine, amniotic fluid, breast milk and cord blood. The anti-androgenic effect of this chemical has been widely researched and is suspected to be related to several disorders, both in childhood and adulthood (3). The main emphases of current studies are the effect of BPA on obesity, gonadal abnormalities, infertility, thyroid function and malignancy (4,5,6,7,8,9,10,11).

Anogenital distance (AGD) is a relatively new measurement parameter showing the distance from anus to the genitals. While it was used for sex determination in animals for a long time (12,13), the effort to implement this measurement

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as an epidemiological marker of genital development in humans is quite recent. Animal studies have shown a shortened AGD in male offspring reflecting decreased *in utero* androgen exposure and conversely a longer distance in females reflecting increased *in utero* androgen exposure (14).

In this study, we aimed to investigate the relationship between cord blood BPA levels and anogenital measurements in healthy newborns.

Methods

Near East Univestity Local Ethics Committee approval was obtained prior to the study (approval number: YDU/2015/32-215) and informed parental consent was obtained for each participant. One hundred and fifty healthy newborns up to three days of age who were born in the period of May-August 2016 were included in the study population. Infants who had congenital anomalies, perinatal asphyxia, major surgical operation and those who were hospitalized in the neonatal intensive care unit were excluded from the study. Pregnancy and birth history together with body mass and length data were obtained from the patients' hospital records.

Cord blood samples from the umbilical vein were obtained at birth and collected into BPA-free polystyrene tubes (BD Diagnostics Preanalytical Systems, BE). Each blood sample was left to coagulate for 30 minutes, then samples were centrifuged at 2000 g for 10 minutes at room temperature to obtain serum, which was stored in aliquots in BPA-free Eppendorf (Eppendorf AG, GE) vials at -80 °C until analysis. On the day of analysis, the aliquots were brought to room temperature and thoroughly vortexed before the analysis. Total serum concentration of BPA was analyzed by sandwich enzyme-linked immunosorbent assays (ELISA) kit (General Bisphenol A ELISA, MyBioSource, Inc., San Diego, California, USA) with a Spectramax M5 Series Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California, USA). The kit is characterized by a limit of detection for BPA of 0.6 ng/mL.

The anogenital measurement technique for the study was standardized as follows. The infants were placed in supine position, with flexed hips and knees to provide a "frog leg" posture. After marking the center of the anus with a pencil, the distances from the anus to the anterior base of the penis; anopenile distance (AGD_{AP}) and to the base of the scrotum; anoscrotal distance (AGD_{AS}) were measured in boys. In girls, the distance from anus to the anterior tip of the clitoral hood; anoclitoral distance (AGD_{AC}) and the posterior fourchette of labia majora; anofourchette distance (AGD_{AF}) were measured. Two blinded (digital screen turned away from the researcher) measurements per patient with digital Vernier caliper were obtained from five newborns by two different researchers as a pilot study to assure the right measurement technique. The study proceeded after the measurement consistency was ensured. All measurements were performed by one blinded researcher and the results were analyzed and interpreted by a second blinded researcher.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 for Macintosh (SPSS Inc., Chicago, Illinois, USA). The results are expressed as mean and standard deviation of the mean. To determine the relationship between principal variables and the other continuous variables, the Pearson correlation test was used. The Mann-Whitney U test was used to determine the relationship between grouped variables. A p value less than 0.05 was considered statistically significant.

Results

Twenty newborns were excluded due to the exclusion criteria, leaving 130 patients in the study group. This consisted of 72 (55%) boys and 58 (45%) girls. The mean birth weight of the group was 3172 ± 492 grams and mean birth length was 48.3 ± 2 cm. In boys, the mean AGD_{AP} was 45.2 ± 6 mm and AGD_{AS} was 21.9 ± 5.4 mm. In girls, the mean AGD_{AC} was 33.8 ± 6.6 mm and mean AGD_{AF} was 12.2 ± 4.9 mm. Mean cord blood BPA level was 4.75 ± 2.18 ng/mL (Table 1). The 90th percentile value of the cord blood BPA was 8.26 ng/mL. None of the patients had any obvious genital development abnormality.

In general, anogenital measurements did not show statistically significant correlations with the cord blood BPA levels ($p > 0.05$). However, a significant negative correlation was found between AGD_{AS} and cord blood BPA levels above the 90th percentile ($p = 0.047$) in boys. AGD_{AS} mean value was significantly lower in the group with cord blood BPA levels above 90th percentile (higher than 8.26 ng/mL). In contrast, an apparent but not statistically significant increase was noted in AGD_{AP} with increased levels of BPA. In girls, a statistically nonsignificant increase in the AGD_{AC} and a similar decrease in the AGD_{AF} was found in the group with high cord blood BPA levels (Table 2).

Table 1. Anthropometric measurements (anopenile distance, anoscrotal distance, anoclitoral distance, anofourchette distance) and cord blood bisphenol A levels

Measurement	Mean ± SD	Range
Birth weight (g)	3172 ± 492	1540-4525
Birth length (cm)	48.3 ± 2	40-52
AGD_{AP} (mm)	45.2 ± 6	10.3-57.9
AGD_{AS} (mm)	21.9 ± 5.4	7.9-36.5
AGD_{AC} (mm)	33.8 ± 6.6	12.3-45.4
AGD_{AF} (mm)	12.2 ± 4.9	7.2-36.5
Cord blood BPA level (ng/mL)	4.75 ± 2.18	1.58-10.8

SD: standard deviation, AGD: anogenital distance, AGD_{AP}: anopenile distance, AGD_{AS}: anoscrotal distance, AGD_{AC}: anoclitoral distance, AGD_{AF}: anofourchette distance, BPA: bisphenol A

Table 2. The relationship between anogenital (anogenital distance, anopenile distance, anoscrotal distance, anoclitoral distance, anofourchette distance) measurements and 90th percentile of cord bisphenol A values

	Mean value in group under 90th percentile for BPA	Mean value in group above 90th percentile for BPA	p
AGD_{AP} (mm)	45.2	45.7	0.956
AGD_{AS} (mm)	22.3	17.7	0.047
AGD_{AC} (mm)	33.5	35.4	0.471
AGD_{AF} (mm)	12.2	12	0.652

AGD: anogenital distance, AGD_{AP}: anopenile distance, AGD_{AS}: anoscrotal distance, AGD_{AC}: anoclitoral distance, AGD_{AF}: anofourchette distance, BPA: bisphenol A

Discussion

BPA, a well-known endocrine disruptor has been investigated for more than a decade. Leaching of this molecule from daily used plastic products is highly dependent on heat, on contact with chemicals and deterioration of the product itself (15,16). Drinking water carries risk of pollution by BPA as plastic bottles used for household water dispensers are being reused and exposed to high temperatures during the cleaning process. BPA is suspected to have an anti-androgenic effect on genital development *in utero*. Studies regarding the prenatal effect of BPA on fetal development have mostly been performed in rodents and confirm adverse effects of increased BPA exposure on the growth and genital development of the offspring (14,17). The mechanism of action of BPA is thought to be through its binding to estrogen receptors thus triggering their activation. However, some authors do not attribute the anti-androgenic action solely to estrogen receptor activation as BPA has the ability to interact with other receptors such as aryl hydrocarbon receptor (the androgen receptor behaving as an antagonist) and the seven transmembrane domain estrogen receptor (G protein-coupled receptor 30) (18,19).

Several human studies have reported significant results regarding the relationship between BPA and genital development. Liu et al (20), in a study that compared maternal, urinary BPA and cord blood sex hormones, maternal urinary BPA was found to be negatively associated with cord blood testosterone levels. The authors proposed that BPA might decrease testosterone levels by affecting both the testes and the pituitary system or by inhibiting the testosterone surge *in utero*. The hypothesis that BPA may reduce testosterone acting as estradiol was also suggested by Nakamura et al (21) in a different study. The effects of endocrine disrupting molecules on androgens are thought to be more profound in the masculinization programming window (8-14 gestational weeks) during intrauterine life, especially in male offspring (22). The findings in our study are coherent with this postulate.

AGD measurement, as an epidemiological marker of sexual development, is still controversial due to conflicting results from different studies. Miao et al (23) showed a significant relationship between AGD in boys and their parents' occupational BPA exposure. In a study in adults, Eisenberg et al (24) found a significant association between serum testosterone levels and AGD. However, Parra et al (25) reported no significant relationship between anogenital measurements, reproductive hormone levels and semen quality.

To our knowledge, this is the first study investigating the relationship between cord blood BPA levels and anogenital measurements in neonatal human subjects. In our study, BPA values in cord blood were found to be higher than those reported in the literature (20,26,27,28). This may be due to poor governmental regulation of water supply companies in our country and the continuous reuse of plastic containers beyond their lifespan. We know that BPA is not the only phenolic endocrine disrupting substance that may be implicated in altering the reproductive development of the fetus, as prenatal exposure to other phenolic molecules and phthalates was also shown to alter AGDs (29). As other environmental pollutants were not measured during our investigation, we can neither confirm nor dismiss their potential adverse effects in our study group.

AGD is measured in different ways in different studies. AGD_{AS} in boys and AGD_{AF} in girls are mostly accepted as AGDs.

We measured the distances from anus to the anterior base of the penis (AGD_{AP}) and the base of the scrotum (AGD_{AS}) in boys; the distance from anus to the anterior tip of the clitoral hood (AGD_{AC}) and the posterior fourchette of labia majora (AGD_{AF}) in girls, as described by Sathyanarayana et al (30). The most important finding in our study population was that male newborns in the group with a cord blood BPA level over 90th percentile (8.26 ng/mL) had significantly shorter AGD_{AS} values compared to the group with lower cord blood BPA levels and this finding may reflect the anti-androgenic effect of BPA on fetal male genital development *in utero*. AGD_{AC} was longer in the group with high cord blood BPA levels, although this finding was not statistically significant. The AGD_{AF} did not show any major change with increased levels of BPA. Despite variations in AGDs, none of the patients in our study population had any external genital abnormality which suggests that these changes did not have a major impact on the development of the external genitalia. However, other adverse effects of BPA need to be further investigated.

Study Limitations

One of the major limitations of our study was the relatively small study sample. Larger series are needed to confirm our results. Another limitation is the high coefficient of variability for which we do not have a clear explanation.

Conclusion

The results of our study showed a significant association between high cord blood BPA levels and shortened AGD_{AS} in healthy male newborns. Based on our findings, we suggest that even if BPA has any effect on genital development *in utero*, this effect is subtle at low dose exposure.

Ethics

Ethics Committee Approval: The study was approved by the Near East Univestity. Local Ethics Committee (approval number: YDU/2015/32-215).

Informed Consent: Informed parental consent was obtained for each participant.

Peer Review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Emil Mammadov, Ceyhun Dalkan, Concept: Emil Mammadov, Ceyhun Dalkan, Design: Emil Mammadov, Ceyhun Dalkan, Data Collection or Processing: Emil Mammadov, Ceyhun Dalkan, Murat Uncu, Analysis or Interpretation: Emil Mammadov, Ceyhun Dalkan, Murat Uncu, Literature Search: Emil Mammadov, Writing: Emil Mammadov, Ceyhun Dalkan.

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The Evaluation of Cases with Y-Chromosome Gonadal Dysgenesis: Clinical Experience over 18 Years

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What is already known on this topic?

Gonadal dysgenesis is rare and is the most complicated subgroup of disorders of sexual development. It results from underdeveloped gonads. Small case series have been published.

What this study adds?

Characteristics of Y-chromosome gonadal dysgenesis are presented in a large group. Specific characteristics of patients that provided important clues for follow-up are discussed.

Abstract

Objective: Y-chromosome gonadal dysgenesis (GD) is a rare subgroup of disorders of sexual development (DSD) which results from underdeveloped testis and may exhibit heterogenous symptoms. These patients are phenotypically classified into two groups - complete and partial, and their karyotypic description is either 46,XY GD or 45,X/46,XY GD. In this study; we aimed to evaluate the characteristics of cases with Y-chromosome GD.

Methods: Thirty eight cases were followed-up between 1998 and 2016. The age of admission ranged between 0 and 17 years. Clinical and laboratory findings as well as follow-up characteristics of the cases were evaluated retrospectively from the patient files.

Results: There were 26 cases (four complete, 22 partial) in the 46,XY GD group, and 12 cases (four complete, 8 patients with complete GD in the 45,X/46,XY. Mean age at admission was 6.2 ± 4.6 years for all cases. Patients with complete GD in the 45,X/46,XY GD group were diagnosed earlier that the patients with complete GD in the 46,XY group [11 years vs. 14.31 years of age ($p < 0.01$)]. There were no additional findings in 55% of all patients. In the remaining 45% additional clinical findings, mainly short stature, were detected in 75% of the patients in the 45,X/46,XY GD and 30% of the patients in the 46,XY GD groups. All patients with complete 46,XY and 45,X/46,XY GD were raised as females. There was no gender dysphoria in patients that were raised as females, except for one case. Gonadectomy was performed in 14 patients, at a mean age of 8.75 ± 2.3 years and pathological assessment of the gonads was reported as normal in all cases.

Conclusion: Y-chromosome GD is a very heterogenous clinical and genetic disorder and requires a multifaceted approach to management. Whether including syndromic features or not, associated clinical features may lead to earlier diagnosis, especially in complete forms of GD. Due to difficulties encountered in the long-term follow-up of these patients, evaluation of appropriateness of sex of rearing decision is not truly possible. Performance of gonadectomy during the first decade appears be a preventive factor for tumor development since these tumors are usually seen during the second decade

Keywords: Gonadal dysgnesis, 46,XY, 45,X/46,XY

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Introduction

Gonadal dysgenesis (GD) is a rare condition and is the most complicated subgroup of disorders of sexual development (DSD) which result from underdeveloped gonads. DSDs are phenotypically classified into two groups, as complete and partial; while their karyotypic description is 46,XY GD and 45,X/46,XY GD which may both occur in either group (1,2,3). The term "Y-chromosome GD" is used for both 46,XY and 45,X/46,XY GD. Histologically 45,X/46,XY individuals can have bilateral streak gonads, bilateral dysgenetic gonads or a unilateral streak and contralateral dysgenetic gonad. This latter form is termed as mixed 45,X/46,XY GD (4,5). Complete Y-chromosome GD is characterized by female external genitalia, bilateral streak gonads and hypergonadotropic hypogonadism in 46,XY GD and 45,X/46,XY GD patients. Absence of anti-Müllerian hormone (AMH) leads to a normally developed Müllerian duct. Patients with partial Y-chromosome GD may present with variable degrees of impaired testicular development and testicular function. Phenotypic appearance is related to the level of functional testicular hormones (6). The amount of AMH production also determines the degree of regression of Müllerian structures. Patients with partial GD have bilateral dysgenetic testis or a unilateral dysgenetic testis and contralateral streak gonad with ambiguous genitalia (7). Sex chromosome abnormalities or mutation in genes of transcription factors which are required for normal development of gonads may lead to Y-chromosome GD. Several transcriptional factors such as SRY, SOX9, NR5A1, MAP3K1, GATA4, FOG2, DHH, CBX2 and ATRX all have roles in testicular development (8). Additional systemic findings are frequently seen in patients with Y-chromosome GD. It is accepted that 46,XY GD and 45,X/46,XY DSD with defective testis development or function are associated with the greatest risk of neoplasia (9). Thus there is extra concern about patients who have the increased risk of germ cell neoplasms in the dysgenetic gonads (7,10,11,12). Early and correct diagnosis of Y-chromosome GD has important clinical implications, not only for gonadal intervention because of the higher potential malignancy risk and for the timing of gonadectomy, but also for sex of rearing. In this study we aimed to evaluate the characteristics of the cases with GD followed-up in our clinic and provide the benefit of our long-standing experience which may be helpful in the diagnosis and treatment of these patients.

Methods

In this retrospective study, patients with Y-chromosome GD admitted to the pediatric endocrinology clinic between June 1998 and December 2016 were evaluated. Patients with Turner syndrome, Klinefelter syndrome, Ovotesticular syndrome and 46,XX GD were excluded from the study. Of patients with gonadal developmental disorders, nine had been diagnosed as ovotesticular syndrome and five as 46,XX GD. Patients with Y-chromosome GD consisted of 38 children and age at presentation ranged from newborn to 17 years old. Clinical and laboratory findings including the patients' phenotypic appearance, karyotypes, imaging of gonads and internal genital structures by ultrasonography and/or magnetic resonance imaging, histopathological evaluation of gonads, functioning of gonads, sex of rearing and additional systemic findings were evaluated. The diagnosis of complete Y-chromosome GD was considered in cases with bilateral streak gonads and female internal and external genitalia. The diagnosis of partial Y-chromosome GD was made by the findings of 46,XY or 45,X/46,XY karyotype, ambiguous genitalia, low testeosterone response to human chorionic gonadotropin (hCG) stimulation test at prepubertal ages, low basal testosterone levels at pubertal ages, low AMH levels, bilaterally or unilaterally dysgenetic gonads and presence of a contralateral streak gonad (13). External genitalia was rated using the Sinnecker classification (14). Laparotomy was performed as required. Pathological studies were obtained by laparotomy and/or gonadectomy and conducted in 20 patients. Histopathological examination of gonads was carried out either on resected or biopsied tissue. Chromosomal analysis was done by evaluating metaphase G bands prepared from peripheral blood lymphocytes. When required, specific molecular analysis [fluorescent *in situ* hybridization, polymerase chain reaction (PCR), gonadal tissue sytology] was performed. Patients with Y-chromosome GD were divided into 46,XY GD and 45,X/46,XY GD karyotypically. Hormonal assessment in serum samples was performed using the immuno-chemiluminescence method for luteinising hormone, follicle-stimulating hormone (FSH) and serum total testosterone levels on the Advia Centaur XP®, Siemens Healtcare GmbH, Germany. AMH concentrations were assessed using an enzyme-linked immunosorbent assay on the Access 2 Immunassay Analyser®, Beckman Coulter, Inc, California, United States. For determination of Leydig cell function, basal and hCG stimulated testosterone levels were determined when required. The hCG stimulation protocol was administration of three intramuscular injection of hCG on successive days in an age dependent daily dose (age <1 year, 500 units; 1-10 years, 1000 units; >10 years, 1500 units) (15). Gender assignment was approved by the DSD Ethics Committee of the University. DSD Ethics Commitee members consisted of a pediatric endocrinologist, an adult endocrinologist, a plastic surgeon, a pediatric surgeon, a medical geneticist, a

child psychiatrist, a pediatric urologist and a medical ethics specialist. Follow-up characteristics of the patients such as appropriateness of sex of rearing by psychiatric evaluation, development of gonadal tumors or additional problems (short stature, cardiac problems, renal abnormalities etc.) were evaluated. Frequencies and percentages represented the descriptive statistics for categorical variables and mean ± standard deviation values were used for continuous variables by using SPSS for Windows v. 22.0 statistical software. Student's t-test were used for parametric variables between groups. $p < 0.05$ was accepted as significant. Inform consent were given by parents after evaluation by DSD Committee. Ethical approved was given by the Ankara University Ethical Comittee for Clinical Research (approval number: 15-639-15).

Results

Admission Characteristics

During the study period, 38 cases with a mean age of presentation of 6.2 ± 4.6 years, were diagnosed as Y-chromosome GD. There were 26 cases of 46,XY GD and 12 cases of 45,X/46,XY GD. At presentation, the mean ages were 6.72 ± 5.2 years for 46,XY GD and 5.14 ± 4.1 years for the 45,X/46,XY GD patients (Table 1 and Figure 1). In our cohort, the number of patients with 46,XY GD was nearly double of the patients with 45,X/46,XY GD (Figure 1). In the 46,XY GD group, four patients phenotypically had complete GD, 22 patients had partial GD and presented with genital ambiguity. Sinnecker scores of patients were from 2 to 5. Müllerian structures were persistent in 12 of 46,XY GD patients. Gonads were bilaterally streak gonad in six patients, bilaterally dysgenetic testis in 14 patients. Two patients were of 46,XY karyotype and phenotypically one was partial GD and the other was complete GD. This latter case had embryonic testicular regression syndrome (ETRS). *SRY* gene deletion was not detected in any of the cases. Precise genetic diagnosis could be made in three patients; two with SF1 and one with WT1 mutations. In the patients with SF1 mutation diagnosis had been made at the age of ten years in the first and in the neonatal period in the second patient. The older of these patients had been raised as female and admitted to our clinic with virilisation of external genitalia. Hormonal analysis showed high FSH and high testosterone levels. The gonads were bilateral

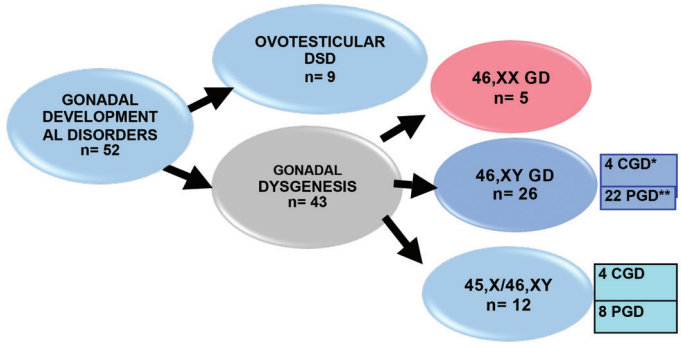

Figure 1. The distribution of patients with gonadal developmental disorders

*CGD: complete gonadal dysgenesis, **PGD: partial gonadal dysgenesis, DSD: disorders of sexual development, GD: gonadal dysgenesis

Table 1. Characteristics of patients

		46,XY	45,X/46,XY	All patients
Number of cases		26	12	38
Age of presentation (years)		6.72 ± 5.20	5.14 ± 4.10	6.2 ± 4.6
Phenotype (complete/partial)		4/22	4/8	8/30
Gonads	**Bilateral dysgenetic**	14	5	19
	Bilateral streak	6	3	9
	Unilateral dysgenetic, contralateral streak	4	4	8
	Testicular regression syndrome	2	0	2
Müllerian duct/remnant		12	12	24
Sex of rearing (M/F)*		17 male 7 female	6 male 6 female	24 male 13 female
Gender dysphoria		0	1	1
Gonadectomy		8	6	14
Gonadal tumor development		0	0	0

*One 46,XY patient died, precise decision was not made in another 46,XY patient
F: female, M: male

Table 2. Additional clinical features of Y-chromosome gonadal dysgenesis patients

	46,XY GD (n = 26) n (%)	45X/46,XY GD (n = 12) n (%)
Syndromic features	2 (Denys-Drash syndrome, camptomelic dysplasia) (7%)	-
Short stature	2 (7%)	6 (50%)
Cubitus valgus	-	4 (33%)
Webbed neck	-	3 (25%)
Bicuspid aorta	-	3 (25%)
Pulmonary stenosis	2 (7%)	-
Secundum atrial secundum defect	1 (3.5%)	-
Horseshoe kidney	-	1 (8%)
Ectopic kidney	-	1 (8%)
Lissencephaly, intractable convulsions	1 (3.5%)	-
Frequency of additional features	*8/26 (30%)	**9/12 (75%)

*$p < 0.05$, **some patients had more than one finding
GD: gonadal dysgenesis

dysgenetic testes and were located in the abdomen. The Müllerian duct had not regressed completely. The other SF1 mutated patient had Sinnecker 2a external genitalia with bilaterally inguinal testis and no Müllerian duct. He was raised as male without any health problems. The patient with a mutation of the *WT1* gene was diagnosed as Denys-Drash syndrome. Phenotypically he was partial GD. During follow-up his nephropathy progressed to end stage renal disease. Although not genetically proven, one patient within the 46,XY group probably had SOX9 mutation. He had partial GD and skeletal abnormalities including craniosynostosis and camptodactyly. A further patient with 46,XY partial GD had lissencephaly with absence of corpus callosum, intractable seizures, and choroid coloboma suggesting the aristaless-related homeobox (*ARX*) gene mutation. Unfortunately this patient died in the neonatal period and no genetic studies could be undertaken. In the 45,X/46,XY GD group, phenotypically four had complete GD and the remaining eight patients had partial GD. Of the 45,X/46,XY patients, four had unilateral dysgenetic testis and contralateral streak gonad, three had bilaterally streak gonads and the rest had bilaterally dysgenetic testis. All cases with 45,X/46,XY GD had Müllerian ducts exhibiting varying degrees of regression. Patients with complete GD in the 45,X/46,XY GD group were diagnosed at a younger age than the patients with complete GD in the 46,XY group (11 years vs. 14.31 years respectively, $p < 0.01$). There were no additional findings in 55% of all patients. Among the syndromic cases, additional clinical findings were detected in 75% in the 45,X/46,XY GD (9/12) and 31% in the 46,XY GD (8/26) groups. Short stature was the most frequently encountered additional finding and was detected in six patients with 45,X/46,XY GD and in two patients with 46,XY GD (Table 2). Mean duration of follow-up was 7.3 ± 3.8 years. Sex assignment was male in 24 patients and female in 12 patients (Table 3). Decision of sex assignement was not made in only one 46,XY infant admitted to our clinic at an age of seven months with Sinnecker 3 external genitalia. The last evaluation of this patient was at age 1.7 years and at this time the patient had been given a female name by the family and psychologic evaluation is continuing.

Follow-up Characteristics

Long term follow-up of patients was complicated by poor attendance in some patients for a number of reasons such as financial problems, families giving more importance to additional systemic findings than the genital problems, transfer to another clinic, etc. One case with severe congenital abnormalities died in the neonatal period.

