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

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Editor's Note

What starts puberty is still intriguing scientists as well as clinicians. This unique developmental event in human life renders not only a reproductive capacity but profoundly influences the psychosocial identity of the individual.

Dr. Topaloglu and colleagues have attempted to answer this question by studying a group of familial cases in whom natural puberty had not occurred, namely, patients with idiopathic hypogonadotropic hypogonadism. The authors interrogate the exact genetic underpinnings of this particular phenotype by whole exome sequencing coupled with autozygosity mapping. Through their previous ground-breaking work, these researchers have identified the roles of such genes as TAC3, TACR3, and KISS1 which constitute the Neurokinin and Kisspeptin signaling leading to our current understanding of the GnRH pulse generator. In their present work, they (in collaboration with Dr. Leygue's group in Canada) report in our Journal an exciting new puberty gene. SRA1 (Steroid receptor RNA activator 1) encodes for a nuclear hormone receptor coregulator.

Nuclear hormone receptors (NRs) mediate the transcriptional responses to a wide range of physiological stimuli and thus function as important regulators of development, metabolism, and reproduction. NR coregulators, by functioning as coactivators or corepressors of NR activity, play pivotal roles in mediating hormone action. SRA1 was originally described as a functional ncRNA involved in the regulation of gene expression by steroid receptors. More recently, SRA isoforms were identified that were also able to encode for a protein now referred to as the Steroid Receptor RNA Activator Protein (SRAP). SRA and SRAP now define a very intriguing bi-faceted genetic system, where both RNA and protein products of the same gene play specific and sometimes overlapping roles in cell biology. *SRA1* gene products functioning as a protein and/or a noncoding functional RNA act as co-regulators of nuclear receptors including sex steroid receptors as well as SF-1 and LRH-1, the master regulators of steroidogenesis.

It is evident from the eloquent studies reported here that inactivating mutations of the *SRA1* gene cause complete normosmic IHH, hence pubertal failure in humans. Further, there are circumstantial evidence to suggest that SRA1 may prove to be of paramount importance in puberty. SRA1 stands as an intriguing gene with its functioning protein as well as noncoding RNA products, which may account for the complexity, versatility, and elusiveness of the pubertal process, especially when one considers the fact that actions of nuclear receptor coregulators can spatially and temporally vary to become activators or repressors of the target nuclear receptors depending on the cellular and promotor context.

Feyza Darendeliler
Editor in Chief

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Review

Maternal Obesity and its Short- and Long-Term Maternal and Infantile Effects

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ABSTRACT

Obesity, in childhood or in adulthood, remains to be a global health problem. The worldwide prevalence of obesity has increased in the last few decades, and consequently, the women of our time suffer more gestational problems than women in the past. The prevalence of obesity is greater in older women than in younger ones and in women with low educational level than in their counterparts with a higher level of education. Maternal obesity during pregnancy may increase congenital malformations and neonatal morbidity and mortality. Maternal obesity is associated with a decreased intention to breastfeed, decreased initiation of breastfeeding, and decreased duration of breastfeeding. We discuss the current epidemiological evidence for the association of maternal obesity with congenital structural neural tube and cardiac defects, fetal macrosomia that predisposes infants to birth injuries and to problems with physiological and metabolic transition, as well as potential for long-term complications secondary to prenatal and neonatal programming effects compounded by a reduction in sustained breastfeeding.

Keywords: Maternal obesity, fetal, neonatal, short- and long-term effects

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Introduction

In the United States and Europe, 20-40% of pregnant women are obese or their weight gain during pregnancy is excessively high. Body mass index (BMI) is the basic criterion when assessing obesity (Table 1) (1,2).

The studies conducted between 2001 and 2007 demonstrated that in the United Kingdom, 20% of pregnant women were obese and that obesity rose in parallel with age, parity, and socio-economic level (3). However, attention should be drawn to the fact that these figures vary in different parts of the world and in different ethnic groups. Obesity negatively affects both contraception and fertility. Maternal obesity is linked with higher rates of cesarean section as well as higher rates of high-risk obstetrical conditions such as diabetes and hypertension.

In recent years, the subject of epigenetic mechanisms having important effects on maternal obesity has become an issue of discussion. The genetic effects of maternal obesity on child development cannot be explained with the Mendelian model, which is based on genes per se. How this interaction occurs is a broad field of research (4). Epigenetics can be used to refer to various factors which collectively regulate gene expression. The most common of these is DNA methylation (5). In addition, in a study on the offspring of rural Gambian women who had been

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eating different diets during pregnancy depending on the season, differences have been found in DNA methylation of metastable epialleles in the peripheral blood during childhood (6).

Another theory as to how metabolic disease and obesity in children are linked is related to the epigenetic differentiation of the genes in ribonucleic acid and DNA transmission, without any change in the sequence of nucleotides induced through the in utero environment owing to inflammatory environment, insulin resistance, and other hormonal factors (7,8). Glucose intolerance due to histone modification encoding the increased glyconeogenesis and enzyme phosphoenolpyruvate carboxykinase-1 has been detected in newborn mice fed on a diet which is high in fat (9). Consistent with these observations, it was assumed that overfeeding in mice was a precursor to the epigenetic programming of insulin receptors. In addition, the programming of the estrogen receptor, mitogen-activated protein kinase, as well as tumor suppressors *BRCA-1*, *P53*, and caveolin-1 is affected by diet-induced changes (10).

Based on all these data, it has been established that birth weight, which can be assumed to reflect the expression of a genetic marker as well as the effect of the environment throughout intrauterine life on this genetic make-up, is positively correlated with adult bone mass.

For this reason, in the subsequent stages of life, maternal obesity may be associated with the risk of osteoporosis. The induction epidemic changes for this disease throughout puberty and pregnancy may play significant roles in determining the risk of cancer in subsequent stages of life (11,12).

Maternal obesity is a problem more frequently encountered in pregnancies of women of advanced age. It also leads to other problems such as gestational diabetes mellitus (GDM), pregnancy-related hypertension, preeclampsia, and sepsis (13,14). Maternal obesity is, in all probability, also associated with pre-gestational diabetes. It has been reported that the weight gain in the 5-year period prior to becoming pregnant increases the risk of GDM development, notably in women who are not obese at the outset (15). Obesity with insulin resistance, may contribute to hyperglycemia, hyperinsulinism, GDM, and other untoward perinatal consequences. However, maternal obesity is correlated with the side effects of pregnancy independent of GDM.

Table 1. Classification of obesity based body mass index (2)

Classification	**BMI (kg/m^2)**	
Normal weight	18.5-24.9	
Overweight	25-29.9	
Obese	30-39.9	
	Grade-1 obesity 30-34.9	Grade-2 or severe obesity 35-39.9
Morbid obese (grade 3 or massive obesity)	40-49.9	
Super obese	≥50	

BMI: body mass index

In obese pregnant women, the rates of births with intervention or by cesarean section, as well as the risk of intrapartum and postpartum complications are also higher (16). The levels of blood lipids, notably of triglycerides, peak at gestational weeks 31-36 in response to such gestational hormones as progesterone, 17 βE2 and placental lactogenic hormone, which also increase during pregnancy (17,18). In obese pregnant women, the increase in triglyceride levels accompanied by a temporary decrease in high-density lipoprotein is particularly interesting (19). The trans-placental transport of lipids is not yet understood. However, normal placental transport and the synthesis of lipids in women with maternal obesity and gestational hyperlipidemia can give rise to disorders that may affect fetal development and growth (20).

Insulin resistance increases progressively throughout pregnancy as a result of the continuous production of counter-regulatory hormones by the placenta. However, obese women have higher insulin resistance (lower insulin sensitivity) than women of normal weight, which results in elevated availability of lipids for fetal growth and development. In fact, there is a higher expression of genes related to lipid metabolism and transport in the placenta of obese women with GDM, which results in a higher birth weight and fat mass in their offspring. More than 50% of women with GDM become diabetic within the first 20 years after giving birth (21,22). As for amino acids, they do not rise in the first trimester, but do so in the second and third trimester by 15% and 25%, respectively, in the pregnancies of women with normal protein synthesis and normal weight (23,24). Although it is not known how changes in protein synthesis during pregnancy affect maternal obesity, there is reduced protein anabolic response, consistent with insulin resistance, in obese women who are not pregnant. In a small scale study in which pregnant women with BMI of 21.0-29.0 kg/m^2 were included, the mass of internal organs was found to positively correlate with the maternal protein cycle, contributing to greater length of the newborn baby (25).

The effective factor in the placental transport of protein is the sodium-dependent transporter family responsible for amino acid transport and expressed in the central nervous system. This factor, as expressed from placenta, affects fetal growth. The decrease in the activity of the system A transporter (*SNAT1, SNAT2, SNAT4*, also known as *SCL38A1*, *SCL38A2*, and *SCL38A4*) is associated with the restriction of fetal growth. Several past studies have demonstrated that the placental system A in pregnant women (BMI 30-39.9) reduced SNAT activity. This reduction of activity runs contrary to the hypothesis that suggests an increase in placental *SNAT*. The authors of the study are of the opinion that such a correlation can exist because the women included in this study were obese participants who had the gestational weight gain

(GWG) recommended. The reduction in placental *SNAT* activity supports the hypothesis that infants born of obese mothers should have reduced body mass (26,27).

Obese women are at high risk of intraoperative and postoperative complications, which can be enumerated as postpartum hemorrhage, anesthesia complications, unsuccessful intubation, and retardation in the healing of postoperative injuries and infection, thromboembolism, and endomyometritis puerperium (28). Studies have shown that 25% of mortalities in pregnant women result from obesity. Regarding maternal mortalities, 18% are related with anesthesia and 80% with obesity (29,30). There are also studies investigating the effects of ethnicity on obesity and its side effects. In one study in African-American and Asian obese women, the rate of birth by cesarean section was found to be higher. The incidence of gestational diabetes in Latin and Asian obese women was reported to be twice as high as compared to other communities. In another study, Latin obese women, when compared with the women of other ethnic groups, were the only group with increased incidence of preeclampsia (31).

In a recent study, it was propounded that increased BMI is strongly correlated with low gestational weight, lower segment cesarean section, preeclamptic toxemia, pregnancy-induced hypertension and that, interestingly, this correlation is independent of maternal glucose levels. In addition, contrary to what is known, there are also studies which demonstrate absence of an important relationship between maternal BMI and losses of pregnancy (32). Sufficient evidence could not be found to indicate the relationship of GWG with neonatal glycemia, neonatal distress, hyperbilirubinemia, neonatal hospitalization, and other cases of infant morbidity. The evidence for the relationship between the major forms of pregnancy complications, such as GDM, and complications of hypertensive pregnancy and GWG is rather flimsy (33,34). In addition, the International Association of Diabetes and Pregnancy Study Group proposed in their study in 2012 that the blood sugar criteria for the diagnosis of GDM at the end of the oral glucose tolerance test in pregnant women should also be revised (35).

There is evidence to link increased maternal obesity with placental ultra-structural changes, accumulation of maternal macrophage and placental weight increase, vascular muscularity, and the expression of inflammatory cytokines. This placental response can help explain the short-term and long-term side effects observed in the children of obese mothers (36,37,38).

In the middle period of pregnancy (weeks 18-28), the relationship between GWG and adiposity in childhood was demonstrated. However, no such relationship was detected between childhood adiposity and GWG in women who had put on more than 500 gm a week after gestational week 28 (39).

In contrast to the findings of some studies, glucose is the main substance to cause obesity in the development of the fetus, a finding which confirms the view that maternal hyperglycemia throughout pregnancy is one of the most important factors which enable pregnancy complications in the obese population to be predicted. Children born to women with GDM are likely to develop macrosomia, shoulder dystocia, childhood obesity, and type 2 diabetes mellitus (DM) (40).

Stillbirth, fetal distress, congenital malformations (three-fold frequent), defects in the jugular vein and abdominal wall, intestinal defects, hydrocephaly, omphalocele, neural tube defect, macrosomia, shoulder dystocia, hypoglycemia, jaundice can be listed among the negative effects of maternal obesity in the fetal and neonatal period. These findings can be explained by the insufficiency in the distribution and absorption of the basic building substances (e.g. folic acid), by the elevation in the blood levels of triglycerides, uric acid, estrogen, and insulin, and hyperglycemia associated with insulin resistance caused by GWG. In a comprehensive retrospective study including obese and non-obese women with macrosomic newborns, obstetric complications in the obese group have been reported to be 3 times more frequent (17% vs. 6%) (41,42,43,44).

Obesity is associated with variations in cytokines and adipokines and with chronic inflammatory conditions. Adipose tissue, an active endocrine organ, is the source of pro-inflammatory cytokines such as adiponectin. Adiponectin is considered to be not only an important mediator which raises glucose sensitivity but also a substance which acts as a stimulant of glucose uptake by skeletal muscle (45,46). Adiponectin levels go down in healthy pregnant women. A low level of adiponectin, in turn, is associated with an increase in fetal growth. Adiponectin binds to receptor-2 in trophoblast cells, activating *P38 MAPKPPAR-α*, which is an insulin/insulin-like growth factor-1 (IGF-1) signal pathway inhibition. For this reason, it is not surprising that maternal serum adiponectin has an inverse relation with fetal growth (47). Oxide lipids can be cytotoxic and affect gene expression by activating cell nucleus. In addition, they can affect antenatal organ development and the response to environmental stimulants in the postnatal period. Moreover, exposure to a high level of lipid in intrauterine life can cause epigenetic changes in the metabolism genes and lipid sensitivity through molecular stimulation and transcriptional activation (48,49). Furthermore, obesity can increase the products of reactive oxygen resulting from increased oxidative stress in mitochondrial tissue. Oxidative stress and excess lipid combination result in increase of oxide lipids which, on the other hand, play role in some of the obesity-related side effects of pregnancy, such as oxide lipids trophoblast invasion and placental development, lipid metabolism, and preeclampsia (48,50).

Maternal obesity is also associated with apoptosis and decreased placental proliferation, increasing the tendency to develop side effects in the course of pregnancy. Experimental studies on animal models have demonstrated that a long-term diet rich in fat results in a change in placental vascularization,

which, in turn, causes oxidative stress and increases the possibility of a hypoxic placenta. Such placental changes account for the increase in the number of stillbirths, the decrease in the number of surviving newborns, and for the increase in abnormal birth weights. Epidemiological studies have also detected a relationship between placental dysfunction and maternal metabolic syndrome (51,52,53). Lipid metabolism becomes impaired in adiposity. When hyperlipidemia arises, it reduces the prostacyclin level while increasing the thromboxane level. It is seriously considered that these changes affect the placenta causing fetal deaths (54). Serum leptin concentration is positively correlated with the storage of fat in the body. Maternal obesity is accompanied by placental leptin resistance and maternal hyperleptinemia which assist amino acid transfer and placental functions. IGF-1:IGFB3 ratio and leptin levels have been found to be higher in the cord blood of fetuses that are large in relation to their gestational age. High ratio of IGF-1:IGFB3 can also be one cause of abnormal fetal growth (26,55).

The incidence of dizygotic twins was found to be 1.1% in the offspring of mothers whose BMI was 30 and above, and 0.5% in those with a BMI below 25. However, no increase was detected in the incidence of monozygotic twins born to obese mothers (56). Maternal obesity disrupts iron transfer to the fetus owing to the increase in the level of hepcidin, in particular, and to the effect of a pro-inflammatory medium. Due to these changes, anemia often occurs in maternal obesity (57).

Maternal obesity increases the risk of early neonatal death and stillbirth. It has been demonstrated that an increase of 3 units or more in BMI during pregnancy considerably increases the risk of stillbirth and pregnancy-related complications including preeclampsia, gestational diabetes, gestational hypertension (21,58,59). In England, one-third of mothers with babies who are stillborn or die during the neonatal period are obese. According to the meta-analyses of 9 studies, stillbirths occur in obese women twice as frequently as they do in non-obese women (60). Again in England, a large-scale cohort study revealed that the incidence of stillbirths among obese women is 6.9/1000, while it is 4/1000 among those who are not overweight. The mechanism underlying this relationship is not clear. However, there are studies which indicate changes in lipid metabolism as a possible cause. Stillbirths may be related with hypertension or maternal obesity with gestational diabetes. Maternal obesity has been found to be related with the risk of mortality in the early neonatal period (Table 2) (21,58).

Significant conclusions have been drawn from studies on the relationship of perinatal mortality with ethnicity. In one such study in recent times, perinatal mortality rate in the offspring of black women proved to be twice as high as that of the offspring of white women. The risk of stillbirth was observed to increase by 30% in the class 1 obese group, and by 50% in the extremely obese group. In a retrospective study on the relationship between mortality in pregnancy with twins and maternal obesity, the researchers compared obesity in all twin pregnancies with women of normal weight and found that the number of stillbirths in the obese mothers was greater by 31%. When obese women pregnant with triplets were compared to

Table 2. Short- and long-term complications associated with maternal obesity (21)

Maternal	Fetal/neonatal	Postnatal
Miscarriage	Congenital anomalies	Obesity
Gestational nonproteinuric	NTDs	Type 2 diabetes
hypertension	Omphalocele	Cardiovascular diseases
Preeclampsia	Congenital heart disease	Osteoporosis
GDM	Fetal distress	Cancer
Urinary infections	Macrosomy (>4500 g)	Metabolic syndrome
Preterm birth	Hydramnios	Neurodevelopmental delay
Assisted vaginal delivery	Shoulder dystocia	Aging
Cesarean section	Hypoglycemia	
Wound infection/breakdown	Jaundice	
Postpartum bleeding		
Postpartum thromboembolism		
Anesthetic complications		
Longer hospitalization		
Intrauterine fetal demise (stillbirth)		

GDM: gestational diabetes mellitus, NTDs: neural tube defects

their counterparts of normal weight, they had a 4 times greater risk of having stillbirths. These findings also demonstrate that the in utero survival rate in women pregnant with singleton or twins is higher than it is in pregnant women pregnant with triplets. This increase in risk suggests the existence of synergistic factors between fetal number and high BMI (61,62).

Although the correlation between obesity and stillbirth cannot be fully explained, some mechanisms regarding the issue have been proposed. It is possible to draw the conclusion that conditions such as apnea-hypoxia, retardation of fetal growth increasing the risk of fetal loss, and pregnancy-induced hypertension, which are all encountered more frequently in obese women than they are in women of normal weight, result in an increase in stillbirths. Impairment of the endocrine system and lipid metabolism causes increases in adiposity, which, in turn, results in hyperlipidemia in obese mothers. Hyperlipidemia increases the production of thromboxane while it reduces the secretion of prostacyclin. These changes lead to reductions in placental thrombosis and placental perfusion. In addition to insulin resistance, the decrease in fibrinolytic activity and perfusion increases the risk of thrombolysis considerably. The impairment of placental blood flow results in fetoplacental dysfunction. This phenomenon may reflect in clinic the increment of risk for stillbirth. Hypertensive diseases and DM are also among the factors which increase the risk of stillbirths (Table 2).

Cohort studies have revealed that, compared with women of average weight, obese women have more frequent elective preterm births; rate of preterm spontaneous births is also higher in the obese (63). This association changes with parity. In nulliparous women, the risk of requirement for elective preterm birth increases, but at the same time, the risk of spontaneous preterm birth decreases. The association of obesity with the risk of preterm birth is weak in multiparous women. However, the risk of preterm birth was shown to be increased in obese multiparous women (64).

Still, the relationship between maternal obesity and preterm birth is complicated, and other factors such as ethnicity, smoking, parity, and age-related factors are potentially involved in this relationship. Preeclampsia is the most important factor to increase the risk of elective birth in nulliparous women. In a large-scale cohort study in Scotland with 187,290 women, it was found that 40% of women who had morbid obesity and were subjected to elective preterm birth were preeclamptic (64). In the same study, it was also found that among multiparous women, the risk of preterm birth increases as BMI increases, while the risk of spontaneous preterm birth decreases, and preterm birth is only a weak correlate of preterm birth in multiparous obese women. How obesity reduces the risk of spontaneous preterm action while all the risks causing prematurity increase in a nulliparous morbidly obese pregnant woman is not fully understood. The mechanisms of obesity in reducing the risk of spontaneous preterm births cannot be explained adequately. It may be speculated that it is related with the reduction in the level of spontaneous uterine activity in obese women compared with normal or thin women (65,66).

It is possible to further analyze the pathogenesis of spontaneous preterm action. In the Scottish cohort study, it was found that the risk of spontaneous preterm action is low in obese women when membranes are intact, but, with the preterm rupture of amniotic membranes, the risk of preterm birth increases. It is thought that this condition may be related with metabolic syndrome (endothelial dysfunction, systematic inflammation, and insulin resistance) associated with acute chorioamnionitis, light or subclinical genitourinary system infection, or obesity. Again in the same study, from the analysis of more than 3000 preterm infants, it was understood that spontaneous preterm births, as a result of the rupture of amniotic membranes, are rather frequent in obese women, and that the risk of spontaneous preterm labor pains, with intact membrane, is reduced in obese women. The mechanism presumed to exist for these relationships has not yet been explained, but the presumed possible mechanisms may be obesity-related metabolic syndromes, subclinical infections of low grade, or acute chorioamnionitis (67).

Preeclampsia, which occurs in obese pregnant women with a greater incidence than in controls, is the most important problem leading to risky births in nulliparous women (Table 3). Obesity increases the birth risk triggered by preeclampsia and hypertension. An increase of 5-7 kg/m^2 in BMI gives rise to twice as great an increase in the rate of preeclampsia (68,69).

Among the congenital structural anomalies resulting from maternal obesity, neural tube defect and congenital heart disease are of particular importance (Table 4) (65). In a comprehensive study in 2011, the rate of children born with congenital malformation to obese mothers was found to be 2% compared to 0.8% for children born to mothers with normal BMI. Talipes equinovarus and facial defects are also among the main congenital malformations encountered in the offspring of obese women. These differences in rate of congenital malformations have been reported to be unrelated with maternal glucose levels. In addition, no relationships were found between maternal BMI or glucose levels and neonatal hypoglycemia, jaundice, respiratory distress, admission to neonatal intensive-care unit, and finally, fetal death (32). In a meta-analysis published in 2009, children born to obese mothers were compared with those born to mothers with normal BMI (Table 4) (65).

The risk of neural tube defects starts to increase with weight gain before pregnancy, and possibly folate supplementation does not diminish this increase. Observational data have indicated that the risk of neural defects resulting from obesity is independent of the effect of standard daily folate supplement (>400 mcg) (70,71). The consensus reached in United Kingdom suggests that women with a BMI above 30 kg/m^2 and planning to become pregnant receive 5 mg of folic acid daily, starting at

least one month before conception and continuing throughout the first trimester. The sensitivity of ultrasonography (USG) decreases in overweight and obese women owing to technical difficulties, which, in turn, detracts from its accuracy. While the risk of congenital anomaly after normal USG is 1/250 in women with BMI below 25, it is 1/100 in obese women (72).

Interestingly, gastroschisis occurs less frequently in children born to obese mothers. However, this is thought to be related with maternal age. The incidence of obesity rises with age and low maternal age is a risk factor in gastroschisis (73). Various mechanisms are thought to contribute to the relationship between structural anomalies and maternal obesity.

While the relationship of congenital anomalies with maternal obesity may be partly due to the fact that some obese women have DM, it was found that the relationship between maternal obesity and neural tube defects is similar in systematic studies which specifically exclude women with DM or provide subgroup data for women without DM (73).

Even when such pregnancy complications as preeclampsia and diabetes are excluded, the intrapartum risk of morbid prognosis in obese women and in their babies is high compared to those who are not overweight (Table 5) (74).

Obese women are at high risk for intrapartum complications. Their need for surgical birth and support is high. Among the reasons for this is the delay in the development of labor pain, the birth being rendered difficult by fetal macrosomia, intrauterine growth retardation, or anus presentation. A wide variety of factors, which include macrosomia, can contribute to these adverse results. Among these factors are inadequate contractions of the uterus and the failure of labor to progress owing to macrosomia (16).

The frequency of fetal macrosomia, independent of diabetes existing previously but arising during pregnancy, is almost two-fold in obese pregnant women compared to those who are not overweight. Dystocia, birth trauma (fractures and nerve paralysis), perinatal asphyxia, and hospitalization occur more frequently in large sized neonates. On the other hand, in one comprehensive case-control study from Canada, which included 45,877 vaginally born babies exclusively, no relationship was found between maternal obesity and fetal weight, which was estimated to be within the normal ranges, nor between maternal obesity and dystocia (75).

Metabolic complications such as hypoglycemia, glucose dysregulation, as well as disorders in insulin, lipid, and aminoacid metabolism play an important role in development of pregnancy complications and increased maternal adiposity, and also of undesired perinatal side effects. The transplacental passage of glucose is significantly correlated with glucose concentration in maternal blood. Therefore, even a slight change in the glucose in the maternal circulation can be transferred to the fetus (76).

The diurnal and nocturnal glucose profiles of obese pregnant women with normal glucose tolerance are higher than those of their counterparts with normal body weight, by virtue of harmony of the potential mechanisms mentioned

Table 3. Maternal obesity and relative risk of developing preeclampsia (68,69)

Parameter	RR
BMI ≥25 kg/m^2	RR 9.3 (95%, CI 2.0-48.0)
Waist circumference ≥80 cm	RR 5.0 (95%, CI 1.3-18.8)
Increase of 1 BMI unit	0.5% increase in preeclampsia incidence
Weight reduction and exercise before and during pregnancy	
BMI reduction by 1.6 kg/m^2	50% decrease in preeclampsia incidence

BMI: body mass index, RR: relative risk, CI: confidence interval

Table 4. Relationships between specific congenital anomalies associated with maternal obesity and overweight state (vs. women with normal body mass index) (65)

Anomaly	Overweight (BMI 25-29.9 kg/m^2)		Obese (BMI ≥30 kg/m^2)	
	OR (95% CI)	Number of studies (cases)	OR (95% CI)	Number of studies (cases)
Neural tube defects	120 (1.04-138)	8 (1523)	1.87 (1.62-2.15)	9 (2093)
Spina bifida	1.12 (0.92-137)	4 (621)	2.24 (1.86-2.69)	5 (863)
Anencephaly	1.12 (0.83-1.50)	3 (233)	1.39 (1.03-1.87)	4 (373)
Cardiac anomalies	1.17 (1.03-134)	6 (9630)	1.30 (1.12-1.51)	7 (9349)
Cleft lip/palate	1.00 (0.87-1.15)	3 (1237)	120 (1.03-1.40)	3 (1188)
Gastroschisis[a]	0.83 (039-1.77)	2 (369)	0.17 (0.10-030)	2 (379)

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

[a]The risk of gastroschisis among obese mothers is significantly reduced, but this is likely to be due to the effect of maternal age since low maternal age is an established risk factor for gastroschisis, whereas the prevalence of obesity increases with age

above, a finding which, in turn, makes the exposure of the fetus to hyperglycemia greater (77).

Obese women have higher insulin resistance than women with normal body weight (22). Apart from this, there is a linear relationship between maternal BMI and the other parameters related to obesity including glucose intolerance. The same relationship exists among elevated cord C-peptide levels and glucose intolerance and increased birth weight.

Kalk et al (78) monitored 505 infants of non-diabetic obese mothers and found that hypoglycemia occurs in 26% of these infants, while it was noted to occur in 9% of infants of mothers who were mildly overweight or of normal body weight and in 6% of babies of thin mothers. These findings suggest that babies born to obese mothers should also be monitored closely for hypoglycemia, as in the case of children born to diabetic mothers.

Vitamin D uptake mechanisms, through the interaction of megaline and eubilin which possess binding protein and receptors, are thought to be immature in the newborn (79). The placental passage of 25-hydroxy (OH) vitamin D in obese women is decreased. Therefore, even if mothers have the same level of 25-OH vitamin D, the levels in the cord blood of their offspring were reported to be lower than those of the offspring of mothers with normal weight. Other researchers have also observed that the levels of cord blood 25-OH vitamin D closely related to maternal 25-OH vitamin D, maternal obesity, maternal age, and neonatal obesity. A correlation has also been detected between maternal obesity, 25-OH vitamin D nutritional status, and adiposity in the neonatal period which is capable of affecting 25-OH vitamin D activity in childhood and adulthood (80).

In various studies, a positive association has been found among the degree of obesity in childhood and adolescence and weight gain during pregnancy. It has also been demonstrated that this adiposity, being increased at birth, is a risk factor for metabolic dysregulation and for childhood obesity (39,81,82,83,84). Obesity is a significant factor for the development of insulin resistance and metabolic syndrome in the adult. In fact, β-cell dysfunction and insulin resistance occur more frequently in obese women than in non-obese women (85). These findings show that obese women with genetic disposition are at higher risk of developing type 2 DM.

