J Clin Res Pediatr Endocrinol 2016;8(1):1-12
DOI: 10.4274/jcrpe.2764

Review

Sex Hormone-Binding Globulin in Children and Adolescents

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ABSTRACT

Sex hormone-binding globulin (SHBG) is a circulating glycoprotein that transports testosterone and other steroids in the blood. Interest in SHBG has escalated in recent years because of its inverse association with obesity and insulin resistance, and because many studies have linked lower circulating levels of SHBG to metabolic syndrome, type 2 diabetes, nonalcoholic fatty liver disease, polycystic ovary syndrome, and early puberty. The purpose of this review is to summarize molecular, clinical, endocrine, and epidemiological findings to illustrate how measurement of plasma SHBG may be useful in clinical medicine in children.

Keywords: Sex hormone-binding globulin, obesity, type 2 diabetes, metabolic syndrome, non-alcoholic fatty liver disease, polycystic ovary syndrome

Conflict of interest: None declared
Received: 23.12.2015
Accepted: 06.01.2016

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Sex hormone-binding globulin (SHBG) is a glycoprotein produced in the liver that transports certain sex steroids in the circulation and regulates their access to target cells. Many studies have linked lower circulating levels of SHBG to obesity, type 2 diabetes, metabolic syndrome, non-alcoholic fatty liver disease, polycystic ovary syndrome, and early puberty.

WHAT THIS STUDY ADDS?

Our review was written to summarize the molecular, clinical, endocrine, and epidemiological findings which illustrate how measurement of plasma SHBG levels may be useful in clinical medicine in children. We believe that this review is novel and will be useful for the physicians who manage pediatric obesity and related comorbidities and for scientists who conduct translational research in this area.

Introduction

Sex hormone-binding globulin (SHBG) is a 90-100 KDa homodimeric glycoprotein that is encoded by a single gene on the short arm of chromosome 17. Variable glycosylation explains the variation in molecular weight and is known to be increased by estrogens, but its significance is unknown. Circulating SHBG is produced primarily by hepatocytes, however, the gene is also expressed in the brain, uterus, prostate, breast, ovary, and testis (1), as well as in certain ovarian and prostate cancers. SHBG is found in the circulation of numerous mammals but is seemingly absent in the plasma of adult rats and mice, guinea pigs, and pigs. SHBG transports testosterone and other steroids in the blood plasma, reduces their metabolic clearance rate, and regulates their access to target tissues (2). While SHBG can sequester steroids from target tissues, there is some evidence that ligand-bound SHBG binds to membrane receptors, and stimulates cyclic

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adenosine monophosphate production (3), and/or enters cells by binding to the membrane protein megalin (4) to initiate a biological effect. Human SHBG binds dihydrotestosterone (DHT) > testosterone > estradiol as well as drugs such as levonorgestrel and fluoxymesterone (2).

Homozygous missense mutation resulting in a complete deficiency of plasma SHBG has been reported in a few cases. An affected adult male complained of low libido, decreased spontaneous morning erections, fatigue, muscular weakness, decreased shaving frequency, and had small testes and a low bone mass. His semen analysis was normal, however. His affected sister had delayed menarche, small breasts, and irregular menstrual periods (3). An adult woman with an undetectable level of SHBG and a compound heterozygote polymorphism had mild hirsutism that increased dramatically during a pregnancy when her free testosterone level was 4-fold elevated (4) suggesting that SHBG functions to protect the pregnant woman from placental hyperandrogenism. Polymorphisms have been reported that more subtly affect SHBG binding of testosterone.

SHBG binds testosterone with high affinity (~1 nmol/L) and much of the SHBG-binding sites in adult male serum are occupied by testosterone such that the level of SHBG is a major determinant of the total testosterone level in adult men. Eugonadal adult men with low SHBG levels have low total testosterone levels, while men with high SHBG levels have higher testosterone levels. Obesity and hyperthyroidism, respectively, are examples of these effects. SHBG and testosterone are also related in newborn boys (5) during minipuberty but not in prepubertal boys with much lower testosterone levels in whom only a small portion of the SHBG in the plasma is occupied by testosterone (Figure 1).

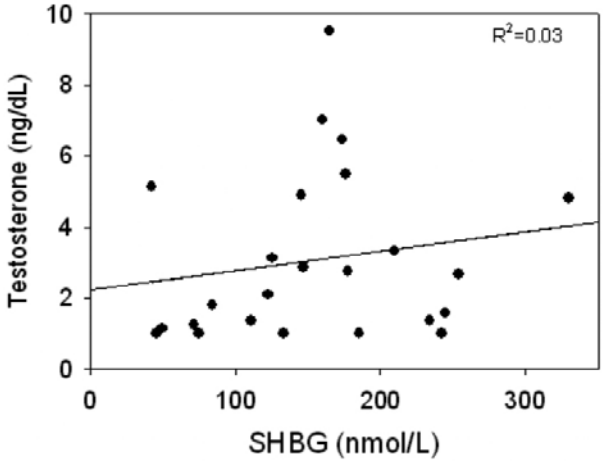

Figure 1. Relationship between sex hormone-binding globulin and total testosterone levels in prepubertal boys age 5-8 years, data from reference (82). SHBG: sex hormone-binding globulin

Sex Hormone-Binding Globulin in Children and Adolescents

SHBG is present in the fetal circulation and in cord blood where levels are similar in males and females (6). SHBG levels are markedly increased in the maternal circulation due to the effect of placental estrogens, whereas the levels in cord blood are low and similar to values on day 2 of life. Whether SHBG plays a physiological role during fetal life is unknown. In one study of women from China, cord blood SHBG levels were lower among babies born to overweight mothers, most of whom had gestational diabetes (7).

As diagrammed in Figure 2, some cross-sectional studies indicate that SHBG levels rise substantially from birth to early childhood (8), whereas other studies indicate unchanged values (9). Longitudinal studies are lacking. During childhood, values are relatively stable but then decline at puberty, more so in boys than in girls (10). The reason for this change is not certain, but it is probably partly from androgens which are known to suppress SHBG levels (11). However, the decline is also seen in boys with idiopathic hypopituitarism (12) suggesting metabolic rather than neuroendocrine control. SHBG levels in adulthood are higher in women than in men, which is probably due to estradiol since estrogen administration is known to increase SHBG (13). Levels then rise slightly in the elderly, especially in men.

Regulation of Sex Hormone-Binding Globulin Production

There is a 20-fold variation in SHBG levels among individuals, while the level of SHBG in a given individual is relatively constant (5). SHBG levels are unrelated to meals or time of day (6). Table 1 lists those factors that are known to influence the level of SHBG in blood. In most cases, the mechanism is unknown.

SHBG levels decrease with increasing obesity (14) and rise with weight loss (15). SHBG is reduced in type 2 diabetes mellitus (T2DM), and the strength of the association is reduced,

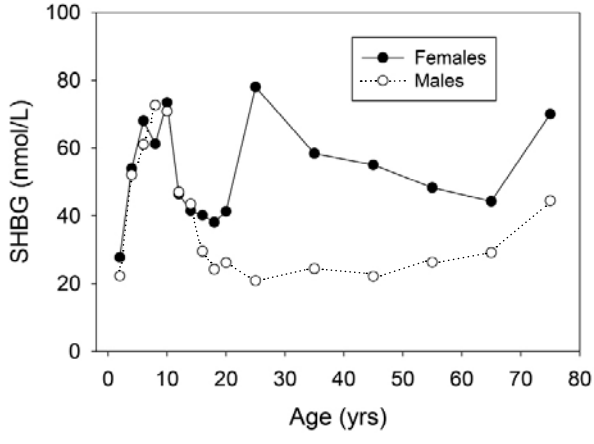

Figure 2. Sex hormone-binding globulin levels from birth to old age in males and females. Redrawn from Elmlinger et al (8). SHBG: sex hormone-binding globulin

but not eliminated, after adjustment for age and body mass index (BMI) (16). Notably, a low level of SHBG is a biomarker for the future development of the metabolic syndrome (MetS) (17), gestational diabetes (18), and T2DM (19).

Table 1. Factors that influence the level of sex hormone-binding globulin in blood

Decrease	Increase
Androgens	Estrogens
Obesity	Pregnancy (Estrogens)
Insulin resistance	Weight loss
Metabolic syndrome	Alcoholic cirrhosis
Type 2 diabetes mellitus	Hepatitis-B and hepatitis-C infection
Gestational diabetes mellitus	Hemochromatosis
Polycystic ovary syndrome	Hyperthyroidism
Non-alcoholic fatty liver disease	Growth hormone deficiency
Acromegaly	Acute intermittent porphyria
Cushing's syndrome	First generation anticonvulsants
Congenital adrenal hyperplasia	
Hyperprolactinemia	
Tumor necrosis factor alpha	
Interleukin-1 beta	

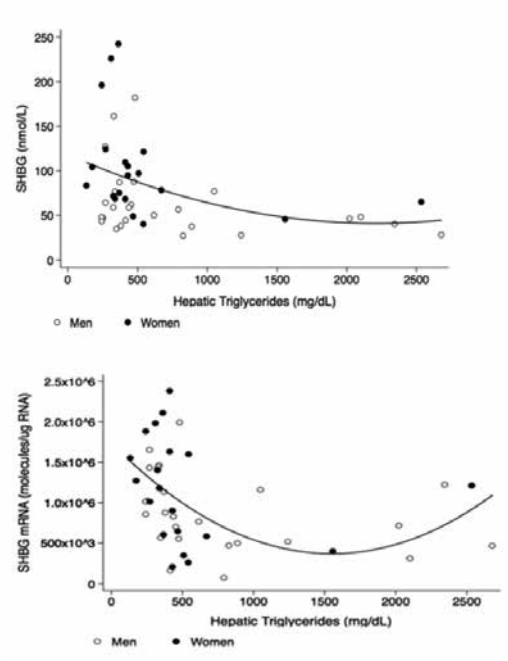

Figure 3. Associations between serum sex hormone-binding globulin levels (A) and sex hormone-binding globulin messenger ribonucleic acid (B) with hepatic triglyceride levels in women and men undergoing partial hepatectomy. Redrawn from reference (38). SHBG: sex hormone-binding globulin, mRNA: messenger ribonucleic acid

Birkeland et al (20) reported that the level of SHBG represents an index of insulin resistance (IR), and many studies have confirmed this result (21). The traditional explanation for low SHBG levels in IR has been hyperinsulinemia (22). Studies have found an inverse correlation between SHBG and fasting (23), glucose-stimulated (24) or 24-hour mean insulin or C-peptide (25,26), and SHBG levels increase when IR improves and insulin levels decline with weight loss (27), resistance exercise (28), or following treatment with insulin sensitizing drugs (29). Moreover, adding insulin to HepG2 hepatocarcinoma cells reduced their production of SHBG (30,31), and insulin was reported to suppress SHBG messenger ribonucleic acid (mRNA) levels (31). A more recent study also using HepG2 cells, however, found no effect of insulin on SHBG secretion or mRNA levels. Instead, SHBG gene expression was reduced by the cytokines tumor necrosis factor-alpha (TNFα) (32) or interleukin-1 beta (IL1β) (33) and in transgenic mice that express SHBG after they were mated with obese, diabetic, hyperlipidemic db/db mice with inactivating leptin receptor mutation (34).

The nuclear receptor hepatic nuclear factor-4α (HNF4α) activates the promoters of many genes that are expressed in the liver and plays a key role in lipid metabolism (35). Functional HNF4α-binding sites are found in over 140 genes, including those involved in the metabolism of glucose, lipids, and amino acids, and in the proximal promoter of the SHBG gene. Moreover, over-expression of HNF4α in HepG2 cells by transient transfection increased the transcriptional rate of a SHBG-luciferase reporter (36). The effect of TNFα to suppress SHBG expression *in vitro* is mediated by HNF4α (37) and there is a strong correlation between the expression levels of HNF4α and SHBG in human liver (38). Thus, HNF4α regulation plays a central role in determining the level of SHBG in plasma.

Hepatic fat is associated with IR (39) and recent studies have linked hepatic steatosis to low SHBG. A study of subjects at risk for T2DM which found no relationship between SHBG and insulin secretion following glucose challenge concluded that the amount of liver fat was the strongest predictor of SHBG (40). Several studies have subsequently found a strong inverse correlation between the amount of liver fat and serum levels of SHBG (41,42), and SHBG levels rise and liver fat decreases with weight loss (43). We recently found that serum SHBG and SHBG mRNA levels are low when the hepatic triglyceride concentration is elevated in a study of adult men and women undergoing hepatic resection as treatment for cancer (Figure 3) (38). In a recent study, Tong et al (44) reported that SHBG levels rose during short-term intensive insulin therapy in adults with newly-diagnosed T2DM which improved their lipid profiles and decreased liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT)] and homeostatic model assessment-IR (HOMA-IR). Thus, the evidence to date suggests that excess hepatic fat is a

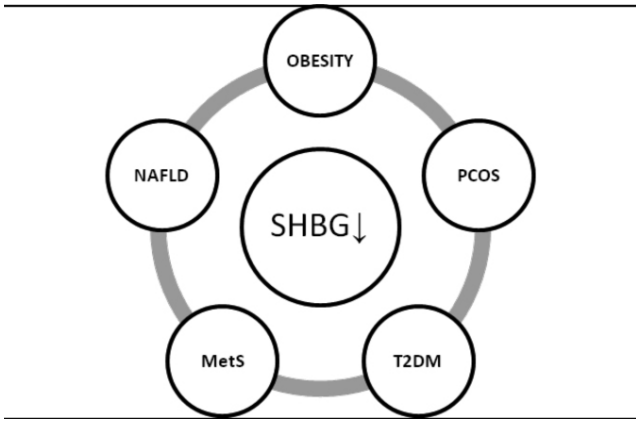

Figure 4. Relationship between obesity-related comorbidities and sex hormone-binding globulin levels. MetS: metabolic syndrome, NAFLD: non-alcoholic fatty liver disease, PCOS: polycystic ovary syndrome, T2DM: type 2 diabetes mellitus, SHBG: sex hormone-binding globulin

key determinant of low SHBG, although more research is needed.

Clinical Disorders Affecting Sex Hormone-Binding Globulin

There are several disorders which affect SHBG levels, and understanding these effects may be important clinically.

Hyper- and Hypothyroidism

SHBG levels increase dramatically in hyperthyroidism in proportion to the levels of thyroxine (T_4) and triiodothyronine (T_3) in children (45) as well as in adults. Values normalize when hyperthyroxinemia is treated. High SHBG levels will result in elevated levels of testosterone in both males and females, and may present a diagnostic challenge and lead to an unneeded evaluation for pituitary, adrenal, or gonadal disorders. High SHBG leads to elevation of luteinizing hormone (LH) and estradiol and may produce breast enlargement in males (46). High SHBG levels result from thyroid hormone activation of the *HNF4α* gene promoter which, in turn, stimulates SHBG expression (47). SHBG is thus a marker of increased thyroid hormone bioactivity. This idea has been used clinically in patients with inappropriate thyroid-stimulating hormone (TSH) secretion with high free T_4/T_3 levels and TSH levels that are not suppressed. Some of these individuals have inactivating mutations of the thyroid hormone receptor, which disrupts feedback control of TSH secretion. These individuals may be recognized by their normal SHBG levels (48) and distinguished from patients with TSH-producing pituitary tumors who have hyperthyroxinemia and high SHBG (49). SHBG levels are reduced in hypothyroidism, which in men may be interpreted as testosterone deficiency.

Adrenal Disorders

SHBG levels are reduced in patients with Cushing's syndrome (50) and in patients treated with glucocorticoids (51). Low SHBG together with adrenocorticotropic hormone (ACTH)-mediated testosterone production may cause virilization in children, and contribute to delayed puberty, and anovulation and oligo-amenorrhea in ACTH-dependent Cushing's syndrome (52). In children treated with prednisone or dexamethasone for leukemia, the fall in SHBG occurred slowly over 4 weeks during which time BMI and leptin levels rose suggesting a connection to IR (51). Perhaps because of a tendency to abdominal adiposity (53), SHBG levels are also low in girls with congenital adrenal hyperplasia (54).

Pituitary Disorders

SHBG levels are elevated in patients with growth hormone deficiency (55) and are decreased in patients with acromegaly. To what extent these changes are mediated by insulin sensitivity and resistance is unknown. Lower SHBG levels have been reported in patients with hyperprolactinemia, but this association may also be influenced by the higher body fat with hypogonadism.

Liver Disease

SHBG is produced in the liver, and SHBG levels are affected by diseases of the liver through a variety of mechanisms. SHBG levels are elevated in patients with alcoholic cirrhosis. Alcohol damages the testis so that LH levels are elevated which in turn stimulate testicular aromatase and thereby estradiol production which increases SHBG. High SHBG may also be due to increased estrone and estradiol from the adrenals that is activated by stress and ACTH (56). Moreover, sulfatase (the enzyme which converts inactive estrogen sulfates to active estrogens) is increased in alcoholic liver. Amenorrheic women with both alcoholic and nonalcoholic cirrhosis, by contrast, tend to have low LH/follicle stimulating hormone and normal SHBG levels (57). SHBG levels are also markedly increased with hepatitis-B or hepatitis-C infection (58), while patients with liver disease due to hemochromatosis develop hypogonadotropic hypogonadism due to pituitary iron deposition and tend to have slightly elevated SHBG levels. Non-alcoholic fatty liver disease (NAFLD), a condition of increased hepatic triglycerides in the absence of excess alcohol consumption, is associated with increased visceral adipose tissue (VAT), IR, and dyslipidemia, and with low SHBG levels (41).

Obesity and Related Comorbidities

Childhood obesity is one of the most important health problems of our era due to its high prevalence and association with many chronic diseases and shorter life expectancy (59,60). Recent studies have found alarming increases in the rates of childhood obesity and related comorbidities, such as T2DM, MetS, peripubertal hyperandrogenemia (HA), polycystic ovary syndrome (PCOS), NAFLD, and early puberty (59,61,62,63,64). These disorders are inter-related, and their etiology and pathogenesis are multifactorial and controlled by

genetic factors, the intrauterine environment, and an unhealthy lifestyle (63). Since these conditions increase the risk of early cardiovascular disease (CVD), finding effective ways to identify at-risk children as early as possible is an important goal. SHBG is a promising biomarker (Figure 4) because SHBG levels are unaffected by meals or time of day, there is no influence of sex hormones in prepubertal children, and SHBG can be readily measured in a finger-stick blood sample. Many studies have linked lower circulating levels of SHBG to obesity, IR, MetS, T2DM, PCOS, and NAFLD (21,60,65). These associations may be explained by the idea that low SHBG is a marker for IR.

Type 2 Diabetes Mellitus and Sex Hormone-Binding Globulin

SHBG levels are low in adults with T2DM, and many studies show that low levels predict diabetes risk (19,66,67). The relationship is reduced, but maintained, after controlling for obesity. T2DM is increasingly diagnosed in children as young as age 10, and now accounts for 20% to 50% of new-onset diabetes in children (64). In the U.S., it disproportionately affects Latino and Black children. Several studies showed that weight loss through calorie restriction and metformin treatment, in combination with lifestyle changes, increases serum SHBG levels in adolescents at risk for developing diabetes (68). In those studies, insulin levels decreased with intervention due to improvement in insulin sensitivity.

It has been suggested that SHBG may have a causal role in the risk of T2DM since Mendelian randomization studies have reported that carrying specific SHBG single-nucleotide polymorphisms (SNPs) affects the risk of T2DM (19,66). Carriers of rs6259 polymorphism were shown to have higher SHBG levels and a lower risk of T2DM, and rs6257 SNP carriers were reported to have lower SHBG levels and higher risk of T2DM (19). In another larger study including 86138 adults, presence of the rs1799941 SNP was associated with increased SHBG concentrations and reduced risk of T2DM after correction for age, sex, and BMI (66). In a recent study, Wang et al (69) showed that circulating SHBG levels were predictive for future IR in healthy young Finnish adults, whereas Mendelian randomization suggested minor, if any, causal effects.

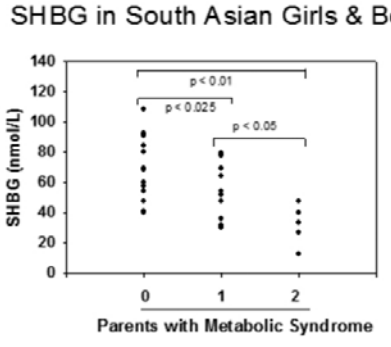

Figure 5. Serum levels of sex hormone-binding globulin in South Asian Indian children according to the diagnosis of Metabolic syndrome in their parents; redrawn from reference (86). SHBG: sex hormone-binding globulin

Metabolic Syndrome and Sex Hormone-Binding Globulin

MetS is a combination of risk factors for increased CVD morbidity and mortality that includes central obesity, hypertension, dyslipidemia, and impaired glucose metabolism. MetS is increasingly recognized in children and adolescents, but the diagnostic criteria for this age group remain controversial (70). Furthermore, no accepted definition applies to all ethnic groups because ethnic variations exist in the distribution of MetS components in children (71,72,73,74).

As in adults, SHBG levels are low in children and adolescents diagnosed with MetS (75,76,77). In a cross-sectional study of 815 school children in Spain by de Oya et al (77), SHBG levels were lower in those adolescents with MetS or with some MetS features, such as abdominal obesity, high blood pressure or high insulin and low high density lipoprotein cholesterol (HDL-C) levels. Agirbasli et al (78) reported that low SHBG was a significant predictor of low HDL-C levels in Turkish children and adolescents. Detailed metabolic profiling of 6475 young adults from two population-based Finnish cohorts revealed a strong association between SHBG and circulating lipids and metabolites reflecting the degree of adiposity and IR. Low SHBG predicted the development of IR in early adulthood, and these associations remained robust after adjustment for baseline adiposity, insulin and testosterone levels (69). Glueck et al (65) demonstrated that low SHBG levels in U.S. schoolgirls at age 14 were a positive predictor for the development of MetS 10 years later. Thus, SHBG may be valuable biomarker for MetS risk in children long before the disease progresses.

There are substantial racial and ethnic differences in body composition for a given BMI between subjects of the same sex and age. Previous studies have documented a genetic predisposition for MetS (79,80) and there is evidence that SHBG levels, like MetS components, vary by ethnicity (Table 2). In the national health and nutrition examination survey (NHANES) study, SHBG levels were lower in Mexican-American males age 12-19 than in non-Hispanic blacks and whites (81). Abdelrahaman et al (82) found that high levels of SHBG are more common among healthy African American prepubertal boys, a racial group with more subcutaneous but less VAT than their white peers. Hergenc et al (83) reported that Turkish middle-aged adults have lower SHBG levels compared with Germans, and most of the difference in HDL-C between Germans and Turks was explained by ethnicity independent of obesity markers, insulin, and sex hormones. The MELEN study of 751 Turkish adult women and men, with a mean age of 55 years, found that 34% had MetS (84), while a recent study of German women reported a prevalence of 23.1% (85). South Asian Indians are an ethnic group at especially high risk for MetS and T2DM even though they have low BMI. Krishnasamy et al (86) found that prepubertal South Asian Indian children

with one parent with MetS had 24% lower SHBG levels, and with both parents affected had 55% lower SHBG levels (Figure 5). Their study also demonstrated that SHBG levels were inversely related to waist circumference and to BMI percentile in those children. Significant associations were reported between SHBG (rs6257), cholesterol ester transfer protein (rs708272) polymorphisms and high triglycerides, low HDL-C and high low-density lipoprotein cholesterol levels in a cohort of 365 Turkish children and adolescents (87). Additionally, White et al (88) reported that SNPs located in the SHBG gene (rs1799941) were associated with MetS in children. They found that association with MetS remained after sequential adjustment for each MetS component, indicating that the identified association was not being driven by any single trait. The A allele of rs1799941 was associated with a significant increase in SHBG levels in control subjects, while there was no association between rs1799941 and SHBG levels in children with MetS.

Peripubertal Hyperandrogenaemia, Adolescent Polycystic Ovary Syndrome and Sex Hormone-Binding Globulin

PCOS is the most common endocrine disorder among reproductive-aged women, and the most common cause of infertility in young women. PCOS is characterized by HA, menstrual dysfunction, and polycystic ovarian morphology, and arises as a complex trait due to inherited and environmental factors. Adolescents with PCOS are more insulin resistant and hyperinsulinemic compared to weight-matched non-hyperandrogenemic girls (89). SHBG levels are reduced in PCOS resulting in a higher portion of biologically active androgen, and an increased number of (TAAAA)n repeats in the SHBG promoter region may be a susceptibility locus for PCOS (90) although this association is controversial (91). The clinical manifestations of PCOS often begin during puberty, but the anovulation and acne that often occur in healthy teenage girls make the PCOS diagnosis challenging in adolescents. Therefore, biochemical evidence of HA, including low SHBG levels, is important in the evaluation of adolescent PCOS (92).

SHBG levels are low in obese and overweight peripubertal girls, and weight loss is associated with a decrease in testosterone and an increase in SHBG levels (93,94). Not all peripubertal obese girls have elevated androgens, however, and not all adolescents diagnosed with PCOS are obese or overweight, suggesting that obesity per se is not sufficient to produce HA (95). Teenage daughters of PCOS patients are more likely to have features of the MetS, and to be hyperinsulinemic (96), have larger ovaries beginning at Tanner stage 1, and by Tanner stage V have lower SHBG levels than daughters born to control women (97).

The prevalence of PCOS in women born small for gestational age (SGA) is twice as high as in women born with normal weight (98). Girls born SGA with catch-up growth were shown to display more visceral fat as compared to age- and BMI- matched children born at normal weight (99) and have lower SHBG concentrations and an exaggerated adrenarche between the ages of 6 and 8 years (100). Longitudinal studies revealed that metformin-treated low birth weight children were leaner, had less IR and higher SHBG levels than placebo-treated children, and in low birth-weight girls, the increase in SHBG was followed by a delay of menarche (68).

Table 2. Studies evaluating ethnic and racial differences in serum sex hormone-binding globulin levels in children and adolescents

Author, year (reference)	Study population (n)	Age range (years)	Conclusion
Hui et al, 2003 (121)	Healthy children with roughly equal numbers of African American and white boys and girls (n=232)	4-16	SHBG did not differ between racial groups
Abdelrahaman et al, 2005 (82)	African American and white boys (n=47)	5-9	High levels of SHBG were more common among African American boys than white boys
Danielson et al, 2010 (122)	Racially/ethnically diverse individuals with T1DM diagnosed at age <18 years (n=79) (32.9% NHW, 46.8% NHB, 12.7% Hispanic, 7.6% other/mixed)	3.2-32.5	Insulin resistance, estimated by eGDR, was greater in minorities with T1DM than in NHW probands; eGDR was negatively associated with SHBG
Hannon and Arslanian, 2012 (123)	African American and white obese females (n=22)	8.8-13.9	SHBG levels were not significantly different between white and blacks
Lopez et al, 2013 (81)	NHB, NHW and Hispanic males (n=134)	12-19	SHBG levels were lower in Hispanic males than in NHB and NHW
Wolfgram et al, 2014 (108)	Non-obese NHW and Hispanic girls (n=32)	11-14	Hispanic girls had significantly lower SHBG than NHW

eGDR: estimated glucose disposal rate, NHB: non-Hispanic black, NHW: non-Hispanic white, T1DM: type 1 diabetes mellitus, SHBG: sex hormone-binding globulin

Non-alcoholic Fatty Liver Disease and Sex Hormone-Binding Globulin

NAFLD has become the most common form of liver disease in childhood. The presence and severity of NAFLD are associated with an increased incidence of CVD, independent of established risk factors, and NAFLD was suggested as not only a marker of CVD risk but also an important player in CVD pathogenesis (101). Early diagnosis and treatment is crucial, but most children with NAFLD remain undiagnosed (63). While liver biopsy is the gold standard for diagnosis, the European Society for Pediatric Gastroenterology, Hepatology and Nutrition instead recommends abdominal ultrasound and liver function tests for all obese children (102). Large-scale ultrasound may not be a cost-effective approach, however, and liver transaminase (ALT, AST) elevations were a poor predictor, especially in the earliest stages (60,102). Additionally, the serum GGT level may be a marker of oxidative stress rather than a specific marker of NAFLD-induced liver disease (103).

Previous studies have shown that liver fat is a stronger predictor of SHBG than is total body fat. Serum SHBG levels were lower in high-grade NAFLD patients with T2DM than in diabetics without NAFLD (104), and lower SHBG levels were found in adult (105) and adolescent (106) PCOS subjects with NAFLD compared with PCOS subjects without NAFLD (40). Moreover, those women with PCOS and NAFLD are more insulin resistant than are PCOS women without evidence for hepatic steatosis by ultrasound (107). Wolfgram et al (108) showed significant correlations between hepatic proton density fat fraction measured by magnetic resonance imaging and SHBG blood levels in non-obese Hispanic middle school girls. Finally, SHBG levels were shown to rise as liver fat decreases with weight loss (43). In the light of these findings, SHBG represents an alternative marker for pediatric NAFLD risk stratification and in certain children at higher risk for NAFLD and MetS, may be a useful biomarker perhaps prior to the development of obesity.

Early Puberty and Sex Hormone-Binding Globulin

There is accumulating evidence that puberty in girls is occurring at an earlier age, and the obesity epidemic is an important factor in this phenomenon (109). SHBG may function during childhood to restrict the actions of sex steroids until puberty at which time sex steroid levels increase in concert with a fall in plasma SHBG levels such that the overall result is a progressive increase in both total and free and hormone levels. The mechanism for the decline in SHBG at puberty is not well understood but appears to be metabolic rather than hormonal since the decrease occurs in boys with hypopituitarism (11). Moreover, insulin sensitivity declines in early normal puberty (110) which could lead to lower SHBG levels. In a cross-sectional study on 132 healthy Caucasian children and adolescents, SHBG was a strong predictor of insulin sensitivity after adjustment for puberty, fat mass, and aerobic fitness (111). In that study, the authors reported a significant negative association between metabolic risk and SHBG levels after adjustment for relevant confounders, and hypothesized that SHBG integrates the marked changes in glucose metabolism and body composition that occur during the pubertal transition and might be valuable in the assessment of CVD risk during puberty. Pinkney et al (10) reported that girls with lower SHBG levels at 5 years of age reached Tanner stage 2 earlier, tended to have earlier increases in LH secretion, and an earlier age at peak height velocity and menarche. They reported negative correlations between SHBG and adiposity, insulin, IGF-I, CRP, and leptin, and positive associations between adiponectin and SHBG (10). Sorensen et al (112) found that, after adjustment for BMI and pubertal stage, girls with central precocious puberty have lower SHBG levels compared with healthy controls, and the decline in SHBG levels during puberty is associated with increasing fat mass in healthy children and adolescents.

Although studies tend to indicate a relationship between obesity and early puberty in girls, the association in boys is controversial. Some authors report advanced sexual maturation in obese boys (113,114), some describe normal pubertal timing (115), while others report delayed testicular development with obesity (116,117). The reason for these contradictory findings is uncertain but might be due to differences in the study populations, pubertal markers, and cut-off points for defining obesity. Studies have shown that SHBG levels are lower in obese boys than in their normal weight peers (23,115). Pinkney et al (10) reported that boys with lower SHBG levels at age 5 years reached Tanner stage 2 earlier, but there was no relationship between SHBG and earlier onset of LH secretion or age at peak height velocity. Most studies report lower total testosterone levels in obese boys during pubertal progression (118) which can be explained by lower SHBG whereas SHBG and total testosterone are unrelated in prepubertal boys (82).

Denburg et al (119) reported lower SHBG levels and decreased insulin sensitivity in boys with premature pubarche (PP) than in age- and BMI-matched peers. They showed significant correlations between SHBG and measures of insulin sensitivity in boys with PP and controls, and suggested that SHBG may be a marker for IR. On the other hand, Potau et al (120) found that SHBG levels and measures of the glucose and insulin response to an oral glucose challenge were comparable in boys with PP and controls, and concluded that PP in boys may be regarded as a variant of normal development.

In conclusion, evidence is accumulating that low SHBG levels are an indicator of IR, and SHBG may be an easy-to-measure and clinically useful biomarker for the early identification of children who are destined to develop obesity-related chronic diseases. Further research is needed to understand how SHBG is regulated in children. Moreover, studies with respect to race

and ethnicity are needed to establish SHBG reference ranges for children and adolescents. Finally, whether SHBG is solely a biomarker or rather participates actively in the pathogenesis of metabolic disease remains to be elucidated.

Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Stephen J. Winters, Design: Stephen J. Winters, Data Collection and/or Processing: Stephen J. Winters, Banu Aydın, Analysis and/or Interpretation: Stephen J. Winters, Banu Aydın, Literature Research: Stephen J. Winters, Banu Aydın, Writing: Stephen J. Winters, Banu Aydın.

Financial Disclosure: BA is supported through The Scientific and Technical Research Council of Turkey (TUBITAK) (International Postdoctoral Research Scholarship Program). SJW is supported in part by a gift from the Walter F. and Avis Jacobs Foundation.

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J Clin Res Pediatr Endocrinol 2016;8(1):13-20
DOI: 10.4274/jcrpe.2219

Original Article

Distribution of RET Mutations and Evaluation of Treatment Approaches in Hereditary Medullary Thyroid Carcinoma in Turkey

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Genetic screening of germline RET mutations provides the early diagnosis of hereditary cases. Prophylactic thyroidectomy (PTx) is the only preventive option for mutation carrier family members.

WHAT THIS STUDY ADDS?

Turkish people has a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries i.e. Italy and France. Despite complimentary *RET* gene testing by the courtesy of Society of Endocrinology and Metabolism of Turkey, the number of the PTx in Turkey is limited and relatively late in the lifespan of the gene carriers. This is mainly due to patient and family incompliance and incomplete family counselling.

ABSTRACT

Objective: This retrospective multicenter study, centrally conducted and supported by the Society of Endocrinology and Metabolism of Turkey, aimed to evaluate the impact of free RET proto-oncogene testing in medullary thyroid carcinoma (MTC) patients. Surgical timing, adequacy of the treatment, and frequency of prophylactic thyroidectomy (PTx) in mutation carriers were also assessed.

Methods: Genetic testing for MTC and pheochromocytoma was conducted between July 2008 and January 2012 in 512 patients. Application forms and RET mutation analyses of these patients whose blood samples were sent from various centers around Turkey were assessed retrospectively. An evaluation form was sent to the physicians of the eligible 319 patients who had confirmed sporadic MTC, familial MTC (FMTC), multiple endocrine neoplasia type 2 (MEN2), or who were mutation carriers. Physicians were asked to give information about the surgical history, latest calcitonin levels, morbidity, mortality, genetic screening, and PTx among family members. Twenty-five centers responded by filling in the forms of 192 patients.

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ABSTRACT

Results: Among the 319 patients, RET mutation was detected in 71 (22.3%). Cys634Arg mutation was the most prevalent mutation (43.7%), followed by Val804Met in 18 patients (25.4%), and Cys634Tyr in 6 patients (8.5%). Among 192 MTC patients, the diagnosis was sporadic MTC in 146 (76.4%), FMTC in 14 (7.3%), MEN2A in 15 patients (7.9%), and MEN2B in one patient. The number of mutation carriers among 154 apparently sporadic MTC patients was 8 (5.2%). Ten patients were submitted to PTx out of twenty-four mutation carriers at a mean age of 35±19 years.

Conclusion: Turkish people have a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries. Despite free *RET* gene testing, the number of the PTx in Turkey is limited and relatively late in the life span of the carriers. This is mainly due to patient and family incompliance and incomplete family counselling.

Keywords: Sporadic medullary thyroid carcinoma, hereditary medullary thyroid carcinoma, multiple endocrine neoplasia, RET mutation

Conflict of interest: None declared
Received: 08.07.2015
Accepted: 30.09.2015

Introduction

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor of the thyroid gland and accounts for 5% of thyroid cancers. The tumor originates from parafollicular 'C' cells and secretes calcitonin (CT). MTC mainly occurs sporadically (70-75%) (1). Hereditary syndromes of germline RET proto-oncogene mutations are the cause of MTC in the remaining 25-30% of patients.

The RET proto-oncogene is located on chromosome 10 (10.q11.2). It is a member of the receptor tyrosine-kinase family and comprises 21 exons. 'C' cells of the thyroid gland, parathyroid glands, adrenal medulla, urogenital system all express RET proto-oncogene (2). Various germline mutations of RET proto-oncogene cause distinct clinical features and influence the course of the disease (3). Multiple endocrine neoplasia type 2 (MEN 2) and familial MTC (FMTC) are both autosomal dominant inherited hereditary cancer syndromes.

MEN2A accounts for 80% of hereditary MTC syndromes; consists of MTC in all patients, pheochromocytoma in 50% and primary hyperparathyroidism in 20-30% of patients (4). Cutaneous lichen amyloidosis (10%) and Hirschprung disease (7%) may also develop in MEN2A patients (5,6). MEN2A prevalence is estimated to be 1 per 50.000 and age of diagnosis is usually 20-30 years. *De novo* mutations may be responsible in up to 5% of MEN2A patients (7). Codon 634 mutations are the most frequent mutations in many of European countries (8,9).

MEN2B is characterized with MTC, pheochromocytoma, mucosal neuromas, ganglioneuromatosis of the gut, and marfanoid habitus. This syndrome is caused by autosomal dominant genetic inheritance and *de novo* mutations equally (10). Prognosis of MEN2B is worse than MEN2A since MTC is more aggressive (11).

FMTC is classically characterized by only hereditary predisposition to MTC for at least three generations (12).

Germline mutations of the RET proto-oncogene are found in 98% of MEN2A, 95% of MEN2B, and in 88% of FMTC patients (13). Germline RET mutations can also be found in 7-10% of apparently sporadic forms of MTC by routine RET screening (14,15). Hence, only genetic testing could rule out hereditary forms of MTC.

Genetic screening of germline RET mutations is also the means for the early diagnosis of hereditary cases. Prophylactic thyroidectomy (PTx) is the only preventive option for mutation carrier family members. The association between RET mutations (genotype) and the biological behavior of tumor (phenotype) is well documented (3). Current guidelines recommend to consider risk stratification, mainly depending on the type of the mutation, in decisions on the timing of PTx (16). Age, family history, and basal/stimulated serum CT levels are the other factors which may have impact on the timing of PTx. In 2009, American Thyroid Association (ATA) recommended a 4-level risk classification for RET mutation carriers. PTx was recommended in the first year of life for MEN2B (M918T, A883F), before 5 years of age for subjects with level C (634 mutations) and also for level B (609, 611, 618, 620, 630, 631 mutations). The lower risk category (level A) involves distal codons (768, 790, 791, 804, 891). Family history of tumor behavior and family preference for these rare mutations with low genetic penetrance also need to be considered in decisions for surgery. However, there is no consensus on the management of these subjects (6,16,17,18).

This retrospective multicenter study aimed to evaluate the impact of free RET proto-oncogene testing of MTC patients. All data were evaluated in one center. The study was supported by the Society of Endocrinology and Metabolism of Turkey (SEMT). Adequacy of early diagnosis, surgical timing, and frequency of PTx in mutation carriers were also assessed.

Methods

Genetic testing for MTC and pheochromocytoma was conducted between July 2008 and January 2012 in 512 patients. Application forms and RET mutation analyses of these 512 patients whose blood samples were sent from various

centers around Turkey were assessed retrospectively. Patients with pheochromocytoma without detected mutations and RET negative relatives of hereditary MTC patients were excluded. A total of 319 patients with familial/sporadic MTC, MEN2, and mutation carriers from known MEN2/FMTC families were found to be eligible for retrospective analysis. An evaluation form including information on the surgical history, latest CT levels, survival of the patients, and PTx had been prepared and sent to several physicians working in centers around the country. Twenty-five centers responded by filling in the forms of 192 patients.

Patients who were considered to be sporadic cases by their physicians before mutation analysis were categorized as 'apparently sporadic' cases. After the final analysis of the RET mutations and of the evaluation forms, the final diagnoses were categorized as MEN2A, familial/sporadic MTC, and mutation carriers from MEN2A/FMTC families.

The study was approved by the Local Ethics Committee of Ankara University Faculty of Medicine (September 5, 2012).

RET Mutation Analysis

RET mutation analyses were performed in Düzen Laboratory Groups. Genomic DNA from patients was isolated from peripheral blood leukocytes automatically by MagNA Pure LC 2.0 (Roche Applied Science). *RET* gene exons 1-20 were amplified by polymerase chain reaction (PCR) using previously reported primers flanking the intron-exon junctions. PCR amplification of the exons 1-20 was carried out in a 25 µl PCR mix containing 5 ng genomic DNA, 0.1 µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl, and 1.25 U Taq polymerase (19,20). PCR [touchdown (TD) TD-PCR] was carried out. TD-PCR cycling program was: initial denaturation at 95 °C for 15 min followed by 10 cycles of 1 min at 95 °C, 1 min at 62 °C with an 0.5 °C decrease of temperature per cycle and 1 min at 72 °C and an additional 25 cycles of 1 min at 95 °C, 1 min at 57 °C, 1 min at 72 °C and 10 min at 72 °C for final extension. The amplicons were analyzed in ethidium bromide-stained agarose gels and showed a single band with expected size. Then, amplicons were sequenced in an automated sequencer (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The sequence data was analyzed by using SeqScape version 2.7 (Applied Biosystems) and Sequencing Analysis version 5.1 (Applied Biosystems) softwares. The results were interpreted by a professor in medical genetics.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software, version 20.0 (IBM Corp, NY, and USA). Categorical data were compared using the chi-square Fisher exact test. Group data with a normal distribution were compared using the Student t-test or analysis of variance, and nonparametric data were compared using the Mann-Whitney U or Kruskal-Wallis tests. Values were expressed as mean ± standard deviation or median as appropriate. A p-value <0.05 was considered statistically significant.

Results

Figure 1, organized as a flow chart, depicts the numbers of patients included in the study at each step of the analysis. Between 2008 and 2012, among the 319 patients with hereditary/sporadic MTC whose blood samples were sent for RET analysis, mutation was detected in 71 patients (22.3%). Codon 634 mutation was detected in 39 (54.9%) patients. Cys634Arg was the most prevalent mutation (n=31) and accounted for 43.7% of all mutations. Cys634Tyr and Cys634Gly mutations were detected in 6 patients (8.5%) and in 2 patients (<1%), respectively. Val804Met germline mutation accounted for 25.4% of mutations. Distribution of RET mutations is summarized in Table 1.

Among the 192 MTC patients with available clinical information, 146 patients had sporadic MTC, 15 patients MEN2A, 14 patients FMTC, and one subject had MEN2B. Sixteen patients were mutation carriers from known families. However, so-called FMTC patients may not fulfill the diagnostic criteria yet since three generations of follow-up is mandatory (12).

Distribution of RET proto-oncogene mutation in 191 patients with available clinical information is summarized in Table 2, and distribution of RET mutations according to final diagnosis is summarized in Table 3. One MEN2B patient with Met918Thr mutation was excluded before statistical analyses.

RET mutation was detected in 8 of 154 patients (5.2%) defined as 'apparently sporadic' cases. Among these patients, three had Cys634Arg, three Val804Met, one Cys618Ser, and one subject had Y790Phe mutation.

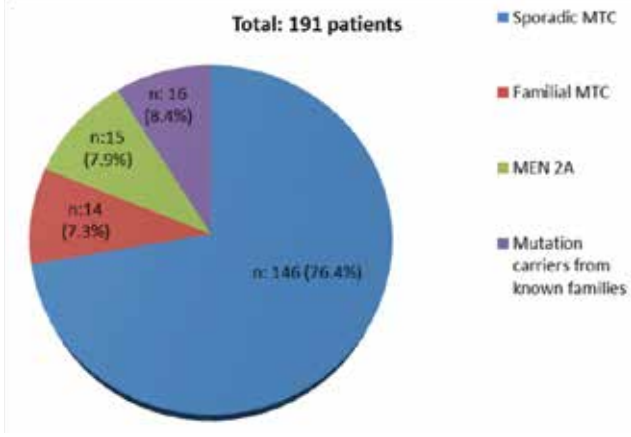

Figure 1. The distrubition of diagnosis for 191 medullary thyroid carcinoma patients with available clinical information (multiple endocrine neoplasia type 2 B patient was excluded). MTC: Medullary thyroid carcinoma, MEN2A: multiple endocrine neoplasia type 2 A

Table 1. Distribution of RET proto-oncogene mutations in 71 out of 319 patients with hereditary sporadic medullary thyroid carcinoma or pheochromocytoma who had been tested for RET proto-oncogene

	N	%	Frequency of mutations % (n=71)
Cys634arg	31	9.7	43.7
Val804Met	18	5.6	25.4
Cys634Tyr	6	1.8	8.5
Tyr791Phe	4	1.2	5.6
Cys618Ser	3	<1	4.2
Met918Thr	2	<1	2.8
Cys634Gly	2	<1	2.8
Y790Phe	1	<1	1.4
Tyr790Phe	1	<1	1.4
Leu790Phe	1	<1	1.4
Ser891Ala	1	<1	1.4
D631Y	1	<1	1.4
Total	71	22.3	100.0

Table 2. Distribution of results of the RET proto-oncogene testing in 191 patients with available clinical information

	N	%	Frequency in mutations % (n=45)
No mutation	146	76.4	-
Cys634Arg	15	8.0	33.5
Cys634Tyr	7	3.6	16.0
Val804Met	15	8.0	33.5
Cys618Ser	2	1.0	4.0
Y790Phe	3	1.5	7.0
Tyr791Phe	2	1.0	4.0
D631Y	1	0.5	2.0
Total	191	100.0	100.0

Mean age at diagnosis was similar in the two genders (44.2±14 years in females vs. 44.6±13.6 years in males, p=0.7), but was significantly lower in the patients diagnosed with genetic testing compared to those diagnosed with classical clinical features (31±18.2 years vs. 46.5±12.7 years, p<0.01). Mean age at diagnosis was also not statistically different between sporadic MTCs (47.6±1.05 years) and FMTCs (41.3±2.9 years) (p>0.05). MEN2A patients were younger (35.6±2.78 years) than sporadic and FMTCs at presentation (p<0.01) (a summary of preoperative clinical data is given in Table 4).