Thirteen patients were raised as females, seven in the 46,XY GD group and six in the 45,X/46,XY GD group. Psychiatric evaluation is ongoing in one infant and the sex assignment has not yet been made. There was no gender dysphoria in patients that were raised as females, except one with 45,X/46,XY GD. This patient had been diagnosed at age 1.5 years and sex assignment was made as female at first evaluation. Female correction surgeries were undertaken. Unfortunately the patient did not reattend clinic until 14

Table 3. Phenotypic characteristics and sex of rearing in all patients

Patient no	Age of diagnosis (years)	Karyotype	Sinnecker score	Right gonad	Left gonad	Müllerian duct	Sex of rearing	Precise genetic diagnosis	Gender dysphoria
1	Newborn	45,X/46,XY	**2**	S	D	Yes	Male	-	No
2	Newborn	45,X/46,XY	**3**	D	D	Yes	Male	-	No
3	7.0	45,X/46,XY	**5**	S	D	Yes	Female	-	No
4	0.9	45,X/46,XY	**2**	S	D	Yes	Male	-	No
5	Newborn	45,X/46,XY	**2**	D	D	Yes	Male	-	No
6	4.8	45,X/46,XY	**5**	S	S	Yes	Female	-	No
7	1.5	45,X/46,XY	**2**	D	D	Yes	Male	-	No
8	15.4	45,X/46,XY	**5**	S	S	Yes	Female	-	No
9	1.5	45,X/46,XYg-	**3**	S	D	Yes	Female	-	Yes
10	12.9	45,X/46,XY	**2**	D	D	Yes	Male	-	No
11	0.6	45,X/47,XYY/46,XY	**3**	D	D	Yes	Female	-	No
12	17.1	45,X/46,XY	**5**	S	S	Yes	Female	-	No
13	Newborn	47,XY-trisomy 21	**2**	D	D	No	Male	-	No
14	Newborn	46,XY	**3**	D	D	Yes	Male	-	Exitus
15	11.5	46,XY	**2**	D	D	No	Male	-	No
16	5.6	46,XY	**4**	D	D	Yes	Female	-	No
17	3.7	46,XY	**3**	D	D	Yes	Male	-	No
18	0.7	46,XY	**2**	D	D	No	Male	WT1 mutation	No
19	Newborn	46,XY	**2**	D	D	No	Male	-	No
20	10.5	46,XY	**3**	S	S	Yes	Female	SF1 mutation	No
21	Newborn	46,XY	**2**	D	S	No	Male	-	No
22	14.4	46,XY	**5**	S	D	No	Female	-	No
23	0.2	46,XY	**1**	D	D	No	Male	-	No
24	16.3	46,XY	**1**	S	S	Yes	Male	-	No
25	0.1	46,XY	**3**	S	D	Yes	Not decided	-	
26	14.8	46,XY	**3**	S	S	Yes	Male	-	No
27	1.5	46,XY	**3**	TR	TR	Yes	Male	-	No
28	14.0	46,XY	**1**	TR	TR	No	Male	-	No
29	14.8	46,XY	**5**	S	S	Yes	Female	-	No
30	7.0	46,XY	**4**	D	D	Yes	Male	-	No
31	1.0	46,XY	**2**	D	D	Yes	Male	-	No
32	14.0	46,XY	**5**	S	S	Yes	Female	-	No
33	2.9	46,XY	**2**	D	S	No	Male	-	No
34	Newborn	46,XY	**3**	S	S	No	Male	-	No
35	14.0	46,XY	**5**	D	D	No	Female	-	No
36	13.6	46,XY	**4**	D	D	No	Female	-	No
37	2.5	46,XY	**2**	D	D	No	Male	SF1 mutation	No
38	7.1	46,XY	**3**	D	D	No	Male	-	No

S: streak, D: dysgenetic, TR: testicular regression

years of age, at which time he had reassigned himself as a male. The patient was reevaluated psychologically and the local ethic committee decided that he had a male gender identity. Apart from this patient, 19 patients (six in the 45,X/46,XY GD and 13 in the 46,XY GD group) who had attained pubertal ages showed no gender dysphoria.

Gonadectomy was carried out in 14 of these patients. Mean age of this group was 8.75 ± 2.3 years. Orchidopexy was performed in eight patients. Gonadal pathology results were normal in all cases and showed no malignancy.

Discussion

Y-chromosome GD shows a wide spectrum of phenotypic, genetic and histopathological characteristics and constitutes a heterogenous group within gonadal developmental disorders. Among the etiological factors, there are a range of genetic abnormalities. During testis development, several genes are activated and expressed at different times (1). Due to this genetic heterogenity in etiology, it is not suprising to observe extremely heterogenous clinical findings in these patients. Despite extensive analysis, no definite etiology can be established in an important proportion of gonadal developmental disorders (16). In our study sample, genetic etiology of cases with Y-chromosome GD could only be established in three patients. Two of these patients had SF1 mutations and the other had WT1 mutation. In patients with 45X/46,XY GD, no additional genetic studies were needed. In the 46,XY GD group, *SRY* gene deletion was not detected in any of the cases. Because analysis of mutations in the *SF1* gene has become available only recently for our patients, this analysis could not be performed in any of the patients. WT1 mutation could be analyzed only in one patient with Denys-Drash syndrome clinically. Future advances in genetic analysis will be helpful in exploring etiology further. SF1 mutation was detected in two patients who showed clinically distinctive features. It is known that the clinical spectrum of the *SF1* gene mutation is very heterogeneous and that it causes either a female phenotype or an ambiguous genital structure in 46,XY individuals. It has been reported that in a small number of cases with SF1 gene mutation, virilization can be observed in puberty (17,18). Although we have only two patients in this series with SF1 mutation, they demonstrate how this disorder can exhibit clinical heterogeneity with one presenting at a young age and the other presenting in late childhood with virilization. These cases also illustrate that clinical heterogenity of Y-chromosome GD patients depends not only on genetically different gene mutations, but also on a variety of presentations in patients with the same mutation. Syndromic Y-chromosome GD was found in two patients (Denys-Drash and Camptomelic Dysplasia syndromes). These patients presented with severe extragenital systemic findings; genital problems may be overlooked in such cases. Thus, genital abnormalities should be given more attention in cases with syndromic features. Low testosterone and low AMH levels also may be a clue in GD patients who have syndromic features.

In our study group, 55% of patients had no additional systemic findings. The presence of any systemic feature, apart from genital abnormalities, may actually be helpful in leading to an early diagnosis in these patients. Complete GD without additional abnormalities may only be diagnosed at pubertal ages due to a delay in pubertal signs (19). In our series, additional findings such as short stature, webbed neck and coarctation of the aorta were more frequent in the 45,X/46,XY GD group than in the 46,XY GD. Short stature was the most frequently detected finding (Table 2). It is well known that stigmata of Turner syndrome are common in children with 45,X/46,XY mosaicism. Monogenic X-chromosome is blamed for most of these features (20,21). In the case of 45,X/46,XY GD, there is a simultaneous presence of a lineage with X monosomy and XY cell lines among the tissues. The different tissue distributions of the 45,X and 46,XY chromosomal cell lines presumably reflect the wide variety of phenotypes observed (22). 45,X/46,XY mosaicism may be seen in cases with completely normal male external genitalia and it has been suggested that this phenotype is the most common among patients with 45,X/46,XY mosaicism (22). Patients with complete GD may only present because of pubertal delay. If there are additional phenotypic features, this may lead to earlier diagnosis, as was true in our series. While three patients with complete 45,X/46,XY GD were diagnosed before 10 years of age, all cases with complete 46,XY GD came to medical attention due to pubertal delay after the age of 14 years. Within the 46,XY GD group, two patients had been diagnosed as ETRS. ETRS has been considered to be a part of the clinical spectrum of partial 46,XY GD (23). The time of testicular regression (early or late) determines the degree of virilization of the external genitalia and regression of the Müllerian duct. Long term follow-up of GD patients involves many difficulties. Precise decision making on sex of rearing, time of gonadectomy and time of corrective surgery can be taxing. Associated clinical features may be present in these patients which will also require investigation and treatment. Sex of rearing may be the most important issue in patients with GD. In fact, in patients with complete GD, the sex of rearing decision is usually female. Controversies occur mostly for patients with partial GD (5). In our study, sex assignment was made in all patients with complete GD,

and no gender dysphoria was seen. In the partial GD group, only one case showed gender dysphoria. Although most of our cases seem to be successfully adapted to their selected gender, difficulties in long term follow-up of cases prevent us from definitively reporting on this issue. Despite a mean duration of follow-up in our cohort of 7.3 ± 3.8 years, a substantial proportion of followed-up patients are still at prepubertal ages. Only 19 of the 38 patients attained puberty. At this stage, we are not able to make a complete evaluation for appropriateness of sex of rearing in this group. It is important to avoid performing radical surgery in patients before they are given their precise sex. It should always be remembered that every GD patient is unique and has to be treated with individualized care and with a multidisciplinary approach (24). Gonadal tumor development is one of the most important challenges in patients with GD. Gonadal tumor risk is highest in 46,XY GD 46,XY DSD (11). The most common tumor observed in complete and/or partial GD is gonadoblastoma (12). The timing of gonadectomy before tumor development is a very important issue in patients with GD (10,11,12). In our series, gonadectomy was performed during the first decade and this seems to be an acceptable regimen for prevention of tumor development, since these tumors are usually seen during the second decade. Risk of gonadoblastoma is high when the early stage of Sertoli cell differentiation is disrupted by mutations in SRY, WT1, SOX9, DMRT1, FOG2/GATA4, FGF9 etc. (8,25). Patients with 45,X/46,XY GD also have disturbed early Sertoli cell development. The presence of gonadoblastoma Y locus is also a prerequiste for development of malignancy. Neoplastic transformation of germ cells in dysgenetic gonads (gonadoblastomas and/or an invasive germ cell tumor) occurs in 20-30% of 46,XY DSD patients (25). Thus, careful evaluation of gonads by imaging and/or histology is critical. The risk of germ cell tumor increases with age and undescended testis is an additional risk factor (11). In complete 46,XY GD patients, it is suggested that bilateral gonadectomy should be performed before pubertal age to avoid degeneration of dysgenetic tissue (5). In partial 46,XY GD, there is inconsistency in opinion with respect to timing of gonadectomy. A more individualized and conservative approach in the decision-making process for gonadectomy, by taking into account certain factors including location of the gonads, internal and external phenotype and sex of rearing, has being emphasized in recently (5). Provided the gonad is functional and easily accessible to palpation and imaging studies, some authors propose that gonadectomy can be postponed and imaging should be performed annually (25). Bilateral gonadectomy was recommended in patients with XY partial GD with nonscrotal gonads that cannot be surgically repositioned into the scrotum (5).

Study Limitations

Limitations of our study are irregular clinic attendance of patients.

Conclusion

Y-chromosome GD is an extremely heterogenous clinical and genetic disorder with variations in diagnosis, treatment and also in approach to additional problems. Each patient should be evaluated individually using a multidiciplinary approach. Whether the patient has syndromic features or not, associated clinical features can lead to earlier diagnosis, especially in complete forms of GD. Difficulties in long-term follow-up constitute an obstacle to judging the appropriateness of the sex of rearing decision. Gonadectomy during the first decade seems to be protective against tumor development which usually occurs during the second decade in these patients.

Ethics

Ethics Committee Approval: Ethical approved was given by the Ankara University Ethical Comittee for Clinical Research (approval number: 15-639-15).

Informed Consent: Informed consent were given by parents after evaluation by DSD Committee.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Merih Berberoğlu, Zeynep Şıklar, Design: Merih Berberoğlu, Zeynep Şıklar, Data Collection or Processing: Merih Berberoğlu, Zeynep Şıklar, Ankara University Faculty of Medicine Disorders of Sexual Development Ethics Committee, Analysis or Interpretation: Merih Berberoğlu, Zeynep Şıklar, Literature Search: Merih Berberoğlu, Zeynep Şıklar, Writing: Merih Berberoğlu, Zeynep Şıklar.

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ORIGINAL ARTICLE

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Effect of Intrahepatic Cholestasis of Pregnancy on Neonatal Birth Weight: A Meta-Analysis

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What is already known on this topic?

Several studies have demonstrated that intrahepatic cholestasis of pregnancy is associated with fetal growth, but the results are inconsistent.

What this study adds?

Neonatal birth weights of intrahepatic cholestasis of pregnancy infants were lower than that of normal pregnancies. Furthermore, early-onset intrahepatic cholestasis of pregnancy is associated with a lower birth weight than late-onset intrahepatic cholestasis of pregnancy.

Abstract

Objective: To evaluate the effect of intrahepatic cholestasis of pregnancy (ICP) on neonatal birth weight.
Methods: Potential articles were identified by searching PubMed and Web of Science databases on April 30th, 2017. Using the Mantel-Haenszel random-effects or fixed-effects model, outcomes were summarized through weighted mean difference (WMD) and 95% confidence intervals (CI). Potential publication bias was tested using a funnel plot and the methods of Egger's regression and Begg's test.
Results: A total of eight studies were included in our meta-analysis. Six studies reported data on neonatal birth weight in ICP and control pregnancies. Pooled data from the six studies showed that the birth weight in the ICP group was significantly lighter than in the control group. The overall pooled WMD was -175 g (95% CI: -301, -48). Meanwhile, pooled data from the other two studies indicated that the birth weight in the late-onset ICP group was heavier than in the early-onset ICP group (WMD: 267 g, 95% CI: 168, 366).
Conclusion: Neonatal birth weights in ICP pregnancies were lower than in normal pregnancies. Furthermore, early-onset ICP is associated with a lower birth weight than late-onset ICP.
Keywords: Intrahepatic cholestasis, pregnancy, birth weight, meta-analysis

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver disease that usually occurs during the late second or third trimesters of pregnancy. The clinical characteristics of ICP are unexplained maternal pruritus, altered liver function and increased fasting serum bile acids (> 10 mmol/L) in previously healthy pregnant women (1,2). There are differences in its prevalence in different regions and countries. It occurs in approximately 0.1% to 1.5% of pregnancies in Europe and the United States (3,4), while its prevalence ranges from 11.8% to 27.6% in Chile and Bolivia, varying by ethnic origin (5,6). Currently, the etiology of this condition is not fully understood and it is estimated that racial, genetic, hormonal, nutritional and environmental factors play a role (7). Although ICP is a benign disease, it can lead to increased fetal morbidity and mortality, particularly with regard to neonatal respiratory Distress syndrome, preterm delivery, fetal distress and sudden intrauterine fetal death (8). Several studies have demonstrated an association between ICP and fetal growth, but the results are inconsistent. A large, population-based cohort study reported a significant increase in the incidence of large for gestational age (LGA) infants in pregnancies

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complicated by ICP even after controlling for diabetes and preeclampsia (9). Martineau et al (10) also reported that ICP was associated with increased fetal growth. However, a study from Turkey found that ICP may lead to low birth weight (11) and a similar result was reported in another study (12). To further investigate the possible association between ICP and neonatal birth weight, we conducted this meta-analysis to summarize all available evidence.

Methods

Search Strategy and Selection Criteria

Relevant literature published before April 30th, 2017 was identified by searching PubMed and Web of Science databases. The search strategy was based on the following keywords: "cholestasis", "intrahepatic cholestasis", "pregnancy", "pregnant", "birth weight", "birthweight", "fetal growth restriction" and "intrauterine growth restriction". Only publications in English or Chinese were included. Relevant eligible literatures were also scanned through cross-references of identification in the reference lists within both original and review articles. In situations where key information relevant to the meta-analysis was missing, the authors were contacted to supply additional data. We employed EndNote for managing bibliographies and references. An essential feature of EndNote is that it allowed us to identify duplicates of studies found through different, overlapping databases. Studies were included in the analysis if the sample included: a) patients diagnosed with ICP; and b) birth weight measurements and if birth weight was measured as a continuous variable. If more than one study was identified for the same population, the more recent study or the one providing more information was selected. Studies were excluded if they were reported as case series, letters, review articles or editorials, and did not meet the above criteria. All analyses were based on previous published studies, thus no ethical approval and patient consent are required.

Data Extraction

After initial evaluation, two reviewers (L.L. and C.Y.H.) independently and carefully evaluated the articles and performed the data extraction according to the selection criteria. The following variables in each study were extracted: first author, year of publication, study country, age, gestational age at delivery, number of pregnancies (ICP and control; early-onset ICP and late-onset ICP), definition of ICP. When discrepancies existed, the case was discussed with another reviewer (Y.Y.Y.) until a consensus was reached.

Quality Assessment

The methodological quality of each study was independently assessed by two reviewers (L.L. and C.Y.H.) using the Newcastle-Ottawa quality assessment scale (13). Ten questions were assessed and each satisfactory answer received one point, resulting in a maximum score of nine. When publications had scores of ≥6, they were graded as high-quality. When there was a disagreement, it was solved by consensus of the whole team.

Statistical Analysis

All statistical analyses were carried out with the Stata 12.0 program (Stata-Corp, College Station, TX USA). Weighted mean difference (WMD) can be used as a summary statistic in meta-analysis when outcome measurements in all studies are made on the same scale. So using the Mantel-Haenszel random-effects or fixed-effects model, outcomes were summarized through WMD and 95% confidence intervals (CI). Statistical heterogeneity was measured using the chi-square test on Q statistic, which was quantified by I-squared values, assuming that I-squared values of 25, 50 and 75% were nominally assigned as low, moderate and high estimates, respectively (14). $P < 0.10$ or I-squared $> 50\%$ indicates that heterogeneity existed among the studies, so a random-effects model (Mantel-Haenszel method) should be used. In order to assess the impact on the results of a single study, we conducted a sensitivity analysis of each study by excluding each study one by one and recalculating the combined estimates on remaining studies. Potential publication bias was tested using the funnel plot and the method of Egger's regression and Begg's test. $P \leq 0.05$ indicated the presence of statistically significant findings.

Results

The initial literature search revealed 365 relevant articles on the association of ICP and neonatal birth weight. After the careful screening process, 108 studies were excluded as they were duplicates. Two hundred twenty-nine studies were rejected because 188 of these reports were irrelevant to our topic, 20 were review articles and 21 were case reports. The remaining 28 relevant studies were selected for detailed evaluation. Of these, 20 publications did not meet the inclusion criteria and were excluded. Finally, a total of eight studies (10,11,12,15,16,17,18,19) were included in our meta-analysis. Figure 1 outlines the literature review and study selection process. The characteristic of each article included in this meta-analysis is shown in Table 1. Two of these studies were performed in Turkey (11,16), two in China (12,19), one in Finland (18), one in the United Kingdom (10), one in USA (15) and one in Poland

Table 1. Characteristics of the studies included in the meta-analysis

Study (reference)	Publication year	Country	Maternal age (years)	Gestational age at delivery (weeks)	Number of group A	Number of group B	Definition of ICP	NOS scores*
(1) Studies comparing ICP (A) with control (B) group								
Alsulyman et al (15)	1996	USA	NR	A: 38.5 ± 1.9 B: 38.8 ± 1.7	79	79	The diagnosis of ICP was based on the presence of generalized pruritus in the absence of other skin or medical conditions that could produce pruritus.	6/9
Cheng et al (12)	2009	China	A: 28.4 ± 2.9 B: 28.2 ± 2.6	A: 37.7 ± 1.2 B: 38.7 ± 1.1	30	30	The diagnosis was based on criteria for intrahepatic cholestasis in pregnancy cited in the 2nd edition of Chinese obstetrics and gynecology	6/9
Papacleovoulou et al (18)	2013	Finland	NR	NR	7808	45	Women with ICP presented with pruritus and hepatobiliary injury, hypercholanemia (elevated serum BA levels) and dyslipidemia.	7/9
Kowalska-Kanka et al (17)	2013	Poland	A: 32.9 ± 3.4 B:31.2 ± 4.3	A: 36.0 ± 1.9 B: 38.1 ± 1.5	40	33	Total BA ≥11 μmol/L; elevated liver enzymes: ALT > 41U/L and/or AST > 40 U/L; and presence of pruritus (current or previous).	7/9
Martineau et al (10)	2015	United Kingdom	A: 30.5 ± 5.7 B:31.1 ± 5.1	A: 37.4 ± 1.6 B: 40.1 ± 1.5	27	26	All cases of ICP were confirmed by demonstration of serum BA ≥10 mmol/L, raised liver transaminase enzymes in association with pruritus, and no additional identifiable cause for their liver dysfunction	8/9
Ersoy et al (16)	2016	Turkey	A:27.8 ± 5.1 B:38.8 ± 5.9	A: 37.4 ± 1.0 B: 39.5 ± 1.4	22	21	ICP was diagnosed when a pregnant woman had pruritus without rash and elevated total BA (≥10 mmol/L) levels in the blood sample.	6/9
(2) Studies comparing early-ICP (A) and late-onset ICP (B) group								
Zhou et al (19)	2013	China	27.3 ± 4.8	NR	108	197	Reference to the diagnosis and treatment of intrahepatic cholestasis in pregnancy (version 1)	6/9
Uyar et al (11)	2015	Turkey	28.1 ± 6.2	36.7 ± 2.0	49	101	The patient files were accessed by the ICD-10 computer recording system and reviewed retrospectively	6/9

*Study quality assessment is listed using the results of the Newcastle-Ottawa questionnaire. All studies were sorted by publication year.

ICP: intrahepatic cholestasis of pregnancy, ICD: International Classification of Diseases, BA: bile acids, AST: aspartate transaminase, ALT: alanine transaminase, NR: not reported, NOS: Newcastle-Ottawa Scale

(17). Six of the studies (10,12,15,16,17,18) were conducted to explore the effect of ICP on birth weight (control vs ICP), and two studies (11,19) were conducted to compare early-onset (<32 weeks gestation) and late-onset (≥32 weeks gestation) ICP pregnancies. The quality of study was assessed by Newcastle-Ottawa quality assessment scale. The quality scores ranged from six to eight and showed that the studies were of acceptable quality.

Meta-Analysis Results

A total of six studies reported data on neonatal birth weight in ICP and control pregnancies. Pooled data from all the six studies showed that the birth weight in the ICP group was significantly lighter than those in the control group. The overall pooled WMD was -175 g (95% CI: -301, -48). The I-squared statistic (I-squared = 50.5%, p = 0.072) indicated moderate heterogeneity (Figure 2). Two studies reported data on neonatal birth weight in early-onset (<32 weeks) and late-onset (≥32 weeks) ICP pregnancies. Combined data from these two studies indicated a significant difference between the groups (Figure 3). The birth weight in the late-onset ICP group was heavier than that in the early-onset ICP group (WMD: 267 g, 95% CI: 168, 366). There was low heterogeneity (I-squared = 0.0%, p = 0.495).

Sensitivity Analysis

To confirm the stability and reliability of the meta-analysis, sensitivity analysis was performed by repeating the calculation of pooled WMD (95% CI) when any single study was deleted. Figure 4 showed that the corresponding pooled WMD (95% CI) ranged from -215 (-355, -75) g to -135 (-255, -15) g and was not substantially altered. The confidence limits of the overall estimate are -301 and -48 and -361 and -11 are the most extreme confidence limits of the estimates, calculated when any one study was omitted. The statistically similar results indicated that no single study had any influence on the stability of the overall WMD estimate in this meta-analysis.

Publication Bias

The graphical funnel plots appeared to be symmetrical (Figure 5), and the Begg's test (z = 1.13, p = 0.260) and Egger's test (t = -1.93, p = 0.126) indicated there was no strong evidence for publication bias.

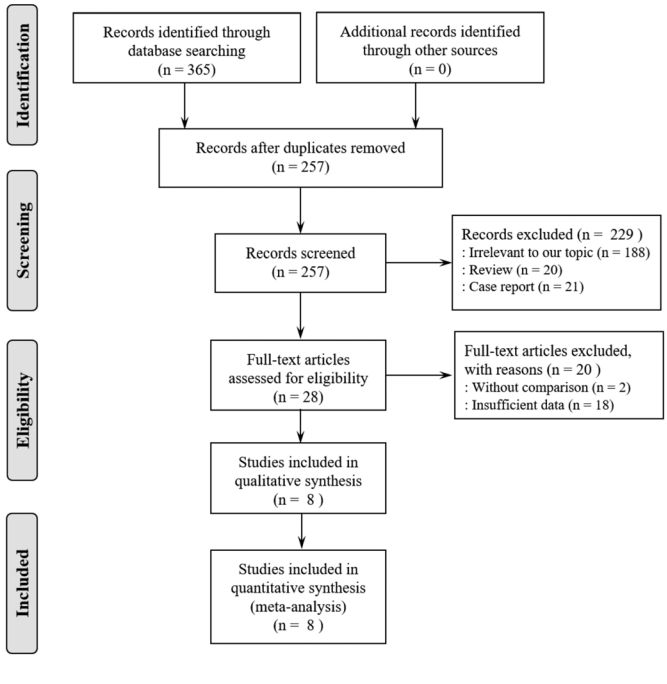

Figure 1. Flow diagram of the study selection process

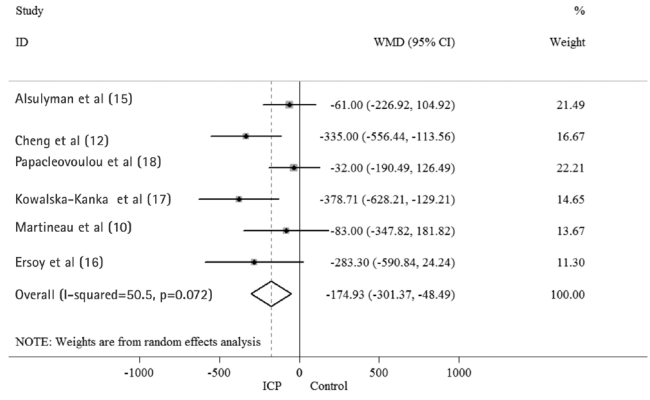

Figure 2. Forest plot of pooled estimated weighted mean difference with 95% confidence interval of birth weight between intrahepatic cholestasis of pregnancy and normal pregnancies. All studies were sorted by publication year

CI: confidence interval, ICP: intrahepatic cholestasis of pregnancy, WMD: weighted mean difference, ID: infectious disease

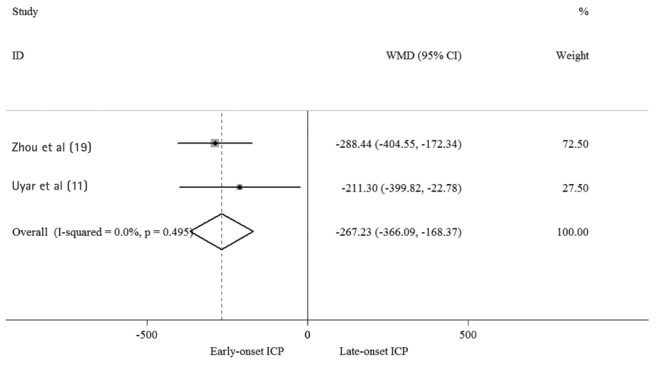

Figure 3. Forest plot of pooled estimated weighted mean difference with 95% confidence interval of birth weight between early-onset and late-onset intrahepatic cholestasis of pregnancy pregnancies. All studies were sorted by publication year.