Table 5. Risk of more common adverse perinatal outcomes in obese women vs. women with normal body mass index (primigravid singleton otherwise uncomplicated pregnancies) (74)

Outcome	OR (95% CI)
Post-dates (>41 weeks) delivery	1.4 (1.2-1.7)
Induction of labor	1.6 (1.3-1.9)
Failed instrumental delivery	1.75 (1.1-2.9)
Cesarean section delivery	1.6 (1.4-2)
Birth weight >4000 g	2.1 (1.6-2.6)
Shoulder dystocia	2.9 (1.4-5.8)
Birth trauma[a]	1.5 (1.1-2.1)
Neonatal unit admission	1.5 (1.09-2.3)
Gastric tube feeding	1.5 (1.08-2.0)

OR: odds ratio, CI: confidence interval

[a]Skin cuts, grazes, bruises, fractures, muscle haematomas, dislocations, cephalhaematomas, and nerve palsies

In utero insulin resistance was first suggested in 2009 in a study on the fetuses of obese women, which included measurements of glucose and insulin concentration in the umbilical cord. The findings corroborate the concept of fetal programming and its possible impact in subsequent periods of life (86). The first child of a woman with high BMI during pregnancy was reported to have a higher proportion of fat than the younger siblings, demonstrating a relationship between adiposity and maternal parity. The cause of the high mass of fat, which occurs more frequently in the first birth, is, in all probability, due to the re- regulation of the leptin and glucocorticoid axis in the adipocyte, which contribute to the increase in adipogenesis throughout the gestational period (87,88).

In another comprehensive longitudinal cohort study with infant participants, the obesity prevalence of 16-year-old children born to obese mothers and mothers with GDM was found as 40% and 26%, respectively. This prevalence has been demonstrated to persist among not only the children of mothers with GDM, but also those of obese mothers. In animal studies, hypothalamic neuronal changes were observed in the young of diabetic obese rats, caused by the intrauterine medium, which increases the risk of diabetogenic state and obesity. These data have not only provided the justification for the hypothesis of an in utero programming of metabolic syndrome in obese women's children but have also provided clues to the researchers on the risk of prenatal disease (89).

At birth, infants born to overweight or obese mothers have a greater amount of adipose tissue than those born to mothers of normal weight. However, there are also data indicating that infants born to overweight or obese mothers are normal in the first 15 days, but fall behind normal children in terms of gaining weight, increase in height, and increase in adipose tissue in the first 3 months. Studies have shown that a programmed tendency to childhood obesity, leading to increase in the risks for childhood obesity, type 2 DM, and metabolic syndrome in adult life, may be related with low socio-economic level which prevents access to healthy food, especially when combined with the paucity of postnatal breast feeding (21,90,91,92,93).

GDM is a predisposing factor in the development of obesity and being overweight throughout the childhood period. The probability that macrosomic infants, in particular, will be obese in their future life is rather high. From the community health perspective, it is crucial that these children should be monitored closely for possible metabolic and cardiovascular risk, and

that the necessary precautions should be taken (21,90). Transcriptional regulation in childhood and programming in the early period of life contribute to retardation in development, deficits in linguistic skills, and other neurological disorders. In recent years, it has been demonstrated that the probability of obese mothers having autistic children has increased 1.7-fold, and the probability of their having children with developmental retardation has increased 2-fold (94,95).

It has been proposed that high maternal BMI, notably early in pregnancy, is a predisposing factor in the development of schizophrenia in children, probably due to the high levels of pro-inflammatory cytokines during the second trimester (96,97). A large number of studies on animal and human subjects have demonstrated that maternal obesity during pregnancy is related with postnatal lifelong programming of children for chronic diseases, including aging, neurodevelopmental retardation, cancer, osteoporosis, type 2 DM, metabolic syndrome, and cardiovascular diseases. In a study in which telomere lengths were measured in 1122 women aged 18-76 years, obesity was found to aggravate the aging process through telomere erosion.

Obesity and excess weight gain before pregnancy raises the rate of miscarriages, as well as the rates of obstetrical and neonatal complications. These complications, in turn, result in a lower quality of health. It has been demonstrated that, in addition to adverse consequences for the mother, exposure to maternal obesity in the intrauterine period is an important risk factor for development of chronic diseases such as cardiovascular diseases, metabolic syndrome, and type 2 DM in childhood and adolescence.

Fetal programming of metabolic functions by means of physiological and epigenetic mechanisms causes interactions between generations, and obesity can acquire permanence in posterity. Therefore, it is of vital importance that ideal weight gain be achieved, care be taken for the prevention and/or right management of obesity if this vicious circle is to be broken, and the short- and long-term serious negative effects on the fetus and mother are to be avoided.

In conclusion, it can be stated that reducing the adverse effects of maternal obesity is a community health problem. More studies are required to assess the effects and reliability of weight management programs in women in the reproductive age group. The need for future studies on the negative side effects of maternal obesity and overweight on fetal growth and development, as well as for studies assessing the approaches which restrict weight gain in pregnancy and the postpartum period is of vital importance.

Ethics

Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Levent Korkmaz, Osman Baştuğ, Selim Kurtoğlu, Design: Levent Korkmaz, Osman Baştuğ, Selim Kurtoğlu, Data Collection or Processing: Levent Korkmaz, Selim Kurtoğlu, Analysis or Interpretation: Levent Korkmaz, Osman Baştuğ, Selim Kurtoğlu, Literature Search: Levent Korkmaz, Selim Kurtoğlu, Writing: Levent Korkmaz.

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Original Article

Idiopathic Hypogonadotropic Hypogonadism Caused by Inactivating Mutations in SRA1

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ABSTRACT

Objective: What initiates the pubertal process in humans and other mammals is still unknown. We hypothesized that gene(s) taking roles in triggering human puberty may be identified by studying a cohort of idiopathic hypogonadotropic hypogonadism (IHH).

Methods: A cohort of IHH cases was studied based on autozygosity mapping coupled with whole exome sequencing.

Results: Our studies revealed three independent families in which IHH/delayed puberty is associated with inactivating *SRA1* variants. *SRA1* was the first gene to be identified to function through its protein as well as noncoding functional ribonucleic acid products. These products act as co-regulators of nuclear receptors including sex steroid receptors as well as *SF-1* and *LRH-1*, the master regulators of steroidogenesis. Functional studies with a mutant *SRA1* construct showed a reduced co-activation of ligand-dependent activity of the estrogen receptor alpha, as assessed by luciferase reporter assay in HeLa cells.

Conclusion: Our findings strongly suggest that *SRA1* gene function is required for initiation of puberty in humans. Furthermore, *SRA1* with its alternative products and functionality may provide a potential explanation for the versatility and complexity of the pubertal process.

Keywords: Hypogonadotropic hypogonadism, puberty, *SRA1*, *PNPLA6*, mutation

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Pubertal development is thought to be a result of a complex interplay among genes and environmental factors including nutrition.

WHAT THIS STUDY ADDS?

SRA1 gene function is required for the initiation of puberty in humans.

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Introduction

What initiates the pubertal process in humans and other mammals is still an enigmatic question (1). The hallmark of puberty is reemergence of a pulsatile gonadotropin-releasing hormone (GnRH) release from the hypothalamus driving the pituitary gonadotropes to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act in concert to stimulate the gonads to bring about sex hormone secretion and gametogenesis. Normosmic idiopathic hypogonadotropic hypogonadism (IHH) is characterized by failure to develop secondary sexual characteristics and a mature reproductive system due to defects in the central part of the hypothalamo-pituitary-gonadal axis. In an effort to gain a greater understanding into the elusive pubertal process, our laboratory has undertaken a search for genes playing important roles in the generation of gonadotropin secretion in a cohort of familial IHH cases via autozygosity mapping. Our previous seminal descriptions of the mutated genes, *TAC3*, *TACR3* (2), and *KISS1* (3) in this same patient cohort led the way to the characterization of the GnRH pulse generator (4). Along the same line, we hypothesize that gene(s), whose products trigger the GnRH pulse generator to restart ticking at the usual time of puberty, can also be identified via autozygosity mapping together with whole exome sequencing.

Methods

As an overall genetic sequencing strategy, we screen probands from consanguineous multiplex pedigrees for known IHH genes in our IHH cohort. If no mutation is found, then we perform autozygosity mapping based on either single nucleotide polymorphism (SNP) microarray genotyping or (lately) whole exome sequencing. Once we identify a likely candidate gene, we then screen the probands for that gene in the entire cohort either with Sanger sequencing or whole exome sequencing.

Case Reports

Family 1

The proband (II-2), a 25-year-old male, grew and developed normally until his early-to-mid-teen years. At the age of 15, he presented with a chief complaint of absent pubertal development. His past medical history was remarkable for undescended testicles, for which he received human chorionic gonadotropin treatment. His testicular volumes were 3 mL bilaterally. His basal and stimulated gonadotropin levels as well as testosterone concentrations remained prepubertal.

The affected sibling (II-1) is a 30-year-old female. She grew and developed normally until her early-to-mid-teen years. At the age of 15, she presented with absent breast development and lack of menses. She had Tanner stage 1 breast development and stage 2 axillary and pubic hair. Her bone age then was 13 years. A pelvic ultrasonography showed a hypoplastic uterus and ovaries.

The parents are healthy and paternal cousins of Turkish origin. The mother experienced menarche at age 12 years, and the father started shaving at age 14 years.

Family 2

The proband (II-3), now an 18-year-old man, first presented to us at age 13 years with a small penis for which he had been given some human menopausal gonadotropin injections elsewhere. His past medical history was remarkable for undescended testicles and inguinal hernia. At age 14, his testicular volumes were 3 mL bilaterally, with stretched penile length of 4 cm. While having a bone age of 13, his gonadotropin and testosterone levels were prepubertal. Shortly afterwards, his testicular volumes increased to pubertal levels with corresponding penile growth and pubertal hormone levels. Both of his sisters have gone through puberty in time.

The parents are healthy, unrelated, and of Turkish origin. The mother experienced menarche at age 12 years, and the father started shaving at age 14 years.

Family 3

The proband (II-3) is a 21-year-old male, who grew and developed normally until his early-to-mid-teen years. At the age of 16, he presented with absent pubertal development. His past medical history was unremarkable. His testicular volumes were 2 mL bilaterally. His bone age was 12 years at presentation. His basal and stimulated gonadotropin levels as well as testosterone concentrations remained prepubertal.

The affected sibling (II-4) is a 17-year-old male. He grew and developed normally until his early-to-mid-teen years. At the age of 12.5 years, he presented with absent pubertal development. His past medical history was remarkable for a small penis and undescended testicles for which he received human chorionic gonadotropin treatment and subsequent orchiopexia at age 4. His right testicle was reportedly not found at the intervention. His bone age was 11.5 years at presentation to us. Later at age 14 years, he has spontaneously started pubertal development to become a young adult with normal hormonal values.

The parents are healthy, unrelated, and of Kurdish origin. The mother experienced menarche at age 12.5 years, and the father started shaving at age 13.5 years.

The clinical and hormonal features of the affected individuals in the three families are shown in Table 1.

The pedigrees of the families are shown along with their genotypes in Figure 1. The patients are otherwise healthy and have a normal sense of smell. They have otherwise normal anterior pituitary functions. In consideration of the known roles of the *SRA1* with other nuclear receptors, any disorders associated with a potential dysfunction of these receptors were actively ruled out. Thus, thyroid receptor was ruled out

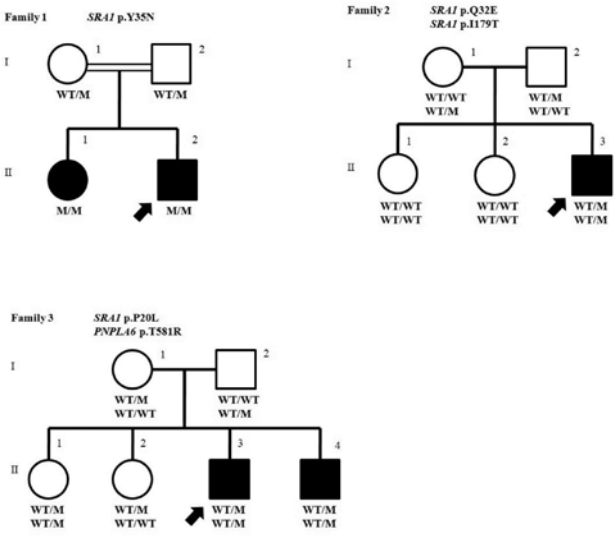

Figure 1. Pedigree profile and corresponding *SRA1* and *PNPLA6* mutations in the three families. Pedigrees are shown to indicate phenotypes and genotypes among family members. Filled circles indicate affected girls or women, open circles unaffected female family members, filled squares affected male family members, and open squares unaffected male family members. The double line indicates consanguinity. Under each available individual is the *SRA1* and *PNPLA6* gene genotype with M indicating mutant and WT indicating wild type

by normal thyroid function test, PPAR γ by normal fasting blood glucose and Hemoglobin A1c, corticosteroid receptors by normal 8 AM cortisol and adrenocorticotropic hormone (ACTH) levels, retinoid receptors by direct examination of the retina by an ophthalmologist, and dilated cardiomyopathy due to abnormal myogenesis by echocardiography and a cardiological examination.

The Ethics Committee of the Çukurova University Faculty of Medicine approved this study, and informed consent was obtained from each participant or from the parents.

Hormonal measurements in the affected individuals: Plasma ACTH, serum LH, FSH, estradiol, dehydroepiandrosterone sulfate, cortisol, and testosterone levels were analyzed by commercial kits based on solid-phase, two-site sequential, or competitive chemiluminescent immunometric assay (Beckman Coulter).

A GnRH stimulation test was performed on the proband of the Family 1 by injecting 0.1 mg GnRH intravenously and drawing blood samples at 0, 15, 30, 45, 60, and 75 minutes for FSH and LH determinations.

A prolonged GnRH stimulation test was also performed on the proband of the Family 1. A daily subcutaneous injection of GnRH at 0.1 mg for one week was administered and on the 7th day, plasma FSH and LH levels were determined 30, 45, and 60 minutes after the injection.

Table 1. The clinical and hormonal features of the affected individuals in the three families

	Family 1		Family 2	Family 3		Normal range
Family member	**II-2**	**II-1**	**II-3**	**II-3**	**II-4**	
Gender	M	F	M	M	M	
FSH (mIU/mL)	<1.8	<1.8	1.0	0.94	2.4	M: 1.4-18.1 F: 2.5-10.2
LH (mIU/mL)	<0.7	<0.7	0.1	0.3	0.8	M: 1.5-9.3 F: 1.9-12.5
Estradiol (ng/dL)	ND	<20.0	ND	ND	ND	M: 0.8-3.5 F: 6.3-16.5
Testosterone (ng/dL)	20.0	ND	16.0	26.0	24.5	175-781
Prolactin (pg/mL)	18.0	9.4	6.6	6.8	ND	M: 2.1-17.7 F: 2.8-29.2
TSH (mIU/mL)	1.1	2.0	2.1	2.3	3.9	0.35-4.2
Free T_4 (ng/dL)	1.1	1.1	1.2	0.9	0.7	0.89-1.8
Cortisol (mcg/dL)	21.0	9.5	ND	14.3	ND	3-25
LHRH stimulation test (max. LH)	<0.7	<0.7	ND	4.1	ND	M: 1.5-9.3 F: 1.9-12.5
LHRH stimulation test (max. FSH)	0.7	3.5	ND	4.4	ND	M: 1.4-18.1 F: 2.5-10.2

M: male, F: female, FSH: follicle-stimulating hormone, LH: luteinizing hormone, TSH: thyroid-stimulating hormone, T_4: thyroxine, LHRH: luteinizing hormone-releasing hormone, ND: not determined

Screening of known genes by Sanger sequencing: Known or strong candidate genes for IHH and Kallmann syndrome including *GNRHR, GNRH1, LHB, FSHB, KISS1R, KISS1, TAC3, TACR3, KAL1, PROK2, PROK2R,* and *FGFR1* were screened by automated Sanger sequencing (5). Briefly, polymerase chain reaction-amplified exons and splice junctions were sequenced on an ABI PRISM 3130 autosequencer (Applied Biosystems).

Genome-wide single nucleotide polymorphism analysis: For genome-wide SNP analysis, we used 250 K NspI SNP microarrays (Affymetrix) and analyzed data using AutoSNPa software (AutoSNPa.org) to identify autozygous regions in Family 1.

Whole exome sequencing: Samples were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2000 Sequencer (Macrogen, Seoul, Korea). The reads are mapped against UCSC hg19.

Site-directed mutagenesis: SRA-D7 and SRA-WT constructs were previously described (6). SRA-Y35N was generated using synthetic oligonucleotides and the QuickChangeII site directed mutagenesis kit (Agilent Technologies) using SRA-WT vector as template following manufacturer's protocol.

Western Blot and immunofluorescent microscopy: Western Blot and Immunofluorescent Microscopy were performed as previously described (7).

Luciferase assay: Twenty-four hours prior to transfection, HeLa cells were seeded into 24 well dishes (7.5x105 cells/well) containing 5% CS-FBS phenol-red free DMEM containing 4.5 g/L D-glucose and 2 mM L-glutamine. Cells were then co-transfected with constructs expressing PS2-ERE (0.4 ug), ERa (0.04 ug), Renilla (0.02 ug), and various SRA (0.34 ug) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. The next day, the medium was changed and replaced with 5% CS-FBS DMEM containing either ethanol vehicle (cont-E) or 10 nM beta-estradiol [+E (Sigma)] for an additional 24 hours before lysis in 100 uL 1xPLB buffer (Promega). Renilla luciferase and luciferase activities were measured using SpectraMaxL Luminometer (Molecular Devices) and SoftMax Pro software using Dual Luciferase Reporter Assay System (Promega) reagents according to the manufacturer's instructions. For each constructs, readings were normalized to control. Results represent the average of four independent experiments performed in triplicate.

Statistical Analysis

HeLa cell transfected with SRA-WT and mutant constructs were compared to control cells transfected with SRA-D7 construct in the absence or presence of estradiol using unpaired 2-tail student's t-test. Graphpad prism 5 software was used for all statistical analyses.

Results

Hormonal results as shown in Table 1 are consistent with the diagnosis of IHH.

Family 1: A genome-wide SNP analysis identified two regions of homozygosity common to the affected siblings but not found in the parents. Two autozygous regions spanned on chromosome 2 from 132159998 to 142605624 and on chromosome 5 from 107034345 to 161513613. Analysis of targeted exome sequencing data for the autozygous regions revealed that the only plausible candidate variant to account for the phenotype is in the *SRA1* gene. A subsequent whole exome sequencing data on the proband were analyzed with particular attention to these autozygous regions by filtering for homozygous variants that are in the coding or splice regions and of minor allele frequency less than 1% or previously undescribed. Our analyses singled out a missense mutation in the *SRA1* gene in the larger autozygous region on chromosome 5. We confirmed the presence of this nonsynonymous mutation in the coding sequence of *SRA1* gene (HGNC: 11281) in the proband (Figure 2) by Sanger sequencing. The proband and his affected sibling were homozygous for a T-to-A change at cDNA nucleotide 103 (NM_001035235.3: c.T103A), leading to the substitution of tyrosine at residue 35 for asparagine (NP_001030312.2: p.Y35N). Their parents both were heterozygous for this mutation. Y35 is among phosphorylated residues. SIFT predicts that this substitution would affect the protein function with a score of 0.00 and PolyPhen-2 predicted this variant probably damaging. In addition, this variant was neither found in 100 ethnically matched healthy adult controls, 110 in-house whole exomes, nor in 1000 genomes, Exome Variant Server or in the ExAC databases. Besides, this variant was not seen in the Turkish

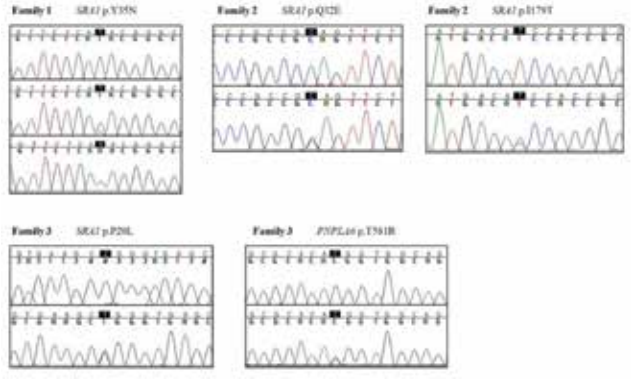

Figure 2. Results of automated DNA sequencing for *SRA1* mutations in the three families. Top, middle, and bottom pictures show mutations in Family 1, 2, and 3, respectively. In Family 1 picture, top, middle, and bottom lines indicate wild type, heterozygous, and homozygous mutations, respectively. In Families 2 and 3 pictures, top and bottom lines indicate wild type and heterozygous mutations, respectively. In addition, the *PNPLA6* mutation in Family 3 is also shown in the same order

whole exome database consisting of over 1000 Individuals' data at TÜBİTAK-BİLGEM. Genotyping by Sanger screening and/or whole exome sequencing in search of additional *SRA1* mutations in 136 IHH families revealed two other families with *SRA1* mutations. In view of the co-occurrence of a *PNPLA6* mutation in family 3, we thoroughly screened the affected individuals in family 1 and 2 for *PNPLA6* mutations by Sanger sequencing and did not find any suspicious variants.

Family 2: A whole exome sequencing in the proband revealed two heterozygous variants: a C-to-G change at cDNA 94 predicting substitution of glutamine at residue 32 for glutamic acid, p.Q32E, rs35610885 and a T-to-C change at cDNA 536 predicting substitution of isoleucine at residue 179 for threonine, p.I179T, rs14810885 (Figure 2). Both of these variant were predicted to be harmful by SIFT and PolyPhen-2. In addition, these variants were not found in 100 ethnically matched healthy adult controls, 110 in-house exomes, or in 1000 genomes, or Exome Variant Server. The allele frequency rates of the p.Q32E and an in p.I179T variants are 0.007 and 0.0007 in ExAC; the allele frequency rates in Turkish whole exome database were 0.004 and 0.006, respectively.

Family 3: A Sanger screening for *SRA1* showed that the proband and his affected brother had a heterozygous mutations (a C-to-T change at cDNA 59 predicting substitution of proline at residue 20 for leucine, p.P20L) (Figure 2). This variant was predicted to be harmful by both SIFT and PolyPhen-2. A whole exome sequencing in the proband revealed a heterozygous variant in *PNPLA6* (HGNC: 16268, NM_006702; a c.C1742G leading to p.T581R) in both siblings (Figure 2) in addition to confirming the *SRA1* variant described above. As can be seen in the pedigrees, the IHH phenotype segregated with co-occurrence of these two heterozygous mutations in the nuclear family, except for the eldest sibling who appears to be unaffected but carries the two variants. The whole exome sequencing in the proband showed no other mutations in known IHH genes. In addition, these variants were neither found in 100 ethnically matched healthy adult controls, 110 in-house exomes, 1000 genomes, or Exome Variant Server. The *PNPLA6* variant was seen once in the ExAC while none in Turkish whole exome database respectively. The *SRA1* variant is seen in 12 (allele frequency <1/1000-10.000) and once in the ExAC and Turkish whole exome database, respectively.

None of the probands from those three families harbored mutations in known IHH and Kallmann genes (5).

Highly conserved positions of the mutations in the ribonucleic acid (RNA) and protein products of *SRA1* are shown in Figure 3.

Y35N Mutation Impairs SRA Mediated Estrogen Receptor Alpha *(ESR1)* Transactivation

SRA1 gene products are known to potentiate ligand-dependent transcription of several nuclear receptor transcription factors as measured by response-element driven luciferase activity under standard luciferase reporter assay conditions (6,8,9,10,11). In particular, SRA has been shown to co-activate estradiol-induced estrogen receptor alpha (ESR1) transcription of luciferase reporters whose expression is under control of the estrogen-response-elements derived from the *PS2* gene (PS2-ERE) (6,9,10). We have addressed whether the mutant found in family 1 (SRA-Y35N) could differentially co-regulated estrogen-dependent ER-alpha activity compared to wild-type SRA (SRA-WT). HeLa cells were co-transfected with an ERE-luciferase, ER-alpha, and either SRA-WT, SRA-Y35N, or SRA-MET7 control construct. This control corresponds to an artificial SRA sequence unable to encode functional SRA RNA due to extensive silent mutations at codon wobble positions, nor SRA protein (SRAP) as a result of site-directed conversion of all SRA methionine residues to leucines (6,12). Cell were subsequently treated with estradiol (+E2) or ethanol (cont) as described in the Material and Methods section.

We checked that SRA-WT and mutant SRA-Y35N levels reached in these experiments were similar. As shown in Figure 4A, Western blot analysis of protein extracts from cells transfected with either SRA-WT or SRA-Y35N showed

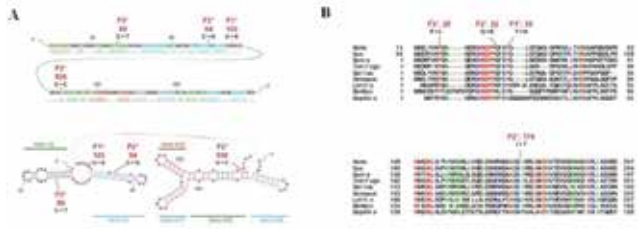

Figure 3. Position of the mutations in the ribonucleic acid and protein products.
A. Positions of mutations in conserved SRA secondary structures.
Conserved SRA secondary structures (Helices H1-2 and H15-18) identified by Novikova et al (28) and corresponding to the regions containing the observed mutations are depicted (28). Nucleotides are numbered using the first human "A" from the first AUG codon as 1. The exact location of the mutations observed in Family 1 (F1*, 103), Family 2 (F2*, 94 and 536), and Family 3 (F3*, 59) are indicated. Please note that all mutations but F1* affect nucleotides involved in conserved helices and might contribute to their stability. Shown are Dot bracket notation (top) and Plain secondary (bottom) structures. Graph generated by The Vienna ribonucleic acid (RNA) website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).
B. Position of mutations in conserved SRAP protein sequences.
Two portions of the sequences of SRAP from Homo sapiens (NP_001030312.2, 236 aa), Susscrofa (XP_003124061.1, 280 aa), Daniorerio (NP_001002047.1, 210 aa), Takifugurubripes (XP_011609562.1, 264 aa), Gallus gallus (NP_001288615.1, 219 aa), Xenopuslaevi (NP_001107371.1, 227 aa), Lottiagigantea (XP_009055386.1, 279 aa), Bombyxmori (XP_004922978.1, 201 aa), and Daphnia pulex (EFX89230.1, 203 aa), which correspond to the region containing the mutations found in this study have been aligned. The numbers correspond to the positions of the side amino acids of the sequence shown. Amino acids identical, strongly similar, and weakly similar are colored in red, green, and blue, respectively. The top and bottom regions depicted correspond to the first and second phylogenetically conserved portion of SRAP, respectively (23). The exact location of the mutations observed in Family 1 (F1*, Y to N, 35), Family 2 (F2*, Q to E, 32 and I to T, 179), and Family 3 (F3*, P to L, 20) are indicated. Please note that these mutations modify amino acids that are identical in all chordata

that transfected cells express similar levels of endogenous SRAP and exogenous mutant proteins. We also checked that SRA-WT, an SRA-Y35N mutant protein, had similar localization

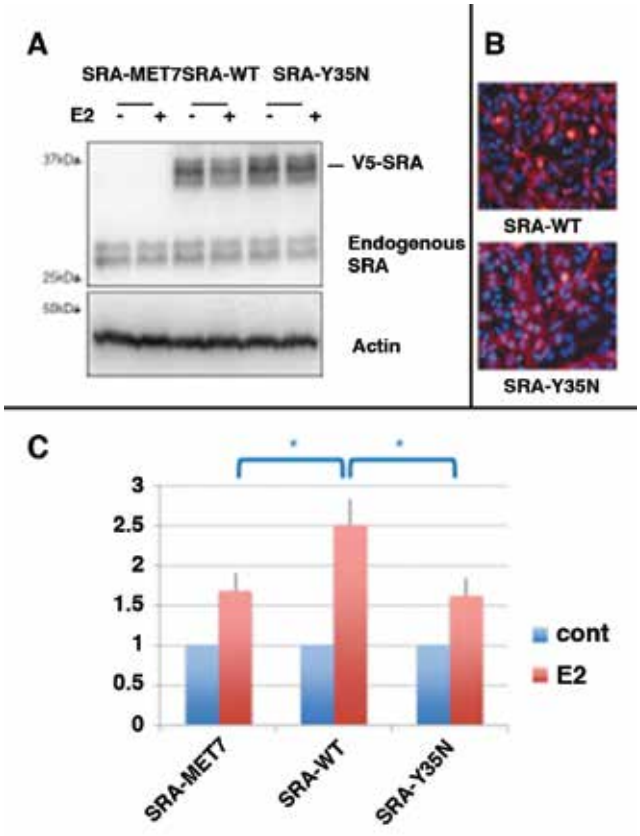

Figure 4. Functional analyses of SRA1 mutant p.Y35N
A) Levels of SRAP expression protein extracts of transfected HeLa cells. Equal volumes of luciferase assay extracts from HeLa cells transfected with Estrogen Receptor-α (ESR1ESR1) and PS2-ERE luciferase reporter plasmids, and either control (SRA-MET7), wild-type (SRA-WT), or mutant (SRA-Y35N) SRA plasmids, were subjected to western blot analysis using anti-SRAP (743, Bethyl Laboratories) and anti-Actin antibodies (Abcam). Shown is a representative blot displaying equal levels of exogenous V5-epitope tagged SRAP (~35kDa) products in both SRA-WT and SRA-Y35N but not control SRA-MET7 transfected cell lysates.
B) Pancellular localization of wild-type versus Y35N SRAP. HeLa cells were transiently transfected with either V5-epitope tagged SRA-WT or SRA-Y35N constructs and exogenous SRAP (Red) expression was observed by indirect immunofluorescent microscopy using anti-V5 (Life Technologies) primary antibody followed by anti-Mouse-Alexa555 (Life Technologies). Cells were counterstained with Dapi to visualize nuclei (Blue).
C) SRA-Y35N mutation results in impaired estradiol induced ESR1 transactivation of PS2-ERE luciferase reporter. HeLa cells were co-transfected with Estrogen Receptor-α (ESR1), PS2-ERE luciferase reporter, and either control (SRA-MET7), wild-type (SRA-WT), or mutant (SRA-Y35N) SRA plasmids 24 h prior to being treated with estradiol (+E2) or ethanol (cont). Data were normalized as detailed in the Materials and Methods section. Error bars represent standard deviation for n=4. Unpaired 2 tailed student's t-test was performed to test for significant difference among different conditions (*represents $p<0.05$)

(Figure 4B), i.e. cytoplasmic and nuclear, as previously described (13,14).