Mean follow-up period was 40±27 months in the whole group. The stage of tumor at diagnosis and preoperative CT levels are summarized in Table 4. Preoperative CT level was significantly higher in sporadic cases compared with the MEN2A and FMTCs (p<0.05) (Table 4).

Follow-up data was available for 138 patients. On their most recent follow-up visit, 80 patients (58%) were in full remission, 21 patients (15%) had locoregional disease (17 operable, 4 inoperable) and 29 (21%) had distant metastases. Seven sporadic and one MEN2A patient (6%) died due to distant metastases.

Sorafenib was the most preferred chemotherapeutic drug in metastatic patients (n=14). Conventional chemotherapeutics were chosen for 9 metastatic patients.

The mean number of surgical procedures was not statistically different between sporadic (1.67±1.05) and hereditary cases (1.31±1.5) (p=0.09).

Table 4. Preoperative clinical data of patients*

	Sporadic MTC	MEN2A	FMTC	p
Age (Mean, years)	47.6±1.05	35.6±2.78	41.3±2.9	p<0.01
Stage at diagnosis				
Stage I	41	6	8	NS
Stage II	7	2	0	NS
Stage III	17	4	3	NS
Stage IV	26	1	2	p<0.01
CT (mean, pg/mL)	1072±1102	704±594	254±569	p<0.05

MTC: Medullary thyroid carcinoma, CT: calcitonin, FMTC: familial medullary thyroid carcinoma, MEN2A: multiple endocrine neoplasia type 2 A, NS: non significant
*Tumor stage at the time of diagnosis was available for 117 patients

Table 3. Distribution of RET mutations according to final diagnosis

Diagnosis	Cys634Arg	Cys634Tyr	Val804Met	Cys618Ser	Tyr791Phe	Y790Phe	D631Y
MEN2A	9	2	1	2	1	-	-
FMTC	2	-	11	-	-	1	-
Mutation carrier	4	5	3	-	1	2	1

MEN2A: multiple endocrine neoplasia 2 A, FMTC: familial medullary thyroid cancer

Table 5. Mutations and age of the patients with known RET mutation who had not yet undergone thyroid surgery

Patient	Age (years)	RET mutation	Patient	Age (years)	RET mutation
1-H.K.	29	Val804Met	8-A.K.	7	Cys634Tyr
2-A.K.	27	Val804Met	9-K.K.	57	Cys634Tyr
3-B.A.	17	Tyr791Phe	10-E.P.	4	Cys634Arg
4-M.B.	34	Val804Met	11-İ.B.	47	Tyr791Phe
5-Ü.Y.	33	Tyr791Phe	12-M.K.	53	Cys634Tyr
6-S.E.	23	Cys634Arg	13-N.K.	56	Cys634Tyr
7-H.E.	46	Cys634Arg	14-D.Ö.	5	Cys634Arg

A significant number of mutation carriers (n=14) had not been operated yet although RET mutation carrier status was known at least for a year. Three out of fourteen subjects had elevated basal CT levels at the time of the study (Table 5). PTx or total thyroidectomy with central lymph node dissection was performed only in 10 out of 24 patients (41.6%). Mean age at thyroidectomy was 35±19 (12-60) years. Our study population included only five pediatric cases, and three of them had not undergone PTx although they had Codon 634 mutation.

Discussion

This retrospective multicenter study aimed to evaluate the impact of complimentary RET proto-oncogene testing in MTC patients. The timing of the surgical intervention, adequacy of diagnosis, treatment in familial/sporadic MTCs, and frequency of PTx for mutation carriers were also assessed.

More than 145 germline mutations of RET proto-oncogene have been identified during the past 20 years, and it has been shown that common mutations are localized in eight exons (exons 5, 8, 10, 11, 13, 14, 15, 16) (21). Previously, the approach for the screening of family members of this autosomal dominant inherited disorder consisted of repeated analyses of basal and/or stimulated CT measurements. In 1993, two different groups identified RET proto-oncogene mutations as the cause of hereditary cases and MEN2 syndromes, and RET genetic testing has since become the method of screening gradually (22,23). The prognosis of MTC is not favorable compared to differentiated thyroid carcinomas. Tumor stage at the time of diagnosis is the most important prognostic factor (24). Curability of metastatic disease is quite difficult. When possible, early diagnosis and treatment is the cornerstone of the management.

In asymptomatic carriers, mutation analysis facilitates early diagnosis and treatment of the disease. The ATA guidelines recommend the use of a four-level risk classification for RET mutations (16). Risk categories are important to decide the timing of PTx for mutation carriers. Although the biological behavior of the disease is usually comparable within the families, it can occasionally be variable within the same family (25,26).

It is well-known that early PTx reduces cancer mortality to lower than 5% in MEN2A patients (27). The primary goal of early prophylactic surgery is to prevent lymph node metastases. Unfortunately, cervical lymph node metastases are found in 70% of patients when the nodule becomes palpable (28). On the other hand, surgery at early ages is associated with increased morbidity (29). Thus, annual follow-up of stimulated CT has been suggested to guide the timing and extent of surgery. Elisei et al (30) achieved cure in RET mutation carriers who were treated early after stimulated CT levels were detected to be elevated. However, in a recent study, it has been reported that basal and stimulated CT levels failed to detect MTC before surgery in three of the 31 (10%) mutation carriers (31). European Multiple Endocrine Neoplasia (EUROMEN) study also demonstrated that 75% (12/16) of codon 634 mutations developed MTC before the age of five. Thus, early diagnosis with RET proto-oncogene testing, risk stratification, and stimulated CT levels are the mainstay for the timing of PTx in hereditary cases.

Our results in this retrospective analysis showed that, although free genetic testing is available with the courtesy of SEMT and conducted centrally by the society itself, the number of PTx is still low in Turkey and the timing of surgical intervention is late. Current data showed that prophylactic/total thyroidectomy was performed only in 10 patients out of 24 mutation carriers identified and mean age of hereditary cases at surgery was relatively late [35±19 years (12-60)]. Fourteen of the known mutation carriers had not yet undergone surgery at the time of the study due to patient and family incompliance and/or incomplete family counselling. Unfortunately, people deny genetic diseases, parents still have hesitations about surgical interventions for their children, especially during younger ages, and healthcare professionals may not be able to overcome these problems.

MEN2A is frequently caused by the mutations in codons 634, 620, 618, 611, 609. Mutations at codon 634 of exon 11 account for 85% of cases and approximately half of them are cysteine to arginine amino acid substitution (Cys634Arg) (32). In FMTC, more than 85% of patients have mutations in exons 10 and 11 (22). Other rare mutations were described in exon 13 (codons 768, 790, 791), exon 14 (codons 804,844) and exon 15 (codon 891).

This is the largest mutation analysis ever performed in Turkish population and most common RET proto-oncogene

mutation reported was codon 634 mutation (54.9%). Val804 mutation was also an important mutation, accounting for 25.4% of RET mutations in Turkish population, especially in FMTC patients (79%) although some of these patients did not fulfill the diagnostic criteria yet. Codon 804 mutation is considered to be a low risk in ATA 2009 guidelines, and individual-, patient-, and family-based management is suggested. In our series, 11 patients with FMTC had Val804Met mutation and they underwent surgery at a mean age of 47.2±10.9. Two patients had local recurrence, one had metastatic disease, and despite relatively late age of surgery, the remaining eight subjects were in remission. Surgical technique could of course be questioned in the local recurrence. Thus, our results are concordant with ATA guidelines, where distal codons (768, 790, 791, 804, 891) were categorized as lower risk mutations with low genetic penetrance.

EUROMEN multicenter study showed that the most prevalent RET mutation in the European population was Cys634 with 67.6% frequency (6). The multicenter ItaMEN network analysis demonstrated that codon 634 at exon 11 was the most affected codon (34.8%) followed by Val804Met mutation (19.6%) among Italian MEN2 syndromes (33). Val804 mutations were threefold frequent in Italian and French when compared to German families. These data are concordant with the results in our series since Val804Met mutations are also prevalent in Turkey (25.4%). Sánchez et al (34) reported that the most frequent RET mutation in MEN2A Spanish families was C634Y, occurring in 73% (22/30) of cases and this finding was attributed to founder effect. A recent German study also demonstrated that RET mutations were distributed fairly similarly among German, French, and Italian families. The mutations in codon 634 were the most prevalent (36%), and codon 790 mutations were also frequent in Germany compared to Italy and France (13% vs. 4% and 4%) (35). In our series, codon 790 mutations were very rare, accounting for 1.4% of all mutations. Other studies from China, India, and Korea also demonstrated that Codon 634 mutations accounted for 60-80 % of hereditary cases. Interestingly, Val 804 mutation was not reported in China (36) and Korea (37) and had a lower frequency in a small Indian study (38) compared to current and other European studies (7% vs. 25.4%) (33,35).

Another important finding in accordance with previous findings of our group and of the others is that genetic testing revealed eight mutation carriers (hereditary cases) (5.2%) among 154 clinically diagnosed as sporadic MTCs (13,14). This data underlines the necessity of genetic screening for all MTC patients (15,16). On the other hand, frequency of mutation carriers among apparently sporadic cases was lower than in the previous TURKMEN study (10.7%), presumably due to increased awareness and genetic testing among physicians in our country. Current guidelines recommend genetic testing for all MTC cases and if positive, clinical screening for pheochromocytoma and primary hyperparathyroidism (16,21). In the rare situation when the clinical criteria for hereditary syndromes are present but RET mutation analyses are negative, clinical screening of siblings is also recommended. As a cost-effective approach, ATA and ETA recommend to test MEN2-specific exons (i.e. exons 10,11) principally and if found negative, continue testing for exons 13, 14, and 15 subsequently (16,21).

The mean number of surgical procedures were higher in our sporadic cases compared to familial cases, but no statistically significant difference was observed. These findings may reflect delayed timing of diagnosis and small number of PTx for hereditary cases.

This study shows that the Turkish people has a similar RET proto-oncogene mutation distribution when compared to other Mediterranean countries such as Italy and France. Despite complementary *RET* gene testing made possible by the courtesy of SEMT, the number of the PTx in Turkey is limited and the surgical intervention is performed at a relatively late stage in the lifespan of the gene carriers. This is mainly due to patient and family incompliance and incomplete family counselling. Healthcare professionals seem to be unsuccessful to overcome these problems for the moment. Physicians and health authorities should be aware of this situation. Legal measures could be considered for the families who refuse healthcare for their children.

Acknowledgements

The authors would like to thank the SEMT for sponsoring the RET genetic screening between the years 2008 and 2012. Special thanks to Professor Fatma Ajlan Tükün, Professor Yahya Laleli, and Düzen Laboratory Group workers for their careful RET mutation analyses and interpretation of the results.

Funding

RET genetic screening is sponsored by SEMT. This research did not receive any grant from any funding agency.

Ethics

Ethics Committee Approval: It was taken (Ethical Committee of Ankara University Faculty of Medicine, September 5, 2012), Informed Consent: It was taken.

Peer-review: External peer-reviewed.

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Financial Disclosure: RET genetic screening is sponsored by SEMT. This research did not receive any grant from any funding agency.

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J Clin Res Pediatr Endocrinol 2016;8(1):21-25
DOI: 10.4274/jcrpe.2456

Original Article

Screening of HHEX Mutations in Chinese Children with Thyroid Dysgenesis

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ABSTRACT

Objective: Congenital hypothyroidism (CH) is a frequent neonatal endocrine disease with an incidence of about 1:2500 worldwide. Although thyroid dysgenesis (TD) is the most frequent cause of CH cases, its pathogenesis remains unclear. The aim of this study was to screen the hematopoietically-expressedhomeobox gene (*HHEX*) mutations in Chinese children with TD.

Methods: Genomic deoxyribonucleic acid was extracted from peripheral blood leukocytes in 234 TD patients from Shandong Province. Mutations in all exons and nearby introns of *HHEX* were analyzed by direct sequencing after polymerase chain reaction amplification.

Results: Sequencing analysis of *HHEX* indicated that no causative mutations were present in the coding regionof the TD patients. However, a genetic variant (IVS2+ 127 G/T, 10.26%) was observed in the intron 2 in *HHEX*.

Conclusion: Our results indicate that the frequency of *HHEX* mutation is very low and may not be the main causative factor in Chinese TD patients. However, these results need to be replicated using larger datasets collected from different populations.

Keywords: Congenital hypothyroidism, thyroid dysgenesis, *HHEX*, mutation

Conflict of interest: None declared
Received: 29.09.2015
Accepted: 30.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

HHEX knock-out mouse strongly suggested that *HHEX* has no role in thyroid specification but is required to maintain Tif-1, Pax8, and Tif-2 expression in the developing thyroid.

WHAT THIS STUDY ADDS?

The frequency of *HHEX* mutation is very low and may not be the main causative factor in Chinese thyroid dysgenesis patients.

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Introduction

Congenital hypothyroidism (CH) is the most frequent endocrine metabolic disease in infancy and affects about 1/2500 newborns (1). This disease, if left untreated, can seriously affect the child's physical and mental growth. CH was, until the introduction of the newborn screening program, one of the most important causes of mental retardation. With the exception of rare cases of central hypothyroidism, CH is characterized by elevated serum thyroid stimulating hormone (TSH) levels resulting from reduced thyroid hormone levels. In nearly 15% of cases, CH is caused by inborn errors of thyroid hormones biosynthesis. The term dyshormonogenesis is generally used for this condition, which is associated with goiter and shows classical Mendelian recessive inheritance. Dyshormonogenesis is often caused by mutations in the genes involved in the synthesis of thyroid hormone, such asiodotyrosinedeiodinase (*IYD*), dual oxidase 2 (*DUOXA2*), DUOX maturation factor 2 (*DUOXA2*), thyroglobulin (*TG*), thyroperoxidase (*TPO*), sodium/iodide symporter (*NIS*), and pendrin (*PDS*) (2). In the remaining 85%, casesare grouped under the term thyroid dysgenesis (TD) due to defects in thyroid gland development, which contains agenesis (35-40%), ectopy (30-45%), and hypoplasia (5%) (3). Some studies have been reported that some genes, such as paired box transcription factor 8 (*PAX8*), thyroid transcription factor 1 (*TTF1*), thyroid transcription factor 2 (*TTF2*), *NKX2-5*, and *TSHR*, play important roles during thyroid morphogenesis (4). Although mutations in these genes can lead to TD, its pathogenesis remains unclear.

Hematopoietically-expressed homeobox gene (*HHEX*), located on human chromosome 10q24, contains a 5.7 kb coding sequence divided into four exons (5,6) and encodes a homeodomain-containing transcription factor, first identified in multipotent hematopoietic cells. *HHEX* is expressed in the primordium of several organs derived from the foregut, including the thyroid bud (7). Studies of *HHEX* knock-out mouse strongly suggested that *HHEX* has no role in thyroid specification but is required to maintain *Tif-1, Pax8, and Tif-2* expression in the developing thyroid (4). In this present study, we hypothesized that the *HHEX* possibly contributed to the development of TD in humans and aimed to identify potential pathogenic *HHEX* mutations in 234 Chinese children with TD, thereby providing insights into its etiology.

Methods

A total of 234 TD patients (94 boys, 140 girls, sex ratio 1:1.5, age 1.7±0.8 years), who were examined to make sure that they did not have other congenital anomalies such as congenital heart disease, congenital deafness, congenital cleft lip, congenital megacolon, were recruited through the neonatal screening program conducted in Qingdao, Yantai, Weifang, Jinan and Liaocheng in Shandong Province, China, from 2008 to 2012. Within the context of this same program, all measurements at five different laboratories in the relevant cities were done using the same assay. Neonatal screening for CH using filter paper was conducted in all of the subjects at 72 hours after birth. The blood samples were collected from the heel and TSH level was measured by enzyme-linked immunosorbent assay (ELISA). Subjects with increased TSH (TSH ≥20 uIU/mL) levels during this neonatal screening were invited for further evaluation. In these subjects, serum TSH (normal range 0.27-4.2 uIU/mL) and free thyroxine (fT_4, normal range 12-22 pmol/L) were determined using electrochemiluminescence assay. The diagnosis of CH was based on a high serum TSH level and a low fT_4 level. The diagnosis of TD was based on thyroid scintiscan or thyroid ultrasound examinations. Mutationsin *PAX8, TTF1*, and *TTF2* in these patients were excludedin our previous studies (8). This present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. The blood samples from the children with TD were collected after written informed consent was obtained.

Deoxyribonucleic Acid Analysis

Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood leukocytes using the phenol-chloroform method. The four exons and nearby introns in *HHEX* were amplified. Three pairs of specific primers were designed by PRIMER 5, polymerase chain reaction (PCR) was performed in 25 uL, using 250 nMdNTPs, 100 ng of template DNA, 0.5 uM of each forward and reverse primer, and 1.25 U AmpliTaq Gold DNA polymerase, in 1× reaction buffer (10 mMTrisHCl, pH 8.3, 50 mMKCl, 2.5 mM $MgCl_2$). Samples were denatured at 94 °C for 5 minutes followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 seconds, at primer specific annealing temperature for 45 seconds, and primer extension at 72 °C for 45 seconds. After the last cycle, the samples were incubated for an additional 10 minutes at 4 °C to ensure that the final extension step was complete. The amplified products were analyzed in 1.5% agarose gel. In order to perform mutational analysis, amplified PCR products were purified and sequenced using the appropriate PCR primers and the DNA sequencing kit-BigDye Terminator Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) and run on an automated sequencer, ABI 3730XL (Applied Biosystems). The same region was sequenced in 168 blood samples from control individuals. All analyses were performed by statistical software package Statistical Package for the Social Sciences 19.0. Differences in the distribution of genotype and allele between case-control groups were analyzed by the chi-square method. The level of statistical significance was defined as a p-value <0.05.

Results

Using thyroid scintiscan or thyroid ultrasound examinations, TD cases were divided into three groups according to the location and size of the thyroid gland, as agenesis (83 cases, 35.5%), ectopy (85 cases, 36.3%), and hypoplasia with normal location (66 cases, 28.2%) (Table 1). In all TD cases, the four exons and nearby introns of *HHEX* were amplified by PCR. The agarose gel electrophoresis indicated that the amplified products were in accordance with the target fragment we wanted. After direct sequencing, the sequences were analyzed using the Chromas and Sequencher and Nucleotide BLAST software programs. Sequence analysis of *HHEX* did not show any non-synonymous variance in the coding regions; however, we found a variant (rs2275729) which results in nucleotide T to G substitution (IVS2+127 G/T, 10.26%) in the intron 2 in 24 TD cases (Figure 1). These cases included 10 ectopy cases, 12 agenesis cases, and 2 hypoplasia cases. The frequency of this variant in the controls was 6.55%. Moreover, the differences in the allelic and genotypic frequencies between patients and controls were not statistically significant (χ^2=1.692, df=2, p=0.193 by genotype; χ^2=1.615, df=1, p=0.204 by allele) (Table 2).

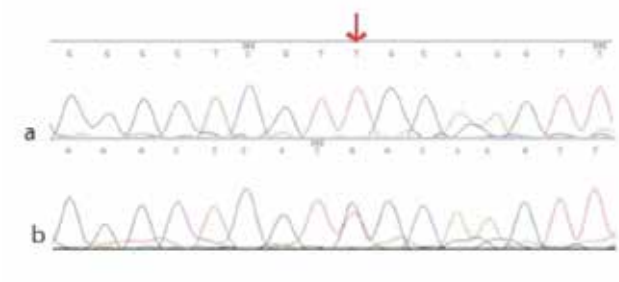

Figure 1. Partial sequences of *HHEX*. The variant is indicated by the arrow and it is 127 from splice site of intron 2.
a: the sequencing diagram of one patient with the homozygous T, b: the sequencing diagram of one patient with the heterozygous G and T

Table 1. Clinical andlaboratoryfindings of patients with thyroid dysgenesis

Diagnosis	Ectopia	Athyreosis	Hypoplasia
F/M*	60/25	45/38	35/31
TSH (uIU/mL)** (NR: 0.27-4.2)	76.2-500.0	36.1-440.0	13.0-100.0
fT_4 (pmol/L)** (NR: 12-22)	0.3-4.7	0.7-11.2	1.3-10.8
PAX8	No mutation	No mutation	No mutation
TTF1	No mutation	No mutation	No mutation
TTF2	No mutation	No mutation	No mutation
HHEX#	10	12	2

*F: female; M: male, **TSH: thyroid stimulating hormone, fT_4: free thyroxine, NR: normal reference values, Minimal and maximal levels, #HHEX: have a variation of the intron 2 of HHEX, PAX8: paired box transcription factor 8, TTF1: thyroid transcription factor 1, TTF2: thyroid transcription factor 2, HHEX: hematopoietically-expressedhomeobox gene

Discussion

The thyroid follicular cells (TFCs), the most numerous cells of the thyroid gland that form the thyroid follicles, are spherical structures serving as a storage site for thyroid hormones and are essential for thyroid morphogenesis (9). The absence of TFCs in orthotopic or ectopic location leads to athyreosis. Lack of formation of the thyroid budor alterations in any of the steps following the differentiation of the thyroid bud such as defective survival and/or proliferation of the precursors of the TFCs can also cause this condition. The developing thyroid is unable to migrate to its definitive location anterior to the trachea, resulting in an ectopic thyroid gland. Up to now, only mutations in the thyroid transcription factors, such as *PAX8, TTF1, NKX2-5, TTF2,* and *TSHR* were associated with TD.

HHEX is a member of the homeobox family of transcription factors which play important roles in regulating the tissue-specific gene expression that is required for tissue differentiation, as well as in determining temporal and spatial patterns of development (10). *HHEX* has been shown to have a significant role in vertebrate thyroid development. In *HHEX* knock-outmouse embryos at E9, the thyroid primordium is

Table 2. The genotypic and allelic frequencies of the HHEX IVS2+127 G/T (rs2275729) in the study and control groups

	Cases	Controls	χ^2	p-value	OR	95% CI
Genotypes						
TT	210 (89.74%)	157 (93.45%)	1.692	0.193	0.613	0.292-1.289
TG	24 (10.26%)	11 (6.55%)				
GG	0 (0%)	0 (0%)				
Alleles						
T	444 (94.87%)	325 (96.73%)	1.615	0.204	0.626	0.302-1.297
G	24 (5.13%)	11 (3.27%)				

OR: odds ratio, CI: confidence interval

present and this does not affect the expression of *Ttf1, Pax8,* and *Ttf2*. At E10, in the absence of *HHEX*, thyroid budding is severely impaired and the thyroid anlage is represented only by a few non-migrating cells which do not express *Ttf1, Pax8*, or *Ttf2* messenger ribonucleic acid (mRNA). At later stages, the anlage disappears (4). These data strongly showed that *HHEX* is required for the development of the thyroid in the embryo. In addition, *HHEX* plays an important roleinzebrafish thyroid development. Elsalini et al (11) injected *HHEX* morpholino antisense RNA into one-cell-stage embryos and found that 82% of *HHEX* morphants with injection of 0.17 mM *HHEX* develop heart edema and have no thyroid follicles. With injection of lower concentrations of *HHEX* morpholino, a higher percentage of larvae were reported to show some follicle differentiation. Coinjection of *HHEX* mRNA restores follicle development in part of the morphants (11).

Further researchers suggested that the survival and growth of thyroid progenitors depend on a thyroid-specific signature of transcription factors. The combined expression of *HHEX*, *Ttf2, Pax8*, and *Ttf1* is important for the development of thyroid (3), which is highlighted by their impact on regulation of thyroid-specific genes and functional differentiation of follicular cells. In thyroid progenitor cells, *HHEX, Pax8, Ttf2,* and *Ttf1* form a regulatory network which probably occurs both at the level of promoter binding and by physical interaction with the other transcription factors (12). Previous studies demonstrated that the *HHEX* promoter is the binding and transactivating site of *Ttf1* (13) and Pax8 (14). *Pax8* interacts physically with *Ttf1* (15), and the transcription of *Ttf2* is activated by *Pax8* (16). Besides, *HHEX* (13) and Ttf1 (16) regulate their own promoters automatically.

In this study, we screened potential causative mutations in *HHEX* in Chinese children with TD. However, we did not find any causative mutations in the *HHEX* coding regions. Al Tajialso found no *HHEX* mutations which were analyzedin a female hypothyroid patient (17). Interestingly, we found a variant which results in nucleotide T to G substitution (IVS2+ 127 G/T, 10.26%) in the intron 2 in TD cases. Although the intronic regions in which the variant located do not translate to amino acid sequences, they could potentially affect the protein product by changing the splicing site in RNA and therefore the final mRNA product. In other words, this part of human genome possesses significant regulatory elements that could affect gene expression. Due to its relatively distant location (IVS2+ 127 G/T) from splice site of intron 2, this variant may not effect on the splicing of the *HHEX* RNA. However, functional assessment of this variant will be needed in future studies. Our results also indicate that whole genome sequencing is a valuable tool for understanding variations in the human genome in our population.

To the best of our knowledge, this is the first attempt to examine the mutation in *HHEX* in a large sample of TD cases. The negative results of direct sequencing may have been caused by the limitations in our study. First, the causative mutation of *HHEX* for TD cases may not exist in the coding regions. Second, this is a highly selected population and sample size is relatively small in this study. Therefore, further studiesare still needed to determine the important role of *HHEX* during thyroid development, which may give an insight to the etiology of thyroid defects.

Acknowledgements

We thank all subjects for their collaborative participation in this study. We also wish to express our appreciation for the support from the National Natural Science Foundation of China (81170812 and 81470044) and from the Shandong province population and family planning committee of science and technology programme (2013-5), without which the finances of our study could not be met.

Ethics

Ethic Committee Aproval: This present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University in 2012, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Shiguo Liu, Design: Yinlin Ge, Data Collection and/or Processing: Huichao Li, Analysis and/or Interpretation: Deguo Lu, Literature Research: Guohua Zheng, Writing: Jian Chai.

Financial Disclosure: The authors declared that this study has received National Natural Science Foundation of China (81170812 and 81470044) and from the Shandong province population and family planning committee of science and technology programme (2013-5) support.

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J Clin Res Pediatr Endocrinol 2016;8(1):26-31
DOI: 10.4274/jcrpe.2152

Original Article

Pediatric Reference Intervals for Free Thyroxine and Free Triiodothyronine by Equilibrium Dialysis-Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Objective: Thyroid hormone concentrations fluctuate during growth and development. To accurately diagnose thyroid disease in pediatric patients, reference intervals (RIs) should be established with appropriate age groups from an adequate number of healthy subjects using the most exact methods possible. Obtaining statistically useful numbers of healthy patients is particularly challenging for pediatric populations. The objective of this study was to determine non-parametric RIs for free thyroxine (fT_4) and free triiodothyronine (fT_3) using equilibrium dialysis-high performance liquid chromatography-tandem mass spectrometry with over 2200 healthy children 6 months-17 years of age.

Methods: Subjects were negative for both thyroglobulin and thyroid peroxidase autoantibodies and had normal thyrotropin concentrations. The study included 2213 children (1129 boys and 1084 girls), with at least 120 subjects (average of 125) from each year of life, except for the 6 month to 1 year age group (n=96).

Results: Non-parametric RIs (95th percentile) for fT_4 were: 18.0-34.7 pmol/L (boys and girls, 6 months-6 years) and 14.2-25.7 pmol/L (boys and girls, 7-17 years). RIs for fT_3 were: 5.8-13.1 pmol/L (girls, 6 months-6 years); 5.7-11.8 pmol/L (boys, 6 months-6 years); 5.7-10.0 pmol/L (boys and girls, 7-12 years); 4.5-8.6 pmol/L (girls, 13-17 years); and 5.2-9.4 pmol/L (boys, 13-17 years).

Conclusion: Numerous significant differences were observed between pediatric age groups and previously established adult ranges. This emphasizes the need for well-characterized RIs for thyroid hormones in the pediatric population.

Keywords: Pediatric, reference interval, free thyroxine, free triiodothyronine, mass spectrometry

Conflict of interest: None declared
Received: 11.06.2015
Accepted: 30.09.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Early detection of thyroid abnormalities is critical in young children. However, the challenges of determining pediatric reference intervals (RIs) are well known. Obtaining sufficient sample numbers in healthy children is often exceedingly difficult and underscores the importance of reporting these types of studies. Free thyroxine (fT_4) and free triiodothyronine (fT_3) RIs have additional complexities, including accurate measurement of free hormones and the necessity of screening for subclinical thyroid disease in the reference population.

WHAT THIS STUDY ADDS?

This work provides nonparametric RIs for fT_4 and fT_3 for children from 6 months through 17 years of age. Uniquely, our study utilized a significant number of samples (n=2213) from both healthy boys (n=1131) and girls (n=1082). All individuals were screened for thyroid stimulating hormone and thyroid autoantibodies prior to inclusion in our study and had no known medical conditions or medication use. This study was performed using an in-house equilibrium dialysis-high performance liquid chromatography-tandem mass spectrometry method. We point out the importance of determining method-specific intervals, which has been recommended, particularly for fT_4. This work will be useful for any laboratory or clinician serving a pediatric population.

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Presented In: Academy of Clinical Laboratory Physicians & Scientists (ACLPS). St. Louis, MO. June 2011. Am J Clin Path 2011;136:458-476.

Introduction

Identifying thyroid dysfunction is critical in young children. An imbalance in thyroid hormone concentrations early in life can have long-term ramifications, such as developmental delays, mental and/or growth retardation. Numerous conditions affecting the thyroid gland in children and adolescents may result in thyroid dysfunction. Primary congenital hypothyroidism is one of the more common thyroid abnormalities that can occur in children, with a prevalence of 1 in 3000 infants (1). It results when the thyroid gland is unable to produce sufficient amounts of thyroxine (T_4) or triiodothyronine (T_3). Graves' disease is the most common cause of hyperthyroidism in children, and although rare, can be fatal if not properly diagnosed and treated (2).

Symptoms of thyroid disease are not always obvious in healthy populations, therefore, laboratory measurements are of increased value. Thyroid stimulating hormone (TSH) is the initial screening test for assessment of thyroid dysfunction. The dynamic equilibrium that exists between free and protein-bound forms of T_3 and T_4 hormones makes the assessment of their *in vivo* concentrations complex. Free thyroxine (fT_4) and free triiodothyronine (fT_3) are analyzed when additional diagnostic information is needed in patients with suspected thyroid disease. Identifying the presence of thyroglobulin autoantibodies (TgAb) or thyroid peroxidase autoantibodies (TPOAb) is also useful in detecting autoimmune disorders that affect thyroid function (3).

Establishing reference intervals (RI) for thyroid function tests in healthy pediatric subjects is essential to effectively diagnose disease in this patient population. The National Academy of Clinical Biochemistry recommends obtaining method-specific RIs for thyroid hormones, particularly for fT_4 (4). Mass spectrometry is considered a more specific method overall, particularly in challenging populations and situations requiring accurate measurement of small concentrations. Separation techniques such as equilibrium dialysis are useful for obtaining more accurate and consistent results in cases where alterations in thyroid hormone binding protein concentrations are suspected (4).

Getting access to samples from truly healthy individuals, particularly children, is often complicated. Challenges include obtaining consent, defining the healthy status in various stages of childhood development, restrictions imposed by institutional review boards, and small sample volumes due to maximum blood draw limits. These complexities often lead to small data sets that lack statistical power. The samples included in this study were from a well-characterized repository (CHILDx® program) that included sufficient information to confirm the health status of over 6000 children in total.

The purpose of this study was to improve the clinical utility of diagnostic tools available to physicians working with pediatric patients suspected of thyroid disease. Non-parametric RIs were established for fT_4 and fT_3 by equilibrium dialysis-high performance liquid chromatography-tandem mass spectrometry (ED-LC-MS/MS) using a well-characterized, healthy population of children ages 6 months-17 years (n=2213). Importantly, the population used for these determinations had normal TSH concentrations, lacked thyroid autoantibodies, and were of sufficient sample size to apply non-parametric statistics.

Methods

Sample Acquisition

Samples used for this study were part of the CHILDx® repository of samples from healthy children. Two different approaches were used for sample acquisition. Children 6 months-6 years of age were assessed for enrollment by a physician assistant at Primary Children's Medical Center (Salt Lake City, Utah) prior to elective, non-invasive, outpatient surgery, such as dental surgery, umbilical hernia repairs, nevus removals, orchiopexies, or orthopedic procedures. No unhealthy, medicated, or inpatient children were enrolled. Blood was drawn while patients were under gas anesthesia but prior to general anesthesia administered by IV. For children 7-17 years of age, subjects volunteered to participate and were recruited by institutional review board (IRB)-approved flyers, advertisements in magazines, and by word of mouth. An evaluation was conducted that included a full physical examination followed by blood and urine collection. Subjects were excluded for known medical conditions, medication use (other than seasonal allergy medication), or had a medical history that would consider them to be unhealthy. Eligible subjects from both age groups were enrolled after obtaining parental permission. All subject enrollment and testing protocols were approved by the University of Utah IRB.

Sample Processing and Testing

Blood was drawn into serum separator tubes and allowed to coagulate for 30 minutes prior to centrifugation. All samples were aliquoted and cryogenically frozen. In an effort to include only subjects with normal thyroid function, all samples were tested for TgAb and TPOAb on the ARCHITECT i2000SR (Abbott Diagnostics, Abbott Park, IL). Upper reference limits of 14.4 IU/mL for TgAb and 3.9 IU/mL for TPOAb were determined previously (5). Autoantibody negative samples were then tested for TSH (MODULAR ANALYTICS E170, Roche Diagnostics, Indianapolis, IN) to establish TSH RIs specific for our population. The central 95% non-parametric RI was established for TSH and samples outside of these ranges were excluded from fT_4 and fT_3 testing. Analysis by ED-LC-MS/MS for fT_4 and fT_3 was performed as previously described (6). Briefly, serum samples were dialyzed 1:1 against a simple protein-free buffer for 20 hours at 37 °C. Thyroid hormones in dialysates were purified by online solid-phase extraction, then

chromatographically separated and quantified in positive ion and multiple reaction monitoring modes. Total imprecision was reported to be <10%. Adult non-parametric fT_4 and fT_3 RIs were previously established as 16.5 (95% confidence interval (CI), <15.1 to 17.5) to 28.6 (26.1 to >36.9) pmol/L for fT_4 and 5.6 (95% CI, <4.8 to 6.4) to 10.4 (95% CI, 10.2 to >10.9) pmol/L for fT_3 (n=67 females, n=70 males) (6).

Statistical Analysis

Non-parametric RIs were determined using EP Evaluator software (Data Innovations, South Burlington, VT). Differences between ages or gender were first identified by determining whether the determined reference limits were contained within the 95% CIs of the adjacent group or other gender. If limits were contained within the adjacent CI, age groups and genders were combined. Statistical significance of the resulting partitions was confirmed by calculating p-values using GraphPad Prism (GraphPad Software, San Diego, CA). Pediatric reference limits were compared to adult CIs to determine whether ranges were different. Dixon's test was used to identify and remove outliers.

Results

A total of 2,540 subjects were initially evaluated for this study. Subjects with thyroid autoantibody concentrations above the established thresholds (n=172) and TSH concentrations outside the determined RIs (n=155) were excluded from RI analysis for fT_4 and fT_3. The central 95% non-parametric RIs for TSH, as determined using the E170 (n=2284), are provided (Table 1). Subjects that were negative for thyroid autoantibodies, within the central 95% of the established RI for TSH, and had sufficient volume (n=2213) were analyzed for fT_4 and fT_3 using ED-LC-MS/MS. More than 120 samples (average n=125) were tested for fT_4 and fT_3 from each year of life, with the exception of the 6 months-1 year age group (n=96).

The established pediatric RIs for fT_4 are summarized in Table 2 along with a dot plot of the data (Figure 1). FT_4 concentrations ranged from 7.7 to 87.7 pmol/L (median=20.6 pmol/L). No significant differences were observed when partitioning by gender for fT_4, therefore, genders were combined. Only differences between the 6 months-6 years and the 7-17 years of age groups were statistically significant and warranted partitioning (p-value <0.0001). Pediatric ranges were compared with RI previously established for adults (see Methods and reference 6) and were considered different if limits did not fall within the 95% CIs of the adult reference limits. For fT_4, there were differences between adult values and both age groups for the lower and upper limits. The lower limit for the 6 months-6 years of age group was higher than that for adults (designated "H" in Table 2), while that value for the 7-17 year old age group was lower (designated "L" in Table 2). The fT_4 upper limit was different only in the 7-17 year olds and found to be lower than that in adults.

The established pediatric RIs for fT_3 are provided (Table 2, Figure 1). FT_3 concentrations ranged from 2.6 to 15.1 pmol/L (median=7.9 pmol/L). Due to significant differences, genders were partitioned for the 6 months-6 years (p-value, 0.038) and 13-17 years age groups (p-value <0.0001). Statistically significant differences were also observed between age groups 6 months-6 years, 7-12 years, and 13-17 years (p-values <0.0001 to 0.002). Within the 6 months-6 years age group, upper limits were higher in girls than boys. In contrast, upper limits were higher for boys than girls in the oldest age group (13-17 year olds). Upper limits were higher for ages 6 months-6 years than for all other age groups. When compared to adult RI, the only difference observed for the fT_3 lower limit was for 13-17 year old girls being lower than that for adults. However, for the fT_3 upper limit, all of the pediatric age groups were different than the adult population; 6 months-6 year olds (both boys and girls) were higher and both of the older age groups were lower than the adult range.

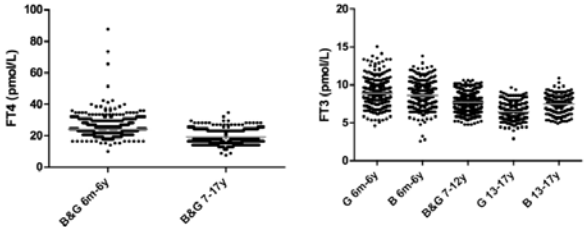

Figure 1. Scatter dot plot representation of the distribution of free thyroxine and free triiodothyronine in children ages 6 months to 17 years. Age groups and genders were combined when no significant differences were observed. The gray line represents the mean. FT_4: free thyroxine, fT_3: free triiodothyronine, B: boys, G: girls, m: months, y: years

Discussion

Thyroid dysfunction during childhood development may result in serious outcomes, including mental impairment and growth delays. Early diagnosis allows for rapid intervention that can almost entirely reverse symptoms. This study was performed to improve clinicians' ability to correctly diagnose thyroid disorders in children by providing population-specific RIs. Providing accurate RIs requires testing of well-characterized, healthy patients from a sample set of adequate size to provide statistical relevance. These criteria are particularly difficult to meet in pediatric populations; however, we were able to address both in this study.

TSH results are often used as the primary indicator for assessing thyroid dysfunction and may be followed by fT_4 and fT_3 testing when TSH results are close to reference limits or if further evidence of thyroid disease is required. Even though our main focus was to establish RIs for fT_4 and fT_3, those results could be skewed by including children with unapparent thyroid disorders. Because symptoms of thyroid disease are not always recognized or diagnosed, we screened samples and

Table 1. Pediatric reference intervals for thyroid stimulating hormone using the Roche E170

Gender	Age range	n	Lower limit (mIU/L)	95% CI (lower limit)	Upper limit (mIU/L)	95% CI (upper limit)
G	6 m-2 y	159	0.85	0.69-1.10	5.78	5.48-6.68
B	6 m-2 y	188	1.07	0.87-1.30	7.57	6.08-9.29
G	3-4 y	122	0.80	0.56-1.00	6.90	5.25-7.56
B	3-4 y	125	1.10	0.99-1.34	6.56	5.32-7.68
G	5-6 y	121	0.85	0.69-1.08	5.83	5.11-7.83
B	5-6 y	126	1.00	0.87-1.12	6.51	5.58-8.35
B&G	7 y	137	1.12	0.97-1.32	5.66	4.82-6.04
G	8-9 y	131	0.94	0.87-1.15	5.40	4.53-5.89
B	8-9 y	133	1.14	0.97-1.25	6.41	4.75-7.34
G	10-11 y	135	0.94	0.80-1.04	4.71	3.78-6.12
B	10-11 y	131	0.78	0.65-0.94	6.11	4.20-6.76
G	12-13 y	129	0.88	0.51-0.89	4.71	4.23-5.71
B	12-13 y	130	0.77	0.71-0.94	4.32	4.14-4.90
G	14-15 y	130	0.47	0.31-0.74	4.56	3.63-6.46
B	14-15 y	127	0.65	0.59-0.92	4.16	3.49-4.63
G	16-17 y	127	0.56	0.10-0.68	4.62	3.19-5.11
B	16-17 y	133	0.63	0.37-0.84	4.58	3.67-5.28

CI: confidence interval, B: boys, G: girls, m: months, y: years

Table 2. Pediatric reference intervals for free thyroxine and free triiodothyronine using equilibrium dialysis-high performance liquid chromatography-tandem mass spectrometry

Analyte	Gender	Age range	n	Lower limit (pmol/L)	95% CI (lower limit)	Upper limit (pmol/L)	95% CI (upper limit)
fT_4	B&G	6 m-6 y	840	18.0^H	16.7-18.0	34.7	33.0-37.3
fT_4	B&G	7-17 y	1373	14.2^L	14.2-14.2	25.7^L	25.7-27.0
fT_3	G	6 m-6 y	401	5.8	5.4-6.3	13.1^H	12.1-13.4
fT_3	B	6 m-6 y	438	5.7	5.4-6.3	11.8^H	11.5-12.1
fT_3	B&G	7-12 y	759	5.7	5.4-5.8	10.0^L	9.8-10.1
fT_3	G	13-17 y	305	4.5^L	4.3-4.8	8.6^L	8.6-9.1
fT_3	B	13-17 y	310	5.2	5.1-5.5	9.4^L	9.1-9.8

CI: confidence interval, fT_4: free thyroxine, fT_3: free triiodothyronine, ^{L,H}: lower (L) or higher (H) than adult reference interval (reference 6), B: boys, G: girls, m: months, y: years

only included those that were thyroid autoantibody negative and were within the determined TSH RI.

Thyroid hormone concentrations are highest immediately following birth (7). TSH concentrations rise in response to the temperature shock of leaving the in utero environment, which in turn increases the concentrations of T_4 and T_3. Higher TSH concentrations are also expected in children due to progressive maturation and modulation of the hypothalamic-pituitary-thyroid axis during development (8). Expectedly, the TSH RIs that were established for children in this study were higher than the recommended range for adults (0.3-3.0 mIU/L) (4) and gradually decreased with age.

Initial partitioning of gender and age groups showed no significant differences for fT_4; therefore, genders and most age groups were combined. In contrast, the fT_3 RIs showed significant differences between age and gender and more partitions were required. Differences were particularly notable in girls where the difference between upper limits of girls' ages 6 months-6 years and girls' ages 13-17 years was 34%. There are conflicting data regarding gender differences for fT_3 in pediatric populations. Similar to our findings, the decline of fT_3 in girls during puberty has been observed previously, whereas concentrations in boys remained seemingly constant (9,10,11). Kapelari et al (12) also established gender-specific

RIs for fT_3 and did not report significant gender differences for fT_4 using the ADVIA Centaur. However, Hübner et al (13) observed gender differences for fT_3 only within the 11-14 age group, which is in contrast to ours and other studies where no statistically significant differences were observed between genders for the 13-17 year age group. Moreover, differences between boys and girls for the upper limit of fT_3 were not reported previously by Soldin et al (14) using similar methodology to our study. The differences described here may be attributed to known differences in thyroid hormone concentrations among populations and/or methods (15,16). Of note, we have previously observed ethnic differences in thyroid hormones in pregnant individuals (17,18), and ethnicity has not been addressed in these pediatric populations. Furthermore, either lower limits, upper limits, or both, from children for fT_4 and fT_3 were significantly different from adult RIs (6), for every age group. The above comparisons and the numerous significant differences observed further emphasize the necessity of establishing reference ranges specific to pediatric populations.

Soldin et al (14) performed testing on pediatric subjects by LC-MS/MS using an ultrafiltration method rather than equilibrium dialysis for isolation of free hormones. For fT_4, our RIs using ED were comparable to those determined by Soldin et al (14) using ultrafiltration performed at 37 °C. Our lower reference limits for fT_3 were higher than the limits determined by ultrafiltration performed at both 37 °C and 25 °C. The upper reference limits showed some similarities for fT_3 with the exception of our younger age groups being higher.