ID: infectious disease, WMD: weighted mean difference, ICP: intrahepatic cholestasis of pregnancy

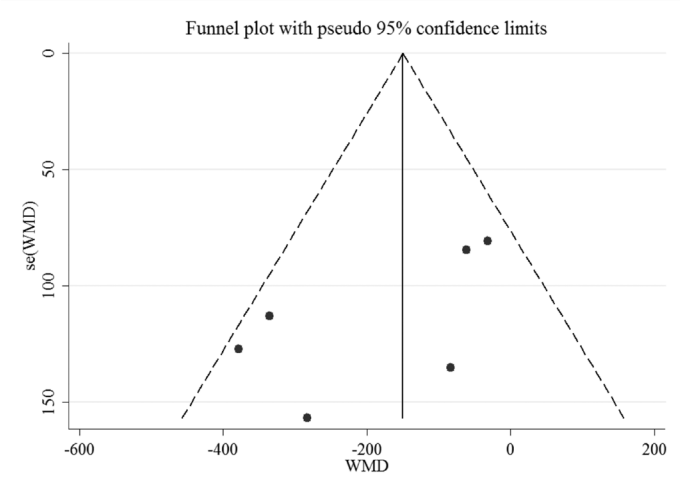

Figure 4. Funnel plot of the 6 studies included in the meta-analysis

WMD: weighted mean difference

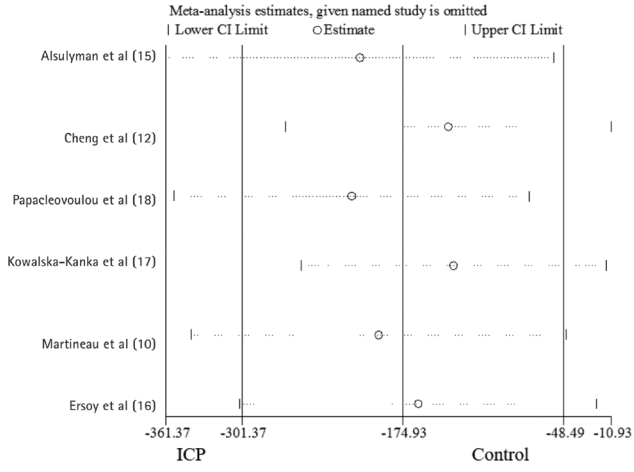

Figure 5. Sensitivity analysis for individual studies on the summary effect. All studies were sorted by publication year

ICP: intrahepatic cholestasis of pregnancy, CI: confidence interval

Discussion

This systematic review and meta-analysis was conducted to assess the effect of ICP on neonatal birth weight. The data showed that birth weight in the ICP group was significantly lighter than that in the control group (WMD: -175g, 95% CI: -301, -48). In addition, birth weight was significantly higher in late-onset compared with early-onset ICP cases. Although ICP is a relatively nonthreatening condition to mothers, there are serious risks for the fetus. It is linked with a higher risk of fetal death, meconium staining of amniotic fluid, fetal distress and preterm delivery (20,21,22). Fetal growth *in utero* is a complex process and involves interactions between mother, fetus and placenta. Maternal and fetal endocrine status, genetic predisposition and available substrates have an impact on fetal growth and also determine birth weight (23). Several studies have demonstrated that ICP has an influence on fetal growth. However, there was a wide variation in the results reported in the conducted studies (10,12,17,24,25). In a study from Poland investigating 73 pregnant women, it was reported that the babies of ICP mothers had a lower birth weight (17). These findings are consistent with a study from China (12). In contrast, a retrospective case-control study reported increasing customized, singleton, birth-weight centiles with advancing gestational age in cholestatic pregnancies (24). In another study, the incidence of LGA infants of ICP mothers was higher, compared with the incidence of SGA infants (25). Some studies have also focused on the association between ICP cases of different gestational onset time and birth weight. In a retrospective analysis (26), it was reported that early-onset ICP is associated with a higher frequency of adverse fetal outcomes than late-onset ICP, especially in severe disease. In this systematic review and meta-analysis, neonatal birth weights were found to be lower in early-onset ICP than late-onset ICP, a finding which indicates that early-onset ICP has greater influence on birth weight. In our meta-analysis, there is no potential risk of publication bias. When we excluded one study per iteration, the range of variation of the overall result is also smaller, which suggests that no one study can significantly alter the findings. Furthermore, the overall quality was acceptable in all of the studies included. However, there are still some limitations. Firstly, some included studies were conducted using medical databases, raising the possibility of coding inaccuracy. Secondly, a heterogeneity between studies was observed in the study. In addition, the results relied on aggregated published data. In the future, large-scale prospective studies will possibly provide a more accurate association between ICP and birth weight.

Study Limitations

There are still some limitations. First, some included studies were conducted using medical databases, raising the possibility of coding inaccuracy. Second, a heterogeneity between studies was observed in the study. In addition, the results relied on aggregated published data. In in the future, large-scale prospective studies will possibly provide a more accurate association between ICP and birth weight.

Conclusion

In summary, this meta-analysis demonstrated that neonatal birth weight is lower in ICP pregnancies than in normal pregnancies. Furthermore, early-onset ICP is associated with a lower birth weight than late-onset ICP.

Ethics

Ethics Committee Approval: All analyses were based on previous published studies, thus no ethical approval is required.

Informed Consent: All analyses were based on previous published studies, thus no patient consents are required.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Concept: Lin Cong, Li Li, Design: Lin Cong, Li Li, Data Collection or Processing: Yuan-Hua Chen, Yuan-Yuan Yang, Analysis or Interpretation: Li Li, Yuan-Hua Chen, Lin Cong, Literature Search: Li Li, Yuan-Hua Chen, Yuan-Yuan Yang, Writing: Li Li, Lin Cong.

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Vitamin D Deficiency in Pregnant Women and Their Infants

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What is already known on this topic?

Despite improvement in the socio-economic level of the population, vitamin D deficiency is still a serious health problem in Turkey. A new vitamin D support programme was launched for pregnant women by the Ministry of Health in 2011.

What this study adds?

Despite the launch of a vitamin D support programme for pregnant women by the Ministry of Health, vitamin D deficiency in pregnant women and their infants continues to be a serious health problem in Turkey.

Abstract

Objective: Vitamin D deficiency is a serious health problem despite a general improvement in socio-economic status in Turkey. The aim of this study was to evaluate maternal vitamin D status and its effect on neonatal vitamin D concentrations after a support programme for pregnant women was introduced. A second aim was to identify risk factors for vitamin D deficiency in a district of İstanbul.
Methods: A total of 97 pregnant women and 90 infants were included in this study, conducted between January and October 2016. The demographic data, risk factors and daily vitamin intake were recorded. Serum levels of vitamin D, calcium, phosphorus and alkaline phosphatase in all subjects were measured. The mothers and newborns were divided into groups based on their vitamin D levels. The relationship between vitamin D levels and risk factors was analyzed.
Results: Mean ± standard deviation vitamin D levels for the women and their infants were found to be 14.82 ± 11.45 and 13.16 ± 7.16 ng/mL, respectively. The number of mothers and infants was significantly higher in the deficient group, and their mean vitamin D levels significantly lower (9.02 ± 1.34 and 8.80 ± 1.06 ng/mL, respectively) ($p < 0.001$, $p < 0.001$). Only 14.4% of pregnant women took 1000-1200 IU/day of vitamin D. When the mother groups were evaluated in terms of risk factors, there were significant differences in daily vitamin intake and clothing style ($p < 0.001$ and $p < 0.001$ respectively).
Conclusion: Vitamin D deficiency in pregnant women and their infants is still a serious health problem in Turkey, although a vitamin D support programme during pregnancy has been launched by the department of health.
Keywords: Vitamin D, neonate, pregnancy

Introduction

Vitamin D is not only a lipid-soluble vitamin, but also a steroid hormone that can be synthesized endogenously. It has an important role in calcium (Ca)-phosphorus (P) homeostasis and its deficiency causes rickets in children and osteomalacia in adults (1,2). Vitamin D deficiency may also result in impairment of immune function, predisposition to cancer, cardiovascular disease, diabetes, rheumatic disease, muscle weakness, chronic pain and neuropsychiatric dysfunction (3,4,5,6,7). The lack of vitamin D during pregnancy is the most important risk factor for infantile rickets and may also result in poor fetal growth and neonatal development (8,9,10,11). In addition, its deficiency in pregnant women may predispose to gestational diabetes mellitus and preeclampsia (12,13).

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Vitamin D deficiency continues to be a serious health problem in Turkey despite a general improvement in socio-economic status in recent years. In 2005, a "Vitamin D prophylaxis augmentation program" was initiated by the Turkish Pediatric Endocrine Society and the Ministry of Health for prevention of rickets. This program included free distribution of supplements to provide 400 IU/day of vitamin D. At the end of this program, the prevalence of rickets decreased in children under three years of age (14). After this success, a new nationwide vitamin D support program was launched for pregnant women by the Ministry of Health in 2011. This program included 1200 IU vitamin D replacement daily to all pregnant women from the first trimester of pregnancy until six months after delivery (15).

The aim of this study was to evaluate maternal vitamin D status and its effect on neonatal vitamin D status, following the introduction of the support programme for pregnant women. A second aim was to identify risk factors for vitamin D deficiency in the Istanbul district of Bağcılar, a low socio-economic neighborhood.

Methods

This prospective study was conducted in cooperation with the Departments of Pediatrics and Obstetrics and Gynecology in Medicine Hospital/Biruni University. The study protocol was approved by the Ethics Committee of the Biruni University (approval number: 2015-KAEK-43). Informed consent was obtained from pregnant women and given by them for the participation of their infants.

It was planned to include one hundred and twenty women in their 3rd trimester of pregnancy and their infants in this study, but 23 women were excluded as they rejected inclusion of their babies. Also, seven infants were excluded from this study because blood samples could not be obtained. Women younger than 20 or over 40 years of age, those with chronic disease, taking medications and those with twin pregnancy were excluded. Being small for gestational age (SGA; defined as a birth weight less than 2500 g), prematurity, having a congenital disease or malformation, age at sampling older than 28 days and refusal of parental consent were exclusion criteria for infants. Thus, a total of 97 pregnant women and 90 infants were included in this study conducted between January and October 2016. Information on the mothers and their infants such as age, gender, weight, height, parity, socio-economic status, daily sun exposure, daily vitamin D intake, style of clothing and season were recorded. Not being exposed to sunlight daily was defined as low sun exposure for the mothers. Using a headscarf and clothes which covered arms and legs were defined as covered clothing style. The mothers were divided into three groups according to daily vitamin D intake; none, 400-600 IU/day and 1000-1200 vitamin D IU/day.

Body mass index (BMI) was calculated by the formula [weight (kg)/height $(m)^2$]. Blood samples for (Ca), P, alkaline phosphatase (ALP) and 25-hydroxyvitamin (OH) D were taken from the mothers within one month, prior to delivery and from the infants within one week after delivery. Samples were examined on the same day. The 25(OH)D levels were measured by enzyme linked fluorescent assay on the Mini Vidas (Biomerieux, France). Ca, P and ALP were measured using photometry on the Cobas Integra 400 Plus (Roche Diagnostics, Germany). Participants were divided into three groups as deficient, insufficient or sufficient according to their vitamin D levels. 25(OH)D levels were defined as <12 ng/mL (<30 nmol/L) deficient, 12-20 ng/mL (30-50 nmol/L) as insufficient and >20 ng/mL (>50 nmol/L) as sufficient (16).

In this study, IBM SPSS v20 and R were used to conduct the analysis. The Statistical G Power program was used to calculate sample size. We estimated a minimum total sample size of 84 to achieve an effect size of 0.35, the power of 0.8 and type 1 error of 0.05. Descriptive statistics are given via tables. Chi-square test of independence was used to detect the significant relationships between nominal variables. To test the differences between means, t-test, one-way ANOVA for normally distributed data and Mann-Whitney U test and Kruskal-Wallis H test for nonparametric data were used. To detect from which groups the difference originated, Tukey's honestly significant difference and Dunn's tests were used.

Results

Ninety-seven pregnant women were included in this prospective study. The mean vitamin D level for all women was 14.82 ± 11.45 ng/mL. When the risk factors were evaluated in pregnant women, there were statistically significant differences in BMI, daily vitamin intake and clothing style ($p=0.02$, $p<0.001$, $p=0.02$ respectively). The characteristics of the groups are shown in Table 1.

The number of women was significantly higher in the deficient group ($p<0.001$), and their mean vitamin D level was significantly lower (9.02 ± 1.34 ng/mL) than insufficient (15.13 ± 2.34 ng/mL) and sufficient groups (33.95 ± 20.71 ng/mL) ($p<0.001$; see Table 2). No significant differences were found between groups in terms of Ca, P or ALP levels ($p=0.07$ $p=0.10$, $p=0.94$). When the groups were evaluated in terms of risk factors, there were statistically significant differences in daily vitamin intake and clothing style ($p<0.001$, $p<0.001$).

Ninety infants were included in this prospective study. Mean vitamin D level was 13.16 ± 7.16 ng/mL for the total group of infants. The mean gestational age and birth weight of infants were 38.45 ± 1.10 weeks and 3.36 ± 0.39 kg respectively. The number of female infants was 48 (53%). Infants were divided into three groups according to their vitamin D levels. The number of infants in the deficient group was significantly higher than that in insufficient and sufficient groups ($p < 0.001$). Mean vitamin D level in the deficient group was 8.80 ± 1.06 ng/mL and this level was significantly lower when compared to the insufficient (15.43 ± 2.33 ng/mL) and sufficient groups (28.84 ± 9.26 ng/mL; $p < 0.001$). Among the groups, there were no differences in Ca, P and ALP levels (Table 3).

When the effect of maternal risk factors on their infants' vitamin D levels was evaluated, there were no statistical differences, with the exception of daily vitamin D intake. Mean vitamin D levels of babies whose mothers wear covered clothing or not were 13.01 and 13.44 ng/mL, respectively. This difference is statistically insignificant (Independent sample t-test, $p = 0.79$). Mean vitamin D level in infants whose mothers took no daily vitamin D, in infants whose mothers took 400- < 1000 IU and 1000-1200 IU daily were 12.13, 12.95 and 16.25 ng/mL, respectively. There were statistically significant differences in the mean values of these three groups (Kruskal-Wallis H test, $p = 0.04$), and this difference originated from the 400- < 1000 IU and 1000-1200 IU groups (Dunn's test, $p = 0.01$).

Table 1. Serum 25-hydroxyvitamin D levels in pregnant women according to their characteristics

	n (%)	25(OH)D levels (ng/mL) (mean ± SD)	p
All pregnant women	97 (100%)	14.82 ± 11.45	
Age (years)			
20-30	51 (52.6%)	15.41 ± 11.79	[1]0.60
> = 30	46 (47.4%)	14.17 ± 11.16	
BMI (kg/m^2)			
< 18.5	3 (3.1%)	8.10 ± 0.25	[2]0.02*
18.5-24.9	56 (57.7%)	15.82 ± 11.42	
25-29.9	26 (26.8%)	14.62 ± 14.15	
≥30	12 (12.4 %)	12.30 ± 3.68	
Number of parity			
1	37 (38.1%)	14.38 ± 5.8	[3]0.83
2	37 (38.1%)	15.19 ± 12.71	
≥3	23 (23.8%)	14.95 ± 15.88	
Socio-economic status			
Low	45 (46.4%)	15.28 ± 12.37	[1]0.71
Moderate	52 (53.6%)	14.42 ± 10.7	
Daily vitamin D intake			
None	12 (12.4%)	11.44 ± 5.27	[3] < 0.001**
400- < 1000 IU	71 (73.2%)	12.49 ± 4.75	
1000-1200 IU	14 (14.4%)	29.56 ± 23.43	
Daily sunlight exposure			
Yes	41 (42.3%)	15.13 ± 11.05	[1]0.76
No	56 (57.7%)	14.41 ± 12.10	
Clothing style			
Covered	65 (67%)	12.96 ± 6.71	[1]0.02*
Uncovered	32 (33%)	18.61 ± 17.06	
Vitamin D by seasons			
Winter	5 (5.2%)	13.45 ± 5.07	[2]0.56
Spring	55 (56.6%)	14.68 ± 10.97	
Summer	35 (36.1%)	15.07 ± 13.2	
Autumn	2 (2.1%)	18.02 ± 5.63	

*statistically significant at 0.05, **statistically significant at 0.01, [1]two-sample t-test, [2]Kruskal-Wallis H test, [3]one-way analysis of variance

SD: standard deviation, BMI: body mass index, 25(OH)D: 25-hydroxyvitamin D

Table 2. Maternal groups and their laboratory results by vitamin D status

	Deficient	Insufficient	Sufficient	p
Number of women (n, %)	48 (49.5%)	35 (36.1%)	14 (14.4%)	[1] < 0.001*
25(OH)D (ng/mL) (mean ± SD)	9.02 ± 1.34	15.13 ± 2.34	33.95 ± 20.71	[2] < 0.001*
Ca (mg/dL) (mean ± SD)	8.84 ± 0.53	9.06 ± 0.45	9.07 ± 0.59	[2]0.07
P (mg/dL) (mean ± SD)	3.67 ± 0.68	3.89 ± 0.45	3.90 ± 0.71	[2]0.10
ALP (U/L) (mean ± SD)	140.71 ± 50.07	142.81 ± 45.63	138.00 ± 22.75	[2]0.94

*Statistically significant at 0.01, [1]chi-square test of independence, [2]one-way analysis of variance

SD: standard deviation, Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, 25(OH)D: 25-hydroxyvitamin D

Table 3. Infant groups and their laboratory results by vitamin D status

	Deficient	Insufficient	Sufficient	p
Number of infants (n, %)	51 (56.7%)	29 (32.2%)	10 (11.1%)	[1] < 0.001*
25(OH)D (ng/mL) (mean ± SD)	8.80 ± 1.06	15.43 ± 2.33	28.84 ± 9.26	[2] < 0.001*
Ca (mg/dL) (mean ± SD)	10.66 ± 5.67	9.86 ± 1.24	10.06 ± 0.64	[2]0.49
P (mg/dL) (mean ± SD)	5.74 ± 0.63	5.61 ± 0.78	5.81 ± 0.63	[2]0.85
ALP (U/L) (mean ± SD)	185.53 ± 50.76	192.48 ± 52.11	166.30 ± 43.95	[2]0.57

*Statistically significant at 0.01, [1]chi-square test of independence, [2]one-way analysis of variance

SD: standard deviation, Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, 25(OH)D: 25-hydroxyvitamin D

Table 4. Number of infants and their vitamin D status according to maternal groups

	Number of infants and their vitamin D status		
Maternal group	**n (%)**	**25(OH)D levels (ng/mL) ± SD**	**p**
Sufficient	13 (14.5%)	24.28 ± 10.33	[1] < 0.001*
Insufficient	29 (32.2%)	13.06 ± 4.09	
Deficient	48 (53.3%)	10.05 ± 3.70	
Total	90 (100%)	13.08 ± 7.16	

*Statistically significant at 0.01, [1]one-way analysis of variance

25(OH)D: 25-hydroxyvitamin D

Also, we evaluated the infants according to mother's vitamin D status. Mean vitamin D level in the infants of mothers who have deficient, insufficient and sufficient vitamin D levels were 10.05 ± 3.70 ng/mL, 13.06 ± 4.09 ng/mL and 24.28 ± 10.33 ng/mL, respectively ($p < 0.001$) (Table 4). The Pearson correlation between the mothers' and their babies' vitamin D levels was significant ($p < 0.001$) and the correlation coefficient was 0.63.

Discussion

Vitamin D deficiency leads to important health problems, not only in mothers but also in their infants, because the vitamin D store of the mother is the major source of vitamin D for the fetus (9). The vitamin D dose that the World Health Organization recommends for pregnant women is 200 IU/day (17). The Institute of Medicine suggested that the "Estimated Average Requirement" and "Recommended Dietary Allowance" (RDA) for pregnant women be 400 and 600 IU/day, respectively (18). Recent studies reported that the daily dose for pregnant women should be greater than 1000 IU/day to achieve adequate levels (8,19). The safety dose during pregnancy is not clear, but Hollis et al (20) showed that vitamin D supplementation of 4000 IU/day for achieving adequate levels was safe and effective in pregnant women.

The International Association of Endocrinology defined a vitamin D level of 21-29 ng /mL as insufficiency and < 20 ng/mL as deficiency in adults (21). However, the levels of vitamin D insufficiency and deficiency are not clearly defined and the discussion about the prevalence vitamin D deficiency is ongoing (22,23). The recommended value for serum vitamin D level is lower in children than adults. The Endocrine Society suggests vitamin D levels of > 20 ng/mL for sufficiency, 12-20 ng/mL for insufficiency and < 12 ng/mL as vitamin D deficiency (16). This recommendation was used in our study.

Studies reported from different countries have shown a prevalence of vitamin D deficiency in pregnant women and in infants ranging from 4% to 60% and from 3% to 86%, respectively (24,25). In a study from Egypt, El Koumi et al (26) reported that only 35.8% of pregnant women had blood levels over 20 ng/mL. In a study from India, it has

been reported that 84% of pregnant women had vitamin D concentrations <22.5 ng/mL (27). In a national survey from Belgium, vitamin D insufficiency (<30 ng/mL) and deficiency (<20 ng/mL) were found as 74.1% and 44.6% (28).

Previous studies have shown that vitamin D deficiency is common in pregnant women in Turkey. In 1998, Alagöl et al (29) found that vitamin D levels were low in 66.6% of women of reproductive age in İstanbul. In 2003, Pehlivan et al (30) reported that 94.8% of the mothers and 24.6% of their infants had levels below 16 ng/mL. In a further study by Ergur et al (2009) (31), only 18.6% of the mothers and 2.9% of the neonates had normal vitamin D levels. In 2008, Halicioglu et al (32) found that 50.4% of pregnant women in İzmir, a city in a sunny region of Turkey, had blood vitamin D levels ≤10 ng/mL. In a study conducted in Ankara in 2010, vitamin D deficiency (≤20 ng/mL) in pregnant women and their infants were found to be 62.6% and 58.6%, respectively (33). It should be noted that all of these studies were conducted prior to the introduction of the national pregnancy vitamin D supplementation programme. In this present study, mean vitamin D level was found to be 14.82±11.45 ng/mL in pregnant women and 13.16±7.16 ng/mL in their infants. Vitamin D deficiency in mothers and infants were 49.5% and 56.7%, respectively. All these data confirm that vitamin D deficiency continues to be a problem in pregnant women and their infants in Turkey, despite the introduction of the supplementation programme.

Although the Ministry of Health has recommended a vitamin D intake of 1200 IU/day, we found that 12.4% of mothers never used vitamin D supplements and 73.2% used irregular or low doses. The proportion of pregnant women who received 1000-1200 IU/day of vitamin D was 14.4% and this low value was statistically significant. Vitamin D levels were significantly lower in mothers who used low dose vitamin D supplements compared to those who used recommended doses. These results show that high dose vitamin D support is necessary during pregnancy and that the support program should be continued.

Limited sun exposure, regular use of sunscreens, living in northern latitudes, dark skin, obesity, extensive clothing cover, aging, poor nutritional status, malabsorption syndromes and medications have been reported as risk factors for vitamin D deficiency (1,19). In previous studies in Turkey, winter season, low socioeconomic status, low educational status and covered clothing style were reported as risk factors for vitamin D deficiency in mothers (31,34,35). However, Pehlivan et al (30) found no significant difference related to factors other than covered clothing. Similarly, Halicioglu et al (32) and Çuhacı-Çakır et al (36) reported that covered clothing style was a risk factor for vitamin D deficiency. In this present study, the difference was not in terms of socioeconomic status and season because all mothers had low or moderate incomes and only five (5.2%) of mothers gave birth in winter. We found no differences in terms of number of parity and sunlight exposure, but vitamin D levels of mothers who had covered clothing had significantly lower blood levels than the uncovered women. These findings show that covered clothing style is an important factor for vitamin D deficiency in pregnant women in Turkey.

When we evaluated the infants according to the vitamin D levels of their mothers, we found no difference between groups in terms of gender, gestational age, birth weight, delivery route and the levels of Ca, P and ALP. However, the infants of mothers in the sufficient group had significantly higher vitamin D level than other infants. As might be expected previous studies have shown a strong correlation between maternal and neonatal levels of vitamin D (8,9). Ergur et al (31) suggested that maternal deficiency was the most important factor for vitamin D deficiency in newborns. Andiran et al (35) reported that the most important risk factor for low level in the newborn was a maternal 25(OH)D level below 10 ng/mL. Similarly, we found a strong positive correlation between the mothers' and their babies' vitamin D levels and low level of vitamin D in mother was an important risk factor for deficiency in infants.

When we evaluated the relationship between the infants' vitamin D level and their mothers' clothing style, together with daily vitamin D intake, we found that there were no significant differences with respect to the mothers' clothing style. However, mothers' low vitamin intake was found as a risk factor for the infants' vitamin D level.

Study Limitations

Our study has some limitations that should be mentioned. First, this study was conducted in a single district of İstanbul and second, this district has a population of low socio-economic level. Therefore, this study may be insufficient to evaluate all socio-economic levels and all other regions in Turkey. Further studies are needed to evaluate the limitations of this study.

Conclusion

Although a vitamin D support programme was launched for pregnant women by the Ministry of Health in 2011, vitamin D deficiency in pregnant women and their infants is still

a serious health problem in Turkey in 2016. Also, the data from this study indicate that the usage rate of the dose recommended by the support programme was very low and the prescribed supplements were generally multivitamin preparations. Therefore, the support programme should be continued, more widely promoted and physicians should be more informed about the content of the support programme in pregnancy.

Ethics

Ethics Committee Approval: The study was approved by the Biruni University Local Ethics Committee (approval number: 2015-KAEK-43).

Informed Consent: Informed consent was obtained from pregnant women and given by them for the participation of their infants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Abdurrahman Avar Özdemir, Yasemin Ercan Gündemir, Concept: Abdurrahman Avar Özdemir, Yasemin Ercan Gündemir, Design: Mustafa Küçük, Deniz Yıldıran Sarıcı, Data Collection or Processing: Yakup Çağ, Günal Bilek, Deniz Yıldıran Sarıcı, Analysis or Interpretation: Abdurrahman Avar Özdemir, Yakup Çağ, Günal Bilek, Literature Search: Abdurrahman Avar Özdemir, Yusuf Elgörmüş, Writing: Abdurrahman Avar Özdemir, Yusuf Elgörmüş, Mustafa Küçük.

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ORIGINAL ARTICLE

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The Relationship Between Blood Pressure and Sleep Duration in Turkish Children: A Cross-Sectional Study

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What is already known on this topic?

Hypertension is an important risk factor for cardiovascular disease in children as well as in adults. Preventive precautions for children and adolescents should be considered in terms of maintaining a healthy lifestyle.

What this study adds?

A sleep duration ≤8 h is an independent risk factor for prehypertension and hypertension in Turkish children aged 11-17 years.

Abstract

Objective: As in adults, hypertension is also an important risk factor for cardiovascular disease in children. We aimed to evaluate the effect of sleep duration on blood pressure in normal weight Turkish children aged between 11-17 years.
Methods: This cross-sectional study was conducted in the primary and secondary schools of the two central and ten outlying districts of Kayseri, Turkey. Subjects were 2860 children and adolescents (1385 boys, 1475 girls). Systolic and diastolic blood pressures were measured according to the recommendations of the Fourth Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents. Sleep duration was classified as follows: ≤8 hours, 8.1-8.9 hours, 9.0-9.9 hours or ≥10 hours.
Results: For short sleeper boys and girls (participants with a sleep duration ≤8 h) the prevalence of prehypertension and hypertension was 35.0% and 30.8%, respectively. In univariate binary logistic regression analyses (age-adjusted), each unit increment in sleep duration (hours) in boys and girls, decreased the prehypertension and hypertension risk by 0.89 [odds ratio (OR)] [confidance interval (CI); 0.82-0.98] and 0.88 (OR) (CI; 0.81-0.97), respectively ($p < 0.05$). In multiple binary logistic regression analyses [age- and body mass index (BMI)-adjusted] the location of the school and sleep duration categories were shown to be the most important factors for prehypertension and hypertension in both genders, while household income was the most important factor, only in boys.
Conclusions: A sleep duration ≤8 h is an independent risk factor for prehypertension and hypertension in Turkish children aged 11-17 years.
Keywords: Adolescent, blood pressure, children, sleep duration

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Introduction

In recent years, the prevalence of both hypertension and prehypertension are increasing worldwide (1,2,3). Increased levels of high blood pressure (BP) in childhood is an indicator for an increase in prevalence of coronary artery disease in adulthood. Sedentary lifestyle, obesity and nutritional habits are modifiable risk factors known to effect development of hypertension in childhood. The relationship between sleep duration and BP is an issue for both experimental and epidemiological studies. Both hypertension and coronary artery disease have been associated with sleep duration in adults (4,5). However, the etiology of this relationship is not fully understood. Changes in hormonal activity, increased activity of the renin-angiotensin-aldosterone system and changes in circadian rhythm have been reported to have a role in this relationship (6,7,8,9,10).