As shown in Figure 4, in the control SRA-MET7 and ESR1 co-transfected HeLa cells, estradiol treatment resulted in an approximate 1.6 fold increase in luciferase activity over that obtained from control transfected cells treated with ethanol vehicle alone (t-test, $p<0.05$) (Figure 4C, bars 1 vs 2). By comparison, co-transfection with SRA-WT resulted in a more pronounced and significant 2.5 fold increase in luciferase activity in the presence of estradiol (t-test, $p<0.01$) (compare bars 3 vs 4). These data are consistent with previously published results that indicate wild-type SRA to be an ESR1 co-activator (6). In contrast, SRA-Y35N did not increase the action of estradiol in this system, as indicated by similar luciferase activity in SRA-MET7 negative control and SRA-Y35N transfected cells (compare bars 2 and 6).

Overall, these data indicate that the mutant Y35N SRA is functionally different than the wild-type SRA and is unable to co-activate ER-alpha under these luciferase reporter assay conditions indicating a loss of function in this specific assay.

Discussion

In this article, we report three independent families in which affected siblings with inactivating mutations in *SRA1* suffer from IHH. We identified and confirmed these mutations by a variety of genetic methods including candidate screening, genome-wide SNP genotyping and autozygosity mapping, targeted exome sequencing, and whole exome sequencing. Extreme rarity of these variants was confirmed not only in the international databases but also in in-house and national ones.

The products of this gene, SRA RNA and SRAP protein, define a very intriguing bi-faceted genetic system where both RNA and protein products of the same gene have the potential to play specific and sometimes overlapping roles in cell biology (15).

The steroid receptor RNA activator (SRA) was originally identified as a functional non-coding RNA involved in the regulation of gene expression by steroid receptors (8,12,16). It is now established that this RNA forms complexes, through critical secondary structures and loops, with a wide range of molecules including, but not limited to, multiple nuclear receptors, nuclear receptors co-regulators, proteins involved in gene silencing and gene insulation (8,11,17,18,19,20,21,22,23,24). Subsequently, some SRA transcripts were found to be able to encode for a protein, now referred to as the Steroid Receptor RNA Activator Protein (SRAP) (13,25,26). This made *SRA1* the first gene able to encode for both a functional RNA and a protein (26). SRAP, as its RNA counterpart, has now been known to positively regulate the activity of steroid receptor such as the androgen receptor (AR) and the estrogen receptor (25,27).

We showed in this study that while WT-SRA acted, as expected, as an ER-alpha co-activator in a reporter assay, the mutation identified in Family 1 patients elicited a significantly decreased estradiol dependent ER-alpha activity. Notably, mutations in this study are located, in the protein sequence, within the two SRAP domains highly conserved among chordate (15,23). These mutations are also positioned in the Helix 1, 2, and 15 of the non-coding SRA sequence, which are highly conserved in all chordate (28). As outlined earlier, it has been shown that SRA conserved secondary structures were critical for its functional properties (12). Overall, the fact that the mutations identified here lay within both RNA and protein conserved regions emphasize the likelihood that they could have a profound effect on SRA and/or SRAP action, whatever the exact relevant mechanisms are involved.

IHH could be caused by defects in the hypothalamus and/or the pituitary. In both tissues, sex steroid receptors, ER-alfa and AR, are expressed. These receptors sense and accordingly respond to peripheral sex steroid levels in a negative feed-back pattern. Thus, inactivating mutations in *SRA1*, a known coactivator of ER-alfa (as once again shown in the control experiments in this study), should result in increased but not decreased gonadotropin levels. This would not be consistent with the phenotype of IHH seen in our patients. Indeed, in the rare cases of male and female patients who have inactivating ER-alfa mutations, there is a clear clinical picture of hypergonadotropic hypogonadism (29,30). Therefore, inactivating *SRA1* mutations in our patients in this study must have caused hypogonadotropic hypogonadism by a mechanism other than via a decreased ER-alfa coactivation either at the hypothalamic or the pituitary level. When we stimulated the proband in Family 1 with GnRH in an extended fashion, the patient did not show a noticeable LH response, suggesting that the response of the pituitary gland to GnRH is compromised, thus we focused on the pituitary as the site of dysfunction leading to IHH (31).

Vertebrate members of the nuclear receptor NR5A subfamily, which includes steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1), regulate crucial aspects of development, endocrine homeostasis, and metabolism. In the pituitary, both LRH1 and SF-1 regulate the expression of the gonadotropins (32,33,34) and of the GnRH receptor (32,35). Mice with pituitary-specific disruption of SF-1 have markedly diminished levels of pituitary gonadotropins modeling hypogonadotropic hypogonadism (36). *DAX-1* (*NR0B1*), a close partner of SF-1, acts as an adaptor that recruits other factors, such as the nuclear receptor corepressors to SF-1. In humans, inactivation mutations of *DAX-1* are well known to cause hypogonadotropic hypogonadism (37). Notably, the endogenous SRA is required for the synergistic enhancement of SF-1 transcriptional activity by *Dax-1*. Taken together, it appears that *SRA1* regulates SF-1 target gene expression by functioning as a coactivator in association with *Dax-1* (11). Thus, reduced *SRA1* activity due to inactivating mutations found in this study would result in diminished SF-1/LRH-1 effect leading to IHH, in parallel to the mechanism by which IHH is caused by inactivating *DAX-1* mutations.

Interestingly, in one of the families (Family 3), we observed mutations in two genes, i.e. *SRA1* and *PNPLA6*. Digenic inheritance is a well-established phenomenon of IHH, accounting for about 10% of all cases (38,39). With the increase in number of unbiased comprehensive genetic studies such as whole exome sequencing, it is now further appreciated that oligogenic inheritance is quite common in Mendelian disorders (40). Recently, a dedicated database for digenic inheritance has been established, in which IHH is listed along with other well-known oligogenic phenotypes such as non-syndromic hearing impairment (41).

In digenic inheritance, gene pairs are associated with one another by sharing pathway membership in about 20% of the time (41). We recently described patients with Gordon-Holmes syndrome (IHH and cerebellar ataxia) due to inactivating *PNPLA6* mutations (42). *PNPLA6* encodes for neuropathy target esterase (NTE), a lysophospholipase that maintains intracellular phospholipid homeostasis by converting lysophosphatidylcholine to glycerophosphocholine (42). We also demonstrated that inhibition of NTE activity in the LβT2 gonadotrope cell line, which represents the pituitary gonadotropes, diminished LH response to GnRH by impaired LH release from pituitary gonadotropes leading to IHH. Thus, the sites of action of *SRA1* and *PNPLA6* seem to overlap at the pituitary gonadotropes, which suggests that dysfunction of these two gene variants potentialize or are additive to each other. Even more interestingly, there is ample evidence in the literature to show that phospholipids including certain species of lysophosphatidylcholine are ligands for SF-1/LRH-1, potentially placing two genes (i.e. *SRA1* and *PNPLA6*) in the same pathway (43,44). In fact, phospholipids may represent a potential link between metabolism and reproductive function (45). Although a pituitary site of action seems probable, a hypothalamic involvement cannot be ruled out as there are publications supporting this contention. Most notably, both LRH-1 and *DAX-1* (46) are expressed in the arcuate nucleus and LRH-1 provides a stimulus for kisspeptin activation in the GnRH pulse generator (47).

As for the unusual clinical and laboratory features of these families, the proband in Family 2 and the younger brother (II-4) in Family 3 had undescended testicles and micropenis in infancy suggesting a profound prenatal undervirilization due to a severe IHH. But later, at a delayed age, they went through puberty with or without intervention. Spontaneous or induced regain of central gonadal function or reversibility is seen in about 10 percent of all IHH cases (48), even in the most severe cases of congenital IHH (49). In Family 3, the older sister (II-1) appears to be unaffected despite carrying the same two variants as her affected brothers. Like her younger brother, she

may have gone through spontaneous recovery. It should be noted however, that similar variability of phenotypes from IHH to delayed puberty to even normal timing in persons carrying the same genotype within the same family has been repeatedly observed (50,51,52,53). Alternatively, a third mutated gene or a copy number variation among others could have provided an explanation for the genotype/phenotype discrepancy in this family, but we have not been able to find one.

Lastly, a global knock-out of SRA in the mouse has been recently reported. This model mouse protects against diet-induced obesity and improves whole body glucose homeostasis probably via its action as a PPARγ coactivator. The SRA–/– mice appeared "normal" with no specific information regarding their reproductive function provided (54).

In conclusion, it is evident from the studies reported here that inactivating mutations of the *SRA1* gene cause complete normosmic IHH, hence pubertal failure in humans, and we would argue that proper function of *SRA1* is a critical element of the central gonadal function in humans. It is tempting to speculate that *SRA1*, an intriguing gene whose products functioning both as a protein and a noncoding RNA, may in part account for the complexity, versatility, and elusiveness of the pubertal process, especially when one considers the fact that actions of nuclear receptor coregulators can spatially and temporally vary to become activators or repressors of the target nuclear receptors depending on the cellular and promoter context.

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Ethics

Ethics Committee Approval: The Ethics Committee of the Çukurova University Faculty of Medicine approved, Informed Consent: Obtained from each participant or from the parents.

Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Leman Damla Kotan, A. Kemal Topaloğlu, Etienne Leygue, Design: Leman Damla Kotan, A. Kemal Topaloğlu, Etienne Leygue, Data Collection and/or Processing: Charlton Cooper, Şükran Darcan, Samim Özen, Yi Yan, Fatih Gürbüz, Eda Mengen, İhsan Turan, Ayça Ulubay, Gamze Akkuş, Bilgin Yüksel, Analysis and/or Interpretation: Ian M. Carr, Charlton Cooper, Mohammad K. Hamedani, Literature Research: Leman Damla Kotan, Fatih Gürbüz, Eda Mengen, İhsan Turan, Writing: Leman Damla Kotan, A. Kemal Topaloğlu, Etienne Leygue.

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Original Article

Association of DENND1A Gene Polymorphisms with Polycystic Ovary Syndrome: A Meta-Analysis

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ABSTRACT

Objective: The rs2479106 and rs10818854 polymorphisms in the *DENND1A* gene have been reported to be extensively associated with risk of polycystic ovary syndrome (PCOS). However, the results from these studies remained inconclusive and conflicting. To detect a true association of rs2479106 and rs10818854 polymorphisms with PCOS risk, a single study may be underpowered, particularly for those studies with inadequate sample size. Therefore, we performed a meta-analysis of all available studies to explore this association.

Methods: All studies published up to March 2015 on the association were identified by searching electronic databases PubMed, EMBASE, Web of Science, and China National Knowledge Infrastructure. Studies containing available genotype frequencies of those 2 polymorphisms were chosen, and the odds ratios and associated 95% confidence intervals were calculated using fixed- or random- effects models.

Results: A total of 8 studies about s2479106 polymorphism (8185 cases and 28675 controls) and 5 studies about rs10818854 polymorphism (6638 cases and 27443 controls) met the inclusion criteria for the meta-analysis. Overall, significant increase of PCOS risk was found between *DENND1A*-rs10818854 and PCOS susceptibility. In addition, we also found an increased risk of PCOS in rs2479106 allele model, heterozygote variant genetic model, and dominant genetic model.

Conclusion: This meta-analysis suggested that rs2479106 and rs10818854 polymorphisms in the *DENND1A* gene were associated with increased risk of PCOS. To validate the association between these polymorphisms and PCOS susceptibility, further large and well-designed studies are needed.

Keywords: Polycystic ovary syndrome, *DENND1A*, rs2479106, rs10818854, meta-analysis

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The rs2479106 and rs10818854 polymorphisms in the *DENND1A* gene have been reported to be extensively associated with risk of polycystic ovary syndrome (PCOS). However, the results from these studies remained inconclusive and conflicting.

WHAT THIS STUDY ADDS?

This meta-analysis suggested that the *DENND1A* gene s2479106 and rs10818854 polymorphisms were associated with increased risk of PCOS.

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Introduction

Polycystic ovary syndrome (PCOS), known as the most common endocrinopathy in women of reproductive age, is a hyperandrogenic and ovulatory disorder (1). It affects about 5-8% of child-bearing women and is also associated with obesity and several cardiometabolic abnormalities, including metabolic syndrome, insulin resistance (IR), diabetes mellitus type 2, dyslipidemia, atherosclerosis, and hypertension (2). Despite PCOS prevalence and health implications, there is no gold standard for long-term treatment of women with PCOS and the etiology of PCOS remain unclear. Interestingly, it has also been demonstrated that PCOS is a multifactorial disease with polygenic nature, and this heterogeneity complicates the effort to investigate additional genetic components of its pathogenesis (3). The DENN domain containing 1a *(DENND1A)* gene, a member of a family of 18 human genes termed "connecdenns", has gained recognition as a strong PCOS susceptibility gene in several studies (4,5).

DENND1A, or connecdenn1, encodes a protein containing domains differentially expressed in normal and neoplastic cells (DENN). The *DENND1A* protein involves in endosomal membrane trafficking and acts as a guanine exchange factor and interacts with members of the Rab family of small GTPases Rab35 (4,6). *DENND1A* is ubiquitously expressed and the protein is present in high levels in the brain and kidneys (7). In addition, *DENND1A* affects a wide range of physiological processes, and it is expected that *DENND1A* might influence the pathogenesis of PCOS through misregulation of endoplasmic reticulum aminopeptidase1 (ERAP1) (5,8).

Over the last two decades, a number of studies have been conducted to investigate the potential association between *DENND1A* genomic region and PCOS risk in humans. Among the many polymorphisms of *DENND1A* genes, rs2479106 and rs10818854 polymorphisms have received much attention. Several studies have previously suggested that the rs2479106 and rs10818854 polymorphisms were associated with an increased risk of PCOS (9). However, other studies have failed to confirm such an association (10,11), presumably due to the relatively small samples of individual studies, possible selective bias, and various genetic backgrounds. Herein, to acquire more comprehensive evidences, we conducted a meta-analysis to assess the effect of the two polymorphisms on the risk of PCOS.

Methods

Search Strategy and Selection Criteria

We conducted a PubMed search, a Google Scholar search, an EMBASE search, and a China National Knowledge Infrastructure (CNKI) search using the keywords "*DENND1A*", "polycystic ovary syndrome", "rs2479106", "rs10818854", and "polymorphism" for the articles. We also supplemented this search by reviewing the reference lists of all retrieved publications. If more than one article was published by the same author using the same case series, we selected the latest research. The relevant search was finished in March 15, 2015. Data on sample characteristics were extracted by 2 independent reviewers who reached a consensus regarding inclusion or exclusion of the article. For the meta-analysis, the following inclusion criteria were considered: 1) Unrelated case-control studies; 2) about rs2479106, rs10818854 polymorphisms and risk of polycystic ovary syndrome; 3) describing useful genotype frequencies; 4) sufficient genotypes data were presented to calculate the odds ratios (ORs); 5) conforming to Hardy-Weinberg (H-W) equilibrium [HWE was tested for genotype frequency distributions of single nucleotide polymorphism (SNP). If there would be deviation from HWE, the results should be interpreted with caution because the observed genotype distribution in control population does not represent genotype distribution in the overall population]; 6) pathological diagnoses and the sources of cases and controls should be clearly described. The exclusion criteria were: 1) Incomplete data; 2) non-case-control studies; 3) duplicated publications; 4) unbalanced matching in patient populations; and 5) lack of approval of local ethics committees.

Data Extraction

Two separate investigators reviewed and extracted data from all of the eligible publications independently according to the inclusion and exclusion criteria listed above. The following information will be collected: Characteristics of the methodological research project, the first author's name, year of publication, country of origin, ethnicity, source of controls, number of cases and controls, PCOS confirmation criteria, genotyping method, genotype frequency for cases and controls, and HWE of controls.

Statistical Analysis

The meta-analysis evaluated the overall association between the *DENND1A* polymorphism and the risk of PCOS using ORs with the corresponding 95% confidence interval (CI) for each study. The significance of the pooled OR was determined by Z test and a p-value of less than 0.05 was considered significant. Different ORs were calculated using the following models: the allele model (A vs. a), the additive genetic model (AA vs. Aa/Aa vs. aa), the dominant genetic mode (AA+Aa vs. aa), the recessive genetic model (AA vs. Aa+aa), heterozygote variant genetic model (Aa vs. aa), and homozygous variant genetic model (AA vs. aa). The heterogeneity of these studies was tested by the $\chi 2$ based Q test and I^2 statistics (12,13). We considered the result of $P_Q<0.1$ or $I^2 \geq 50\%$ as indicative of heterogeneity according to Cochrane

Handbook, a random-effects (REs) model (the DerSimonian and Laird method) was used to estimate the summary ORs (14); Otherwise, $P_Q \geq 0.1$ or $I^2 < 50\%$, indicating the absence of heterogeneity, the fixed-effects the (Mantel-Haenszel method model) was used (15). If heterogeneity was presented in this meta-analysis, meta-regression and subgroup analyses were conducted by grouping studies that showed similar characteristics, such as ethnicity, control sources, and genotyping methodology. We also performed meta-analysis using REs models as a sensitivity analysis to examine the robustness of our findings to alternative methods of pooling. If potential publication bias was found in the meta-analyses, contour-enhanced funnel plots and Egger's test were performed to explore the probable source of publication bias. All statistical analyses were performed using Stata 8.0 (TX, USA) and RevMan 4.2 (Cochrane Collaboration, Oxford, UK).

Results

Characteristics of Studies

A total of 64 articles relevant to search keywords were identified based on the search criteria. After removing 27 duplications, 37 articles remained for which the full-text article was retrieved. By reading the titles and abstracts, we excluded 11 articles, including non-relevant studies, reviews, and meta-analysis. After retrieving the full text of the remaining 26 articles, we excluded 16 articles because of the following reasons: non-case-control studies, incomplete data, unbalanced matching in patients, and not relevant to rs2479106 and rs10818854 polymorphisms. Of these 10 articles, the Han Chinese samples (828 participants) in the Zhao et al (16) study might have overlapped subjects used in the Cui et al (17) study, and the Han Chinese groups (GWAS: 744 cases and 895 controls, Replication 1: 2840 cases and 5012 controls, Replication 2: 498 cases and 780 controls) in the Chen et al (8) study were also likely overlapped with those in the Shi et al (9) study. Finally, 8 relevant articles were included in the final meta-analysis (Flow diagram shown in Figure 1).

Overall, a total of 8 case-control studies about rs2479106 with 8185 cases and 28675 controls were included. Simultaneously, 5 studies about rs10818854 with 6638 cases and 27443 controls were analyzed. The main characteristics of these studies are summarized in Table 1. These studies focused on different populations of various ethnicities: 2

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

	First Author	Country	Year	Ethnicity	Study design	Case/control	Genotyping method	Source of controls	HWE
rs2479106	Shi	China	2012	Han Chinese	GWAS I	744/895	Affymetrix Array 6.0	Hospital	Yes
				Han Chinese	GWAS II	1510/2016	Axiom	Hospital	Yes
	Cui	China	2012	Han Chinese	GWAS	1614/4744	Affymetrix Array 6.0	Hospital	Yes
	Eriksen	Denmark	2012	Denmark	Replication	168/248	TaqMan	Hospital	Yes
	Lerchbaum	Austria	2011	Caucasian	Replication	503/311	-	Hospital	Yes
	Goodarzi	USA	2012	European	Replication	939/957	Taqman	Hospital	Yes
				European	Replication	535/845	Illumina	Hospital	Yes
	Welt	USA	2012	Iceland	Replication	376/16947	Illumina	Hospital	Yes
				Boston	Replication	559/477	Centaurus	Hospital	Yes
				Chicago	Replication	201/188	Centaurus	Hospital	Yes
	Meredith	European	2014	White subjects	Replication	845/845	Illumina	Population	Yes
	Gammoh	Bahrain	2015	Arab	Replication	191/202	Taqman	Population	Yes
rs10818854	Shi	China	2012	Han Chinese	GWAS I	744/895	Affymetrix Array 6.0	Hospital	Yes
					GWAS II	1510/2016	Axiom	Hospital	Yes
	Cui	China	2012	Han Chinese	GWAS	1583/4916	Affymetrix Array 6.0	Hospital	Yes
	Goodarzi	USA	2012	European	Replication	939/957	Taqman	Hospital	Yes
				European	Replication	535/845	Illumina	Hospital	Yes
	Welt	USA	2012	Iceland	Replication	376/16947	Illumina	Hospital	Yes
				Boston	Replication	559/477	Centaurus	Hospital	Yes
				Chicago	Replication	201/188	Centaurus	Hospital	Yes
	Gammoh	Bahrain	2015	Arab	Replication	191/202	Taqman	Population	Yes

GWAS: genome-wide association study, Replication: repeated verification experiment, HWE: Hardy-Weinberg (H-W) equilibrium

studies of Chinese, 1 study of Bahrain, 1 study of European, 1 study of Denmark, 1 study of Austria, and 2 studies of the USA. The distribution of the genotypes in the control subjects was consistent with HWE.

Meta-analysis Results

The determined association between rs2479106 polymorphism and the risk of PCOS are shown in Table 2 and Supplementary 1. Overall, individuals carrying the rs2479106 G allele had significantly increased risk for PCOS compared to those carrying A allele (OR=1.202, 95% CI: 1.070-1.350, p=0.002) in the allele model. This was also the case for GA vs. AA in the heterozygote variant genetic model (OR=1.266, 95% CI: 1.140-1.407, p<0.001) and for GG/GA vs. AA in the dominant genetic model (OR=1.277, 95% CI: 1.155-1.411, p<0.001). In the stratified analysis by ethnicity, significantly elevated PCOS risk was found in Asians when all studies were pooled into the meta-analysis (G vs. A: OR=1.301, 95% CI: 1.219-1.389, p<0.001; GG vs. AA: OR=1.559, 95% CI: 1.234-1.970, p<0.001; recessive genetic model: OR=1.417, 95% CI: 1.127-1.782, p=0.003; additive genetic model: OR=1.264, 95% CI: 1.156-1.382, p<0.001). However, no increased risk was detected in Caucasians in the allele model, the homozygote contrast, the recessive model, or the additive model (all p>0.05). Further subgroup analysis by ethnicity was not done due to the small number of studies.

The meta-analysis findings of the correlation between rs10818854 and PCOS risk are summarized in Table 3 and Supplementary 2. A significant association was found between the rs10818854 A allele and PCOS risk in the allele model (OR=1.395, 95% CI: 1.148-1.694, p=0.001). Similarly, this association was found in the additive genetic model (OR=1.543, 95% CI: 1.381-1.725, p<0.001). In the subgroup analysis of ethnicity, rs10818854 A was significantly associated with an increased risk of PCOS in Caucasians or Asians in the allele model (OR=1.530, 95% CI: 1.170-2.000, p=0.002; OR=1.362, 95% CI: 1.073-1.729, p=0.011), potentially suggesting that the A variant might exhibit a higher risk of PCOS between different ethnical populations.

Tests of Heterogeneity

We reckoned the heterogeneity between each of the studies using the Q-test. For rs2479106-related PCOS risk (Table 2), significant heterogeneity between studies was observed in some comparisons (G vs. A: P_Q=0.002; GG vs. AA: P_Q=0.016; recessive genetic model: P_Q=0.011; additive genetic model: P_Q=0.038). Then, the REs models were used to evaluate the combined ORs when necessary. Vice versa, the other models used fixed-effects model to analyze the ORs. After stratifying by

Table 2. Pooled odds ratio with 95% confidence interval for the association between rs2479106 and Polycystic ovary syndrome risk in the meta-analysis

	Genetic model	OR (95% CI)	p	p for Heterogeneity	I^2 (%)	Model	p for Egger's test
Overall							
(n=7)	G vs. A	1.202 (1.070-1.350)	0.002	0.002	71.9%	Random	0.905
(n=4)	GG vs. AA	1.181 (0.700-1.994)	0.533	0.016	70.8%	Random	0.757
(n=4)	GA vs. AA	1.266 (1.140-1.407)	0.000	0.969	0.0%	Fixed	0.673
(n=4)	Dominant	1.277 (1.155-1.411)	0.000	0.550	0.0%	Fixed	0.624
(n=4)	Recessive	1.077 (0.637-1.821)	0.783	0.011	73.3%	Random	0.789
(n=7)	Additive	1.092 (0.987-1.209)	0.088	0.038	54.9%	Random	0.413

OR: odds ratio, CI: confidence interval

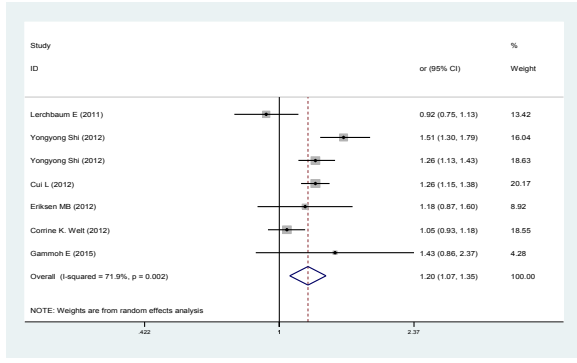
Table 3. Pooled odds ratio with 95% confidence interval for the association between rs10818854 and Polycystic ovary syndrome risk in the meta-analysis

	Genetic model	OR (95% CI)	p	p for Heterogeneity	I^2 (%)	Model	for Egger's test
Overall							
(n=5)	A vs. G	1.395 (1.148-1.694)	0.001	0.004	74.0%	Random	0.887
(n=4)	Additive	1.543 (1.381-1.725)	0.000	0.157	42.4%	Fixed	0.777
Ethnicity							
Caucasian (n=1)	A vs. G	1.530 (1.170-2.000)	0.002	-	-	Random	-
Asian (n=4)	A vs. G	1.362 (1.073-1.729)	0.011	0.002	79.9%	Random	0.825

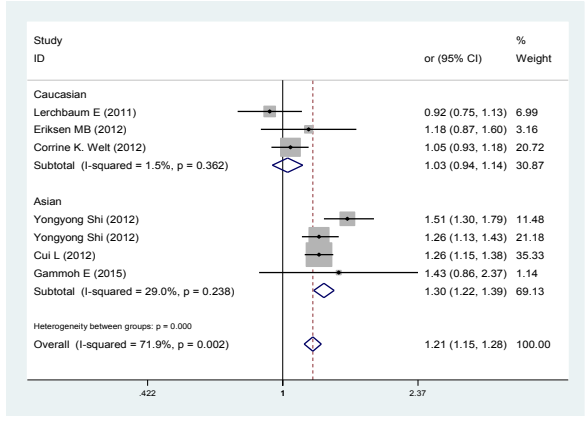
OR: odds ratio, CI: confidence interval

Supplementary 1. Pooled odds ratio with 95% confidence interval for the association between rs2479106 and Polycystic ovary syndrome risk in all genetic model comparisons (risk allele: G)

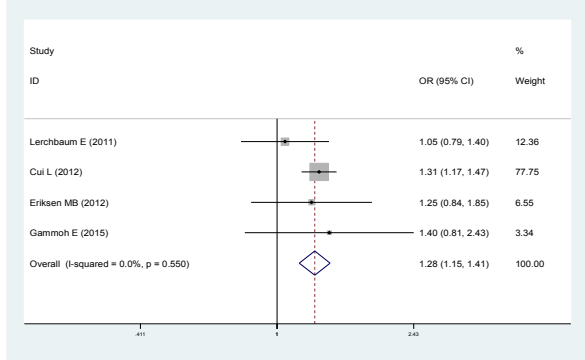
A. Allele model (G vs. A) and estimate of the pooled odds ratio



Subgroup analysis and estimate of the pooled odds ratio

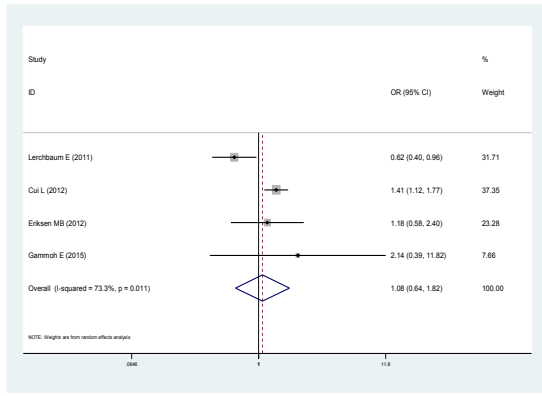


B. Dominant model (GG/GA vs. AA) and estimate of the pooled odds ratio

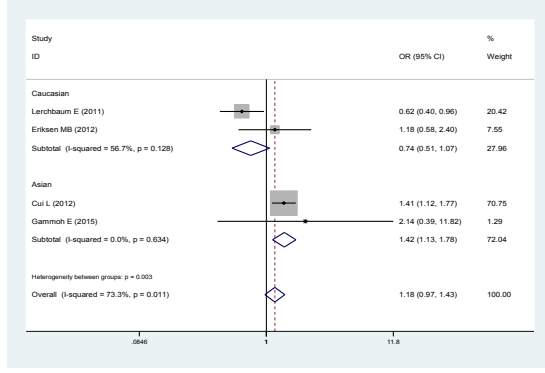


Subgroup analysis and estimate of the pooled odds ratio
The dominant genetic models only comprised four groups, and the *p*-values of the test of heterogeneity was not significant ($p>0.1$), thus we did not perform subgroup analysis.