In comparison to a candidate international reference method, our ED-LC-MS/MS method demonstrated a positive bias for both fT_4 and fT_3 (19). Until standardization efforts of free thyroid hormone assays/methods are established, caution should be used when interpreting RIs. Laboratories need to verify RIs specific to the method and population they are using and evaluating, particularly for thyroid function testing.

A limitation of these RIs is that all samples were taken from patients living in the region surrounding Salt Lake City, UT. This resulted in a less diverse population (96% Caucasian). Due to IRB limitations, children under 6 months of age were excluded. Although thyroid testing is part of most newborn screening programs, additional studies would be needed to determine RIs for these analytes using this ED-LC-MS/MS method in preterm and newborn populations.

Detecting thyroid dysfunction early in development is critical to reversing long-term symptoms. This study provides useful RIs established from over 2200 healthy children using equilibrium dialysis and mass spectrometry, the preferred methods of analysis for fT_4 and fT_3 hormones. Having RIs for this specific patient population improves the ability of physicians to properly diagnose and treat children suspected of these often reversible thyroid abnormalities.

Acknowledgements

The authors wish to acknowledge the late William L. Roberts, MD, PhD for the generation of the healthy child repository and the contributions to development of the ED-LC-MS/MS assay. The authors also gratefully acknowledge John Simmons, P.A. and Ashley Bunker for patient enrollment and specimen collection as well as Primary Children's Medical Center for allowing us to enroll children at their facility. Support for this study was provided by the ARUP Institute for Clinical and Experimental Pathology.

Ethics

Ethics Committee Approval: University of Utah Institutional Review Board; (protocols: 9200&15079), Informed Consent: Yes, by subject or guardian.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: William Roberts (late), Design: William Roberts (late), Sonia L. La'ulu, Data Collection or Processing: Sonia L. La'ulu, Kyle J. Rasmussen, Analysis or Interpretation: Sonia L. La'ulu, Kyle J. Rasmussen, Joely A. Straseski, William Roberts (late), Literature Search: Sonia L. La'ulu, Kyle J. Rasmussen, Joely A. Straseski, Writing: Sonia L. La'ulu, Kyle J. Rasmussen, Joely A. Straseski.

Financial Disclosure: ARUP Institute for Clinical and Experimental Pathology.

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J Clin Res Pediatr Endocrinol 2016;8(1):32-39
DOI: 10.4274/jcrpe.2313

Original Article

Relationship between Neck Circumference and Non-Alcoholic Fatty Liver Disease in Childhood Obesity

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ABSTRACT

Objective: The aim of this study was to establish the association between anthropometric parameters and non-alcoholic fatty liver disease (NAFLD) and to determine the most reliable measurement as a parameter in predicting NAFLD.

Methods: Two-hundred fifty-three obese children of ages 10 to 18 years were enrolled in this study. Anthropometric data and metabolic parameters such as fasting blood glucose, insulin and lipid levels, were measured. Liver function tests were assessed. NAFLD was determined by ultrasound.

Results: Most metabolic parameters and anthropometric indices were significantly higher in children with NAFLD. A univariate logistic regression analysis was performed, taking NAFLD status as the dependent variable and anthropometric parameters as the independent variables. NAFLD was affected significantly by the anthropometric values. The multiple logistic regression analysis showed that neck circumference (NC) was the only parameter which determined the risk in both genders. Each 1 cm increase in the NC increased the risk of NAFLD 1.544-fold (p<0.001, 95% confidence interval (CI): 1.357-2.214) in the boys and 1.733-fold (p=0.001, 95% CI: 1.185-2.012) in the girls. Receiver operating characteristic analysis was performed to compare the reliability of anthropometric measurements. NC was observed to be a better indicator.

Conclusion: Measurement of the NC was shown to be associated with NAFLD in children. We suggest the use of NC as a novel, simple, practical, and reliable anthropometric index in predicting children at risk for NAFLD.

Keywords: Non-alcoholic fatty liver disease, obesity, metabolic values, anthropometric measurements

Conflict of interest: None declared
Received: 07.08.2015
Accepted: 12.11.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

In obesity, central body fat is strongly linked to risk of non-alcoholic fatty liver disease (NAFLD) and metabolic complications rather than total body fat. Anthropometric measurements such as body mass index, waist circumference, mid-upper arm circumference providing information about body fat and fat distribution can be used to predict the risk of NAFLD in obese children.

WHAT THIS STUDY ADDS?

Besides other anthropometric measurements, neck circumference was significantly related to upper body fat and NAFLD. Neck circumference may be used as an additional useful screening being an inexpensive, practical and reliable anthropometric measure to assess NAFLD in obese children.

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Introduction

One of the complications of obesity is non-alcoholic fatty liver disease (NAFLD). As in adults, NAFLD has become the most common cause of chronic liver disease in childhood (1,2). Additionally, NAFLD is closely related with insulin resistance, type 2 diabetes mellitus, dyslipidemia, hypertension, metabolic syndrome, and severe cardiovascular complications (3). In obesity, central body fat, rather than total body fat, is strongly linked to risk of NAFLD and metabolic complications (4,5).

Various anthropometric parameters have been developed to determine total body fat and central body fat accumulation. Body mass index (BMI) is used as major index in the evaluation of obesity. Waist circumference (WC), mid-upper arm circumference (MUAC), and waist-height ratio (WHR) are recommended in determining central body fat (6,7,8,9). Recently, a few studies have been reported suggesting that upper body fat accumulation and visceral fat may contribute to the development of risk factors for metabolic disease (5). Neck circumference (NC) has been suggested as a useful tool to determine the upper body fat accumulation (10).

Based on this information, anthropometric measurements providing information about body fat and fat distribution can possibly be used to predict the risk of NAFLD in obese children at a young age. Thus, it would be possible to prevent fatty liver disease in its early stages.

The aims of this study were to determine the relationship between NAFLD and metabolic disorders and to show the reliability of anthropometric measurements including BMI, WC, MUAC, NC, and WHR in detecting cases with NAFLD. We also aimed to find the most reliable and practical measurement among these anthropometric criteria.

Methods

A total of 248 children (114 boys and 134 girls between the ages of 6 and 18 years) admitted to our endocrine outpatient clinic because of obesity were enrolled. All children who participated in the study had BMI levels above the 95th percentile according to our reference values (11). The present study was approved by the local ethics committee. Signed consent was obtained from all parents of the children participating in the study. Patients with diseases which may cause obesity such as hypothyroidism, Cushing's syndrome, those with diseases/deformity affecting anthropometric measurements, patients with hepatitis (viral, congenital) or a history of alcohol use, and children who were using any kind of medicine were excluded. None of the participants had a previous diagnosis of type 2 diabetes or NAFLD.

Chronological age was calculated as the decimal age by subtracting the observation date from the birth date. All anthropometric measurements were performed by the same endocrinologist. Weight, height, WC, NC, and MUAC were measured twice, and the averages were recorded for reference charts. Weights were measured with subjects in minimal (without shoes and with light clothing) underclothes, using a standard beam balance sensitive to 0.1 kg. Heights were determined to the nearest 1 mm using a portable Seca stadiometer.

Body mass index was calculated by dividing weight to the square of height (kg/m^2). WHR was calculated by waist circumference divided by height. WC and MUAC were measured as previously described in detail (12). NC was measured using a non-stretch plastic tape measure while the child's head was being held erect, with the eyes facing forward, and the neck in a horizontal plane at the level of the most prominent portion, the thyroid cartilage. All measurements were taken with the subjects standing upright, with the face directed forward, and shoulders relaxed (8). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice in a sitting position after 20 min of rest and the average measurement was recorded.

The Tanner staging was used in the evaluation of pubertal development. However, since the number of our pubertal subjects was limited, subjects at pubertal stages 3 and 4 were combined in the analysis (13).

Blood samples were collected after a 10-hour overnight fast for determination of fasting blood glucose (FBG), insulin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride (TG) levels as metabolic function tests and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) as liver function tests. Biochemical parameters were determined by using enzymatic kits from Roche Diagnostics with a Cobas Integra 800 autoanalyzer. Insulin was measured by the electrochemiluminescence immunoassay method using Roche kits (Roche Diagnostics, Mannheim, Germany).

Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the equation: HOMA-IR=Fasting insulin (μU/mL)xfasting glucose (mg/dL)/405 (14).

The ultrasonographic (USG) examinations of all the children were performed using a 3.5 MHz convex transducer (Xario TOSHIBA). All children were evaluated in supine position by the same radiologist. The echogenicity of the liver parenchyma was compared with the right kidney parenchymal echogenicity. USG evidence of NAFLD was based on the bright hepatic echo pattern, increased echo attenuation, and loss of intrahepatic architecture (15).

Statistical Analysis

The Student's t-test was used to compare the findings in subjects with or without NAFLD. All statistical analyses were adjusted for pubertal stage and chronological age.

The relationship between anthropometric parameters (BMI, WC, NC, MUAC, and WHR) and metabolic parameters (FBG, insulin, TG, and HDL-cholesterol levels, HOMA-IR, and liver

function tests) were evaluated by parietal Spearman correlation test adjusted for age and pubertal stage. Univariate and multivariate logistic regression analysis was performed in which NAFLD was dependent and anthropometric parameters were independent variables in each gender. The univariate and multivariate models were also adjusted for age and pubertal stages. Independent variables without significant effect on NAFLD were eliminated by utilizing the backward stepwise elimination (p>0.1).

In order to test the reliability of anthropometric data to diagnosed NAFLD, receiver operating curves (ROC) analysis was made for each pubertal stage.

Results

The frequency of a fatty liver in USG examinations was 35.5%. The subjects with and without fatty liver in the two sexes were evaluated separately. In the boys, significant differences in BMI, WC, NC, MUAC and WHR measurements, DBP, insulin, liver function tests, and HOMA-IR were found between subjects with and without NAFLD. HDL levels were lower in patients with NAFLD. In girls, all anthropometric parameters and biochemical values except GGT, total cholesterol and TG were higher and HDL levels were lower in patients with NAFLD as compared to those without NAFLD (Table 1).

Adjusting for age and pubertal stage, the correlation analysis between the anthropometric measurements and metabolic risk factors/liver function tests were performed by gender. According to the results of this analysis, of all anthropometric measurements, positive correlations were detected only between NC and liver function tests (ALT, AST, GTT) in boys. This relationship was not found in the girls. The results of correlation analysis between anthropometric measurements and metabolic parameters are given in Table 2.

Regression analysis was performed to determine the relationship between NAFLD and anthropometric measurements. A univariate logistic regression analysis was performed taking NAFLD status as the dependent variable and BMI, WC, NC, and MUAC as the independent variables adjusted for age and pubertal stages (Table 3). In both boys and girls, NAFLD status was affected significantly by the anthropometric values. After adjusting for age and pubertal stages, the multiple logistic regression analysis and the backward elimination method showed that only NC determined the risk in both genders. Each 1 unit increase in the NC increased the risk of NAFLD 1.551-fold and 1.846-fold (p<0.001, B: 0.613, 95% confidence interval (CI): 1.385-2.462) (p<0.001, B: 0.439, 95% CI: 1.284-1.875) in boys and girls, respectively.

Table 1. Comparison of subjects with and without non-alcoholic fatty liver disease in terms of metabolic and anthropometric parameters in boys and girls

	Boys					Girls				
	Non-NAFLD		NAFLD		p	Non-NAFLD		NAFLD		p
	Mean-SD	Min-Max	Mean-SD	Min-Max		Mean-SD	Min-Max	Mean-SD	Min-Max	
Age (years)	10.9±2.5	6.1-17.0	11.8±2.4	6.0-18.7	0.059	12.3±2.6	6.0-18.2	13.3±2.3	8.1-17.9	0.028
BMI (kg/m^2)	27.6±3.1	23.0-36.0	30.1±3.5	24.0-39.0	<0.001	29.1±4.3	22.1-41.7	33.9±5.7	23.0-46.7	<0.001
WC (cm)	85.6±9.2	71.0-109.0	94.0±8.5	76.5-115.0	<0.001	87.7±8.9	69.5-112.0	98.4±11.3	76.0-124.0	<0.001
NC (cm)	33.8±2.8	29.0-42.0	36.9±2.8	32.2-45.0	<0.001	33.9±2.5	25.4-40.0	37.1±3.2	30.0-43.0	<0.001
MUAC (cm)	28.1±2.9	23.0-34.0	30.1±2.9	25.3-37.0	0.001	29.4±3.4	22.1-40.5	31.9±4.0	26.0-41.0	0.001
WHR	0.6±0.1	0.5-0.7	0.6±0.04	0.5-0.7	0.005	0.6±0.1	0.5-0.7	0.6±0.1	0.5-0.8	<0.001
SBP (mmHg)	109.5±14.3	80.0-150.0	113.4±13.7	80.0-150.0	0.150	108.9±14.3	80.0-150.0	119.5±13.9	90.0-150.0	<0.001
DBP (mmHg)	68.7±10.3	40.0-90.0	74.0±10.5	50.0-90.0	0.010	70.7±11.0	50.0-90.0	78.3±12.2	60.0-100.0	0.001
FBG (md/dL)	90.2±7.4	74.0-116.0	87.6±6.6	66.8-104.0	0.052	86.4±7.3	64.0-104.0	91.6±9.5	73.1-120.0	0.001
Insulin (mU/mL)	13.0±6.5	4.7-30.0	20.6±9.0	5.3-40.7	<0.001	21.3±12.0	5.5-79.3	33.0±19.0	10.2-201.0	<0.001
ALT (U/L)	22.9±9.1	3.0-53.0	48.3±30.6	13.0-151.0	<0.001	19.8±8.9	3.0-58.0	41.6±29.6	12.0-127.5	<0.001
AST (U/L)	26.3±5.9	15.0-46.0	34.1±14.8	15.0-90.0	<0.001	23.5±7.0	8.0-59.0	30.3±15.2	14.0-80.1	0.001
GGT(U/L)	18.3±5.9	8.2-40.0	25.4±8.4	11.0-46.0	<0.001	18.7±16.8	9.0-119.0	24.9±11.6	10.0-67.0	0.080
TG (mg/dL)	111.0±60.4	35.0-370.4	149.0±78.1	42.0-407.7	0.004	123.5±58.1	39.1-312.7	144.4±75.6	40.0-486.2	0.087
HDL (mg/dL)	49.3±13.1	23.3-93.8	44.0±9.4	26.5-74.0	0.019	44.2±9.9	23.8-93.3	40.4±9.9	22.1-64.8	0.041
HOMA-IR	2.9±1.6	0.9-8.6	4.5±2.0	1.1-9.2	<0.001	4.6±2.7	0.9-16.9	7.6±5.2	2.1-30.2	<0.001

NAFLD: non-alcoholic fatty liver disease, NC: neck circumference, BMI: body mass index, WC: waist circumference, MUAC: mid-upper arm circumference, WHR: waist-height ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, FBG: fasting blood glucose, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma-glutamyl transpeptidase, TG: triglyceride, HDL: high-density lipoprotein, HOMA-IR: homeostasis model assessment for insulin resistance, SD: standard deviation, Min-Max: minimum-maximum

Similarly, aiming to compare reliability of BMI, WC, NC, WHR, and MUAC measurements for determining NAFLD risk, ROC analysis was performed separately by pubertal stages. While it is possible to use all anthropometric measurements for the assessment of risk in all of pubertal stages, area under curve (AUC) for the NC was higher than the others, except for Tanner stage 3-4 (Table 4, Figure 1, 2). In this pubertal stage, AUC for the WC was found to be higher than the other criteria and similar to NC. Table 5 shown NC cut-off values for determining NAFLD according to pubertal stages.

Discussion

In the present study, the relationships between NAFLD and metabolic and anthropometric measurements were evaluated. Most of the metabolic parameters and measurements including

Table 2. Correlations between anthropometric and metabolic parameters after adjusting for age and pubertal stage

	BMI		WC		NC		MUAC	
	r	p	r	p	r	p	r	p
Boys								
SBP (mmHg)	0.367	0.013	0.337	0.024	0.123	0.421	0.216	0.099
DBP (mmHg)	0.202	0.182	0.250	0.097	0.238	0.116	0.327	0.521
FBG (mg/dL)	-0.295	0.049	-0.010	0.947	-0.018	0.907	-0.319	0.826
Insulin (mU/mL)	0.356	0.017	0.494	0.001	0.319	0.033	0.113	0.005
ALT (U/L)	0.136	0.372	0.263	0.080	0.303	0.043	0.045	0.042
AST (U/L)	0.076	0.626	0.199	0.191	0.318	0.033	0.116	0.129
GGT(U/L)	0.076	0.621	0.269	0.074	0.433	0.003	0.139	0.136
TG (mg/dL)	0.036	0.816	0.080	0.603	-0.069	0.652	0.118	0.371
HDL (mg/dL)	0.033	0.832	0.080	0.601	-0.013	0.934	-0.032	0.996
HOMA-IR	0.315	0.035	0.489	0.001	0.325	0.029	0.072	0.005
Girls								
SBP (mmHg)	0.319	0.013	0.322	0.012	0.338	0.008	0.148	0.061
DBP (mmHg)	0.338	0.008	0.370	0.004	0.455	<0.001	0.201	0.053
FBG (mg/dL)	0.324	0.011	0.386	0.004	0.316	0.014	0.129	0.052
Insulin (mU/mL)	0.382	0.003	0.454	<0.001	0.481	<0.001	0.145	0.016
ALT (U/L)	0.069	0.602	0.109	0.409	0.190	0.147	0.138	0.534
AST (U/L)	-0.033	0.800	-0.068	0.605	0.111	0.400	-0.022	0.936
GGT (U/L)	0.145	0.270	0.223	0.087	0.205	0.116	0.077	0.097
TG (mg/dL)	0.105	0.424	0.210	0.108	0.120	0.369	0.006	0.261
HDL (mg/dL)	-0.381	0.003	-0.408	0.001	-0.378	0.003	-0.258	0.043
HOMA-IR	0.421	0.001	0.500	<0.001	0.515	<0.001	0.163	0.006

NC: neck circumference, BMI: body mass index, WC: waist circumference, MUAC: mid-upper arm circumference, WHR: waist-height ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, FBG: fasting blood glucose, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma-glutamyl transpeptidase, TG: triglyceride, HDL: high-density lipoprotein, HOMA-IR: homeostasis model assessment for insulin resistance

Table 3. Univariate logistic regression analysis between having non-alcoholic fatty liver disease and anthropometric parameters in boys and girls

	Boys			Girls		
	OR	95% CI	p	OR	95% CI	p
BMI (kg/m^2)	1.253	1.075-1.461	0.004	1.216	1.106-1.337	<0.001
WC (cm)	1.115	1.035-1.201	0.004	1.118	1.060-1.180	<0.001
NC (cm)	1.826	1.350-2.470	<0.001	1.626	1.309-2.020	<0.001
MUAC (cm)	1.160	0.944-1.426	0.159	1.194	1.043-1.368	0.010

OR: odds ratio, CI: confidence interval, NC: neck circumference, WC: waist circumference, BMI: body mass index, MUAC: mid-upper arm circumference

Table 4. Comparison of anthropometric parameters by receiver operating curves analysis in defining non-alcoholic fatty liver disease according to gender and pubertal stage

	Boys n=114		Girls n=134		Tanner stage 1 n=61		Tanner stage 2 n=62		Tanner stage 3-4 n=44		Tanner stage 5 n=81	
	AUC	95% CI	AUC	95% CI	AUC	95% CI	AUC	95% CI	AUC	95% CI	AUC	95% CI
NC	0.822	0.729-0.894	0.791	0.707-0.860	0.915	0.754-0.984	0.854	0.669-0.957	0.749	0.571-0.881	0.814	0.704-0.896
BMI	0.684	0.580-0.777	0.744	0.655-0.819	0.634	0.439-0.801	0.631	0.429-0.804	0.667	0.485-0.818	0.797	0.685-0.883
WC	0.751	0.650-0.835	0.762	0.676-0.836	0.759	0.569-0.895	0.736	0.536-0.883	0.786	0.612-0.907	0.791	0.678-0.879
MUAC	0.678	0.573-0.771	0.682	0.591-0.765	0.578	0.385-0.755	0.685	0.483-0.845	0.569	0.388-0.737	0.738	0.620-0.835
WHR	0.683	0.578-0.775	0.705	0.615-0.785	0.605	0.411-0.777	0.641	0.439-0.812	0.693	0.512-0.839	0.766	0.651-0.859

AUC: area under the curve, SE: standard error, CI: confidence interval, NC: neck circumference, BMI: body mass index, WC: waist circumference, MUAC: mid-upper arm circumference, WHR: waist-height ratio

Table 5. Neck circumference cut-off values for determining non-alcoholic fatty liver disease according to pubertal stages

Tanner stage	Cut-off	Sensitivity	95% CI	Specificity	95% CI
1	33	75.00	42.8-94.2	83.33	68.6-93.0
2	34.9	85.19	66.3-95.7	88.00	68.8-97.3
3-4	35.2	75.00	35.0-96.1	71.43	51.3-86.7
5	36.5	75.00	53.3-90.2	78.72	64.3-89.3

CI: confidence interval

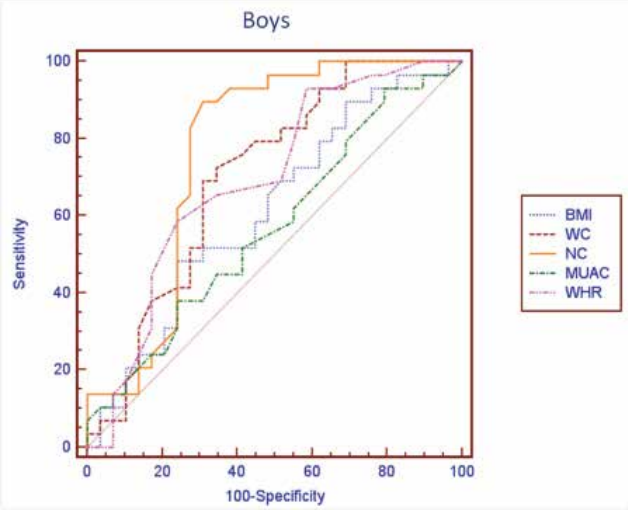

Figure 1. Receiver operating characteristic curves of anthropometric measurements in defining non-alcoholic fatty liver disease in boys. NC: neck circumference, BMI: body mass index, WC: waist circumference, MUAC: mid-upper arm circumference, WHR: waist-height ratio

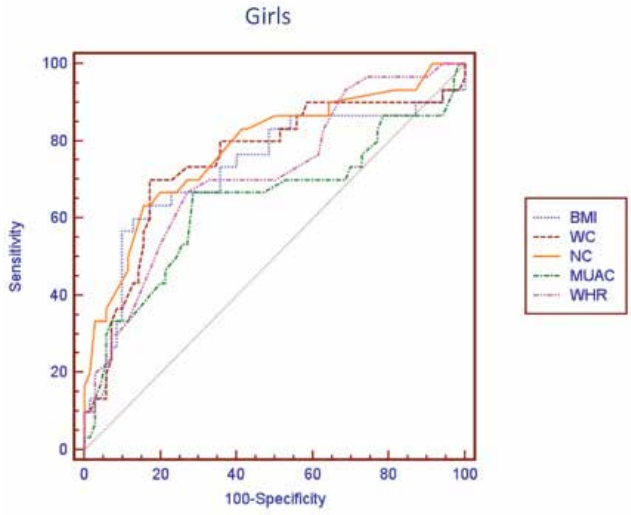

Figure 2. Receiver operating characteristic curves of anthropometric measurements in defining non-alcoholic fatty liver disease in girls. NC: neck circumference, BMI: body mass index, WC: waist circumference, MUAC: mid-upper arm circumference, WHR: waist-height ratio

BMI, WC, NC, MUAC, and WHR were found to be significantly higher in children with fatty liver. In further analysis, we found that NC is the most discriminative measurement that can predict the development of NAFLD.

The global epidemic of childhood obesity has become a serious public health problem and recent studies show that the prevalence of NAFLD in obese children increased (16,17,18,19,20). While the incidence of NAFLD in the general population is 2.6%, this rate increased to 53% in obese children (21). Additionally, a correlation between the degree of obesity and hepatic steatosis has been reported (22). The definition of

NAFLD includes a spectrum from simple fatty liver disease to steatohepatitis which is potentially fatal (23).

Even in children with steatohepatitis, NAFLD may still be asymptomatic and is often detected incidentally. Although confirmation of diagnosis can be established by imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), MRI spectroscopy, and USG and by increased liver enzyme levels, liver biopsy continues to be the gold standard in the diagnosis of NAFLD (24,25,26). However, liver biopsy is an invasive diagnostic method and it may cause serious complications such as peritoneal hemorrhage (27). Abdominal USG is a safe, non-invasive, and non-expensive diagnostic tool and is applied by most clinicians as the most practical and widely used technique (28,29). In this study, we used USG to identify hepatic steatosis.

Metabolic complications are much more common when body fat is accumulated in the upper body. BMI is an indicator of total body fat, whereas other measurement such as WC, NC, WHR, and MUAC are indicators of body fat accumulation in central and upper body (4,5,6,7,8,9). The relationships of NAFLD with measurements of BMI, WC, WHR, and MUAC have been reported in several publications (4,30,31,32,33,34).

Although both BMI and WC are predictors of NAFLD severity, indicators of central obesity such as WC and WHR are proposed as independent predictors for steatosis (32,33,34). In another study which aimed to determine the relationship between body fat distribution and steatosis, a positive correlation was found between trunk body fat and NAFLD and a negative correlation between thigh fat and liver enzymes (35). In a study on 2111 patients, Ishibashi et al (36) reported that WC shows a positive correlation with visceral adiposity in both genders and that WC may be used as an indicator of fatty liver in males. In a study conducted on Korean adults, it was shown that WHR is as useful as WC to determine NAFLD and as useful as dual x-ray absorptiometry and CT in diagnosis (37).

Compared to reports on adult subjects, there are relatively limited publications about the relationship between NAFLD and anthropometric measurements in childhood. In 69 children with non-alcoholic steatohepatitis, BMI was proposed as a predictor of hepatic fibrosis (38). Oliveira et al (39) showed that each 5 cm increase in WC or a one unit increase in BMI Z-score increases ALT 1.3-fold. Similarly, Lin et al (16) found that the odds ratio of diagnosing NAFLD with USG increased 1.391 times for each 5 cm increase of WC. In a study conducted by el-Karaksy et al (40), a relationship between BMI, subscapular thickness, hip circumference, and WHR, metabolic parameters, and hepatic steatosis is reported in 2-15 years old children. As in adult studies, a relationship between WC and NAFLD has been shown in childhood (30,41).

Neck circumference is accepted as an alternative measurement to detect fat accumulation in the upper body, a finding which is considered to be indicative of a significant metabolic risk factor for type 2 diabetes mellitus and hyperlipidemia in adults (42,43,44). On the other hand, studies about the significance of NC in childhood are relatively new. Recently, both age- and gender-specific NC reference and cut-off values were published in which cardio-metabolic risks related with NC were mentioned (8,45,46,47,48).

This is the first study indicating that there may be a relationship between NAFLD and NC as an indicator of accumulation of fat in the upper body. The only study that can be considered to bear similarity to our rationale showed that dorsocervical lipohypertrophy is related with NAFLD. Dorsocervical lipohypertrophy is also the most reliable measure to estimate the severity of liver inflammation resulting from fatty liver (49).

In our study, we found that NC is correlated with parameters of metabolic risk for the development of NAFLD and also with elevated liver enzymes in males. The results of univariate logistic regression analysis showed that with the exception of MUAC, all parameters were significant to determine NAFLD. In multivariable logistic regression analysis that is independent of puberty and age, we also detected that NC is the most reliable measure to assess fatty liver. The one unit increase in NC has the odds ratio of 1.846 in males and 1.551 in females for NAFLD. In ROC analysis, we found that among other anthropometric parameters and indices, NC is the most reliable parameter indicating the presence of NAFLD except for the midpubertal stage (Tanner stages 3-4). We consider this finding to be related to a change in body fat distribution occurring in this pubertal stage, or to the low sample size. Due to the small size of the sample, we were not able to assess the anthropometric measurements separately in the boys and the girls. In our previous study, the NC cut-off value as an indicator for metabolic syndrome was calculated as 36 cm in boys and 35 cm in girls. In this present study, NC cut-off values to determine NAFLD were calculated according to pubertal stage. The ranges of NC cut-off values were 33 cm for Tanner stage 1 and 36.5 for Tanner stage 5 for both genders.

Body mass index, WC, MUAC, and WHR have been commonly used as indices to determine metabolic risk factors. However, all of these parameters may vary from one person to another. Also, the percentile curves need to be used in the evaluation. NC appears to be a reliable alternative anthropometric parameter to be applied in the assessment of metabolic risk situations. NC, which is an easily measured and practical anthropometric index, may be used to assess upper body fat, and especially for screening NAFLD. The differences between intra-and inter-individual measurements are lower in NC than the other parameters, thus, NC appears to be a reliable and accurate index. In addition, there is no need to take off clothes during the NC measurement (50).

The relatively low sensitivity and specificity of USG analysis to show steatosis or its low capacity to discriminate between hepatitis and steatohepatitis may be considered as a limitation of this study. The fact that anthropometric measurements are indirect measures rather than direct indicators of metabolic risk

situations can be listed as another limitation of the study. Finally, our inability to make a gender- and pubertal stage-specific evaluation in ROC analysis because of the smallness of the sample can be considered another limitation of the study.

The present study indicates that NC was significantly related to upper body fat and NAFLD. Since NC is an inexpensive, practical, and reliable anthropometric measurement, we recommend that it can be used as an additional useful screening method to assess NAFLD in the primary evaluation of obese children.

Ethics

Ethics Committee Approval: The present study was approved by the local ethics committee, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Nihal Hatipoğlu, Design: Nihal Hatipoğlu, Selim Kurtoğlu, Data Collection or Processing: Nihal Hatipoğlu, Selim Kurtoğlu, Radiological Examination: Serap Doğan, Analysis or Interpretation: M. Mümtaz Mazıcıoğlu, Nihal Hatipoğlu, Literature Search: Nihal Hatipoğlu, Serap Doğan, Writing: Nihal Hatipoğlu, M. Mümtaz Mazıcıoğlu.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):40-47
DOI: 10.4274/jcrpe.2136

Original Article

Alpha-Melanocyte-Stimulating Hormone and Agouti-Related Protein: Do They Play a Role in Appetite Regulation in Childhood Obesity?

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ABSTRACT

Objective: The hypothalamus plays a crucial role in the regulation of feeding behavior. The anorexigenic neuropeptide alpha-melanocyte-stimulating hormone (α-MSH) and the orexigenic neuropeptide agouti-related protein (AgRP) are among the major peptides produced in the hypothalamus. This study investigated the plasma concentrations of α-MSH and AgRP in underweight and obese children and their healthy peers. The associations between α-MSH and AgRP levels and anthropometric and nutritional markers of malnutrition and obesity were also assessed.

Methods: Healthy sex-matched subjects aged 2 to 12 years were divided into 3 groups, as underweight (n=57), obese (n=61), and of normal weight (n=57). Plasma fasting concentrations of α-MSH and AgRP were measured by enzyme-linked immunosorbent assay. The differences between the three groups as to the relationships between plasma concentrations of α-MSH and AgRP and anthropometric data, serum biochemical parameters and homeostatic model assessment of insulin resistance were evaluated.

Results: Obese children had significantly lower α-MSH levels than underweight (1194±865 vs. 1904±1312 ng/mL, p=0.006) and normal weight (1194±865 vs. 1762±1463 ng/mL, p=0.036) children; there were no significant differences in the α-MSH levels between the underweight and normal weight children (p=0.811). Also, no significant differences were observed between the underweight and obese children regarding the AgRP levels (742±352 vs. 828±417 ng/mL, p=0.125). We found a significant positive correlation between plasma α-MSH and AgRP levels across the entire sample.

Conclusion: This study is the first to demonstrate body weight-related differences in α-MSH and AgRP levels in children. Circulating plasma α-MSH levels in obese children were markedly lower than those of underweight and normal-weight children. This suggests that α-MSH could play a role in appetite regulation.

Keywords: Alpha-melanocyte-stimulating hormone, agouti-related protein, underweight, childhood obesity

Conflict of interest: None declared
Received: 02.06.2015
Accepted: 17.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Children require sufficient nutrients to support the immune system and to help the body maintain health and normal bodily functions. Appetitive hormones are of interest in human populations because they are implicated in appetite regulation, weight loss and gain, malnutrition, and obesity. Circulating levels of alpha-melanocyte-stimulating hormone (α-MSH) and agouti-related protein (AgRP), and the potential role of these proteins in childhood malnutrition and obesity, have not yet been studied; scant data exist regarding their circulatory function in children.

WHAT THIS STUDY ADDS?

We assessed the peripheral concentrations of α-MSH and AgRP in three different groups (underweight and obese children and their healthy peers) and investigated the changes in the levels of these peptides with respect to body mass index, insulin, and homeostatic model assessment of insulin resistance. We hope this study will be useful for our colleagues.

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Introduction

Two major public health problems in the adult population that involve energy balance, namely obesity and anorexia, also appear in childhood. Body weight and fat levels are low in anorexic children who eat slowly, consume a limited number of foods, lack interest in food, display an irregular eating pattern, and experience loss of appetite and less frequent hunger episodes (1). Obesity, a multifactorial disorder resulting from the interactions among genetic, psychological, physical, environmental, and socioeconomic factors, develops only if energy intake from feeding chronically exceeds total energy expenditure. Feeding behavior is regulated by a system, with the hypothalamus at its center, in which the amount eaten is determined by the body's response to the internal energy status (2). There are complex interconnections between the hypothalamic nuclei that maintain energy homeostasis by regulating food intake and energy expenditure; the latter includes physical activity, basal metabolism, and adaptive thermogenesis (3,4).

One of the major regulators of food intake is leptin, a hormone released by adipose tissue that induces satiety via receptors located in the arcuate nucleus. Leptin crosses the blood-brain barrier and acts directly on two populations of neurons within the arcuate nucleus that express agouti-related protein (AgRP) or proopiomelanocortin (POMC). The POMC system plays a crucial role in the regulation of feeding behavior. Alpha-melanocyte-stimulating hormone (α-MSH) is a potent anorexigenic neuropeptide (5); leptin stimulates the production of α-MSH, which is an agonist for melanocortin-4 receptors (MC4R) and melanocortin-3 receptors (MC3R), and inhibits the production of AgRP (an antagonist for these receptors) in a coordinated manner to regulate the energy balance by inhibiting food intake and stimulating energy expenditure (6).

Recent research indicates that α-MSH is produced in the human pituitary by cells of the pars distalis of the pituitary gland and numerous extrapituitary cells, including monocytes, astrocytes, gastrointestinal cells, and keratinocytes (7). AgRP is among the most potent and long-lasting appetite stimulators and exerts its effects primarily by opposing the anorexigenic/catabolic actions of POMC by competitively inhibiting melanocortin receptors (specifically MC3-R and MC4-R) at the postsynaptic level. It is encoded by the AgRP gene. AgRP expression has been detected in a range of human tissues including the brain, adrenal glands, testis, lung, and kidney. α-MSH plays a role in thermal regulation (hypermetabolic/hyperthermic) by increasing free fatty acid oxidation in skeletal muscle (8,9). Five melanocortin receptors are known; two of these, namely, MC3-R and MC4-R, are believed to be involved in energy balance signaling. Mutations in MC4-R have been shown to play a major role in the genetics of obesity (10). However, circulating levels of MSH and AgRP, and the potential role of these proteins in childhood malnutrition and obesity, have not yet been studied; scant data exist regarding their effects on circulatory function in children. Taking the information listed above into consideration, we aimed to assess the peripheral concentrations of α-MSH and AgRP in three groups (underweight and obese children and their healthy peers) and to investigate the differences in the levels of these peptides with respect to body mass index (BMI), insulin, and homeostatic model assessment of insulin resistance (HOMA-IR).

Methods

This cross-sectional study was conducted at the Bezmialem Vakıf University Hospital in İstanbul, on randomly selected pediatric patients who attended the outpatient clinic of the pediatrics department for routine examinations between October 2014 and March 2015. The study groups comprised 57 underweight prepubertal children (26 males and 31 females with a BMI for age and sex <18.5th percentile due to loss of appetite and infrequent hunger episodes), 61 obese children (28 males and 33 females with a BMI for age and sex ≥95th percentile), and 57 healthy children of normal weight (26 males and 31 females with a BMI for age and sex between the 18.5th to 95th percentiles). The inclusion criterion did not comprise a dietary history of inadequate nutrient intake (in quality or quantity). Only children with no health problems, except for anorexia or obesity, were included in the study. The underweight patients had the opportunity to obtain food, but had poor appetite; they were diagnosed with malnutrition on admission, but no patient was receiving therapy or nutritional support during the evaluation. The exclusion criteria included presence of an endocrine disease or a syndromal problem, of an acute or chronic inflammatory disease, a malabsorption syndrome such as celiac disease or cystic fibrosis. Presence of an infectious or systemic disease, use of prescription medications, vitamins, or mineral supplements for any reason, were also reasons for exclusion. None of the subjects had a history or any current evidence of metabolic, cardiovascular, or hepatic disease.

Anthropometric measurements were performed for all patients; height was measured to the nearest 0.1 cm using a Harpenden fixed stadiometer, and weight was measured to the nearest 0.1 kg using a SECA balance scale with the subject dressed only in light underwear without shoes. Using this information, age- and sex-specific BMI percentiles were calculated. BMI expresses the relationship between weight and height as a ratio (weight in kg divided by height in m^2) and is strongly correlated with the percent body fat. In the present study, childhood BMI was calculated as described above and the subjects were classified as thin, normal, or obese according to Cole's recently published BMI cut-offs for children aged 2 to 18 years for thinness/overweight/obese according to sex and age (11).

After 12 hours of fasting, venous blood samples were collected into tubes (Vacuette; Greiner Labor Technic GmbH, Germany) between 8:00 am and 9:00 am The samples were then separated by centrifugation (10 min at 4500 rpm, 4 °C) and stored at -80 °C until subsequent use. A complete blood count,

serum iron level, serum total iron-binding capacity, ferritin, thyroid stimulating hormone, thyroxine, vitamin B12, folic acid, glucose, albumin, insulin, total immunoglobulin A (IgA), tissue transglutaminase antibody IgA (anti tTG-IgA), total cholesterol, triglycerides, C-reactive protein (CRP), plasma α-MSH and AgRP levels, and erythrocyte sedimentation rate were assessed in all subjects. Insulin resistance (IR) was estimated from fasting plasma measurements using HOMA-IR [insulin (mU/L)×glucose (mmol/L)/22.5]. The criterion for IR in prepubertal children is a HOMA-IR of >2.5 (12). Total cholesterol and triglycerides were measured using the homogeneous colorimetric enzyme technique (Roche Cobas 8000 modular analyzer; Roche Diagnostics, Mannheim, Germany). Glucose was measured using the glucose oxidase technique (Advia 1800; Siemens Healthcare Diagnostics, Tarrytown, NY, USA); insulin levels were analyzed using the direct chemiluminescence technique (Advia Centaur, Siemens). Plasma α-MSH and AgRP concentrations were measured using commercially available enzyme-linked immunosorbent assay kits purchased from SunRed (SRB/ Shanghai; cat no. 201-12-5500; sensitivity: 14.068 ng/L, range: 15-4200 ng/L, intraassay CV, 7.4%; cat no. 201-12-1479, sensitivity: 4.776 ng/L, range: 5-1500 ng/L, intraassay CV, 4.3%, respectively) according to the manufacturer's protocol. Standards and samples were incubated with antibody-coated 96-well plates for 2 hours. Enzyme-linked antibodies for the proteins were then incubated for 1 hour. Finally, the substrate solution was added; the reaction stopped after a short while. The intensity of the color in each well was measured using a microplate reader (Varioskan™ Flash Multimode Reader; Thermo Scientific, Hudson, NH, USA) at 450 nm.

Analyses were conducted using the IBM Statistical Package for the Social Sciences for Windows software package (version 20.0; IBM Corp., Armonk, NY, USA). The results are presented as means ± standard deviation (SD), with categorical variables presented as frequencies and percentages. Comparison of group means was performed using one-way ANOVA for parametric tests with Tukey's honestly significant difference (HSD) post-hoc test applied for multiple comparisons. Pearson's correlation was used to determine relationships between variables. Categorical data were compared using the chi-squared test. A p-value of <0.05 was taken to indicate statistical significance.

The study protocol was carried out in accordance with the ethical principles of the Declaration of Helsinki, 1989. Information concerning the aim of the study was provided to the children's parents at the time of enrollment; written informed consent was also obtained. Ethical approval was granted by the Bezmialem Vakıf University Local Research Ethics Committee.

Results

The experimental groups were randomly selected and comprised prepubertal underweight children (n=57; 26 males and 31 females, mean age: 7.7±2.4 years, range: 2-12 years) with a mean ± SD score for BMI of -1.91±0.7 (thinness grade of 1-3); obese children (n=61; 28 males and 33 females, mean age: 8.1±2.2 years, range: 2-12 years) with a mean ± SD score for BMI of 2.35±0.6; and healthy children of normal weight (n=57; 26 males and 31 females, mean age: 7.4±2.7 years, range: 2-12 years) with a mean ± SD scores for BMI of 0.26±0.8. In the underweight group, 32 (56.1%) subjects had a thinness grade of 1, 20 (35.0%) had a thinness grade of 2, and 5 (8.7%) had a thinness grade of 3. The anthropometric and metabolic characteristics of the three groups are summarized in Tables 1 and 2. The age and sex distribution did not differ

Table 1. Comparison of demographic characteristics of underweight, normal weight and obese children

Characteristics	Underweight children	Healthy children	Obese children	
	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	p
Number of children	57	57	61	
Female	31 (54.1)	31 (54.1)	33 (54.1)	
Male	26 (45.6)	26 (45.6)	28 (45.9)	0.999
Age, years	7.7±2.4 (2-12)	7.4±2.7 (2-12)	8.1±2.2 (2-12)	0.720
Weight, kg	21.2±7.2 (9.5-36)	26.5±10.6 (14-59)	51.1±16.8 (20-78)	<0.001
Weight, z-score	-1.36±0.86 (-3.89:0.48)	0.24±0.67 (-1.01:1.7)	2.12±0.76 (-0.04:4.13)	<0.001
Height, cm	122.2±18.0 (83-153)	123.3±17.4 (90-159)	142.8±15.6 (95-163)	<0.001
Height, z-score	-0.25±1.06 (-2.9:2.3)	-0.01±0.84 (-1.5:2.0)	0.86±0.9 (-1.6:2.7)	<0.001
BMI, kg/m^2	13.77±0.90(11.7-16.3)	16.75±1.90 (14.2-21.8)	25.78±4.2 (19.6-43.6)	<0.001
BMI-z score	-1.91±0.7 (-4.9:-0.6)	0.26±0.8 (-1.5:1.7)	2.35±0.6 (0.9:4.5)	<0.001
BMI percentile	5.6±5.7 (0-24.3)	55±2.5 (13.3-91.6)	98.3±1.6 (95-1009	<0.001

BMI: body mass index, SD: standard deviation

The data are expressed as number, mean values ± standard deviation (range)

One-way ANOVA test was used for correlation analysis, p<0.05

among the groups (p=0.720 and 0.999, respectively). The underweight group had a significantly lower mean weight, weight z-score, mean height, height z-score, BMI, BMI z-score, and BMI percentage as compared to the control and obese groups (p<0.001). There were no significant group differences in the hemoglobin, albumin or ferritin levels (p=0.770, 0.680, 0.409, respectively). There were no significant differences between the underweight and normal-weight children in fasting glucose, insulin, total cholesterol, triglyceride, CRP or HOMA-IR (p=0.993, 0.247, 0.913, 0.494, 0.999, and 0.391, respectively) levels. The obese group had higher serum fasting glucose, insulin, total cholesterol, triglycerides, CRP and HOMA-IR than did the other two groups (p<0.001). HOMA-IR was positively correlated with glucose and insulin levels in all groups (all p<0.001). The level of vitamin B12 was higher in normal-weight children than in underweight or obese children (p=0.021 and 0.030, respectively), but there were no differences between the underweight and obese children. In the obese group, 46 (75%) subjects had a HOMA-IR of >2.5.

Obese children had significantly lower α-MSH levels than did the underweight (1194±865 vs. 1904±1312 ng/mL, p=0.006) and normal-weight (1194±865 vs. 1762±1463 ng/mL, p=0.036) children; there were no significant differences in α-MSH levels between the underweight and normal-weight children (p=0.811). There were also no significant differences between the underweight and obese children regarding the AgRP levels (742±352 vs. 828±417, p=0.125) (Table 3). Furthermore, the plasma AGRP and α-MSH levels were not significantly different between males and females across the entire sample (Figure 1).