The best approach to prevent hypertension-related mortality or morbidity is to prevent and treat the hypertension (11). However the treatment of hypertension is difficult and complex. Despite major advances in treatment of hypertension in the past few decades, the majority of patients fail to achieve treatment goals. Lifestyle changes including a low salt diet, weight loss, regular physical activity, cessation of smoking and diet therapy are important in decreasing the risk of hypertension-related disease (12,13).

Much current epidemiologic research, related to lifestyle, focuses on the relationship between short sleep duration and hypertension prevalence (11,13). In the current study, we aimed to evaluate the effects of lifestyle characteristics on BP among children living in one of the biggest city in Central Anatolia, Kayseri.

Methods

This cross-sectional study was performed on a large cohort of children, aged 11-17 years. All children attended primary and secondary schools located centrally (two schools) and in the outlying districts (ten schools) of the town of Kayseri in Turkey. Kayseri is one of the largest three cities in Central Anatolia with more than 1.000.000 inhabitants. Data were obtained from the "Determination of Anthropometric Measurements of Turkish Children and Adolescents" survey (14,15).

The study was approved by the Ethics Committee of Erciyes University School of Medicine and Kayseri Province Educational Board (approval number: 04/312, approval date: 07/09/2004). Written consent was taken from the parents at the beginning of the study in accordance with the Declaration of Helsinki. Decimal age was calculated by subtracting the birth date from the observation date (16).

Anthropometric Indices

All anthropometric indices were measured twice by experienced health professionals and the mean value was recorded for analyses. All inter-observer correlation coefficients were ≥0.91.

Weight was measured using a standard beam balance sensitive to 0.1 kg, using a Tefal Ultraslim (France), in minimal clothing (bare feet and with light clothing).

Height was measured with a portable Seca stadiometer with a sensitivity of 0.1 cm. Daily calibration was made to the device. Measurements were made with the subject barefoot, the heels, hip and shoulders touching the stadiometer and the head in neutral position with eyes gazing forward.

Body mass index (BMI) was calculated by dividing body weight in kilograms by the square of body height in meters (kg/m^2).

Systolic BP (SBP), and diastolic BP (DBP) were measured according to the recommendations of the Fourth Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents (15,17). According to BP charts for age, gender and height, normal, pre- and hypertension were defined as SBP and DBP below the 90^{th} percentile, between 90^{th}-$< 95^{th}$ percentile and equal to or higher than 95^{th} percentile respectively (15).

Several anthropometric indices were measured: waist circumference (WC), mid-upper arm circumference (MUAC), triceps skinfold thickness, arm fat area, MUAC fat percentage (14). WC was divided by height to yield the WC to height ratio.

Demographics

A questionnaire form constructed by the researchers with socio-demographic variables was used. Computer use (hours), sleep duration (hours), TV viewing (hours), location of residence (urban/suburban), mode of transport to school (walking or by car), self-reported household income (good, fair, poor), house size (m^2), parental education, elevator use (none/habitual), maternal employment (housewife/employed) were asked (14).

The questionnaire was sent to parents and the question related to sleep was asked as, "How many hours of sleep does your child usually get?", aiming to provide information to the researchers on the child's habitual sleep duration. Sleep duration was classified into four groups as: ≤8 h, 8-9 h, 9-10 h, and ≥10 h and these categories were selected to compare the results of the current study with similar studies (18).

The following sociodemographic and behavioral effectors of obesity were analysed: computer use, sleep duration,

viewing duration of TV, place of residence, appetite, mode of transport to school, household income, house size, parental education, maternal employment and elevator use (14).

Statistical Analysis

Chi-square tests were used to determine significant differences in proportions among categorical variables and to compare continuous variables independent sample's t-test was used. Anthropometric indices were compared with sleep duration categories by analysis of covariance (adjustment for multiple comparisons: Bonferroni), where age or age and BMI were the covariates. Univariate (adjusted age) and multiple (the backward stepwise procedure; adjusted age or age and BMI) binary logistic regression analyses were used to examine the risk factors to influence whether SBP or DBP≥90th percentile (prehypertension and hypertension). All statistical analyses were performed by R2.14.0 program (www.r-project.org). Two-tailed p-values of <0.05 were considered to be statistically significant.

Results

The study included 2860 children. Of the sample 1385 (48.4%) were boys and 1475 (49.6%) were girls. Prehypertension and hypertension together (DBP≥90th) was found in 485/1385 (35.0%; 95% CI 0.32.5-0.37.6) of boys and 455/1475 (30.8%; 95% CI 0.28.5-0.33.3) of girls, respectively. Prehypertension prevalences in this group were 26.3% and 22.4% for boys and girls, respectively. In both genders, we found that increased sleep duration was significantly related with decreased prehypertension and hypertension (Table 1). In Table 2, the relationship between sleep duration (≤8 h, 8.1-8.9 h, 9.0-9.9 h, ≥10 h), anthropometric indicators of metabolic risk and BP is shown. The unique significant finding in this comparison was the reduction in mean SBP from 112.9 mmHg to 107.9 mmHg in girls and the reduction in mean DBP from 72.7 mmHg to 69.4 mmHg in boys as sleep duration increases.

In Tables 3A and 3B, the effects of prehypertension on sociodemographic and behavioural variables for children and adolescents (by univariate and multiple binary logistic regression analyses) are shown. Increase in sleep duration and decrease in BMI are two indicators of normal BP in both genders. The corresponding odds for sleep duration were OR: 0.89, CI; 0.82-0.98 and OR: 0.88, CI; 0.81-0.97, respectively for boys and girls. The corresponding odds for BMI were OR: 1.12, CI; 1.08-1.16 and OR: 1.09, CI; 1.06-1.14 respectively for boys and girls (Table 3A).

We adjusted our dependent variables (normotensive/prehypertensive and hypertensive) for BMI and age in multiple binary logistic regression. Independent variables in this categoric analysis that were statistically significant ($p<0.05$) were; location of the school (in both genders), sleep duration (in both genders) and household income (only in boys). There was no relationship between maternal employment, paternal education, house size, elevator use and mode of transport to school ($p>0.05$). Sleeping 8-9 hours compared to sleeping less than 8 hours in boys and sleeping more than 10 hours compared to sleeping less than 8 hours a day in girls were the risk factors for increased BP. Residing in urban versus rural areas was shown to be a risk factor for increased prehypertension and hypertension risk; OR: 1.43, CI: 1.11-1.84, OR: 1.77; CI; 1.37-2.27, respectively in boys and girls (Table 3B, $p<0.05$).

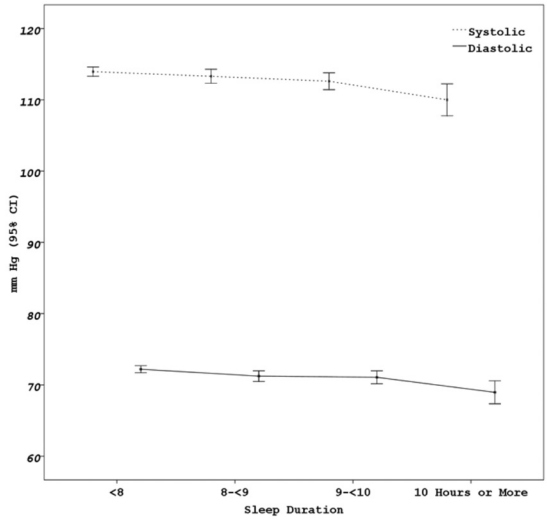

Figure 1. Systolic and diastolic blood pressure values according to sleep duration

Table 1. The relationship between sleep duration and blood pressure for boys and girls

Gender	Blood pressure	Sleep duration categories (hours)				p
		≤8 n (%)	8.1-8.9 n (%)	9.0-9.9 n (%)	≥10 n (%)	
Boys	Prehypertensive and hypertensive	292 (38.9)	110 (30.6)	64 (31.2)	19 (27.1)	0.010
	Normotensive	458 (61.1)	250 (69.4)	141 (68.8)	51 (72.9)	
	Prehypertensive and hypertensive	278 (32.9)	97 (29.0)	67 (30.2)	13 (17.6)	
Girls	**Normotensive**	566 (67.1)	238 (71.0)	155 (69.8)	61 (82.4)	0.037

Table 2. The comparison of several variables by sleep duration categories in boys and girls

Variables	Boys (n = 1385) Sleep duration categories (hours)					Girls (n = 1475) Sleep duration categories (hours)				
	≤8 (n = 750)	**8.1-8.9 (n = 360)**	**9.0-9.9 (n = 205)**	**≥10 (n = 70)**	**p**	**≤8 (n = 844)**	**8.1-8.9 (n = 335)**	**9.0-9.9 (n = 222)**	**≥10 (n = 74)**	**p**
BMI	20.7 ± 0.1	20.4 ± 0.2	20.1 ± 0.2	20.4 ± 0.4	0.073	20.9 ± 0.1	20.7 ± 0.2	20.6 ± 0.2	20.2 ± 0.4	0.177
WC	70.4 ± 0.4	69.8 ± 0.4	69.9 ± 0.6	69.7 ± 0.9	0.714	65.7 ± 0.2	65.2 ± 0.4	65.1 ± 0.4	64.3 ± 0.8	0.206
WHtR	0.43 ± 0.003	0.42 ± 0.003	0.43 ± 0.003	0.43 ± 0.006	0.936	0.42 ± 0.001	0.41 ± 0.002	0.42 ± 0.003	0.41 ± 0.005	0.376
MUAC	22.3 ± 0.1	22.0 ± 0.2	22.0 ± 0.2	21.9 ± 0.4	0.223	22.1 ± 0.1	22.2 ± 0.1	22.1 ± 0.2	22.0 ± 0.3	0.973
TSF	9.2 ± 0.3	9.7 ± 0.2	9.7 ± 0.3	9.2 ± 0.6	0.641	14.3 ± 0.2	14.3 ± 0.3	14.1 ± 0.4	13.6 ± 0.6	0.752
AFA	10.3 ± 0.2	10.1 ± 0.3	9.7 ± 0.4	9.6 ± 0.4	0.562	14.4 ± 0.2	14.4 ± 0.5	14.2 ± 0.4	13.6 ± 0.7	0.790
SBP	114.9 ± 0.5	114.5 ± 0.7	113.9 ± 0.9	112.5 ± 1.5	0.436	**112.9 ± 0.5[a]**	**112.1 ± 0.7[ab]**	**111.5 ± 0.9[ab]**	**107.9 ± 1.5[b]**	**0.013**
DBP	**72.7 ± 0.4[a]**	**71.0 ± 0.5[ab]**	**71.0 ± 0.7[ab]**	**69.4 ± 1.2[b]**	**0.007**	71.5 ± 0.3	71.7 ± 0.5	71.4 ± 0.7	69.1 ± 1.1	0.235
Fat %	24.7 ± 0.3	25.2 ± 0.5	24.3 ± 0.7	24.3 ± 1.1	0.698	35.6 ± 0.4	35.6 ± 0.6	35.1 ± 0.7	34.4 ± 1.2	0.751

Values are expressed as age-adjusted means (standard error of the mean), in accordance with where age or age and body mass index were the covariates for multiple comparisons (Bonferroni). Statistical significancy was ($p < 0.05$)

BMI: body mass index, WHtR: waist/height ratio, WC: waist circumference, MUAC: mid-upper arm circumference, TSF: triceps skinfold thickness, AFA: arm fat area, SBP: Systolic blood pressure, DBP: diastolic blood pressure, Fat %: arm fat area

[a, b]: According to post-hoc tests, statistically significant ($p < 0.05$) different anthropometric indices between sleep duration categories were labelled with different letters

Figure 2. The distribution of sleep duration by age groups in boys and girls

SBP and DBP values according to sleep duration are shown in Figure 1. Longer sleep duration was associated with lower BP. The distribution of sleep duration for age groups in boys and girls is given in Figure 2. In both genders, sleep duration showed a decrease with increase in age.

Discussion

Previous studies on adults indicate that there is a strong relationship between sleep duration and increased BP (19,20,21). To the best of our knowledge, the current study is the first to demonstrate a relationship between short sleep duration and high BP in children and adolescents aged 11-17 years. According to the univariate binary logistic regression analyses, sleep duration less than 8 h is a significant risk factor for hypertension in both genders. With each increment in sleeping hours for boys and girls, the risk of increased BP decreases (OR:0.89, CI: 0.82-0.98 and OR: 0.88, CI: 0.81-0.97 respectively in boys and girls). In multiple binary logistic regression analyses (adjusted for age and BMI) and by categorising sleep duration into four groups (≤8 h, 8.1-8.9 h, 9.0-9.9 h, ≥10 h), shorter sleep duration was also shown to be a risk factor for increased BP. There are several studies examining the relationship between increased BP and various environmental conditions, other than sleep duration (22,23,24,25,26). In this present study, the only other significant environmental risk factor identified was location of the school the children or adolescents were attending and this was only true of the girls. No other environmental risk factors included in this study were found to be an independent risk factor in our data set.

Table 3A. Univariate and multiple binary logistic regression of the likelihood of prehypertension on sociodemographic and behavioural variables for children and adolescents [with odds ratio and 95% confidence intervals]

Variables	Gender					
	Boys		Girls		Boys	Girls
	Normotensive	Prehypertensive	Normotensive	Prehypertensive	Univariate logistic regression (Adjusted for age)	
Continuous Variables	$\overline{X}$ + SD	$\overline{X}$ + SD	$\overline{X}$ + SD	$\overline{X}$ + SD	OR (CI)	OR (CI)
Computer use (h)	0.75 ± 0.05	0.83 ± 0.06	0.43 ± 0.03	0.46 ± 0.06	1.04 (0.97-1.13)	1.02 (0.93-1.12)
Sleep duration (h)	**8.48 ± 0.04**	**8.31 ± 0.06**	**8.47 ± 0.04**	**8.27 ± 0.06**	**0.89 (0.82-0.98)**	**0.88 (0.81-0.97)**
Viewing TV (h)	2.94 ± 0.05	2.96 ± 0.07	2.70 ± 0.00	2.75 ± 0.07	1.01 (0.94-1.08)	1.02 (0.95-1.10)
BMI (kg/m^2)	**20.13 ± 0.09**	**21.20 ± 0.15**	**20.50 ± 0.09**	**21.44 ± 0.16**	**1.12 (1.08-1.16)**	**1.09 (1.06-1.14)**
Categorical Variables	**n (%)**	**n (%)**	**n (%)**	**n (%)**		
Sleep duration categories (h)						
≤8.0	**458 (61.1)**	**292 (38.9)**	**566 (67.1)**	**278 (32.9)**	1.0	1.0
8.1-8.9	**250 (69.4)**	**110 (30.6)**	**238 (71.0)**	**97 (29.0)**	**0.69 (0.53-0.90)**	0.83 (0.63-1.09)
9.0-9.9	**141 (68.8)**	**64 (31.2)**	**155 (69.8)**	**67 (30.2)**	**0.71 (0.51-0.99)**	0.88 (0.64-1.21)
≥10.0	**51 (72.9)**	**19 (27.1)**	**61 (82.4)**	**13 (17.6)**	**0.58 (0.34-0.99)**	**0.43 (0.23-0.80)**
Location of the school						
Suburban	**601 (62.5)**	**360 (37.5)**	**646 (65.5)**	**341 (34.5)**	**1.0**	**1.0**
Urban	**299 (70.5)**	**125 (29.5)**	**374 (76.6)**	**114 (23.4)**	**1.43 (1.12-1.83)**	**1.73 (1.35-2.22)**
Transportation to school						
On foot	582 (66.1)	299 (33.9)	657 (69.8)	284 (30.2)	1.0	1.0
By bus/private car	318 (63.1)	186 (36.9)	363 (68.0)	171 (32.0)	1.14 (0.91-1.43)	1.09 (0.87-1.37)
Household income						
Poor	**174 (72.2)**	**67 (27.8)**	140 (71.4)	56 (28.6)	**1.0**	1.0
Fair	**512 (64.3)**	**284 (35.7)**	549 (67.9)	259 (32.1)	**1.63 (1.14-2.32)**	1.06 (0.73-1.53)
Good	**214 (61.5)**	**134 (38.5)**	331 (70.3)	140 (29.7)	**1.44 (1.05-1.99)**	1.18 (0.84-1.66)
House size (m^2)						
< 100	262 (66.2)	134 (33.8)	291 (70.5)	122 (29.5)	1.0	1.0
100-200	591 (10.4)	329 (35.8)	683 (68.6)	312 (31.4)	0.92 (0.53-1.58)	1.09 (0.62-1.90)
> 200	47 (68.1)	22 (31.9)	46 (68.7)	21 (31.3)	1.88 (1.18-3.54)	1.09 (0.85-1.39)
Parental schooling (years)						
Father						
< 5	423 (65.9)	219 (34.1)	440 (70.2)	187 (29.8)	1.0	1.0
6-8	208 (65.8)	108 (34.2)	241 (69.3)	107 (30.7)	1.37 (0.92-2.03)	1.06 (0.72-1.56)
9-11	197 (64.8)	107 (35.2)	235 (67.3)	114 (32.7)	1.05 (0.79-1.40)	1.14 (0.86-1.51)
> 12	72 (58.5)	51 (41.5)	104 (68.9)	47 (31.1)	1.01 (0.76-1.33)	1.05 (0.79-1.40)
Mother						
< 5	715 (65.8)	371 (34.2)	814 (68.8)	370 (31.2)	1.0	1.0
6-8	101 (60.5)	66 (39.5)	106 (70.2)	45 (29.8)	0.64 (0.20-2.00)	0.35 (0.10-1.18)
9-11	72 (62.1)	44 (37.9)	81 (68.6)	37 (31.4)	1.18 (0.79-1.75)	1.01 (0.67-1.51)
> 12	12 (75.0)	4 (25.0)	19 (86.4)	3 (13.6)	1.26 (0.90-1.76)	0.93 (0.65-1.35)

Table 3A. Continue						
Maternal employment						
Unemployed (Housewife)	858 (65.1)	459 (34.9)	975 (68.8)	442 (31.2)	1.0	1.0
Employed (Has a regular job)	42 (61.8)	26 (38.2)	45 (77.6)	13 (22.4)	1.16 (0.70-1.19)	0.64 (0.34-1.19)
Elevator use						
None	664 (66.4)	336 (33.6)	737 (70.7)	306 (29.3)	1.0	1.0
Habitual	236 (61.3)	149 (38.7)	283 (65.5)	149 (34.5)	1.25 (0.98-1.59)	1.27 (0.99-1.61)

Values represent odds ratio 95% confidence interval adjusted for age or age and body mass index

BMI: body mass index, SD: standard deviation, OR: odds ratio, CI: confidence interval

Table 3B. Multiple logistic regression analysis of the likelihood of prehypertension on sociodemographic and behavioural variables for children and adolescents

Variables	Multiple logistic regression analysis (the backward stepwise procedure)			
	Adjusted for age		Adjusted for age and BMI	
	Boys	Girls	Boys	Girls
	OR (CI)	OR (CI)	OR (CI)	OR (CI)
Sleep duration categories (hours)				
≤8	1.0	1.0	1.0	1.0
8.1-8.9	**0.68 (0.52-0.89)**	0.80 (0.61-1.06)	**0.72 (0.55-0.95)**	0.84 (0.63-1.11)
9.0-9.9	**0.70 (0.50-0.97)**	0.84 (0.61-1.15)	0.77 (0.55-1.07)	0.89 (0.64-1.23)
≥10	**0.55 (0.32-0.95)**	**0.43 (0.23-0.79)**	0.59 (0.34-1.04)	**0.47 (0.25-0.89)**
Region of the school				
Suburban	1.0	1.0	1.0	1.0
Urban	**1.45 (1.13-1.86)**	**1.76 (1.37-2.26)**	**1.43 (1.11-1.84)**	**1.77 (1.37-2.27)**
Household income				
Poor	1.0	-	1.0	-
Fair	**1.64 (1.15-2.35)**	-	**1.66 (1.15-2.38)**	-
Good	**1.43 (1.04-1.97)**	-	**1.38 (1.00-1.91)**	-

Values represent odds ratio 95% confidence interval adjusted for age or age and body mass index

BMI: body mass index, OR: odds ratio, CI: confidence interval

The American Academy of Sleep Medicine developed new consensus recommendations for the amount of sleep needed to promote optimal health, including avoidance of hypertension in children and adolescents. According to these recommendations, teenagers 13-to-18 years of age should sleep 8-to-10 hours per 24 hours on a regular basis to promote optimal health (27).

In a recent review on sleep characteristics and cardiovascular risk in children and adolescents, sleep pattern is indicated as a significant risk factor for cardiovascular disease, however the sleep pattern may differ between different geographic locations (28). Similarly, Meininger et al (29), demonstrated that long sleep duration is associated with a decrease both in SBP and DBP. In the study by Archbold et al (30) in 334 children aged 6-11 years, both a decrease in sleep duration and an increase in BMI were associated with increased BP. Prehypertension and hypertension were both associated with short sleep duration (less than eight hours) among Lithuanian children and adolescents (n = 6940) aged 12 to 15 years; when adjusted for age, gender, BMI, physical activity and smoking (31).

However, a recent longitudinal analysis from early-to-late adolescence found no association between sleep duration and BP in females. However, in males longer sleep duration was associated with lower values of BP (32).

Our study was conducted in a large province of central Anatolia in Turkey. In other previous studies, higher salivary cortisol levels were detected in individuals with short sleep when compared with normal or high sleep duration (33). In healthy young adults, impaired vascular endothelial adhesion markers and also endothelial-dependent/independent microvascular reactivity were detected in acute total sleep deprivation (34). Additionally, decreased endothelial-dependent vasodilatation, representing impaired endothelial function, was reported

in participants with short sleep duration. In another study, participants with a sleep duration of less than five hours were found to have significant changes in heart rates and BP values (35). Significant increases in catecholamine levels measured in 24-hour urine samples were reported in adults, suggesting an increase in sympathetic activity related to short sleeping hours (36).

In accordance with previous studies, we found a relationship between short sleep duration and hypertension in children and adolescents of both genders (32,33). In the current study, with the decrement in sleep duration, hypertension risk is increased. However, in previous studies, the relationship betwen short sleep duration and the risk of hypertension in adult women were explained by structural characteristics (BMI and other body composition variables) and menopause (37,38,39).

Peach et al (40), examined BMI as a possible mediator of the effect of sleep duration on risk for hypertension in a sample of sixth graders. Among boys, all three sleep characteristics (school-night sleep duration, weekend night sleep duration, and daytime sleepiness) predicted BMI and yielded significant indirect effects on risk for hypertension. In girls on the other hand, only daytime sleepiness predicted BMI and yielded a significant indirect effect on risk for hypertension.

The primary contribution of the current study may be revealing the age- and BMI-adjusted risk factors for increased BP associated with decreased sleeping duration.

Study Limitations

However, the main limitation of our study is that we did not use a validated scale to assess sleep duration and relied on self-reports of the parents for sleep duration of their children (41,42,43,44,45,46,47,48). The cross-sectional measurement of BP to assess increased BP is also a limitation of this study.

Conclusion

To the best of our knowledge, the current study is the the first to demonstrate that short sleep duration is a risk factor for increased BP in non-obese, Turkish children. We believe this issue requires further exploration.

Ethics

Ethics Committee Approval: The study was approved by the Erciyes University Local Ethics Committee (approval number: 04/312 approval date: 07/09/2004).

Informed Consent: Consent form was filled out by the parents of all children and adolescents.

Peer-reviewed: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Mümtaz M Mazıcıoğlu, Selim Kurtoğlu, Concept: Ahmet Öztürk, Betül Çiçek, Demet Ünalan, Dinçer Göksülük, Sevda İsmailoğulları, Selim Kurtoğlu, Design: Cengiz Bal, Ahmet Öztürk, Betül Çiçek, Vahap Eldem, Data Collection or Processing: Betül Çiçek, Ahmet Öztürk, Emine Erdem, Selim Kurtoğlu, Analysis or Interpretation: Ahmet Öztürk, Gökmen Zararsız, Gözde Ertürk Zararsız, Selçuk Korkmaz, Dinçer Göksülük, Literature Search: Ahmet Öztürk, Betül Çiçek, Gözde Ertürk Zararsız, Ahmet Özdemir, Mümtaz M Mazıcıoğlu, Writing: Betül Çiçek, Cengiz Bal, Ahmet Öztürk, Ahmet Özdemir, Sevda İsmailoğulları, Mümtaz M Mazıcıoğlu.

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A Meta-Analysis and an Evaluation of Trends in Obesity Prevalence among Children and Adolescents in Turkey: 1990 through 2015

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What is already known on this topic?

Obesity in childhood and adolescence is one of the most serious public health problems due to a remarkable increase in prevalence in recent years and its close relationship with non-communicable diseases, such as diabetes and hypertension, resulting in increased adult morbidity and mortality.

What this study adds?

The results of this present study reveal that further national, population-based surveys on the prevalence of obesity in children and adolescents are definitely needed in Turkey.

Abstract

Objective: Obesity in childhood and adolescence is one of the most serious public health problems due to a remarkable increase in prevalence in recent years and its close relationship with non-communicable diseases, such as diabetes and hypertension, resulting in increased adult morbidity and mortality. This study aims to quantify the secular trend in different regions of Turkey from 1990 to 2015 by performing a meta-analysis of childhood and adolescent obesity prevalence studies conducted.

Methods: Uludag University Library Database was searched for relevant articles published prior to March 2017. The heterogeneity of the studies in the meta-analysis was tested by the I2 statistic and Cochran's Q test. The obesity trend analyses were examined by chi-square trend analysis with respect to five year periods. The statistical significance level was taken as $\alpha = 0.05$.

Results: A total of 76 papers were initially identified addressing childhood and adolescent obesity in Turkey. Fifty-eight papers were selected for analysis. The prevalence of obesity increased from 0.6% to 7.3% with an 11.6-fold increase between the periods 1990-1995 to 2011-2015. The prevalence of obesity increased in both genders. However, boys were more likely to be obese than girls.

Conclusion: Studies on obesity prevalence in the 5-19 age group in Turkey have gained importance, especially in the 2000s. While a remarkable number of prevalence studies, mostly regional, have been conducted between 2005-2011, a gradual decline was observed thereafter. Further national and population-based surveys on prevalence of obesity in children and adolescents are definitely needed in Turkey.

Keywords: Childhood, adolescence, obesity

Introduction

Obesity in childhood and adolescence generally manifests itself in school years. Even when it does not continue into adulthood, it is correlated with increased adult morbidity and mortality by causing chronic disease states such as diabetes and hypertension (1). The prevalence of obesity has been increasing rapidly and because of these facts it is now one of the most serious public health problems for the 21st century. The World Health Organization (WHO) has reported that the percentage of overweight children under five years increased from 5% in 2000 to 6% in 2010. This increase has been estimated to result in over 42 million (6.3%) of children being overweight in 2013. This problem

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currently affects many low and middle-income countries, and especially urban residential areas (2). The increasing trend of being overweight is a worldwide problem as the number of adults with a body mass index (BMI) of >25 kg/m^2 increased from 29.8% to 36.9% in men and from 29.8% to 38.0% in women between the years 1980 and 2013 (3).