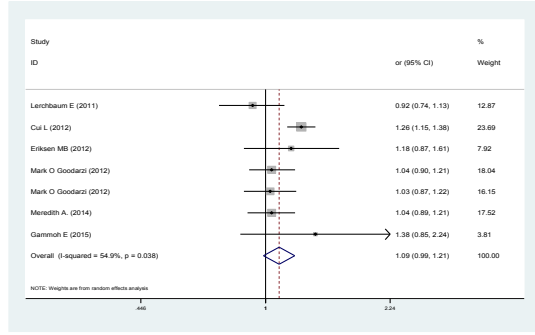
C. Recessive model (GG vs. AA/GA) and estimate of the pooled odds ratio



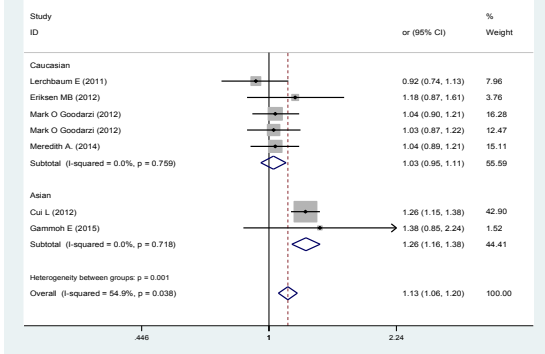
Subgroup analysis and estimate of the pooled odds ratio



D. Additive model (GG vs. GA/GA vs. AA) and estimate of the pooled odds ratio



Subgroup analysis and estimate of the pooled odds ratio



ethnicity, no heterogeneity was found between studies (P_Q>0.05). For rs10818854 (Table 3), there was significant heterogeneity between studies under the allele model (G vs. A: P_Q=0.004), and ORs were pooled under REs model. Subsequently, we performed subgroup analyses stratified by ethnicity. However, heterogeneity still existed among Asians (G vs. A: P_Q=0.002).

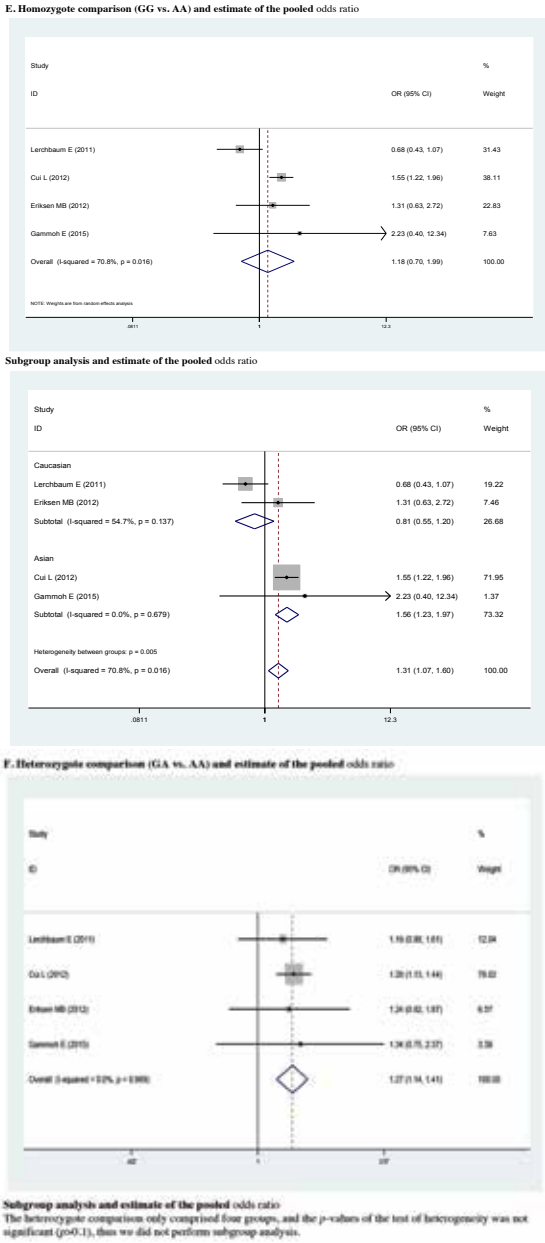
Sensitivity Analysis

Since significant heterogeneity across studies was observed for some models, we performed a sensitivity analysis to assess the influence of each individual study on the pooled OR by sequentially removing the individual study (statistics of study removal). The results suggested that no single study dramatically change the pooled ORs, indicating that our results were robust and reliable (Figures Supplementary 1 and Supplementary 2).

Publication Bias

The results of Egger's test did not show any evidence of publication bias in any comparison model (p>0.05).

Supplementary 1.



Supplementary 2. Pooled odds ratio with 95% confidence interval for the association between rs10818854 and Polycystic ovary syndrome risk in genetic model comparisons (risk allele: A)

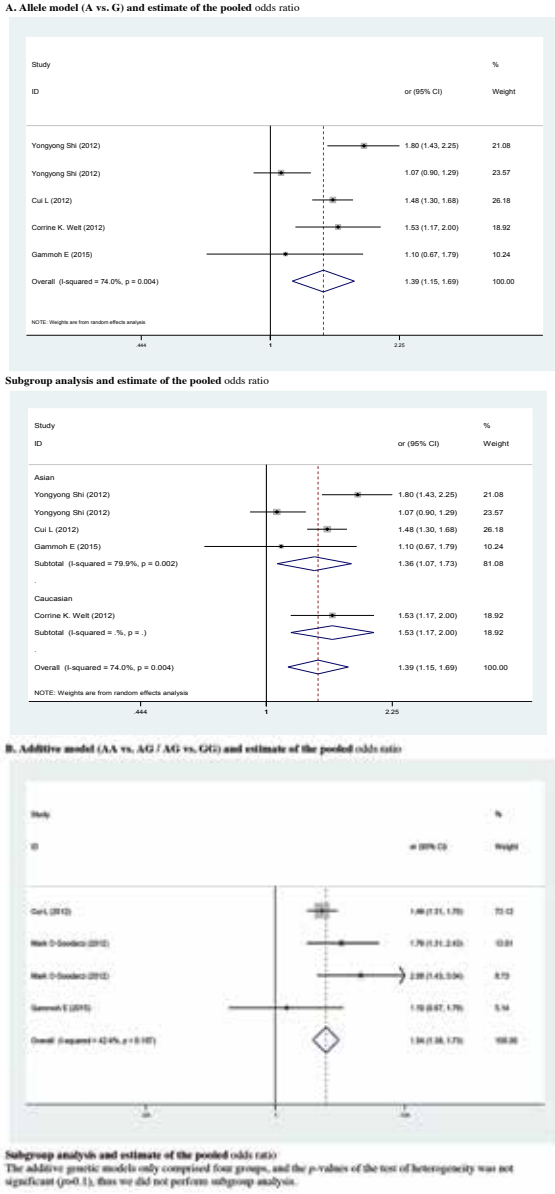
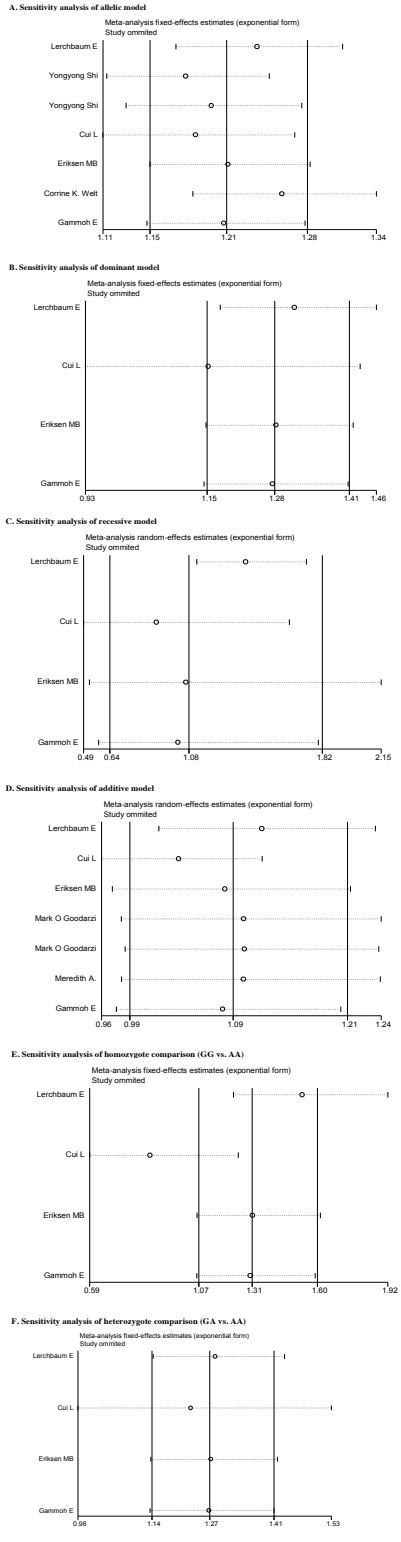


Figure Supplementary 1. Sensitivity analysis of rs2479106 polymorphism. The analysis was performed by omitting each study in turn. The two ends of the dotted lines represent the 95% confidence interval



Discussion

The *DENND1A* gene has been mapped to chromosome 9q22.32 and consists of 22 exons extending over 500,000 bases (4). Rs2479106 and rs10818854 are located in intron regions of the *DENND1A* gene and encode the protein *DENND1A*, and as such, SNPs located within *DENND1A* introns may influence *DENND1A* expression by interacting with upstream or downstream chromosomal regions (18). Expression of this gene has been reported in testes, ovarian theca cells, and H295 adrenal carcinoma cells (19).

To date, a number of epidemiological studies have evaluated the association between *DENND1A* rs2479106 and rs10818854 polymorphisms and PCOS risk, but the results remain inconclusive. Meta-analysis can comprehensively evaluate and quantitatively analyze multiple research results from a critical and objective perspective, thereby improving the efficiency of statistical analysis and tests.

In this study, we found a significant association between the variant rs10818854 and increased risk of PCOS. Nevertheless, the results were conflicting in previous studies. Shi et al (9) found a correlation between rs10818854 variant allele and elevated PCOS risk in genome-wide association study (GWAS 1), and Cui et al (17), Goodarzi et al (11) and Welt et al (20) also identified an increased risk. Contrary to these results, Shi et al (9) GWAS 2 and Gammoh et al (21) did not detect an association between rs10818854 variant allele and PCOS risk. In addition, in the subgroup analysis by ethnicity, the association between rs10818854 variant allele and PCOS risk was also found in Asians and Caucasians. Taken together, it may be concluded that *DENND1A* rs10818854 polymorphism was associated with PCOS risk in common population. Although

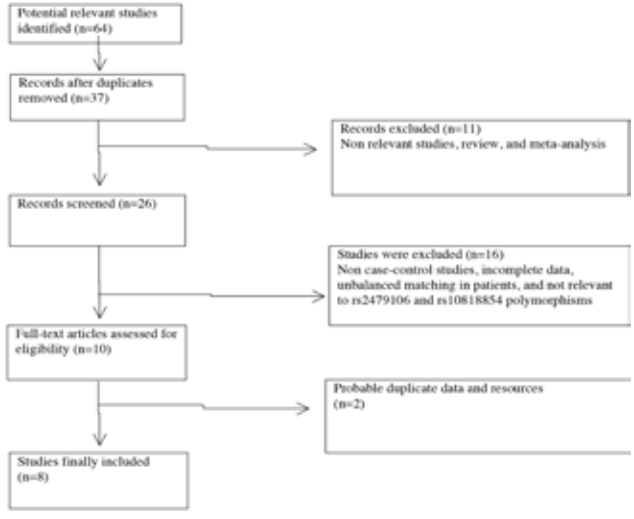
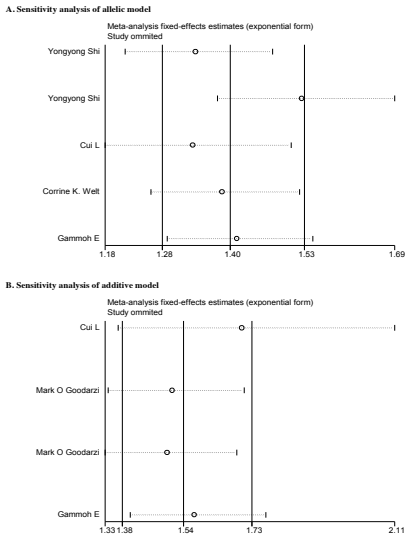


Figure 1. The flow chart of the study selecting process

Figure Supplementary 2. Sensitivity analysis of rs10818854 polymorphism. The analysis was performed by omitting each study in turn. The two ends of the dotted lines represent the 95% confidence interval



we herein confirmed the association between the rs10818854 polymorphism and PCOS risk, whether this SNP is causative is still uncertain. Significantly, previous study have shown rs10818854 is in high linkage disequilibrium with rs10986105 in European populations (r2=0.83 in HapMap CEU) (11). Thus, a systematically functional validation study is necessary to clarify the role of the rs10818854 polymorphism in the development of PCOS risk.

Reviewing the past studies about *DENND1A*-rs2479106 polymorphism and the risk of PCOS, Shi et al (9) and Cui et al (17) found an increased risk for PCOS associated with rs2479106 variant allele, Lerchbaum et al (22) and Eriksen et al (18) identified a decreased risk, and the other studies did not detect an association between rs2479106 variant allele and PCOS risk (20,21,23). In this study, we analyzed the data from eight available case-control studies. We also found that the rs2479106 polymorphism was associated with elevated PCOS risk in allele model (CC vs. CG), heterozygote variant genetic model (GA vs. AA), and dominant genetic model (GG/GA vs. AA). After stratifying by ethnicity, significantly elevated risk associated with the rs2479106 G allele was only found for PCOS risk among Asians but not in Caucasians in all genetic models. This suggested that *DENND1A*-rs2479106 may have different effects in different populations. The possible explanation for these discrepancies could be different genetic backgrounds. However, the number of studies in this subgroup was a little small (n=4), which may have insufficient statistical power to detect a slight effect or may reduce the reliability of the results. Notably, the results should be interpreted with caution.

Heterogeneity is a potential problem when interpreting the results of a meta-analysis, and the obvious evidence of between-study heterogeneity in this meta-analysis should be issued. In the present meta-analysis, there were modest heterogeneities in some comparisons for *DENND1A*-rs2479106 polymorphism and PCOS risk. After stratifying by ethnicity, the subgroup did not show heterogeneity anymore, reflecting that ethnicity might influence the heterogeneities. Nevertheless, for rs10818854-related PCOS risk, there were still notable heterogeneities in Asians in subgroup analyses, which may be caused by the different ethnicities, study quality, genotyping methods, and experimental designs.

There are several limitations in the present study. First, the sample size of our case-control study was relatively small. Some non-English articles, unpublished reports were not included in our meta-analysis, which may bias our results. Second, our meta-analysis was based on unadjusted estimates, whereas a more precise analysis of the various groups should be conducted according to potentially confounding factors, such as age, gender, obesity, drinking and smoking status, menopausal status, use of contraceptives, environment factors, and so on.

Conclusion

In summary, this meta-analysis suggests that the rs10818854 polymorphism may be associated with increased risk of PCOS in the allele model and additive model. In addition, increased risk of PCOS was found in rs2479106 allele mode, the heterozygote variant genetic model, and dominant genetic model. Our findings comprehensively evaluate the association between *DENND1A* SNPs and PCOS risk and provide the basis for subsequent research of molecular mechanisms underlying the identified association. Further large sample size and well-designed studies in different ethnic populations are needed to verify our observations.

Ethics

Ethics Committee Approval: Human Research Committee of Hainan Provincial People's Hospital for Approval of Research Involving Human Subjects, Informed Consent: Each participant gave written informed consent.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Shan Bao, Jun-Hong Cai, Concept: Shan Bao, Jun-Hong Cai, Shu-Ying Yang, Design: Tianbo Jin, Zhuo-Ri Li, Data Collection or Processing:

Yongchao Ren, Tian Feng, Analysis or Interpretation: Yongchao Ren, Tian Feng, Literature Search: Yongchao Ren, Tianbo Jin, Writing: Yongchao Ren.

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Original Article

Investigation of SHOX Gene Mutations in Turkish Patients with Idiopathic Short Stature

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ABSTRACT

Objective: The frequency of mutations in the short stature homeobox (*SHOX*) gene in patients with idiopathic short stature (ISS) ranges widely, depending mostly on the mutation detection technique and inclusion criteria. We present phenotypic and genotypic data on 38 Turkish patients with ISS and the distinctive features of 1 patient with a *SHOX* deletion.

Methods: Microsatellite markers (MSMs) DXYS10092 (GA repeats) and DXYS10093 (CT repeats) were used to select patients for fluorescent *in situ* hybridisation (FISH) analysis and to screen for deletions in the *SHOX* gene. The FISH analysis was applied to patients homozygous for at least one MSM. A Sanger sequencing analysis was performed on patients with no deletions according to FISH to investigate point mutations in the *SHOX* gene.

Results: One patient (2.6%) had a SHOX mutation.

Conclusion: Although the number of cases was limited and the mutation analysis techniques we used cannot detect all mutations, our findings emphasize the importance of the difference in arm span and height when selecting patients for *SHOX* gene testing.

Keywords: Idiopathic short stature, *SHOX* gene, pseudoautosomal region 1, height, arm span-height difference

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The frequency of mutations in the short stature homeobox (*SHOX*) gene in patients with idiopathic short stature ranges widely, depending mostly on the mutation detection technique and inclusion criteria.

WHAT THIS STUDY ADDS?

Short children should be carefully investigated with respect to these mutations, even if they have only mildly disproportionate stature.

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Introduction

Idiopathic short stature (ISS) is defined as a condition where a person's height is more than two standard deviations (SDs) below the average height for a specific age, gender, and population with no other systemic, endocrine, nutritional, or chromosomal abnormalities, nor a history of intrauterine growth retardation and low weight for gestational age (1,2). ISS excludes other identifiable conditions not based on positive specific signs of ISS.

Height has a high degree of heritability and is a polygenic quantitative trait that shows complex and monogenic Mendelian inheritance patterns (3). One study reported that hundreds of variants clustered in specific genomic loci play roles in the human height trait (4). A clearly relevant gene that strongly affects height is the short stature homeobox *(SHOX)* gene, mapped to pseudoautosomal region 1 (PAR1) of the X and Y chromosomes. The *SHOX* gene has been reported to cause ISS and the short stature seen in patients with Turner's syndrome, Leri-Weill dyschondrosteosis, and Langer mesomelic dysplasia (5,6,7,8). A high recombination rate in PAR1 is associated with mandatory crossover between the X and Y chromosomes during meiosis (9,10,11). All 24 genes in the PAR1 region escape X inactivation (12). As a result, all genes located in the PAR1 region have two functional copies in humans and show a pseudoautosomal inheritance pattern (10,13). The only gene in the PAR1 region clearly associated with a disease is *SHOX* (14).

The frequency of mutations in the *SHOX* gene in patients with ISS varies widely, depending mainly on the mutation detection technique and inclusion criteria. In one study, approximately 2.4% of a large cohort of patients with ISS had *SHOX* mutations, of which 80% were complete gene deletions (15). Stuppia et al (16) reported a 12.5% frequency of *SHOX* mutations in 56 patients with ISS.

In this study, we evaluated the frequency of mutations in the *SHOX* gene in patients with ISS and discussed the distinctive clinical and radiological features of patients with such mutations.

Methods

The study was approved by the Ethics Committee of the Ankara University Faculty of Medicine. Written informed consent was obtained from all patients and their legal guardians. In all, 38 patients (34 females and 4 males; mean age, 11.84 years; range, 6.5-17 years) were included in the study. We used the following criteria based on the definition of ISS: height <-2 SD of the mean height for a given age, sex, and population group; normal karyotype (for girls); no evidence of chronic disease (e.g., chronic renal failure, chronic anaemia, celiac disease, malabsorption, malnutrition, chronic hepatic disease, chronic infectious disease, or congestive heart failure); no growth hormone (GH) deficiency and/or GH resistance based on the routine provocation test (peak GH>10 ng/mL) and normal insulin-like growth factor-1 level; no history of low birth weight; and no apparent skeletal disease.

The clinical assessment included measurements of height, weight, and sitting height, as well as the lengths of the upper segment (US), lower segment (LS), forearm, upper arm, hands, and feet. Furthermore, the degree of short stature, US/LS ratio, difference between arm span and height, assessed body proportions, extremities/trunk ratio (ETR; sum of leg length and arm span divided by sitting height), relative body mass index (RBMI), and the presence of additional features (e.g., appearance of muscular hypertrophy, cubitus valgus, forearm bowing) were evaluated.

Mutation Analysis

Genomic DNA was extracted from 1 mL peripheral blood using the Magna Pure LC instrument (Roche Applied Science, Manheim, Germany). We used an approach similar to the study of Chen et al (17) in which microsatellite markers (MSMs) were used to select patients for multiplex ligation-dependent probe amplification (MLPA) analysis to screen deletions in the *SHOX* gene. We used DXYS10092 (GA repeats) and DXYS10093 (CT repeats) to select patients for fluorescent *in situ* hybridisation (FISH) analysis to screen for *SHOX* gene deletions (Figure 1). Benito-Sanz et al (18) reported heterozygosity values of 0.96 and 0.69 for DXYS10092 and DXYS10093, respectively, and the repeat ranges were 18 and 14, respectively. Both MSMs were amplified by polymerase chain reaction and analysed on 8% polyacrylamide gels (see Supplementary Material).

The FISH analysis was applied to patients homozygous for at least one MSM using lymphocyte metaphase spreads and the Aquarius *SHOX* probe (cat no: LPU 025; Cytocell, Cambridge, UK).

Supplementary Material

We used 100 ng genomic DNA, 20 pmol DXYS10092 (F/R: TTC GTG ACA AAG GCC TTT GC/CTA CAA GTC CTA GTA CCT AC) and DXYS10093 (F/R: GCC CGT GAT CCC AGT ACT G/CAA CTT CCT TGG AAA TCT TC) primers, 2 U DNA polymerase, 10 pmol dNTP, and 75 mM MgCl2 to amplify each microsatellite marker.

SH0X Gene Sanger Sequencing: Exons 2, 3, 4, 5, and 6a and their exon-intron junction sites were amplified using 100 ng genomic DNA, 20 pmol of each of the appropriate primers, 2 U DNA polymerase, 10 pmol dNTP, and 75 mM MgCl2. The following primer sets were used: exon 2-F/R: CGC GGG GAG ACG CGC GCA TCC/GGC GGC GAA CCC CAG GAG GGC, exon 3-F/R: GCC ACG TTG CGC AAA ACC TC/CCC GAG GAC CAG GCG ATG, exon 4-5-F/R: GGG AGG CTG GGC TGG GTT C/GGA AGG GAG CAG CAG GTC C, exon 6a-F/R: GTC CCC ATC CTG CGC CCT CAC CC/GCG

CGG AGC CCG GGA GTC CG. The ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) instrument, SeqScape ver. 2.7 (Applied Biosystems) and Sequencing Analysis ver. 5.1 (Applied Biosystems) software were used for the sequencing analysis.

Sanger sequencing was applied to patients with no deletions detected by the FISH analysis to investigate point mutations in exons 2, 3, 4, 5, and 6a and their exon-intron junction sites in the *SHOX* gene (see Supplementary Material).

Results

In all, 36 index cases and an additional two children (patient 2 was a monozygotic twin brother of patient 1, and patient 34 was a sister of patient 33) were evaluated. All patient heights were <-2 SD (Figure 2). Mean height SD was -2.76±0.46. Height measurements and additional anthropometric data are shown in Figure 2 and Table 1.

One patient (2.6%, patient 12) had a *SHOX* deletion detected by FISH analysis (Figure 3). Patient 12 was an 11.5-year-old girl. She had a sister and two brothers with normal height, and her parents were first cousins. Her mother's height was 153 cm and the father's height was 178 cm. The mother's *SHOX* FISH analysis was normal. Patient 12's main clinical findings were short stature (height, 137 cm; -2.02 SD), disproportionate body measurements (arm span/height difference: -7, <-2 SD), obesity (RBMI, 126.1%), short forearms, cubitus valgus, muscular hypertrophy, genu valgus, micrognatia, high palate, and bilateral epicanthus. Hand and forearm radiography of the patient showed minimal bowing and mild wedging of the radius (Figure 4).

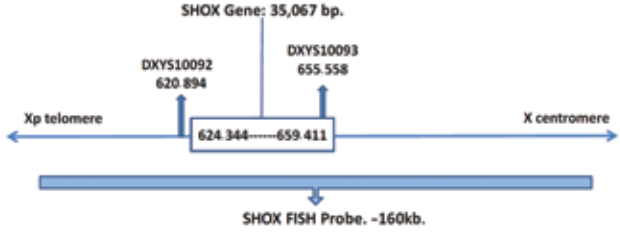


Figure 1. Schematic presentation of *SHOX* gene, *SHOX* fluorescent *in situ* hybridisation probe, and microsatellite markers DXYS10092 and DXYS10093 (according to Human GenomeAssembly GRCh38). FISH: fluorescent *in situ* hybridisation

Discussion

GH treatment is quite effective for patients with ISS and a mutation in the *SHOX* gene (19). Thus, it is important to demonstrate genetic aetiology in these cases. The frequency of mutations in the *SHOX* gene in patients with ISS is 2-15% (15,16,20,21,22,23). According to our results, this frequency was 2.6% in children with ISS.

Rappold et al (15) screened intragenic mutations using single-strand conformation polymorphism analysis in 900 patients followed by sequencing of 750 patients and detected 3 patients (0.4%) with functional mutations. They also analysed complete gene deletions using FISH in 150 patients and detected 3 patients (2%) with deletions. Another study on 56 patients with ISS reported a 12.5% (n=7) frequency of *SHOX* mutations (16). Jorge et al (21) reported a rate of 3.2% (2/63 patients with ISS). A large study that included 1534 patients with ISS reported a rate of 2.2% (n=34) (22). This wide range is mainly due to the mutation detection technique and the case inclusion criteria. Our results are compatible with the findings in these studies.

The clinical expression of *SHOX* deficiency is highly variable, as short stature is frequently nonspecific in preschool children. *SHOX* deficiency is more severe in females than males. Young children with *SHOX* deficiency may not have any specific clinical findings, but the phenotype usually becomes more pronounced with age, and characteristic signs appear over time (21,24,25). The most prominent features besides short stature are a Madelung deformity, short fourth and fifth metacarpals, high arched palate, increased carrying angle of the elbow, scoliosis, and micrognathia.

Rappold et al (22) investigated the presence of *SHOX* defects in a large cohort of 1608 children with short stature. The mean SD in height was not different between the participants

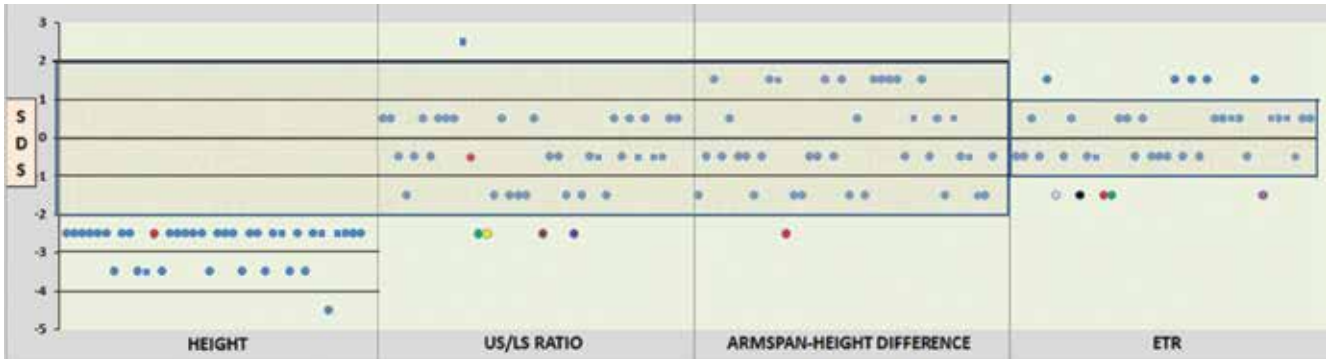


Figure 2. Height, upper segment/lower segment ratio, arm span-height difference and extremities-trunk ratio representations together with standard deviation score for all patients. Males are illustrated by square, whereas females by round. Patients lined up in order to patient number from left to right. Grey colour for P6, black for P9, red for P12, green for P13, yellow for P14, brown for P21,purple for P25, pink for P32. US: upper segment, LS: lower segment, ETR: extremities-trunk ratio

with short stature with or without identified defects in the *SHOX* gene in that study. The authors created an evidence-based scoring system based on the clinical features of 68 patients with *SHOX* defects to identify the most appropriate children for testing. They concluded that some clinical findings were useful as clues to distinguish patients with a *SHOX* mutation among patients with short stature and that the presence of any combination of reduced arm span/height ratio, increased sitting height/height ratio, above average body mass index (BMI), a Madelung deformity, cubitus valgus, short or bowed forearms, dislocation of the ulna at the elbow, or muscular hypertrophy should prompt the clinician to conduct a molecular analysis for the *SHOX* gene. An increased sitting height/height ratio, above average BMI, cubitus valgus, short forearms, and muscular hypertrophy were noted in our case with an *SHOX* gene deletion.