There was a significant positive correlation between plasma α-MSH and AgRP levels across the entire sample. The

Table 2. Laboratory findings in underweight, normal-weight, and obese children

	Underweight children	Healthy children	Obese children	
	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	p
Hb (g/dL)	12.46±0.9 (9.6-14.2)	12.34±0.9 (10.4-14)	12.73±0.9 (10.7-14.2)	0.770
fT_4 (pmol/L)	15.40±1.9 (11.4-19.9)	14.01±2.54 (10.5-18.6)	13.91±2.09 (3.19-18.9)	0.011
TSH (IU/mL)	2.25±0.92 (0.9-4.57)	3.10±1.37 (0.9-6.7)	3.24±2.08 (1.08-14.1)	<0.001
Albumin (g/dL)	4.48±0.25 (4-5.2)	4.30±0.23 (4-4.9)	4.46±0.25 (4.1-5.2)	0.680
CRP mg/dL)	0.16±0.19 (0-0.9)	0.17±0.17 (0-0.9)	0.33±0.25 (0-1)	<0.001
ESR (mm/h)	7.3±3.4 (2-16)	6.6±2.3 (3-12)	6.5±2.5 (2-14)	0.218
Vitamin B12 (pg/mL)	399.6±146.2 (101-717)	481.2±184.1 (173-970)	404.6±154.3 (104-721)	0.012
Ferritin (ng/mL)	34.69±24.05 (5.6-107.7)	31.08±22.4 (3.2-107.7)	29.57±16.7 (5.4±80.3)	0.409
Folic acid (ng/mL)	10.51±3.3 (3.26-18.6)	10.09±2.7 (5.2-18.4)	10.52±4.25 (1.2-24.1)	0.705
Glucose (mg/dL)	89.1±9.9 (59-108)	89.04±7.7 (74-112)	93.72±6.3 (79-119)	0.002
Insulin (IU/mL)	8.5±5.6 (1.03-26.5)	11.83±5.6 (1.64-30.4)	19.14±12.4 (3.9-81.8)	<0.001
HOMA-IR	1.94±1.4 (0.19-6.7)	2.38±1.39 (0.3-6.9)	4.49±3.18 (0.9-21.9)	<0.001
Triglyceride (mg/dL)	70.2±21.6 (40-135)	64±15.5 (42-112)	92.2±36.4 (32-237)	<0.001
Total cholesterol (mg/dL)	152±18.2 (109-188)	154.5±20 (108-223)	168±30.5 (109-247)	<0.001

Hb: hemoglobin, fT_4: free thyroxin, TSH: thyroid stimulating hormone, CRP: C-reactive protein, HOMA-IR: homeostatic model of assistance of insulin resistance, ESR: erythrocyte sedimentation rate, SD: standard deviation

The data are expressed as number, mean values ± standard deviation (range)

One-way ANOVA test was used for correlation analysis, p<0.05

Table 3. Study parameters of underweight, normal-weight, and obese children

	Underweight children	Healthy children	Obese children	
Parameters	Mean ± SD (range)	Mean ± SD (range)	Mean ± SD (range)	p
α-MSH (ng/mL)	1904.6±1312 (407-5424)	1762.0±1463 (296-5914)	1194.9±865(116-3486)	0.005
AgRP (ng/mL)	742.7±352 (273-1605)	641.9±462 (123-1921)	828.2±417 (159-2500)	0.125

α-MSH: alpha-melanocyte-stimulating hormone, AgRP: agouti-related protein, SD: standard deviation

The data are expressed as mean values ± standard deviation (range)

One-way ANOVA test was used for correlation analysis, p<0.05

results of Pearson's correlation of α-MSH, AgRP, and HOMA-IR with age, glucose and insulin levels are summarized in Table 4. When the entire sample was evaluated together (n=175), there was a significant positive correlation between the plasma α-MSH and AgRP levels (p<0.001) and a negative correlation between the α-MSH levels and weight z-score (p=0.017), BMI (p=0.019), BMI z-score (p=0.014), BMI percentile (p=0.017), insulin (p=0.045), and CRP (p<0.001).

Table 4. The results of Pearson's correlation of alpha-melanocyte-stimulating hormone, agouti-related protein and homeostatic model of assistance of insulin resistance with age, glucose, and insulin levels between the groups

	Underweight children		Healthy children		Obese children	
α-MSH correlations with	**r**	**p**	**r**	**p**	**r**	**p**
AgRP	0.621	<0.001	0.888	<0.001	0.570	<0.001
AgRP correlations with	**r**	**p**	**r**	**p**	**r**	**p**
α-MSH	0.621	<0.001	0.888	<0.001	0.570	<0.001
HOMA-IR correlations with	**r**	**p**	**r**	**p**	**r**	**p**
Age	-0.073	0.591	-0.052	0.699	0.252	0.05
Glucose level	0.546	<0.001	0.457	<0.001	0.477	<0.001
Insulin level	0.989	<0.001	0.982	<0.001	0.992	<0.001

α-MSH: alpha-melanocyte-stimulating hormone, AgRP: agouti-related protein, HOMA-IR: homeostatic model of assistance of insulin resistance
Statistical analysis by Pearson's correlation

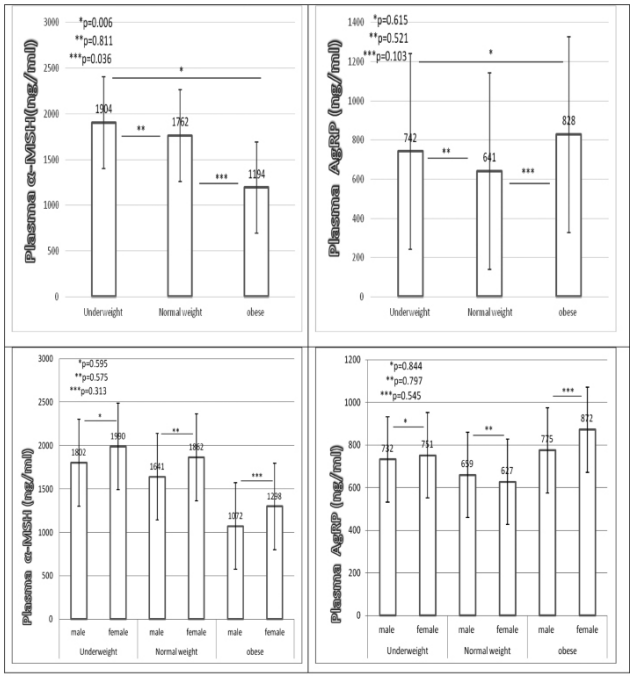

Figure 1. Plasma alpha-melanocyte-stimulating hormone and agouti-related protein levels in underweight, normal-weight, and obese children. The data are expressed as mean values ± standard error of mean. One-way ANOVA followed by Post Hoc Tukey's test was used for correlation analysis; p<0.05

Discussion

Appetitive hormones are of interest in human populations because they are implicated in appetite regulation, weight loss and gain, malnutrition, and obesity. There are several reports on plasma α-MSH and AgRP levels in adults and children (13,14). The present study demonstrated that circulating plasma α-MSH levels in obese children are markedly lower than those in underweight and normal-weight children. We also found that AgRP levels were relatively lower in underweight children than in controls, but not significantly. We found no significant differences between females and males in α-MSH or AgRP levels. When the entire sample was evaluated together, α-MSH levels were negatively correlated with various parameters of obesity, including weight z-score, BMI, BMI z-score, BMI percentile, as well as insulin and CRP levels.

As the central feeding organ, the hypothalamus mediates the regulation of short and long-term dietary intake via synthesis of orexigenic and anorectic neuropeptides. Previous studies in animals demonstrated that α-MSH in the peripheral circulation plays a role in metabolism regulation, fat storage, and glucagon secretion (15,16). Studies investigating the role of peripheral α-MSH and AgRP in humans have reported equivocal results. Hoggard et al (17) showed that both plasma α-MSH and AgRP levels were elevated in 18 obese subjects relative to 11 lean adults; in both cases there was also a close correlation with both BMI and body fat mass. Furthermore, there were no significant changes in plasma α-MSH levels in response to either food deprivation or food restriction in either the lean or obese subjects. Similarly, Katsuki et al (18) demonstrated that both plasma α-MSH and AgRP levels were higher in obese than non-obese men; furthermore, plasma AgRP levels were significantly correlated with plasma α-MSH levels, suggesting a degree of peripheral involvement in energy balance regulation. Another study found that plasma α-MSH levels were similar between normal-weight, overweight, and obese subjects, although there was a weak trend toward higher α-MSH levels in obese than in lean men (19). In contrast, Nam et al (20) found that plasma and cerebrospinal α-MSH levels in obese women did not differ significantly from those of controls at baseline or after a 5% weight loss. Gavrila et al (21) demonstrated no relationship between peripheral α-MSH levels and body composition parameters in 108 healthy, normal-weight adults. We encountered only one comparable study of plasma α-MSH levels in children published previously (22). In this study, Roth et al (22) reported no significant differences in α-MSH levels between obese and normal-weight children, although children with craniopharyngioma had lower α-MSH levels than did

obese and lean children. Furthermore, the authors found no significant differences in the α-MSH levels between females and males, similar to our study.

α-MSH binds to hypothalamic neurons that express MC4-R, leading to appetite suppression (23). Despite the fact that α-MSH is an anorexigenic neuropeptide, as mentioned above, many studies on adults have demonstrated that obese subjects had higher levels of α-MSH than do normal-weight or lean subjects. In contrast, our data showed that circulating plasma α-MSH levels in obese children were markedly lower than those in underweight and normal-weight children, which suggests that α-MSH plays a role in appetite regulation. More frequent hunger episodes due to α-MSH deficiency could lead to increased feeding signals, which may in turn increase the risk of developing obesity.

In the present study, when all three groups were analyzed together, there were significant negative correlations between α-MSH levels and parameters including the weight z-score, BMI, BMI z-score, BMI percentile, and insulin and CRP levels. These results suggest that α-MSH levels are probably more closely related to weight status in children, supporting a probable role of α-MSH in the peripheral regulation of energy homeostasis. Thus, it would appear that α-MSH levels decrease with increased BMI in children, which leads to diminished appetite suppression. Whether the observed differences are due to the more accurate effect estimates due to the relatively larger sample size of this study or to genetic differences in the studied populations requires further investigation.

The hypothalamic melanocortin system, which comprises the MC4-R, its agonist α-MSH, and its antagonist AgRP, is considered to be the main central anorexigenic pathway controlling energy homeostasis (24). Attention has focused particularly on the roles of peripheral α-MSH and AgRP in obesity, and several studies have reported on the peripheral actions of α-MSH and AgRP in the pathophysiology of eating disorders in adults (25). We found no previous studies on the levels of these proteins in underweight children, but studies in patients with anorexia nervosa suggest abnormal expression of appetite-regulating hormones. Moriya et al (26) showed that plasma AgRP levels were significantly higher in patients with anorexia nervosa than in controls, whereas plasma α-MSH levels did not differ between the two groups. These authors suggested that elevated plasma AgRP may be related to energy homeostasis disturbances in anorexia nervosa. Furthermore, a previous study showed that patients with acute anorexia nervosa had higher AgRP levels than those of healthy controls, but the AgRP levels of weight-restored anorexia patients were similar to those of healthy controls (27). In our study, no differences were found between obese and underweight children with respect to AgRP levels.

In patients with anorexia nervosa, decreased food intake is accompanied by hyperactivity and activation of the hypothalamic-pituitary-adrenal axis. Polymorphism in the gene encoding AgRP is associated with the development of both anorexia nervosa and obesity (28). Vink et al (29) suggested that genetic defects resulting in chronic activation of the melanocortinergic system could lead to anorexia nervosa. Variations in AgRP could be due to suppression of MC4-R, leading to a decreased feeding signal and thereby increasing the risk of developing anorexia nervosa. Interestingly, synthetic MC4-R antagonists, which act as AgRP mimetics, relieve different kinds of anorectic conditions. MC4-R antagonists have also been shown to be effective against cancer-induced anorexia (30). In the present study, our anorexic underweight children (due to loss of appetite and infrequent hunger episodes) had lower AgRP levels than their obese peers, although not significantly. Recently, there has been a growing interest in a number of genes related to appetite regulation (31). The importance of the melanocortin signaling pathway in humans for the control of appetite and energy balance is suggested by numerous monogenetic mutations identified in genes involved in the synthesis or processing of the glycoprotein POMC, or in mutations that lead to defects in POMC signaling via the melanocortin receptors. In particular, MC4-R mutations result in a severely obese phenotype and may be responsible for up to 4% of all cases of severe obesity in certain populations (31,32,33).

However, previous studies have also shown that peripheral α-MSH possesses a number of functions, such as anti-inflammatory and antimicrobial effects, and probably contributes to innate immunity. Recent research has shown that α-MSH is produced in the human pituitary by cells of the pars distalis and by numerous extrapituitary cells including monocytes, astrocytes, gastrointestinal cells, and keratinocytes (34). Human studies have demonstrated significant changes in endogenous peptide levels in pathological states. α-MSH is extremely effective in the treatment of animal models of local and systemic inflammatory disorders, including sepsis syndrome, adult respiratory distress syndrome, respiratory arrest, rheumatoid arthritis, inflammatory bowel disease, and encephalitis (35,36). Obese children have been shown to have elevated levels of inflammatory markers, of which CRP is strongly and positively associated with weight status in children (37). Our study demonstrated that obese children had higher CRP levels than their normal-weight and underweight peers; there was also a negative correlation between α-MSH and CRP levels, which suggests that a decreased anti-inflammatory effect of α-MSH in obese children increases the risk of elevated CRP levels.

The present study showed for the first time that circulating plasma α-MSH levels are lower in obese children than in underweight and normal-weight children and that they are also negatively correlated with body weight. The study also demonstrated that AgRP levels were lower in underweight children than in controls, but not significantly so. Decreased α-MSH levels in obese children appear to be important for understanding the physiology of energy homeostasis; further research in this area may lead to the development of novel treatment strategies for pediatric malnutrition and obesity.

Neuropeptides that are involved in appetite regulation and energy expenditure could be important in future weight loss interventions.

Acknowledgements

The authors of this manuscript would like to thank the mothers and children who participated in the study.

Ethics

Ethics Committee Approval: Bezmialem Vakıf University Local Research Ethics Committee (Approval number: 12-09-14), Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Aysel Vehapoğlu, Design: Aysel Vehapoğlu, Data Collection or Processing: Aysel Vehapoğlu, Serdar Türkmen, Şule Terzioğlu, Analysis or Interpretation: Aysel Vehapoğlu, Literature Search: Aysel Vehapoğlu, Serdar Türkmen, Şule Terzioğlu, Writing: Aysel Vehapoğlu.

Financial Disclosure: This study was supported by a grant from University Research Council, Bezmialem Vakıf University.

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J Clin Res Pediatr Endocrinol 2016;8(1):48-54
DOI: 10.4274/jcrpe.2266

Original Article

Impact of Vitamin D Status on Cardiometabolic Complications among Children and Adolescents with Type 1 Diabetes Mellitus

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ABSTRACT

Objective: There is an ongoing interest in the relationship between vitamin D status and diabetes control and complications. However, data from Saudi Arabia are limited. To determine the impact of vitamin D status on glycemic control and cardiometabolic complications of children and adolescents with type 1 diabetes mellitus (T1DM) attending a tertiary care diabetes clinic in Saudi Arabia.

Methods: Demographic, clinical, and laboratory data of 301 children and adolescent subjects with T1DM (53.5% females) of a mean age of 13.9 years attending King Abdulaziz Medical City-Jeddah during 2010-2013 were retrospectively collected. Relationships between vitamin D status and frequency of hypoglycemia, hemoglobin A1c (HbA1c) level, body mass index (BMI), blood pressure, and lipid profile were evaluated.

Results: The mean duration of diabetes was 7.7±3.7 years. Mean BMI value was 21.1±4.5 kg/m^2 and HbA1c was 9.6±1.9% in both genders. Only 26.2% of the patients had a satisfactory HbA1c level. The mean level of 25-hydroxyvitamin D [25(OH)D] was 35.15 and that of cholesterol was 4.75. Vitamin D deficiency [25(OH)D≤37.5 nmol/L] was detected in 63.6% of the male and 67.7% of the female subjects. In males, it was inversely associated with frequency of hypoglycemia ($p<0.01$), BMI ($p<0.05$), diastolic blood pressure ($p<0.05$), and triglyceride levels ($p<0.01$), while in females, it was inversely associated with current age ($p<0.05$), age at diagnosis ($p<0.01$), and triglyceride levels ($p<0.01$). No significant correlation between HbA1c and vitamin D deficiency was observed.

Conclusion: Vitamin D deficiency was highly prevalent in our study sample and was found to be associated with frequency of hypoglycemic episodes and with adverse cardiometabolic control.

Keywords: Type 1 diabetes mellitus, vitamin D deficiency, metabolic control
Conflict of interest: None declared
Received: 25.07.2015
Accepted: 10.11.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Vitamin D deficiency is common in Saudi children and has been linked to several cardiometabolic risk factors.

WHAT THIS STUDY ADDS?

Vitamin D deficiency is highly prevalent in our study and it is associated with frequent hypoglycemia and adverse cardiometabolic control.

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Introduction

The current global prevalence of type 1 diabetes mellitus (T1DM) under the age of 15 years is estimated to be around 500 000, with the largest demographics found in Europe and North America (1). Epidemiological data also point to an increased incidence of T1DM globally with a rate of around 3-4% per year and with an age of onset younger than previous estimates (2). These observations were confirmed in developed and developing countries, specifically the US, Latin America, Europe, Australia, India, South-east Asia, and China (2,3,4,5,6,7,8,9). Over the last 3 decades, the incidence rate of T1DM is growing also in Saudi Arabia (10). The most recent reports of incidence of T1DM in Saudi children is 27.5/100 000 (11) and 29/100 000 (12) which are high rates. The prevalence of T1DM in Saudi children and adolescents is 109.5 per 100 000 (13).

Moving on to vitamin D, accumulating evidence in recent years has pointed to a significant link between vitamin D deficiency and auto-immune disease as well as endocrine disorders in children (14). Furthermore, vitamin D deficiency is common in Saudi children and has been linked to several cardiometabolic risk factors outside its conventional role in bone homeostasis (15).

To date, studies on T1DM in the Saudi population are limited. Also, emerging risk factors such as vitamin D deficiency for several chronic diseases have been modestly addressed in this specific population. At present, vitamin D deficiency is highly prevalent among the Saudi T1DM patients, both children and adults (16). A national study by Bin-Abbas et al (17) showed an overall prevalence of vitamin D deficiency in 84% of T1DM children and in 59% of healthy children. In the present study, we aimed to describe the clinical presentation and the level of metabolic control in Saudi children and adolescents with T1DM attending the pediatric endocrine clinic at King Abdulaziz Medical City in Jeddah (KAMC-J) and to determine differences in anthropometric and metabolic indices of those with and without vitamin D deficiency.

Methods

The study followed the Helsinki declaration recommendations and was approved by the Institutional Review Board of King Abdullah International Medical Research Center. In this retrospective cross-sectional study, we included all children and adolescents between the ages of 1 and 18 years with known T1DM and regular follow-up for more than 3 months attending the pediatric endocrine clinic at KAMC-J from January 2010 to January 2013. Gender, puberty staging, duration of T1DM, symptoms of early presentation were collected as well as clinical information such as blood pressure (BP) using the Dinamap automated oscillometric device and body mass index (BMI) using Center for Disease Control and Prevention charts. Data on hemoglobin A1c (HbA1c), 25-hydroxyvitamin D [25(OH)D] levels, lipid profile, and thyroid function were also collected from the medical records. We followed the American Diabetes Association (ADA) 2014 Guidelines for target HbA1c levels per age group, namely, ≤8.5% in toddlers, ≤8% in school children, and ≤7.5% in adolescents and young adults (18).

HbA1c was measured using ion-exchange high-performance liquid chromatography technique. HbA1c values were based on measurement at regular intervals (3 months) and the average of the last 3 readings in the last year follow-up. Other variables (BP, BMI, lipid profile, and thyroid function) were recorded from the last follow-up visit.

25(OH)D measurements are done routinely for T1DM patients in our center, measured by chemiluminescent micro particle immunoassay for the quantitative determination of serum 25(OH)D in human serum and plasma. For the purpose of this study, vitamin D deficiency was defined as a serum 25(OH)D level of <37.5 nmol/L based on the Drug and Therapeutics Committee of the Lawson Wilkins Pediatric Endocrine Society (19).

Statistical Analysis

The data were analyzed using Statistical Package for the Social Sciences version 16.5 (SPSS Inc., Chicago, IL, USA). The results were presented as percentages (%) for frequencies and means ± standard deviations for continuous variables. Independent t-test was done to compare variables that are normally distributed, and a Mann-Whitney U-test for non-Gaussian variables. Chi-square test was used to compare frequencies. Spearman correlation was done to determine associations of vitamin D status to measured variables. Linear regression using log-transformed vitamin D and HbA1c was done to determine the association between glycemic and vitamin D status. Significance was set at p-value <0.05.

Results

A total of 301 T1DM patients (161 females) were studied. Table 1 shows the clinical characteristics of males and females. The mean age for the total group was 13.9±3.8 years (13.86±3.88 for males and 14.06±3.86 for females). Mean age of T1DM at diagnosis was slightly younger in males (6.01±3.65) than females (6.33±3.45). Pubertal signs were noted in 50.7% of male and 57.8% of female subjects. Symptoms of hyperglycemia were the most common presentation (57.9% in males; 51.6% in females). Frequency of symptomatic hypoglycemic attacks was relatively higher in males (47.1%) than in females (42.9%).

Table 2 shows the anthropometric and metabolic findings and reveals that females have a slightly higher BMI than males (p=0.07). There was no significant gender difference in systolic and diastolic blood pressure, HbA1c, and lipid profile. The overall mean value for HbA1c was 9.67±1.93 (9.7±1.8 in males and

9.66±1.98 in females). Acceptable HbA1c (≤8%) values for glycemic control were found only in 26.2% (79 out of 301) of the subjects. When stratified by age, only 28.6% of toddlers, 15.6% of children, and 12.8% of adolescents had acceptable HbA1c levels. The mean level of 25(OH)D was 35.1±15.9 nmol/L. The mean 25(OH)D level was higher in males than females (36.93±14.69 versus 33.37±17.28 nmol/L; p=0.02) (Table 3).

Table 3 shows the comparison of parameters according to vitamin D status and shows that subjects in the ≤37.5 nmol/L group had a significantly higher BMI and serum triglycerides than the >37.5 nmol/L group (p-values 0.019 and 0.044, respectively). In Table 4, vitamin D deficiency was noted to be 63.6% in males and 67.7% in females. In males, those who were vitamin D deficient subjects had significantly higher BMI values than those who were not deficient (p<0.01). In females, serum triglycerides were significantly higher in those who were vitamin D deficient than those who were not (p<0.05). As expected, 25(OH)D levels were higher in the sufficient than in the deficient group in both genders. There were no significant differences between the groups in the remaining parameters (Table 4). The vitamin D status of subjects was assessed for its associations to the different metabolic parameters included in the study (Table 5). In males, vitamin D status was inversely associated with frequency of hypoglycemia (p<0.01), BMI (p<0.05), diastolic BP (p<0.05), and triglyceride levels (p<0.01). In females, vitamin D status was inversely associated with age (p<0.05), age at diagnosis (p<0.01), and triglyceride levels (p<0.01). In all subjects, there was no association between circulating 25(OH)D and HbA1c (Figure 1) (r=0.04; p=0.60).

Discussion

To our knowledge, this is the first study from Saudi Arabia on the relationship between vitamin D status and metabolic control and complications in children with T1DM. The main finding of the present study is the alarmingly high prevalence of vitamin D deficiency among Saudi diabetic children and adolescents and the adverse effects of this deficiency on some cardiometabolic parameters which are gender-specific.

The high prevalence of vitamin D deficiency in our patients was an expected finding in view of the high

Table 1. Clinical characteristics of the subjects

Parameter	Males	Females	p-value
Number	140	161	
Age (years)	13.86±3.88	14.06±3.86	0.67
Age of diagnosis (years)	6.01±3.65	6.33±3.45	0.44
Duration of DM (years)	7.83±3.81	7.71±3.6	0.78
Duration of DM symptoms prior to DM diagnosis (weeks)	2.03±1.14	2.05±1.13	0.78
Pubertal stage (%)			
Pre-Pubertal	49.3	42.2	0.22
Pubertal	50.7	57.8	
Symptoms of DM at onset			
DKA	42.1	48.4	0.27
Hyperglycemia symptoms	57.9	51.6	
Reasons for admission			
DKA	27.9	38.8	0.22
Education	32.9	27.5	
Other reasons	39.2	33.7	
Number of missed clinic visits/year			
0	42.1	44.1	0.72
1	40.7	37.9	
2	10.7	13.7	
3	6.4	4.3	
Hypoglycemia attacks (symptomatic)	47.1	42.9	0.46

DM: diabetes mellitus, DKA: Diabetic keto acidosis
Data presented as mean ± standard deviation and as percentages (%); significant at p<0.05

prevalence of vitamin D deficiency in the Saudi general population, including children (20). In Saudi children, risk factors for vitamin D deficiency include gender, diet, and physical activity (21,22). Furthermore, the inverse associations of vitamin D to anthropometric and clinical parameters are in line with previous reports on non-DM, apparently healthy Saudi children (15). Nevertheless, the study supports an existing theory that expressions of vitamin D and its receptors are differentially expressed in males and females. In a recent study, differences in the modulation of different proteins at a proteomic level, associated with various pathways, including vitamin D function and activation of 1α 25(OH)D signaling, were observed to be more pronounced in males relative to

Table 2. Anthropometric and metabolic characteristics of the subjects

Parameter	Males	Females	p-value
Number	140	161	
BMI (kg/m^2)	20.69±4.45	21.63±4.63	0.07
BMI Z-score	-0.11±0.98	0.10±1.01	0.07
Systolic blood pressure (mmHg)	114.19±13.0	112.96±10.80	0.44
Diastolic blood pressure (mmHg)	68.5±8.14	68.29±8.89	0.86
HbA1c (%)	9.7±1.8	9.6±1.98	0.29
Triglycerides (mmol/L)	1.12±0.7	1.14±0.57	0.92
Total Cholesterol (mmol/L)	4.68±1.12	4.83±1.1	0.53
HDL-Cholesterol (mmol/L)	1.19±0.31	1.26±0.31	0.30
LDL-Cholesterol (mmol/L)	2.87±1.3	2.99±0.86	0.61
25-hydroxyvitamin D (nmol/L)	36.93±14.69	33.37±17.28	0.02
TSH (mIU/L)	1.81±1.94	2.0±2.05	0.20
fT_4 (pmol/L)	9.49±6.83	11.09±6.02	0.11

BMI: body mass index, HbA1c: hemoglobin A1c, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TSH: thyroid stimulating hormone, fT_4: free thyroxine
Data presented as mean ± standard deviation and as percentages (%), #denotes non-Gaussian variable significant at p<0.05

Table 3. Anthropometric and metabolic characteristics by vitamin D status

Parameter	25-hydroxyvitamin D ≤37.5 nmol/L	25-hydroxyvitamin D >37.5 nmol/L	p-value
Number	103	198	
Age (years)	14.1±3.4	13.1±4.2	0.054
BMI (kg/m^2)	21.6±4.5	20.1±3.9	**0.019**
Systolic blood pressure (mmHg)	113.2±11.2	111.9±12.8	0.531
Diastolic blood pressure (mmHg)	67.8±9.0	67.9±8.5	0.912
HbA1c (%)	10.6±7.8	9.6±2.0	0.289
Triglycerides (mmol/L)	1.25±0.6	1.0±0.6	**0.044**
Total Cholesterol (mmol/L)	4.9±1.1	4.7±1.0	0.419
HDL-Cholesterol (mmol/L)	1.3±0.3	1.2±0.4	0.535
LDL-Cholesterol (mmol/L)	3.1±1.2	2.9±0.8	0.622
25-hydroxyvitamin D (nmol/L)	26.7±11.1	51.1±12.4	**<0.001**
TSH (mIU/L)	2.6±2.1	2.5±1.8	0.741
fT_4 (pmol/L)	13.9±2.0	14.0±1.8	0.872

BMI: body mass index, HbA1c: hemoglobin A1c, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TSH: thyroid stimulating hormone, fT_4: free thyroxine
Data presented as mean ± standard deviation; significant at p<0.05.

Table 4. Anthropometric and metabolic characteristics by gender and vitamin D status

Parameter	Males		Females	
	25-hydroxyvitamin D >37.5 nmol/L	25-hydroxyvitamin D ≤37.5 nmol/L	25-hydroxyvitamin D >37.5 nmol/L	25-hydroxyvitamin D ≤37.5 nmol/L
Number	51	89	52	109
Age (years)	12.6±4.3	13.7±3.5	13.3±4.4	14.5±3.3
BMI (kg/m^2)	18.8±3.2	21.8±4.8**	21.0±4.2	21.4±4.3
Systolic blood pressure (mmHg)	112.2±15.4	113.0±12.9	111.3±11.5	113.5±9.6
Diastolic blood pressure (mmHg)	67.1±10.4	67.4±7.9	67.9±7.9	68.3±9.5
HbA1c (%)	9.7±1.2	10.2±1.8	9.8±2.2	10.9±9.9
Triglycerides (mmol/L)	1.0±0.7	1.2±0.5	1.0±0.5	1.3±0.3*
Total cholesterol (mmol/L)	4.4±1.0	4.7±0.94	4.8±0.10	5.0±1.2
HDL-Cholesterol (mmol/L)	1.3±0.4	1.2±0.3	1.2±0.2	1.3±0.3
LDL-Cholesterol (mmol/L)	2.7±0.8	3.2±1.5	3.1±0.8	3.1±0.8
25-hydroxyvitamin D (nmol/L)	51.8±13.5	28.4±6.0**	52.4±17.1	24.4±7.2**
TSH (mIU/L)	2.3±1.9	2.3±2.0	2.3±1.9	2.2±2.2
fT_4 (pmol/L)	12.9±4.5	11.5±5.8	11.9±5.2	12.1±5.0

BMI: body mass index, HbA1c: hemoglobin A1c, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TSH: thyroid stimulating hormone, fT_4: free thyroxine
Data presented as mean ± standard deviation; #denotes non-Gaussian variable; *denotes p<0.05; **denotes p<0.01; significant at p<0.05

Table 5. Significant correlations between 25-hydroxyvitamin D levels and clinical/metabolic parameters

Parameter	Males	Females
Age (years)	-0.13	-0.21*
Age at diagnosis (years)	-0.14	-0.26**
Frequency of hypoglycemia	-0.28**	0.08
BMI (kg/m^2)	-0.23*	-0.14
Diastolic BP (mmHg)	-0.21*	-0.11
Triglyceride levels	-0.35**	-0.38**

BMI: body mass index, BP: blood pressure
Only significant associations were presented. Data presented as coefficient (r);
*denotes p<0.05; **denotes p<0.01; significant at p<0.05

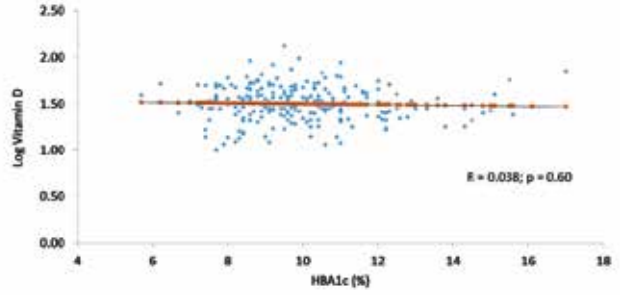

Figure 1. Linear correlation between log vitamin D and hemoglobin A1c

females (23). This can probably explain why there were more inverse cardiometabolic associations elicited in males than the female cohort. Another explanation could be the cardioprotective effect of estrogen inherent in premenopausal women including adolescent girls (24). While vitamin D status does not seem to exert any effect in the glycemic control of our T1DM cohort, the inverse cardiometabolic associations of vitamin D in T1DM males and females warrant recommendations for vitamin D correction in this specific population. The mean age of T1DM diagnosis in Saudi Arabia, specifically in the Western region of the country, is comparatively younger than that reported for European countries, with a median age of 7.2 years in the present study (versus 6.0) (23).

There were modest gender-specific differences in Saudi patients with T1DM, with males having more symptoms than females. With regard to the clinical presentation of subjects, findings of the present study confirm the findings in previous reports from Saudi Arabia, namely, the relatively poorer metabolic control as compared to other populations, as well as the use of multiple daily insulin regimens as the most common therapy for T1DM (25).

In the present study, only 28.6% of toddlers, 15.6% of children, and 12.4% of adolescents were found to have

achieved acceptable HbA1c levels indicating good glycemic control. This is an alarming situation and needs to be addressed aggressively since T1DM patients with poor metabolic control, Arab children in particular, are at highest risk for complications and cardiovascular disorders even at an early age (26,27).

Our study has some limitations. The retrospective design limits the findings to what is available in records. As such, several variables including the autoantibodies were excluded from further data analysis. Furthermore, the single-center approach limits making generalizations from the results of the study. Nevertheless, the sample size is arguably one of the largest cohorts assembled for a T1DM study in Saudi Arabia.

Metabolic control among Saudi children with T1DM is less satisfactory compared to other countries. The high prevalence of vitamin D deficiency in this population and its inverse cardiometabolic associations support the recommendation of vitamin D status correction in T1DM subjects. Further studies in a larger cohort are needed to confirm our findings.

Acknowledgments

The authors are grateful to the staff and faculty of the Pediatric Endocrine section in KAMC-J for their support in data collection.

Ethics

Ethics Committee Approval: The study followed the Helsinki declaration recommendations and was approved by the Institutional Review Board of King Abdullah International Medical Research Center, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Adnan Al Shaikh, Abdullah M. Al Zahrani, Study Design: Adnan Al Shaikh, Abdullah M. Al Zahrani, Data Collection or Processing: Adnan Al Shaikh, Abdullah M. Al Zahrani, Analysis or Interpretation: Adnan Al Shaikh, Abdullah M. Al Zahrani, Literature Search: Adnan Al Shaikh, Abdullah M. Al Zahrani, Writing: Adnan Al Shaikh, Abdullah M. Al Zahrani.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):55-60
DOI: 10.4274/jcrpe.2338

Original Article

Assessment of Anti-Müllerian Hormone Level in Management of Adolescents with Polycystic Ovary Syndrome

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**Prof. Dr. Güven and Dr. Dursun have equal rights in this study.*

ABSTRACT

Objective: This study was oriented to investigate the benefit of anti-Müllerian hormone (AMH) level in the management of polycystic ovary syndrome (PCOS). To assess the impact of metformin and oral contraceptives (OC) on serum AMH levels in a cohort of adolescents with PCOS.

Methods: Forty-nine adolescents with PCOS were recruited to the study. Twenty-nine patients without insulin resistance were treated with OC (group 1), and 20 patients with insulin resistance were treated with metformin and OC (group 2). AMH and androgen levels were measured prior to and 6 months after the initiation of treatment.

Results: AMH levels were significantly decreased with treatment in both group 1 (p=0.006) and group 2 (p=0.0048). There was a significant correlation between pre- and post-treatment AMH and left ovarian volume (pretreatment: rho=0.336, p=0.018; post-treatment: rho=0.310, p=0.034).

Conclusion: This study investigated two different treatment regimens in adolescents with PCOS and revealed that AMH levels decreased with treatment. AMH levels were correlated with ovarian volume.

Keywords: Polycystic ovary syndrome, adolescents, anti-Müllerian hormone, treatment

Conflict of interest: None declared
Received: 14.08.2015
Accepted: 03.11.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Anti-Müllerian hormone (AMH) levels decreased with treatment in adult patients with polycystic ovary syndrome (PCOS).

WHAT THIS STUDY ADDS?

This study revealed that AMH levels decreased with treatment in adolescent patients with PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age (1). PCOS is characterized by hyperandrogenism and oligomenorrhea, and is also highly associated with obesity and insulin resistance (IR) (1). Hyperandrogenism is the key element of the physiopathology responsible for the interruption of

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This manuscript was previously presented as a poster at ESPE 2013 in Milan.

physiologic feedback mechanisms that are fundamental for the establishment of ovulatory cycles, which leads to chronic anovulation. However, primary ovarian dysfunction, and disorders of the production and action of growth factors and anti-Müllerian hormone (AMH) are other mechanisms associated with possible changes in follicular recruitment and development (2).

AMH is produced in the granulosa cells of early developing follicles. AMH expression in the ovary starts at the end of the third trimester of pregnancy, although its levels at birth can be almost undetectable. Serum levels of AMH increase after puberty, probably as a result of follicular growth, and it remains detectable until the end of ovarian activity. Serum levels of AMH were higher in patients with PCOS than in women with normal cycles in several studies (3,4). This hormone is thought to reflect the continuous, non-cycling growth of small follicles in the ovary. The level of AMH is not influenced by fluctuations in other reproductive hormones, and its level does not change throughout the menstrual cycle, which makes it a promising marker (5). Women with PCOS are usually treated with an oral contraceptive (OC), and women with PCOS who are obese and have IR might benefit from treatment with metformin. Treatment with OC is known to normalize menstrual function and to ameliorate hirsutism, although the effects on IR are controversial. There are scant data available on the impact of these treatments on serum AMH levels in women with PCOS. In the studies by Streuli et al (6) and Somunkıran et al (5), AMH levels did not decrease with OC use; conversely, Panidis et al (1) reported reduced AMH levels with OC (35 mg ethinylestradiol and 2 mg cyproterone acetate). Many studies have demonstrated that AMH levels decrease with metformin treatment (3,7). However, to our knowledge, no studies have evaluated AMH levels with metformin and OC treatment in adolescent patients with PCOS.

The objective of this study was to evaluate whether treatment with OC and OC plus metformin would reduce AMH levels in adolescents with PCOS.

Methods

Patients

The study was conducted with 49 adolescent patients (aged 13-17.5 years) who were being followed-up for PCOS in Göztepe Education and Research Hospital, Pediatric Endocrinology Clinic patients who had been recently diagnosed as having PCOS were divided into two groups, those receiving OCs (group 1) and those receiving OCs plus metformin (group 2), and their treatments were evaluated. G power 3.1.4 was used for the sample size, which was calculated as 54 to give a power of 0.95 (1-β probability of error), effect size f: 0.5, and α probability of error: 0.05.

Thirty patients were recruited for each group at the start of the study but 9 were excluded due to treatment incompliance, and 2 due to failure to report for follow-up visits. Thus, group 1 included only 29 patients and group 2 comprised 20. This was planned as a two-stage prospective study; the study compared AMH, androgen levels, and pelvic ultrasonography (USG) before and after treatment, as well as between the two groups.

PCOS diagnosis was based on the Rotterdam (8) criteria (European Society of Human Reproduction and Embryology/American Society of Reproductive Medicine Consensus Workshop group). Accordingly, patients who met at least 2 of the 3 criteria below were included in the study (8). 1) Oligomenorrhea (cycle interval >45 days or amenorrhea (absent menses >3 months; 2) evidence of clinical and/or biochemical hyperandrogenism; and 3) polycystic ovaries (presence of more than 10 follicles with a diameter of at least 2-9 mm and ovarian volume more than 10 cm^3 with USG). In addition, if at least 2 years had passed since the last menstruation, this was also considered as a diagnostic criterion for PCOS (8). Patients who were treated for hirsutism over the past 6 months were excluded. Further exclusion criteria included current chronic disease and drug use. For the differential diagnosis of hirsutism, patients with congenital adrenal hyperplasia, hyperprolactinemia, hypothyroidism, adrenal and ovarian tumors, or Cushing's syndrome were also excluded. The standard adrenocorticotropic hormone test (Synacthen 0.25 mg/1 mL; Novartis Pharma, Rueil-Malmaison, France) was performed to exclude congenital adrenal hyperplasia in all patients with 17-hydroxy-progesterone (17-OHP) levels >2 ng/mL.

Approval from the hospital's Scientific Research Evaluation Commission (approval no. 26.01.2011/9/A) and written consents from the patients and their relatives were received for the study.

Method

Patients who presented to our clinic who had previously been diagnosed as having PCOS were measured pre-treatment and at least 6 months post-treatment (Pt) for AMH, total testosterone, 1,4 androstenedione (AS), dehydroepiandrostenedione sulfate (DHEAS), sex-hormone binding globulin (SHBG), insulin, glucose, prolactin (PRL), luteinizing hormone (LH), and follicle stimulating hormone (FSH) levels. Each patient was evaluated for age, menarche age, body weight, height, body mass index (BMI), hirsutism and menstruation disorders. From these measurements, BMI was calculated using the body weight (kg)/height (m^2) formula. Based on the resulting BMI, patients with values above the 95th percentile of the age- and sex-matched curve were considered as obese in accordance with Bundak et al's (9) data. Ferriman-Gallwey scores (FGS) greater than

>8 were considered as clinical hyperandrogenism (10). All blood samples were collected between the second and fifth days of menstruation, in the morning after an overnight fast. For subjects with oligomenorrhea, blood was collected regardless of day, again in the morning after an overnight fast.

Prior to treatment initiation, each patient underwent oral glucose tolerance tests (OGTT) to investigate IR and to establish whether the patient had type 2 diabetes mellitus (DM). The test was performed by administering 1.75 g/kg (75 g) oral glucose following a 12-hour overnight fast.

OGTT-based diagnoses of IR, glucose tolerance, and type 2 DM were established based on the American Diabetes Association (ADA) criteria (11). Patients with normal OGTT results were started on OC only (ethinyl estradiol 0.03 mg+drospirenone 3 mg) (group 1), while those with impaired glucose tolerance and IR with OGTT were started on an OC and metformin combination (2000 mg/day, 2 doses) (group 2). Clinical and biochemical evaluations were repeated at month 3, and after 6 to 11 months of treatment. Trans-abdominal pelvic ultrasound was used to measure ovarian size and to assess the presence of PCO. All ultrasounds were performed by the same radiologist. Ovarian volume was calculated as V: width (mm) x length (mm) x thickness (mm) x 0.523/1000 (mL). Polycystic ovary was diagnosed if the ovarian size was larger than 10 cm^3 or in the presence of more than 10 peripheral cysts with diameters of 2-9 mm (12,13).

LH (IU/L), FSH (IU/L), total testosterone (TT, ng/mL), DHEAS (DHEASO4, mcg/dL), insulin, and PRL were analyzed at the central laboratory of our hospital using an immunoenzymatic method (device: Beckman Coulter, DXI 800 USA). AS (ng/mL), 17-OHP (ng/mL) were studied using radioimmunoassay with the Immunotech (Beckman Coulter) kit and an ICN ISO DATA Gamma Counter, SHBG (nmol/L) was studied using a chemiluminescence assay with a Siemens kit and Siemens Immulite 2000XPI analyzer at the Gelişim Laboratory.

AMH (ng/mL) was studied by the Gelişim Laboratory using enzyme-linked immunosorbent assay (ELISA) with an Immunotech Gen II (A73818) Beckman Coulter kit and a Pasteur ELISA reader. The minimum sensitivity of the kit was 0.08 ng/mL. The intra-assay variability coefficient was 5.4% for 4.42 ng/mL and 3.6% for 14.03 ng/mL, and the inter-assay variability coefficient was 5.6% for 4.42 ng/mL and 4.5% for 14.03 ng/mL.

Statistical Analysis

The results were transferred to 'Statistical Package for the Social Sciences 15.0 for Windows' software, which was used for the descriptive analyses of the study group. The Shapiro-Wilk test was used to establish whether the data were normally distributed. Insulin, Pt-insulin, Pt-LH, Pt-FSH, Pt-E2, Pt-SHBG, Pt-right ovary, and Pt-left ovary without normal distribution were expressed as median (IQR) and other data with normal distribution were expressed as mean ± standard deviation values.

The pre- and Pt data differences between groups 1 and 2 were studied using the independent samples t-test for data with normal distribution and Mann-Whitney U test for data without normal distribution.

The differences between pre- and Pt measurements of patients in groups 1 and 2 were calculated separately using the Paired Sample test for data with normal distribution and Wilcoxon's signed-rank test for data without normal distribution. The difference in AMH values before and after treatment was designated as delta AMH. Correlations between the data were calculated using Spearman's correlation (rho and p provided). The results were expressed with 95% confidence interval and statistical significance was set at $p<0.05$.

Results

The clinical characteristics of the study population are summarized in Table 1. With the exception of SHBG, which was significantly higher in group 1, groups 1 and 2 did not differ significantly in respects to pre-treatment LH, FSH, E2, AMH, AS, TT, and DHEAS. Following treatment, no significant differences were noted between the groups regarding hormonal values or AMH (Table 1).

Patients' pre- and Pt AMH and other androgen levels were compared between the groups (Table 1), and significant decreases of AMH only were observed for both groups (Figure 1).

AMH serum levels were significantly decreased in group 1 from 6.7±3.7 ng/mL at baseline to 4.3±3.1 ng/mL after 6 months of treatment (p=0.006). In group 2, AMH serum levels ranged from 4.7±3.07 ng/mL at baseline to 3.1±2.05 ng/mL after 6 months of treatment (p=0.048).

Delta AMH was used to determine whether AMH reductions were significant between the groups; no significant difference was found (p=0.315, t=1.017). SHBG increased significantly in both groups. TT levels decreased in both groups but without statistical significance. AS decreased significantly in group 2 (p=0.02).

Evaluation of correlations between AMH, androgens, insulin, FGS and ovarian volumes did not yield any correlations between AMH, FGS, and androgens. There was only a significant correlation between AMH and left ovarian volume (rho=0.336, p=0.018) (Figure 2). DHEAS was correlated with AS (rho=0.347, p=0.026) and TT (rho=0.775, p<0.001).