This is also true in Turkey where rapid changes in lifestyles, including dietary and physical activity habits have contributed to a remarkable increase in the prevalence of obesity which is now accepted as a serious threat to public health. Turkish studies have reported different results for the prevalence of obesity in children and adolescents, depending upon geographic and cultural differences. According to the latest national representative data, the obesity prevalence in the 6-18 age group is 8.2% overall. The difference between genders and for area of residence are 7.3% for girls, 9.1% for boys and 9.7% in urban, 4.5% in rural settings respectively (4). Adoption of a Western lifestyle amongst children and living in an urban setting in a developing country are considered probable risk factors (4,5).

Prevalence Studies of Diabetes, Hypertension, Obesity and Endocrine Diseases in Turkey data have confirmed the scale of the public health problem associated with childhood obesity. Notably, the results show that as the education level of women rises, the obesity risk decreases independent of other factors, a finding which emphasizes the importance of the education of girls in improving community health status (6).

The primary aim of this study is to identify the secular trend in the prevalence of childhood and adolescent obesity by performing a meta-analysis of studies conducted in different regions of the country between 1990 and 2015. We also aim to review the prevalence and changing trends of obesity among Turkish school children aged 5-19 years.

Methods

Search Strategy

The Uludağ University Library Database was searched to identify relevant papers in Turkish and in English published prior to March 2017 (7). The following key words were used: ['incidence' OR 'frequency' OR 'prevalence' OR 'epidemiology'] AND ['obesity' OR 'body mass index' OR 'BMI' OR 'weight gain'] AND ['Turkey' OR 'Turkish'] AND ['childhood' OR 'children' OR 'adolescence' OR 'adolescents' OR 'youth' OR 'teen' OR 'teenager'] for obesity in Turkish children and adolescents.

Studies were selected according to the following criteria:

i) A sample that included school children (5-19 years of age),

ii) Cross-sectional design,

iii) Original studies on prevalence of obesity,

iv) Studies conducted within the borders of Turkey,

v) Studies that defined obesity categories according to BMI calculated by dividing body weight (kg) by the square of height (m^2) and those that used the age and sex specific BMI percentile tables by Neyzi et al (8), or those of the centers for disease control and prevention (9) or WHO (10).

Studies which lacked sufficient data, or were repetitive studies based on the same database were considered as not meeting the inclusion criteria and were excluded.

Figure 1 summarizes the flow chart for selection of studies for inclusion in this meta-analysis for obesity.

Data Extraction

On the basis of pre-defined inclusion criteria, titles and abstracts were examined for inclusion by two independent reviewers (ZA and YU) and disagreements were resolved by consensus or, if necessary, by referral to a third reviewer (IE). The full text forms were evaluated for the articles with titles and/or abstracts with insufficient information. Publication year; study time, period and place; study design; representativeness of target population; sample selection; sample size; data source; data collection; description of obesity; sex; age; study objectives; criteria for obesity; and figures that allowed calculation of obesity prevalence were extracted from the studies. We assessed the quality of all included studies on the basis of the following: study design, representativeness of target population, sample selection, sample size, response rate, data source and study objectives, data collection, description of obesity, sex, and age. Studies were rated (++) if all or most of checklist criteria were

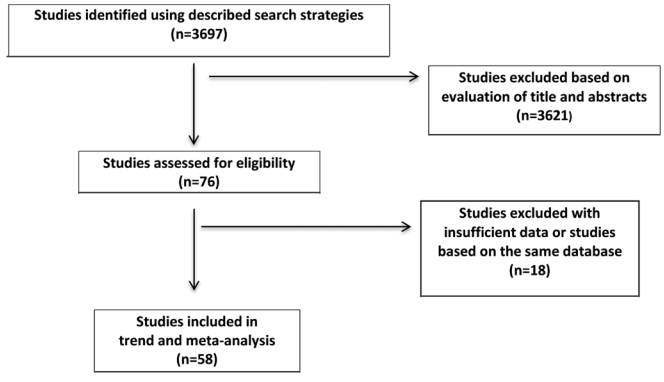

Figure 1. Flow chart for selection of studies for inclusion in this trend and meta-analysis for obesity

fulfilled; (+) if some criteria were fulfilled; and (-) if few or no criteria were fulfilled. All data extractions were ratified by one researcher (IE). Missing raw data were requested from authors by email or by phone calls.

Statistical Analysis

A meta-analysis was made for determining the summary statistics oriented towards the prevalence of obesity. The heterogeneity of the studies in the meta-analysis was tested by the I^2 statistic and Cochran's Q test. In the heterogeneity test, α was taken as 0.10. For the estimation of the summary statistics, the fixed effect model in case of homogeneity and the random effect model in the contrary case were used. The publication bias was assessed by inspection of Funnel plots. Statistics concerning the meta-analysis results are given in tables and by Forest plots.

The obesity trend analyses were examined by chi-square trend analysis with respect to 5-year periods, as there were insufficient studies conducted on a yearly basis. Thus, five blocks of 5-year periods were defined as 1990-1995, 1996-2000, 2001-2005, 2006-2010 and 2011-2015 and compared. In the trend analysis, instead of publication year, the year in which the field study was performed was used. For the studies in which the exact research periods were not stated, the necessary information was obtained by communicating with the author via e-mail and by phone. Any studies in which precise information was not available concerning the year the research was made were excluded from the research. By taking the 1990-1995 period as a baseline, the statistical significance levels for the next 5-year periods and the odds ratio values were calculated. The statistical significance level was taken as $\alpha = 0.05$.

Ethics

Information reported in this retrospective study was collected by references to published works. Ethical responsibility is related to the authors of the studies made.

Results

The analysis included studies which were conducted in different cities and regions of Turkey on school children aged between 5-19 years. While evaluating each of the studies one by one in the meta-analysis, in the trend analysis we evaluated the total of the studies made in different regions in the same 5-year periods, instead of representing only one location, with the aim of evaluating the trends in obesity in the 5-year periods. When all of the studies done between the years 1990-1995 were used as a baseline, we observed that there was an increase in the prevalence of obesity in the following 5-year periods.

After screening 76 papers, we included 58 papers in the analysis. Figure 2 shows the Forest and Funnel plots of 58 studies of obesity prevalence with a total number of

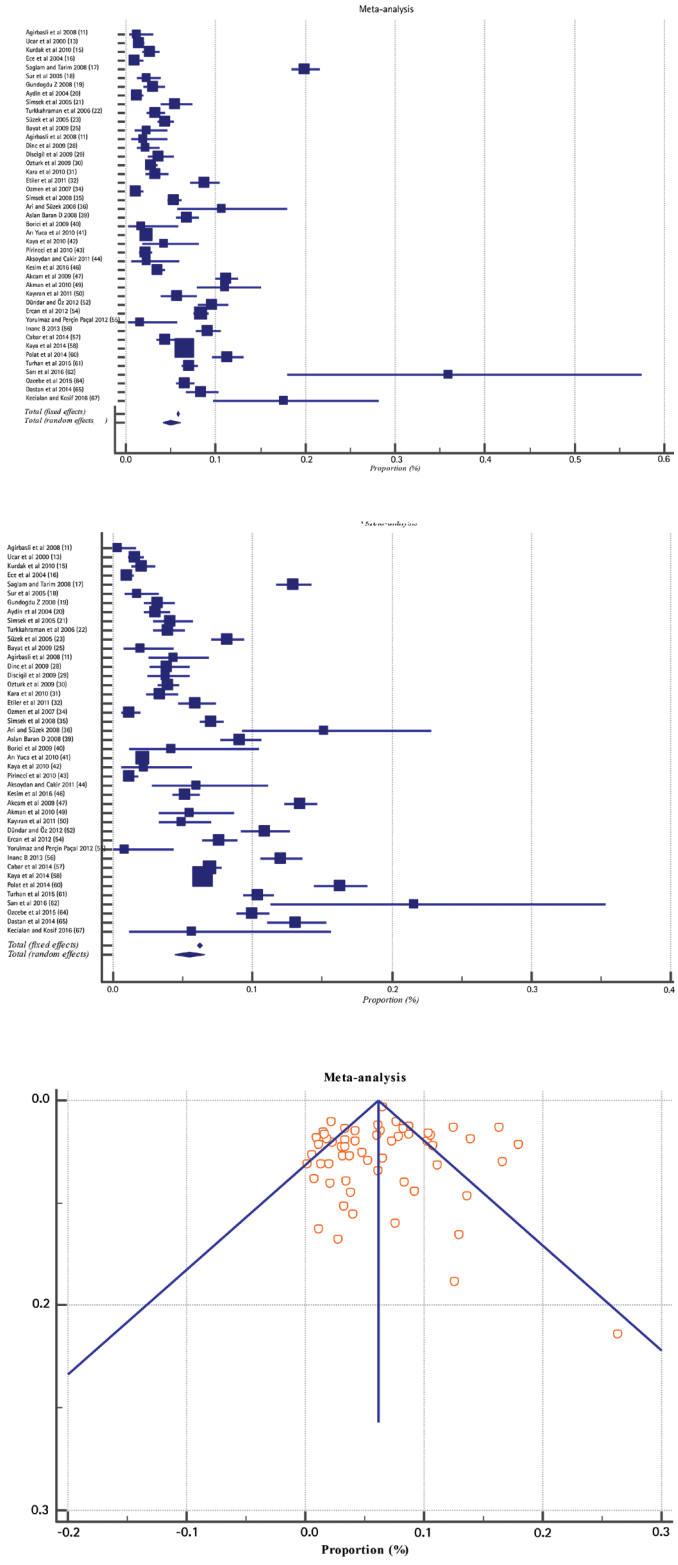

Figure 2. Pooled analysis for obesity in children and adolescents in Turkey, overall, girls and boys respectively

subjects of 230 252 Turkish school children aged 5-19 years to evaluate overall obesity prevalence. For assessing gender specific obesity prevalence 43 papers with a total of 100 086 girls and 108 491 boys aged 5-19 years were assessed. The prevalence of obesity was found as 5.7% [95% confidence interval (CI), 4.8-6.6] totally, 5.0% (95% CI, 4.1-6.1) in girls and 5.5% (95% CI, 4.4-6.6) in boys (Table 1).

Time trend analyses based on data collection years showed that obesity increased 11.6-fold (5.8-fold for girls, 24.5-fold for boys) from 1990-1995 to 2011-2015. The prevalence of obesity increased in both genders, but boys were more likely to be obese than girls (Table 2).

While the prevalence of obesity increased from 0.7% to 7.1% between 1990-1995 and 2011-2015 (for girls: 1.2% to 6.8%; for boys: 0.3% to 7.4%) according to the data obtained from 43 publications in which the data are given separately as overall, girls and boys, we observed that the overall prevalence of obesity increased from 0.6% to 7.3% by analysing the trend with 58 publications. (Table 2,3) (Figure 3,4).

Discussion

This meta-analysis indicates that the prevalence of obesity has increased significantly among both girls and boys in Turkey since 1990 and that this increase is much more marked in boys.

In the present study, we consulted 58 different studies conducted on prevalence of obesity between 1990 and 2015 in school children aged 5-19. All studies confirmed an increase in obesity, though the magnitude of this increase varied.

Study Limitations

The meta-analysis reported here combines data across studies conducted in different cities and groups in Turkey in order to estimate trends in obesity in school children aged 5-19 with more precision than is possible in a single study. The main limitations of this meta-analysis. as with any overview. are the differences between the age groups of the study population. insufficient age-specific data and regional and cultural differences. Among these studies. there were publications whose aim was not to determine obesity prevalence and publications which did not discriminate between obesity prevalence according to gender. despite the fact that they were well designed. So. the quality of the data cannot go beyond the quality of the individual studies included and the results can only be representative of the studies that have been included and are unable to provide a representation of all studies published.

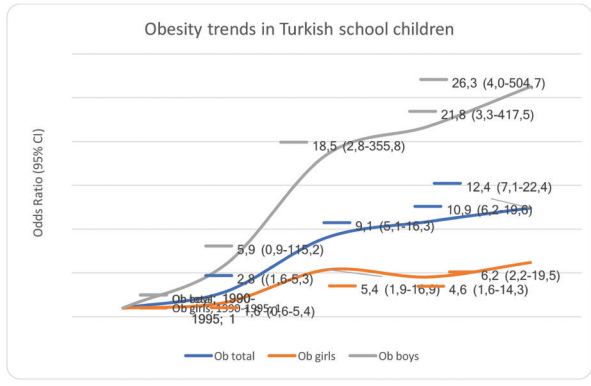

Figure 3. Trends in obesity prevalence [OR (95% CI)] in Turkish children and adolescents aged 5-19 years from 1990-1995 through 2011-2015. Data obtained from all 58 publications with and without gender discrimination

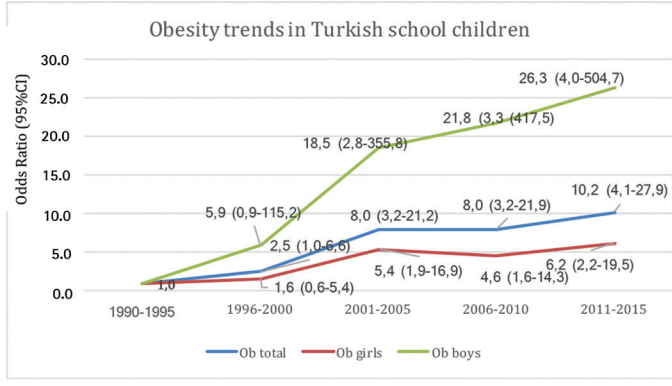

Figure 4. Trends in obesity prevalence [OR (95% CI)] in Turkish children and adolescents aged 5-19 years from 1990-1995 through 2011-2015. Data obtained from 43 publications in which the data are given separately as overall. girls and boys

Conclusion

However, the results of this present study reveal that further national. regular population-based surveys representing Turkey on the prevalence of obesity in children and adolescents are definitely needed. We. as authors. wish to increase awareness of this global public health concern in order to develop comprehensive public health policies and strategies to improve the prevention and management of obesity and related diseases. We also wish to provide baseline data for monitoring the effectiveness of national programs for control of obesity in the future. which we suggest should be a high priority public health initiative for Turkey. It should not be forgotten that obesity. and obesity-related non-communicable diseases. will negatively affect immediate health. quality of life and educational attainment in childhood and adolescence and will likely have a permanent negative effect on the future life of the child.

Table 1. Meta-analysis for obesity in children and adolescents in Turkey

Study, publication year, (city, study year)	Overall sample size	Overall proportion % (95% CI)	Overall weight (%) random	Girls sample size	Girls proportion % (95% CI)	Girls weight (%) random	Boys sample size	Boys proportion % (95% CI)	Boys weight (%) random
Agirbasli et al 2008 (Ankara,1990)(11)	673	0.7 (0.2-1.7)	1.7	341	1.2 (0.3-3.0)	2.3	332	0.3 (0.0-1.7)	2.2
Agirbasli et al 2006 (Ankara,1994)(12)	1385	0.6 (0.3-1.1)	1.8	NA	NA	NA	NA	NA	NA
Ucar et al 2000 (Eskişehir, 1997)(13)	4026	1.5 (1.2-2.0)	1.8	2065	1.5 (1.0-2.1)	2.5	1961	1.6 (1.1-2.2)	2.5
Soylu et al 1999 (İzmir, 1998)(14)	1024	1.4 (0.7-2.3)	1.7	NA	NA	NA	NA	NA	NA
Kurdak et al 2010 (Adana, 2000)(15)	2352	2.3 (1.8-3.0)	1.8	1173	2.6 (1.8-3.7)	2.5	1179	2.0 (1.3-3.0)	2.5
Ece et al 2004 (Diyarbakır, 2001)(16)	3040	1.0 (0.7-1.4)	1.8	810	1.0 (0.4-1.9)	2.4	2230	1.0 (0.6-1.5)	2.5
Saglam and Tarim 2008 (Bursa, 2001)(17)	5368	16.3 (15.3-17.3)	1.8	2559	20.0 (18.4-21.6)	2.5	2809	1.7 (0.8-3.3)	2.5
Sur et al 2005 (İstanbul, Ankara, İzmir, 2002)(18)	1044	2.0 (1.2-3.1)	1.7	528	2.3 (1.2-3.9)	2.4	516	3.2 (2.2-4.5)	2.3
Gundogdu Z 2008 (Kocaeli, 2002)(19)	1899	3.1 (2.4-4.0)	1.8	866	3.0 (2.0-4.4)	2.4	1033	3.0 (2.2-4.1)	2.4
Aydin et al 2004 (Bursa, 2003)(20)	2793	1.8 (1.3-2.4)	1.8	1379	1.2 (0.7-2.0)	2.5	1414	4.1 (2.8-5.8)	2.5
Simsek et al 2005 (Ankara, 2003)(21)	1510	4.8 (3.7-6.0)	1.8	732	5.5 (3.9-7.4)	2.4	778	3.9 (2.9-5.1)	2.4
Turkkahraman et al 2006 (Antalya, 2003)(22)	2645	3.3 (2.7-4.1)	1.8	1232	3.2 (2.3-4.4)	2.5	1233	8.2 (7.0-9.4)	2.5
Süzek et al 2005 (Muğla, 2004)(23)	4260	6.3 (5.6-7.1)	1.8	2040	4.3 (3.5-5.3)	2.5	2220	2.0 (0.7-4.3)	2.5
Nur et al 2008 (Sivas, 2004)(24)	1020	0.2 (0.0-0.7)	1.7	NA	NA	NA	NA	NA	NA
Bayat et al 2009 (Kayseri, 2004)(25)	610	2.1 (1.1-3.6)	1.7	310	2.3 (0.9-4.6)	2.2	300	4.4 (2.6-6.9)	2.2
Anamur Uguz and Bodur 2007 (Konya, 2005)(26)	496	3.8 (2.3-5.9)	1.7	NA	NA	NA	NA	NA	NA
Discigil G 2008 (Aydın, 2005)(27)	826	6.2 (4.6-8.0)	1.7	NA	NA	NA	NA	NA	NA
Agirbasli et al 2008 (İstanbul, 2005)(11)	640	3.4 (2.2-5.2)	1.7	251	2.0 (0.7-4.6)	2.2	389	3.9 (2.6-5.5)	2.3
Dinc et al 2009 (Manisa, 2005)(28)	1346	3.2 (2.3-4.3)	1.8	580	2.2 (1.2-3.8)	2.4	776	3.8 (2.4-5.5)	2.4
Discigil et al 2009 (Aydın, 2005)(29)	1348	3.7 (2.8-4.9)	1.8	683	3.7 (2.4-5.4)	2.4	665	3.8 (2.4-5.5)	2.4
Ozturk et al 2009 (Kayseri, 2005)(30)	5358	3.3 (2.9-3.9)	1.8	2737	2.8 (2.2-3.5)	2.5	2621	3.9 (3.2-4.7)	2.5
Kara et al 2010 (Diyarbakır, Mardin, 2005)(31)	1912	3.3 (2.6-4.3)	1.8	872	3.3 (2.2-4.7)	2.4	1040	3.4 (2.4-4.7)	2.4
Etiler et al 2011 (Kocaeli, 2005)(32)	2491	7.3 (6.3-8.4)	1.8	1217	8.7 (7.2-10.4)	2.5	1274	5.9 (4.7-7.3)	2.5
Ozturk and Akturk 2011 (Kayseri, 2005)(33)	1226	6.5 (5.2-8.1)	1.8	NA	NA	NA	NA	NA	NA
Ozmen et al 2007 (Manisa, 2006)(34)	2101	1.1 (0.7-1.7)	1.8	1051	1.1 (0.6-2.0)	2.5	1050	1.1 (0.6-2.0)	2.4
Simsek et al 2008 (Düzce, 2006)(35)	6924	6.2 (5.6-6.8)	1.8	3643	5.4 (4.7-6.2)	2.5	3281	7.0 (6.2-8.0)	2.5
Ari and Süzek 2008 (Muğla, 2006)(36)	231	13.0 (8.9-18.0)	1.5	112	10.7 (5.7-18.0)	1.8	119	15.1 (9.2-22.8)	1.9

Table 1. Continue

Akis et al 2009 (Bursa, 2006)(37)	2478	4.2 (3.5-5.1)	1.8	NA	NA	NA	NA	NA	NA
Calisir and Karacam 2011 (Aydın, 2006)(38)	460	13.7 (10.7-17.1)	1.7	NA	NA	NA	NA	NA	NA
Aslan Baran D 2007 (Bursa, 2007)(39)	3066	7.9 (7.0-8.9)	1.8	1510	6.8 (5.5-8.1)	2.5	1556	9.1 (7.7-10.6)	2.5
Borici et al 2009 (İstanbul, 2007)(40)	216	2.8 (1.0-5.9)	1.5	121	1.7 (0.2-5.8)	1.8	95	4.2 (1.2-10.4)	1.7
Arı Yuca et al 2010 (Van, 2007)(41)	9048	2.2 (1.9-2.5)	1.8	4184	2.3 (1.8-2.8)	2.5	4864	2.1 (1.7-2.6)	2.5
Kaya et al 2010 (İstanbul, 2007)(42)	369	3.3 (1.7-5.6)	1.6	190	4.2 (1.8-8.1)	2.1	179	2.2 (0.6-5.6)	2
Pirincci et al 2010 (Elazıg, 2007)(43)	3642	1.6 (1.3-2.1)	1.8	1782	2.1 (1.5-2.9)	2.5	1860	1.2 (0.7-1.8)	2.5
Aksoydan and Cakir 2011 (Kocaeli, 2007)(44)	319	4.1 (2.2-6.9)	1.6	169	2.4 (0.6-5.9)	2.0	150	6.0 (2.8-11.1)	2
Duzova et al 2013 (Turkey, 2008)(45)	3622	8.8 (7.9-9.7)	1.8	NA	NA	NA	NA	NA	NA
Kesim et al 2016 (Kayseri, 2008)(46)	4534	4.3 (3.7-4.9)	1.8	2516	3.6 (2.9-4.4)	2.5	2018	5.2 (4.2-6.2)	2.5
Akcam et al 2009 (Isparta, 2009)(47)	5716	12.5 (11.6-13.3)	1.8	2454	11.2 (9.9-12.5)	2.5	3262	13.4 (12.3-14.6)	2.5
Akan et al 2010 (İstanbul, 2009)(48)	499	9.2 (6.8-12.1)	1.7	NA	NA	NA	NA	NA	NA
Akman et al 2010 (İstanbul, 2009)(49)	625	8.3 (6.3-10.8)	1.7	316	11.1 (7.8-15.1)	2.2	309	5.5 (3.2-8.7)	2.2
Kayıran et al 2011 (İstanbul, Iğdır, Muğla, 2009)(50)	1134	5.3 (4.1-6.8)	1.7	581	5.7 (3.9-7.9)	2.4	553	4.9 (3.2-7.0)	2.4
Albayrak and Kutlu 2012 (İstanbul, 2009)(51)	276	7.6 (4.8-11.4)	1.6	NA	NA	NA	NA	NA	NA
Dündar and Öz 2012 (Samsun, 2009)(52)	2477	10.3 (9.1-11.5)	1.8	1206	9.6 (8.0-11.4)	2.5	1271	10.9 (9.2-12.7)	2.5
Onsuz and Demir 2015 (Sakarya, 2010)(53)	2166	18.0 (16.4-19.7)	1.8	NA	NA	NA	NA	NA	NA
Ercan et al 2012 (Ankara, 2011)(54)	8848	7.7 (7.1-8.2)	1.8	4408	8.4 (7.6-9.2)	2.5	1755	7.6 (6.4-8.9)	2.5
Yorulmaz and Perçin Paçal 2012 (İstanbul, 2011)(55)	250	1.2 (0.2-3.5)	1.6	124	1.6 (0.2-5.7)	1.9	126	7.6 (6.4-8.9)	1.9
Inanc B 2013 (Mardin, 2011)(56)	3460	10.6 (9.6-11.7)	1.8	1667	9.1 (7.7-10.5)	2.5	1793	0.8 (0.0-4.3)	2.5
Cabar et al 2014 (Sinop, 2011)(57)	3352	6.0 (5.2-6.9)	1.8	1597	4.3 (3.4-5.4)	2.5	4440	7.0 (6.2-7.7)	2.5
Kaya et al 2014 (Kütahya, 2011)(58)	92933	6.5 (6.3-6.7)	1.8	44126	6.5 (6.3-6.7)	2.6	48807	6.5 (6.3-6.7)	2.5
Demirci et al 2013 (Bursa, 2012)(59)	1000	11.2 (9.4-13.4)	1.7	NA	NA	NA	NA	NA	NA
Polat et al 2014 (Ankara, 2012)(60)	2826	13.9 (12.7-15.2)	1.8	1330	11.3 (9.6-13.1)	2.5	1496	16.2 (14.4-18.2)	2.5
Turhan et al 2015 (İzmir, 2012)(61)	6191	8.8 (8.1-9.5)	1.8	3058	7.1 (6.2-8.1)	2.5	3133	10.4 (9.4-11.5)	2.5
Sarı et al 2016 (İzmir, 2012)(62)	76	26.3 (16.9-37.7)	1.2	25	36.0 (18.0-57.5)	0.9	51	21.6 (11.3-35.3)	1.4
Gokler et al 2015 (Eskişehir, 2013)(63)	3918	10.4 (9.5 - 11.4)	1.8	NA	NA	NA	NA	NA	NA
Ozcebe et al 2015 (Turkey, 2013)(64)	4958	8.3 (7.5-9.1)	1.8	2475	6.6 (5.6-7.6)	2.5	2483	9.9 (8.8-11.2)	2.5
Dastan et al 2014 (İzmir, 2014)(65)	2009	10.8 (9.4-12.1)	1.8	992	8.4 (6.7-10.3)	2.5	1017	13.1 (11.1-15.3)	2.4
Cam and Top 2015 (Giresun, 2014)(66)	1109	16.7 (14.5-19.0)	1.7	NA	NA	NA	NA	NA	NA
Kecialan and Kosif 2016 (Bolu, 2015)(67)	127	12.6 (7.4-19.7)	1.4	74	17.6 (9.7-28.2)	1.6	53	5.7 (1.1-15.7)	1.4
Total (random effects)	230252	5.7 (4.8-6.6)	100.0	100086	5.0 (4.1-6.1)	100	108491	5.5 (4.4-6.6)	100
	Q = 4554.2913, df = 57, p < 0.0001, I^2 = 98.75 %, 95 % CI: 98.62 < I2 < 98.87			Q = 1699.6219, df = 42, p < 0.0001, I^2 = 97.53 %, 95 % CI: 97.14 < I2 < 97.87			Q = 1957.553, df = 42. p < 0.0001, I^2 = 97.85 %, 95 % CI: 97.53 < I2 < 98.14		

NA: not available, CI: confidance interval

Table 2. Trend analysis for obesity in children and adolescents in Turkey

Study year*	Sample	Reference	Obese n (%)	Total	OR (95% CI)	χ^2; p
1990-1995	Overall	Ref. 11	5 (0.7)	673	**1.00**	**χ^2 = 354.588; p < 0.001**
	Girls		4 (1.2)	341	**1.00**	**χ^2 = 124.201; p < 0.001**
	Boys		1 (0.3)	332	**1.00**	**χ^2 = 235.979; p < 0.001**
1996-2000	Overall	Ref. 13,15	117 (1.8)	6 378	**2.5 (1.0-6.6)**	-
	Girls		62 (1.9)	3 238	**1.6 (0.6-5.4)**	-
	Boys		55 (1.8)	3 140	**5.9 (0.9-115.2)**	-
2001-2005	Overall	Ref. 11,16,17,18,19,20, 21,22,23,25,28,29,30, 31,32	2 026 (5.6)	36 094	**8.0 (3.2-21.2)**	-
	Girls		1 004 (6.0)	16 796	**5.4 (1.9-16.9)**	-
	Boys		1 022 (5.3)	19 298	**18.5 (2.8-355.8)**	-
2006-2010	Overall	Ref. 34,35,36,39,40,41, 42,43,44,46,47,49,50, 52	2 285 (5.7)	40 402	**8.0 (3.2-21.9)**	-
	Girls		1 017(5.1)	19 835	**4.6 (1.6-14.3)**	-
	Boys		1 268 (6.2)	20 567	**21.8 (3.3-417.5)**	-
2011-2015	Overall	Ref. 54,55,56,57,58,60,61, 62,64,65,67	8 890 (7.1)	125 030	**10.2 (4.1-27.9)**	-
	Girls		4 098 (6.8)	59 876	**6.2 (2.2-19.5)**	-
	Boys		4 792 (7.4)	65 154	**26.3 (4.0-504.7)**	-

*Year during which the study was conducted

OR: odds ratio, CI: confidance interval

Table 3. Trend analysis for obesity in children and adolescents in Turkey

Study year*	Sample	Reference	Obese n (%)	Total	OR (95% CI)	χ^2; p
1990-1995	Overall	Ref. 11, 12	13 (0.6)	2 058	**1.00**	**χ^2 = 518.326; p < 0.001**
1996-2000	Overall	Ref. 13, 14, 15	131 (1.8)	7 402	**2.83 (1.6-5.3)**	-
2001-2005	Overall	Ref. 11,16,17,18,19, 20,21,22,23,24,25, 26,27,28,29,30,31, 32,33	2 168 (5.4)	39 832	**9.05 (5.1-16.3)**	-
2006-2010	Overall	Ref. 34,35,36,37,38, 39,40,41,42,43,44, 45, 46,47,48,49,50,51, 52,53	3 228 (6.5)	49 903	**10.88 (6.2-19.6)**	-
2011-2015	Overall	Ref. 54,55,56,57,58, 59,60,61,62,63,64, 65, 66,67	9 594 (7.3)	131 057	**12.43 (7.1-22.4)**	-

*Year during which the field study was conducted

OR: odds ratio, CI: confidance interval

Ethics

Ethics Committee Approval: Information reported in this retrospective study was collected by references to published works.