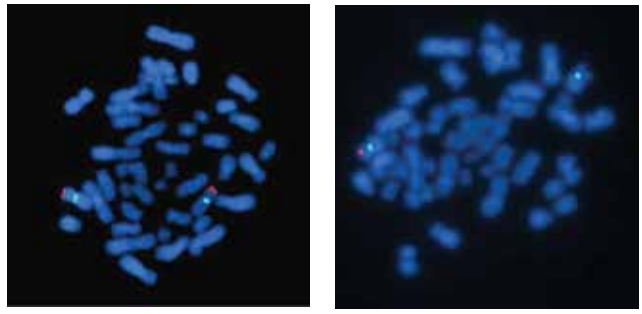
Binder et al (24) used ETR to select patients more likely to have a *SHOX* mutation. They suggested that screening for *SHOX* mutations should be limited to patients whose ETR is <1.95 + ½ height (m) and close inspection of a hand radiograph to detect the main characteristics of *SHOX* deficiency (pyramidalisation of the carpal row, radiolucency of the distal radius at the ulnar border, and triangularisation of the distal radius) in school-age children. Jorge et al (21) confirmed the usefulness of this approach and recommended using the sitting height/height ratio because it is easier to use than ETR. Our results suggest that the ETR and the difference in arm span and height are useful parameters. The US/LS ratio was not reliable alone, as this parameter was normal in our patients (Figure 2).

A radiographic examination of a patient with an *SHOX* gene mutation may demonstrate abnormal carpal wedging, triangularisation of the distal radial epiphysis, radial lucency, shortening of fourth and fifth metacarpals, and radial bowing (26). We did not detect any striking findings on a radiograph of the left hand in our patient, and she had only minimal bowing of the radius and mild wedging. It is not possible to analyse every child with ISS for a *SHOX* gene mutation because of its low incidence. Phenotypic variation in short children can affect the decision to perform a genetic analysis. Beyond the typical dysmorphic signs, a positive family history, careful anthropometric measurements and an x-ray evaluation of the hand and wrist can be used to support this decision.

Table 1. Evaluation of anthropometric measurements in the patients

Features	Mean (± SD) n=38	(Minimum/ Maximum)
Chronological age (year)	11.84 (±1.99)	6.5/17
Height (cm)	132.28 (±9.6)	104.5/154
Height SDs	-2.76 (±0.46)	(-4.05)/(-2.01)
BMI (kg/m^2)	16.44 (±3.7)	12.03/25.01
RBMI (%)	85.61 (±15.69)	64.24/134.39
Maternal height (cm)	154.16 (±6.44)	141/167
Paternal height (cm)	166.32 (±6.55)	155/185
Target height SDS	-1.15 (±0.97)	(-3.46)/0.75
Height SDS-Target height SDS	-1.62 (±1.02)	(-3.78)/0.66
Arm span (cm)	131.79 (±11.01)	98.5/160
Arm span-Height difference (cm)	-0.49 (±3.7)	(-7)/6
Sitting height (cm)	70.11 (±5.37)	57/81
Upper segment (cm)	63.63 (±5.13)	52.5/79
Lower segment (cm)	68.64 (±5.41)	52/76
Upper/Lower ratio	0.93 (±0.06)	0.77/1.07
Extremity/Trunk ratio	2.76 (±0.12)	2.52/3.06
Arm (cm)	27.09 (±2.57)	19.5/32
Forearm (cm)	20.54 (±1.76)	15/25
Hand (cm)	15.34 (±1.36)	11.5/18
Feet (cm)	21.37 (±1.7)	17/25

SDS: standart deviation score, SD: standart deviation, RBMI: relative body mass index, BMI: body mass index



a b

Figure 3. (a and b) Fluorescent *in situ* hybridisation images from P12 and P20. P20 showed two blue and two red signals meaning normal female. P12 showed two blue but 1 red signal meaning *SHOX* gene deletion (right). Probe specification: *SHOX* probe; Xp22.33/Yp11.2, (Red)/DYZ1 probe; Yq12, (Green) and DXZ1 probe; Xp11.1-q11.1, (Blue)

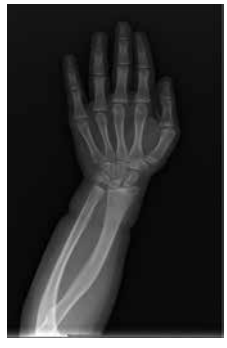


Figure 4. Hand and forearm radiography of the patient with *SHOX* deletion (P12) showing minimal bowing and mild wedging of the radius

Although we had a limited number of cases and the mutation analysis techniques used could not detect all mutations, our findings emphasize the importance of the difference between arm span and height when selecting patients for *SHOX* gene testing. Nevertheless, more extensive studies with larger groups of patients and a wider range of mutation screening techniques are needed.

Deletions are the most frequently detected *SHOX* gene mutations (15). In our study, we first performed MSM and then a FISH analysis to screen for *SHOX* gene deletions. Funari et al (27) suggested that MLPA should be the first molecular method used to screen for *SHOX* gene deletions. We also suggest using MLPA first because *SHOX* deletions are highly heterogeneous, so numerous MSM loci may need to be studied, and MLPA can detect smaller deletions than FISH.

In summary, our patient with a *SHOX* mutation had no obvious findings associated with such a gene deletion. She had a disproportionate body, which could easily go unnoticed, but she had no obvious Madelung deformity.

In conclusion, we detected an *SHOX* gene deletion in 1 of 38 children with ISS. Short children should be carefully investigated with respect to these mutations, even if they have only mildly disproportionate stature.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of the Ankara University Faculty of Medicine Ankara University, 04/10/2010, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Kenan Delil, Halil Gürhan Karabulut, Zeynep Şıklar, Merih Berberoğlu, Gönül Öçal, Ajlan Tükün, Hatice Ilgın Ruhi, Design: Kenan Delil, Halil Gürhan Karabulut, Zeynep Şıklar, Merih Berberoğlu, Gönül Öçal, Ajlan Tükün, Hatice Ilgın Ruhi, Data Collection and/or Processing: Kenan Delil, Halil Gürhan Karabulut, Bülent Hacıhamdioğlu, Zeynep Şıklar, Ajlan Tükün, Analysis and/or Interpretation: Kenan Delil, Halil Gürhan Karabulut, Bülent Hacıhamdioğlu, Zeynep Şıklar, Literature Research: Kenan Delil, Halil Gürhan Karabulut, Writing: Kenan Delil, Halil Gürhan Karabulut, Bülent Hacıhamdioğlu, Zeynep Şıklar, Ajlan Tükün, Hatice Ilgın Ruhi.

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Original Article

Effects of Thyroid Autoimmunity on Early Atherosclerosis in Euthyroid Girls with Hashimoto's Thyroiditis

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ABSTRACT

Objective: In the current study, we aimed to investigate whether thyroid autoimmunity (TA) had any effect on carotid intima-media thickness (cIMT) and enhanced the risk of cardiovascular disease (CVD) independent of thyroid function (TF) in pubertal girls with Hashimoto's thyroiditis (HT).

Methods: Sixty-six newly diagnosed euthyroid girls with HT with a mean age of 14.4±2.4 years were included in the study. The control group consisted of 41 age- and body mass index (BMI)-matched healthy girls. At enrollment, all subjects underwent physical examination including blood pressure, standing height, weight, waist circumference (WC), and hip circumference measurements. The lipid profile, high-sensitivity C-reactive protein (hs-CRP), homocysteine, blood glucose, insulin, TF, and thyroid antibodies were measured, and thyroid ultrasound and cIMT were performed.

Results: There were no significant differences in anthropometric variables between the two groups, but the patients with HT had significantly higher waist-to-hip ratio (WHR). Thyroid hormones, insulin, homocysteine, and homeostatic model assessment-insulin resistance were not different between the two groups. Serum hs-CRP levels were significantly higher in patients than controls (3.4 ng/mL vs. 2.03 ng/mL), ($p<0.001$). Patients were also characterized by significantly higher total cholesterol (166.4±27 mg/dL vs. 151±22 mg/dL), ($p<0.01$) and low-density cholesterol (95.8±24.4 mg/dL vs. 82.6±20.7 mg/dL), ($p<0.01$) levels. Patients, regardless of TF, had significantly increased cIMT compared with controls [0.28 mm vs. 0.25 mm, ($p<0.001$)], and cIMT was correlated with weight-standard deviation score (SDS), BMI-SDS, WC-SDS, and WHR. This increase in cIMT was associated independently with BMI-SDS and hs-CRP levels.

Conclusion: TA may be related to chronic inflammation, which may cause endothelial dysfunction, a promoter of atherosclerosis in girls with HT. cIMT is a good tool for the early detection and the monitoring of early atherosclerosis in euthyroid patients with HT. Early detection of risk factors of CVD, may be helpful for planning treatment and interventions, so as to prevent complications from the disease in adulthood.

Keywords: Hashimoto's thyroiditis, carotid intima-media thickness, adolescent girls, atherosclerosis

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Hashimoto's thyroiditis (HT) increases the intima-media thickness of the carotid artery (cIMT), regardless of thyroid dysfunction and traditional cardiovascular risk factors.

WHAT THIS STUDY ADDS?

Our study is the first to investigate the association between cIMT and thyroid autoimmunity in euthyroid children with HT. The importance of the current study is that although childhood is accepted as an insidious period for atherosclerosis, we found that the euthyroid girls with HT have increased cIMT.

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Introduction

As the most common organ-specific autoimmune disorder, Hashimoto's thyroiditis (HT) is characterized by infiltration of the thyroid gland by inflammatory cells and production of autoantibodies to thyroid-specific antigens such as thyroglobulin (Tg) and thyroid peroxidase (TPO) (1). HT is associated with various degrees of thyroid dysfunction, and hypothyroidism is a well-known cardiometabolic risk factor (2). However, the influence of thyroid autoimmunity (TA) on the cardiovascular system (CVS) in the absence of overt thyroid dysfunction is still unclear.

Although atherosclerosis manifests clinically in adulthood, in recent years, it has been accepted that the disorder has a long insidious course and has its onset in childhood (3). The classical risk factors of cardiovascular disease (CVD) are accepted to be positive family history of early CVD, hypertension, obesity, hyperinsulinemia, and dyslipidemia (2).

Recently, evidence has been put forward indicating that chronic inflammation is an important pathogenic feature in atherosclerotic lesion formation. Cellular and humoral inflammatory responses are involved in the initiation and progression of atherosclerotic lesions (4). There are various inflammatory markers that have been shown to predict cardiovascular events. The high-sensitivity C-reactive protein (hs-CRP), main marker of inflammation, recently emerged as a major cardiovascular risk factor (5). High serum homocysteine concentration is also a new risk factor for atherosclerosis. The atherogenic effect of homocysteine is related to cytotoxin action on the endothelial cells and their function (6). Because inflammation causes impaired endothelium-dependent vasodilation, endothelial dysfunction (ED) could be a mechanism underlying the atherosclerosis (7). Since ED occurs early in the development of atherosclerosis, demonstration of ED could possibly lead to an early diagnosis of cardiac pathology (8).

Measurement of the carotid intima-media thickness (cIMT) of the common carotid artery is a non-invasive and effective procedure for evaluation of subclinical atherosclerosis. Increased cIMT is an indicator of early structural atherosclerosis and a strong predictor of future cardiovascular morbidity (9).

There are a number of reports in the literature that have shown the association between increased cIMT and overt or subclinical hypothyroidism in adults (10). There have also been reports regarding the impact of TA on the CVS. Ciccone et al (11) reported that autoimmunity has been associated with an increase in carotid atherosclerosis in obese women independent of thyroid function, obesity, and cardiovascular risk factors.

To the best of our knowledge, there are no data about the effects of TA on atherosclerosis among euthyroid children and adolescents with HT. In the present study, we aimed to evaluate whether TA is associated with carotid atherosclerosis and other cardiovascular risk markers in euthyroid pubertal girls with HT.

Methods

At the outpatient clinic of the Department of Pediatric Endocrinology of the Sakarya University Faculty of Medicine, 66 euthyroid, newly diagnosed pubertal girls with HT who were positive for TPO and/or Tg antibodies and who mostly had parenchymal heterogeneity according to thyroid ultrasound (US) were included to the study. Mean age was 14.7±2.4 (range 10-18) years. The control group consisted of 41 age- and body mass index (BMI)-matched healthy pubertal girls with negative serum thyroid autoantibodies and normal thyroid function. The girls in the control group had come to the hospital to get a report of good health. Samples were drawn between January 2015 and March 2015. For all participants, the inclusion criteria consisted of having normal serum free thyroxine (fT_4) concentrations (normal range [NR]: 10.3-24.4 pmol/L) and thyroid-stimulating hormone (TSH) levels (NR: 0.3-5 μU/mL). Thyroid antibodies were considered to be positive if anti-TPO antibodies were greater than 35 IU/mL and anti-Tg antibodies were greater than 40 IU/mL, as indicated by the testing kit. Subjects with thyroid dysfunction, a history of cardiovascular or muscle disease, documented diabetes mellitus, severe dyslipidemia, any chronic or autoimmune disease other than HT, or subjects who had been on L-thyroxin treatment or any medication with a possible effect on body weight and lipid levels were excluded. All patients and controls had serum fasting glucose levels <110 mg/dL.

The study was approved by the local ethics committee, and all participants and their families provided written informed consent.

At enrollment, all subjects underwent a physical examination including blood pressure, standing height, weight, waist, and hip circumference (HC) measurements. Blood pressure was measured using an automated sphygmomanometer after the subjects had rested for nearly 10 minutes. Height was measured without shoes using a Seca 264 wireless stadiometer (UK). Weight was measured to the nearest 0.1 kg on a standard electronic scale with the subject wearing only underwear and no shoes. BMI was calculated as body weight (kg) divided by square of height (m^2). With the patient in standing position, waist circumference (WC) was measured at the level of the umbilicus and HC was measured at the widest part of the gluteal region. We used population-specific data for calculation of the anthropometric values to define as standard deviation score (SDS) (12,13,14,15).

Lipid profile, hs-CRP, homocysteine, blood glucose, insulin, free triiodothyronine (fT_3), fT_4, TSH, anti-TPO, anti-Tg, thyroid US, and cIMT were estimated in all patients and controls.

The venous blood samples were obtained in the morning by venipuncture after overnight fasting. Plasma glucose was

measured using the glucose-oxidase method. Insulin, serum fT_3, fT_4, TSH, anti-TPO, and anti-Tg concentrations were measured by chemiluminescent microparticle immunoassay (CMIA) method using Abbott Architect, USA kits with Abbott I2000 analyzer. Total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TG) were measured with routine enzymatic methods using Abbott C 16000 analyzer. Hs-CRP (NR: 0-5 ng/mL) was measured with an immunonephelometric assay using a BNII Nephelometer (Siemens Healthcare Diagnostics, Deerfield, IL). Total plasma homocysteine (NR: 5-12 µmol/L) was measured by chemiluminescent immunoassay (IMMULITE 2000 Siemens, Healthcare Diagnostics, Germany). All assays were performed according to manufacturers' instructions.

cIMT was determined by a real-time B-mode ultrasound (Toshiba Aplio 400, Japan) using a linear transducer (7.5-10 MHz). To avoid variations, the cIMT examination was performed by the same radiologist (Y.G.), who was blind to the subjects' characteristics.

Statistical Analysis

Number Cruncher Statistical System (NCSS) 2007&Power Analysis and Sample Size (PASS) 2008 Statistical Software (Utah, USA) were used for statistical analyses. Descriptive statistical methods (mean, standard deviation, median, frequency, ratio, minimum, maximum) as well as Student's t-test for paired group comparison of normally distributed parameters and Mann-Whitney U test for paired group comparison of non-normally distributed parameters for quantitative data were used in the evaluation of the data. In the evaluation of relationships between parameters, Pearson correlation analysis was used for parameters that showed a normal distribution and Spearman's correlation analysis for parameters that showed a non-normal distribution. The parametric data has been shown as mean ± SD and non-parametric data-as median (minimum-maximum).

Risk factors affecting cIMT were determined using the multivariate linear regression analysis. Statistical significance was set at $p<0.01$ and $p<0.05$.

Results

Anthropometric characteristics and thyroid-related values of patients with HT and controls are summarized in Table 1. There were no significant differences between the two groups in terms of anthropometric variables, except for the waist-to-hip ratio (WHR) which was significantly higher in patients with HT. There was no difference between the patients and the control group in thyroid hormone levels.

Cardiovascular risk factors and cIMT are summarized in Table 2. Systolic and diastolic blood pressures were not different between the two groups ($p>0.05$). Serum fasting glucose level was significantly higher in the patient group ($p<0.01$). Insulin, glucose-insulin ratio, and homeostatic model assessment-insulin resistance (HOMA-IR) were not different between the groups ($p>0.05$). Serum hs-CRP levels were significantly higher in patients than in controls ($p<0.01$). Serum homocysteine levels were similar between the two groups ($p>0.05$). Patients were also characterized by significantly higher TC and LDL-C values than the controls ($p<0.01$). As seen in Figure 1, cIMT was increased significantly in the patient group as compared to the control group ($p<0.01$). None of the patients and controls had atherosclerotic plaques.

cIMT was positively correlated with body weight SDS ($r=0.39$; $p<0.001$), WC SDS ($r=0.41$; $p<0.001$), BMI SDS ($r=045$; $p<0.001$), WHR ($r=0.37$; $p<0.003$), and hs-CRP ($r=0.39$; $p<0.003$) in the patient group. There were no correlations between cIMT and thyroid-related markers in either group.

The multiple regression analysis has shown that only hs-CRP and BMI SDS were the independent variables for cIMT in girls with HT (Table 3).

Discussion

It is well established that hypothyroidism is associated with a higher risk for CVD. cIMT measurement is an effective and non-invasive procedure for evaluation of cardiovascular risk. Increased cIMT has been reported in patients with overt or subclinical hypothyroidism compared to healthy controls (16). HT is the most common cause of hypothyroidism, but it is not clear if TA is a risk factor for atherosclerosis independent of thyroid function. The pathogenic mechanisms underlying the increased cIMT in euthyroid patients with HT have not been studied extensively. This study has shown that euthyroid pubertal girls with HT, regardless of thyroid function, have significantly increased cIMT compared to healthy controls.

It is well known that hypercholesterolemia is one of the major risk factors of atherosclerosis and thus of increased cIMT (17). There is a close association between thyroid

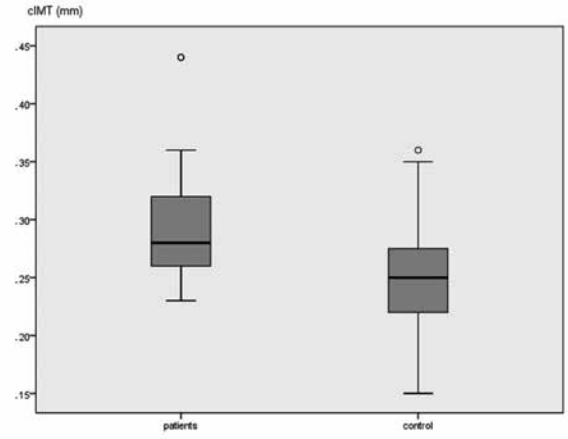

Figure 1. Mean carotid intima-media thickness values of patients and controls. cIMT: carotid intima-media thickness

function and dyslipidemia in overt hypothyroidism (18). Autopsy findings in children and adolescents with hypothyroidism have demonstrated an increase in atherosclerotic lesions in the coronary artery with increasing TC and LDL-C and decreasing HDL-C (19). Because of diminished number of LDL-C receptors in the liver, the fractional excretion of LDL-C is reduced in hypothyroidism (20). In our study, even though fasting TC and LDL-C levels were within normal limits, they were higher in the patients than in the controls. Although elevated TSH is suggested as a major factor in the dyslipidemia (21), in the present study, there was no difference in mean TSH levels between patients and controls. The reason for the differences between lipid values was not clear.

Increased cIMT has been reported in children with diabetes, hypertension, and childhood obesity (22). The common pathogenetic factor involved in endothelial damage in obese and diabetic children and adolescents seems to be reduced insulin function. Insulin acts by modulating the release of vasodilator substances (i.e. nitric oxide and prostaglandins) from the vascular endothelium (10). In the current study, fasting serum glucose levels were elevated in the patients compared to the controls, but serum fasting insulin, glucose-insulin ratio, and HOMA-IR were not different between the groups. Also, no relationship was found between cIMT and HOMA-IR.

The harmful effects of elevated glycemia and mild dyslipidemia on the vasculature, affecting endothelial function are well documented (22). However, insulin function and fasting TC and LDL-C levels were within normal limits in our patients. Therefore, these harmful effects on the vasculature might not be significant.

On the other hand, individuals who suffer from systemic autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus also have a greater and earlier incidence of atherosclerotic CVD (23). Therefore, as an autoimmune disease, HT itself may be responsible for autoimmune, inflammation-based ED. Taddei et al (24) demonstrated in adult

Table 1. Anthropometric characteristics, thyroid antibody levels, and results of thyroid function tests and thyroid ultrasonography in the patients and in controls

		Patients	**Controls**	**p**
		Mean ± SD/Median (minimum-maximum)	**Mean ± SD/Median (minimum-maximum)**	
Age (years)		14.6±2.7	14.7±1.9	0.887
Height (cm)		154.9±9.8	157.7±8.0	0.136
Height SDS		-0.4 (-4.8-2.5)	-0.3 (-3.4-2.8)	0.647
Weight (kg)		56.4±17.7	56.5±15.0	0.979
Weight SDS		0.07 (-3.6-3.8)	0.04 (-3.5-3.9)	0.793
BMI (kg/m^2)		23.2±6.0	22.6±5.4	0.632
BMI SDS		0.73 (-3.7-3.8)	0.59 (-3.7-3.7)	0.794
WC (cm)		73.6±14	72.5±11.3	0.655
WC SDS		0.91 (-2.9-3.2)	0.83 (-2.7-2.9)	0.782
HC (cm)		94.0±14.4	96.8±11.4	0.297
WHR		0.78±0.0	0.75±0.0	**0.014***
fT_3 (pmol/L)		4.9±0.8	4.6±0.7	0.680
fT_4 (pmol/L)		14.3±4.9	12.9±3.1	0.645
TSH (μU/mL)		2.4±2.2	2.2±1.1	0.850
		n (%)	**n (%)**	
Anti-TPO (IU/mL)	(-)	15 (22.7)	41 (100.0)	**0.001****
	(+)	51 (77.3)	0 (0.0)	
Anti-Tg (IU/mL)	(-)	6 (9.1)	41 (100.0)	**0.001****
	(+)	60 (90.9)	0 (0.0)	
Thyroid US	Normal	8 (18.2)	33 (89.2)	**0.001****
	Heterogeneous	36 (81.8)	4 (10.8)	

BMI: body mass index, SDS: standard deviation score, WC: waist circumference, HC: hip circumference, WHR: waist to hip ratio, fT_3: free triiodothyronine, fT_4: free thyroxine, TSH: thyroid-stimulating hormone, TPO: thyroid peroxidase, Tg: thyroglobulin, US: ultrasound, SD: standard deviation, *p<0.01, **p<0.001

patients with HT that ED comes only from the autoimmune disease, independent from other cardiovascular risk factors. In another study from Turkey, it was demonstrated that TA may have some effect on hyperlipidemia, obesity, and abdominal obesity independent of thyroid function (25). Topaloglu et al (26) have found that euthyroid, premenopausal women with HT have increased cIMT independent of the thyroid function tests. In the Rotterdam study, a greater incidence of atherosclerosis was observed in anti-TPO-positive hypothyroid patients (27). These observations suggest an atherogenic role of thyroid antibodies. A mechanism explaining this link may be a state of chronic inflammation in thyroid antibody-positive patients, which causes ED, ultimately resulting in atherosclerosis (28).

According to Volpe's (29) hypothesis, in HT, helper T cells are not suppressed because of defective suppressor T cells, and thus are able to produce a lot of cytokines such as interferon gamma, interleukin-2, and tumor necrosis factor alpha. Sultan et al (30) suggested that these cytokines might cause weight gain and hyperlipidemia. Therefore, it is likely that the higher serum levels in glucose and lipids are at least in part explained by TA in these patients.

Factors involved in inflammatory processes may be important determinants of increased cIMT, including hs-CRP and homocysteine (26). The predictive association between CRP and coronary artery disease has been extensively confirmed (31). Kaptoge et al (32) in a meta-analysis of 160,309 patients without antecedents of CVD found that hs-CRP concentration shows a continuous association with the risk of coronary disease and cardiovascular mortality. In the present study also, hs-CRP levels were higher in patients than in controls and there was a positive correlation between hs-CRP and cIMT in the patient group. CRP may contribute to inflammation in atheroma and also may be actively involved in early atherogenesis. It has been shown that native CRP deposition displays calcium-dependent *in vitro* binding to LDL in the arterial wall and induces classical pathway of

Table 2. Differences in the variables analyzed in the patient and control groups

Variables	Patient group (n=66)	Control group (n=41)	p
	Mean ± SD/Median (minimum-maximum)	Mean ± SD/Median (minimum-maximum)	
Systolic blood pressure (mmHg)	110.0 (80-125)	105.0 (82-115)	0.461
Diastolic blood pressure (mmHg)	70 (55-92)	68 (54-90)	0.354
cIMT (mm)	0.28 (0.23-0.44)	0.25 (0.15-0.36)	**0.001***
Glucose (mg/dL)	96.4±8.4	90.8±10.0	**0.004***
Insulin (µU/mL)	9.6 (1.8-45.0)	11.7 (4.5-36.7)	0.479
Glucose/Insulin	9.5 (1.7-33.9)	8.7 (2.62-22.5)	0.794
HOMA-IR	2.8±2.2	2.8±1.5	0.287
Inflammation markers			
Hs-CRP (ng/mL)	4.5±6.7	2.9±2.3	**0.001***
Homocysteine (µmol/L)	11.5±7.7	11.4±3.5	0.398
Lipids			
TG (mg/dL)	81 (39-139)	81.5 (41-144)	0.355
TC (mg/dL)	166.4±27.0	150.9±22.2	**0.002***
LDL-C (mg/dL)	95.8±24.4	82.6±20.7	**0.004***
HDL-C (mg/dL)	48.3±9.6	49.2±12.1	0.687

cIMT: carotid intima-media thickness, HOMA-IR: homeostatic model assessment-insulin resistance, Hs-CRP: high-sensitivity C-reactive protein, TG: triglycerides, TC: total cholesterol, LDL-C: low-density lipoprotein-cholesterol HDL-C: high-density lipoprotein-cholesterol, SD: standard deviation, *p<0.01

Table 3. The multiple regression analysis coefficients between carotid intima-media thickness and the other parameters in the patient group

Patient group	β coefficient	95% CI	t	p
(Constant)		0.203±0.452	5.305	**0.001****
Fasting glucose	-0.095	-0.002±0.001	-0.743	0.461
Hs-CRP	0.268	0.000±0.003	2.100	**0.041***
BMI-SDS	0.388	0.003±0.017	3.064	**0.004****

CI: confidence interval, Hs-CRP: high-sensitivity C-reactive protein, BMI: body mass index, SDS: standard deviation score, *p<0.05, **p<0.001

complement activation, and that stimulation of monocyte chemotaxis and inhibition of neutrophil chemotaxis may be important inflammatory mechanisms (31).

Several studies have demonstrated hyperhomocystinemia in overt hypothyroidism, with serum fT_4 being an independent determinant of homocysteine concentrations (6). In the present study, there was no significant difference between the homocysteine levels of the two groups. This might be due to the fact that both groups consisted of euthyroid girls.

Kollias et al (33) examined the association between cIMT and several cardiovascular risk factors in 448 apparently healthy adolescents, and central obesity and systolic blood pressure appeared to be independently associated with cIMT. While BMI remains the most commonly used obesity measure, its main limitation compared to WC and WHR is that it does not take into account body fat distribution (34). Findings of previous studies comparing the ability of BMI and measures of abdominal obesity to identify cardiometabolic risk factors are conflicting. WC has been found to be related to cardiometabolic risk factors independent of BMI in some studies (35), while other studies have found that WC and WHR are not better than BMI for the identification of cardiometabolic risk (36). Previous studies done among Swedish and Turkish children showed that BMI was a better predictor of WHR and of skinfolds (37,38). In our study, the patients had higher WHR, which points to central obesity. The cIMT was significantly correlated with WC-SDS, BMI-SDS, and WHR, but among them, only BMI-SDS was the independent variable for the cIMT in girls with HT.

Our study is the first to investigate the association between cIMT and TA in euthyroid children with HT. The importance of the current study is that although childhood is accepted as an insidious period for atherosclerosis, we found that the girls with HT have increased cIMT.