Evaluation of correlations between Pt FGS, AMH, androgens, insulin, and ovarian volume demonstrated a correlation between AMH and left ovarian volume (rho=0.310, p=0.034). There was no correlation between Pt AMH, FGS, and androgens.

Table 1. Clinical and hormonal characteristics of the patients with polycystic ovary syndrome at baseline and after-treatment

	Group 1			Group 2		
	Before treatment	**After treatment**	**p**	**Before treatment**	**After treatment**	**p**
Age (years)	15.6±1.3			15.2±1.4		
BMI (kg/m^2)	23.5±3.9	23.3±3.8	0.17	30.6±4.8	28.3±4.2	0.23
FGS	14±5.8	9.4±4.6	0.29	15.5±9.2	10.4±5.2	0.016
FSH (IU/L)	5.7±1.8	3.2 (7.2)*	0.014	5.8±1.5	2.6 (2.2)*	0.002
LH (IU/L)*	6.4 (7.4)	2.3 (5)	0.006	6.3 (9)	1.7 (2.6)	0.003
FG (mg/dL)	84.9±9.1	83.8±7.6	0.32	77±11.2	83.6±9.5	0.35
Insulin (µU/mL)*	8.7 (3.9-20)	8.3 (3.4)	0.60	15.9 (5.7-64)	10.7 (11.6)	0.198
AMH (ng/mL)	6.7±3.7	4.3±3.1	0.006	4.7±3.07	3.1±2.05	0.048
TT (ng/mL)	0.51±0.2	0.38±0.13	0.25	0.46±0.16	0.37±0.14	0.22
AS (ng/mL)	2.8±1.5	1.9±0.99	0.62	2.9±1.5	2.1±1.26	0.02
DHEAS (mcg/dL)	229±46	191±103	0.051	209±101	200±81	0.50
SHBG (nmol/L)*	38±14.8	180 (158)*	0.004	27.9±10.7	190 (131)*	0.002
ROV (mL)	11.3±3.4	5.3 (5.2)*	<0.001	8.6±3.3	5.9 (4)*	0.085
LOV (mL)	9.3±4	4.9 (3.7)*	<0.001	8.4±3.1	6.2 (3.8)*	0.048

*Without normal distribution. BMI: body mass index, FGS: Ferriman-Gallwey score, FSH: follicle stimulating hormone, LH: luteinizing hormone, FG: fasting glucose, AMH: anti-Müllerian hormone, TT: total testosterone, AS: 1,4 androstenedione, DHEAS: dihydroepiandrostenedione sulfate, SHBG: sex hormone binding globulin, ROV: right ovarian volume, LOV: left ovarian volume

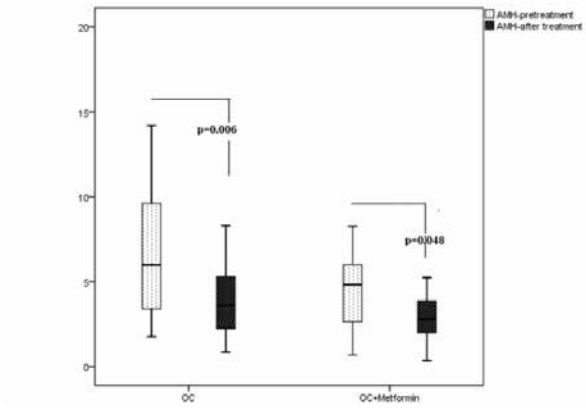

Figure 1. Box and whisker plots depicting the pre and post-treatment of serum anti-Müllerian hormone levels in patients of group 1 and group 2. Solid lines inside boxes depict the median anti-Müllerian hormone level, whereas the upper and lower limits of the boxes and whiskers indicate 75th, 25th, and 95th, and 5th percentiles. AMH: anti-Müllerian hormone, OC: oral contraceptive

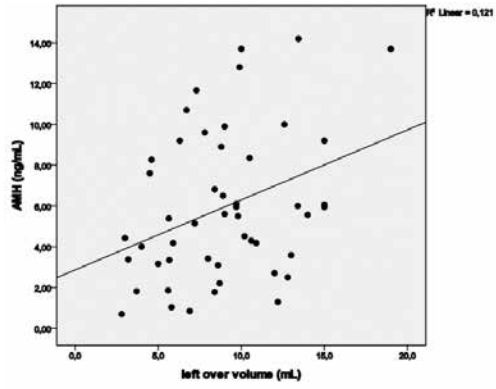

Figure 2. Relationships between serum anti-Müllerian hormone level and total left ovarian volume in patients with polycystic ovary syndrome. AMH: anti-Müllerian hormone

Discussion

This study aimed to investigate whether AMH was a good indicator for treatment and monitoring of adolescent patients with PCOS. For this purpose, levels of AMH and other androgens were studied in patients who were newly diagnosed as having PCOS, after which the patients were divided into two groups to investigate changes in AMH levels in groups and treated with OC or OC plus metformin.

Several studies have demonstrated increased levels of AMH in adult women and adolescent girls with PCOS (3,4,14,15,16). However, few studies have shown decreased levels of AMH, like other androgens, with metformin treatment and OC in adult women with PCOS (3). Moreover, no studies have reported a relationship between OC or OC plus metformin treatment and AMH levels in adolescents with PCOS. Therefore, our study is the first in the literature.

Studies on the relationship of AMH with insulin in PCOS are contradictory. Some studies found no correlation between insulin and AMH levels (17), whereas others identified a positive relationship between them (18). Tomova et al (19)

found no relationship between AMH and insulin although a positive correlation between insulin and androgens was found. Pigny et al (4) identified no relationships. This is supported by the fact that there were no relationships between BMI and AMH in the present study. There was a negative relationship between AMH and insulin in our study but no relationship between BMI and AMH was found. Insulin may lead to hyperandrogenism by reducing SHBG production in the liver and also by increasing the amount of circulating free testosterone, a biologically active androgen (20). Likewise, our patients in the IR group had significantly lower SHBG compared with group 1.

The relationship between AMH and ovarian volume has been investigated in several studies because attempts are being made to ascribe AMH elevations in patients with PCOS to increased numbers of small follicles. A positive relation between AMH and mean ovarian volume was found in the study of Li et al (21). Healthy adolescents without PCOS but with polycystic ovary morphology also had higher AMH levels (22), which indicated that AMH was related to an increased follicle count. Hart et al (23) failed to demonstrate in a general adolescent study population that serum AMH was a reliable predictor of PCO morphology or for the presence of PCOS. However, we found a significant relationship between pre- and Pt AMH levels and left ovarian volume. Additionally, reductions in right and left ovarian volumes with treatment were noted.

Piltonen et al (3) were the first to demonstrate reduced levels of AMH with metformin treatment in adult women with PCOS. In the study by Tomova et al (19), 17 patients with PCOS were treated with metformin at 2550 mg/day and at the end of 6 months, correction of irregular menstruation and reduced AMH levels were shown in 13 patients; the remaining 4 had no clinical improvement and their AMH levels increased. Nascimento et al (2) observed significant reductions in insulin and testosterone with no changes in AMH levels. In the study by Somunkiran et al (5), AMH levels of 30 adult patients with PCOS were measured before and after 6 months of treatment with OC (35 mcg ethinyl estradiol+2 mg cyproterone acetate), with no significant changes detected; however, reduction in ovarian volume and follicle count were observed. Panidis et al (1) randomized adult subjects with PCOS into 3 groups and administered an OC regimen to group 1 (35 mcg ethinylestradiol+2 mg cyproterone acetate), another OC regimen to group 2 (30 mcg ethinylestradiol+3 mg drospirenone), and metformin to group 3 (2x850 mg). AMH levels decreased significantly only in group 1 with no significant changes in the other two groups. These are studies with adult subjects with PCOS but no studies in the literature have demonstrated AMH changes in adolescent subjects with PCOS. In the present study, we investigated changes in AMH levels with OCS and whether changes in AMH levels would differ in adolescent PCOS patients with IR when given OC plus metformin versus OC alone. AMH levels were significantly reduced in both groups but without a significant difference between the groups; metformin did not induce an additional alternation in AMH levels.

We did not measure free testosterone because free testosterone measurements are not very reliable, thus we preferred to measure total testosterone. TT levels decreased in both groups but without statistical significance. In our study, we found no correlation between AMH level and FGS or testosterone levels. In group 1, where FGS was not statistically significant, there was a decline on AMH and ovarian volumes. This finding indicates that the decline in AMH was independent of hyperandrogenism and was mostly related with ovarian volumes. Rosenfield et al (24) showed that AMH levels were independently related to ovarian androgenic function. In the absence of hyperandrogenism, moderate AMH elevation in women with normal-variant polycystic ovaries seems to indicate an enlarged oocyte pool (24).

One of the weaknesses of our study was that it did not incorporate a patient group treated with metformin alone because it was not possible to distinguish which agent was more effective in reducing AMH levels in group 2, although it is likely that AMH levels were decreased with OC only. A second weakness is the lack of a control group. This was because the approval of the local ethics board could not be obtained for healthy controls. Another limitation of our study was the inadequate number of patients.

In conclusion, the present study demonstrated that treatment reduced AMH levels in adolescents with PCOS and it was not associated with hyperandrogenism. AMH correlated with ovarian volume and both AMH and ovarian volume decreased after treatment. We think that AMH could be used instead of transabdominal pelvic USG, independent of hyperandrogenism. AMH seems to be a good parameter for monitoring adolescent patients with PCOS because it is easy to measure at any period during the cycle. However, in the absence of adequate relevant studies, larger studies are needed.

Ethics

Ethics Committee Approval: Medeniyet University Göztepe Training and Research Hospital (Approval number: 26.01.2011/9/A),

Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Ayla Güven, Fatma Dursun, Design: Fatma Dursun, Ayla Güven, Data Collection or Processing: Fatma

Dursun, Ayla Güven, Metin Yıldız, Analysis or Interpretation: Fatma Dursun, Ayla Güven, Literature Search: Fatma Dursun, Metin Yıldız, Writing: Fatma Dursun.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):61-66
DOI: 10.4274/jcrpe.2406

Original Article

Evaluation of Iodine Deficiency in Children with Attention Deficit/ Hyperactivity Disorder

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ABSTRACT

Objective: To investigate the incidence of iodine deficiency (ID) and its effects on mental function in children referred to the Dr. Sami Ulus Maternity and Children's Training and Research Hospital with a prospective diagnosis of attention deficit/hyperactivity disorder (ADHD).

Methods: The study was conducted on 89 children referred in the period from September 2009 to June 2010 with a diagnosis of ADHD. A questionnaire was given to all parents. Conners' rating scales were applied to the parents (CPRS) and teachers (CTRS), and revised Wechsler intelligence scale for children (WISC-R) to the children. Serum thyroid-stimulating hormone, free triiodothyronine and free thyroxine, thyroglobulin, anti-thyroid peroxidase, anti-thyroglobulin, and urinary iodine levels were measured in all children.

Results: Median age was 9.41±1.95 years, and 83.1% of subjects were male. The mean urinary iodine level of the children was 92.56±22.25 μg/L. ID was detected in 71.9% of subjects and all were mild ID. There was no significant relationship between urinary iodine levels with WISC-R subtest scores and CPRS. However, a significant association was found between urinary iodine levels and hyperactivity section of CTRS ($p<0.05$). Likewise, a significant relationship was found between learning disorder/mental retardation diagnosis and freedom subtest of WISC-R ($p<0.05$).

Conclusion: This study highlights the effects of ID on comprehension, perception, attention, and learning. However, the results need to be supported by new randomized controlled trials.

Keywords: Iodine deficiency, attention deficit/hyperactivity disorder, children

Conflict of interest: None declared
Received: 09.09.2015
Accepted: 02.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Studies in recent years suggested that moderate iodine deficiency (ID) negatively affects the cognitive development even in children with normal thyroid hormones, and may be the cause of decline in school performance. However, the mechanism of cerebral damage due to ID has not been elucidated.

WHAT THIS STUDY ADDS?

This study, which has not been made previously in this regard to our knowledge, is important to highlight the effects of ID on comprehension, perception, attention and learning, which are less known and less conspicuous signs of ID. However, the results should be supported by new randomized controlled trials in order to contribute to clinical applications.

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Introduction

Iodine is an essential element in the synthesis and regulation of thyroid hormones that are required for normal metabolic functions (1). Iodine deficiency (ID) may affect individuals of all ages and is the most common cause of preventable mental retardation, infants being at most risk during the fetal period (2,3). In a state of iodine insufficiency, the synthesis of thyroid hormones is impaired and major health problems ensue. This group of diseases is known as ID disorders (4). While goiter is a well-known symptom of ID disorders, learning, perception, and comprehension disorders also develop in these patients, but these aberrations may at times be difficult to detect in school children and in adults (4,5). The majority of children with ID disorders brought to psychiatric outpatient units by their families or referred to these units by their teachers present with complaints of lack of attention and consequent failure (6). In a substantial portion of these children, attention deficit/hyperactivity disorder (ADHD) needs to be considered in the differential diagnosis (7). ADHD is a disorder in the etiology of which neurological, genetic, environmental, dietary, biological, and psychosocial factors are possibly involved (8,9).

In this present study, we aimed to investigate the incidence of ID in a group of children diagnosed with ADHD and its effect on mental functions such as perception, attention, comprehension, and learning.

Methods

Eighty-nine school-age children who were referred to the psychiatric outpatient unit of Dr. Sami Ulus Maternity and Children's Training and Research Hospital with a diagnosis of ADHD in the time period from September 2009 to June 2010 were prospectively evaluated. Patients with a history of chronic systemic disease, neurological deficit, and chronic drug usage were excluded. None of the patients had a history of identified TSH abnormality by neonatal screening, or a previous history of goiter or an endocrine disease. Ethical approval was obtained for the study as well as verbal and written consent from the families after detailed information was given to them about the subject and purpose of the research. All parents were subjected to a questionnaire which included descriptive information about the child and the family, iodized salt usage status, and the state of awareness of the parents about iodized salt. ADHD and other possible additional disorders such as oppositional defiant disorder (ODD), conduct disorder (CD), and learning disorders/mental retardation (LD/MR) were evaluated according to Diagnostic and Statistical Manual of Mental Disorders-IV criteria (10). At the beginning of the study, both the parents (CPRS) (11) and the teachers (CTRS) (12) of the children were subjected to the Conners' rating scale in order to examine the developmental processes of ADHD. Revised Wechsler intelligence scale for children (WISC-R) (13), which is the only standardized intelligence assessment test in Turkey, was used to assess the mental condition of the children. The crude scores from the test sections were translated into standard scores according to the age of the children and assessed with improved intelligence quotient (IQ) tables. Anthropometric measurements and physical examination of the thyroid gland were performed in all children.

Serum thyroid-stimulating hormone (TSH), free triiodothyronine (fT_3) and free thyroxine (fT_4) (all listed before; chemiluminescence method, Immulite BioDPC, Los Angeles), thyroglobulin (Tg), anti-thyroid peroxidase (anti-TPO), and anti-Tg levels (using the electro-chemiluminometric assay method, Abbott Architect, USA) were determined in venous blood samples in all patients. Urinary iodine levels in a morning spot urine sample were also measured using the (inductively-coupled plasma mass spectrophotometer method). Evaluation of iodine excretion in the urine was performed according to the World Health Organization (WHO) criteria (14) (mild [50-99 µg/L], medium [20-49 µg/L], severe [<20 µg/L], normal [100-200 µg/L], and higher than normal [>200 µg/L]). Thyroid gland palpation was performed in all patients and the results based on WHO goiter staging were recorded (3). Thyroid ultrasonography by an experienced radiologist was performed in all patients in whom goiter had been detected.

Statistical Analysis

The Statistical Package for the Social Sciences version 16.0 (SPSS Inc., Chicago, IL, USA) was used in the evaluation of the data. Arithmetic average of the values (x), standard deviations (SD), and significance levels (p-values) were shown. Data of the questionnaire specified by count were evaluated as number and percentage. Categorical variables were compared using the chi-square test. Comparison of continuous variables in the groups with and without ID was made by one-way variance analysis. The relationship between rating scores obtained from parents/teachers and the urine iodine levels were assessed by Pearson's correlation coefficient. A p-value of 0.05 was considered statistically significant.

Results

Descriptive Characteristics

The age of children ranged from 6 to 15.5 years (mean 9.41±1.95), and 83.1% (74/89) were male (male:female t-ratio 4.9). Mean height standard deviation score (SDS) of the group was 0.16±1.02 and mean SDS for body weight was 0.07±1.09. The majority of the families were from central and northern parts of Turkey (84.26%). The rate of families living in central Turkey was 89.9%, and 83.1% of them were living in a city center. The majority of parents (44.95%) had completed compulsory basic education. Only 7.85% had completed

high school. Most families were of a middle (49.43%) or low (41.57%) socioeconomic status.

Iodized Salt Usage and State of Consciousness

According to the questionnaire results, 96.6% of the households were using iodized salt and rock salt was consumed by all non-iodized salt users. Salt was stored in a glass lid jar (46.06%) or an opaque lid jar (31.46%). Salt was generally added to the food during cooking (71.91%).

Goiter, Thyroid Tests and Urine Iodine Levels

Goiter was detected in 5.61% (5/89) of cases and showed different stages (stage Ia in 1 of the 5 cases [ID-related], stage Ib in 2 [Hashimoto thyroiditis in one, normal thyroid volume by ultrasonography in one], and stage II in 2 cases [ID-related]) by palpation. TSH, fT_3 and fT_4, Tg, thyroid auto-antibody levels, and urine iodine levels of the patients are given in Table 1. A high TSH level was detected in 2.2% of the subjects. fT_3 and fT_4 levels were within normal limits in all subjects. All detected ID cases were in the mild ID group.

In the comparison of the groups with normal iodine levels and those with mild ID, no significant relationship was found with place of residence or of educational level of the families ($p>0.05$). Also, no correlation was found between ID with salt type used or with time of salt adding to foods ($p>0.05$).

Attention Deficit/Hyperactivity Disorder Diagnosis, Revised Wechsler Intelligence Scale for Children Test and Conners' Scales

The distribution of ADHD subtypes was as follows: ADHD-combined type was seen in 83.1% of patients, ADHD-predominantly inattentive type- in 10.1%, and ADHD-predominantly hyperactive-impulsive type was found in 6.7% of subjects. Rates of additional diagnosis were as follows: only one additional diagnosis in 30.7% of patients, two additional diagnosis in 27.3%, three additional diagnosis in 9.1%, and no additional diagnosis in 32.9%. Anxiety was present in 13/89 (14.6%), LD or MR in 26/89 (29.21%), and ODD/CD in 33/89 (37.07%).

Table 1. Iodine status of the children (n=89)

	Normal values	Mean ± SD (min-max)
TSH (μIU/mL)	0.6-4.5	2.15±1.14 (0.59-9.1)
fT_3 (pg/dL)	2.0-6.5	4.77±0.69 (2.12-6.49)
fT_4 (ng/dL)	0.8-1.9	1.26±0.16 (0.95-1.68)
Tg (ng/mL)	0-60	15.49±14.25 (0.20-113.1)
Urinary iodine level (μg/L)	100-200	92.56±22.25 (61-147)
	n	**%**
High anti-TPO	3	3.37
High anti-Tg	1	1.12
Iodine deficiency	64	71.91

TSH: thyroid-stimulating hormone, fT_3: free triiodothyronine, fT_4: free thyroxine, Tg: thyroglobulin, anti-TPO: anti-thyroid peroxidase, SD: standard deviation

Mean scores of the WISC-R subtests were as follows: information 6.25±2.72 (1-14), similarities 7.35±3.64 (0-19), arithmetic 7.62±2.66 (1-12), block design 8.60±2.54 (3-18), freedom 23.53±6.04 (8-35), verbal IQ 81.09±14.65 (50-114), performance IQ 88.94±14.53 (51-127), and total score 84.01±13.60 (51-118). Mean scores of CPRS were as follows: hyperactivity (HA) section 8.68±2.82 (3-12), attention deficit (AD) section 7.66±3.04 (1-14), and behavior problems (BP) section 13.67±8.86 (1-52). And mean scores of CTRS were resulted in HA as 10.68±3.88 (1-17), in AD 13.84±4.57 (6-23), and in BP 7.96±4.34 (0-15). Comparison of urinary iodine levels of patients with Conners' and WISC-R results are given in Table 2. In correlation analysis of the CPRS and CTRS scores with urinary iodine levels, there was a significant negative correlation in HA section of CTRS ($p=0.038$) with urinary iodine levels (Figure 1), but there was no significant result in other sections. Comparison of freedom section scores of WISC-R and HA section scores of CTRS with the age, gender, and additional diagnosis are shown in Table 3.

Table 2. Comparison* of urinary iodine levels with Conners' scales and revised Wechsler intelligence scale for children results of children

Subtest	Urinary iodine status (n=89)		p-value
	Normal (n=25) mean ± SD	Iodine deficiency (n=64) mean ± SD	
WISC-R subtests			
Information	6.33±1.87	6.23±2.93	0.904
Similarities	8.28±3.26	7.09±3.73	0.284
Arithmetic	7.85±2.31	7.56±2.76	0.716
Block design	8.14±2.47	8.72±2.57	0.447
Freedom	24.15±3.55	23.38±6.53	0.685
Verbal IQ	84.05±9.20	80.18±15.92	0.344
Performance IQ	91.70±10.69	88.09±15.51	0.374
Total	86.70±8.46	83.18±14.80	0.354
CTRS			
HA	9.10±3.49	11.20±3.88	0.04**
AD	14.10±4.60	13.75±4.60	0.777
BP	7.47±3.77	8.12±4.53	0.576
CPRS			
HA	8.0±3.27	8.94±2.63	0.193
AD	7.71±3.08	7.64±3.05	0.934
BP	12.09±7.66	14.27±9.27	0.342

WISC-R: revised Wechsler intelligence scale for children, IQ: intelligence quotient, HA: hyperactivity, AD: attention deficit, BP: behavior problems, CTRS: Conners' teacher rating scale, CPRS: Conners' parent rating scale, SD: standard deviation

*Pearson's correlation coefficient was applied, **was considered significant because of $p<0.05$

Table 3. Comparison of freedom section scores of revised Wechsler intelligence scale for children and hyperactivity section scores of Conners' teacher rating scale with the age, gender and additional diagnosis of children (n=89)*

Dependent variable	Urinary iodine level	Gender	Age	Additional diagnosis		
				Anxiety	LD/MR	ODD/CD
	p values					
Freedom	0.651	0.075	0.277	0.193	0.001**	0.520
HA section of CTRS	0.057	0.532	0.003**	0.421	0.608	0.761

LD/MR: Learning disorder/mental retardation, ODD/CD: oppositional defiant disorder/conduct disorder, HA: hyperactivity,
CTRS: Conners' teacher rating scale
*Regression analysis was applied, **was considered significant because of p<0.05

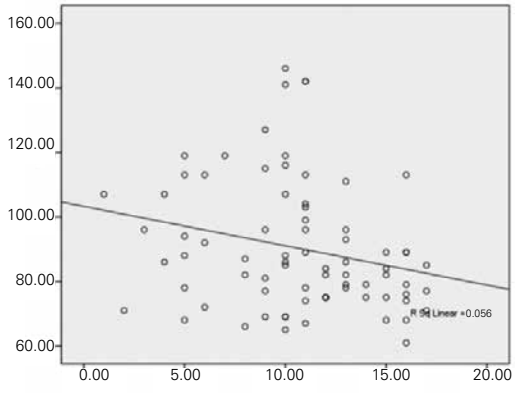

Figure 1. The relationship between hyperactivity section scores (horizontal values) of Conners' teacher rating scale and urinary iodine levels (vertical values [µg/L])

Discussion

Iodine is required in the synthesis and regulation of thyroid hormones and it is known that these hormones have an essential importance for normal metabolic functions. Iodine is an element which should be ingested regularly and in sufficient amounts at all ages. It is not stored in the human body (1,15). About 25% of the world population, including the Turkish population, is faced with ID, a serious public health problem (2). Low intelligence test scores, decrease in intellectual level, deficiency in visual perception and visual motor coordination, speech disorders, incompetence in fine motor skills, balance disorders, isolated deafness, neurological symptoms such as irregular electroencephalogram (EEG) findings were reported in individuals living in ID regions (16,17,18).

Deficiency in intellectual functions varies depending on the time, duration, and degree of exposure to ID. Effects of ID on brain function at various stages of human life has been shown in several studies (4,5). By way of example, the most significant impacts of endemic cretinism on intelligence components are low intelligence and deterioration in visual perception. It has been shown that neurological abnormalities increase and overall intelligence quotients decrease in children exposed to ID, especially in those exposed in the neonatal period (19,20). Studies in recent years suggest that moderate ID negatively affects the cognitive development even in children with normal thyroid hormone levels and may be the cause of decline in school performance (21,22). However, the mechanism of cerebral damage due to ID has not been elucidated.

Tiwari et al (16) investigated the effect of prolonged ID on lack of attention, poor motivation, and learning disorders and reported that while visual learning and memory are affected by severe ID, moderate ID affects motivation more than severe ID does. In another study which aimed to examine the benefits of iodine support in children with euthyroid ID, significant differences in cognitive and motor skills were not detected in repeated tests after iodine supplementation (21). In contrast, improvement in mental function and intelligence scores was reported after iodine supplementation in some similar studies (22,23,24). In an interesting study from Italy, regardless of the iodine levels of the children, ADHD has been claimed to be a syndrome caused by a generalized resistance to thyroid hormone and associated with maternal hypothyroxinemia related to ID in the early gestational period (5). In a systematic review and meta-analysis, technical and methodological limitations of the previous studies on this issue were highlighted, indicating the need for new studies (25). In our study, a significant relationship between ID and verbal performance or performance in other areas of intelligence was not detected. We are aware that lack of a control group is a limitation of our study. New controlled trials including a greater number of patients with moderate or severe ID are needed in order to yield more accurate results. However, we think that the negative correlation we found between urinary iodine levels and HA symptoms requires attention as an important finding in ID-related disorders.

Our study was conducted in an age-compliant target group using a screening method in accordance with WHO

recommendations for determining the prevalence of ID (26). Although it is known that the prevalence of goiter is relatively high in school-age Turkish children (27), there are no detailed studies showing the effects of iodine levels on mental functions. Our study was conducted on a limited patient group with disproportionate gender distribution and patient density from the city center. These limitations also need to be considered in the assessment of the study. On the other hand, our study shows that, despite the high rate of iodized salt usage, there is a lack of awareness in Turkish families on the correct storage and usage of iodized salt (2,3). This fact may have caused the high levels of ID in our patients despite the high rate of iodized salt usage. Our results also show that the growth of our patients had not be affected by ID. However, it should be noted that all of our patients detected to have ID were in the mild ID group and short stature is an expected finding in severe ID (3).

The results of our study were similar to reported literature as to sex distribution (28), ADHD subtypes, and additional diagnosis (29,30). Considering the scores of each section of Conners' rating scales, there is an incompatibility between reported symptoms by the teachers and parents. More precise and accurate observations made by teachers than parents are not surprising because of the low socioeconomic status and low educational levels of the families. Additionally, the results of our study about performance and verbal IQ scores of WISC-R include incompatibility with previous studies (31). However, if the results are analyzed in the light of information that children with ADHD had low scores from the subtests of freedom (the sum of the arithmetic, password and number series sections; also an indicator of distractibility of the attention), cube pattern and image editing of WISC-R, our results are consistent with the literature (32,33).

Unlike other previous studies such as screening of ID in the general population and in school-age children or assessment of the intelligence functions in ID groups, our study aimed to measure actual incidence of ID in lack of attention, comprehension, and perception as well as to assess the effect of ID on learning disorders. This study is important because the effects of ID in children with ADHD have not been examined in previous studies. In our study, a significant negative correlation was detected in HA section of CTRS (p=0.038) with urinary iodine levels. Additionally, statistically significant relationships were determined between age and HA section of CTRS as well as between freedom subtests of WISC-R and additional diagnosis of LD/MR. However, due to the lack of a control group, the confirmation of the detected results is not possible at this stage.

We believe that our results need to be confirmed by randomized controlled studies involving large patient series.

Ethics

Ethics Committee Approval: Dr. Sami Ulus Maternity and Children's Training and Researh Hospital Ethics Committee (Approval number: 06.2009/049), Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Saliha Kanık Yüksek, Design: Saliha Kanık Yüksek, Özgür Öner, Data Collection or Processing: Saliha Kanık Yüksek, Analysis or Interpretation: Özgür Öner, Saliha Kanık Yüksek, Literature Search: Saliha Kanık Yüksek, Zehra Aycan, Writing: Saliha Kanık Yüksek.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):67-73
DOI: 10.4274/jcrpe.2051

Original Article

Health-Related Quality of Life and Metabolic Control in Children and Adolescents with Type 1 Diabetes Mellitus

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ABSTRACT

Objective: The burdens imposed on a child and his/her parents by a diagnosis of type 1 diabetes mellitus (T1DM) adversely affect their health-related quality of life (HRQoL). HRQoL is important for prognosis and is related to metabolic control. To evaluate the HRQoL of Turkish children and adolescents with T1DM and to assess the correlation of HRQoL subscales (including physical and psychosocial health) with metabolic control, and particularly with hypo- and hyperglycaemic episodes.

Methods: This cross-sectional study included 70 participants with T1DM aged between 8 and 18 years (study group) and 72 healthy controls who were matched to the study group in terms of age, gender, and sociodemographic characteristics (control group), and their parents. HRQoL was determined by the Pediatric Quality of Life Inventory. As an indicator of metabolic control, the most recent hemoglobin A1c (HbA1c) levels were obtained and the number of hypo- and hyperglycaemic episodes over the past one month were checked.

Results: The study group had similar HRQoL scores for children's self-reports and parents' proxy-reports to the control group apart from a decreasing psychosocial health score for parents' proxy-reports in the study group. Although HbA1c level was not related to HRQoL scores, lower number of hypo- and hyperglycaemic episodes were associated with an increase in psychosocial health scores and physical health scores as well as an increase in the total score for parents' proxy-reports.

Conclusion: Although there was no correlation between metabolic control and HRQoL in children's self-reports, the improving HRQoL levels in parents' proxy-reports were associated with good metabolic control.

Keywords: Child, adolescent, type 1 diabetes mellitus, quality of life, nutrition

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Health-related quality of life (HRQoL) is important for prognosis and is related to metabolic control of type I diabetes mellitus.

WHAT THIS STUDY ADDS?

The higher scores of HRQoL subscales including physical and psychosocial health are associated with metabolic control, particularly hypo- and hyperglycaemic episodes, in Turkish children and adolescents.

Conflict of interest: None declared
Received: 09.04.2015
Accepted: 05.10.2015

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Introduction

Diabetes mellitus is a group of metabolic diseases characterised by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (1,2). Type 1 diabetes mellitus (T1DM) continues to be the main type of diabetes encountered in children and adolescents. Over 85% of all diabetes cases in individuals aged <20 years worldwide are T1DM (3). Being diagnosed with T1DM permanently changes the life of children and adolescents. Treatment has some requirements such as frequent insulin injections, daily blood glucose monitoring, diet plan, and regular physical activity. Also, acute and chronic complications related to diabetes may occur. All of these factors can adversely affect the health-related quality of life (HRQoL) in children and adolescents with T1DM (4).

HRQoL is a multidimensional concept including well-being in terms of patient's physical, emotional, mental, and social behaviours and is defined as the way the effects of a disease and/or its treatment are perceived by the patient (5,6). Well-being can be described in different forms by individuals, and the disease process may also be experienced differently. When evaluating quality of life, it should be considered that there are objective and subjective areas of HRQoL. Two people in the same situation objectively may have different perceptions of their HRQoL subjectively (7). Some researchers suggest that subjective assessment is more valuable because it reflects self-perception about the situation of individuals. However, other researchers indicate that parental forms are more relevant because of objective consequences (7,8,9). Therefore, the evaluation of HRQoL perceived by parents as well as by the children is important to understand the children's and adolescents' HRQoL correctly.

The aims of this study were to evaluate the HRQoL of Turkish children and adolescents with T1DM as perceived by both child and parent, and to assess the correlation of HRQoL subscales (including physical health and psychosocial health) with metabolic control, and particularly with hypo- and hyperglycaemic episodes.

Methods

This cross-sectional study was conducted between September 2012 and February 2013 on patients attending the Pediatric Endocrinology Outpatient Clinic at Erciyes University Hospital. The study was performed in accordance with the declaration of Helsinki, after informed oral and written consent was obtained from the parents. It was approved by the University of Erciyes Clinical Research Ethics Board (date: 07.08.2012 and reference number: 2012/479).

Sample size was decided as a distinction between intergroup scale scores ≥5 points, statistical power= 0.80 and fallibility= 0.05. The study group consisted of 72 children and adolescents aged between 8 and 18 years diagnosed with T1DM at least 1 year previously and using multiple daily injection insulin therapy and one parent of each child. Exclusion criteria were mental retardation of a severity that made communication difficult and/or having another chronic disease such as coeliac disease, hypothyroidism, etc. In addition, two children with T1DM were excluded from the study because of missing data. The control group consisted of 72 healthy children and adolescents, who were matched to the study group in terms of age, gender and sociodemographic characteristics, and the parents of these children/adolescents. All subjects in the study and control groups were recruited from primary and high schools.

Body weight and height were measured by an experienced dietician, and body mass index (BMI) was calculated with the "weight (kg)/height (m)^2" equation (10). The measurements were made with the participants in their underwear, without shoes, standing erect, and placing their head in the Frankfurt plane. An automatic height gauge scale (DENSİ GL150, İstanbul) (11) sensitive to 10-200 kg±50 g and 90-200 cm±1 mm was used for the measurements.

Demographic data including age, gender, socioeconomic status, parents' education and job, breast feeding duration, eating habits, and physical activity level were collected via a face-to-face interview and recorded on a pre-prepared form. Physical activity level was assessed by the following questions: "Do you perform a physical activity regularly?", "Define the nature, frequency, and duration of this activity", and "How many times in the past 7 days did you exercise or participate in sports activities which made you sweat and breathe hard for at least 20 minutes?" (12). Data on diabetes-related information such as age at diagnosis, duration of diabetes, total length of stay in hospital due to diabetes, most recent hemoglobin A1c (HbA1c) value, and the number of hypo- and hyperglycaemic episodes over a period of one month were also collected during these interviews.

The HRQoL levels of the study and control groups were determined by the Pediatric Quality of Life Inventory™ (PedsQL™) 4.0 Generic Core Scale (13). The validity and reliability of the scale have been tested for Turkish children (7,14). Quality of life is evaluated by the PedsQL™ 4.0 Generic Core Scale in four areas: physical function (eight items), emotional function (five items), social function (five items), and school function (five items). However, three scores are derived from this scale: total score and two subscales' scores including physical health and psychosocial health covering emotional, social, and school function. When calculating scores, each item is given a score from 0 to 100. Subscales or total scores are calculated by adding the score of each item in a section or total and dividing by the total number of items in the section or total. As the score increases, HRQoL improves. The PedsQL™ 4.0 Generic Core Scale also has appropriate forms for children's self-reports and parents' proxy-reports.

After being given information about the purpose and content of the study, participants' demographic characteristics, eating habits, and physical activity status were determined by a researcher using the above-mentioned questionnaire. Children's and adolescents' body weight and height were measured. Participants' HRQoL levels were assessed using the PedsQL™ 4.0 Generic Core Scale (7,14). Various studies have indicated that the assessment of both parent and child should be considered to understand a child's HRQoL accurately (7,8,9). Therefore, in this study, the PedsQL™ 4.0 Generic Core Scale was applied to one of the parents as well as their offspring.

In the study group, diabetes-related information including the age at diagnosis, duration of diabetes, and total length of stay in hospital due to diabetes were examined. As an indicator of metabolic control, the most recent HbA1c level was obtained from the medical records of the children. These values were classified as <7.5% optimal, 7.5-9.0% suboptimal, and >9.0% high risk (15). Furthermore, self-monitoring of blood glucose was scanned to the number of hypo- and hyperglycaemic episodes over a period of one month (16). Before starting the study, the patients' glucometer measurements were checked with their simultaneous laboratory results. The patients were asked to measure their fasting blood glucose at least 4 times per day and to record those values. At the end of one month, the patients' self-records were checked with automatically records from their glucometers. Hypoglycaemic episodes were defined as when blood glucose levels fall below 70 mg/dL and hyperglycaemic episodes as when blood glucose levels rise above 145 mg/dL-without seizures or coma (15).

Statistical Analysis

Data were evaluated using IBM Statistical Package for the Social Sciences Statistics 21 statistical software package program. Quantitative variables were analysed with the Shapiro-Wilk test for normality. Because the data did not show a normal distribution, two independent group comparisons were performed by Mann-Whitney U test, and more than two independent groups were compared by Kruskal-Wallis analysis. Also, summary statistics were given as the number (n) and percentage (%) for categorical variables, and median and 25^{th}-75^{th} percentile (Q1-Q3) for numeric variables. When quantitative variables were compared with each other, Spearman correlation analysis was used. Categorical variables were compared by the exact method of chi-square test. Values of $p<0.05$ were considered statistically significant.

Results

The demographic characteristics of the participants are given in Table 1. The study and control groups were well matched for age, gender, and socioeconomic status. Also, BMI and parents' education, job and marital status were similar in the two groups ($p>0.05$). In the study group, while breastfeeding duration (exclusive plus partial) was shorter than in the control group ($p=0.020$), birth weight, exclusive breastfeeding duration, and starting age for introduction of cow's milk were similar to the control group ($p>0.05$). Children and adolescents in the study group consumed a greater number of meals and had breakfast more regularly than those in the control group ($p=0.001$).

Table 1. Baseline characteristics

Variables	Study group (n=70)	Control group (n=72)	p
Age in years (median and Q1-Q3)	13.00 (11.00-15.00)	12.00 (10.00-15.75)	0.280
Gender (male %)	54.3	50.0	0.609
Birth weight (kg) (median and Q1-Q3)	3.30 (2.98-3.61)	3.45 (3.00-3.79)	0.177
Exclusive breastfeeding duration (months) (median and Q1-Q3)	5.00 (3.00-6.00)	6.00 (3.50-6.00)	0.618
Breastfeeding duration (exclusive plus partial) (months) (median and Q1-Q3)	12.00 (5.25-18.00)	17.00 (9.00-24.00)	0.020*
Starting age of cow's milk (months) (median and Q1-Q3)	8.00 (6.00-12.00)	12.00 (6.00-13.00)	0.282
Number of meals	6.00 (4.00-6.00)	4.00 (3.00-5.00)	<0.001*
Regular breakfast (%)	84.3	48.6	<0.001*
Regular physical activity (%)	67.1	29.2	<0.001*
Physical activity frequency (times/week)	7.00 (3.00-7.00)	2.00 (2.00-3.00)	<0.001*
Physical activity duration (min)	50.00 (30.00-60.00)	60.00 (30.00-105.00)	0.384
Exercise frequency during past week (times)	5.00 (3.00-7.00)	2.00 (1.00-4.25)	<0.001*

*p<0.05

Moreover, performance of physical activity regularly, physical activity frequency, and exercise frequency during the past week were more common among children and adolescents in the study group than in the control group ($p<0.05$). However, physical activity duration was similar in both groups ($p>0.05$).

Diabetes-Related Characteristics of Children and Adolescents with Type 1 Diabetes Mellitus

There was no statistically significant difference between girls and boys in terms of diabetes-related characteristics, as shown in Table 2. The median percentage of HbA1c was 7.80% (7.10-9.03), and the median number of hypo- and hyperglycaemic episodes was 2.5 (0.00-5.25) and 38 (22.00-57.25), respectively.

Quality of Life Assessment

In the analysis of the children's self-reports, no significant differences were found in total scores for HRQoL ($p=0.694$), for physical health ($p=0.359$), and psychosocial health ($p=0.922$) between the study and control groups. However, in the parents' proxy-reports, the study group parents reported a lower psychosocial health score than the control group parents ($p=0.030$), while the total scores for HRQoL ($p=0.071$) and physical health ($p=0.269$) were similar in both groups (Figure 1).

The Relationship between Quality of Life and Metabolic Control

In the study group, the correlations between HRQoL scores and metabolic control were evaluated and are shown in Table 3. There was no correlation between HRQoL scores (including total, physical health, and psychosocial health scores) for children's self-reports and metabolic control. On the other hand, the lower number of hypoglycaemic episodes was associated with increase in psychosocial health scores for parents' proxy-reports ($p=0.031$). Also, the lower number of hyperglycaemic episodes was related to increase in the total score of HRQoL ($p=0.021$) and physical health score ($p=0.018$) for parents' proxy-reports. Furthermore, there was no significant difference among the optimal, suboptimal, and high risk groups in terms of HRQoL scores for children's self-reports and parents' proxy-reports ($p>0.05$).

Table 2. Diabetes-related information in children and adolescents with type 1 diabetes mellitus [median and 25th-75th percentile (Q1-Q3) values]

Variables	Boys (n=38)	Girls (n=32)	Total group (n=70)	p
Age at T1DM diagnosis (years)	8.25 (5.13-11.00)	10.21 (7.00-12.19)	8.79 (6.13-11.08)	0.148
Duration of diabetes (years)	4.00 (2.50-6.00)	3.00 (1.63-5.38)	3.50 (2.00-6.00)	0.139
Total length of stay in hospital (days)	23.00 (14.75-44.25)	18.50 (15.25-25.00)	20.00 (15.00-30.75)	0.516
HbA1c (%)	8.40 (7.08-9.28)	7.60 (7.10-8.45)	7.80 (7.10-9.03)	0.100
Number of hypoglycaemic episodes	2.00 (0.00-6.00)	3.00 (0.00-5.00)	2.50 (0.00-5.25)	0.644
Number of hyperglycaemic episodes	40.00 (17.75-58.00)	35.50 (23.25-46.50)	38.00 (22.00-57.25)	0.612

T1DM: type 1 diabetes mellitus, HbA1c: hemoglobin A1c

Table 3. Correlations of health-related quality of life scores with metabolic control indicators and diabetes-related data

Variables	CTS	CPhyS	CPsyS	PTS	PPhyS	PPsyS
Age at T1DM diagnosis (years)	-0.156	-0.116	-0.103	0.099	0.061	0.037
Duration of diabetes (years)	0.171	0.188	0.074	0.056	0.141	0.049
Total length of stay in hospital (days)	0.145	0.157	0.079	0.007	0.098	-0.031
HbA1c (%)	0.074	0.113	0.024	-0.004	-0.016	0.033
Number of hypoglycaemic episodes	0.006	0.100	-0.058	-0.196	-0.016	-0.258*
Number of hyperglycaemic episodes	-0.023	-0.127	-0.045	-0.275*	-0.281*	-0.188

*p<0.05

T1DM: type 1 diabetes mellitus, HbA1c: hemoglobin A1c, CTS: Child Total Score, CPhyS: Child Physical Health Score, CPsyS: Child Psychosocial Health Score, PTS: Parent Total Score, PPhyS: Parent Physical Health Score, PPsyS: Parent Psychosocial Health Score

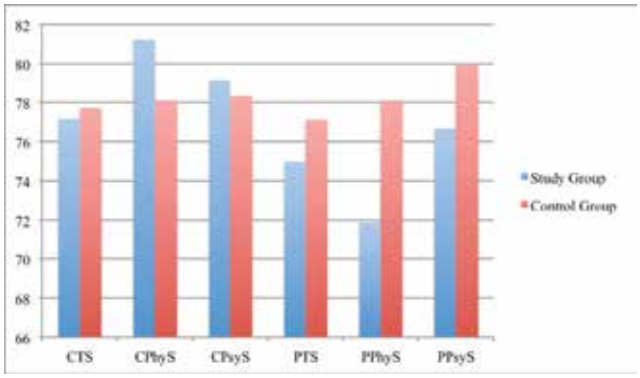

Figure 1. Comparison of groups in terms of health-related quality of life scores. p>0.05 for CTS, CPhyS, CPsyS, PPhyS, PTS and p<0.05 for PPsyS. CTS: Child Total Score, CPhyS: Child Physical health Score, CPsyS: Child Psychosocial Health Score, PTS: Parent Total Score, PPhyS: Parent Physical Health Score, PPsyS: Parent Psychosocial Health Score

Discussion

In the treatment of T1DM, intensive therapy programmes are implemented to reduce complications. These intensive therapy programmes place a burden on the children and their family relationships. They usually limit the children's daily activity, influence the behaviour of the children and their families in a way that is focused on illness and consequently, quality of life may be adversely affected (17). On the other hand, it is known that a diagnosis of T1DM per se creates difficulties for children and adolescents and they usually have difficulties in adapting to the loss of their health and the change in their lives. However, they usually adapt to their disease in the course of time despite their initial perception that their quality of life is impaired (18,19).

In this study, it was shown that Turkish children and adolescents with T1DM have similar HRQoL scores for children's self-reports and parents' proxy-reports as healthy controls, apart from a decreasing psychosocial health score noted in the proxy-reports of parents of children with T1DM. The similar perceptions of children with and without T1DM for quality of life are thought to result from the fact that they may have adapted to living with a chronic disease, because of continually living with diabetes for at least a year. However, one individual's perception of living with a disease may differ from that of another (20). This difference can be demonstrated by looking at the perception of families regarding their children's status. Unlike the children and adolescents who often have an optimistic view, families tend to indicate that they are adversely influenced by their children having a chronic disease (16,20). These data may explain why HRQoL scores for parents' proxy-reports in the T1DM group are lower than that of parents of the healthy group, while HRQoL scores for children's self-reports are similar in the two groups.