Authorship Contributions

Concept: Züleyha Alper, Yeşim Uncu, İlker Ercan, Design: Züleyha Alper, İlker Ercan, Yeşim Uncu, Data Collection or Processing: Züleyha Alper, İlker Ercan, Analysis or Interpretation: İlker Ercan, Literature Search: Züleyha Alper, Yeşim Uncu, Writing: Züleyha Alper.

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CASE REPORT

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A Patient with Proopiomelanocortin Deficiency: An Increasingly Important Diagnosis to Make

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What is already known on this topic?

Proopiomelanocortin (POMC) deficiency is an extremely rare disorder characterized by early-onset obesity, adrenal insufficiency, red hair and decreased skin pigmentation. Hyperphagia, cholestasis, exponential weight gain and adrenal insufficiency are typically observed during the first months of life. In some children, the diagnosis may only be established later.

What this study adds?

This study presents clinical and molecular features of a child with POMC deficiency. We also provide a brief summary of the clinical and genetic features of POMC deficiency based on previously published patient reports and describe how these are providing insight into the role of POMC in the regulation of human metabolism.

Abstract

Proopiomelanocortin (POMC) deficiency is a rare monogenic disorder with early-onset obesity. Investigation of this entity have increased our insight into the important role of the leptin-melanocortin pathway in energy balance. Here, we present a patient with POMC deficiency due to a homozygous c.206delC mutation in the *POMC* gene. We discuss the pathogenesis of this condition with emphasis on the crosstalk between hypothalamic and peripheral signals in the development of obesity and the POMC-melanocortin 4 receptors system as a target for therapeutic intervention.

Keywords: Obesity, melanocortin 4 receptors, paediatric obesity, proopiomelanocortin deficiency

Introduction

Proopiomelanocortin (POMC) is a 241-amino acid polypeptide that is cleaved via prohormone convertase (PC) to produce the peptides γ-, β-, α-melanocyte stimulating hormone (MSH), adrenocorticotropin hormone (ACTH), γ-, β-lipotrophin and endorphins (1). These peptides stimulate five different melanocortin receptors (MCR) with varying affinity and specificity. Cortisol secretion is regulated through MC2R in the adrenal gland while MC1R regulates skin pigmentation. MC3R and MC4R regulate body weight.

Congenital POMC deficiency develops due to genetic defects in the *POMC* gene located at *Chr.*2p23.3. This disorder is characterized by early-onset obesity, adrenal insufficiency, red hair and decreased skin pigmentation (1,2,3,4). Obesity develops as a result of inadequate production of α- and β-MSH, which normally activate the MC3R in the arcuate nucleus and the MC4R in the paraventricular nucleus and

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antagonize the action of agouti-related peptide (AgRP) (4). The hypocortisolaemia and hypopigmentation are due to inadequate stimulation of MC2R and MC1R by POMC-derived peptides in the adrenal gland and skin, respectively. POMC deficiency is rare, but has increased our insight into the important role of the leptin-melanocortin pathway in energy balance.

Here, we present the clinical characteristics of a patient with POMC deficiency due to a mutation in the POMC gene, hoping to contribute to a better understanding of the leptin-melanocortin pathway and to introduce possible treatment options.

Case Report

This female patient presented at age 2.5 months with restlessness, cyanosis, and spasms. She was found to be hypoglycaemic with a blood glucose of 31 mg/dL. She was born at 39 weeks gestation with a birth weight of 3000 grams and had no problems during the prenatal or early postnatal period. Her mother and father were not related and she had a healthy brother aged five years. There was no history of relevant disease in the family. Examination findings at presentation revealed growth failure (body weight: 3700 g, 3rd percentile; height: 51 cm, < 3rd percentile; head circumference: 35 cm, < 3rd percentile) (Figure 1A), red eyebrows and hair and normal female genitalia. Results of further laboratory investigations confirmed the hypoglycaemia (blood glucose: 19 mg/dL) and revealed mild hyponatraemia with a sodium of 132 mmol/L (135-143), accompanied by a potassium of 4.8 mmol/L (3.1-5.5), mildly elevated aspartate transaminase: 123 U/L (< 36) and creatinine kinase: 419 U/L (34-204).

Concomitant with the hypoglycaemic state (34 mgl/dL), serum and urinary ketones were low and there was no evidence of hyperinsulinaemia or any other metabolic cause for the hypoglycemia (serum insulin: 0.11 U/L, C-peptide: < 0.03 nmol/L, lactate: 2.37 mmol/L (0.49-2.19), ammonium: 93.5 μmol/L (13.5-42.8), urinary and blood amino acids and organic acid profile normal). Total/indirect bilirubin levels were 3.7/2.4 mg/dL. However, the child was hypocortisolaemic (cortisol: < 5.51 nmol/L) with an undetectable ACTH level (ACTH: < 1.1 pmol/L). Other anterior pituitary hormones were as follows: growth hormone 14.8 μg/L; thyroid-stimulating hormone 1.73 U/L; free thyroxine 14.02 pmol/L; prolactin 390 mIU/L (3-24); follicle-stimulating hormone < 3 U/L (0.1-3.3); luteinizing hormone < 0.07 U/L (0-1.9). A low-dose ACTH stimulation test showed an insufficient cortisol response at 40 minutes (12.1 nmol/L). A magnetic resonance imaging (MRI) scan of the pituitary gland was normal. A diagnosis of isolated central (secondary) ACTH insufficiency, rather than panhypopituitarism was made.

Hydrocortisone treatment was initiated which subsequently enabled successful control of the hypoglycemia. Due to the presence of central adrenal insufficiency together with red hair, a genetic analysis of the *POMC* gene was undertaken. A homozygous frameshift mutation, c.206delC (p.P69Lfs*2) in the *POMC* gene was detected (5). This mutation results in a downstream frameshift and premature protein truncation, removing ACTH and other important peptides and most likely completely disrupting POMC function (Figure 1B).

Following this initial presentation, in subsequent months, the child showed rapid increase in growth and developed

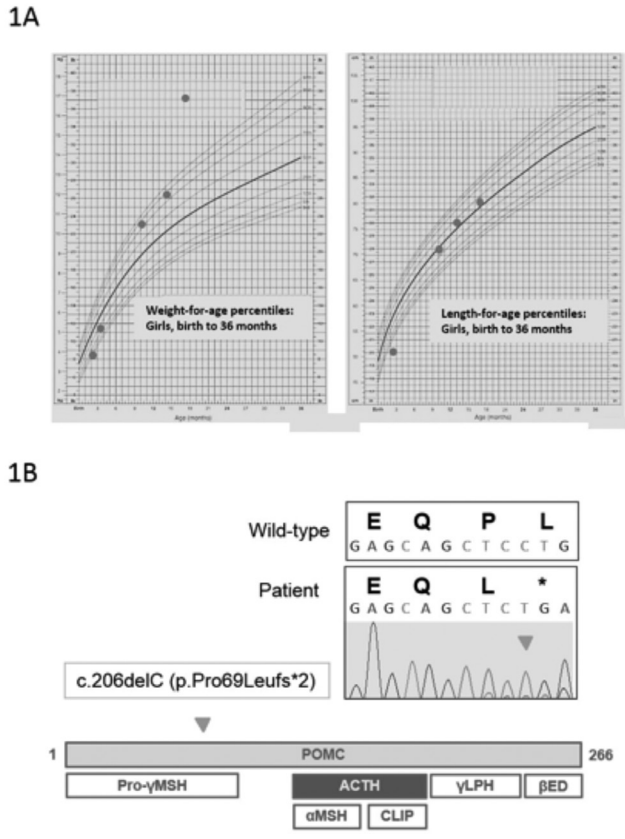

Figure 1. Weight (left) and height chart (right) of the patient showing rapid weight gain after infancy (A). Chromatogram showing the homozygous c.206delC change that results in a leucine residue (CTG) being replaced by a stop codon (TGA) (upper). This mutation causes disruption of proopiomelanocortin and prevents cleavage of proopiomelanocortin into key peptides such as adrenocorticotropin hormone and α-melanocyte stimulating hormone (lower) (B)

ACTH: adrenocorticotropin hormone, POMC: proopiomelanocortin, CLIP: corticotropin-like intermediate lobe peptide, γLPH: γ-lipotropin, βED: β-endorphin, MSH: melanocyte-stimulating hormone

obesity. At 17 months of age her weight was 16.9 kg (> 97th percentile), height 80 cm (50th percentile), and head circumference 40 cm (75-90th percentile) (Figure 1A). Her eyebrows and hair were red (Figure 2). The final steroid treatment dose was 8 mg/m^2/day. An informed consent form for publication was given by the parents.

Discussion

Congenital isolated ACTH deficiency is a rare condition and the symptoms and signs can be nonspecific. However, it can be life threatening unless appropriate steroid replacement is initiated. POMC is synthesized in the corticotropic cells of the pituitary gland by the action of the transcription factor TBX19/TPIT. POMC is then cleaved to form ACTH by the enzyme PC1 [PC1/3, proprotein convertase subtilisin/kexin (PCSK) type 1] following corticotropin-releasing hormone stimulation (Figure 1B) (6). Isolated ACTH deficiency can result from pathogenic variations of the *TBX19* (TPIT), *PCSK1* (PC1/3) and *POMC* genes (6).

Although POMC defects were first reported in 1998, relatively few children with the condition have been reported to date (4). The classic triad of POMC deficiency consists of early-onset obesity, central adrenal insufficiency and red hair. Hyperphagia (80-99%), cholestasis (30-79%, at onset), exponential weight gain (100%) and adrenal insufficiency (30-79%, at onset) are typically observed during the first months of life, but the diagnosis may only be established later in some children. Linear growth is initially normal, as in our patient, and weight gain may not occur initially in a child with uncontrolled adrenal insufficiency. However, weight often increases to above the 90th percentile by the end of the first year. This process likely reflects an insufficiency of hypothalamic POMC. Normally, nutrition and energy hemostasis is balanced by the complex interaction of POMC and AgRP/neuropeptide Y (NPY) with MCR in the hypothalamus (Figure 3) (1,2,3,4). This system is also regulated by peripheral polypeptides such as leptin and ghrelin. Our patient showed a rapid and early-onset weight gain as a result of this process. The weight gain was independent of steroid treatment as only a physiological replacement dose of hydrocortisone was used and subsequent linear growth rate was stabilized on the 50th percentile line, despite ongoing rapid weight gain.

The red hair associated with POMC deficiency is an important sign, especially in children from an ancestral background of dark hair. However, there are a few reports of children with POMC deficiency who do not have red hair or where only the roots of the hair are red (6,7,8,9). Other children may have red hair initially but this turns brown in the first three to four years of life (10).

Other reported features potentially associated with POMC deficiency include pale skin (Fitzpatrick type 1) due to reduced stimulation of MC1R by MSH; central hypothyroidism, possibly due to interactions between POMC and thyrotropin-releasing hormone in the hypothalamus (10,11,12,13); and hypogonadotropic hypogonadism with pubertal growth hormone deficiency reflecting a possible direct interaction between POMC and gonadotropin-

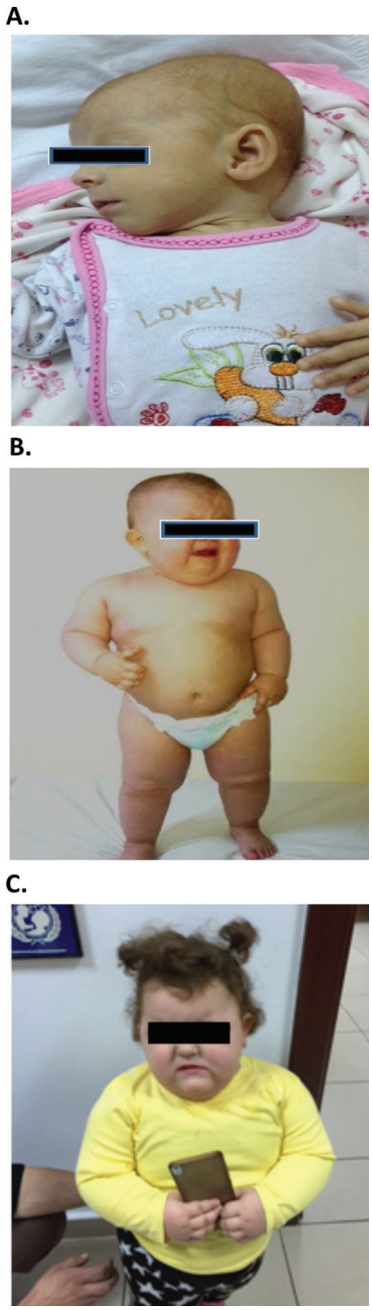

Figure 2. General appearance [At appointment (red eyebrows and hair) (A), at 1.5 years old (obesity) (B), at 2.5 years old (C)]

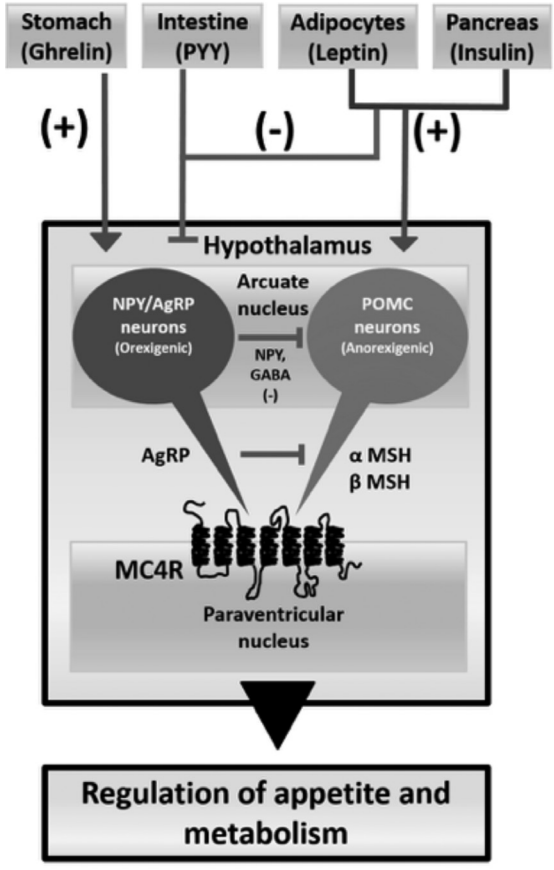

Figure 3. Cartoon showing potential interactions of proopiomelanocortin neurons in appetite regulation. In the hypothalamus, nutrition and energy hemostasis is balanced by proopiomelanocortin and agouti-related peptide/neuropeptide Y through melanocortin receptors. This system is regulated by peripheral polypeptides such as leptin, ghrelin, insulin and peptide YY. In proopiomelanocortin deficiency, the appetite-stimulating effect of agouti-related peptide is not balanced by the appetite-suppressing effect of proopiomelanocortin. AgRP is co-expressed with neuropeptide Y. This peptide increases appetite and decreases energy use and metabolism. This system is mainly inhibited by leptin and stimulated by ghrelin

NPY: neuropeptide Y, AgRP: agouti-related peptide, POMC: proopiomelanocortin, MSH: melanocyte-stimulating hormone, MC4R: melanocortin 4 receptors, PPY: peptide YY, GABA: gamma-amino butyric acid

releasing hormone neurons or indirectly via kisspeptin and NPY/AgRP. Our patient did not show hypothyroidism, but did have pale skin and had a low gonadotropin level in early infancy at around the time of the typical "minipuberty". This finding may reflect impaired gonadotropin release so it is important to monitor the development of puberty in these patients. Transient hyponatraemia has been reported with central ACTH insufficiency, as was seen in our patient (14). This may reflect decreased free water clearance due to hypocortisolaemia, especially with intercurrent infections, or a supportive mineralocorticoid effect of cortisol at times of stress. Detecting hyponatraemia can sometimes lead to a misdiagnosis of primary adrenal insufficiency instead of a secondary or central defect. Finally, developmental delay with abnormal MRI changes has been reported in one child with POMC deficiency. This finding is likely to represent the effects of recurrent hypoglycaemia rather than the underlying condition itself (15).

Genetic analysis was useful to establish the diagnosis of POMC deficiency. Using a custom adrenal array coupled with next generation sequencing we identified a homozygous c.206delC mutation in the child (5). This nucleotide deletion was confirmed by Sanger sequencing and causes a frameshift and premature truncation of the protein at codon 70 (p.Pro69Leufs2*) (Figure 1B). This mutation results in a POMC product that lacks ACTH, α-MSH and other small peptides, and may be subject to non-sense mediated decay. A review of the literature shows that all reported patients with POMC deficiency have homozygous or compound heterozygous mutations in the amino-terminal region of the protein that result in defective ACTH and α-MSH synthesis. The c.206delC change has been reported in two other families in Turkey suggesting a founder effect (7). The only established point mutation in POMC causing a similar phenotype is p.Arg145Cys change that corresponds to a p.Arg8Cys mutation in the ACTH peptide (16). This point mutation results in a bioinactive form of POMC/ACTH with a clinical phenotype of red hair, obesity and central adrenal insufficiency but with elevated ACTH levels on biochemical testing. In addition, isolated obesity has been reported in carriers of POMC mutations or in association with heterozygous point mutations in POMC (especially p.Arg236Gly) (7,17,18,19). Overview of the clinical and molecular features of patients with POMC insufficiency published to date were presented in Table 1 (Table 1). However, the parents of our child had BMIs of 23 kg/m^2 and 27 kg/m^2.

Treatment of POMC deficiency can be challenging. Patients require life-long glucocorticoid treatment using replacement doses. Mineralocorticoid replacement is not required. Hypothyroidism should be monitored and treated if present. Early onset obesity can be very difficult to treat beyond standard dietary and lifestyle measures, but the hyperphagic component is especially challenging. Krude et al (10) attempted intranasal ACTH treatment in two index cases with the ACTH peptide fragment identical to α-MSH. However, ACTH treatment at low doses during the first six weeks followed by a high dose (5 mg/day) did not produce a significant response in weight loss. Recently, setmelanotide (Rhythm Pharmaceuticals, Boston, Massachusetts, USA) has been developed as a novel MC4R agonist for the treatment of rare genetic disorders of obesity associated with defects in the MC4 pathway. This novel therapy is currently in Phase II trials in patients with POMC deficiency.

Table 1. The clinical and molecular features of patients with POMC insufficiency reported to date

cDNA	Protein	Ancestry	Age	ACT H/AI	Red hair	Obesity	Other features	Reference
c.-11C>A	Alternative translation	Dutch	4 weeks	↓	+ Changed to brown at age 2-3 yrs	Early	Conjugated hyperbilirubinemia	10
c.-11C>A	Alternative translation	German	5 years	↓	+	Early	Subclinical central hypothyroidism	3
c.-11C>A/ c.403_404dupGG	Alt. Trans/ p.Lys136Alafs*23	Swiss	6 months	↓	+ Changed to brown at age 2-3 yrs	Early		10
c.64delA	p.Met22Trpfs*49	Turkish	3.5 years	↓	ND	Early	Developmental delay, ataxia	15
c.151A>T/ c.296delG	p.Lys51*/ p.Gly99Alafs*59	Slovenian	Neonatal	↓	+	Early		10
c.202C>T	p.Gln68*	Egyptian	9 months (transient hypo early)	↓	-	Early		9
c.206delC	p.Pro69Leufs*2	Turkish	2 years	↓	(+) Red roots	Early		7
c.206delC	p.Pro69Leufs*2	Turkish	2 weeks	↓	+ Brown later	Early	Central hypothyroidism	20
c.206delC	p.Pro69Leufs*2	Turkish	2.5 months	↓	+	Early		This report
c.223dupC	p.Arg75Profs*44	North African (Kabilian)	4 weeks	↓	-	Early	GH deficiency hypogonadism	8
c.231C>A	p.Tyr77*	Hispanic	9 months	↓	-	Early	Apnea, neonatal jaundice; transient hyponatremia	6
c.256C>T	p.Arg86*	Indian	1 week	↓	(+) Skin and hair lighter than expected	Early	Central hypothyroidism	21
c.296delG	p.Gly99Alafs*59.	Turkish	3 months	↓	+	Early	Transient hypoglycaemia; transient salt-wasting during UTI	14
c.313G>T/ c.433delC	p.Glu105*/ p.Arg145Alafs*13	German	3 years	↓	+	Early	Subclinical central hypothyroidism	3

cDNA: complementary DNA, ND: not described, GH: growth hormone, UTI: urinary tract infection, ACTH: adrenocorticotropin hormone, AI: adrenal insufficiency

In summary, we present a Turkish child with POMC deficiency due to a potential founder POMC mutation. Routine genetic analysis in patients suspected of POMC deficiency is recommended not only to guide long-term prognosis and tailor the personalized management of these patients *per se*, but also to enable discovery of breakthrough treatments for important public health problems such as obesity.

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Of note, the authors have no links with Rhythm Pharmaceuticals or any company developing novel anti-obesity therapeutics.

Ethics

Informed Consent: The inform consent was taken from the patient's parents for publication.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Semra Çetinkaya, Erdal Kurnaz, Melikşah Keskin, Elif Sağsak, Şenay Savaş Erdeve, Zehra Aycan, Concept: Semra Çetinkaya, Tülay Güran, John C. Achermann, Design: Semra Çetinkaya, Tülay Güran, John C. Achermann, Data Collection or Processing: Semra Çetinkaya, Erdal Kurnaz, Analysis or Interpretation: Jenifer P. Suntharalingham, Federica Buonocore, Tülay Güran, John C. Achermann, Literature Search: Semra Çetinkaya, Tülay Güran, John C. Achermann, Writing: Semra Çetinkaya, Tülay Güran, John C. Achermann.

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CASE REPORT

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46,XY Disorder of Sex Development due to 17-Beta Hydroxysteroid Dehydrogenase Type 3 Deficiency in an Infant of Greek Origin

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What is already known on this topic?

17β-hydroxysteroid dehydrogenase type 3 (17βHSD-3) enzyme deficiency is a rare cause of ambiguous genitalia in XY neonates due to inadequate testosterone production that leads to undervirilization in utero.

What this study adds?

This study describes a neonate with ambiguous genitalia that proved to be a compound heterozygote for the gene responsible for 17βHSD-3 production and could offer some further validation for the idea of a founder effect for 655-1;G→A mutation in the Greek population.

Abstract

17-beta hydroxysteroid dehydrogenase type 3 (17βHSD-3) enzyme catalyzes the conversion of androstenedione (Δ4) to testosterone (T) in the testes of the developing fetus, thus playing a crucial role in the differentiation of the gonads and in establishing the male sex phenotype. Any mutation in the encoding gene (*HSD17B3*) can lead to varying degrees of undervirilization of the affected male, ranging from completely undervirilized external female genitalia to predominantly male with micropenis and hypospadias. We present here an infant who was referred to our clinic because of ambiguous genitalia at birth. Gonads were palpable in the inguinal canal bilaterally and no Müllerian structures were identified on pelvic ultrasound. Because of a low T/Δ4 ratio after a human chorionic gonadotropin stimulation test, a tentative diagnosis of 17βHSD-3 deficiency was made which was confirmed after genetic analysis of the *HSD17B3* gene of the patient. The molecular analysis identified compound heterozygosity of two previously described mutations and could offer some further validation for the idea of a founder effect for 655-1;G→A mutation in the Greek population.

Keywords: Disorder of sex development, 17-β-hydroxysteroid dehydrogenase type 3 deficiency, *HSD17B3* gene, androstenedione, testosterone

Introduction

The development of the male internal and external genitalia in a 46,XY fetus requires a complex interplay of several crucial genes, hormones and enzymes. At the time of fertilization the *chromosomal sex* is established, which in turn defines *gonadal sex* and in the final phase of development, *phenotypic sex* is established by the production and action of specific hormones (1).

Among these hormones, testosterone, together with its 5α-reduced end-product dihydrotestosterone (DHT), plays a crucial role in the development of internal and external genitalia of the male fetus. Testosterone is biosynthesized from cholesterol in five enzymatic steps,

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the last being the conversion of androstenedione (Δ4) to testosterone that takes place in the testes. This step of testosterone biosynthesis is catalyzed by the enzyme 17-beta hydroxysteroid dehydrogenase type 3 (17βHSD-3). This enzyme is expressed solely in the testes and belongs to a large enzyme family called 17-β-hydroxysteroid dehydrogenase enzymes. Any damaging mutation in the *17βHSD-3* gene (*HSD17B3*) can cause a 46, XY disorder of sex development (DSD), i.e. a child with a 46,XY karyotype and atypical gonadal or anatomical sex. These mutations can be either homozygous or compound heterozygous and can cause variable 17βHSD-3 enzyme deficiency (2).