In conclusion, in girls with HT, TA may be responsible for chronic inflammation, which may cause ED, a promoter of atherosclerosis. cIMT is a good tool for the early detection and the monitoring of early atherosclerosis in euthyroid patients with HT. Hs-CRP level and BMI can be early leading indicators for cardiovascular risk in young girls with HT. The combination of these factors with biochemical markers like hypercholesterolemia and hyperglycemia may play an important role in the prediction of cardiovascular risk in young girls with euthyroid HT. Early detection of risk factors of CVD may be helpful for planning treatment and interventions, so as to prevent complications from the disease in adulthood. Finally, further longitudinal studies are needed to help establish the link between accelerated atherosclerosis and autoimmunity during childhood.

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Ethics

Ethics Committee Approval: The study was approved by the local ethics committee December 2014, Informed Consent: Informed consent was obtained.

Peer-review: External peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Pınar İşgüven, Yasemin Gündüz, Concept: Pınar İşgüven, Design: Pınar İşgüven, Data Collection or Processing: Pınar İşgüven, Mukaddes Kılıç, Analysis or Interpretation: Pınar İşgüven, Yasemin Gündüz, Literature Search: Pınar İşgüven, Writing: Pınar İşgüven.

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Original Article

Thyroid Function and Thyroid Autoimmunity in Relation to Weight Status and Cardiovascular Risk Factors in Children and Adolescents: A Population-Based Study

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ABSTRACT

Objective: In obese subjects, slight increases have been observed in thyrotropin [thyroid-stimulating hormone (TSH)] levels, but data in children are scarce. The aim of this study was to evaluate whether thyroid function and autoimmunity vary with weight status in a healthy population of children and adolescents and to determine whether hyperthyrotropinemia is associated with any cardiovascular risk factor.

Methods: This cross-sectional epidemiological study was conducted in Almería (Spain) on a representative sample of 1317 healthy subjects aged 2-16 years. Thyroid function, thyroid autoimmunity and cardiovascular risk factors were measured. Chi-square test, analysis of variance and multiple linear regression were used in the statistical analyses.

Results: The obese children and adolescents had thyrotropin levels (mean ± standard deviation) of 3.12±2.44 mU/L. These levels were higher than those of overweight subjects (2.79±1.51 mU/L) and of normal weight subjects (2.73±1.30 mU/L) (p=0.02). Levels of free thyroxine and urinary iodine did not differ significantly between the groups. The prevalence (95% confidence interval) of thyroid autoimmunity was lower in the individuals with normal weight (2.9%; 2.0-4.2) than in the overweight (6.3%; 3.9-9.9) and obese subjects (5.6%, 2.5-11.3) (p=0.02). TSH levels were associated with obesity (β=0.36; p<0.001) and thyroid autoimmunity (β=1.10; p<0.001). They were not associated with any cardiovascular risk factor.

Conclusion: Obese children and adolescents had higher levels of thyrotropin than those who were overweight and of normal weight. The differences among the groups were of very little clinical significance and could possibly be linked to the higher prevalence of thyroid autoimmunity in obese subjects. The hyperthyrotropinemia in these subjects was not associated with any cardiovascular risk factor.

Keywords: Thyrotropin, hyperthyrotropinemia, obesity, overweight, child

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

In obese subjects, slight increases have been observed in thyrotropin, or thyroid-stimulating hormone levels.

WHAT THIS STUDY ADDS?

Populational studies about this topic have been conducted only in adults. To the best of our knowledge, no previous populational study in this field has been conducted in pediatrics.

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This study was presented in the 53rd ESPE Annual Meeting 2014.

Introduction

The mechanisms that interrelate thyroid function and body weight status are still not completely understood. Hyperthyroid patients lose a lot of weight and regain it when treated. However, in replacement therapy for hypothyroidism, the weight loss is very modest and due more to water deficit than to changes in body fat. Moreover, treatment of obese euthyroid patients with levothyroxine does not induce weight changes (1,2,3,4,5).

In obese subjects, slight increases have been observed in thyrotropin, or thyroid-stimulating hormone (TSH), but these are far from being the cause of overweight and could rather be a consequence of it (1,2,3,4,5). Although the origin of this increase in TSH in obese subjects is not fully understood and may be multifactorial, rather than being an indicator of subclinical hypothyroidism or of thyroid hormone resistance, it is currently considered to be an adaptive change, an attempt to increase basal metabolism in order to avoid further weight gain. This conclusion has been derived from longitudinal studies of children, in which those who lost weight by modifying their lifestyles were observed to decrease their levels of TSH (4,6,7,8,9), while those treated with levothyroxine to normalize TSH did not lose weight (4,8). This adaptive hyperthyrotropinemia is accompanied by a slight increase in free triiodothyronine (T_3) and by absence of antithyroid antibodies (4,10,11).

Increases in TSH and T_3 in obese subjects could be mediated in part by leptin (12). This hormone, produced by adipose tissue, promotes the synthesis of thyrotropin-releasing hormone in the hypothalamic paraventricular nucleus and the conversion of thyroxine (T_4) to T_3 in the peripheral tissues (2,4,13), which in turn is the direct result of the action of TSH (14). A low-grade inflammatory reaction mediated by interleukins and other adipokines may also be involved in these changes. In obese subjects, an ultrasound thyroid pattern of chronic low-grade inflammation (irregular hypoechoic areas) unaccompanied by any signs of autoimmunity and termed as "non-autoimmune thyroiditis from obesity" has been reported (1,2,3,5,15).

The aims of this study were to examine whether thyroid function and thyroid autoimmunity vary with weight status in a healthy population of children and adolescents and to determine whether hyperthyrotropinemia in obesity is associated with any cardiovascular risk factor.

Methods

This observational cross-sectional epidemiological study was conducted on a population of children aged 2-16 years living in Almeria (southern Spain). A representative sample of 1317 children and adolescents was analyzed. The selection criteria were described in a previous study (16).

The study was carried out in accordance with the guidelines of the 1975 Declaration of Helsinki and was approved by the Research and Ethics Committees of Torrecárdenas Hospital. Written informed consent of the parents or tutors and of the individuals themselves (if they were older than 12) has been obtained after full explanation of the purpose and nature of all procedures used.

Anthropometric measurements were obtained and a physical examination was carried out in all subjects. Wearing light clothing and no shoes, body weight was recorded as the mean of two determinations, using a digital *Seca 861* scale with an accuracy of 100 g. Height was recorded as the average of two measurements, measured to the nearest millimeter using a height rod attached to the weighing scale, with the child standing upright. Body mass index (BMI) was calculated. Signs of pubertal onset were determined (testes of at least 4 mL in the males and breast buds in the females), as was the presence of goiter. Waist circumference and systolic and diastolic blood pressure were also measured using calibrated equipment and following standard methods. The examinations were made by six physicians who had previously completed a training and standardisation programme. Obesity, overweight, and normal weight were defined according to thresholds proposed for childhood and adolescence by the International Obesity Task Force (17). The definition for excess weight encompassed the first two of these categories.

Under fasting conditions, blood samples were obtained for determination of glucose, insulin levels, and of the lipid profile [triglycerides, total LDL (low-density lipoprotein), HDL (high-density lipoprotein), and cholesterol], in addition to thyroid hormone and thyroid antibody levels. A urine sample was also collected. Serum concentrations of free T_4 (normal range 0.9-1.7 ng/dL), TSH (normal range 0.2-4.2 mU/L), antiperoxidase antibodies (normal less than 34 U/mL), and thyroglobulin antibodies (normal values <115 U/mL) were analyzed by chemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland). Urinary iodine was determined by the Benotti method (18). A positive result for any antibody was considered to indicate thyroid autoimmunity.

Statistical analysis was performed using SPSS 17.0, and the sample size was determined using Epidat 3.0. The qualitative variables are expressed as percentages with a 95% confidence interval, and the quantitative variables as the mean (standard deviation). Among other statistical tests, chi-square, analysis of variance, and multiple linear regression analyses were performed. In all cases, statistical significance was taken as $p<0.05$.

Results

The study population was composed of 1317 children and adolescents with a mean age of 8.8 (4.3) years. 48.8% were female and 38.4% pubescent. The following age groups were

established: 402 subjects aged 2-6 years, 504 aged 6-12, and 411 aged 12-16 years. Of the 333 individuals invited to take part in the study, 20.2% refused. The rejection rate was higher among the youngest (26.9%) and the oldest (25.3%) age groups than in the intermediate one (8.4%), but did not differ by gender, ethnicity, or geographic area.

In the study group, 1.2% of the children and adolescents presented with excess weight, 9.8% being obese and 21.4% overweight. The prevalence of thyroid autoimmunity in this population was 3.7% (Table 1).

Comparison of the three subgroups defined in terms of weight status (obesity, overweight, and normal weight) showed that the obese children and adolescents presented TSH levels (3.12±2.44 mU/L) higher than those of the subjects with overweight (2.79±1.51 mU/L) and also higher than those of the subjects with normal weight (2.73±1.30 mU/L) (p=0.02). Free T_4 concentrations were not significantly different among the three subgroups (Table 2). There were also no differences between genders, Tanner stage groups, or age groups (data not shown).

The prevalence of thyroid autoimmunity was significantly lower among subjects with normal weight (2.9%) than in the overweight (6.3%) and obese subjects (5.6%) (p=0.02) (Table 2).

TSH levels showed a correlation with obesity after correction for age, Tanner's stage, gender, ethnic group, iodine intake, and thyroid autoimmunity. However, the magnitude of the association (linear regression coefficient) was quite low after adjusting for presence of thyroid autoimmunity (Table 3).

Table 1. Characteristics of the study sample. The qualitative variables are expressed as percentages (%) with a 95% confidence interval, and the quantitative variables as means ± standard deviation

Females (%)	48.8 (46.1-51.5)
Age (years)	8.80 (4.3)
Pubescents (%)	38.8 (36.2-41.5)
Obesity (%)	9.8 (8.5-11.2)
Overweight (%)	21.4 (19.2-23.7)
Blood thyrotropin level (mU/L)	2.79 (1.48)
Blood free thyroxine level (ng/dL)	1.32 (0.22)
Thyroid autoimmunity (%)	3.7 (2.8-5.0)
Urinary iodine level (µg/L)	209.0 (101.2)

Among the excess-weight children and adolescents with hyperthyrotropinemia, none of the clinical and laboratory variables studied (including major cardiovascular risk factors) were significantly different from those of the excess-weight subjects with normal TSH concentrations. After correction for the rest of the variables including weight status, thyrotropin was not associated with any of the cardiovascular risk factors (Table 4).

Discussion

In our study population, the obese children and adolescents had slightly higher levels of thyrotropin, a finding which is in accord with most recent publications (3,6,10). However, population-based studies with relatively large numbers of subjects have been conducted only in adults. Thus, one study carried out in the United States analyzed 3114 subjects, relating BMI and waist circumference with elevated TSH levels (19), while in a Norwegian publication conducted on 1500 individuals, weight gain was associated over time with this elevation (20). However, these findings are not unanimous: a population-based study on adults in our own region and also other studies reported absence of any relationship between thyrotropin and BMI (21,22,23). Increased BMI has also been associated with slightly elevated free T_3 (10,19,22,23) and with a slight decrease in free T_4 (23,24).

To the best of our knowledge, no previous population-based study in this field has been conducted in pediatric age groups. Reports have been published of samples of obese children and adolescents, most of them from outpatient clinics, presenting a positive association between BMI and TSH (3,6, 25,26,27,28,29,30,31,32,33), a positive association with free T_3 (3,6,7,14,26,27), and a negative one between free T_4 and waist circumference (26) and between free T_4 and visceral fat assessed by ultrasound (34). In accordance with our own findings, none of these studies have reported any significant difference by gender or pubertal status (3).

According to these earlier studies, differences in TSH values between obese and normal weight individuals range from 0.2 to 0.8 mU/L, among both adults and children (3,7,10). Given the fact that current clinical practice guidelines recommend treatment when thyrotropin levels exceed 10 mU/L, the reported increased concentrations are not clinically significant (35).

Table 2. Comparison of variables in three subgroups of children and adolescents by weight status. The qualitative variables are expressed as percentages (%) with a 95% confidence interval, and the quantitative variables as means ± standard deviation

Variable	Obese	Overweight	Normal weight	p-value
Thyrotropin (mU/L)	3.12 (2.44)	2.79 (1.51)	2.73 (1.30)	0.02
Free thyroxine (ng/dL)	1.27 (0.15)	1.31 (0.30)	1.33 (0.20)	0.52
Thyrotropin >4.2 mU/L	14.4 (9.2-21.7)	11.5 (8.1-11.8)	10.4 (8.5-12.6)	0.38
Thyroid autoimmunity	5.6 (2.5-11.3)	6.3 (3.9-9.9)	2.9 (1.9-4.2)	0.02
Urinary iodine (µg/L)	194.2 (89.1)	202.0 (99.2)	213.4 (104.8)	0.14

Whether the differences in TSH concentrations are due to a poor iodine status or to a higher frequency of autoimmunity associated with excess weight remains to be determined. No relationship between iodine intake and weight status has been found in this present study nor has it been reported in any previous studies (30). However, the prevalence of thyroid autoimmunity is known to increase in obese children and adolescents (30) and in obese adults (13,21,36). In accordance with other authors, we believe that autoimmunity might be the main factor responsible for the increased concentration of TSH in obese subjects, as the correlation between thyrotropin and BMI is weaker when correcting for it (21). However, other studies on children and adolescents, an age range in which thyroid autoimmunity is much less frequent than in adults, have reported that only a small proportion (<10%) of obese subjects present elevated TSH levels (27,28).

In our opinion, whether or not increased concentrations of thyrotropin in obese subjects without autoimmunity are statistically significant, their clinical significance is negligible. Most previous studies corroborate our findings, in that no cardiovascular risk factor is aggravated, nor is there any increase in the index of insulin resistance in children with hyperthyrotropinaemia (2,7,27,37). This leads us to consider the condition a physiological one and to discard the possibility of using levothyroxine to lower TSH levels in overweight or obese children and adolescents in order to reduce associated comorbidities. On the other hand, there are studies which report a significant association between thyrotropin and metabolic syndrome (38,39), carbohydrate intolerance (24), elevated total and LDL cholesterol (21,40), and elevated triglycerides (24,29,33,40) as well as between low levels of free T_4 and increased insulin concentration (34).

In our study population, the obese group of children and adolescents had slightly higher TSH levels than the overweight and normal weight subjects, but these differences, although statistically significant, were of very little or no clinical significance. The increased concentration of TSH is also not associated with any cardiovascular risk factor. This study has also shown that the prevalence of thyroid autoimmunity is higher in children and adolescents with excess weight. The hyperthyrotropinaemia associated with obesity could possibly be linked to a state of autoimmunity.

Table 3. Multiple linear regression model showing the relationship between thyrotropin (response variable) and predictor variables

Predictor variable	Coefficient	Standard error	p-value
Constant	3.11	0.09	<0.001
Thyroid autoimmunity	1.10	0.21	<0.001
Age	-0.05	0.01	<0.001
Obesity	0.36	0.14	0.009

Table 4. Multiple linear regression models showing the relationship between any cardiovascular risk factor (response variable) and thyrotropin and obesity as predictor variables

Response variable: triglycerides			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	61.13	2.66	<0.001
Thyrotropin	0.27	0.51	0.60
Obesity	16.45	0.59	<0.001
Response variable: high-density lipoprotein cholesterol			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	51.20	1.27	<0.001
Thyrotropin	0.31	0.25	0.20
Obesity	-2.30	1.24	0.20
Response variable: low-density lipoprotein cholesterol			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	95.2	2.04	<0.001
Thyrotropin	0.69	0.39	0.18
Obesity	0.13	1.98	0.94
Response variable: glucose			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	70.86	0.74	<0.001
Thyrotropin	0.31	0.25	0.20
Obesity	0.29	0.15	0.09
Response variable: insulin			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	1.51	0.70	0.03
Thyrotropin	0.01	0.14	0.92
Obesity	3.79	0.68	<0.001
Response variable: systolic blood pressure			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	89.8	1.16	<0.001
Thyrotropin	0.22	0.22	0.32
Obesity	6.62	1.14	<0.001
Response variable: diastolic blood pressure			
Predictor variable	**Coefficient**	**Standard error**	**p-value**
Constant	57.6	0.88	<0.001
Thyrotropin	0.16	0.16	0.32
Obesity	3.42	0.86	<0.001

Ethics

Ethics Committee Approval: Research and Ethics Committees of Torrecárdenas Hospital, Almeria, Spain, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Emilio García-García, María A. Vázquez-López, Design: Emilio García-García, María A. Vázquez-López, Data Collection and/or Processing: Eduardo García-Fuentes, Rafael Galera-Martínez, Carolina Gutierrez-Repiso, Icíar García-Escobar, Analysis and/or Interpretation: Emilio García-García, María A. Vázquez-López, Antonio Bonillo-Perales, Literature Research: Emilio García-García, Writing: Emilio García-García.

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Original Article

Bone Mineral Density in Adolescent Girls with Hypogonadotropic and Hypergonadotropic Hypogonadism

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ABSTRACT

Objective: Deficiency of sex steroids has a negative impact on bone mineral content. In studies conducted on postmenopausal women and animal studies, elevated follicle-stimulating hormone (FSH) levels were found to be correlated with a decrease in bone mineralization and osteoporosis. The aim of the present study was to evaluate bone mineral density (BMD) in adolescent girls with hypogonadotropic and hypergonadotropic hypogonadism and also to investigate the correlation between FSH level and BMD.

Methods: The study group included 33 adolescent girls with hypogonadism (14 with hypogonadotropic hypogonadism and 19 with hypergonadotropic hypogonadism). FSH, luteinizing hormone, estradiol levels, and BMD (using dual energy x-ray absorptiometry) were measured.

Results: There were no statistically significant differences between the chronological age and bone age of the two patient groups, namely, with hypogonadotropic and hypergonadotropic hypogonadism. There was also no significant difference between BMD z-score values obtained from measurements from the spine and the femur neck of patients in the two groups (p-values were 0.841 and 0.281, respectively). In the hypergonadotropic group, a moderately negative correlation was detected between FSH level and BMD z-score measured from the femur neck (ρ=-0.69, p=0.001), whilst no correlation was observed between FSH levels and height adjusted BMD-z scores measured from the spine (ρ=0.17, p=0.493). FSH level was not found to be an independent variable affecting BMD z-score.

Conclusion: BMD z-scores were detected to be similar in adolescent girls with hypogonadotropic and hypergonadotropic hypogonadism, and FSH levels were not found to have a clinically relevant impact on BMD.

Keywords: Hypogonadism, adolescent, follicle-stimulating hormone, osteoporosis

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Role of estrogen deficiency and elevated follicle-stimulating hormone (FSH) on bone health have been studied in postmenopausal females and in experimental studies. However, there is a lack of studies conducted on bone mineral density (BMD) in adolescent hypogonadal girls.

WHAT THIS STUDY ADDS?

The present study evaluates the BMD in girls with hypergonadotropic and hypogonadotropic hypogonadism and assesses the role of FSH in the development of osteopenia/osteoporosis.

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Introduction

Estrogen has a positive impact on bone mineralization and its deficiency plays a key role in the development of osteoporosis (1). Bone mineral density (BMD) of women with primary ovarian failure and that of adolescents with hypogonadism has been reported to be low as compared to their healthy counterparts (2).

In gonadectomized mice, estrogen replacement has been shown to increase trabecular BMD (3). Furthermore, in mice model including intact controls, hypophysectomized (HX), ovariectomized (OV), and HX+OV mice, ovariectomy was found to be related with profound bone loss, while hypophysectomy blunted this bone loss due to ovariectomy (4). In addition, Sun et al (5) have shown that elevated follicle-stimulating hormone (FSH) levels enhance osteoclastic bone resorption and lead to hypogonadal bone loss. Likewise, FSH was shown to increase bone loss in OV mice by increasing tumor necrosis factor-alpha (TNF-α) and despite having a severe estrogen deficiency, mice deficient in the beta-subunit of FSH (FSH beta) had lower TNF-α and were thereby protected against bone loss (5,6).

A negative correlation has been reported between FSH and BMD in healthy women (7). On the contrary, some studies showed that BMD was correlated with race and body mass index (BMI) rather than FSH and luteinizing hormone (LH) levels (7,8,9).

In children, bone mass increases with age and reaches 90% of the maximum adult bone mass during the adolescent period (10). BMD reaches peak values in the axial skeleton by age 20 in women, while bone mass in the appendicular skeleton reaches its peak values by ages 17-35 years (10). Sex steroids play a substantial role in the increase of BMD and up to 60% of osteoporosis in adult life may be due to the defect in bone mineralization during early adulthood. In female subjects, estrogen deficiency during puberty causes inadequate bone mineralization with increased risk of osteoporosis (11). In studies conducted on postmenopausal women and in animal studies, elevated FSH has been found to be related with a decrease in bone mineralization and development of osteoporosis. However, to the best of our knowledge, the impact of elevated FSH and LH on BMD has not been evaluated in adolescents. The aim of the present study was to evaluate BMD in adolescent girls with hypogonadotropic hypogonadism and hypergonadotropic hypogonadism and to investigate the relationship between FSH level and BMD.

Methods

Hypogonadotropic hypogonadism is defined as a low estrogen level with inappropriately normal or low gonadotropin levels (FSH and LH). Hypergonadotropic hypogonadism is defined as inappropriately low estrogen levels in the presence of elevated gonadotropins (FSH and LH) and absence of secondary sexual characteristics. A total of 33 adolescent girls with either hypogonadotropic (n=14) or hypergonadotrophic hypogonadism (n=19) were included in this retrospective study. There was no history of bone fracture in any of the subjects. The group with hypergonadotropic hypogonadism included adolescent females with Turner syndrome (TS) except for one patient with 46,XX gonadal dysgenesis. The cases with hypogonadotropic hypogonadism were being followed with a diagnosis of idiopathic normosmic hypogonadotropic hypogonadism.

The data were collected from hospital files. Anthropometric measurements (height, weight) were performed using standard methods and devices. Bone age was assessed using the Greulich-Pyle method (12). Serum calcium, phosphorus, alkaline phosphatase, parathormone, 25 hydroxy vitamin D, free thyroxine, thyrotropin, FSH, LH, and estradiol levels were measured.

Patients with concomitant diseases that potentially affect bone mineral content such as Cushing syndrome, severe malnutrition, anorexia nervosa, hyperthyroidism, obesity, disorders in calcium metabolism (e.g. vitamin D deficient rickets), or those receiving medication that have a positive or negative impacts on bone mineralization (growth hormone, estrogen replacement, corticosteroid therapy etc.) were excluded.

BMD was measured using dual energy X-ray absorptiometry (DXA) method (Hologic QDR-4500, USA) from the anteroposterior L1-L4 spine and femur neck. Total bone mineral content in g/cm^2 (BMD) and age-adjusted z-scores (BMD z-score) were calculated. Since the hypergonadotropic hypogonadism group included TS patients who were shorter than the hypogonadotropic group, BMD z-scores were adjusted according to height [height adjusted (HA) BMD- z score]. A BMD z-score <-2 standard deviation (SD) was considered as low for age, a low BMD associated with pathological bone fractures was considered as osteoporosis (13,14,15).

Statistical Analysis

Statistical analysis was carried out using IBM Statistical Package for the Social Sciences (SPSS) 21.0 for Windows statistical software (Armonk, New York, USA). Shapiro-Wilk test was used to test the normality of distribution of the data. Ratios were compared using χ^2 test or Fischer's exact test. Means were compared using independent sample t-test in normally distributed data and medians using Mann-Whitney U test for non-normally distributed data. Spearman rank test was performed for correlations. A multivariate linear regression analysis was performed to test the effect of independent variables on the BMD z-scores. Data were expressed as mean ± SD (range). A p-value <0.05 was considered as statistically significant.

The study was approved by the Institutional Board of Diyarbakır Gazi Yaşargil Training and Research Hospital.

Results

The auxological, biochemical, and hormonal characteristics of the patients are shown in Table 1. There was no statistically significant difference between BMD z-scores of patients with hypogonadotropic hypogonadism and hypergonadotropic hypogonadism (Table 1 and Figure 1). A low BMD z-score (<-2 SD) was detected in 10 of the 14 (71.4%) patients with hypogonadotropic hypogonadism and in 17 of the 19 (89.5%) patients with hypergonadotropic hypogonadism (p=0.363). When the BMD z-score was adjusted for height, the frequency of having a BMD z-score <-2 SD was only 3/14 (21.4%) for the hypogonadotropic group and 3/19 (15.8%) for the hypergonadotropic group (p=0.510).

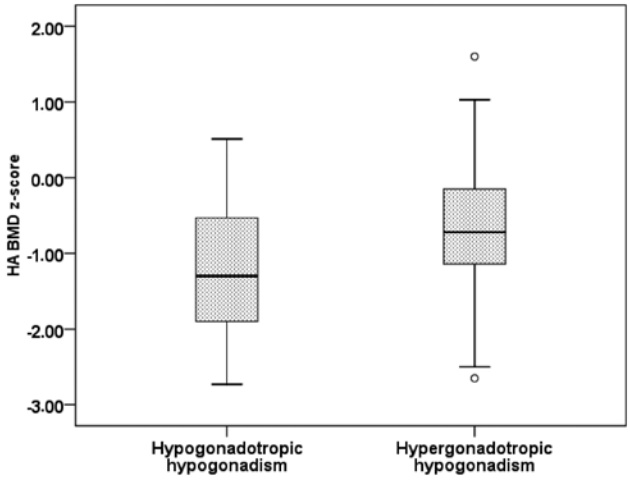

Figure 1. Height adjusted bone mineral density -z scores of patients with hypogonadotropic and hypergonadotropic hypogonadism measured from the spine (L1-L4) were not statistically different. HA BMD: height adjusted bone mineral density

Spearman rank test revealed that chronologic age moderately negatively correlated with HA BMD z-score in the measurements from the spine, while such a correlation was not found in BMD z-score measurements from femur neck. A weak positive correlation was detected between height standard deviation score (SDS) and BMD z-score measured from femur neck. FSH was weakly negatively correlated with BMD measured from femur neck, whereas a weak positive correlation was found between FSH and HA BMD z-score measured from the spine. No correlations were detected between BMI, estradiol, LH values and BMD z-scores measured from either spine or femur neck (Table 2).

When Spearman rank test was performed in patients with hypogonadotropic hypogonadism, a strong negative correlation was detected between chronologic age and HA BMD z-score measured from the spine, whilst no correlation was detected with BMD z-score measured from femur neck. In the hypergonadotropic group, chronologic age demonstrated a weak negative correlation with HA BMD z-score measured from the spine, whilst no correlation was detected with BMD z-score measured from the femur neck. Besides, in the hypergonadotropic group, BMD measured from the femur neck showed a moderate positive correlation with height SDS and a moderate negative correlation with gonadotropin (FSH, LH) levels (Figure 2A). However, there was no correlation between FSH level and HA BMD z-score measured from the spine (Figure 2B and Table 3). In addition, the correlation analysis performed in patients with hypogonadotropic hypogonadism did not show any significant correlation between FSH level and BMD z-score measured neither from spine nor from femur neck (Figure 2C, 2D).

Multivariate linear regression analysis revealed height SDS as an independent factor affecting BMD z-score measured

Table 1. Clinical and laboratory characteristics of patients with hypogonadotropic hypogonadism and hypergonadotropic hypogonadism

	Hypogonadotropic hypogonadism (n=14)	Hypergonadotropic hypogonadism (n=19)	p-value
Chronological age (years)	14.9 ±1.5	14.2±1.3	0.183
Bone age (years)	10.9±1.6	11.1±1.2	0.498
Height SDS	-3.3±1.4	-4.1±1.4	0.035
BMI	17.9±3.6	19.7±3.8	0.155
Serum FSH level (mIU/mL)	1.0±0.9	109.1±51.8	<0.0001
Serum LH level (mIU/mL)	0.2±0.19	21.8±12.1	<0.0001
Serum estradiol level (pg/mL)	4.2±3.1	4.6±2.6	0.447
BMD (spine) (g/cm^2)	0.649±0.11	0.602±0.10	0.155
BMD-z score (spine)	-3.07±1.63	-3.37±1.08	0.841
HA BMD-z score (spine)	-1.21±1.0	0.57±0.5	0.142
BMD (femur neck) (g/cm^2)	0.602±0.10	0.574±0.11	0.259
BMD-z score (femur neck)	-2.39±0.96	-3.13±1.00	0.281

SDS: standard deviation score, BMI: body mass index, FSH: follicle-stimulating hormone, LH: luteinizing hormone, HA BMD: height adjusted bone mineral density

Table 2. Spearman rank test for chronologic age, height standard deviation score, body mass index, follicle-stimulating hormone, luteinizing hormone, and estradiol levels vs. bone mineral density z-score measured from spine and femur neck

	HA BMD z-score (Spine)		BMD z-score (Femur neck)	
	ρ	p	ρ	p
Chronological age	**-0.61**	**0.000**	-0.171	0.342
Height SDS	-0.14	0.438	**0.49**	**0.004**
BMI	0.56	0.758	0.27	0.120
FSH	**0.35**	0.050	**-0.36**	**0.038**
LH	0.32	0.068	-0.26	0.140
Estradiol	0.058	0.747	-0.32	0.070

SDS: standard deviation score, BMI: body mass index, FSH: follicle-stimulating hormone, LH: luteinizing hormone, HA BMD: height adjusted bone mineral density

from spine and femur neck, whereas BMI was found as an independent factor affecting only BMD z-score measured from femur neck (Table 4). FSH was not found as an independent factor affecting the BMD measured neither from spine nor from femur neck.