The findings of this study are consistent with some previous reports (17,21,22), but are in conflict with others (23,24). Jafari et al (23) showed a statistically significant difference between Iranian children and adolescents with T1DM and healthy controls in terms of HRQoL scores for children's self-reports and parents' proxy-reports. In the group with T1DM, these authors reported lower HRQoL scores than in the healthy group. A similar result was found in Kuwaiti children and adolescents (24). These findings have been attributed to be due families not being well informed on the needs of their children, a consequence of the inadequacies in the health system in these communities. Moreover, both studies were conducted at a shorter time after diagnosis as compared to this present study, thus the T1DM patients may not yet have completed the process of adapting to their disease. Thus, the HRQoL scores of children and adolescents with T1DM may differ from healthy controls in some studies.

HRQoL is considered to be an important indicator of prognosis. Children with diabetes experience chronic psychosocial stress and they frequently show more behavioural difficulties and less social competence as compared to healthy children. Therefore, improving HRQoL is important to prevent secondary morbidity and to achieve good metabolic control in the management of diabetes (5,25). In this study, HbA1c level was considered as an indicator of metabolic control. In the majority of studies, the most recently measured HbA1c value was used (16,26,27,28,29,30,31,32,33), although the mean HbA1c during the previous year was also used by some (5,34). In this present study, HbA1c values measured on the day that the questionnaire and PedsQL^{TM} scale were administered were used as an indicator of metabolic control. We found no relationship between HRQoL and HbA1c level, and this finding is consistent with some previous studies (5,26). However, in contrast to this finding, an inverse relationship between HRQoL and HbA1c, decreasing HRQoL scores with increasing HbA1c levels have been reported in many recent studies (27,30,31,33,35,36). At this point, we should note that the lower sample size in our study may have been responsible for our results. Moreover, the fact that the generic HRQoL scale was used to assess the HRQoL of children and adolescents with T1DM in this study, while the diabetes-specific HRQoL scale was used in other studies may also have affected this finding.

Patients with symptoms of hypoglycaemia are more affected by diabetes and they also have more fear and anxiety of hypoglycaemic episodes compared to patients who experience no hypoglycaemia episodes. The potential impact of hypoglycaemia on patients can be explained by considering hypoglycaemic episodes as a barrier to glycaemic control. Increase in the number of hypoglycaemic episodes is associated with reduced glycaemic control, increased cost and also reduced HRQoL (37). The dilemma between tight glycaemic control and risk of hypoglycaemia imposes quite a large burden of disease on young people with diabetes

and their families. Treatment and follow-up requirements and the continuous risk of hypoglycaemia (particularly nocturnal hypoglycaemia) adversely affect the HRQoL of patients and their families. As fear of hypoglycaemia increases, the HRQoL of both children and families decreases. Families have a fear of hypoglycaemia which is associated with episodes of severe hypoglycaemia. During severe hypoglycaemia, the child is unconscious or in a coma, not aware of attacks at that moment, and may remember nothing about the incident afterwards. On the other hand, the families witness the incident and hence are perhaps more affected (32).

Hypoglycaemia is a psychosocial barrier as well as a physical barrier for optimal metabolic control and HRQoL. Although some level of fear is a normal response, higher levels are detrimental to HRQoL. Moreover, psychosocial factors often determine self-management behaviours, and psychosocial inconsistencies such as depression are often more powerful predictors of medical outcomes such as hospitalisation and mortality than physical and metabolic measurements such as the presence of complications or high BMI and HbA1c values (32,38). Therefore, the management of T1DM patients should include the evaluation of psychosocial burden including fear of hypoglycaemia imposed by diabetes on children and their families. On the other hand, a reduction in symptoms of hyperglycaemia reported individually is associated with a decrease in diabetes burden and an increase in treatment satisfaction (38). Also, it has been reported that increased HRQoL as perceived by children and their parents, particularly physical health, is related to fewer symptoms of hyperglycaemia (39).

Based on this information, the relationship between number of hypo- and hyperglycaemic episodes and HRQoL subscales including psychosocial and physical health was investigated in this study, and the findings were consistent with those in the literature. It was shown that the increased psychosocial health score for parents' proxy-reports is related to a decrease in hypoglycaemic episodes, and increased total and physical health scores for parents' proxy-reports are associated with a reduction in hyperglycaemic episodes. While previous studies reported that total HRQoL score is associated with hypo- and hyperglycaemic episodes (16,40), to the best of our knowledge, this is the first report showing that relationships exist between HRQoL subscales including psychosocial and physical health, especially as perceived by parents, and the number of hypo- and hyperglycaemic episodes.

The findings of this study highlight that poor glycemic control in children and adolescents with T1DM is associated with lower HRQoL scores. These results suggest that it is easier to motivate a child or an adolescent to reach optimal blood glucose levels for improving his/her HRQoL than for preventing long-term diabetes-related complications. In this perspective, routine clinical assessment of HRQoL as an important part of diabetes management may be especially useful in individualizing care and determining the most appropriate interventions.

Ethics

Ethics Committee Approval: It was approved by the University of Erciyes Clinical Research Ethics Board (date: 07.08.2012 and reference number: 2012/479), Informed Consent: It was taken.

Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Neriman İnanç, Selim Kurtoğlu, Design: Zeynep Caferoğlu, Nihal Hatipoğlu, Data Collection or Processing: Zeynep Caferoğlu, Analysis or Interpretation: Zeynep Caferoğlu, Nihal Hatipoğlu, Literature Search: Neriman İnanç, Selim Kurtoğlu, Writing: Zeynep Caferoğlu.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):74-79
DOI: 10.4274/jcrpe.2308

Original Article

Evaluation of Periaortic Adiposity and Metabolic Disorders in Obese Children

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ABSTRACT

Objective: To evaluate the relationship between periaortic fat thickness (PAFT) and parameters involved in the development of metabolic complications of the cardiovascular system in obese children and to assess the usefulness of echocardiographic measurements of PAFT in correlation with cardiovascular risk factors.

Methods: The study was conducted with 263 obese and 100 healthy children and adolescents. PAFT was measured with echocardiography method which was recently performed in obese children and adolescents.

Results: PAFT was significantly higher in the obese group (0.258±0.031 mm) than in the control group (0.137±0.032 mm) ($p<0.001$). In multivariable regression analysis, body mass index-standard deviation score and total body fat were predictors of PAFT. The area under the receiver operating characteristic curve was 0.989 and was quite significant at $p<0.001$. PAFT above 0.179 mm was determined as the cut-off value in obese children and adolescents (sensitivity=1, specificity=0.97).

Conclusion: The measurement of PAFT in obese children and adolescents may be a good method to reveal the presence of early cardiovascular risk.

Keywords: Obesity, periaortic fat thickness, atherosclerosis, children, adolescents

Conflict of interest: None declared
Received: 07.08.2015
Accepted: 26.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Periaortic adiposity is a strong and new risk factor for cardiovascular disease. Studies were carried out in adult groups using multidetector computed tomography or magnetic resonance imaging.

WHAT THIS STUDY ADDS?

Applying echocardiography in childhood for measuring periaortic fat thickness. Determining the presence of early cardiovascular risk in childhood with a non-invasive technique beside classic methods.

Introduction

Obese children are candidates for accelerated development of vascular disease due to obesity-induced risk factors. Atherosclerosis, an inflammatory condition, lies at the foundation of cardiovascular conditions. Inflammation also develops on the vessel wall, similar to fatty tissue (1,2).

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Atherosclerosis can be identified in its early stage by ultrasonographic or echocardiographic measurement of the intima media thickness of the carotid artery or of other large arteries. The thickness of the carotid intima media is increased in obese children, however, there are conflicting data and findings in the literature with regard to the factors behind this increase (3).

Although subcutaneous fatty tissue contains the majority of body fat, visceral adiposity due to obesity plays an important role in the development of the metabolic syndrome and in the pathogenesis of atherosclerosis. Periadventitial fat accumulation is the localized form of body fat in large blood vessels. Periaortic adiposity is a subtype of perivascular adiposity and is an important indicator of atherosclerosis, which is a critical complication of obesity (2,4,5,6). In adult studies using multidetector computed tomography or magnetic resonance imaging (MRI), periadventitial fat accumulation was considered to be strong and a new risk factor for cardiovascular disease.

The main purpose of this study was to measure periaortic fat thickness (PAFT) by echocardiography and to reveal the relationships between PAFT and metabolic data. MRI examination is expensive and not easily available in most clinical settings. Echocardiography is a noninvasive method which can be used to image aortic fat without radiation exposure. We believe our study will be helpful in the evaluation of endocrinological and cardiovascular complications in the monitoring of obese children.

Methods

The study population consisted of 263 obese children and adolescents (129 females, 134 males, aged 11.42±2.69) who presented to the Pediatric Endocrinology Outpatient Clinic of the Faculty of Medicine at Necmettin Erbakan University in Konya, Turkey. Obesity was defined as a body mass index (BMI) greater than the 95th percentile for age and gender (7). Exclusion criteria were the presence of chronic diseases, having genetic or endocrinological diseases, having heart disease, or use of any medication. The control group in this study consisted of 100 children and adolescents (45 females and 55 males, aged 12±2.51 years). Healthy children and adolescents with normal percentiles of weight and height were selected as control group.

The study was approved by the local ethics committee (2010/034) and designed prospectively. The study was conducted in accordance with the guidelines proposed in the declaration of Helsinki.

All participants underwent a thorough physical examination by the same pediatric endocrinologist. Tanner stage based on breast stage and pubic hair development in girls and on genitalia development in boys was assessed in each child.

Height was measured to the nearest 0.5 cm on a standard height board, and weight was determined to the nearest 0.1 kg on a standard physician's beam scale with the subject dressed only in light underwear and no shoes. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference (WC) was measured at the level of the umbilicus with the patient standing and breathing normally. WC was evaluated using the percentile curves for WC of healthy Turkish children (8). The hip circumference (HC) was estimated on the basis of the widest diameter passing through the most protruding point of the gluteus maximus and over the symphysis pubis. Waist/hip (WC/HC) ratio was determined by dividing WC to HC. Blood pressure was measured with a standard mercury sphygmomanometer after the subjects had rested for at least 10 minutes. Blood pressure threshold values were evaluated with reference to the normal values reported for children in the National High Blood Pressure Education Program Working Group in 2004. Casual systolic blood pressure (SBP) and diastolic blood pressure (DBP) values more than 95th percentile for age, sex, and height were defined as hypertension (9).

Serum fasting glucose, fasting plasma insulin, total cholesterol, triglyceride, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol levels were estimated.

The homeostasis model assessment of insulin resistance (HOMA-IR; fasting insulinxfasting glucose/22.5) was used as an index of insulin resistance (10). Insulin resistance is defined as a HOMA-IR of greater than 2.5 in the prepubertal group and 3.16 in the pubertal group (11,12).

Total body fat was measured by bioelectricimpedance analysis (Model MC 180, MA Multi-Frequency Body Composition Analyzer; Tanita, London, UK) with correction for light indoor clothing.

Echocardiographic examinations were performed with a Philips Hewlett-Packard Sonos 5500, using 12 MHz flat probes, according to the American Association of Echocardiography Pediatric and Congenital Heart Disease Council's Standard imaging techniques (13). Periaortic adipose tissue was measured from the periaortic tissue to the muscular layer of the abdominal aorta which represents periaortic tissue with adventitial layer of the abdominal aorta. Measurement of periaortic adipose tissue should be taken with adventitia (Figure 1) because in deep tissue it couldn't be differentiated exactly with echocardiography and ultrasonography.

PAFT was measured in sagittal and axial planes at L1-2 level, proximal to the iliac bifurcation in the supine position.

Evaluations were made three times by pediatric cardiologists. The mean PAFT values were recorded. Reliability tests were also performed.

Statistical Analysis

Normality was checked. Data are expressed as means ± standard deviation. Student's t-test and chi-square test were used.

Multiple regression analysis was performed.

Reliability testing was done to evaluate PAFT measurements in the obese and control groups. Compliance (reliability) within observers and between observers was assessed for axial and sagittal measurements. Measurement of PAFT were coherent for both intra class and inter class evaluation determined by intraclass correlation coefficient with 95% confidence intervals.

Results

Reliability of axial and sagittal measurements of PAFT are shown in Table 1 for inter-observer and in Table 2 for intra-observer differences.

Interpretation of calculated levels of compliance by intraclass correlation coefficients:

0-40: measurements compatible (consistent),

41-60: measurements of harmony (consistency) low,

61-80: measurements sufficiently compatible (consistent),

81-100: measurements quite consistent.

The mean age of the subjects in the obese group was 11.42±2.69 years and that of the control group was 12±2.51 years. 65% of the obese group and 74% of the control group were pubertal. Demographic and anthropometric parameters of obese andcontrol groups are shown in Table 3.

PAFT was 0.258±0.031 mm in the obese group and 0.137±0.032 mm in the control group and this was statistically significant difference (p<0.001) (Figure 2). PAFT was not statistically different according to sex or pubertal status. Cardiovascular and laboratory parameters of obese and control groups are shown in Table 4.

In the obese group, 99 cases (37.6%) had SBP elevation. In the control group, there was no blood pressure elevation. SBP and DBP values in the obese group were statistically significantly higher than in the control group. Dyslipidemia was detected in 46.1% of the patients. Between the groups with and without dyslipidemia, PAFT was not statistically significantly different (p=0.95). In 41.7% of obese patients, insulin resistance was detected. PAFT in patients with insulin resistance was not significantly higher than in the group without insulin resistance (p=0.44).

The significant correlations between PAFT and clinical and laboratory parameters are shown in Table 5.

In multivariate regression analysis, the only predictors of PAFT were BMI-SDS (β:0.47, p<0.001) and total fat percentage (β:0.37, p<0.001).

The area under the receiver operating characteristic curve was 0.989 and was quite significant at p<0.001. PAFT above 0.179 mm was determined as the cut-off value for obese children and adolescents (sensitivity=1, specificity=0.97).

Table 1. Intra-observer reliability table

	ICC	%95 CI	p-value
First observer			
Axial	67.7	47.5-83.0	<0.001
Sagittal	66.8	46.4-82.4	<0.001
Second observer			
Axial	63.9	42.6-80.7	<0.001
Sagittal	71.0	52.1-84.9	<0.001

ICC: intraclasscorrelation coefficient, CI: confidence interval

Table 2. Inter-observer reliability table

	ICC	%95 CI	p-value
Axial			
First measurement	76.0	52.0-88.8	<0.001
Second measurement	77.8	55.3-89.8	<0.001
Third measurement	79.6	58.4-90.6	<0.001
Sagittal			
First measurement	75.4	51.0-88.5	<0.001
Second measurement	72.1	45.5-86.9	<0.001
Third measurement	83.2	65.1-924	<0.001

ICC: intraclasscorrelation coefficient, CI: confidence interval

Discussion

The most important cardiovascular problem observed in obesity is early development of atherosclerosis. Facilitators of development of atherosclerosis are type 2 diabetes and the presence of hypertension and dyslipidemia. An increase in visceral adipose tissue disrupts metabolic balance, enhances

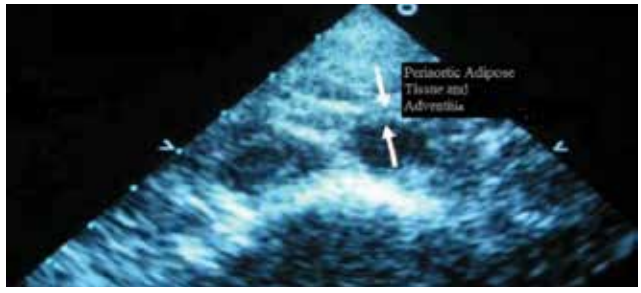

Figure 1. Image of periaortic adipose tissue and adventitia on echocardiography

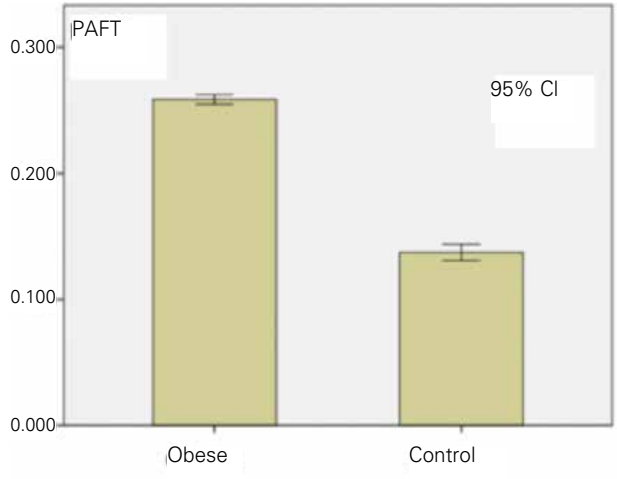

Figure 2. Periaortic fat thickness of obese and control groups. PAFT: periaortic fat thickness, CI: confidence interval

generation of proinflammatory and prothrombotic substances, and increases the risk of atherosclerosis (14).

Coronary atherosclerosis is the most well-known pathology. This process begins in childhood and can be irreversible at this stage. Irreversible fatty lines, rather than atherosclerosis, generally develop in children. Studies indicate that the severity of atherosclerosis in children and young adults is associated with the same risk factors determined in adults. Many studies have shown that fatty lines and fibrous plaques in coronary arteries of adolescents and thickening in vessel intima were determined from the age of 5 years (15,16).

Periaortic adiposity is an important indicator of atherosclerosis that also begins at an early period (17). Another component of abnormal body fat accumulation is accumulation of ectopic fatty tissue. It surrounds the organs and vessel structures. Perivascular adiposity is a type of ectopic adiposity. It is believed to have a local pathogenic effect on blood vessels. Periaortic adiposity is a subtype of perivascular adiposity and only publications using measurement by multi-sectional computer tomography are currently available (4,6). Measurements in these studies are in limited numbers and are experimental.

In this research PAFT values were taken with

Table 3. Demographic and anthropometric parameters of obese and control groups

	Obese	Control	p-value[a]
Demographic parameters	**n=263**	**n=100**	
Age (years)	11.42±2.69	12.00±2.51	0.06
Sex (female/male)	129/134	45/55	0.49[b]
Prepubertal/pubertal	170/93	74/26	0.09[b]
Anthropometric parameters			
Weight (kg)	63.88±19.99	39.28±12.09	**<0.001**
Height (cm)	148.42±15.27	145.38±14.21	0.08
BMI (kg/m^2)	28.09±6.48	18.11±2.78	**<0.001**
BMI-SDS	2.12±0.32	-0.19±1.08	**<0.001**
Total body fat percentage (%)	33.39±6.46	17.02±3.77	**<0.001**
WC (cm)	90.49±12.45	64.92±10.42	**<0.001**
HC (cm)	98.40±13.03	76.75±10.67	**<0.001**
WC/HC ratio	0.92±0.07	0.86±0.19	**<0.001**

BMI: body mass index, SDS: standard deviation score, WC: waist circumference HC: hip circumference
[a]Student's t-test
[b]Chi-square test

Table 5. Correlations between periaortic fat thickness and clinical/ laboratory parameters

	r	p-value
BMI-SDS	0.73	**<0.001**
WC(cm)	0.66	**<0.001**
HC (cm)	0.59	**<0.001**
WC/HC ratio	0.21	**<0.001**
Total body fat (%)	0.69	**<0.001**
SBP (mmHg)	0.33	**<0.001**
DBP (mmHg)	0.26	**<0.001**
Insulin (mcIU/mL)	0.24	**0.004**
Total cholesterol (mg/dL)	0.11	**0.031**
Triglyceride (mg/dL	0.15	**0.004**
HDL cholesterol (mg/dL)	-0.26	**<0.001**
LDL cholesterol (mg/dL)	0.20	**<0.001**

BMI: body mass index, SDS: standard deviation score, WC: waist circumference HC: hip circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL: low-density lipoprotein, HDL: high-density lipoprotein

Table 4 . Cardiovascular and laboratory parameters in the obese and control groups

Cardiovascular parameters	Obese	Control	p-value[a]
SBP (mmHg)	117.53±16.40	105.93±16.93	**<0.001**
DBP (mmHg)	74.19±12.59	65.48±14.84	**<0.001**
Periaortic fat thickness (mm)	0.258±0.031	0.137±0.032	**<0.001**
Laboratory parameters			
Glucose (mg/dL)	91.55±9.61	90.56±17.47	0.50
Insulin (mcIU/mL)	15.59±14.97	7.44±3.71	**<0.001**
HOMA-IR	3.60±3.72	1.67±0.91	**0.001**
Total cholesterol (mg/dL)	170±32.26	156.96±30.39	**0.001**
Triglyceride (mg/dL)	120.75±62.53	96.74±44.75	**0.001**
HDL cholesterol (mg/dL)	41.45±11.43	50.62±20.22	**<0.001**
LDL cholesterol (mg/dL)	103.14±26.91	86.52±26.07	**<0.001**

[a]Student's t-test, SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL: low-density lipoprotein, HDL: high-density lipoprotein, HOMA-IR: homeostasis model assessment of insulin resistance

echocardiography method. PAFT was found to be 0.258±0.031 mm in the obese group and 0.137±0.032 mm in the control group ($p<0.001$). Obese group had significantly higher PAFT values. The threshold value for PAFT was determined to be 0.179 mm in obesity.

Lehman et al (18) reported in their study that thoracic adiposity was related with metabolic risk factors. The relationship between visceral adipose tissue and periaortic adiposity is not known. In our study, supporting the findings of Lehman et al (18) a positive correlation was found between PAFT and cardiovascular risk factors, namely, SBP and DBP, total cholesterol, LDL cholesterol, and triglyceride. Ruberg et al (19) found PAFT to be higher in an obese group than a control group and to have a positive correlation with BMI and a negative correlation with HDL cholesterol in their study performed with MRI. Schlett et al (20) found PAFT to positively correlate with BMI and WC.

Britton et al (4) used computed tomography in adult studies and showed the relation of thoracic aortic fat, cardiac and metabolic disorders.

In cases who had increased aortic adiposity, BMI, WC and visceral adiposity were markedly higher. In this present study, we also found a positive correlation between PAFT and WC, an important indicator of visceral adiposity. All our data support the aforementioned studies.

Thanassoulis et al (5) reported that increased periaortic adiposity is related to aortic remodeling. They emphasized that local adiposity in the aorta caused aortic remodeling to a greater extent than the systemic effects of obesity. We found that among the risk factors only BMI-SDS and total fat had effects on PAFT, which is consistent with this hypothesis.

The fact that periaortic adiposity is correlated with all cardiovascular risk factors in our study indicates that it only defines the cases who are metabolically obese and who carry cardiovascular risks. As shown in our study, a lack of differences based on gender or pubertal stage indicates that periaortic adiposity may possibly be used as a standard method.

The mechanisms responsible for the development of local adiposity in the vessels are not currently clear and the role of this local adiposity in the development of insulin resistance and metabolic syndrome is still being reviewed. In support of thisrelationship, PAFT was found to have a positive correlation with serum insulin level and HOMA-IR in our study.

Insulin resistance provides groundwork for the development of atherogenic dyslipidemia, prothrombotic and proinflammatory conditions. It is reported that insulin resistance observed in obesity also contributes to the development of hypertension (21). Coronary artery disease is significantly associated with fasting insulin levels. Other findings in our study, such as differences between the obese and control groupsregarding insulin level and HOMA-IR, findings independent of blood glucose level, also support the importance of insulin resistance in the development of these pathologies.

In obesity, sympathetic nervous system activation occurs and catecholamine secretion increases as a result of excessive intake of calories through foods rich in fat and carbohydrates. Blood pressure elevates with increased catecholamines (22). In our study,elevated SBP was detected in 37.6% of obese subjects andwas statistically significantly higher than in the control group.

Reinehr et al (23) reported the frequency of hypertension in obese children as 38%, and Maggio et al (24) as 47%. In other studies, conducted by the monitoring of blood pressure for 24 hours, this rate was reported to be between 47-60% (24).

Saha et al (25) reported the frequency of insulin resistance as 63%in obese subjects. Viner et al (26) reported a frequency of 30% for dyslipidemia. In our study, we found 46.1% of patients to have dyslipidemia in the obese group. LDL cholesterol, total cholesterol, and triglyceride levels in obese cases were higher and HDL cholesterol level were lower than in the control group. This suggests that dyslipidemia is important in the development of obesity.

Various studies indicate that BMI and WC are important indicators of obesity and body fat distribution and that WC is an important indicator of cardiovascular risk (27). That WC is correlated with PAFT in our study also supports these findings.

The relationship between local fat distribution and cardiometabolic complications is not known. Increased WC-HC ratio is believed to represent an increase in abdominal adipose tissue. Compared to other anthropometric measurements, it is reported to have a positive correlation with cardiovascular disease. Many studies support this relation (21). In our study, WC/HC ratio was found to be increased in obese cases and a positive correlation was determined with PAFT. These findings support the reports in the literature.

Our results also indicate that evaluation of PAFT is important for early diagnosis of atherosclerosis in obese cases, the most important factors for PAFT, independently from additional metabolic risks, being BMI and total fat mass.

The limitation of our study was that the findings were not validated with standard techniques such as MRI.

This study showed the usefulness of echocardiographic measurement of PAFT in correlation with cardiovascular risk factors. Echocardiography allows good delineation of normal abdominal aortic anatomy including the recognition of vessel layers, especially when they are thick and contain fat. We also showed that the measurement of PAFT had good intra-operator and inter-operator reliability. PAFT measurement with conventional echocardiography in obese cases may be useful for assessing cardiovascular risk at earlier ages.

Ethics

Ethics Committee Approval: Necmettin Erbakan University Ethics Committee (Approval number: 2010/034), Informed Consent: Verbal consent.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Beray Selver Eklioğlu, Mehmet Emre Atabek, Design: Beray Selver Eklioğlu, Mehmet Emre Atabek, Data Collection or Processing: Beray Selver Eklioğlu, Nesibe Akyürek, Hayrullah Alp, Analysis or Interpretation: Beray Selver Eklioğlu, Mehmet Emre Atabek, Nesibe Akyürek, Hayrullah Alp, Literature Search: Beray Selver Eklioğlu, Nesibe Akyürek, Hayrullah Alp, Writing: Beray Selver Eklioğlu, Mehmet Emre Atabek, Nesibe Akyürek, Hayrullah Alp.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):80-85
DOI: 10.4274/jcrpe.2248

Original Article

Prediabetes and Cardiovascular Parameters in Obese Children and Adolescents

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ABSTRACT

Objective: In this study, our aim was to determine cardiovascular risk and cardiac function in prediabetic obese children and adolescents.

Methods: The study was conducted on 198 obese children and adolescents 6-18 years of age. Anthropometric measurements, blood pressure measurements, oral glucose tolerance test, lipid profile, and HbA1c levels of patients were assessed. Prediabetes was defined according to American Diabetes Association criteria. Left ventricular mass index (LVMi), carotid intima-media thickness (c-IMT), and tissue Doppler measurements records were used.

Results: LVMi was found to be significantly higher in the prediabetes group (p=0.03). There were no statistically significant differences in right ventricular tissue Doppler measurements between the prediabetic and non-prediabetic groups. Left ventricular tissue Doppler measurements were significantly higher in the prediabetes group: LVEEM (left ventricular E/e ratio) (p=0.04); LVEM (left ventricular myocardial velocity cm/s) (p=0.035). LVMi was found to positively correlate with triglyceride level, diastolic blood pressure, waist circumference, body weight standard deviation score and to negatively correlate with high-density lipoprotein cholesterol (p=0.043, r=0.15; p=0.039, r=0.15; p=0.025, r=0.17; p=0.009, r=0.19; p=0.038, r=-0.15, respectively). LVEM was correlated with glucose (p=0.046, r=0.15) and LVEEM was correlated with systolic blood pressure (p=0.035, r=0.15). In linear regression analysis for clinical cardiovascular risk factors, fasting glucose level was the best predictor of LVEM.

Conclusion: In this study, deterioration of cardiac function in prediabetic obese children and adolescents was shown. We recommend determining cardiovascular risk and cardiac dysfunction at early stages in prediabetic obese children and adolescents.

Keywords: Obesity, prediabetes, children, adolescent

Conflict of interest: None declared
Received: 21.07.2015
Accepted: 15.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Childhood obesity causes subclinical impairment of cardiac function. Left ventricular structural changes have already been demonstrated in obese children.

WHAT THIS STUDY ADDS?

Ventricular dysfunction studies have not been performed in obese children and adolescents with prediabetes. Studies have generally been performed in adult population.

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Introduction

Obesity causes several co-morbidities. Insulin resistance, type 2 diabetes mellitus (T2DM), and cardiovascular impairment are the most important obesity-related complications. When insulin secretion cannot maintain the degree of hyperinsulinemia required to overcome the resistance, prediabetes [impaired glucose tolerance (IGT), impaired fasting glucose] and subsequently T2DM develop (1).

Obesity in children is associated with early structural myocardial disturbances in adulthood. Childhood obesity has been shown to be a cause of subclinical impairment of cardiac function in childhood. Left ventricular structural changes have been demonstrated in obese children and adolescents (2). Increased values for left ventricular mass index (LVMi) and carotid intima-media thickness (c-IMT) as well as abnormal results of Doppler imaging have been reported in childhood obesity (3,4,5,6,7).

Left ventricular dysfunction is a determinant for the development of future heart failure. Tissue Doppler parameters are less load dependent compared to traditional Doppler parameters (3). In particular, markers of ventricular dysfunction, as shown by myocardial tissue Doppler velocities, have not been clearly examined in obese children and adolescents with prediabetes. Studies have generally been performed with the adult population (8).

This study aimed to assess the relationship between prediabetes and ventricular function in obese children and adolescents beyond traditional echocardiographic parameters.

Methods

One hundred ninety-eight obese children and adolescents were included in this study. The study was approved by the Necmettin Erbakan University Faculty of Medicine Local Ethics Committee. The boys and girls included in the study were 6 to 18 years of age, free of known diseases, and not taking any medication. Anthropometric parameters were assessed in all patients. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Patients with a BMI greater than the 95th percentile for age and gender were considered as obese (9). Waist circumference (WC) was measured at the level of the umbilicus with the patient standing and breathing normally. WC was evaluated using the percentile curves for WC of healthy Turkish children (10). Pubertal development stages were assessed using the Tanner criteria (11,12). Blood pressure was measured with a standard mercury sphygmomanometer. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values more than the 95th percentile for age, sex, and height were defined as hypertension (13).

After overnight fasting, blood samples were taken for determination of glucose, insulin, total cholesterol, triglyceride, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol, and hemoglobin A1c (HbA1c) levels. The homeostasis model assessment of insulin resistance (HOMA-IR; fasting insulinxfasting glucose/22.5) was used as an index of insulin resistance. Insulin resistance was defined as a HOMA-IR of greater than 2.5 in the prepubertal group and greater than 3.16 in the pubertal group (14,15). HOMA2-IR was calculated (16). An oral glucose tolerance test (OGTT) was performed in all subjects with 1.92 g/kg glucose monohydrate and samples taken at 0, 30, 60, 90 and 120 minutes after glucose loading.

Prediabetes was defined according to the American Diabetes Association guidelines (17). Accordingly, impaired fasting plasma glucose was defined as a fasting plasma glucose level of 100 mg/dL to 125 mg/dL, or IGT as shown by a 2-hour plasma glucose of 140 mg/dL to 199 mg/dL in the OGTT, or a HbA1c level between 5.7% and 6.4%.

Echocardiography was done with a Sonos 5500 with a 5.0 MHz transducer in the pediatric cardiology department and the echocardiography data previously done were used in the assessments. All measurements were done according to the criteria defined by the American Society of Echocardiography (18,19). Patients with any congenital or acquired heart disease were discarded from the study group.

LVM was estimated using the formula of Devereux and Reichek (20). The LVMi was calculated by dividing LVM by height$^{2.7}$ [de Simone et al formula (21)]. Intima-media thickness of the common carotid artery (c-IMT) far wall was measured with the electronic calipers of the machines, as previously described (22).

Tissue Doppler velocities were obtained from three locations in the right and left ventricles. The sample volume was positioned on the lateral aspect of each atrioventricular valve annulus and the basal portion of the interventricular septum. Early (E) diastolic velocities, peak early diastolic myocardial (e') (LVEM, RVEM), late myocardial velocity (LVAM, RVAM), and peak systolic (s') (LVSM, RVSM) myocardial velocities were measured by this technique. The E/e' ratios were calculated (LVEEM, RVEEM) (23).

Statistical Analysis

Normality was tested. The data were expressed as mean ± standard deviation. Differences were assessed using the Student's t-test and chi-square test. Correlation and regression analysis were performed. A p-value of <0.05 was accepted to be of statistical significance.

Results

The prevalence of prediabetes was 40.9% in the obese study population. The mean age was 11.84±2.95 years in prediabetic children and 11.88±2.97 years in non-prediabetic children. 74% of the prediabetic children were pubertal. There was no difference in the presence of prediabetes

according to puberty (p=0.82) and sex (p=0.77). There were no statistically significant differences between the two groups with respect to age, gender, or BMI. Traditional risk factors such as SBP, DBP, WC, LDL, total cholesterol, and triglycerides were not statistically different according to prediabetic status. The baseline characteristic features of the two groups are shown in Table 1.

Cardiovascular parameters of patients according to prediabetes are shown in Table 2. The c-IMT difference was not statistically significant (p=0.37), whereas the LVMi was 43.98±10.95 in the prediabetes group and 40.63±10.33 in the non-prediabetes group, findings which were significantly different (p=0.036). LVMi was positively correlated with triglycerides, SBP, WC, and weight standard deviation score, and negatively correlated with HDL cholesterol (p=0.043, r=0.15; p=0.039, r=0.15; p=0.025, r=0.017; p=0.009, r=0.19; p=0.038, r=-0.15, respectively). The statistically significant tissue Doppler findings in prediabetic patients were LVEM and LVEEM (p=0.035, p=0.043, respectively).

In the prediabetes group, LVEM was correlated with fasting glucose (p=0.046) and LVEEM was correlated with SBP (p=0.035). In linear regression analysis, the best predictor of LVEM was fasting glucose in the prediabetes group.

Table 1. Characteristic features of the prediabetes positive and negative groups

	Prediabetes (+)	Prediabetes (-)	
	(n=81)	(n=117)	p
Gender (female/male)	46/35	64/53	0.88
Age	11.84±2.95	11.88±2.97	0.93
Puberty (n)	60	85	0.87
Weight (kg)	68.04±19.81	66.5±21.72	0.61
Height (cm)	150.71±14.11	150.48±15.71	0.91
BMI (kg/m^2)	29.99±9.03	28.05±5.19	0.05
BMI-SDS	2.16±0.36	2.11±0.28	0.32
Waist circumference (cm)	91.55±12.91	91.85±13.39	0.87
Systolic blood pressure (mmHg)	115.70±17.39	116.08±16.34	0.88
Diastolic blood pressure (mmHg)	75.91±12.81	72.49±12.46	0.07
Fasting glucose (mg/dl)	95.64±10.98	89.98±9.97	<0.001
Glucose 120. minute (mg/dl)	125.30±25.56	111.24±14.39	<0.001
Fasting insulin (mU/ml)	17.52±15.34	13.79±9.56	0.04
HOMA-IR	4.60±4.32	3.05±2.22	0.001
HOMA2-IR	2.16±1.62	1.73±1.15	0.034
HbA1C (%)	5.80±0.23	5.40±0.19	<0.001
Triglyceride (mg/dl)	114.46±59.21	112.82±60.62	0.85
HDL cholesterol (mg/dl)	39.40±8	41.93±11.95	0.10
LDL cholesterol (mg/dl)	104.17±27.21	103.07±39.58	0.83
Total cholesterol (mg/dl)	166.43±33.40	169.71±43.16	0.57

LDL: low-density lipoprotein, HDL: high-density lipoprotein, HbA1c: hemoglobin A1c, HOMA-IR: homeostasis model assessment of insulin resistance, BMI: body mass index, SDS: standard deviation score

Discussion

With the increasing prevalence of obesity, there is also an increase in prevalence of T2DM, prediabetes, and insulin resistance in pediatric ages. Effective prevention and treatment of T2DM is already a debate in pediatric populations. Today, the diagnosis of prediabetes and T2DM is increasing, and there is an interest in earlier identification and prevention (24). In Turkey, Kurtoglu et al (25) found the prevalence of prediabetes to be 37% in the boys and 27.8 % in the girls before puberty and 61.7% in the boys and 66.7% in the girls during puberty in obese children and adolescents. In another study, the prevalence of prediabetes was 15.2% in obese adolescents and 25.5% in obese children (1). In other countries, the prevalence of prediabetes in obese adolescents is reported to range from 19% to 39%. In this study, we found a prevalence of 40.6%, a figure similar to previous reports (26).

Studies have recently demonstrated that higher fasting plasma glucose levels within the normoglycemic range might be a predictor of diabetes. Nguyen et al (27) found a significantly increased risk for developing adult prediabetes and T2DM in children in the 86 to 99 mg/dL plasma glucose range after controlling for other traditional cardiometabolic risk factors. In our study, we found fasting glucose levels similar to this in

Table 2. Cardiovascular parameters of patients according to prediabetes

	Prediabetes (+)	Prediabetes (-)	
	(n=81)	(n=117)	p
c-IMT (cm)	0.099±0.015	0.101±0.020	0.37
LVMi	43.98±10.95	40.63±10.33	0.036
LVEM	17.17±3.36	16.23±2.80	0.035
LVEEM	5.51±1.10	5.18±1.12	0.043
LVAM	8.53±2.23	8.70±1.98	0.57
LVSM	10.09±1.71	10.27±1.81	0.48
RVEM	14.65±2.98	13.90±2.77	0.07
RVEEM	4.84±1.20	4.87±1.17	0.88
RVAM	12.09±3.73	11.49±2.91	0.20
RVSM	13.82±2.31	13.35±2.33	0.16

c-IMT: carotide-intima media thickness, LVMi: left ventricle mass index, LVEM: left ventricle early myocardial velocity cm/sn, LVEEM: left ventricle E/Em ratio, LVAM: left ventricle late myocardial velocity, LVSM: left ventricle systolic velocity, sn, RVEM: right ventricle early myocardial velocity cm/sn, RVEEM: right ventricle E/Em ratio, RVAM: right ventricle late myocardial velocity, RVSM: right ventricle systolic velocity

the prediabetes group. There was no significant difference for other cardiometabolic parameters (such as lipid profile, blood pressure). Similar to other studies, our study showed that a high fasting glucose level, a high insulin resistance index, and a high BMI might be potential risk factors for diabetes (27,28).

Tfayli and Arslanian (29) reported that adolescents with T2DM had significantly lower insulin-stimulated total and oxidative glucose disposal, suggesting that a defect in first-phase insulin response is seen early in the development of T2DM in youths, and that defects in second-phase response cause overt diabetes mellitus. IGT or prediabetes is an intermediate phase of altered glucose metabolism that is part of the process of the development of T2DM. Weiss et al (30) mentioned in their manuscript that IGT in obese youth is associated with severe insulin resistance, beta cell dysfunction, and altered abdominal and muscle fat partitioning. In their study, they found that all children who developed T2DM on follow-up had IGT at baseline. Developing diabetes from IGT in adults takes at least 5-10 years. It is speculated that there is an accelerated process in youth. Haemer et al (24) highlighted that insulin levels may be a poor predictor of diabetes risk because while insulin resistance may be accompanied by high insulin levels initially, insulin secretion may decrease later in the progression toward diabetes as a result of glucotoxicity and beta-cell failure In our study, we found both HOMA-IR and insulin level to be significantly different in the prediabetes group.

Studies have shown that prediabetes significantly increases the risk of developing diabetes, but it is reversible. In one study, nearly 50% of severely obese adolescents with prediabetes subsequently reverted to normal glucose tolerance, whereas 24% progressed from prediabetes to diabetes (24). Although the former group appeared to be clinically normal, the effects of the prediabetic state on tissue levels in the cardiovascular system remain unknown.

Obesity and cardiometabolic risk factors have been shown to be associated with vascular changes indicative of early atherosclerosis, or with ventricular hypertrophy, dilatation, and dysfunction. These cardiovascular consequences may be evident in young ages, but childhood obesity is also predictive of similar consequences in adulthood (31).

Several studies have shown that c-IMT is increased in children with cardiovascular risk factors, possibly making it a useful tool for assessing cardiovascular risk in children. Another cardiovascular risk factor is LVM. As shown from the Bogalusa Heart Study, childhood adiposity is related to LVM in adults. Although left ventricular hypertrophy is rare in obese children, cardiac remodeling might be present, which in adults has also been found to predict adverse cardiovascular outcomes (32). In a recent study, Pires et al (32) found both LVMi and c-IMT to be increased in obese children.

Left ventricular hypertrophy is an enlargement related to an increased load on the heart. The most common cause is hypertension. Obesity and prediabetes are additional risk factors for left ventricular hypertrophy (33). In previous studies, increased LVMi was reported in obese patients. With hyperglycemia, glycation end products are produced and irreversible cross-linking with collagen polymers in the myocardial and arterial walls occurs. In this way, myocardial compensation decreases, leading to left ventricular hypertrophy (34). Left ventricular hypertrophy may also reflect neurohormonal and metabolic stimuli causing left ventricular growth. Recent studies showed that LVMi was also increased in children with prediabetes, as in our study (35). c-IMT values were similar to obese patients in the literature but not significantly different from prediabetic patients. c-IMT values were high in obese children and there were variable values for c-IMT in literature. In our study we found c IMT higher according to some studies (36,37). However c-IMT wasn't statistically different in prediabetes group according to non prediabetes group. This may be due to the small number of patients.

Studies have shown left ventricular diastolic dysfunction to be the first manifestation of myocardial involvement in diabetic patients. The development is multifactorial, including metabolic disturbances, changes in extracellular matrix components, small vessel disease, autonomic dysfunction, and insulin resistance. Therefore, patients with prediabetes may have decreased ventricular function due to prolonged exposure to elevated glucose levels (8,38). Aslan et al (8) showed ventricular impairment in the adult population and suggested detailed evaluation of cardiovascular changes in prediabetic patients.

In a study in prediabetic youth, Shah et al (39) showed deterioration in metabolic profiles with higher BMI z-score, higher SBP, and fasting insulin as well as with increased c-IMT. To our knowledge,there are two studies recently carried out concerning the relationship between prediabetes and cardiac function in obese children and adolescents. Shah et al (39) showed that youth with prediabetes have worse cardiometabolic risk factors and display evidence of increased arterial thickness and stiffness. De Marco et al (35) reported early preclinical systolic and diastolic dysfunction with early cardiovascular alterations also being present in prediabetic adolescents.

In this study, our aim was to confirm that there is a deterioration in ventricular function (not seen in conventional echocardiographic measurements) in prediabetic children,using tissue Doppler echocardiography (39).

Tissue Doppler echocardiography can show subclinical alterations (both the diastolic and systolic impairment) of ventricular functions in obese patients. In one study, obesity-related increased preload volume was considered to be involved in the impairment of diastolic myocardial velocity (7). Harada et al (40) reported that the E/e' ratio showed the strongest correlation with LV diastolic filling pressure. Van Putte-Katier et al (41) reported a positive correlation between E/e' ratio and BMI These data suggest that a greater E/e' ratio is due to impaired ventricular relaxation and is associated

with early and subclinical higher ventricular filling pressures in obese young subjects. We speculate that increased glucose levels worsen the function of the left ventricle.

In our data, children with prediabetes were characterized by significantly higher LVEM e' tissue velocity and higher E to- e' ratio (LVEEM), compared to non-prediabetic children. Also, early LV diastolic and systolic dysfunction were determined to be present in prediabetic children and adolescents.

Our results indicate that obese prediabetic children are characterized by a higher frequency of increased LVMi and impaired ventricular function. With this study, we also want to emphasize the importance of glucose and blood pressure monitoring in the follow-up of obese children and to state that the assessment of Doppler imaging might be useful in detecting subclinical impairment of cardiac function in prediabetic obese patients at a pediatric age.

Ethics

Ethics Committee Approval: Necmettin Erbakan University Ethics Committee (Approval number: 2014/572), Informed Consent: Retrospectively designed.

Peer-review: External peer-reviewed.