Here, we report the case of an infant who presented with ambiguous external genitalia and gonads that were bilaterally palpable in the inguinal region. A 46,XY karyotype was found and 17βHSD-3 deficiency was suspected after measuring a lower than normal testosterone/Δ4 ratio, after a human chorionic gonadotropin (hCG) stimulation test. *HSD17B3* gene was genetically analyzed confirming the diagnosis and further validating the idea of a founder effect for 655-1;G→A mutation in the Greek population.

The concept of the founder effect is as follows. When a new population is established in a new area by a very small number of individuals, the descendants of this population will show loss of genetic variation. This can be expressed by specific gene mutations being present in the given population, in a much higher frequency than the rest of the world.

Case Report

The patient was the third baby born to a healthy, non-consanguineous couple of Greek origin. He was born at 37 weeks of gestation by caesarean section with a birth weight of 2860 g and with no perinatal problems. He was referred to our hospital at the age of 23 days due to his ambiguous genitalia at birth. Before referral, his karyotype was determined as 46,XY.

Institutional review board approval was obtained and both the child's parents signed an informed consent in accordance with the national laws. Parents were verbally informed during the investigation regarding the purpose of the study.

On physical examination, he had a phallus-like structure of 1.5 cm, perineoscrotal hypospadias, a perineal blind vaginal pouch and posterior labioscrotal fusion (Figure 1). Two masses, assumed to be the gonads, were palpable in the inguinal canal bilaterally. The rest of the physical examination was normal. Complete blood count and urine analysis were normal. An ultrasound examination confirmed the testicular structure of the masses in the inguinal canals and showed the absence of female internal genitalia.

Baseline LH, FSH, ACTH and cortisol concentrations, measured upon admission, were 12.1 IU/L (0.02-7.0 IU/L), 0.58 IU/L (0.16-4.1 IU/L), 24 pg/dL (5-90 pg/dL) and 11.20 μg/dL (2.8-23 μg/dL), respectively (Table 1). Androgen levels were as follows: Δ4: 10.68 ng/dL (10-37 ng/dL), testosterone: 25 ng/dL (60-400 ng/dL), DHT: 11 ng/dL (12-85 ng/dL) (all normal values given are age-specific). Since baseline hormonal values of various causes of 46,XY undervirilization can significantly overlap, the patient underwent a three-day hCG stimulation test. The results of the test showed a significant increase in Δ4 compared to testosterone and the low testosterone/Δ4 ratio in combination with a normal testosterone/DHT ratio was constistent with a diagnosis of 17βHSD3 deficiency (3).

Mutation analysis of the *HSD17B3* gene in the patient was performed using real-time PCR and identified compound

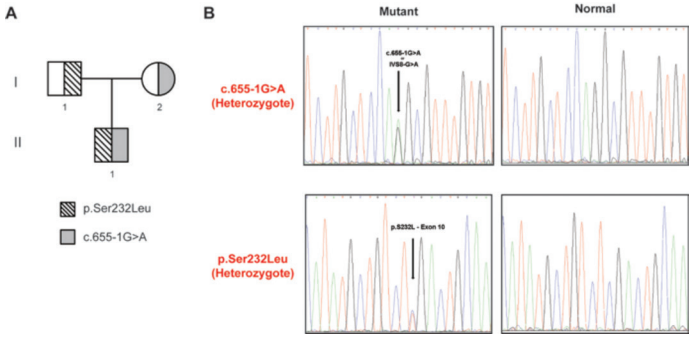

Figure 1. Identification of HSD17B3 mutations associated with 17-β-Hydrogenase type 3 deficiency. Pedigree of the family with HSD17B3; p.Ser232Leu and c.655-1G > A mutations. Grey shading indicates the presence of the c.655-1G > A mutation and filled line shading indicates the presence of the p.Ser232Leu mutation (A). Part of the sequencing electropherograms of the *HSD17B3* gene showing the heterozygous mutations (p.Ser232Leu, c.655-1G > A) detected in individuals with HSD17B3 deficiency. The non-mutated sequences (normal) are depicted (B)

Table 1. Serum androgen concentrations before and after human chorionic gonadotropin stimulation

Hormone levels	Pre-hCG	Post-hCG
Δ4 (ng/dL)	136.8	486.4
T (ng/dL)	25	75
DHT (ng/dL)	19.8	22.6
T/Δ4 (n = > 0.8)	0.18	0.15
T/DHT (n = < 20)	1.26	2.88

hCG: human chorionic gonadotropin, Δ4: androstenedione, T: testosterone, DHT: dihydrotestosterone

heterozygosity of the previously reported missense p.Ser232Leu and the splice junction variant 655-1;G→A (Figure 1). Further genetic analysis in both parents revealed the p.Ser232Leu mutation to be inherited from the father and the 655-1;G→A from the mother...” (i.e. the double space after A should be corrected).

After discussion with the parents, the decision to raise the child as male was reached. A clinical trial of three doses of 25 mg testosterone enanthate intramuscularly, at four-week intervals was initiated which resulted in an increase in penis size from 1.5 cm to 3 cm (Figure 2). It was proposed that surgical repair of the undescended testes and for correction of the hypospadias be delayed until an appropriate age.

Discussion

DSD is a group of congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical. Its incidence is 1 in 5000-5500 (0.018%) (4). The most recent consensus statement on management of intersex disorders classifies DSD in three major categories: sex chromosome DSD, 46,XX DSD and 46,XY DSD (5,6,7).

46,XY DSD comprise cases in which individuals with male chromosomal sex (XY) have atypical gonadal or anatomical sex. 46,XY DSD can be caused by several different defects most commonly involving defective androgen production and/or action. If the testes are normally developed in patients with 46,XY DSD, androgen insensitivity syndrome is usually the cause. Less frequently, 46,XY DSD can be the result of several different mutations involving any of the five enzymes responsible for the conversion of cholesterol to testosterone and its 5α-reduced end-product, DHT. Among these defects in testosterone production, the most frequent is a deficiency in the 17βHSD-3 enzyme (8). The 17βHSD-3 enzyme is expressed solely in the testes and belongs to a large group of enzymes, the 17-β-hydroxysteroid dehydrogenase enzymes. This family comprises 14 isoenzymes, all of which play a significant role in androgen and estrogen production (3).

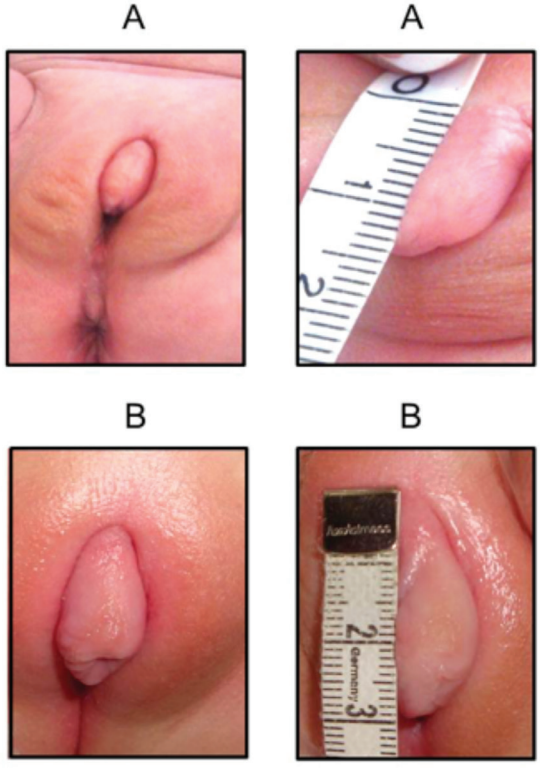

Figure 2. Phallus size in cm before (A) and after (B) administration of testosterone

17βHSD-3 enzyme deficiency leads to an autosomal recessive form of 46,XY DSD, which was first described in 1971 (9,10). Its precise incidence is unknown, but in a study from the Netherlands it was calculated to be around 1 in 147,000 live births with a heterozygote frequency of 1 in 135 (11). Among populations with a high intermarriage rate, such as the Arabs of the Gaza Strip, the incidence has been reported to be much higher, up to 1 in 100-300 (12,13).

The clinical presentation of individuals with 17βHSD-3 deficiency can vary greatly. Affected males usually present with female external genitalia, fusion of the labia and blind ending vagina, with or without clitoromegaly (Sinnecker types 5 and 4). Less frequently, external genitalia can be ambiguous (Sinnecker type 3), or mainly male with hypospadias and micropenis (Sinnecker type 2) (11,14). Furthermore, phenotypic variation can occur between families with the same homozygous mutation and it seems clear that no specific phenotype is associated with a specific mutation (11). Our patient presented with ambiguous genitalia, with a phallus-like midline structure of 1.5 cm, hypospadias, perineal blind vaginal pouch, posterior labioscrotal fusion, while both his testes were localized in the inguinal canals (Sinnecker type 2 to 3).

Either homozygous or compound heterozygous mutations in the *HSD17B3* gene can cause variable 17βHSD-3 enzyme deficiency (4). This gene spans at least 60 kb, consists of 11 exons and is localized on the 9q22 chromosome. To our knowledge, 37 HSD17B3 gene mutations have been reported so far. These include duplication, amplification, intronic splice site and exonic deletion as well as missense and nonsense mutations (2). Some of these appear to be *de novo* mutations while others are apparently ancient. Since some of the latter have been identified with higher frequency in specific countries or populations, a founder effect has been suggested in several cases (11,15,16).

The phenomenon of founder effect is rather common in the Greek population, and several reports have documented examples of founder mutations in the Mediterranean basin (16,17,18,19). Many previously published reviews cluster the Greeks together with the Turks and the Syrians to show a founder effect regarding the 655-1;G→A mutation. According to these papers, this mutation might have spread through the above-mentioned populations during the Ottoman Empire, which extended across most of the Eastern Mediterranean and contributed to the racial admixture in this area (20).

So far, the only reported case of a Greek individual with 17βHSD-3 enzyme deficiency was a paper published in 1978 (21). It describes a 12-year old 46,XY patient reared as a female, with both parents being Greek immigrants residing in New York. The patient was diagnosed with 17βHSD-3 deficiency (termed 17-ketosteroid reductase deficiency at the time) and, later, he was identified to be a homozygote for the 655-1;G→A mutation (22). Our patient was a compound heterozygote for mutations in the *HSD17B3* gene, having inherited the mutant p.Ser232Leu allele from his father and the 655-1;G→A allele from his mother.

In order to have robust evidence of a founder effect in any population, a critical number of individuals presenting with the same mutation for a given genetic trait is needed. Unfortunately, we are describing a rare genetic disease in a rather small (11 million) population which is shown by the fact that our patient is only the second Greek individual ever described with 17βHSD-3 deficiency. This makes it very difficult to have more solid evidence.

Nevertheless, the specific mutation (655-1;G→A) appears to be present in high frequency in Turks and Syrians, populations that reside in areas that (together with present-day Greece) used to be part of the Ottoman Empire. Thus, it is plausible that these areas of the Eastern Mediterranean were populated by a rather small group of people, which caused some degree of loss of genetic variability. This would lead to an increased number of descendants with specific mutations in some of their genes, even though robust evidence for such founder effects, especially in rare conditions, are yet to be found.

The gender assignment of patients with 17βHSD-3 deficiencies can be quite challenging and necessitates the collaboration of several different medical professionals for the initial decision and the subsequent management plan and follow-up (23). If gonadectomy is withheld, individuals who are reared as females can present in late adolescence with marked virilization due to conversion of the increased amount of Δ4 to testosterone (24). Our patient was partly undervirilized at presentation and showed a good response to the clinical trial of testosterone enanthate as shown by the increase in penis size from 1.5 cm to 3 cm. His parents have been informed about the need for surgical correction of cryptorchidism (around the end of the first year) and hypospadias and microphallus (at least two surgical procedures in the first 2-3 years of life) and are currently satisfied with the development of their son.

Acknowledgments

We would like to thank the proband and its parents for participating in the study.

Ethics

Informed Consent: Informed consent from both parents of the proband participating in this paper was obtained in accordance with the national laws.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Assimina Galli-Tsinopoulou, Design: Assimina Galli-Tsinopoulou, Anastasios Serbis, Nicos Skordis, Data Collection or Processing: Anastasios Serbis, Eleni P. Kotanidou, Eleni Litou, Vaia Dokousli, Konstantina Mouzaki, Analysis or Interpretation: Vassos Neocleous, Pavlos Fanis, Assimina Galli-Tsinopoulou, Nicos Skordis, Literature Search: Anastasios Serbis, Eleni P. Kotanidou, Eleni Litou, Vaia Dokousli, Konstantina Mouzaki, Vassos Neocleous, Writing: Assimina Galli-Tsinopoulou, Anastasios Serbis, Eleni P. Kotanidou, Eleni Litou, Vaia Dokousli, Konstantina Mouzaki, Pavlos Fanis, Vassos Neocleous, Nicos Skordis.

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CASE REPORT

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Transient Neonatal Diabetes due to a Mutation in KCNJ11 in a Child with Klinefelter Syndrome

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What is already known on this topic?

Klinefelter syndrome is the most frequent chromosomal aneuploidy in males occurring in about 1 in 660 males. Epidemiological studies suggest increased risk of type 1 diabetes and type 2 diabetes in adults with Klinefelter syndrome. There is only one previous report of neonatal diabetes in a patient with Klinefelter syndrome.

What this study adds?

To our knowledge, this is the second reported case of neonatal diabetes in an infant with Klinefelter syndrome. This case is the first due to a mutation in the KCNJ11 as the previously reported case of transient neonatal diabetes and Klinefelter syndrome had uniparental heterodisomy of chromosome 6.

Abstract

Klinefelter syndrome is the most frequent chromosomal aneuploidy in males occurring in about 1 in 660 males. Epidemiological studies have demonstrated increased risk of type 1 diabetes and type 2 diabetes in adults with Klinefelter syndrome. There is only one previous report of neonatal diabetes in a patient with Klinefelter syndrome. We report transient neonatal diabetes due to a pathogenic heterozygous variant in KCNJ11 in a male infant with Klinefelter syndrome. A 78-day old male infant was noted to have sustained hyperglycemia with serum glucose ranging between 148 mg/dL (8.2 mmol/L) and 381 mg/dL (21.2 mmol/L) three days after undergoing a complete repair of an atrioventricular defect. Hemoglobin A1c was 6.6%. The patient was born at term with a birth weight of 2.16 kg following a pregnancy complicated by gestational diabetes that was controlled with diet. The patient was initially started on a continuous intravenous insulin drip and subsequently placed on subcutaneous insulin (glargine, human isophane and regular insulin). Insulin was gradually decreased and eventually discontinued at seven months of age. Chromosomal microarray at 11 weeks of age showed XXY and a panel-based, molecular test for neonatal diabetes revealed a pathogenic heterozygous variant c.685G > A (p.Glu229Lys) in KCNJ11. The patient is now 34 months old and continues to have normal fasting and post-prandial glucose and HbA1C levels. The patient will need prospective follow up for assessment of his glycemic status. To our knowledge this is the second reported case of neonatal diabetes in an infant with Klinefelter syndrome and the first due to a mutation in the KCNJ11 in a patient with Klinefelter syndrome.
Keywords: Neonatal diabetes, Klinefelter syndrome, KCNJ11

Introduction

Klinefelter syndrome (KS) is characterized by a 47XXY genotype and is the most frequent chromosomal aneuploidy in males occuring in about 1 in 660 males (1). Epidemiological studies have demonstrated increased risk of type 1 diabetes and other autoimmune diseases in adults with KS (2,3,4). Recent studies have also shown an increased risk of type 2 diabetes with accumulation of body fat and a concomitant decrease in insulin sensitivity in KS (5,6). There is only one previous case report of neonatal diabetes in a child with KS (7).

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Neonatal diabetes is a monogenic form of diabetes, with an estimated incidence of 1 in 100,000 to 1 in 260,000 live births (8,9,10,11). It is defined as persistent hyperglycemia occurring in the first six months of life that lasts more than two weeks and requires insulin for management (9,12,13). Transient neonatal diabetes mellitus (TNDM) represents 50-60% of cases of neonatal diabetes, with the other 40-50% being permanent neonatal diabetes mellitus (14). The majority of cases of TNDM have paternal uniparental disomy of chromosome 6 or an unbalanced duplication of paternal chromosome 6 (15,16,17). Less frequent genetic abnormalities noted in patients with TNDM include activating mutations in the ATP-sensitive K^+ channel encoding genes (*KCNJ11* and *ABCC8*)(12). We report the first case of TNDM due to a mutation in the *KCNJ11* gene in an infant with KS.

Case Report

A male infant was born at 39+3 weeks gestation via non-spontaneous vaginal delivery with a birth weight of 2.16 kg and length of 48 cm. Pregnancy was complicated by gestational diabetes that had been controlled with diet and labor was induced for intrauterine growth restriction. There were no complications in the neonatal period. External genitalia were normal. The infant was noted to have bilateral inguinal hernia at one month of age and underwent bilateral inguinal hernia repair at age 39 days. In the postoperative period, he developed tachypnea and respiratory distress. Physical examination was significant for a loud S1/S2 with no splitting of S2 and a harsh 2-3/6 systolic murmur, loudest at the left sternal border. Echocardiogram revealed a type A complete atrioventricular canal defect with moderate regurgitation of left and right sides of the common atrioventricular valve, multiple ventricular septal defects and a small secundum atrial septal defect. At age 42 days, a random blood glucose level was found to be 175 mg/dL and repeat blood glucose estimation on day 43 was 190 mg/dL. The mild hyperglycemia was attributed to stress. Furosemide and digoxin were started to improve acute heart failure. Subsequently, the infant exhibited poor weight gain and poor feeding. He underwent complete atrioventicular canal repair and secundum atrial septal defect closure at age 74 days. Postoperatively, on day 74, blood glucose values were noted to be between 148 mg/dL and 381 mg/dL. The patient was extubated by post-operative day three and weaned off vasopressors and inotropes by post-operative day four. Hyperglycemia persisted and hemoglobin A1C was noted to be 6.6%. C peptide was 2.7 ng/mL (reference range 1.1-4.4 ng/mL). Family history was significant for diabetes in father since his 20s who was successfully treated with metformin. Additionally, a maternal uncle had been diagnosed with diabetes at age 18 years and he was now on insulin but had been on oral hypoglycemic agents previously.

Intravenous insulin infusion was started on day 78 at an initial dose of 0.03 units/kg/hour and was substituted with subcutaneous insulin injections (insulin glargine and regular insulin) at age 91 days. Though insulin glargine is not approved for use in infants, the release pattern of insulin glargine with no "peaks" makes it attractive during early infancy when infants are feeding frequently. There have been several reports regarding use of insulin glargine in infants with neonatal diabetes (18,19,20). Maximum hemoglobin A1c (HbA1C) was 7.2% at age five months. Glargine was subsequently replaced with intermediate acting isophane insulin due to insurance coverage reasons. Due to improvement in glucose values, insulin doses were gradually decreased and insulin was eventually discontinued at seven months of age. Oral sulfonylurea would be the preferred treatment in our patient given the KCNJ11 mutation. We planned to switch the patient from insulin to oral sulfonylurea after the results of the genetic analyses became available but elected not to do so when the insulin requirements began to decrease. We were able to discontinue insulin successfully with normal serum glucose values after discontinuation of insulin.

Fasting and post-prandial blood glucose values as well as HbA1C have subsequently been monitored and have been in the normal range. HbA1C was 6% at eight months of age, 6.2% at 11 months, 5.7% at 14 months, 5.4% at 19 months and 5.5% at 25 months of age. At the most recent follow up at 31 months of age, fasting glucose was 93 mg/dL and HbA1C was 5.4%. The patient has been gaining weight and growing normally and continues to make slow developmental progress. He started walking at 19 months of age and began receiving speech therapy for speech delay.

Genetic Analysis

Chromosomal microarray was performed at 11 weeks of age due to the complete atrioventricular canal defect (Cytogenetics Laboratories, Mayo Clinic, Rochester, MN). Chromosomal microarray revealed gain of the entire X chromosome. Limited chromosome study confirmed a diagnosis of KS/47, XXY. As external genitalia were unremarkable, gonadotropins and testosterone levels were not measured. The platform (Affymetrix CytoScan HD platform) used for microarray laboratory was single-nucleotide polymorphism based and did not detect stretches of homozygosity that would lead to possibility of uniparental disomy of chromosome 6. The laboratory was specifically asked to look for homozygosity at 6q24. Simultaneously, sequence analysis of 27 genes associated with neonatal

diabetes was performed at the Genetic Services Laboratories of University of Chicago (neonatal diabetes/Maturity Onset Diabetes of the Young sequencing panel). This panel based testing revealed a pathogenic heterozygous variant c.685G>A (p.Glu229Lys) in KCNJ11. There was also a heterozygous variant of unknown significance, c.713G>A (p.Arg238Gln) in *BLK*. Other genes included in the panel were *ABCC8, AKT2, CEL, CISD2, CP, EIF2AK3, FOXP3, GATA6, GCK, GLIS3, GLUD1, HADH, KCNJ11, KLF11, INSR, INS, IER3IP1, NEUROD1, NEUROG3, PAX4, PDX1, PTF1A, RFX6, SLC2A2, WFS1,* and *ZFP57*.

Genetic testing for parents was recommended. The rationale for testing was discussed with the parents, as they may also have the KCNJ11 mutation detected in the patient and how this would influence their own medical care, but they have declined testing.

Discussion

We report the second case of TNDM in an infant with KS. Genetic testing in this patient revealed a pathogenic heterozygous variant in KCNJ11. This is the first reported case of neonatal diabetes due to a mutation in the KCNJ11 in a patient with KS.

The *KCNJ11* encodes Kir6.2 subunit of the ATP-sensitive potassium channel in several tissues including pancreatic β cells, brain, heart and skeletal muscles (9,14). Mutations in KCNJ11 lead to a permanent opening of the potassium channel in the pancreatic β cells, thus preventing any activation of voltage-dependent calcium channel and glucose-induced insulin secretion leading to diabetes (21,22,23). The results of the genetic testing in this patient are noteworthy since heterozygous activating mutations in KCNJ11 are seen in only a small number of patients with TNDM (12) and instead comprise the most common cause of permanent neonatal diabetes (24,25,26). However, the pathogenic heterozygous variant c.685G>A (p.Glu229Lys) in *KCNJ11* found in this patient has been previously associated with TNDM (27).

Mutations in KCNJ11 are *de novo* in 80% of cases and inherited in an autosomal dominant pattern in the remaining cases (28). Family history in our patient was significant as his father had been diagnosed with diabetes since his 20s and he was doing well on metformin. Additionally, there was a history of gestational diabetes in the patient's mother and diabetes requiring insulin therapy in a maternal uncle. Unfortunately, inheritance of the KCNJ11 variant in our child could not be determined as parents have declined testing. We continue to revisit with the parents with our recommendations on parental genetic analysis for better understanding of the significance of the KCNJ11 mutations in our patient. In contrast to our patient, the previously reported case of TNDM and KS had uniparental heterodisomy of chromosome 6 (7), the most common abnormality found in children with TNDM (7,15,16,17). Our patient did not have evidence for uniparental disomy on chromosome 6.

There are several similarities between our case and the previous report (7). Both infants had low birth weight as is expected in children with neonatal diabetes (8). The age at discontinuation of insulin was also similar, with insulin being discontinued at six months of age in the previously reported case and at seven months in our patient (7). Follow up data on both patients is limited and is available only until age two and a half years. These data suggest that there has been no recurrence of hyperglycemia. 40% of patients with TNDM have recurrence of hyperglycemia later in life (8,17,29). Additionally, the hypogonadism associated with KS may lead to changes in body composition and a risk of developing metabolic syndrome and type 2 diabetes (2,6). Therefore, prospective follow up for assessment of glycemic status is warranted in our patient. If hyperglycemia were to recur, this patient would be a candidate for oral sulfonylurea therapy as oral sulfonylureas have been shown to result in improved glycemic control in patients with diabetes due to KCNJ11 mutations (9,23,30).

Another remarkable feature in our patient was the presence of an atrioventricular canal defect, ventricular septal defects and an atrial septal defect. Congenital cardiovascular anomalies are quite uncommon in children with KS. Those that have been reported include Tetralogy of Fallot and transposition of the great arteries (31,32). Parental genetic analyses will lead to better understanding of the significance of the KCNJ11 mutation.

Ethics

Informed Consent: Since this was a single case report, informed consent is not required by Mayo Clinic IRB guidelines.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Amanda Dahl, Seema Kumar, Concept: Amanda Dahl, Seema Kumar, Design: Amanda Dahl, Seema Kumar, Data Collection or Processing: Amanda Dahl, Radhika Dhamija, Seema Kumar, Analysis or Interpretation: Amanda Dahl, Radhika Dhamija, Seema Kumar, Alaa Al Nofal, Siobhan Pittock, Literature Search: Amanda Dahl, Seema Kumar, Writing: Amanda Dahl, Radhika Dhamija, Seema Kumar, Alaa Al Nofal, Siobhan Pittock, W Frederick Schwenk.

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CASE REPORT

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CYP24A1 Mutation in a Girl Infant with Idiopathic Infantile Hypercalcemia

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What is already known on this topic?

In 2011 new research found that Idiopathic infantile hypercalcemia (IIH) was associated with mutations in the *CYP24A1* gene involved in vitamin-D metabolism. *CYP24A1* is responsible for the degradation of both 1,25-dihydroxyvitamin-D3 and the precursor 25-hydroxyvitamin-D3. Thus, reduced activity will increase the level of active vitamin-D and can lead to hypercalcemia. So far only a limited number of mutations have been reported.

What this study adds?

The patient presented here reveals a unique clinical presentation and a new mutation expanding the mutational spectrum of *CYP24A1* associated IIH.

Abstract

Idiopathic infantile hypercalcemia (IIH) was associated with vitamin-D supplementation in the 1950's. Fifty years later, mutations in the *CYP241A* gene, involved in the degradation of vitamin-D, have been identified as being a part of the etiology. We report a case of a 21-month old girl, initially hospitalized due to excessive consumption of water and behavioral difficulties. Blood tests showed hypercalcemia and borderline high vitamin-D levels. Renal ultrasound revealed medullary nephrocalcinosis. An abnormality in vitamin-D metabolism was suspected and genetic testing was performed. This revealed the patient to be compound heterozygous for a common (p.E143del) and a novel (likely) disease-causing mutation (p.H83D) in the *CYP24A1* gene. The hypercalcemia normalized following a calcium depleted diet and discontinuation of vitamin-D supplementation. Increased awareness of the typical symptoms of hypercalcemia, such as anorexia, polydipsia, vomiting and failure to thrive, is of utmost importance in diagnosing IHH early and preventing long-term complications such as nephrocalcinosis. Further identification of as many disease-causing mutations in the *CYP24A1* gene as possible can help identification of predisposed individuals in whom vitamin-D supplementation should be reconsidered.
Keywords: Idiopathic infantile hypercalcemia, *CYP24A1*, nephrocalcinosis, vitamin-D supplementation

Introduction

IIt took almost 50 years for idiopathic infantile hypercalcemia (IIH), from first identification to the discovery of its etiology by Schlingmann et al (1) who identified mutations in the *CYP24A1* gene in patients with IIH (1). *CYP24A1* is responsible for the degradation of oth 1,25-dihydroxyvitamin D_3 and the precursor 25-hydroxyvitamin-D_3. Thus, reduced activity will increase the level of active vitamin-D and can lead to hypercalcemia. So far only a limited number of mutations have been reported (2). The patient presented here reveals a unique clinical presentation and a new mutation, expanding the mutational spectrum of *CYP24A1* associated IIH.