Discussion

In the present study, a low age-adjusted BMD z-score (<-2 SD) measured from the spine was detected at a high rate in patients with both hypogonadotropic and hypergonadotropic hypogonadism. HA BMD z-score values were found to be about 6 fold better than the age-adjusted values. In the hypergonadotropic group, FSH levels were negatively correlated with BMD z-scores measured from the femur neck. This relationship did not exist in the hypogonadotropic group. Also, in the hypergonadotropic group, FSH levels were not correlated with BMD measured from the spine and these levels were not found to be an independent factor affecting BMD z-score.

Table 3. Spearman rank test for chronologic age, height standard deviation score, body mass index, follicle-stimulating hormone, luteinizing hormone, and estradiol vs. bone mineral density z-score/height adjusted bone mineral density z-score measured from spine and femur neck in hypogonadotropic and hypergonadotropic hypogonadism groups

	Hypogonadotropic hypogonadism (n=14)				Hypergonadotropic hypogonadism (n=14)			
	HA BMD z-score (Spine)		BMD z-score (Femur neck)		HA BMD z-score (Spine)		BMD z-score (Femur neck)	
	ρ	p	ρ	p	ρ	p	ρ	p
Chronological age	**-0.77**	**0.001**	-0.47	0.088	**-0.45**	**0.053**	-0.02	0.930
Height SDS	0.24	0.418	0.13	0.671	-0.18	0.464	**0.67**	**0.002**
BMI	-0.01	0.982	0.50	0.068	-0.04	0.887	0.25	0.306
FSH	0.39	0.172	0.18	0.546	0.17	0.493	**-0.69**	**0.001**
LH	0.39	0.161	0.49	0.074	0.06	0.805	**-0.56**	**0.014**
Estradiol	-0.20	0.482	-0.39	0.162	0.10	0.682	-0.24	0.323

SDS: standard deviation score, BMI: body mass index, FSH: follicle-stimulating hormone, LH: luteinizing hormone, HA BMD: height adjusted bone mineral density

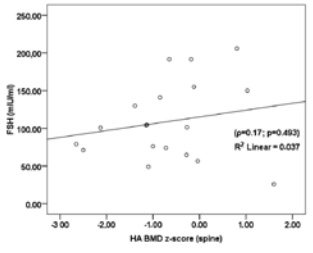

Figure 2A

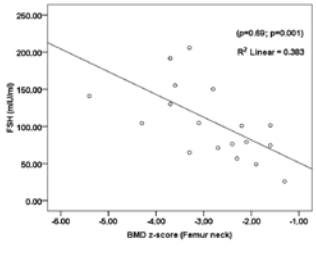

Figure 2B

Figure 2. (A, B) In patients with hypergonadotropic hypogonadism, there was no correlation between follicle-stimulating hormone levels and bone mineral density z-score measured from the spine, whereas a statistically significant negative correlation was detected between follicle-stimulating hormone levels and bone mineral density z-score measured from the femur neck. HA BMD: height adjusted bone mineral density, FSH: follicle-stimulating hormone

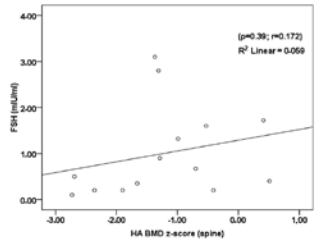

Figure 2C

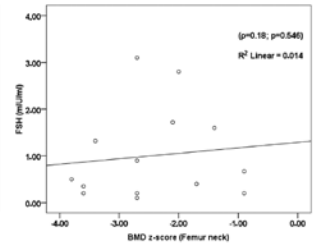

Figure 2D

Figure 2. (C, D) In patients with hypogonadotropic hypogonadism, there was no correlation between follicle-stimulating hormone levels and neither height adjusted bone mineral density z-score measured from the spine nor bone mineral density z-score measured from the femur neck. HA BMD: height adjusted bone mineral density, FSH: follicle-stimulating hormone

Age and height have previously been reported to be negatively correlated with BMD z-scores. A positive correlation has been reported between weight and BMI and BMD (9,16). Similarly, in our study, chronologic age was negatively correlated with HA BMD z-score measured from the spine whilst not correlated with BMD z-score measured from the femur neck. However, since we have adjusted BMD z-score (spine) according to height, we did not detect a correlation between height SDS and HA BMD z-score. This was consistent with the results of previous pediatric age group studies (13,17,18). The reason that BMD z-score was negatively correlated with chronologic age in our patients with hypo- and hypergonadotropic hypogonadism might be the longer duration of the lack of estrogen, thereby inadequate bone mineralization. On the other hand, prolonged hypogonadism, delayed puberty, and lack of pubertal growth spurt may also have caused the short stature and this may have affected the BMD z-score results (15). Thus, it is recommended that measurements of BMD in children with delayed growth or puberty be adjusted for height or height-age or compared with reference values of age-, gender-, and height-specific z-scores (15). Similarly, height- and age-adjusted BMD z-scores in our cohort were found to be better than only age-adjusted values. Also, the frequency of <-2 SD BMD z-scores was significantly lower when the scores were adjusted by age and height.

It is stated that increase in BMI primarily affects the BMD of the body regions, such as femur neck, which has a higher exposure rate to physical stress compared to the other regions (19). Similarly, in our study, BMI was found to be an independent factor affecting BMD z-scores measured from the femur neck. However, femur neck is not recommended for BMD measurement in children and this relationship between BMI and BMD may not have clinical relevance.

Although not statistically significant, a lower BMD was detected in hypergonadotropic hypogonadism patients, most of whom were cases of TS. This finding was consistent with the results from previous reports indicating an association of TS with increased risk of osteopenia and osteoporosis, while the pathogenesis of this association is still controversial (16,19,20). The impact of low estrogen levels on BMD have been shown in TS patients who demonstrated an improvement in BMD with estrogen replacement and also attained spontaneous puberty (19,20). TS is also characterized with short stature, metaphyseal changes, and vertebral abnormalities that can affect the BMD measurements. The mechanisms through which estrogen deficiency and chromosomal abnormality affect bone mineralization in TS have not yet been clarified (20). By contrast, with due regard to the above-mentioned queries, when BMD z-scores were adjusted for height, our results surprisingly revealed better BMD z-scores in the hypergonadotropic group. This has brought new challenges to our understanding of osteopenia/osteoporosis in TS subjects. Therefore, due to the risk of underestimating osteopenia/osteoporosis in this group of patients, adjusting the BMD z-score according to height in TS patients still remains a controversial issue.

Table 4. Multivariate linear regression analysis of effect of independent variables on bone mineral density z-scores

	HA BMD z-score (Spine)		**BMD z-score (Femur neck)**	
	t	**p-value**	**t**	**p-value**
Chronological age	-1.887	0.071	-1.167	0.254
BMI	1.260	0.219	**3.459**	**0.002**
Height SDS	-0.209	0.836	**2.378**	**0.025**
FSH	0.439	0.665	-0.732	0.471
LH	-0.021	0.983	-0.216	0.831
Estradiol	0.652	0.520	-1.409	0.171

SDS: standard deviation score, BMI: body mass index, FSH: follicle-stimulating hormone, LH: luteinizing hormone, HA BMD: height adjusted bone mineral density

As osteoporosis stands as a major health issue in post-menopausal females who have a typical hormone profile of hypergonadotropic hypogonadism, the relationship between osteoporosis, estrogen, and gonadotropins has been a research subject for many years. Thus, there are studies demonstrating the direct effects of FSH on bone turnover and bone mass in animal models, followed by further studies evaluating this relationship in human female subjects (5,6,7). A negative correlation has been reported between FSH and BMD in large scale studies conducted on adult females most of whom were post-menopausal subjects with hypergonadotropic hypogonadism (7,21,22). While the detection of a negative correlation between FSH level and BMD z-score measured from the femur neck in the subjects of our study was consistent with the results in adult studies, lack of a correlation with HA BMD z-score measured from the spine in our hypergonadotropic group and a lack of correlation with BMD measured from both spine and femur neck in the hypogonadotropic group deviated from these previous studies. The negative relationship between FSH and BMD in the hypergonadotropic group may be due to the more severe estrogen deficiency in cases with higher gonadotropin levels. The reason why estradiol levels were not found to be related to BMD in our cohort was thought to be due to the low estrogen levels in both groups and also to the low sensitivity of estrogen assays. In addition, we did not have age-matched controls to compare the estradiol levels and their impact on BMD z-scores.

Nevertheless, although there was no correlation between FSH and BMD when measured from the spine, a negative correlation was detected between FSH and BMD when the measurements were made from the femur neck. This finding was consistent with the results in adult studies indicating a predominance of FSH effect in physically stressed body regions compared to other regions. However, in children, measurement of BMD from the spine is thought to reflect bone mineral content better than measurements obtained from the femur

neck and is a preferred location (15). Therefore, the negative correlations with FSH and BMD derived from measurements from the femur neck may not have any clinical relevance.

In recent studies performed in mice, FSH given as daily injection or infusion did not affect bone mineral content. These results are in accord with findings indicating that FSH has no direct effect on BMD (23,24). In these recent studies, it was shown that FSH has no effect on human mononuclear cell precursors or on the osteoclastogenesis cell line RAW 264.7 (23,24). Similarly, in our study, the negative correlation between FSH and BMD z-score (femur neck) in our hypergonadotropic group and the lack of such correlation in our hypogonadotropic group was attributed to the more severe estrogen deficiency in patients with the higher FSH rather than a direct effect of FSH.

Limitations of this current study was the smallness of the sample and also the lack of an age-matched healthy control group. Our inability to measure the bone mineral markers of osteoclastogenic and osteoblastogenic activities and their relationship with hormonal and DXA measurements was another limitation. We also did not have the means to evaluate the microarchitecture of the bones, an exploration which might have yielded a key on bone quality.

In conclusion, this study which evaluated BMD in adolescent girls with hypogonadotropic and hypergonadotropic hypogonadism revealed a low BMD z-score in the majority of cases in both groups. However, when adjusted for height, a marked improvement was observed, particularly in BMD z-scores of the hypergonadotropic group. FSH level was not found as an independent factor affecting BMD z-score. The negative correlation between FSH and BMD z-score measured from the femur neck was attributed to the more severe estrogen deficiency. There is still a need for larger scale future studies to further elucidate the role of estrogen, gonadotropins, and auxological parameters on bone mineralization in children and adolescents with hypogonadism.

Ethics

Ethics Committee Approval: Institutional Ethical Comittee of Diyarbakır Gazi Yaşargil Research and Training Hospital, Informed Consent: Obtained from the legal guardians of participants.

Peer-review: External peer-reviewed.

Authorship Contributions

Medical Practices: Mehmet Nuri Özbek, Hüseyin Demirbilek, Rıza Taner Baran, Ahmet Baran, Concept: Mehmet Nuri Özbek, Hüseyin Demirbilek, Design: Mehmet Nuri Özbek, Hüseyin Demirbilek, Data Collection or Processing: Mehmet Nuri Özbek, Hüseyin Demirbilek, Rıza Taner Baran, Ahmet Baran, Analysis or Interpretation: Mehmet Nuri Özbek, Hüseyin Demirbilek, Rıza Taner Baran, Ahmet Baran, Literature Search: Mehmet Nuri Özbek, Hüseyin Demirbilek, Rıza Taner Baran, Ahmet Baran, Writing: Mehmet Nuri Özbek, Hüseyin Demirbilek, Rıza Taner Baran, Ahmet Baran.

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Original Article

Transient Congenital Hypothyroidism in Turkey: An Analysis on Frequency and Natural Course

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ABSTRACT

Objective: Prevalence of congenital hypothyroidism (CH) in Turkey at birth was reported to be as high as 1:650 in 2008-2010. Incidence rates of permanent and transient CH separately are unknown due to lack of follow-up data. We aimed to evaluate the impact of transient hypothyroidism on increasing incidence of CH and to determine the natural course and the clinical, biochemical, and imaging characteristics of transient CH.

Methods: Baseline and follow-up data of the infants with CH detected at screening in six provinces in the Black Sea Region were analyzed retrospectively during a time period covering the years 2008-2010.

Results: Among 138 cases (48% female), 16 (12%) showed transient hyperthyrotropinemia which resolved without intervention. Of the treated 122 cases, 63 (52%) had transient CH. While its frequency was 35% in 2008, it increased to 56% in 2009-2010, following a lowering of the thyroid stimulating hormone cutoff value. The frequency was higher in inland provinces than in coast (67% vs. 43%; p=0.01). Clinical characteristics of permanent and transient cases were similar except female-to-male sex ratios (1.5:1 vs. 0.6:1; p=0.02). L-thyroxine was discontinued in 70% of transient cases before 3 years of age at a median age of 19 (2-36) months. The only indication for early discontinuation of treatment was a low L-thyroxine dose, which was 1.25±0.27 μg/kg/day at withdrawal time.

Conclusion: Our regional follow-up data showed that more than half of newborns with primary CH had transient thyroid dysfunction. In the majority of cases, discrimination between transient and permanent CH can be made before age 3 years, as indicated by cessation of L-thyroxine treatment.

Keywords: Congenital hypothyroidism, incidence, iodine deficiency, neonatal screening, transient hypothyroidism

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Birth prevalence of primary congenital hypothyroidism (CH) was found to be very high in Turkey after the national screening. However, the incidence rates of permanent and transient CH are unknown due to lack of follow-up data. To distinguish between transient and permanent forms, the guidelines recommend that the children with unexplained CH be re-evaluated after 3 years of age through a trial of treatment withdrawal.

WHAT THIS STUDY ADDS?

Transient hypothyroidism exists in more than half of the newborns detected at screening in our region and most probably throughout the country. Lowering of thyroid stimulating hormone cutoffs has led to increased birth prevalence owing to detection of a higher number of mild cases of both permanent and transient CH. In the majority of cases, discrimination between transient and permanent CH could be possible before 3 years of age. This study has shown that unnecessary treatment of transient CH can be avoided in many infants owing to early cessation of low dose L-thyroxine.

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Introduction

Congenital hypothyroidism (CH) is one of the most common causes of mental retardation that can be prevented by early detection and treatment. Many developed countries have largely eliminated intellectual disability caused by CH owing to newborn screening programs (NSPs) (1). In Turkey, the National Newborn Screening Program (NNSP) for CH was started at the end of 2006 by the Turkish Directorate of Public Health. In 2013, Dilli et al (2) published the first NNSP data that showed very high CH incidence rates (1:888 in 2008, 1:592 in 2009, and 1:469 in 2010). The overall incidence rate of CH during this period (2008-2010) was 1:650.

The data cited above reflect the birth prevalence of primary CH, which might be potentially permanent or transient. Several studies (3,4,5,6,7) showed that the frequency of transient CH in Turkey ranged between 25-65%, indicating that transient cases might have played a substantial role in the high incidence of CH. Unfortunately, a nationwide registry that collects information on the results of re-evaluation of CH diagnosis at the follow-up centers is not yet available in Turkey. For that reason, current incidence rates for permanent and transient CH are unknown. A recent study reported for the first time that among cases detected at national screening, the frequency of transient CH was 30% (7). However, this study was not intended to be representative of a region. Therefore, there is still no nationwide or regional follow-up data that can be used to estimate the incidence rates for permanent and transient CH.

Transient primary hypothyroidism is a heterogeneous disorder that may be caused by iodine deficiency or excess, maternal thyroid stimulating hormone (TSH) receptor blocking antibodies, maternal antithyroid drugs, and genetic defects such as dual oxidase 2 (DUOX2) and TSH receptor mutations (8,9). However, the underlying mechanism remains often unknown (10,11). To discriminate permanent and transient forms, the guidelines recommend that children with unexplained CH with gland *in situ* be re-evaluated after 3 years of age through a trial of treatment withdrawal (12,13). However, some cases of transient CH caused by identifiable factors such as iodine deficiency or excess may also frequently require a short-term therapy. The prevalence of transient CH in Turkey is much higher than the expected rate of 5-10% reported for iodine-sufficient populations (14). Numerous studies carried out in newborns (15,16,17) and pregnant women (18,19,20) have already demonstrated that iodine deficiency is a continuing problem of Turkey. Also, a recent study revealed that iodine excess could be a problem in newborns due to use of iodine-containing antiseptics during delivery despite maternal iodine deficiency (20). Overall, little is known about the natural course of transient CH in Turkey (6,7). It is possible that treatment withdrawal before 3 years of age may be more commonly applicable.

The aims of our study were to evaluate the impact of transient hypothyroidism on the increasing incidence of CH as well as to investigate the natural course and clinical, biochemical, and imaging characteristics of transient CH in the cases detected at national screening in Turkey.

Methods

Study Area: The Turkish Statistical Institute (TSI) publishes demographic data by dividing Turkey into 12 regions (http://www.tuik.gov.tr/). Our study area includes six neighboring provinces comprising Samsun, Amasya, Tokat, Çorum, Sinop, and Ordu. With the exception of Ordu which is a province on the eastern part of the Black Sea coast, these provinces are located in the West Black Sea Region, which is one of the 12 regions of Turkey. Of the whole population in the West Black Sea Region and Ordu, 70% (n=3 645 836) live in our study area. The Clinic of Pediatric Endocrinology at Ondokuz Mayıs University in Samsun is a tertiary referral center for the patients residing in this area.

Potential CH Cases in the Area: According to the NNSP data (2), the birth prevalence of CH in the West Black Sea Region was about 1:600 between 2008 and 2010. Also, according to the annual birth statistics of six provinces obtained from the website of TSI, the total number of live-born infants was 157 839 during the years of 2008-2010. By multiplying the birth prevalence of CH (1:600) and the number of live-born infants (157 839), we have estimated that around 263 CH cases should have been detected at screening in our area during the study period. While three provinces (Samsun, Ordu, Sinop) in our region are on the seaside, the other three (Amasya, Tokat, Çorum) are with inland location. According to TSI data, 59% of the infants were born in the coastal provinces and the remaining 41% were born in the inland area. Therefore, the potential CH cases should have been scattered proportionally between coastal and inland provinces (Table 1).

Patients: After approval of the Ondokuz Mayıs University Institutional Ethics Committee, we carried out a retrospective analysis of the medical records of patients who were consecutively diagnosed as CH in our clinic between January 2008 and December 2010. These patients were transferred to our clinic by the Provincial Directorates of Public Health carrying out the NNSP. Patients born and resident in the 6 provinces listed above were included in the study. Patients who were from outside the study area (n=6), those who had incomplete data sets (n=12), and those who were lost to follow-up before discrimination between transient and permanent CH (n=21) were excluded from the study. Therefore, the study cohort consisted of 138 cases with CH (Figure 1).

Study Protocol: The newborns with a high serum TSH level (>10 μU/mL) were referred to our institution by the NNSP team as suspected primary CH cases (2). Based on the serum levels of TSH and free thyroxine (fT_4) measured at our

endocrine clinic (or at a local hospital for a few infants who had been referred after initiation of treatment), the patients were classified as primary overt (TSH>10 μU/mL, fT_4<0.9 ng/dL) or subclinical CH (TSH>10 μU/mL, fT_4≥0.9 ng/dL) (21).

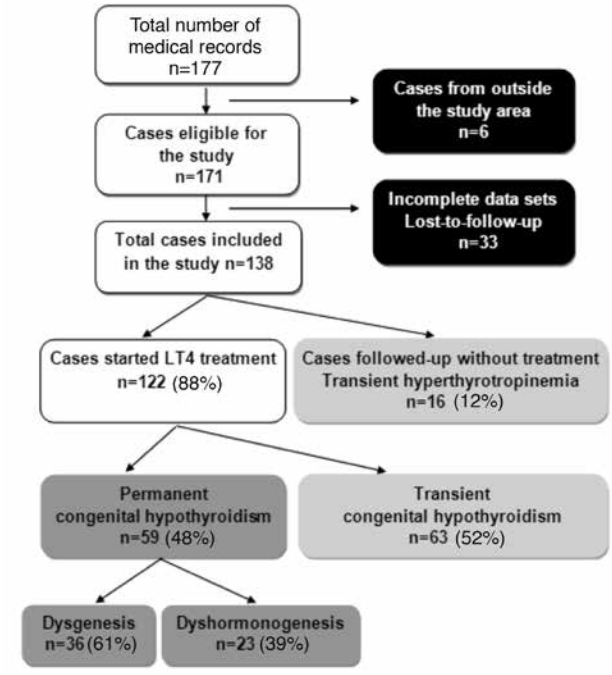

Figure 1. Flow diagram of the follow-up study

L-thyroxine (L-T4) treatment (in a dose of 8-15 μg/kg) was started in patients with low fT_4 and/or high TSH levels (fT_4<0.9 ng/dL and/or TSH>25 μU/mL) (21). Infants with normal fT_4 and slightly elevated TSH (10-25 μU/mL) were followed-up without treatment and rechecked at 2-week intervals. If serum TSH concentration was found to be >25 μU/mL at any time or >10 μU/mL after 4-6 weeks of age, these infants were also started on L-T4 treatment (12,21). On the other hand, infants whose TSH levels returned to normal within 4-6 weeks without intervention were considered to have transient hyperthyrotropinemia (Figure 1) (12,21).

At baseline or during follow-up, CH was categorized as permanent or transient. The criteria for a diagnosis of permanent CH were: 1- absent or ectopic thyroid on imaging; or 2- an increase in medication dosage over time or elevated TSH due to non-compliance to treatment; or 3- elevated TSH (>10 μU/mL) after a 30-day trial off L-T4 therapy at age ≥3 years (12,22,23). If a suppressed serum TSH level was observed despite low doses of L-T4, a trial off medication was done before 3 years of age in the cases with ectopic glands. The presence of normal thyroid function tests over 6 months following a withdrawal of L-T4 was considered to indicate transient CH (22).

In order to determine the distinguishing characteristics and natural course of transient hypothyroidism, the infants with permanent and transient CH were evaluated comparatively in terms of demographic, clinical, laboratory, and treatment parameters at baseline and during follow-up. In addition,

Table 1. Distribution of the live-born infants, potential congenital hypothyroidism cases, and patients with permanent and transient congenital hypothyroidism by provinces (located at seaside and inland)*

Provinces	Live-born infants n	Potential CH cases‡ n (%)	Patients included in the study n (%)	Permanent CH n (%)	Transient CH n (%)
Coastal					
Samsun	54 980	92	42	25 (60)	17 (40)
Ordu	30 920	52	24	13 (55)	11 (45)
Sinop	7 794	13	11	6 (55)	5 (45)
Subtotal	93 694	157 (59)‡‡	77 (63)‡‡	44 (57)	33 (43)†
Inland					
Amasya	12 851	21	17	7 (41)	10 (59)
Çorum	23 503	39	13	4 (31)	9 (69)
Tokat	27 791	46	15	4 (27)	11 (73)
Subtotal	64 145	106 (41)‡‡	45 (37)‡‡	15 (33)	30 (67)†
Total	157 839	263	122 (100)	59 (48)	63 (52)

‡The numbers of potential congenital hypothyroidism cases were calculated by multiplying the number of live-born infants with the incidence rate of congenital hypothyroidism (1:600) in our region.
‡‡The ratio of the patients living in the coastal and inland provinces was not significantly different than that of the potential congenital hypothyroidism cases in the same areas (p=0.48).
*There is no significant difference among the rates of transient congenital hypothyroidism cases from six provinces in the study area (p=0.19).
†A comparison based on coastal and inland provinces shows a significant difference between the rates of transient congenital hypothyroidism (p=0.01).
CH: congenital hypothyroidism

to assess the impact of TSH cutoff and geography on the frequency of transient CH, the cases were also evaluated by year (2008 vs. 2009-2010) and by province (inland vs. coastal).

Laboratory and imaging methods: Serum TSH, fT_4, and thyroglobulin levels were measured by electrochemiluminescence immunoassay using Elecsys 2010 modular analytics E170 (Roche Diagnostics, Indianapolis, IN, USA). Reference ranges of TSH, fT_4, and thyroglobulin for infants at ages between 6 days and 3 months were 0.72-11.0 µU/mL, 0.9-2.2 ng/dL, and 20-228 ng/mL, respectively. Thyroid ultrasound (US) was performed with an Aplio XG SSA-790A US scanner (Toshiba Medical Systems Co., Tokyo, Japan) and a 12-MHz linear-array transducer. The volume of thyroid lobes was calculated using the following formula: V (mL)=0.479xlengthxwidthxthickness (all in cm). Total thyroid volume was the sum of the volumes of two lobes (24). Thyroid gland was considered goitrous when its total volume exceeded +2 standard deviation (SD) score of the volume found by Kurtoglu et al (25) for Turkish newborns of different gestational ages. Thyroid scintigraphy (scan) with ^{99m}Tc-pertechnetate was performed using a Siemens Orbiter gamma camera equipped with a pinhole collimator (Siemens, Erlangen, Germany).

Statistical Analysis

The data were analyzed using SPSS software (Statistical Package for the Social Sciences, version 15.0, SPSS Inc, Chicago, IL, USA). Values in the text were presented either as means ± SD or, if not normally distributed, as medians (ranges). Statistical analysis was performed using parametric (chi-square and student's t-test) or non-parametric (Fisher exact and Mann-Whitney U) tests, when appropriate. A p-value <0.05 was accepted as statistically significant.

Results

Table 1 shows distribution of the live-born infants, the potential CH cases, and the patients with permanent and transient CH in our study group by geography, namely, by provinces located at seaside and inland. According to the numbers of live-born infants, 59% of the potential CH cases should have been detected in the coastal provinces. Of the patients in our study group, 63% came from the coastal area. A comparison between expected and observed ratios of CH infants by the provinces did not show a significant difference (p=0.48), indicating that our study group was representative for all potential CH cases living in both seaside and inland. Also, there was no significant difference among the frequencies of transient CH in the six provinces, ranging between 40% and 73% (p=0.19). However, the frequency of transient CH was higher in the inland provinces compared to that in the coastal ones (67 vs. 43%, p=0.01).

Figure 1 shows a flow diagram of the follow-up study. After exclusion of 33 cases due to incomplete data set and lack of follow-up, a total of 138 children were included in the study. Demographic characteristics (birthplaces, sex ratios, and median ages at presentation) of the excluded infants were not different from those of the study cases (data not shown). Thus, among 263 probable cases of CH detected in our area, 65% (n=171) were evaluated in our clinic, and 53% (n=138) were included in the study. As depicted in Figure 1, sixteen (12%) of these cases showed transient hyperthyrotropinemia. Among 122 cases treated with L-T4, 63 (52%) were found to have transient CH. Permanent CH was identified in 48% of the cases.

All treated infants underwent a thyroid scan and/or an US (53 scan, 46 US, and 21 both) at presentation (n=119) or after 3 years of age (n=3). Based on the findings of thyroid imaging, the etiology of permanent CH was classified as follows: dysgenesis, 61% (22 ectopia, 10 agenesis, 4 hypoplasia) and dyshormonogenesis, 39% (14 normal and 9 hyperplastic glands). In transient CH cases, thyroid imaging revealed a normal (n=34), hyperplastic (n=26), or hypoplastic (n=3) gland *in situ*. Three patients including one transient case showed a discordant result; while a thyroid gland was reported to be present at US, the scan revealed no uptake (Table 2). Maternal autoimmune thyroiditis was reported in 6 cases (3 transient and 3 permanent). Two of them were receiving L-T4 therapy, whereas there was no history of antithyroid or iodine-containing drug use.

The baseline clinical characteristics of the cases with permanent and transient CH are shown in Table 2. Our cohort consisted of 59 females and 63 males, giving a female-to-male sex ratio of 0.9:1. While the female-to-male ratio was 1.5:1 in the permanent group, this ratio was 0.6:1 in the transient group (p=0.02). The female-to-male ratio was higher in the permanent CH cases with thyroid dysgenesis (1.8:1) than those with dyshormonogenesis (1.1:1). There were no statistically significant differences between permanent and transient CH groups in terms of birth weight, gestational age, frequency of prematurity, and median ages at presentation and starting therapy.

The mean serum TSH level was higher in the permanent group than the transient one (91±62 vs. 67±33 µU/mL, p=0.009). But, the dispersion of TSH levels largely overlapped in both groups [100 (12-420) vs. 69 (10-100) µU/mL]. Mean fT_4 levels were equally low in the two groups. Also, the frequencies of overt and subclinical hypothyroidism were not different between the two groups. However, serum thyroglobulin levels were higher in the cases with transient CH compared to those with permanent CH (241±90 vs. 134±113 ng/mL, p=0.001). Within the permanent CH group, serum thyroglobulin levels of the patients with dysgenesis and dyshormonogenesis were 80±89 and 192±112 ng/mL, respectively (p=0.01). A comparison between the patients with transient CH and permanent dyshormonogenesis did not show a significant difference (p=0.48).

The whole blood TSH cutoff value was 10 μU/mL in 2008 and was lowered to 7.5 μU/mL in 2009 (2). In our study group, the number of permanent and transient CH cases has gradually increased from 2008 to 2010, but the increment in the transient group has been more evident (Table 3). The frequency of transient CH was significantly higher in the years of 2009-2010 than in the previous year at which time TSH cutoff was higher (56 vs. 35%, p=0.05).