Author Contributions

Concept: Beray Selver Eklioğlu, Mehmet Emre Atabek, Design: Beray Selver Eklioğlu, Mehmet Emre Atabek, Data Collection or Processing: Beray Selver Eklioğlu, Nesibe Akyürek, Hayrullah Alp, Analysis or Interpretation: Beray Selver Eklioğlu, Mehmet Emre Atabek, Nesibe Akyürek, Hayrullah Alp, Literature Search: Beray Selver Eklioğlu, Nesibe Akyürek, Hayrullah Alp, Writing: Beray Selver Eklioğlu, Mehmet Emre Atabek, Nesibe Akyürek, Hayrullah Alp.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):86-91
DOI: 10.4274/jcrpe.2512

Case Report

Delayed Adrenarche may be an Additional Feature of Immunoglobulin Super Family Member 1 Deficiency Syndrome

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ABSTRACT

Immunoglobulin super family member 1 (IGSF1) deficiency syndrome is characterized by central hypothyroidism, delayed surge in testosterone during puberty, macro-orchidism, and in some cases, hypoprolactinemia and/or transient growth hormone (GH) deficiency. Our patient was a 19-year-old male adolescent who had been treated since the age of 9 years with GH and thyroxine for an idiopathic combined GH, thyroid-stimulating hormone (TSH), and prolactin (PRL) deficiency. His GH deficiency proved to be transient, but deficiencies of TSH and PRL persisted, and he had developed macro-orchidism since the end of puberty. Brain magnetic resonance imaging and *PROP1* and *POU1F1* sequencing were normal. A disharmonious puberty (delayed genital and pubic hair development, bone maturation, and pubertal growth spurt, despite normal testicular growth) was observed as well as a delayed adrenarche, as reflected by very low dehydroepiandrosterone sulfate and delayed pubarche. Direct sequencing of the *IGSF1* gene revealed a novel hemizygous mutation, c.3127T>C, p.Cys1043Arg. Pathogenicity of the mutation was demonstrated *in vitro*. Male children with an idiopathic combined GH, PRL, and TSH deficiency, showing persistent central hypothyroidism but transient GH deficiency upon retesting at adult height, should be screened for mutations in the *IGSF1* gene, especially when macro-orchidism and/or hypoprolactinemia are present. We suspect that delayed adrenarche, as a consequence of PRL deficiency, might be part of the clinical phenotype of patients with IGSF1 deficiency.

Keywords: Immunoglobulin super family member 1 deficiency syndrome, central hypothyroidism, macro-orchidism, delayed adrenarche, novel mutation

Conflict of interest: None declared
Received: 17.10.2015
Accepted: 10.11.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Loss of function of the immunoglobulin super family member I (*IGSF1*) gene is characterized in males by central hypothyroidism, delayed testosterone rise in puberty despite normal timing of testicular enlargement, and adult macro-orchidism. Approximately 15% of male patients have transient growth hormone deficiency and 65% have hypoprolactinemia. Normally, the mature glycoform of IGSF1 is localized at the cell surface, and most loss-of-function mutations impair its glycosylation or trafficking to the cell membrane.

WHAT THIS STUDY ADDS?

In addition to a delayed pubertal surge in testosterone, we documented a delayed increase in dehydroepiandrosterone sulfate and delayed pubarche in our patient. We suspect that a delayed adrenarche might be part of the clinical phenotype of patients with IGSF1 deficiency and contributes to delayed bone maturation.

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Introduction

Loss-of-function of the immunoglobulin super family member 1 gene (*IGSF1*, OMIM#300888) causes an X-linked syndrome, characterized in males by congenital central hypothyroidism, delayed testosterone rise in puberty despite normal timing of testicular enlargement, adult macro-orchidism, and in some cases deficiencies of prolactin (PRL) and/or growth hormone (GH) (1,2,3). A small proportion of heterozygous females show central hypothyroidism, PRL deficiency, and/or delayed menarche (3). Human *IGSF1* messenger ribonucleic acid is abundantly expressed in the adult and developing anterior pituitary gland and testis. The gene encodes a plasma membrane glycoprotein (1).

With 31 patients described to date, the variability of the phenotype may not yet be fully characterized (1,4,5). In the present case, IGSF1 deficiency was diagnosed at the age of 19 years, based on a history of transient GH deficiency, persistent central hypothyroidism, and hypoprolactinemia, and the finding of macro-orchidism. The close follow-up during GH replacement, which was started one year before the onset of puberty, permitted a detailed recording of the genital and pubic hair development, pubertal growth spurt, and bone maturation. Besides a delayed pubertal surge in testosterone, a delayed increase in dehydroepiandrosterone sulfate (DHEAS) was documented in our patient. We suspect that delayed adrenarche might be part of the clinical phenotype of patients with IGSF1 deficiency.

Case Report

The boy was first seen at the pediatric endocrinology clinic at the age of 9 years for reduced growth velocity since the age of 3 years. He was born after 39 weeks of gestation, with a birth weight of 3750 grams and a birth length of 52 cm. A slightly prolonged neonatal jaundice was noted. His neurocognitive development was normal. From 3 years on, he intermittently received standard corticoid inhalation therapy for allergic asthma and corticoid ointments for chronic eczema.

His initial work-up showed a delayed bone age (6.25 years at a chronological age of 9.1 years) and a low IGF-1 concentration of 72 ng/mL (reference range for age: 74-388 ng/mL). Serum thyroid-stimulating hormone (TSH) was normal (1.6 µU/mL, reference range 0.28-4.3 µU/mL), free thyroxine (fT_4) was on the lower limit of the reference range (0.9 ng/dL, reference range 0.9-1.7 ng/dL), and free triiodothyronine (fT_3) was normal (3.4 ng/dL, reference range 2.57-4.43 ng/dL). Serum PRL level was unmeasurable (<0.5 ng/mL), while basal cortisol was normal (15 µg/dL). He was treated with levothyroxine, inducing a temporary catch-up growth (Figure 1). After 6 months, partial GH deficiency was suspected based on a GH peak of 9.4 ng/mL after glucagon stimulation. The low GH reserve was confirmed at insulin tolerance testing after priming with testosterone (peak GH 6.6 ng/mL). Brain magnetic resonance imaging, including the hypothalamic-pituitary region, was normal. Because of the combination of central hypothyroidism, GH deficiency, low PRL status, and normal pituitary imaging, genetic testing of *PROP1* and *POU1F1* was performed, but no mutations were found.

Based on these findings, he was diagnosed with idiopathic combined GH, TSH, and PRL deficiency. GH replacement therapy (0.03 mg/kg/day) was initiated at the age of 10 years and 4 months, resulting in rapid catch-up growth. Follow-up examinations (Table 1) revealed a disharmonious puberty with delayed genital and pubic hair development and testosterone surge, but normal timing of the increase in testicular size. Excessive testicular growth became evident at the end of puberty (Table 1). DHEAS measurements were repeatedly low. Bone maturation progressed slowly. Low dose adrenocorticotropic hormone (ACTH) testing at 14 years and 7 months old showed a normal cortisol increase (serum cortisol 20.3 µg/dL at 30 minutes). PRL levels remained undetectable (<0.5 ng/mL) throughout the whole follow-up.

Z-scores for height increased during pubertal development, in accordance with increasing serum IGF-1 concentrations, while fT_4 concentrations remained normal during treatment with levothyroxine. Pubertal growth slowed down around the age of 16.5 years (height increase <3 cm/year). After stopping GH treatment for 3 months, combined insulin-thyrotropin-releasing hormone (TRH)-gonadotropin-releasing hormone testing showed a low normal TSH (peak value: 6.3 mU/L) and very low PRL reserve (peak value: 5.8 ng/L), but a normal GH (peak value 15.3 ng/mL), cortisol, follicle stimulating hormone (FSH) and luteinizing hormone (LH) response. Basal serum FSH (6.6 U/L) was higher than LH (3.0 U/L), although both were within the reference ranges and testosterone was normal (512.1 ng/dL). Basal fT_4 concentration was low (0.8 ng/dL) due to poor compliance in the last month. At his last visit, at the age of 19 years, his height was 182.8 cm (0.5 standard deviation score [SDS]) and his weight 89.3 kg (body mass index of 27 kg/m^2, 1.5 SDS). His orchidometric testicular volume was >30 mL bilaterally, and his pubertal status A3P5G5. Total pubertal height gain was 41 cm.

The combination of persistent central hypothyroidism, transient partial GH deficiency, and macro-orchidism led to the suspicion of IGSF1 deficiency (1). The patient gave his informed consent for *IGSF1* gene analysis and publication of his clinical history. His mother declined carrier testing.

Mutation analysis of the *IGSF1* gene was performed by direct sequencing. The glycosylation and expression at the plasma membrane was examined in heterologous HEK293 cells, as described in (1). To assess plasma membrane trafficking, HEK293 cells were transfected with pcDNA3.0 (empty vector), or with expression vectors for C-terminal

Table 1. Auxological and hormonal data during adolescence

Age (y m)	Height (SDS)	BMI (SDS)	Puberty (Tanner)	Testis volume (Prader) (mL)	DHEAS (µg/dL)	Testosterone (ng/dL)	Bone age	Treatment
10 y 1 m	-2.0	+1.1	P1G1	2x2 mL	5.5		8 y 6 m	L-thyroxine 75 µg GH 0.85 mg
11 y 7 m	-1.1	+0.8	P1G2	2x4 mL	2.06	<10		L-thyroxine 75 µg GH 1 mg
11 y 10 m	-1.1	+0.5	P1G2	2x5 mL	10.7	13.5		L-thyroxine 75 µg GH 1 mg
12 y 1 m	-1.2	+0.5	P1G2	2x6 mL	14.22	<10	10 y 6 m	L-thyroxine 75 µg GH 1.1 mg
12 y 4 m	-1.1	+0.6	P1G2	2x8 mL	17.07	<10		L-thyroxine 75 µg GH 1.1 mg
12 y 7 m	-1.0	+0.7	P1G2	2x8 mL				L-thyroxine 75 µg GH 1.1 mg
12 y 10 m	-0.9	+0.7	P1G3	2x10 mL	17.93	51.7		L-thyroxine 75 µg GH 1.2 mg
13 y 3 m	-0.9	+0.9	P2G3	2x12 mL	15.53	41.4	11 y 6 m	L-thyroxine 75 µg GH 1.2 mg
13 y 6 m	-0.8	+1.0	P3G4	2x15 mL				L-thyroxine 75 µg GH 1.3 mg
13 y 10 m	-0.6	+0.7	P4G5	2x25 mL	31.75	322		L-thyroxine 75 µg GH 1.4 mg
14 y 6 m	-0.1	+1.1	P5G5	2x30 mL	45.08	377	13 y 9 m	L-thyroxine 75 µg GH 1.8 mg

Testosterone reference values for Tanner stages (stage 1: <12 ng/dL, stage 2: <12-430 ng/dL, stage 3: 65-780 ng/dL, stage 4: 180-760 ng/dL, stage 5: 138-1050 ng/dL).
Dehydroepiandrosterone sulfate reference values for age (10-14 y: 24.4-247 µg/dL, 15-19 y: 70-490 µg/L). DHEAS: dehydroepiandrosterone sulfate, GH: growth hormone, BMI: body mass index, SDS: standard deviation score, m: months, y: years

HA-tagged forms of the wild-type (*IGSF1*-HA wt) or mutant *IGSF1* (*IGSF1*-HA C1043R; c.3127T>C, p.Cys1043Arg). Plasma membrane proteins were biotinylated prior to cell lysis. IGSF1 protein was immunoprecipitated (IP) with an HA antibody and then resolved by SDS-PAGE under reducing conditions. Biotinylated IGSF1 at the plasma membrane was detected by streptavidin-horseradish peroxidase. Efficacy of the IP was assessed by HA immunoblot.

As observed previously for other pathogenic missense mutations, IGSF1 harboring the Cys1043Arg mutation does not acquire mature glycosylation and fails to traffic from the endoplasmic reticulum to the plasma membrane (Figure 2).

Discussion

We described the first Belgian patient with a novel *IGSF1* mutation and presented detailed longitudinal data on the patient's growth, pubertal development as well as his testicular and adrenal functions. The main characteristics of this newly described genetic syndrome are congenital hypothyroidism of central origin and macro-orchidism. The diagnosis of central hypothyroidism is rarely made at birth as most neonatal screening programs for congenital hypothyroidism are based solely on the measurement of TSH. In the Netherlands, where neonatal screening of both thyroxine and TSH levels allows for an early diagnosis of central hypothyroidism, the incidence of

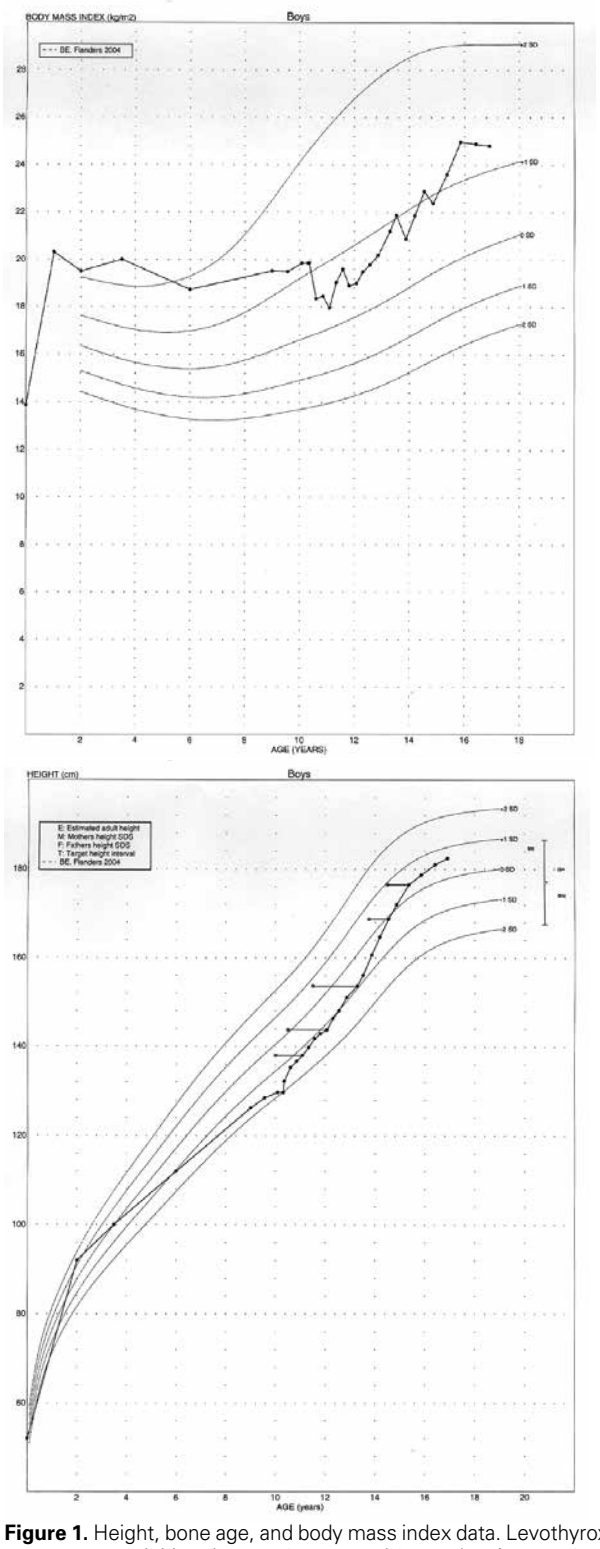

Figure 1. Height, bone age, and body mass index data. Levothyroxine treatment was initiated at age 9 years and 3 months. At age 10 years and 4 months, growth hormone replacement therapy was started

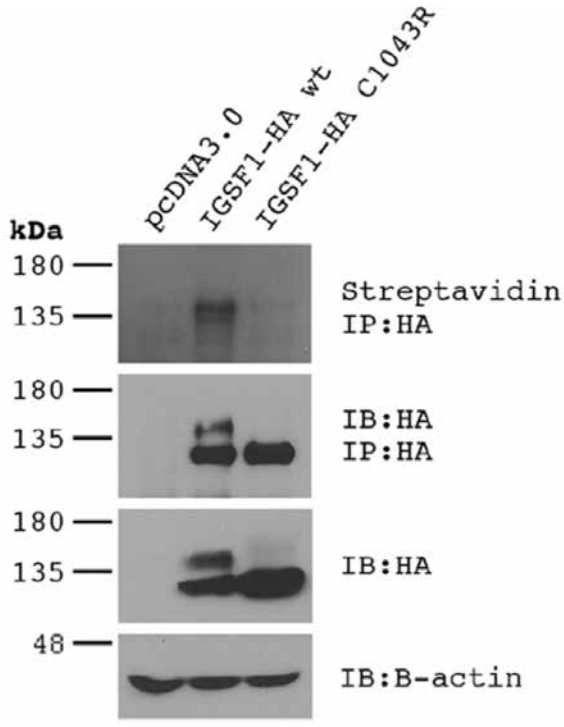

Figure 2. Results of immunoblotting assays. Biotinylated Immunoglobulin super family member 1 (IGSF1) at the plasma membrane was detected by streptavidin-horseradish peroxidase (top panel). Note the appearance of a band exclusively in the wild-type lane. Efficacy of the immunoprecipitated was assessed by HA immunoblot (IB; second panel from top). Note the appearance of a doublet in the wild-type lane and a single band in the mutant IGSF1 lane, indicating that the latter fails to acquire mature carbohydrates. The same banding pattern was observed when the proteins were analyzed by direct immunoblotting (third panel from top). The bottom panel confirmed equal loading of the samples in the HA:IB panel

IGSF1 deficiency is estimated at approximately 1:100 000 (2). Slow linear growth and increased adiposity can be the presenting sign of central hypothyroidism during childhood, as observed in our case. In the reported cases, the hypothyroidism was mild, with a mean serum fT_4 level at the lower limit of the reference range (1,3). TRH stimulation testing in our patient in young adulthood showed a weak TSH response, as has been reported in most cases, although delayed and exaggerated responses have also been observed (1,5).

In a small proportion (15%) of patients with the *IGSF1* deficiency syndrome, a partial and transient GH deficiency also contributes to the growth delay. Our case showed a decreased GH reserve despite correction of his hypothyroid state and priming with testosterone, and his GH reserve normalized only after reaching young adulthood. This observation is in line with the initial report on four patients with transient partial GH deficiency (1). Based on murine expression of IGSF1 protein in thyrotropes, somatotropes, and lactotropes in the pituitary gland, a role for *IGSF1* in pituitary GH production and/or secretion appears likely.

We recorded by regular examinations a disharmonious pubertal development (normal timing of testicular growth, but a delayed surge of serum testosterone) in our patient. In patients with the *IGSF1* deficiency syndrome, prepubertal testicular size is usually normal and testicular enlargement starts at a normal age. However, testicular volumes exceeds the reference range during puberty and enlargement may continue further in adulthood (2). Testicular size is determined by Sertoli cell number and is mainly dependent on FSH and thyroid hormone levels (6). In reported cases of boys with *IGSF1* deficiency, serum FSH concentrations were always higher than serum LH values, although still within the normal range. As a consequence of the delayed testosterone production, pubic hair development and the pubertal growth spurt are delayed in IGSF1 deficiency. Nevertheless, normal testosterone levels are observed in young adulthood (1).

Interestingly, in our case we also observed delayed adrenarche, as reflected by DHEAS levels below the reference range and the absence of pubarche until the age of 13.8 years. Although delayed pubic hair development has been observed previously, no data on adrenal androgens have been reported in patients with IGSF1 deficiency. However, delayed adrenarche has also been observed in boys with mutations in the *PIT1/POU1F1* gene causing GH, TSH, and PRL deficiency and therefore resembling *IGSF1* deficiency (7). It is unlikely that GH or TSH deficiency are responsible for the delayed adrenarche, since our patient received replacement therapy for both deficiencies. Furthermore, patients with isolated GH deficiency are known to have normal adrenal androgen levels (8) and the expression of the TSH receptor in the adrenal cortex is very low (9). We suspect that the delayed adrenarche in both IGSF1 and *POU1F1* defects might be caused by the low PRL secretion. PRL receptors are highly expressed in the adrenal cortex and synergize with ACTH to augment secretion of adrenal androgens (10,11,12). Also, inducing decreased PRL by exogenous dopamine reduces DHEAS levels, whereas hyperprolactinemia is associated with elevated DHEAS (13,14). The low DHEAS production might contribute to the delayed pubic hair growth, but also to the delayed bone maturation, which was observed before as well as during GH therapy.

Up to now, systematic IGSF1 mutation analysis has not been performed in larger cohorts of patients with transient or persistent GH deficiency in combination with central hypothyroidism and low PRL levels. Most institutions start with analysis of the *PROP1* gene in cases of combined pituitary hormone deficiency. However, molecular changes in many patients currently remain unexplored (15). More comprehensive and faster genetic screening techniques will gain importance in the diagnosis and management of these pituitary hormone deficiencies and will detect family members at risk. *IGSF1* deficiency is inherited in an X-linked pattern with reduced penetrance in females. However, since the deficiency may manifest itself as central hypothyroidism, hypoprolactinemia, and delayed menarche in female carriers, mutation analysis in at risk family members is recommended.

Male children with an idiopathic combined GH and TSH deficiency, showing a persistent central hypothyroidism but a transient GH deficiency, should be screened for loss-of-function mutations or deletions of the *IGSF1* gene, especially when delayed puberty and macro-orchidism are present. IGSF1 deficiency may be associated with delayed adrenarche, possibly caused by PRL deficiency.

Ethics

Informed Consent: obtained.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Concept: Severine Van Hulle, Jean De Schepper, Design: Jean De Schepper, Bert Callewaert, Data Collection or Processing: Severine Van Hulle, Margarita Craen, Bert Callewaert, Sjourd Joustra, Marc Olivier Turgeon, Daniel J. Bernard, Jean De Schepper, Analysis or Interpretation: Wilma Oostdijk, Monique Losekoot, Jan Maarten Wit, Marc Olivier Turgeon, Daniel J. Bernard, Sjourd Joustra, Literature Search: Severine Van Hulle, Jean De Schepper, Sjourd Joustra, Writing: Severine Van Hulle, Bert Callewaert, Sjourd Joustra, Jan Maarten Wit, Daniel J. Bernard, Jean De Schepper.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):92-95
DOI: 10.4274/jcrpe.2297

Case Report

An Adolescent Boy with Comorbid Anorexia Nervosa and Hashimoto Thyroiditis

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ABSTRACT

Low triiodothyronine syndrome is a physiological adaptation encountered in anorexia nervosa (AN) and generally improves with sufficient weight gain. However, when a primary thyroid pathology accompanies AN, both the evaluation of thyroid hormone levels and the management of the co-morbid disease become more challenging. Hashimoto thyroiditis could complicate the management of AN by causing hyper- or hypothyroidism. AN could also negatively affect the treatment of Hashimoto thyroiditis by altering body weight and metabolic rate, as well as by causing drug non-compliance. We present the case of a 15-year-old boy with comorbid AN restrictive sub-type and Hashimoto thyroiditis. In this case report, we aimed to draw attention to the challenges that could be encountered in the diagnosis, treatment, and follow-up of patients with AN when accompanied by Hashimoto thyroiditis.

Keywords: Anorexia nervosa, Hashimoto thyroiditis, adolescent, hypothyroidism

Conflict of interest: None declared
Received: 04.08.2015
Accepted: 15.10.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Review of the literature for the association of primary thyroid pathologies with eating disorders offers case reports evaluating the deteriorating effect of hyperthyroidism on the psychological and physiological symptoms of anorexia nervosa (AN) such as anxiety, irritability, and fear of losing control over eating due to an increased appetite. In these cases, hyperthyroidism is mostly due to graves' disease. Also, a recently published case report of a 16-year-old girl with a 4-year history of AN diagnosed with Hashimoto thyroiditis suggests an association between weight and thyroid-stimulating hormone levels.

WHAT THIS STUDY ADDS?

Although Hashimoto thyroiditis is accepted as one of the most common autoimmune endocrine disorders, its effect on eating disorders seems to be lacking in the literature. This case of AN with Hashimoto thyroiditis is informative as it highlights the challenges faced during the diagnosis, treatment, and follow-up of Hashimoto thyroiditis in the presence of AN and attracts attention to the effects of hypothyroidism and hyperthyroidism on the patient.

Introduction

Anorexia nervosa (AN) is a psychiatric disorder with significant neuroendocrine consequences. In patients with eating disorders (EDs), the hypothalamic-pituitary-thyroid axis is possibly the most studied neuroendocrine pathway. Thyroid function tests of patients with AN resemble the sick euthyroid state which is characterized by low total triiodothyronine (T3) levels, usually normal thyroid-stimulating hormone (TSH) levels, and normal or slightly low thyroxin (T_4) levels (1,2). This physiological adaptation state is also known as low T_3 syndrome. Decreased deiodination of T_4 to T_3, increased peripheral conversion to reverse T_3, and decreased thyroidal T_3

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secretion in response to endogenous TSH are the probable causes of this disorder (3,4). T_3 is a negative measure of the nutritional status, and low T_3 levels are necessary for the body to protect itself from starvation by limiting resting energy expenditure and conserving energy for vital functions. With nutritional rehabilitation and a sufficient amount of weight gain, T_3 levels are expected to increase and to attain the normal range (5).

Hashimoto thyroiditis, which can lead to hypothyroidism and sometimes to hyperthyroidism, is a condition which needs to be considered in the differential diagnosis of AN. The laboratory and clinical resemblance of hypothyroidism and AN could complicate the diagnosis and management of both diseases, while a hyperthyroid state could deteriorate the psychological and physiological symptoms of AN.

In this case report, we present these challenges encountered in the diagnosis, treatment, and follow-up of a patient with AN restrictive sub-type accompanied by Hashimoto thyroiditis.

Case Report

We present a 15-year-old boy who was admitted to our hospital with significant weight loss, malaise, and cold intolerance. Written informed consent for presentation was obtained from the patient and his parents.

The patient weighed 102 kg until four years ago, at which time, with the help of a dietitian, he started to lose weight. However, after losing 2 kg, he discontinued the dietary regime. Two years later, when he weighed 100 kg, he decided to lose weight again because he felt uncomfortable with the way he looked and felt very overweight. Within a year, he lost 20 kg by eating less and playing basketball every day for approximately two hours a day. At that time, he had to quit basketball due to a busy school schedule which led to a fear of gaining weight, causing him to restrict his diet even more. By restricting his daily intake to 500 kcal, he had lost 17.5 kg within the last two months before presenting to our clinic. The patient denied having body image problems but agreed that he had an intense fear of gaining weight. Past medical history was unremarkable except for an appendectomy performed when he was 7 years old. The family history revealed that two of his aunts have Hashimoto thyroiditis.

At admission, the patient's body weight was 60.7 kg (50-75th percentile). Height was 186 cm (>97th percentile) and body mass index was 17.55 kg/m^2 (<3rd percentile). His body temperature was 36.1 °C and respiratory rate was 22/min. His supine blood pressure was 100/60 mmHg and heart rate was 40 bpm. His standing blood pressure was 95/60 mmHg and heart rate was 66 bpm. Cardiac examination was normal except for the bradycardia, and other systems were also normal on his physical examination. Meeting the diagnostic criteria of the fourth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM IV), he was diagnosed with AN-restrictive type and hospitalized due to bradycardia. His laboratory investigations which included complete blood count, liver and kidney function tests, glucose and electrolyte levels, sedimentation rate, cortisol, cholesterol levels, and urinary analysis were all within normal ranges. Thyroid function tests revealed very low TSH levels (0.025 uIU/mL, normal range: 0.27-4.20 uIU/mL), low free T_3 (fT_3) levels (2.87 pmol/L, normal range 3.10-6.70 pmol/L), and normal free T_4 (fT_4) levels (21.9 pmol/L, normal range: 12.00-22.00 pmol/L). Thyroid peroxidase antibodies and thyroglobulin antibodies were high, while TSH receptor antibodies were negative. With these findings, he was additionally diagnosed with Hashimoto thyroiditis. Thyroid ultrasonography confirmed the diagnosis.

In the inpatient unit, the patient was followed by an interdisciplinary team consisting of a child and adolescent psychiatrist, an adolescent medicine specialist, a pediatric endocrinologist, and a dietitian with special training and experience in adolescent EDs. During his three-week stay, he gained 4 kg. Despite the weight gain and the improvement in his nutritional status, bradycardia continued (40-50 bpm). Echocardiography findings were normal, and Holter monitoring only revealed sinus bradycardia. Thyroid functions were monitored closely without any medical treatment, along with his vitals. Before discharge, while TSH levels were still low (0.018 uIU/mL), fT_3 levels were thought to be relatively high (3.76 pmol/L) considering his metabolic status. The patient was discharged with a weight of 64.7 kg. At his follow-up visit 2 months later, the boy had gained weight and weighed 75.1 kg. Due to the gradual increase noted in his TSH levels (from 8.91 to 28.84 uIU/mL), levothyroxine treatment was started. At that time, fT_3 and fT_4 levels were measured as 7.74 and 4.42 pmol/L, respectively. Two months later, it was learned that he had been using levothyroxine in doses three times higher than the recommended dose. Although monitored closely, due to the drug compliance problems and weight changes with severe body image issues, it was hard to maintain the thyroid levels within a stable course. Stabilization occurred after ten months of therapy when he started using a proper medication schedule and succeeded in preserving his target weight. The course of the thyroid function tests is given in Table 1. Bradycardia also improved with the recovery in thyroid hormone levels.

Discussion

In the presence of an unexplained weight loss, decrease in appetite, and abnormal eating attitudes, EDs should be considered. However, other medical diseases leading to a similar clinical status such as hyperthyroidism, Addison's disease, diabetes mellitus, malignancy, inflammatory bowel disease, immunodeficiency, malabsorption, chronic infections,

Table 1. Thyroid function test results of the patient (initial values and subsequent monthly results)

	Initial	1st	2nd	4th	5th	6th	7th	10th	12th	18th
TSH (uIU/mL)	0.025	8.91	28.84	0.03	3.33	6.90	0.03	3.28	5.78	6.26
T_3 (pmol/L)	2.87	3.57	4.42	4.50	2.80	3.50	3.42	3.56	3.43	4.13
T_4 (pmol/L)	21.19	7.79	7.74	15.38	7.49	8.68	14.31	9.96	9.29	8.77

Normal ranges for initial values of thyroid-stimulating hormone (TSH): 0.27-4.20 uIU/mL, free triiodothyronine (T_3): 3.10-6.70 pmol/L, free thyroxin (T_4): 12.00-22.00 pmol/L
Normal ranges for subsequent values of TSH: 0.34-5.60 uIU/mL, free T_3: 3.80-6.00 pmol/L, free T_4: 7.86-14.41 pmol/L

and collagen vascular diseases, should also be included in the differential diagnosis (6). Although concern about the cause of weight loss is helpful in the differentiation of EDs from other diseases, coexistence of a medical disease and an ED complicates the diagnosis and management of both conditions. Within the group of diseases listed above, hypothyroidism is of particular importance as its laboratory findings as well as its clinical findings such as cold intolerance, constipation, hypothermia, and bradycardia highly resemble those of ED's.

Hashimoto thyroiditis is a chronic autoimmune inflammation of the thyroid gland and it is the most common cause of acquired hypothyroidism in adolescents (7). The hypothesis that autoimmune mechanisms are involved in the pathogenesis of EDs has become more acceptable in recent years, supported by the results of several studies indicating that autoimmune diseases occur in higher ratios in ED cases (8,9). Hashimoto thyroiditis may exist before or it can develop after the diagnosis of AN. Although patients with Hashimoto thyroiditis generally present at the euthyroid state, Hashimoto thyroiditis either by causing hyper- or hypothyroidism could complicate the management of EDs.

Hypothyroidism caused by Hashimoto thyroiditis may complicate the management of AN by increasing the susceptibility to gain weight, thus inducing the AN patient to eat less. Hashimoto thyroiditis could also disrupt the management of AN by causing hyperthyroidism. The literature contains many reports on hyperthyroidism, mostly due to Graves' disease, worsening AN either by leading to weight loss itself or by increasing the psychological distress by causing hyperphagia, weight gain, and a fear of losing self-control over eating (10,11,12). In the clinical course of our patient, Hashimoto thyroiditis disrupted the management of AN first by causing hyperthyroidism and then by leading to hypothyroidism. In the presence of AN, treatment of Hashimoto thyroiditis is challenging as well. AN could negatively affect the treatment of Hashimoto thyroiditis by altering the body weight and the metabolic rate, as well as by causing drug non-compliance.

While following patients with AN, because malnutrition could mask the clinical manifestations, the diagnosis of hyper- or hypothyroidism might be overlooked. Therefore, further analysis is needed when the major complaint does not improve with sufficient weight gain or when the levels of fT_3, fT_4, and TSH are incompatible with the body weight and nutritional status. Initially, our case was thought to have hyperthyroidism as the autoantibodies were positive, TSH level was lower than expected, and fT_4 level was at the upper limits. However, during follow-up and within a short period of time, TSH suppression disappeared, T_4 level started to decrease, and findings consistent with hypothyroidism became evident. Bradycardia being resistant to weight gain but improving after levothyroxine treatment was probably related to hypothyroidism as well.

Differentiating between primary thyroid diseases and low T_3 syndrome is important in the achievement of best treatment outcome. Levothyroxine treatment is not recommended in low T_3 syndrome, because it may cause weight loss, which is often mediated by loss of muscle mass (13). Also, thyroid hormone therapy, by leading to a decrease in weight, could obstruct the evaluation of patient's response to treatment or could lead to the deterioration of symptoms (14,15). On the other hand, levothyroxine treatment could be given when hypothyroidism due to a primary thyroid disease accompanies AN. This raises the question of when to start levothyroxine in the coexistence of these two conditions. For our patient, thyroid functions tests were monitored closely during his hospital admission, and levothyroxine was initiated two months after discharge when he had gained enough weight and had become metabolically stable. Another problem is that, depending on the beliefs and attitudes of patients with AN, they could either reject using levothyroxine as it may lead to hyperphagia and weight gain or misuse it to lose weight (7,16). In addition, weight fluctuations may complicate the dose adjustment of thyroid hormone therapy in these patients. Our patient`s thyroid hormone levels could only be stabilized after he began to take the recommended dosage of levothyroxine and preserved his target weight.

Review of the literature for the association of Hashimoto thyroiditis with EDs revealed only one recently published case report of a 16-year-old girl with a 4-year history of AN whose TSH levels had increased in response to weight gain and shown a decrease with weight loss. This unexpected pattern of TSH and markedly elevated levels of antithyroperoxidase antibodies suggested Hashimoto thyroiditis (17). The association between body weight and TSH levels observed in this case report was also present in our patient. With weight restoration, an elevation in TSH

levels occurred and levothyroxine treatment was started after TSH levels continued to rise with increasing weight similar to the case cited above. However, since levothyroxine had to be started in an earlier phase due to the hormonal status, the pattern of weight loss associated with decreases in TSH was not observed in our patient. Also, unlike the cited case, our patient was diagnosed with AN and Hashimoto thyroiditis at the same time. Moreover, initial tests of our patient being consistent with hyperthyroidism and misusage of levothyroxine created additional challenges during the follow-up.

Although Hashimoto thyroiditis is accepted as one of the most common autoimmune endocrine disorders, its effect on EDs seems to be lacking in the literature. This case of AN in conjunction with Hashimoto thyroiditis is informative as it highlights the challenges faced during the diagnosis and treatment of Hashimoto thyroiditis in the presence of AN and attracts attention to the effect of this coexistence on the patient.

Ethics

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

Authorship Contributions

Concept: Nuray Kanbur, Sinem Akgül, Ayfer Alikaşifoğlu, Design: Melis Pehlivantürk Kızılkan, Nuray Kanbur, Sinem Akgül, Ayfer Alikaşifoğlu, Data Collection or Processing: Melis Pehlivantürk Kızılkan, Analysis or Interpretation: Melis Pehlivantürk Kızılkan, Nuray Kanbur, Literature Search: Melis Pehlivantürk Kızılkan, Writing: Melis Pehlivantürk Kızılkan.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):96-100
DOI: 10.4274/jcrpe.2070

Case Report

Growth Hormone Deficiency in a Child with Neurofibromatosis-Noonan Syndrome

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ABSTRACT

Neurofibromatosis-Noonan syndrome (NFNS) is a distinct entity which shows the features of both NF1 (neurofibromatosis 1) and Noonan syndrome (NS). While growth hormone deficiency (GHD) has been relatively frequently identified in NF1 and NS patients, there is limited experience in NFNS cases. The literature includes only one case report of a NFNS patient having GHD and that report primarily focuses on the dermatological lesions that accompany the syndrome and not on growth hormone (GH) treatment. Here, we present a 13-year-old girl who had clinical features of NFNS with a mutation in the NF1 gene. The case is the first NFNS patient reported in the literature who was diagnosed to have GHD and who received GH treatment until reaching final height. The findings in this patient show that short stature is a feature of NFNS and can be caused by GHD. Patients with NFNS who show poor growth should be evaluated for GHD.

Keywords: Growth hormone deficiency, growth hormone, neurofibromatosis-Noonan syndrome, NF1 gene, neurofibromatosis type 1, Noonan syndrome

Conflict of interest: None declared
Received: 19.04.2015
Accepted: 27.09.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Neurofibromatosis-Noonan syndrome (NFNS) is a distinct entity which has variable features of both NF1 (neurofibromatosis 1) and Noonan syndrome (NS). Mutations in the *NF1* gene were identified in majority of NFNS cases. Growth hormone deficiency (GHD) has been relatively frequently identified in NF1 and NS, there is limited experience with GHD in NFNS cases.

WHAT THIS STUDY ADDS?

Short stature is a feature of NFNS; however, in some cases it can be caused by GHD and patients with NFNS who are not growing sufficiently should be evaluated for GHD. The case presented herein had clinical features of NFNS with a mutation in the NF1 gene. It is the first NFNS case reported in the literature with GHD, receiving growth hormone (GH) treatment, and reaching a successful final height under GH treatment.

Introduction

Neurofibromatosis-Noonan syndrome (NFNS, OMIM 601321) was first defined as a distinct entity in 1985 by Allanson et al (1) who reported four unrelated patients with neurofibromatosis (NF) who also had manifestations of Noonan syndrome (NS). These cases had presented with clinical findings such as short stature, ptosis, midfacial hypoplasia, webbed neck, learning disabilities, and muscle weakness (1). Opitz and Weaver (2) also reported a similar syndrome, defined as a separate clinical entity which they named NFNS. This entity bore the features of both NF type 1 (NF1) and NS. These early reports were followed by others (3,4,5,6,7,8,9,10,11). When the genetic studies performed on

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NFNS are reviewed, it is noted that a mutation was identified in the *NF1* gene in the majority of these studies. The co-occurrence of NF1 and PTPN11 mutations has been shown in very few studies and has been attributed to a *de novo* mutation either in *NF1* or *PTPN11* gene (12,13). Today, the opinion that NFNS originates from different mutations at distinct genes affecting a common intracellular signal transduction pathway called RAS-MAPK (mitogen-activated protein kinase) pathway is more widely accepted. This pathway plays roles in cell proliferation, differentiation, and apoptosis. The number of affected genes in the RAS-MAPK pathway and the diversity of the mutations in these genes result in various different phenotypic characteristics and different syndromes. Since these syndromes are associated with the effects on the same pathway, they are called "RASopathies" or RAS-MAPK syndromes and NFNS is an important RASopathy.

Growth hormone deficiency (GHD) has been relatively frequently identified in NF1 and NS patients. Those receiving growth hormone (GH) treatment have been published as case reports and the growth pattern, GH responses, near-adult, and adult heights of these cases have been reported (14,15,16,17). However, the literature includes only one study that shows GHD in NFNS cases and that report primarily focuses on the dermatological lesions that accompany NFNS (18). GH treatment in NFNS is still a matter of debate. To our knowledge, the case presented herein is the first reported NFNS patient with GHD who received GH treatment and was followed until she reached final height under GH treatment.

Case Report

A 13-year-old girl presented with short stature. Physical examination showed dysmorphic facial features, a short and webbed neck, low posterior hairline, cubitus valgus, brachy- and clinodactyly, and widely spaced nipples suggesting NS and multiple café-au-lait spots (>15 mm, 8 spots), axillary freckling, and relative macrocephaly suggesting NF1 syndrome. Dysmorphic facial features included midfacial hypoplasia, prominent nasolabial folds, low-set and posteriorly rotated ears, hypertelorism, downslanted palpebral fissures, and low nasal root (Figure 1). The patient did not have any neurofibroma. Cardiovascular examination revealed no cardiac murmur and echocardiography was normal. The ocular examination did not reveal Lisch nodules. There was no sign of developmental delay, and the nervous system examination was completely normal. The patient's pubertal stage was evaluated as Tanner stage 2. Her arm span was 124.8 cm and upper/lower ratio was 0.9 suggesting no skeletal deformity. Karyotype analysis was 46,XX. The auxological parameters of the case at diagnosis are given in Table 1.

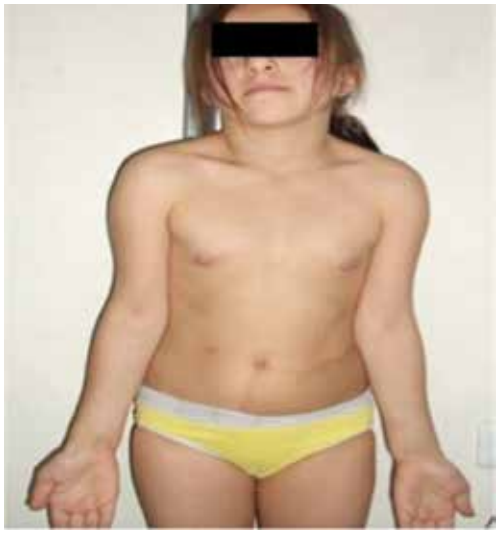

Figure 1. Physical findings of the patient suggestive of both neurofibromatosis 1 and Noonan syndrome

Complete blood count, routine biochemistry, and urine analysis were within the normal limits. The celiac antibodies were negative and thyroid function tests were normal. Both serum insulin-like growth factor-1 (IGF-1) and IGF binding protein 3 (IGF-BP3) levels were below -3 standard deviation score (SDS). Peak GH response to L-dopa and clonidine stimulation tests were 3.9 ng/mL and 4.2 ng/mL, respectively. Other pituitary hormone levels were all within normal ranges. The serum pituitary hormone levels at diagnosis are given in Table 2.

The size of the pituitary gland was measured as 3.5 mm in the pituitary magnetic resonance imaging (MRI) and this was considered to be consistent with anterior pituitary hypoplasia according to the age group of the patient. NFNS syndrome was suspected, and cranial MRI was performed to evaluate the neurological involvement. Cranial MRI showed a hyperintense mildly swollen appearance in T2 at the cerebral peduncles and globus pallidus that may be attributed to NF. T1-weighed images also showed hyperintense lesions associated with T1 limitation; after administration of intravenous contrast substance, these lesions did not show any uptake of the contrast (Figure 2).

Since the patient fulfilled the criteria of GHD, GH therapy was initiated with a dosage of 0.3 mg/kg/week. The height

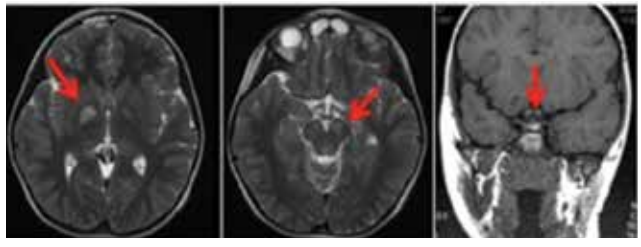

Figure 2. Cranial magnetic resonance imaging of the patient showing the lesions of neurofibromatosis in the cerebral peduncles and globus pallidus and adenopituitary hypoplasia in the pituitary

velocity during the first year of GH treatment was 9.8 cm/year and was 7.2 cm and 4.5 cm on the second and third years of GH therapy, respectively. The patient's height was 147.3 cm (height SDS: -2.3) at the end of the third year of GH therapy. The patient was 16.5 years old when the GH therapy was discontinued; she had had three regular menstruation cycles and her bone age was 14.5 years. Her final height was 148.5 cm, 7.5 cm above the mid-parental height (Figure 3).

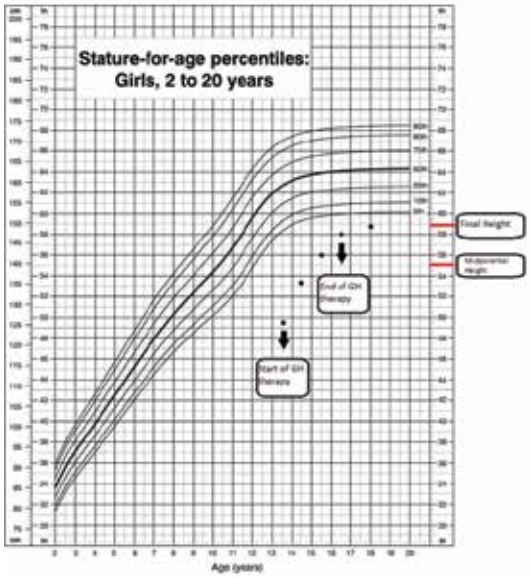

Figure 3. Growth chart of the patient under growth hormone therapy. Source: http:/www.cdc.gov/growth charts (Centers for Disease Control and Prevention (CDC) 2000)

Table 1. Auxological data of the case at the time of diagnosis	
Auxological parameters	
Age at diagnosis (years)	13.5
Bone age (years)	11
Height (cm)	125.8
Height SDS	-4.8
Weight (kg)	26.3
BMI (kg/m^2)	16.6
BMI-SDS	-0.94
Height velocity at the beginning of GH therapy (cm/yr)	1.3
Height velocity SDS at the beginning of GH therapy (cm/yr)	-2.8
Father's height (cm)	150
Father's height SDS	-4.1
Mother's height (cm)	145
Mother's height SDS	-2.9
Midparental height (cm)	141
Midparental height SDS	-3.5
Predicted adult height (cm)	137
Predicted adult height SDS	-4.2
SDS: standard deviation score, BMI: body mass index, GH: growth hormone	

The patient's father also had features representing both NF1 and NS such as multiple café-au-lait spots, short stature, relative macrocephaly, and axillary freckling, suggesting NF syndrome, and findings such as prominent nasolabial folds, low-set ears, low nasal root, dysmorphic facial features, short neck, and cubitus valgus suggesting NS. It was learned that some members of the father's family also had multiple café-au-lait spots. Genetic analyses were performed for the patient and her father to investigate NFNS and the genetic analysis of both the patient and the father revealed a truncating mutation c.7846C>T (M82814), p.Arg2616X (AAA59924) in the *NF1* gene. No mutation was found in *PTPN11* gene.