Case Report

A 21-month old girl was hospitalized in order to observe her excessive thirst and failure to thrive. For five months, the girl had been drinking extensively day and night and

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had been less interested in eating solid foods. Restriction of drinking made the girl refuse to eat altogether. Her general practitioner had ruled out diabetes mellitus by blood tests and diabetes insipidus by evaluating concentration of urine.

The patient's history revealed a healthy pregnancy and birth. However, severe problems with vomiting occurred in the first seven months of life. The infant had been breastfed but the feedings had been supplemented with formula, as she always seemed hungry. After introduction of solid foods, the hunger and vomiting diminished. There was no family history of similar problems. There was no consanguinity: the mother was of Iranian and the father of Danish descent. The infant received no medication besides the recommended 400 IU/day of vitamin-D.

Physical examination was normal and there were no syndromic stigmata nor signs of physical disease. Her growth chart revealed normal height for age but her weight had decreased by one standard deviation within the three preceding months.

When hospitalized, blood tests showed high levels of both total calcium (3.42 mmol/L; Ref. range 2.17-2.66 mmol/L) and free calcium (1.68 mmol/L; Ref. range 1.18-1.32 mmol/L). Parathyroid hormone (PTH) level was undetectable (<4 ng/L; Ref. range 14.0-72.0 ng/L). 25 vitamin-D and 1.25 vitamin-D levels were in the high-to-normal range. 25 vitamin-D level was 107 nmol/L (Ref. range >50 nmol/L), and similarly 1.25 vitamin-D level was 146 pmol/L (Ref. range 51-177 pmol/L). Serum phosphate level, liver, kidney and thyroid tests were all normal. Ultrasound of the kidneys revealed medullary nephrocalcinosis (Figure 1). Her urine was found to be hypercalciuric with a Ca/creatinine ratio of 1.9 mmoL/mmoL (Ref. range: <0.7 mmoL/mmoL). There were no signs of other diseases or malignancy. Both parents had blood calcium levels tested, and the mother had a calcium level of 2.53mmol/L (Ref. range: 2.15-2.51mmol/L) that was considered normal and no further examinations were initiated, including renal ultrasound or renal testing.

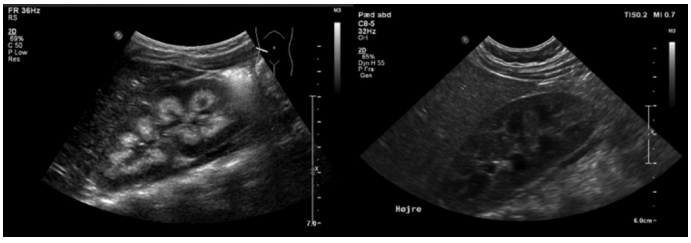

Figure 1. Ultrasound of the kidneys of the index patient and her brother. To the left, ultrasound of the index patient revealed kidney enlargement with hyperechogenic pyramids and signs of medullary nephrocalcinosis. To the right, ultrasound of the younger brother showed normal echogenicity of pyramids

Genetic testing showed the girl to be compound heterozygous for the following CYP24A1 mutations: c.428_430delAAG (p.E143del) and c.247C>G (p.H83D) (Figure 2). Testing of the non-symptomatic parents revealed that the well-known p.E143del mutation was inherited from the heterozygotic mother and the novel p.H83D mutation was inherited from the heterozygotic father.

The patient was started on a low calcium diet and vitamin-D supplementation was stopped resulting in the calcium and PTH levels returning to normal within the subsequent five months. Since then the patient's serum calcium level has remained within the acceptable range, while milk and other high calcium products have been slowly reintroduced. Informed consent was obtained from the family.

Discussion

More than 20 different mutations affecting the *CYP24A1* gene have been reported (2). Of the 37 symptomatic cases recently published, 14 were adults (older than 18 years), three were between three and 13 years of age and the remainder were less than one year old at diagnosis. The average age at diagnosis (excluding the adults) was 11 months, ranging from one month to 13 years.

Symptoms such as polyuria, polydipsia, anorexia, fatigue and depression are all known to be associated with hypercalcemia, but are easily misinterpreted (3). In small children, failure to thrive is a common symptom of IIH and within the 23 pediatric cases, 74% were described as having weight loss and failure to thrive at presentation (1,4,5,6,7,8). In older children and adults urolithiasis can be the only symptom (6,8,9). Though all patients with

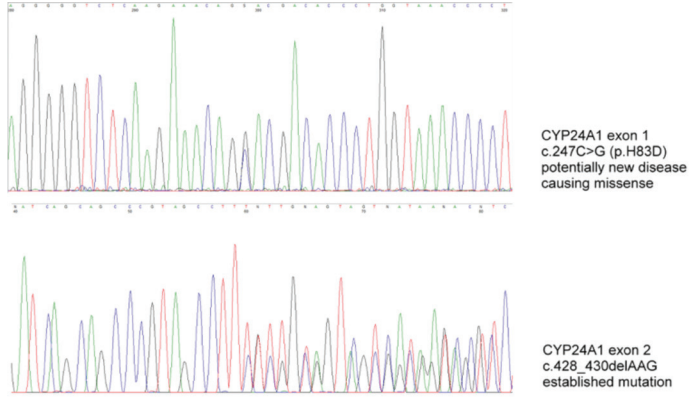

Figure 2. Electropherogram after genetic analysis of the index patient. Genetic investigation revealed the girl to be a compound heterozygote with two different mutations in the *CYP24A1* gene: the previously reported c.428_430delAAG (p.E143del) mutation, and the novel c.247C>G (p.H83D) mutation

symptoms had signs of nephrocalcinosis, no correlation was reported between type of mutation and clinical presentation (1,4,5,6,7,8,9).

Our patient showed several symptoms of hypercalcemia as an infant, but had no blood tests performed and was diagnosed as a case with simple regurgitation. Blood tests were performed when weight gain was affected. The finding of increased levels of calcium and high-to-normal 1,25(OH) D in the face of suppressed PTH levels, usually points to vitamin-D related disease and can be considered typical for IIH (1). There was no history of vitamin-D overdosing as our patient received the recommended supplementation of 400 IU/day. As there were no signs of William's syndrome, familial hypercalcemia or other diseases, the hypercalcemia had earlier been described as idiopathic.

The well-known p.E143del mutation is clearly pathogenic (2), whereas the p.H83D missense variant is a novel finding in a patient with IIH. The fact that c.247C>G is a rare variant that cannot be found in any known genome variant databases (ExAC genome browser; Exome variant server, dbSNP), together with the typical recessive segregation pattern in the family with asymptomatic, heterozygous parents, all suggest biallelic loss of CYP24A1 function, caused by compound heterozygosity for p.E143del; p.H83D in our patient. This conclusion is further supported by the bioinformatic prediction performed with PolyPhen-2 and MutationTaster-2 (10,11). Both programs predict the p.H83D variant to be probably damaging and disease causing.

Dinour et al (6) described four heterozygous children all carrying p.E143del mutations. Even though none of them displayed symptoms of hypercalcemia, all of them had blood levels of calcium at the very upper end of the normal range. This is interesting since none of them received vitamin-D supplementation. This clearly indicates that the role of vitamin-D supplementation, in carriers of CYP24A1 mutations, is not fully understood. Further these cases supports the pathogenicity of the newfound mutation, since our patient had clearly elevated calcium levels. At birth, the younger brother of our patient was tested and found to be carrier of only the p.E143del mutation. At four months of age, blood tests revealed both elevated total calcium levels of 2.75 mmol/L (Ref. range: 2.10-2.62) and free calcium levels of 1.48 mmol/L (Ref. range: 0.18-1.32). However, 25(OH) D was low with a value of 31 nmol/L (Ref. range: >50 nmol/L). The urine was hypercalciuric with a Ca/creatinine ratio of 1.7 mmol/mmol (Ref. range: <0.7 mmol/mmol), but ultrasound revealed no signs of nephrocalcinosis (Figure 1). At 12 months of age the brother was still healthy and had normal urinary calcium excretion, but regular follow-up to observe for development of nephrocalcinosis continues.

Treatment of hypercalcemia has differed significantly in patients with IIH. Oral hydration and discontinuation of vitamin D supplementation was sufficient to normalize the calcium levels in our patient. However most of the reported patients have been treated with both iv hydration, furosemide, and in some severe cases with corticosteroids and bisphosphonates (1,3,4,5,6,7,8). One patient, who was found to be homozygous for the well-known R396W mutation, even had to undergo hemodialysis (4). Accordingly, the severity of hypercalcemia at diagnosis is not only related to the underlying mutation, but also dependent upon the time delay to diagnosis as well as vitamin-D supplementation (5).

Though treatment can often be limited to calcium restriction, calcium metabolism probably remains affected throughout life (5,6). In two publications, periodically high calcium levels, 11 and 18 years post diagnosis, were reported and in one case the nephrocalcinosis remained unchanged after 18 years (1,5).

Since CYP24A1 mutations clearly result in a genetic disposition to hypercalcemia, we recommend genetic testing in siblings of IIH patients, so that vitamin-D supplementation can be reconsidered. A similar conclusion was reached by Ammenti et al (12) after reviewing data on nephrocalcinosis (not solely caused by IIH), stating that early recognition was associated with catch-up growth and stabilization of glomerular function.

Further, all patients with mutations should undergo regular follow-up, since hypercalcemia and nephrocalcinosis have been demonstrated in an asymptomatic compound heterozygote boy, even though he was not receiving any vitamin-D supplementation (1). Certainly it is interesting to follow these predisposed patients to obtain data on long-term prognosis and specifically so in situations where vitamin-D supplementation is normally considered, such as in treatment of osteoporosis (9).

Ethics

Informed Consent: Informed consent was obtained from both parents, who has shared custody of the child in our case.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Jesper Johannesen, Jens Otto Broby Madsen, Concept: Jesper Johannesen, Bodo Beck, Jens Otto Broby Madsen, Design: Jens Otto Broby Madsen, Data Collection or Processing: Jens Otto Broby Madsen, Jesper Johannesen, Sabrina Saur, Analysis or Interpretation: Sabrina Saur, Bodo Beck, Literature Search: Jens Otto Broby Madsen, Writing: Jens Otto Broby Madsen.

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CASE REPORT

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Metachronous Synovial Sarcoma After Treatment of Mixed Germ Cell Tumor in a Child with Complete Gonadal Dysgenesis

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What is already known on this topic?

Complete 46,XY gonadal dysgenesis (GD) patients show a high predisposition to germ cell tumors. Gonadoblastomas and dysgerminomas are the most frequent histotypes.

What this study adds?

This is the first report of synovial sarcoma in patients with GD.

Abstract

Patients with complete XY gonadal dysgenesis (GD) show a high predisposition to germ cell tumors (GCT). Patients with coexistence of GCT and GD have been reported previously. Here we present a 15-year-old girl with mixed GCT and GD who also developed an intra-abdominal synovial sarcoma one year after the treatment. This is the first report, to our knowledge, of synovial sarcoma associated with XY GD.

Keywords: Gonadal dysgenesis, synovial sarcoma, dysgerminoma, gonadoblastoma, embryonal carcinoma

Introduction

A complete 46,XY gonadal dysgenesis (GD) syndrome, known as Swyer syndrome, is characterized by a female phenotype with bilateral streak gonads, normal, female, external genitalia, presence of Müllerian duct and deficient secondary sexual characteristic development with primary amenorrhea (1). Patients with Swyer syndrome show a high predisposition to ovarian cancer. The most frequent observed histotypes are gonadoblastomas and dysgerminomas, followed by Brenner tumors, malignant teratomas and mixed endodermal sinus tumors (2). The lifetime risk of gonadal tumors is in the range of 15-35% in patients with GD (3,4). Germ cell tumors (GCT) were reported in patients with GD but not in conjunction with other types of malignancy. In this report we present a 15 years-old girl patient with mixed GCT and GD who underwent a metachronous somatic malignant transformation (SMT) resulting in intra-abdominal synovial sarcoma one year after the treatment.

Case Report

A 15-year old girl was admitted to our hospital with a complaint of abdominal distension. Her abdominal

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examination revealed the presence of a large pelvic mass. Menstrual history revealed that she had never attained menarche. Her height was 171 cm [+1.95 standard deviation score (SDS)], and her weight was 73 kg (+2.98 SDS). Her body mass index was 25 kg/m^2. Systemic examination showed normal female external genitalia, a "rough" voice, small breasts and a hypoplastic vagina.

Initial hormonal assays showed elevated levels of serum follicle stimulating hormone at 54.3 IU/mL (3.5-12.5) and luteinizing hormone at 50.82 IU/mL (2.4-12.6). Other endocrinological evaluations were as follows: progesterone 0.706 ng/mL (0.4-1.4); estradiol 45 pg/mL (13-71); testosterone 0.26 ng/mL (8-80); DHEA-SO4 327 µg/dL (65-368); and androstenodione 2.02 ng/mL (0.5-4.8). On contrast-enhanced, computed tomography (CT), there was a mass, separate from the uterus, which was 12 cm in diameter and which filled the rectouterine space. The mass was mostly multicystic, but also contained solid areas and coarse calcifications (Figure 1A). Lateral to this lesion, there was another solid mass with a diameter of 5 cm and internal calcifications (Figure 1B). In the left para-aortic region, there were two soft tissue lesions adjacent to each other, with dimensions of 10 cm and 3 cm (Figure 1C). The attenuation feature of the larger lesion was similar to that of the lesion in the recto-uterine space, while the smaller appeared to be solid. By radiological appearance, the masses were evaluated as a bilateral malignant ovarian tumor with lymphatic metastases. Laboratory tests revealed high levels of serum α-fetoprotein (AFP) (19931 IU/mL) and Cancer Antigen 125 (Ca 125) (566.3 U/mL) with normal lactic acid dehydrogenase and beta human chorionic gonadotropin (β-hCG) levels. Cytogenetic studies revealed a 46 XY genotype. No germ line deletion or translocation of the sex-determining region Y (*SRY*) gene was detected by fluorescence *in situ* hybridization. Bilateral gonadectomy, Müllerian duct extraction and tumor resection were performed. Pathological investigation showed dysgerminoma (90%), embryonal carcinoma (7%) and gonadoblastoma (3%) on the left side, a dysgenetic gonad and pure gonadoblastoma on the right side (Figure 1D,E,F). Adjuvant chemotherapy was performed with six cycles of cisplatin/etoposide/bleomycin. The patient was in remission after chemotherapy.

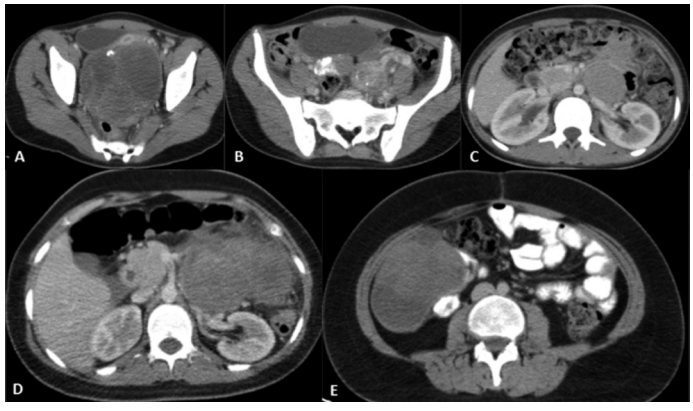

Figure 1. A huge mass with internal cystic components and calcifications, within the cul de sac. Note that the bladder and uterus are displaced anteriorly and the rectum is displaced posteriorly by the lesion (A). Another lesion with calcifications. A small cyst is also visible at the upper level (B), retroperitoneal lymphadenopathy with mild compression on the left renal vein (C). Axial computed tomography image at the renal sinus level shows a huge mass with irregular borders and heterogeneous enhancement within the left retroperitoneal localization, anterior to the left kidney (D). A hypo dense mass with mild irregular contours in the right abdomen at the posterolateral aspect of the caecum and ascending colon (E)

Eighteen months later she was admitted to the emergency department with abdominal pain and distension. A left abdominal mass was detected on physical examination. Abdomino-pelvic CT showed a huge mass with irregular borders and heterogeneous enhancement within the left retroperitoneal region, anterior to the left kidney (Figure 2A). Serum AFP, Ca125, and β-hCG levels were within the respective normal ranges. The mass was near totally resected. Pathological investigation showed a monophasic synovial sarcoma (Figure 2B). Ifosfamide, mesna and doxorubicin chemotherapy protocol was given for six cycles and radiotherapy was added at a dose of 45 Gy. Twelve

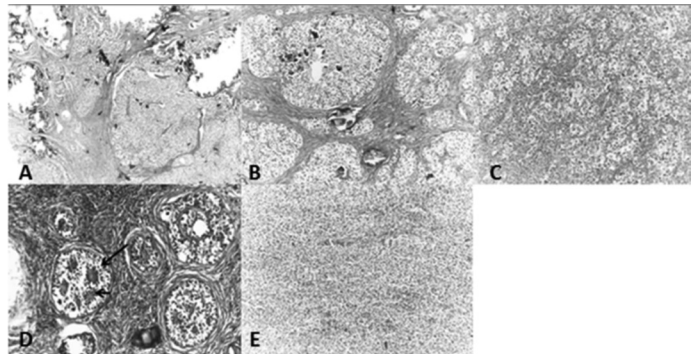

Figure 2. The growth of the gonadoblastoma occurred as rounded nests separated by fibrous stroma that contained significant calcification (hematoxylen and eosin, x40) (A). The germ cells, similar to dysgerminoma cells, in the gonadoblastoma (hematoxylen and eosin, x200) (B). Dysgerminoma. Nests of dysgerminoma cells were separated by fibrous septa containing lymphocytes and plasma cells. The tumor cells had round vesicular nuclei with prominent nucleoli and abundant pale cytoplasm (hematoxylen and eosin, x400) (C). Gonadoblastoma was detected within the streak ovary. The tumor contained a nest of predominantly sex cord-like cells distributed around hyalinized acini (arrow) (hematoxylen and eosin, x400) (D). Synovial sarcoma (monophasic component). Sheets of uniform small spindle cells with ovoid nuclei and scanty cytoplasm (hematoxylen and eosin, x200) (E)

months later the patient had a further relapse on the right side of the abdomen. Abdominal CT showed a hypodense mass, with mild irregular contours at the posterolateral area of the caecum and ascending colon (Figure 2C). The tumor was partially resected. Ifosfamide/Carboplatin/Etoposide chemotherapy was initiated. Forty-five months after initial diagnosis and 27 months after diagnosis of the synovial sarcoma, the patient died due to resistant/progressive disease.

Discussion

Patients with GD are phenotypically female with unambiguously female genital appearance at birth and with normal Müllerian structures. The condition typically presents at an age when puberty would be normally expected with primary amenorrhoea and delayed puberty. Although the etiology is not completely understood, 46,XY GD results from failure of testicular development due to disruption of the underlying genetic pathways and several genes, including *SRY* (gene deletion or loss of function mutations; Yp11.3), *NR5A1* (9q33) and *DHH* (homozygous or compound heterozygous mutations; 12q13.1) have been implicated. In addition, patients with partial duplications of Xp (including the *NR0B1* gene) and chromosome 9p deletions (involving the *DMRT1* and *DMRT2* genes) may also present with isolated 46,XY complete gonadal dysgenesis (CGD). Rarely mutations in the CBX2 gene and also dublication of DAX1 gene have been considered responsible for the development of 46, XY CGD(5,6,7). Mutations in the *MAP3K1* gene (located on chromosome 5q) that cause downstream alterations in the MAP kinase signaling pathway have been identified (8).

Patients with GD have a 30% risk of development of gonadoblastoma with a 50-60% risk of malignant transformation, typically to dysgerminoma (9). The risk of malignancy in patients with GD increases with age; it has been reported that the risk is 50-70% in the third decade and as high as 80% in the fourth (10). In patients with primary amenorrhoea, GD should always be kept in mind due to the high risk of malignant transformation. Bilateral gonadectomy is advised as soon as the diagnosis is made (11). Unfortunately, our patient had bilateral gonadectomy and Müllerian duct extractions only after the disease had progressed to bilateral GCT.

Gonadoblastomas are rare, mixed, germ cell, sex cord, stromal tumors, almost exclusively seen in patients with underlying gonadal disorders. Germ cells in dysgenetic gonads are genetically unstable and tumorigenic because patients with GD have a higher risk of development of germ cell tumor. It has been hypothesized that the Y chromosome contains a gonadoblastoma locus, responsible for this benign tumor, that may also develop bilaterally and coexist with other neoplasms, such as dysgerminoma (12). Radaković et al (13) reported that 55% of patients suffering from GD were diagnosed with gonadoblastoma or dysgerminoma. Cases of co-occurrence of gonadoblastoma with dysgerminoma and gonadoblastoma with choriocarcinoma have also been reported (12). It is assumed that gonadoblastomas are unstable and may result in choriocarcinoma (14). Our patient had a pure gonadoblastoma in her right ovary and dysgerminoma, embryonal carcinoma and gonadoblastoma on the left side.

GCTs include a diverse group of tumors that arise from primordial germ cells, either in the gonads or in non-gonadal sites. SMT of a GCT means the occurrence of somatic non-germ cell malignancy. Faure Conter et al (15) reported that SMT associated with GCT in children is rare and that these are aggressive tumors with various primary lesions, various GCT histologic subtypes, and poor overall prognosis. Although the presentation is mostly synchronous in children, most of the adult cases are metachronous. Giannatempo et al (16) reported that the median delay between SMT and GCT was four years with a maximum of 18 years. They also reported that SMT occurred concurrently with GCT in only 40% of patients. Different studies showed that 78.5-100% of SMT were diagnosed at the same time as the GCT diagnosis. Different malignant tumors such as rhabdomyosarcoma, peripheral primitive neuroectodermal tumors, adenocarcinoma, squamous cell carcinoma, osteosarcoma, angiosarcoma, leukemia, neurosarcoma, undifferentiated sarcoma, myxoid sarcoma, fusiform cell sarcoma, bronchoalveolar sarcoma and thyroid carcinoma have been reported to arise in GCT (17). Our patient had a metachronous presentation, similar to that reported in adult cases, after 18 months from the initial diagnosis and she had no GCT at that time. Tumor markers were negative at the diagnosis of synovial sarcoma.

In most of the reported cases with SMT, primary diagnoses were teratoma (15,16,18). GCTs have a capacity to display totipotential differentiation. Some authors explain this condition with malignant transformation of the yolk sac or with teratoma cells while others propose a divergent differentiation of primordial stem cells toward GCT and SMT (15,16,18,19). Specific chromosomal changes trigger this transformation. The presence of isochromosome 12p, a specific marker of GCT and of chromosomal abnormalities in MT of non-GCT, strongly favor this hypothesis. We did not investigate the presence of isochromosome 12p in our patient. It is possible that this chromosomal aberration may have caused the metachronous tumor development. The patient received cisplatin/etoposide/bleomycin for the treatment of GCT. The most well known late complications of etoposide are dose related myelodysplastic syndrome and secondary acute myeloid leukemia (20,21,22). A similar complication (leukemia) is reported for cisplatine in many

studies (23,24). The most important late complication of bleomycin is pulmonary toxicity. These complications, especially the secondary cancers usually occur five years after treatment. In our patient, synovial sarcoma occurred 18 months after the treatment and we believe that the tumor developed metachronously, rather than as a complication of the chemotherapeutics given in this case.

We could find no other case reports of patients with GD developing synovial sarcoma after GCT. Thus, to the best of our knowledge, this is the first report of GD with synovial sarcoma following GCT.

Bilateral gonadectomy and Müllerian duct extractions have to be considered for newly diagnosed patients with GD as a risk reducing strategy for development of malignancy. Patients with GCT and with chromosomal or genetic defects must be carefully followed and observed because of the high risk for development of synchronous or metachronous SMT.

Ethics

Informed Consent: Written inform consent for publication of the data was given by the patient's parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Elvan Çağlar Çıtak, Emel Yaman, Design: Elvan Çağlar Çıtak, Feryal Karahan, Mehmet Alakaya, Data Collection and Processing: Eda Bengi Yılmaz, Funda Kuş, İclal Gürses, Yüksel Balcı, Analysis and Interpretation: Elvan Çağlar Çıtak, Emel Yaman, Fatih Sağcan, Eda Bengi Yılmaz, Feryal Karahan, Mehmet Alakaya, Yüksel Balcı, Literature Research: Fatih Sağcan, Feryal Karahan, Mehmet Alakaya, Writing: Elvan Çağlar Çıtak, Eda Bengi Yilmaz.

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Letter to the Editor Regarding "Assessment of Retinal Nerve Fiber Layer Thickness in Non-Diabetic Obese Children and Adolescents"

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To the Editor,

I read the manuscript entitled "Assessment of Retinal Nerve Fiber Layer Thickness in Non-Diabetic Obese Children and Adolescents" recently published online by Ozen et al (1) in the J Clin Res Pediatr Endocrinol with great interest. I would like to thank Ozen et al (1) for their comments on our publication entitled "The assessment of peripapillary retinal nerve fiber layer and macular ganglion cell layer changes in obese children: a cross sectional study using optical coherence tomography" (2). I would like to clarify some of the misunderstandings raised by Ozen et al (1). They said that our manuscript included patients with high refractive status (up to 5 D) and that this situation could have affected retinal nerve fiber layer (RNFL) values by optical coherence tomography (OCT). However, in our study, both the study group and the control group had rather low refraction values. Our study demonstrated that spherical equivalent was -0.04 ± 0.61 D in the obese group and -0.05 ± 0.53 in the non-obese group. Although not mentioned in the paper, patients' spherical equivalents ranged from + 2.0 to -1.5 D. We completely agree with the authors that the presence of a refraction error can cause inaccurate measurement of OCT and RNFL values. The measurement errors of OCT parameters due to differences in axial length or refractive error causing ocular magnification effects have been documented by previous studies (3,4,5). OCT scans are typically angular. Hence, a 20 degree projection on a longer eye covers a larger area than on a hyperopic eye. The difference in scanned region (magnification), and the path used to quantify thickness is what causes differences in thickness versus refractive error or axial length.The transverse mirror in OCT is calibrated for an axial length of 24.46 mm. Inter-individual differences in axial length which vary from 24.46 mm would result in magnification errors in the measurements made on OCT (3,5,6). Therefore, to remove the effect of ocular magnification, the clinicians have to adjust the RNFL results using Littmann's method (3,4,5,6,7). In this regard, the data present actual RNFL thickness in high refractive error.

Keywords: Obese children, optical coherence tomography, retinal nerve fiber layer

Ethics

Peer-review: Internally peer-reviewed.

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