Discussion

NFNS is an entity presenting with clinical characteristics of both NF1 and NS. The frequency of NFNS is thought to be higher than the current estimates, since these cases may be missed due to their being inadvertently diagnosed as classic NF1 or NS. In the study of Colley et al (11), the reassessment of 94 cases diagnosed with NF1 has demonstrated that 12 of these cases actually met the criteria for NS. This reassessment has shown that some

Table 2. Pituitary hormone levels of the case at diagnosis		
	Hormone levels of the patient	**Reference hormone levels**
fT_4 (pmol/L)	12.8	7.86-14.41
fT_3 (pmol/L)	4.8	3.8-6.0
TSH (µIU/mL)	2.31	0.34-5.6
FSH (IU/L)	1.6	>0.3 (pubertal)
LH (IU/L)	0.5	>0.3 (pubertal)
Estradiol (pg/mL)	22	>10 (pubertal)
ACTH (pg/mL)	45.8	0-90
Cortisol (µg/dL)	15.1	4.3-22.4
PRL (ng/mL)	12.1	3.3-18.7
IGF1 (µg/L)	150	203-831
IGF-BP3 (µg/L)	2430	2710-6340
Peak GH level in L-dopa test (ng/mL)	3.9	≥10
Peak GH level in clonidine test (ng/mL)	4.2	≥10
GH: growth hormone, fT_4: free thyroxin fT_3: free triiodothyronine, TSH: thyroid-stimulating hormone, FSH: follicle stimulating hormone, LH: luteinizing hormone, ACTH: adrenocorticotropic hormone, PRL: prolactin, IGF1: insulin-like growth factor-1, IGF-BP3: insulin-like growth factor binding protein 3		

patients who have been clinically diagnosed with NF1 or NS can indeed be NFNS.

The genetic studies that have been undertaken to identify the gene causing NFNS have shown that the majority of these cases have a mutation in the NF1 gene. These studies have revealed that the mutations responsible for classic NF1 can also cause NFNS (19,20,21). The mutation identified in the present case is indeed a mutation that is seen in classical NF1 cases. Additional studies are required to clarify which mutations cause classic NF1, which mutations cause NFNS, and which mutations have the potential to cause both.

Short stature is a common feature of NFNS as it is of NF1 and NS (22). The frequent causes of short stature in these syndromes are skeletal deformities and nutritional problems. Presence of suprasellar lesions is also a frequent cause, but GHD can develop in some of these patients in the absence of an underlying suprasellar lesion (15). While many studies have reported presence of GHD in NF1 and NS, to our knowledge, there is only one case report on NFNS receiving GH therapy (18). It is known that NS patients are of normal height and weight at birth and that growth deficiency develops later, with almost 80% of the cases eventually being of short stature (23). Several studies have shown that GH treatment increases the final height in NS cases (24,25,26). Among these studies, the one with the highest number of cases is the National Cooperative Growth Study (27). This study involves a large cohort of 252 NS cases who have received GH therapy for 5.6 years on average. GHD can also be seen in NF1 cases in the absence of suprasellar lesions. Some researchers suggested that there could be a relationship between GHD and NF1 in the absence of an organic pituitary damage, and they agree that larger cohort studies are required to decide whether NF1 is a cause of GHD (15,28). GHD-specific screening was recommended in NF1 cases with insufficient growth. An impairment in the cellular signal transduction was suggested as the reason of GHD in NF1 cases without suprasellar regions (29). Hegedus et al (30) showed that neurofibromin provides somatic growth by affecting the hypothalamic-pituitary axis. In their study, body weight and anterior pituitary gland size were found to decrease in mice with an inactivated NF1 gene. It was also shown that the decrease in anterior pituitary size reduces neurofibromin expression in the hypothalamus, thereby decreasing the production of GH-releasing hormone, that of GH and IGF-1 as well.

There is only one case report in the literature about GHD and GH treatment in NFNS. As mentioned above, this one report focuses on the dermatological lesions in NFNS and gives no detailed information about GH treatment. Thus, the present manuscript is first to provide details of GH treatment in a NFNS case. The growth pattern of our patient showed that short stature had been a problem since early childhood, but that the problem had gradually increased within the last 2 years, during which her peers entered puberty and had pubertal growth. There was no underlying reason, such as severe skeletal deformities, suprasellar lesions, or nutritional deficiency to explain the short stature observed in the present case, thus initially, the short stature was considered to be related to the delay in puberty, as in NS cases. At admission, the height of our patient was <-2 SDS and her growth rate was very low. GHD was considered to be a possible reason for short stature, and GH stimulation tests were performed. The peak GH level was calculated as <5 ng/mL in two GH stimulation tests suggesting that the patient had severe GHD. While the final height predicted based on bone age at the beginning of GH therapy was 137 cm, the final height after GH therapy was 148.5 cm. GH therapy resulted in an 11.5 cm (1.8 SDS) gain in the final height.

Recent studies indicate that NS and NF1 patients also benefit from GH treatment. In one study, the height gain based on CDC standards was 8.9 cm for boys and 10.0 cm for girls in NS cases receiving GH treatment and this gain was similar to that in Turner syndrome cases (27). In another study evaluating NF1 cases receiving GH treatment, the growth rate, which was 5 cm/year before GH therapy, increased to 9 cm/year at the first year of therapy, was 8.3 cm/year in the second year, and decreased to 6 cm/year between the third and the fifth years of treatment (15). Although the final height of our patient was 7.5 cm greater than the midparental height, it was still short due to the underlying familial short stature and the relatively short duration of GH therapy. The reason the father had a short stature was likely due to the fact that he also had NFNS, was not assessed with respect to GHD, and did not receive any treatment. When the patient was referred to our clinic, her puberty had already started. A better final height could possibly be achieved if GH treatment could have been started at a younger age. Studies have shown that the earlier that GH therapy is initiated in NF1 and NS cases, the better the final height that can be achieved (14,15).

There is limited experience with GHD in NFNS cases, since it is a rare condition that is clinically difficult to identify. Our patient had the clinical features of NFNS and was found to have a mutation in the NF1 gene. She also had GHD and responded very well to GH treatment. It may be argued that short stature is a feature of NFNS, but it is evident that in some cases, short stature can be caused by GHD. For this reason, patients with NFNS who are not growing sufficiently should be evaluated for GHD. Those diagnosed to have GHD can benefit from GH treatment. However, it is obvious that more studies are needed on the use and benefits of GH therapy in NFNS cases, and also in NF1 and NS cases.

Ethics

Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Doğuş Vurallı, Design: Doğuş Vurallı, Nazlı Gönç, Data Collection or Processing: Doğuş Vurallı, Dominique Vidaud, Analysis or Interpretation: Doğuş Vurallı, Nazlı Gönç, Dominique Vidaud, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir, Literature Search: Doğuş Vurallı, Nazlı Gönç, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir, Writing: Doğuş Vurallı, Nazlı Gönç.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):101-104
DOI: 10.4274/jcrpe.2254

Case Report

A Novel Homozygous Mutation in the Transient Receptor Potential Melastatin 6 Gene: A Case Report

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ABSTRACT

Hereditary hypomagnesemia with secondary hypocalcemia (HSH) is a rare autosomal recessive disease caused by mutations in the transient receptor potential melastatin 6 (*TRPM6*) gene. Affected individuals present in early infancy with seizures caused by the severe hypocalcemia and hypomagnesemia. By presenting this case report, we also aimed to highlight the need for molecular genetic analysis in inbred or familial cases with hypomagnesemia. A Turkish inbred girl, now aged six years, had presented to another hospital at age two months with seizures diagnosed to be due to hypomagnesemia. She was on magnesium replacement therapy when she was admitted to our clinic with complaints of chronic diarrhea at age 3.6 years. During her follow-up in our clinic, she showed an age-appropriate physical and neurological development. In molecular genetic analysis, a novel homozygous frame-shift mutation (c.3447delT>p.F1149fs) was identified in the *TRPM6* gene. This mutation leads to a truncation of the TRPM6 protein, thereby complete loss of function. We present the clinical follow-up findings of a pediatric HSH case due to a novel mutation in the *TRPM6* gene and highlight the need for molecular genetic analysis in inbred or familial cases with hypomagnesemia.

Keywords: Hypocalcemia, hypomagnesemia, infantile seizures, transient receptor potential melastatin 6

Conflict of interest: None declared
Received: 22.07.2015
Accepted: 04.09.2015

WHAT IS ALREADY KNOWN ON THIS TOPIC?

Hereditary hypomagnesemia with seconday hypocalcemia is a rare disease, which present at infancy with hypocalcemic seizures, is caused by transient receptor potential melastatin 6 (*TRPM6*) gene mutations.

WHAT THIS STUDY ADDS?

In this study, we report a novel mutation in the *TRPM6* gene.

Introduction

Magnesium is a cofactor for a group of enzymes and transporters. It also plays an essential role in the synthesis of nucleic acids and proteins (1). Intestinal absorption of magnesium mainly occurs in the jejunum and ileum. About 30-40% of the dietary magnesium is absorbed primarily by passive transport

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(1,2). Regulation of serum magnesium is controlled mainly by renal magnesium reabsorption. Approximately 20% of filtered magnesium is reabsorbed in proximal tubule, 60% in the cortical thick ascending limb of Henley's loop, and 5-10% in the distal convoluted tubules (3,4).

Hypomagnesemia is defined as a serum magnesium level <1.8 mg/dL (<0.74 mmol/L) (2). Shift of magnesium from the extracellular fluid into cells or bone (Refeeding syndrome, Hungry bone syndrome), increased gastrointestinal or renal loss, reduced absorption, and use of a variety of drugs including antibiotics and chemotherapeutics, may cause hypomagnesemia (2).

Clinical manifestations of hypomagnesemia are carpopedal spasm, muscle cramp, muscle weakness, tremor, convulsions, athetoid movements, and cardiac abnormalities including atrial tachycardia, fibrillation, and supraventricular arrhythmia (1,2).

Hereditary hypomagnesemia with secondary hypocalcemia (HSH) is an autosomal recessive disease caused by the transient receptor potential melastatin 6 (*TRMP6*) gene mutations. It is characterized by severe hypomagnesemia and hypocalcemia which lead to seizures and muscle spasms presenting in the first months of life (5).

The *TRMP6* gene, encoding the epithelial Mg^{2+} channel TRPM6, is mapped to chromosome 9q22. TRMP6 messenger ribonucleic acid, which is expressed in intestinal epithelial cells and kidney tubules, has a crucial role for transcellular Mg^{2+} absorption from the intestine and from the distal convoluted tubules. Existence of a mutant TRMP6 channel leads to impaired intestinal Mg^{2+} reabsorption and enhanced renal loss (5,6,7,8).

Here, we report the clinical characteristics and genetic analysis of a Turkish inbred girl with HSH due to a novel *TRMP6* gene mutation.

Case Report

The female patient, now aged 6 years, had presented to another clinic with seizures due to hypomagnesemia at the age of 2 months. She was born at term with normal birth weight and length after an uneventful pregnancy. Her parents were cousins. There was no family history of hypomagnesemia, hypocalcemia, or seizures (Figure 1). At the time of her first seizure, total serum calcium was 6 mg/dL (normal, 9-11 mg/dL), potassium 4.1 nmol/L (normal, 3.4-4.5 nmol/L), phosphate 6.3 mg/dL (normal, 2.3-4.7 mg/dL), and magnesium <0.6 mg/dL (normal, 1.6-2.6). Serum vitamin D level was 32 ng/mL (normal, 20-100 ng/mL) and parathormone (PTH) was 5 pg/mL (normal, 15-68 pg/mL). Intravenous Mg^{2+} sulfate was administered, and she was discharged with a treatment schedule of oral magnesium (elemental magnesium oxide 40 mg/kg/day) and calcium gluconate. Calcium therapy was stopped when the normal calcium levels were achieved. On clinical follow-up, and while receiving oral magnesium therapy, her serum magnesium levels varied between 1.2-1.4 mg/dL, serum calcium levels between 8.5-9 mg/dL, and serum PTH levels between 20-40.2 pg/mL (N=15-68 pg/mL). 24-hour urinary magnesium excretion (Fe Mg^{2+}) was 3.9-5.5% (normal, 3-5%), spot urinary calcium/creatinine ratio was 0.05-0.08 mg/mg (normal, 0.21 mg/mg), and urinary phosphate concentration was 25.1 mg/dL (normal, 40-136 mg/dL). At the age of 3.6 years, the patient has been admitted to our clinic with complaints of chronic diarrhea. She was on magnesium hydroxide therapy, but the daily dose of magnesium varied due to the changes in the severity of her diarrhea. Her weight was 14 kg (-0.94 standard deviation score [SDS]), height was 97.5 cm (-0.69 SDS), body mass index was 14.7 (-0.54 SDS). Midparental height was 156.5 cm (-1.12 SDS). Systemic evaluation was normal and there were no dysmorphic features. Laboratory evaluation revealed a normal complete blood count, as well as normal thyroid, kidney, and liver function tests. Serum total calcium was 8.5 mg/dL, alkaline phosphatase (ALP) 356 U/L, magnesium 1 mg/dL (0.41 mmol/L), and PTH was 43.7 pg/mL (15-68 pg/mL). Spot urinary calcium/creatinine ratio was 0.09 mg/mg (normal, <0.2). Diarrhea was considered to be related with magnesium hydroxide therapy which is normally prescribed as a laxative in constipated individuals, thus the treatment was switched to magnesium oxide tablets at a dosage of 26 mg/kg/day (1 mmoL/kg/day of elemental magnesium) and the stools became normal. On her follow-up for a period of two years, serum calcium levels varied between 8.9-9.2 mg/dL, magnesium levels varied between 1.2-1.4 mmol/dL. Muscle spasms, as reported by the mother, were rare and were thought to be due to the irregular usage of magnesium. A satisfactory growth rate was achieved.

Molecular genetic analysis of *TRMP6* was performed by direct sequencing of the coding region and the intron/exon boundaries. A homozygous frame-shift mutation (c.3447delT>p.F1149fs) was identified in the *TRMP6* gene (Figure 2). This mutation led to a truncated TRPM6 protein causing a complete loss of function. We have searched the database (http://www.

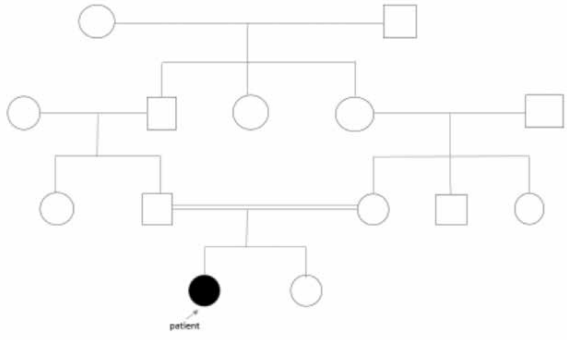

Figure 1. Family pedigree of the patient

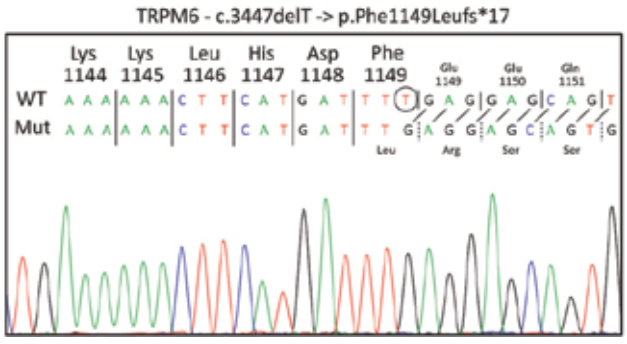

Figure 2. Mutation in the *TRMP6* gene

HGMD.cf.ac.uk/ac/index.php) and could not find this mutation. To the best of our knowledge, this is a novel mutation. Genetic counselling and molecular analysis of the parents were planned, but not yet realized.

Discussion

In this article, we describe the clinical phenotype and follow-up of a patient with HSH due to a novel frame-shift mutation in the *TRMP6* gene. Similar to previous reports which state that the onset of the disease is in early infancy at an average age of 4.9 weeks (4-12 weeks), our patient had also presented in early infancy (8,9,10,11,12,13,14,15,16, 17,18). Also, similar to previously reported cases, our patient had presented with seizures, a symptom which is the most common manifestation of primary hypomagnesemia in children (8,9,10,11,12). High-dose oral magnesium was successful in our patient to achieve a Mg^{2+} level providing a convulsion-free state. A normal neurodevelopmental outcome and a normal growth were achieved. Failure to thrive and mental retardation are the most frequently reported complications of the HSH. These complications have been attributed to non-compliance to treatment and/ or to refractory convulsions due to delayed diagnosis. However, if the patients adhere to the treatment, long-term prognosis seems to be good. Astor et al (19) reported 5 patients without serious complications who had the longest disease duration (over 40 years) in the literature.

Hypomagnesemia in HSH patients results from impaired intestinal magnesium absorption. Active transport of Mg^{2+} via TRPM6 channels situated within the apical membrane of enterocytes prevails when the intestinal Mg^{2+} concentration is low (18). This finding supports that HSH patients fail to absorb Mg^{2+} when the intraluminal Mg^{2+} is low. However, there is a controversy regarding the pathophysiology of urinary magnesium excretion and the role of the magnesium leak. The physiologic range of fractional excretion of magnesium (Fe-Mg^{2+}) has been reported to be 3-5% (3). In the presence of hypomagnesemia, Fe-Mg^{2+} is expected to be lower than 0.5-1 % (2,20). Maintaining a Fe-Mg^{2+} above 2% in the presence of hypomagnesemia is considered as a renal leak (20). In previous studies, initial Fe-Mg^{2+} values of patients with homozygous TRPM6 mutations were reported to be between 0.1-2.3% at the time of diagnosis (9). Additionally, in patients with subnormal serum magnesium levels (1.28 mg/dL), with high dosage of oral magnesium therapy, Fe-Mg^{2+} was reported to vary between 0.2 and 1.6% (8). In contrast to these findings, increased renal Mg^{2+} leak in HSH patients has also been reported (6,10). In our patient, Fe-Mg^{2+} varied between 3.5 % and 5% when serum magnesium levels were subnormal and the patient was receiving magnesium therapy in high doses. This finding supports the role of increased renal Mg^{2+}excretion in the pathophysiology of the disease.

To the best of our knowledge, to date, fewer than 80 cases with TRPM6 gene mutation and 48 different mutations have been reported worldwide (7,8,9,10,11,12,13,14,15,16, 17,19). The identified TRPM6 mutations were distributed over the whole gene, without clustering in any specific domain, consistent with the allelic heterogeneity (7,8,9,10,11, 12,13,14,15,16,17,19). Genotype-phenotype correlation has not been evaluated properly. Until now, 10 Turkish patients with 7 different mutations were reported. Six of them had splice site and the remaining 4 had non-sense mutations (9,10,11,13). Herein, we report a case who presented with findings consistent with a classical phenotype of HSH and in whom the diagnosis was confirmed by detection of a novel homozygous frame-shift mutation (c.3447delT>p.F1149fs) in the molecular genetic analysis of the *TRPM6* gene. Frame-shift mutations have been reported in nine cases with a widespread ethnic distribution including Pakistani, Greek, Indian, and Chinese (10,15). These mutations led to preterm stop codon and loss of function of TRPM6 protein. Reported patients with frame-shift mutations showed similar phenotypic features with a classical clinical presentation of HSH.

In conclusion, HSH is a rare cause of hypomagnesemia. Early diagnosis and proper treatment will prevent complications which may result in irreversible neurological outcomes. Molecular studies in familial or inbred cases with hypomagnesemia are critical to further improve our knowledge of magnesium homeostasis.

Ethics

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

Authorship Contributions

Concept: Ayça Altıncık, Design: Ayça Altıncık, Data Collection or Processing: Ayça Altıncık, Mahya Sultan Tosun, Analysis or Interpretation: Ayça Altıncık, Karl Peter Schlingmann, Literature Search: Ayça Altıncık, Mahya Sultan Tosun, Writing: Ayça Altıncık.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):105-106
DOI: 10.4274/jcrpe.2686

Coexistence of Kabuki Syndrome and Autoimmune Thyroiditis

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Dear Editor;

Kabuki syndrome (KS) is a multiple congenital anomalies/intellectual disability syndrome characterized by developmental delay, specific facial features, skeletal and visceral abnormalities. This syndrome is caused by mutations in MLL2 and KDM6A gene. Autoimmune abnormalities such as idiopathic thrombocytopenic purpura, hemolytic anemia, thyroiditis, and vitiligo have been described very rarely in patients with KS (1,2,3). Herein, we present a very rare condition, KS in association with autoimmune thyroiditis and vitiligo due to a *de novo* heterozygous p.R2471* mutation in *MLL2* gene.

Case report: A female patient aged 7 7/12 years presented with short stature. There was no consanguinity between her parents. She was born as a term neonate weighing 2100 g as the product of a twin pregnancy and had no perinatal complications. On physical examination, the patient was noted to have large and low-set ears, broad and arched eyebrows, elongated palpebral fissures with eversion of the lateral third of the lower eyelid, as well as high and narrow palate. She also had other phenotypic malformations such as numerous vitiligo lesions of different sizes in the neck, brachydactyly, prominent fetal finger pads, and hyperlaxity in her joints. Her height was 116.3 cm (-1.85 standard deviation score [SDS]) and weight was 21.7 kg (-0.78 SDS). She had sensorineural hearing loss (left 65 dB, right 55 dB) and moderate mental retardation (Stanford-Binet intelligence scale total score was 43). She had a normal female karyotype (46,XX). A clinical diagnosis of KS was considered.

Additionally, laboratory findings revealed autoimmune thyroiditis [thyroid-stimulating hormone (TSH): 242 mIU/L (reference range: 0.55-6.7) and free thyroxine (fT_4) was 0.42 ng/dL (reference range: 0.91-1.92), Anti-microsomal antibody: 450.6 U/mL (reference range: 0-9), anti-thyroglobulin antibody: 2766 U/mL (reference range: 0-4)]. Thyroid ultrasonography demonstrated a rough pattern, consistent with thyroiditis. Levothyroxine (50 µg/day) replacement therapy induced a euthyroid state (TSH: 3.65 mIU/L and fT_4: 1.15 ng/dL).

The main causes of KS are point mutations with large intragenic deletions and duplications of the histone methyl transferase *MLL2* gene (1,4,5). We detected a *de novo* heterozygous p.R2471* (c.7411C>T) mutation in this patient. No mutations were detected in *MLL2* gene in her parents and her brother. This mutation causes an early stop codon and a truncated protein. It severely affects the protein structure. To our knowledge, this mutation was not reported in KS patients to date. As this is a truncating mutation, it is most probably a disease causing mutation.

In several patients, KS was reported to be associated with autoimmune abnormalities such as idiopathic thrombocytopenic purpura, hemolytic anemia, thyroiditis, and vitiligo (3,5). The autoimmune disorders may be manifestations of abnormal immune regulation. Ming et al (3) conclude that KS is associated with an increased incidence of autoimmune disorders. In our patient, the vitiligo lesions in the neck and thyroiditis were considered to be signs revealing an autoimmune condition. We recommend that all KS patients be investigated for possible coexistence of autoimmune disorders.

Keywords: Kabuki syndrome, *MLL2* gene, autoimmune thyroiditis, vitiligo
Conflict of interest: None declared
Received: 02.12.2015
Accepted: 18.12.2015

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Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Fatih Gürbüz, Design: Fatih Gürbüz, Data Collection and/or Processing: Fatih Gürbüz, Özge Özalp Yüreğir, Serdar Ceylaner, Ali Kemal Topaloğlu, Bilgin Yüksel, Analysis and/or Interpretation: Fatih Gürbüz, Özge Özalp Yüreğir, Serdar Ceylaner, Ali Kemal Topaloğlu, Bilgin Yüksel, Literature Research: Fatih Gürbüz, Özge Özalp Yüreğir, Serdar Ceylaner, Ali Kemal Topaloğlu, Bilgin Yüksel, Writing: Fatih Gürbüz, Özge Özalp Yüreğir, Serdar Ceylaner, Ali Kemal Topaloğlu, Bilgin Yüksel.

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

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J Clin Res Pediatr Endocrinol 2016;8(1):107
DOI: 10.4274/jcrpe.2765

Reply; Testotoxicosis: Report of Two Cases, One with a Novel Mutation in LHCGR Gene

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Dear Editor,

I have reviewed the letter to the editor regarding "Testotoxicosis: Report of Two Cases, One with a Novel Mutation in *LHCGR* Gene".

I appreciate authors' interest and contributions to our report about 2 cases with *LHCGR* gene mutations. We have reported two cases with similar clinical features suggesting peripheral precocious puberty, one with a novel and one with a known mutation in *LHCGR* gene leading to activation of the receptor. It is well known that the management of these patients is quite difficult because of uncontrolled testosterone secretion from the testes which is the main reason for precocious pubertal signs. We initially started bicalutamide and anastrozole treatments in both of our patients, as bicalutamide treatment had been tried in similar cases as a potent antiandrogen (1). However, in the follow-up, bone age advancement continued rapidly with the pubertal progression in both patients. Additionally, serum testosterone levels were still in high range (~400-800 ng/dL) without significant decline. We changed our treatment regimen to ketoconazole and anastrozole because we could not cease the pubertal and bone age advancement that were also associated with high testosterone levels. Unequivocally, we treat the patient not the laboratory results, but we cannot deny the testosterone effect on the clinic. Testosterone is responsible for the appearance of secondary sexual characteristics, whereas estrogen is the hormone responsible for the epiphyseal maturation which is converted from testosterone by aromatization. High testosterone levels are associated with bone age advancement. Evaluation of pubertal progression and bone age advancement associated with serum testosterone levels are important indicators for treatment monitoring (2,3).

In conclusion, we could not get any benefit from bicalutamide treatment in our patients, whereas ketoconazole treatment is promising in short term. Overall, 'successful treatment' can only be evaluated in long-term follow-up of these patients.

Keywords: Testosterone, *LHCGR* gene, novel mutation
Peer-review: Internal peer-reviewed.
Conflict of interest: None declared
Received: 23.12.2015
Accepted: 23.12.2015

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

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J Clin Res Pediatr Endocrinol 2016;8(1):108-113
DOI: 10.4274/jcrpe.2016.001

Averting the Legacy of Kidney Disease-Focus on Childhood

"For in every adult there dwells the child that was, and in every child there lies the adult that will be."
- John Connolly, The Book of Lost Things

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*on behalf of the World Kidney Day Steering Committee**

ABSTRACT

World Kidney Day 2016 focuses on kidney disease in childhood and the antecedents of adult kidney disease that can begin in earliest childhood. Chronic kidney disease (CKD) in childhood differs from that in adults, as the largest diagnostic group among children includes congenital anomalies and inherited disorders, with glomerulopathies and kidney disease in the setting of diabetes being relatively uncommon. In addition, many children with acute kidney injury will ultimately develop sequelae that may lead to hypertension and CKD in later childhood or in adult life. Children born early or who are small-for date newborns have relatively increased risk for the development of CKD later in life. Persons with a high-risk birth and early childhood history should be watched closely in order to help detect early signs of kidney disease in time to provide effective prevention or treatment. Successful therapy is feasible for advanced CKD in childhood; there is evidence that children fare better than adults, if they receive kidney replacement therapy including dialysis and transplantation, while only a minority of children may require this ultimate intervention Because there are disparities in access to care, effort is needed so that those children with kidney disease, wherever they live, may be treated effectively, irrespective of their geographic or economic circumstances. Our hope is that World Kidney Day will inform the general public, policy makers and caregivers about the needs and possibilities surrounding kidney disease in childhood.

Introduction and Overview

The 11th World Kidney Day will be celebrated on March 10, 2016, around the globe. This annual event, sponsored jointly by the International Society of Nephrology (ISN) and the International Federation of Kidney Foundations (IFKF), has become a highly successful effort to inform the general public and policymakers about the importance and ramifications of kidney disease. In 2016, World Kidney Day will be dedicated to kidney disease in childhood and the antecedents of adult kidney disease, which can begin in earliest childhood.

Children who endure acute kidney injury (AKI) from a wide variety of conditions may have long-term sequelae that can lead to chronic kidney disease (CKD) many years later (1,2,3,4). Further, CKD in childhood, much of it congenital, and complications from the many non-renal diseases that can affect the kidneys secondarily, not only lead to substantial morbidity and mortality during childhood but also result in medical issues beyond childhood. Indeed, childhood deaths from a long list of communicable diseases are inextricably linked to kidney involvement. For example, children who succumb to cholera and other diarrheal infections often die, not from the infection, but because of AKI induced by volume depletion and shock. In addition, a substantial body of data indicates that hypertension, proteinuria and CKD in adulthood have childhood antecedents-from as early as in utero and perinatal life (see Table 1 for definitions of childhood). World Kidney Day 2016 aims to heighten general awareness that much adult renal disease is actually initiated in childhood. Understanding high risk diagnoses and events that occur in childhood have the potential to identify and intervene preemptively in those people at higher risk for CKD during their lifetimes.

Worldwide epidemiologic data on the spectrum of both CKD and AKI in children are currently limited, though increasing in scope. The prevalence of CKD in childhood is rare-and has been variously reported at 15-74.7 per million children (3). Such variation is likely because data on CKD are influenced by regional and cultural factors, as well as

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Table 1. Definitions of stages of early life	
Perinatal Period-	**22 completed weeks of gestation to day 7 of postnatal life**
Neonatal Period-	Birth to day 28 of postnatal life
Infancy	Birth to 1 year of age
Childhood	1 year of age to 10 years of age
Adolescence	10 years of age to 19 years of age
Notes: The data in this table are as defined by the World Health Organization. The perinatal period is defined as 22 completed weeks of gestation to Day 7 of life; the neonatal period, as up to 28 days of life; infancy as up to one year of age; childhood as year 1 to 10; and adolescence from 10 years to age 19. There is variation worldwide in how these stages of early life are defined. Some would define "young people" as those age 24 or less. In the United States, childhood is as a whole defined as going to age 21.	

by the methodology used to generate them. The World Health Organization (WHO) has recently added kidney and urologic disease to mortality information tracked worldwide, and should be a valuable source of such data over time-yet WHO does not post the information by age group (5). Databases such as the North American Pediatric Renal Trials and Collaborative Studies (NAPRTCS) (6) the U.S. Renal Data System (USRDS) (7) and the EDTA registry (8) include data on pediatric ESRD, and some on CKD. Projects such as the ItalKid(9) and Chronic Kidney Disease in Children (CKiD) (10) studies, the Global Burden of Disease Study 2013, as well as registries that now exist in many countries provide important information, and more is required (11).

AKI may lead to CKD, according to selected adult population studies (12). The incidence of AKI among children admitted to an intensive care unit varies widely-from 8% to 89% (1). The outcome depends on the available resources. The results from projects such as the AWARE study, a five-nation study of AKI in children are awaited (13). Single center studies, as well as meta-analyses indicate that both AKI and CKD in children account for a minority of CKD worldwide (2,3). However, it is increasingly evident that kidney disease in adulthood often springs from a childhood legacy.

Spectrum of Pediatric Kidney Diseases

The conditions that account for CKD in childhood, with a predominance of congenital and hereditary disorders, differ substantially from those in adults. To date, mutations in more than 150 genes have been found to alter kidney development or specific glomerular or tubular functions (14). Most of these genetic disorders present during childhood, and many lead to progressive CKD. Congenital anomalies of the kidney and urinary tract (CAKUT) account for the largest category of CKD in children (see Table 2) and include renal hypoplasia/dysplasia and obstructive uropathy. Important subgroups among the renal dysplasias are the cystic kidney diseases, which originate from genetic defects of the tubuloepithelial cells' primary cilia. Many pediatric glomerulopathies are caused by genetic or acquired defects

Table 2. Etiology of chronic kidney disease in children			
CKD		**ESRD**	
Etiology	**Percentage (Range)**	**Etiology**	**Percentage (Range)**
CAKUT	48-59%	CAKUT	34-43%
GN	5-14%	GN	15-29%
HN	10-19%	HN	12-22%
HUS	2-6%	HUS	2-6%
Cystic	5-9%	Cystic	6-12%
Ischemic	2-4%	Ischemic	2%
Rare causes include congenital NS, metabolic diseases, cystinosis/Miscellaneous causes depend on how such entities are classified. CAKUT: Congenital anomalies of the kidney and urinary tract, GN: Glomerulonephritis, HN: hereditary nephropathy, HUS: Hemolytic uremic syndrome. *from Harambat et al. CKD data are from NAPRTCS, the Italian Registry and the Belgian Registry. ESRD data are from ANZDATA, ESPN/ERA-EDTA, UK Renal Registry and the Japanese Registry.			

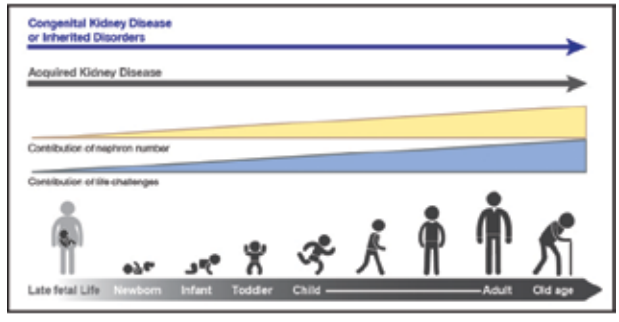

Figure 1. Legend: The types and risks of kidney disease change across the lifecycle. The contribution of nephron number increases over the life cycle, in concert with events that provide direct insults and challenges to kidney health

of the podocytes, the unique cell type lining the glomerular capillaries. Less common but important causes of childhood CKD are inherited metabolic disorders such as hyperoxaluria and cystinosis, and atypical hemolytic uremic syndrome, a thrombotic microangiopathy related to genetic abnormalities of complement, coagulation or metabolic pathways.

In various classifications it is not clear how to categorize children who have suffered AKI and apparently recovered, or how and whether to include those children who have had perinatal challenges, likely resulting in a relatively low nephron number.

Among children with childhood-onset end-stage renal disease (ESRD) glomerulopathies are slightly more and congenital anomalies less common (Table 2), due to the typically more rapid nephron loss in glomerular disease. However, recent evidence suggests that many patients with milder forms of CAKUT may progress to ESRD during adulthood, peaking in the fourth decade of life (15).

There are national and regional differences in the types and course of both AKI and CKD during childhood and beyond. Death from kidney disease is higher in developing nations, and national and regional disparities in care and outcome must be addressed. Further, access to care is variable, depending on the region, the country and its infrastructure. By focusing on kidney disease in childhood, cost-effective solutions may be reached, as treating disease early and preemptively may prevent later, more advanced CKD. Expectations depend on the availability of care and management. Treating children, even from infancy, who have AKI and CKD that requires renal replacement therapy can be effective in mitigating the burden of kidney disease in adulthood. Doing so requires resources that focus on the most expeditious and cost-effective ways to deliver acute RRT in childhood.

Congenital Kidney Disease and Developmental Origins of Health and Disease, Renal Endowment and Implications

In regions where antenatal fetal ultrasounds are routine, many children with urologic abnormalities are identified antenatally, which permits early intervention. However, in much of the world, children with structural abnormalities are not identified until much later, when symptoms develop. While generalized screening for proteinuria, hematuria and urinary tract infections are carried out in some countries and regions, there is a lack of consensus as to its effectiveness. However, there is general agreement that children with antenatal ultrasound studies that indicate possible genitourinary anomalies, children with a family history of kidney disease, and children with signs such as failure to thrive or a history of urinary tract infection, voiding dysfunction or an abnormal appearing urine should be examined. Initial screening would include a focused physical examination and a urine dipstick, formal urinalysis and a basic chemistry panel, followed by a more focused evaluation if indicated.

Depending on the diagnosis, definitive therapy may be indicated. However, the evidence that therapy will slow progression of CKD in childhood remains limited. Angiotensin converting enzyme inhibitors, angiotensin receptor blockers, antioxidants and, possibly, dietary changes may be indicated, depending on the diagnosis. However, dietary changes need to permit adequate growth and development. The ESCAPE trial provided evidence that strict blood pressure control retards progression of CKD in children irrespective of the type of underlying kidney disease (16).

Some very young children may require renal replacement therapy in early infancy. Recent data pooled from registries world wide indicate good survival, even when dialysis is required from neonatal age (2,17). Kidney transplantation, the preferred renal replacement therapy in children, is generally suitable after 12 months of age, with excellent patient and allograft survival, growth and development.

Evidence is accumulating that childhood-onset CKD leads to accelerated cardiovascular morbidity and shortened life expectancy. Ongoing large prospective studies such as the (Cardiovascular Comorbidity in Children with CKD (4C) Study are expected to inform about the causes and consequences of early cardiovascular disease in children with CKD (18).

In addition to those children with congenital kidney disease, it is now known that perinatal events may affect future health in the absence of evident kidney disease in early life (19). Premature infants appear to be particularly at risk for kidney disease long after they are born, based both on observational cohort studies, as well as on case reports. Increasingly premature infants survive, including many born well before nephrogenesis is complete (20). The limited data available indicate that in the process of neonatal ICU care, such babies receive many nephrotoxins, and that those dying prior to discharge from the nursery have fewer and larger glomeruli (21). Additionally, those surviving have evidence of renal impairment that may be subtle (22). Even more concerning, abundant epidemiologic data indicate that persons born at term but with relatively low birth weights may be at high risk for hypertension, albuminuria and CKD in later life (23). When direct measurements are pursued, such persons, as adults, may have fewer nephrons, thus a low cardiorenal endowment.

In focusing on children for World Kidney Day, we would note that it is key to follow kidney function and blood pressure throughout life in those persons born early or small-for-dates. By doing so, and avoiding nephrotoxic medications throughout life, it may be possible to avert CKD in many people.

Resources and Therapeutics for Children-Differences from Therapeutics in Adults

Disparities exist in the availability of resources to treat AKI in children and young people; consequently, too many

children and young adults in developing nations succumb if AKI occurs. To address the problem the ISN has initiated the Saving Young Lives Project, which aims both to prevent AKI with prompt treatment of infection and/or delivery of appropriate fluid and electrolyte therapy, and to treat AKI when it occurs. This ongoing project in SubSaharan Africa and South East Asia, in which four kidney foundations participate equally (IPNA, ISN, ISPD and SKCF),* focuses on establishing and maintaining centers for the care of AKI, including the provision of acute peritoneal dialysis. It links with the ISN's 0 by 25 project, which calls on members to ensure by 2025 that nobody dies from preventable and acute kidney injury.

In view of the preponderance of congenital and hereditary disorders, therapeutic resources for children with CKD have historically been limited to a few immunological conditions. Very recently, progress in drug development in concert with advances in genetic knowledge and diagnostic capabilities has begun to overcome the long-standing 'therapeutic nihilism' in pediatric kidney disease. Atypical HUS, long considered ominous, with a high likelihood of progression to ESRD and post-transplantation recurrence, has turned into a treatable condition-with the advent of a monoclonal antibody that specifically blocks C5 activation (24). Another example is the use of vasopressin receptor antagonists to retard cyst growth and preserve kidney function in polycystic kidney disease (25). First proven efficacious in adults with autosomal dominant polycystic kidney disease, therapy with vaptans holds promise also for the recessive form of the disease, which presents and often progresses to ESRD during childhood.

However, patient benefit from pharmacological research breakthroughs is jeopardized on a global scale by the enormous cost of some of the new therapeutic agents. The quest for affordable innovative therapies for rare diseases will be a key issue in pediatric nephrology in the years to come.

The identification of children likely to benefit from novel therapeutic approaches will be greatly facilitated by the development of clinical registries that inform about the natural disease course, including genotype-phenotype correlations. Apart from disease-specific databases, there is also a need for treatment-specific registries. These are particularly relevant in areas where clinical trials are difficult to perform due to small patient numbers and lacking industry interest, as well as for therapies in need of global development or improvement. For instance, there is currently a large international gradient in the penetration and performance of pediatric dialysis and transplantation. Whereas pediatric patient and technique survival rates are excellent and even superior to those of adults in many industrialized countries, it is estimated that almost half of the world's childhood population is not offered chronic renal replacement therapy (RRT) at all. Providing access to RRT for all children will be a tremendous future challenge. To obtain reliable information on the demographics and outcomes of pediatric RRT, the International Pediatric Nephrology Association (IPNA) is about to launch a global population-based registry. If successful, the IPNA RRT registry might become a role model for global data collection.

Transition from Pediatric to Adult Care

Transition of care for adolescents with kidney disease into an adult setting is critical both for patients and their caregivers. Non-adherence is a too-frequent hallmark of transition from pediatric to adult care for young patients with chronic disease states (26,27,28). Hence, considered steps combined with systematically defined procedures supported by validated pathways and credible guidelines must be in place to ensure successful outcomes.

In the process of change from pediatric to adult care "transition," which should occur gradually, must be distinguished from "transfer," which is often an abrupt and mechanistic change in provider setting. Introducing the concept of transition should be preemptive, starting months to years prior to the targeted time, as children move into adolescence and adulthood. The ultimate goal is to foster a strong relationship and individualized plan in the new setting that allows the patient to feel comfortable enough to report non-adherence and other lapses in care.

A transition plan must recognize that the emotional maturity of children with kidney disease may differ widely. Assessment of the caregiver and the family structure as well as cultural, social, and financial factors at the time of transition are key, including a realistic assessment of caregiver burden (4). The appropriate timing and format of transition may vary widely among different patients and in different settings; therefore, a flexible process without a set date and even without a delineated format may be preferred.

Importantly, transition may need to be slowed, paused or even reversed temporarily during crises such as disease flares or progression, or if family or societal instability occurs. A recent joint consensus statement by the International Society of Nephrology (ISN) and International Pediatric Nephrology Association (IPNA) proposed steps consistent with the points just outlined, aiming to enhance the transition of care in kidney disease in clinical practice (29,30).

Call for Generating further Information and Action

Given vulnerabilities of children with kidney disease including impact on growth and development and future life as an adult, and given the much greater proportion of

children in developing nations facing resource constraints educating everyone involved is imperative in order to realign communications and actions (31,32). These efforts should foster regional and international collaborations and exchange of ideas between local kidney foundations, professional societies, other not-for-profit organisations, and states and governments, so as to help empower all stakeholders to improve the health, well-being and quality of life of children with kidney diseases and to ensure their longevity into adulthood.

Until recently, however, the WHO consensus statement on non-communicable diseases (NCD) included cardiovascular disease, cancer, diabetes and chronic respiratory disease, but not kidney disease (33,34). Fortunately, due, in part, to a global campaign led by the ISN, the Political Declaration on NCDs from the *United Nations* Summit in 2011 mentioned kidney disease under Item 19 (35).

Increasing education and awareness about renal diseases in general and kidney disease in childhood in particular is consistent with the objectives of the WHO to reduce mortality from NCD with a 10 year target population level initiatives focusing on changes in life style (including tobacco use reduction, salt intake control, dietary energy control, and alcohol intake reduction) and effective interventions (including blood pressure, cholesterol and glycemic control). Heightened efforts are needed to realign and expand these multidisciplinary collaborations with more effective focus on early detection and management of kidney disease in children. Whereas the issues related to kidney disease may be overshadowed by other NCDs with apparently larger public health implications such as diabetes, cancer, and cardiovascular diseases, our efforts should also increase education and awareness on such overlapping conditions as cardiorenal connections, the global nature of the CKD and ESRD as major NCDs, and the role of kidney disease as the multiplier disease and confounder for other NCDs. White papers including consensus articles and blueprint reviews by world class experts can serve to enhance these goals (36).

Footnote: *The four partners are (in alphabetical order): IPNA (International Pediatric Nephrology Association), ISN (International Society of Nephrology), ISPD (International Society for Peritoneal Dialysis), SKCF (Sustainable Kidney Care Foundation).

Comprehensive Style of the References (with PMID and PMCID)

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CONGRESS CALENDAR

ENDO 2016 (98th Annual Meeting and Expo of the Endrocrine Society)
1-4 April 2016, Boston, Massachusetts, USA

ECE 2016 (8th European Congress of Endocrinology)
28-31 May 2016, Munich, Germany

ECO 2016 (23rd European Congress on Obesity)
1-4 June 2016, Gothenburg, Sweden

ESPE 2016 (55th Annual Meeting of the European Society for Pediatric Endocrinology)
10-12 September 2016, Paris, France

EASD 2016 (52nd European Association for the Study of Diabetes)
12-16 September 2016, Munich, Germany

ISPAD 2016 (42nd Annual Conference, International Society for Pediatric and Adolescent Diabetes)
26-29 October 2016, Valencia, Spain