Table 4 summarizes the follow-up and treatment data of the cases with permanent and transient CH. L-T4 therapy was discontinued at a median age of 19 months (ranges 2-36 months) in the cases with transient CH. While 54% of the infants with permanent CH were diagnosed at presentation owing to abnormal thyroid imaging, 27% received a final diagnosis during follow-up due to either increased L-T4 dosage over time or elevated TSH caused by non-compliance to treatment. Only 19% of permanent cases required a trial off medication after 3 years of age for definitive diagnosis. On the other hand, L-T4 was discontinued before 3 years of age in 70% (n=44) of transient cases. Finally, L-T4 doses were significantly lower in the transient CH cases than the permanent ones at the onset of treatment (p=0.03) and at the 6 months and thereafter (p<0.001). In the transient CH cases (n=44) whose treatment was discontinued early, L-T4 dose was 1.25±0.27 μg/kg/day at the time of withdrawal. All of them were receiving L-T4 at a dose of <25 μg/day (39 patients, 12.5 μg/day; 2 patients, 18.75 μg/day, and 3 patients 6.25 μg/day).

Discussion

The results of our study have revealed that 52% of the cases detected at screening in our area had transient CH apart from transient hyperthyrotropinemia resolved without intervention. Also, the present study showed that in 70% of transient CH cases, L-T4 treatment was discontinued before 3 years of age, at a median age of 19 months.

Birth prevalence of primary CH was reported as 1:650 in Turkey during the period of 2008-2010 (2). This prevalence was around 1:600 in the West Black Sea Region comprising our study area. However, due to lack of follow-up data, it is still unknown how transient hypothyroidism has made a contribution to the high incidence rate of CH. Owing to a coverage ratio of 65%, we believe that our study data can be used to estimate the incidence rates of permanent and

Table 2. Baseline characteristics of infants with permanent and transient congenital hypothyroidism

Baseline characteristics	Permanent CH (n=59)	Transient CH (n=63)	p-value
Sex ratio (Female:Male)	1.5:1	0.6:1	0.02
Birth weight, grams	3316±561	3120±586	0.08
Birth weight <2500 g, n (%)	9 (15)	11 (17)	0.74
Gestational age <37 w, n (%)	5 (9)	9 (14)	0.31
Median age at presentation, days	18 (7-72)	18 (3-58)	0.94
Median age at start of therapy, days	18 (4-72)	18 (3-58)	0.82
TSH (μU/mL)	91±62	67±33	0.009
fT_4 (ng/dL)	0.59±0.37	0.61±0.28	0.79
Thyroglobulin (ng/mL)*	134±113	241±90	0.001
Subclinical hypothyroidism, n (%)	15 (25)	11 (18)	0.28
Thyroid imaging†, n			
Normal	14	34‡	<0.001
Abnormal	45	29	
Agenesis of the gland	10	0	
Ectopic gland	22	0	
Hypoplastic gland	4‡	3	
Hyperplastic gland	9	26	

Data are expressed as number (percent), median (min-max), or mean ± standard deviation.

*Serum thyroglobulin levels were measured in 25 and 29 cases in the permanent and transient congenital hypothyroidism groups, respectively.

†Thyroid scintigraphy (scan, n=53), ultrasound (ultrasound, n=46), or both (n=21).

‡Discordance between ultrasound and scan was observed 3 patients. Two patients in the permanent group had hypoplastic thyroid glands on ultrasound, but scan revealed no uptake. In a transient case, the thyroid gland was visualized at normal size on ultrasound, but showed no uptake on scan.

CH: congenital hypothyroidism, TSH: thyroid stimulating hormone, fT_4: free thyroxine

transient CH in our region. If transient CH cases of 52% are excluded, the birth prevalence that is 1:600 will be reduced to nearly 1:1250 that corresponds to the incidence of permanent CH in our region. Through a similar approach, the incidence of transient CH can be estimated as 1:1154.

Prior to the onset of NNSP, the regional studies from Turkey reported the incidence of CH in a range of 1:2736 to 1:2326 (3,26,27). The NNSP data indicated that the incidence of CH has increased dramatically over the last two decades. In fact, several NSPs around the world have reported approximately a twofold increase in the incidence of CH (1). Lowering of the screening TSH cutoffs in these programs has been associated with the doubling of CH incidence, primarily explained by detection of milder cases. While the whole blood TSH cutoff value in Turkey was 20 μU/mL in the past (3,26,27), it was chosen as 10 μU/mL at the start of NNSP, and was lowered to 7.5 μU/mL in 2009 (2). With the reduction of TSH cutoff levels, an increase in CH incidence would be expected, but in Turkey the increase has been beyond that expected (approximately 4-fold) compared to other countries including Italy, England, and Greece (28,29,30). Thus, the rise in CH incidence in Turkey was not explicable in the basis of an enhanced detection of milder cases of true CH alone. Our study has shown that transient and permanent forms of hypothyroidism have contributed jointly to the increased CH incidence in our region. Comparison of the previous 1:2326 (3) and the current estimated incidence rates (1:1250) indicates a nearly 2-fold increase in permanent CH incidence in the West Black Sea Region. This is compatible with a worldwide trend towards doubling of CH incidence, which is usually explained by the lower TSH cutoff level (1,28,29,30).

On the other hand, the impact of lowering of the TSH cutoff on the incidence of transient CH has been more dramatic with an approximate 5-fold increase, which is calculated by comparing the earlier incidence (1:6202 in Ref. 3) and our current estimation (1:1154). The frequency of transient CH was 27% in our region in the period of 2000-2002 (4). Our study revealed that this frequency has increased to 35% in 2008 and to 56% in 2009-2010. Such a high increment in the frequency and incidence of transient CH has not been observed in other European countries nor in the USA (28,30,31). However, in

Table 3. Distribution of the permanent and transient congenital hypothyroidism cases by years*

Years	Permanent CH n (%)	Transient CH n (%)	Total n (%)
2008	17 (65)	9 (35)†	26 (21)
2009-2010	42 (44)	54 (56)†	96 (79)
2009	19 (45)	23 (55)	42 (35)
2010	23 (43)	31 (57)	54 (44)
Total	59 (48)	63 (52)	122 (100)

*There is no significant difference among the years of 2008 to 2010 regarding the frequencies of transient congenital hypothyroidism cases (p=0.14).
†A comparison based on the varied capillary thyroid stimulating hormone, cutoff levels (10 μU/mL in 2008 and 7.5 μU/mL in 2009 and 2010) shows a significant difference between the frequencies of transient congenital hypothyroidism in 2008 and 2009-2010 (p=0.05).
CH: congenital hypothyroidism

Table 4. Follow-up and treatment data of the cases with permanent and transient congenital hypothyroidism

Parameter	Permanent CH (n=59)		Transient CH (n=63)		p-value
Follow-up duration, months	54 (37-80)		24 (8-66)		<0.001
Age at withdrawal of therapy, months	36 (36-38)		19 (2-36)		<0.001
Age at decision time to final diagnosis, months	2 (0.1-39)		25 (8-42)		<0.001
Decision time to final diagnosis, n (%)					<0.001
At presentation	32 (54)		0		
During follow-up before 3 years of age	16 (27)		44 (70)		
After a trial off therapy at 3 years of age	11 (19)		19 (30)		
Dose of L-thyroxine (μg/kg/day)	n		n		
At onset	59	12.1±2.5	63	11.0±2.8	0.03
At 6th month	59	4.3±1.4	59	3.1±0.9	<0.001
At 12th month	59	3.4±1.0	47	2.0±0.7	<0.001
At 24th month	59	3.3±0.9	28	1.6±0.3	<0.001
At 36th month	59	3.3±1.0	19	1.4±0.3	<0.001
At withdrawal time of L-thyroxine	11	2.0±0.5	63	1.3±0.4	<0.001

Data are expressed as number (percent), median (minimum-maximum), or mean ± standard deviation.
CH: congenital hypothyroidism

Iran, where the cutoff level for TSH is 5 μU/mL, the birth prevalence of CH was recently reported as high as 1:307 (32). After re-evaluation, the incidence rates of permanent and transient CH were 1:581 and 1:628, respectively. The frequency of transient CH in Markazi Province of Iran was 48%, a ratio similar to that (52%) in our cohort. In Turkey, Dilli et al (2) reported the birth prevalence of CH as 1:469 for the year 2010. Because the TSH cutoff was recently lowered to 5.5 μU/mL whole blood in NNSP, it will not be surprising to see a report on a much higher birth prevalence of CH in the near future in Turkey, similar to Iran. In short, the rate of transient CH in our country is comparable to that reported for Iran, our neighbor in the Middle East, but it is much higher than the rates reported for European countries and the USA.

Past experience in Italy showed that the frequency of transient hypothyroidism was 58% in the high CH incidence (>1:2000) areas affected historically by iodine deficiency. As CH incidence decreased, lower percentages of transient CH were observed in several districts of Italy (33). In Turkey, the 2010 NNSP data revealed very high CH incidence rates varying between 1:996 and 1:250 in 12 regions (2). In our study region that was shown to be affected by mild-to-moderate iodine deficiency in the past (3,34), CH incidence was 1:418 in 2010 (2), and we found that 57% of this could be attributed to transient cases. In accordance with the previous Italian experience, these data suggest a continuing iodine deficiency problem in Turkey.

In fact, transient CH in Turkey is an old problem and its frequency varies between 25-65% from one region to another (3,4,5,6,7). The present study has shown that the frequency of transient CH ranged between 40% and 75% in six provinces in our area, indicating that it was largely affected by the geography. While the frequency was 40% to 45% among infants from the coastal provinces, it was as high as 60% to 75% among those in the inland provinces. Given that the frequency of transient CH is between 5-10% in iodine-sufficient populations (14), the ratios varying between 25-75% reported in the present and other studies possibly reflect variations in iodine status in different regions of Turkey.

Before salt iodization program implemented was initiated in 1998, Turkey was a mild to severe iodine deficiency area (34,35). After iodization program, based on the results of monitoring studies on urinary iodine concentration among school-age children, iodine deficiency has been considered to be eliminated in most urban areas of Turkey (34,36). The World Health Organization has previously recommended the measurement of neonatal TSH in addition to urinary iodine as an indicator for population iodine status. The criterion for iodine sufficiency is that the frequency of whole blood TSH values >5 μU/mL should be under 3% in a population (37). The NNSP data of 2010 showed that the recall rate for the whole blood TSH cutoff of 7.5 μU/mL is 3.8% (2). It is obvious that the rate of newborns with a screening TSH value >5 μU/mL would be much greater than 3% in Turkey. Some experts have proposed that the data on neonatal TSH screening for monitoring population iodine deficiency should be interpreted with caution due to technical issues including the time of sampling after birth (38). Nevertheless, many studies have shown an inverse relationship between neonatal TSH and maternal urinary iodine concentration, supporting the notion that the frequency of neonatal TSH concentrations >5 μU/mL was a sensitive indicator of iodine nutrition during pregnancy (39,40,41). In recent years, several studies have already demonstrated insufficient iodine intake of pregnant women living in different regions of Turkey (17,18), including apparently iodine-sufficient areas (19). As a result, the finding of the high frequency of transient CH is in line with the high recall rate in the NNSP. These data should be interpreted also considering nutritional iodine deficiency in pregnant women, which is clearly different from the iodine status among school-age children in Turkey.

Beside iodine deficiency, other environmental and genetic factors might have contributed to the occurrence of transient CH in our cohort. Iodine excess induces transient hypothyroidism by the Wolff-Chaikoff effect lasting usually about 10 days, and it can be caused by the use of iodine-containing antiseptics, contrast agents and amiodarone (8). In our study group, there was no history of exposure to antithyroid or iodine-containing drugs. However, topical iodine exposure cannot be excluded as a cause of transient CH in our cohort. In fact, newborns in iodine-deficient regions might be more susceptible to the Wolff-Chaikoff effect of topical iodine exposure (9). In a recent study carried out in Zonguldak, a city in our region, iodine excess was observed in 61% of 116 healthy newborns, while their nursing mothers showed a iodine-deficient nutritional status (20). In that study, the recall rate at screening was found to be 9.5% and three newborns required L-T4 therapy. Iodine excess in newborns was attributed to the use of iodine-containing antiseptics during delivery. This same study has demonstrated that iodine deficiency remained an unresolved problem in nursing mothers in our region, and that iodine excess contributed to the high recall rates, as well the increased incidence of transient CH and hyperthyrotropinemia (20).

Maternal TSH receptor blocking antibodies may lead to transient hypothyroidism, but this is a rare condition that occurs in 1-2% of all newborns with CH or 1 in 180,000 live births (42). Under conditions of exposure to maternal blocking antibodies or excess iodine, the thyroid usually can be identified in a normal location by ultrasonography, but radioisotope uptake might be blocked partially or completely (9,14). In our study, only one case in the transient group has shown such discordance between US and scan, implying that blocking antibodies or excess iodine are not major contributing factors. The thyroid gland was enlarged in 41% of the newborns with transient CH. In addition, as compared to the patients with permanent CH, transient CH cases had higher serum

thyroglobulin levels despite the lower TSH and the equally low fT_4 levels. This biochemical profile together with a significant proportion of goiter supports a possibility of iodine deficiency in our cohort. However, high thyroglobulin levels and goiter may be also caused by iodine organification defects, which have been detected in nearly 20% of the patients with transient CH (10). The etiology remains unexplained in the majority of such patients albeit DUOX2 mutations were demonstrated in some (10).

Mutations in thyroid peroxidase (TPO), *DUOX2* and TSH receptor genes may cause permanent or transient CH (8,9,10,43). In the present study, thyroid imaging showed normal, enlarged, or hypoplastic gland *in situ* in 46% of the patients with permanent CH, indicating a higher possibility of recessively inherited genetic defects in our region where consanguineous marriages were relatively frequent. Therefore, genetic background might also have contributed to the development of transient thyroid dysfunction in our cohort. Given that iodine intake may alter the phenotype of TPO and DUOX2 mutations causing iodine organification defects (8,43,44,45), it is even possible that iodine deficiency in our region might have increased the expressivity of gene defects. In Turkey, permanent CH due to dyshormonogenesis is mainly caused by TPO mutations (46), but there is no study investigating the genetic background in transient CH.

As another important finding, the present observational study has shown that early discrimination between transient and permanent CH could be possible in the majority of children with gland *in situ*. This finding was consistent with the data of latest reports (11,47,48). Imaging studies revealed ectopic gland or athyreosis in only 25% of the cases with CH, and thereby let us to make a definitive diagnosis on admission in a small group. The majority of patients who had an ectopic gland received a final diagnosis during follow-up. In transient group, no case required an increment in L-T4 doses and therapy was discontinued at a median age of 19 months with a range of 2-36 months. Thus, we reached a definitive diagnosis in 70% of transient cases before 3 years of age, the recommended time for re-evaluation of the thyroid axis through a trial off treatment (12,13). For discrimination between transient and permanent CH patients with gland *in situ*, there was no clinical or laboratory parameter including gestational age, birth weight, presentation time, age at onset of therapy, or the degree of hypothyroidism based on serum levels of TSH, fT_4, and thyroglobulin. The only indication for early discontinuation of treatment was the L-T4 dose. We could stop therapy early owing to low doses of L-T4 (usually <1.5 µg/kg/day or <18.75 µg/day). This experience is in agreement with the data of a recent study from Turkey, which has shown that L-T4 dose is the sole criterion that can be used to distinguish permanent and transient CH (49). Messina et al (48) proposed that L-T4 requirements <1.7 µg/kg/day at 12 months or <1.45 µg/kg/day at 24 months were highly suggestive of transient CH. On the other hand, Cho et al (47) suggested that infants with CH requiring L-T4 doses <3.25 µg/kg/day at 12 and 24 months were likely to have transient CH. In our study, mean L-T4 doses among the transient CH cases were 2.0±0.7 and 1.6±0.3 µg/kg/day at 12 and 24 months, respectively. In the patients whose treatment was stopped before age 3 years, the dose was 1.25±0.27 µg/kg/day at the time of withdrawal. Although the mean duration of treatment in our cohort was shorter than the recommended usual time, we believe that L-T4 substitution could possibly have been discontinued even earlier, at least in some patients.

In cases with transient hypothyroidism caused by easily identifiable factors including maternal thyroid diseases and iodine deficiency or excess, L-T4 treatment is frequently stopped within the first year of life (11,14). Although iodine intake appears to be the most likely explanation for the high frequency of transient CH in our region, 75% of transient cases were still receiving L-T4 treatment at the end of the first year. Hence, other mechanisms, especially monogenic defects might have played a role in the development of transient CH. Nevertheless, it must be pointed out that even in iodine-sufficient populations, the underlying mechanism of transient CH remains unexplained in the majority of the cases (10,11,22,23). The major limitation of our study was the lack of direct evidence on possible explanations for transient CH including iodine status and genetic background of the patients. This limitation was a result of the retrospective design of the study. Therefore, additional prospective studies will be necessary to investigate iodine status and known or possibly novel genetic defects in the CH population in Turkey. But during this period, to reduce the high frequency of transient CH, it appears reasonable to suggest that pregnant women be supplemented with iodine-containing preparations in addition to iodized salt consumption in iodine-deficient areas in Turkey.

In conclusion, our study showed that more than half of the newborns with primary CH had transient thyroid dysfunction. Lowering of TSH cutoffs has led to the increased birth prevalence of CH owing to the detection of a higher number of mild cases of both permanent and transient CH. In the majority of cases, discrimination between transient and permanent CH appears to be possible before age 3 years.

Ethics

Ethics Committee Approval: Ondokuz Mayıs University Institutional Ethics Committee 2012, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Cengiz Kara, Murat Aydın, Design: Cengiz Kara, Murat Aydın, Data Collection and/or Processing: Figen Günindi, Gülay Can Yılmaz, Analysis and/or Interpretation: Cengiz Kara, Figen Günindi, Literature Research: Cengiz Kara, Figen Günindi, Writing: Cengiz Kara.

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Original Article

Cellular Trace Element Changes in Type 1 Diabetes Patients

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ABSTRACT

Objective: Type 1 diabetes mellitus (T1DM) may lead to deficiencies in trace elements that have substantial functions in the human organism. Changes in serum magnesium (Mg), copper (Cu), and zinc (Zn) levels are correlated with metabolic control and diabetes complications. The aim of this study was to evaluate the intra-erythrocyte levels of trace elements and urinary Mg excretion following intravenous (iv) Mg tolerance testing in children with T1DM.

Methods: A total of 43 children aged 2-18 years with T1DM and age/gender-matched 25 healthy children were included in the study. The iv Mg tolerance test was performed following the measurement of intra-erythrocyte Mg (eMg_1), Cu (eCu_1), and Zn (eZn_1) levels using the atomic absorption spectrophotometer method. The Mg retention ratio was estimated from measurements in 24 h urine samples.

Results: No statistically significant difference was found for eMg_1, eCu_1, and eZn_1 levels between the patient and control groups ($p>0.05$). In the patient group, the eMg_1, eCu_1, and eZn_1 levels measured after the iv Mg tolerance test significantly increased compared with the baseline levels ($p<0.05$), and the Mg excretion ratio measured from the urine collected after the iv $MgSO_4$ infusion was >50%.

Conclusion: The increased retention value following the iv Mg tolerance testing indicates intracellular Mg deficiency in children with T1DM.

Key words: Type 1 diabetes mellitus, trace elements, magnesium tolerance test

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Magnesium (Mg) is the most commonly seen trace element deficiency in patients with type 1 diabetes mellitus (T1DM). Moreover, Mg deficiency is involved in the pathogenesis of diabetes complications that inhibit the prostacyclin receptor function and cause increased thrombocyte activation and aggregation.

WHAT THIS STUDY ADDS?

Intra-erythrocyte Mg (eMg_1) measurement cannot reveal Mg deficiency, but increased retention following intravenous Mg tolerance test indicates intracellular Mg deficiency in patients with T1DM.

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease that occurs with increasing frequency in children. Recently, the effects of trace elements on glucose metabolism have been reported, suggesting their role in the etiopathogenesis and complications of diabetes (1,2,3).

In many studies conducted on diabetic patients and experimental animals, zinc (Zn) has been reported to have positive

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effects on hyperglycemia by increasing phosphoinoside-3 kinase activation, serine/threonine kinase phosphorylation, GLUT4 (glucose transporter type 4) translocation and insulin sensitivity and to enhance prevention of development of complications due to diabetes by decreasing oxidative stress (4,5,6,7). Magnesium (Mg) is the most commonly seen trace element deficiency in patients with T1DM. Decreased plasma and tissue Mg levels have been reported among diabetic patients (1,2,8). Mg plays an important role in whole reactions, including cellular energy transfer, glycolysis and phosphorylation, and it prevents free radical generation required to ensure increased glutathione syntheses (1). Moreover, Mg deficiency is involved in the pathogenesis of diabetes complications that inhibit the prostacyclin receptor function and cause increased thrombocyte activation and aggregation (9,10). Body mineral values are ideally determined by measuring their tissue levels.

In the present study, we aimed to evaluate the tissue trace element levels by measuring the intra-erythrocyte Mg (eMg_1), Zn (eZn_1), and copper (eCu_1) levels and the urinary Mg excretion level following the intravenous (iv) Mg tolerance test in children and adolescents diagnosed with T1DM in our pediatric endocrinology department.

Methods

Forty-three children and adolescents diagnosed with T1DM between June 2012 and March 2013 in the Department of Pediatric Endocrinology at Eskişehir Osmangazi University Faculty of Medicine (group 1) were included in the study. The inclusion criteria were as follows: (1) age under 18 years, (2) diagnosis of T1DM at least six months prior to admission, and (3) no concomitant disease apart from T1DM. The study also included 25 healthy children and adolescents without any chronic disease as a control group (group 2).

The study protocol was approved by the Ethics Committee of Osmangazi University Faculty of Medicine. Informed consent was obtained from all included children and their parents. A detailed physical examination was performed both in the study and control groups. Systolic/diastolic blood pressure (BP) was measured, and cases with a BP ≥95th percentile were considered hypertensive (11). Body weight (BW) and height were measured in all cases. Height was measured by length gauge scale (Harpenden, Holtain, Crymych, UK), and BW was measured by portable scale (SECA 762; Vogel&Halke, Hamburg, Germany). Body weight and height percentiles were estimated using the age- and gender-appropriate growth curves for Turkish children (12). Body mass index (BMI) was calculated as weight in kilograms divided by the square of length. Cases with ≥95th percentile of BM were considered obese (13).

In both the study and control groups, following a 12-hour fasting period, baseline venous blood specimens were taken for determination of fasting blood glucose, urea nitrogen (BUN), creatinine, calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and serum Mg levels. Blood specimens were also taken into 2 mL tubes containing EDTA for measurement of eMg_1, eZn_1, and copper (eCu_1) levels.

Hemoglobin A1c (HbA1c) levels were measured in all cases in the study group. HbA1c levels of 6.06%-7.4%, 7.5%-9.0%, and >9.0% indicated satisfactory, mediocre, and poor control, respectively (14). Among the group 1 cases, retinal examination was performed by an ophthalmologist.

In group 1, the 24 h urine specimens were collected into plastic tubes that did not contain any metal for determination of microalbumin, creatinine, and urinary Mg (uMg_1) levels. In the control group, urinary Mg level was estimated in spot urine samples.

Following the 24 h urine collection, the patient group was administered 0.2 mEq/kg of elemental Mg. This was given as a 4 h iv infusion of a 15% magnesium sulfate ($MgSO_4$) in a 5% dextrose solution. For ethical reasons, iv $MgSO_4$ was not given to the control group. In the patient group, for measurements of volume, creatinine, microalbumin, and uMg_2 levels, 24 h urine specimens were again collected after starting the $MgSO_4$ infusion. At the end of the second 24 h urine collection, eMg_2, eZn_2, and eCu_2 levels were measured again. At the end of the

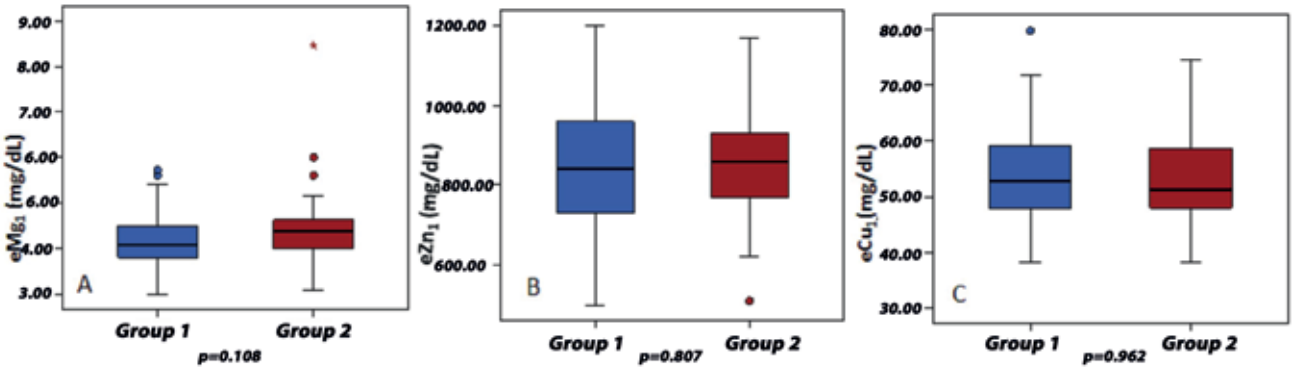

Figure 1. Intergroup (A) intra-erythrocyte magnesium, (B) intra-erythrocyte zinc, and (C) intra-erythrocyte copper levels

iv $MgSO_4$ infusion, Mg retention was estimated as follows (15):

$$\text{Mg retention (\%)} = 1 - \frac{A - (B \times C)}{D} \times 100$$

A: Mg level in 24 h urine after iv $MgSO_4$
B: Mg/creatinine ratio in 24 h urine before iv $MgSO_4$
C: Urinary creatinine level after iv $MgSO_4$
D: Amount of total elemental Mg infusion

A Mg retention level of >50% indicated deficiency. A possible Mg deficiency was considered when the Mg retention level was 25%-50%. A normal Mg level was considered when the retention level was <25%.

Laboratory Analysis

eMg_1, eCu_1, eZn_1, and urinary Mg levels were measured using the atomic absorption spectrophotometer (Analyst 100 Flame, Perkin-Elmer). Glucose, Ca, P, ALP, BUN, TG, HDL-C, LDL-C, and Mg levels were estimated using the enzymatic calorimetrical method with Roche Modular Equipment. Serum and 24 h urinary creatinine levels were measured using the kinetic colorimetric method, HbA1c was measured using the turbidimetric inhibition method, and the microalbumin level was measured using the immune turbidimetric method in 24 h urine.

Statistical Analysis

Data were analysed using the Statistical Package for the Social Sciences 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). The variable distribution was specified using the Kolmogorov-Smirnov test. The parametrical variables were expressed as mean ± standard deviation, and the non-parametrical variables were expressed as median (minimum-maximum). For intergroup comparison, the normally distributed variables were compared using independent samples t-test, and the non-normally distributed variables were compared using the Mann-Whitney U-test. For intragroup comparison, paired samples t-test and Wilcoxon rank test were performed, respectively. The inter-variable associations were determined by the Pearson and Spearman correlations. The intergroup comparison for the qualitative parameters was performed using the chi-square test. The cut-off value was estimated using the receiver operating characteristic (ROC) curve analysis. $p<0.05$ was considered statistically significant.

Results

Among the T1DM patients (group 1), 19 (44.2%) were girls and 24 were boys (55.8%). Mean age was 12.8±3.2 years. In the control group (group 2), 17 (68%) were girls and 8 were boys (32%). Mean age was 12.4±4.5 years. Table 1 shows the demographic and anthropometrical characteristics of the two groups. The patient group received insulin lispro three times daily and insulin glargine once a day, and the daily total insulin dose was 0.7 U/kg/day-1.5 U/kg/day. Of these patients, according to HbA1c levels, 4 (9.3%) had well-controlled diabetes, 20 (46.5%) had mediocre-controlled diabetes, and 19 (44.2%) had poorly controlled diabetes.

No retinopathy was seen in the cases with T1DM. Fourteen of the cases (32.6%) had nephropathy at the microalbuminuric level. Table 2 shows the comparison between serum Mg and other blood parameters by group. No significant difference was found in the baseline eMg_1, eZn_1, eCu_1, and uMg_1 levels between the two groups (Figure 1, Table 2). When the ROC curve analysis was performed for eMg_1, eZn_1, eCu_1, and uMg_1, the area under curve was 0.613 [p=0.073, 95% confidence interval (CI): 0.470-0.756] for Mg, 0.526 (p=0.727, 95% CI: 0.383-0.668) for Zn, and 0.488 (p=0.869, 95% CI: 0.345-0.631) for Cu. Based on these values, the cut-off was estimated as 4.21 mg/dL for Mg and 845 µg/dL for Zn. When the normal and low levels were determined based on the cut-off values, the rate of low eMg_1 level was higher in group 1 than in group 2. The rate of low eZn_1 did not differ between the groups (Table 3).

Table 4 shows the eMg_1, Zn, and Cu levels before and after iv $MgSO_4$ infusion in group 1. These levels were significantly increased ($p<0.001$) after the iv Mg tolerance test. The Mg excretion ratio of 90.2±6.1% was measured from the urine collected after the iv $MgSO_4$ infusion. In the study group, the overall ratio of Mg excretion was >50%.

No statistically significant difference was found in the eMg_1, eZn_1, eCu_1, and uMg_1 levels among patients based on their diabetic metabolic control state according to HbA1c levels ($p>0.05$), as shown in Table 5. Mg excretion ratios were similar in patients with well-controlled (89.3±6.9%) and poorly controlled (91.3±4.5%) diabetes (p=0.287). A positive correlation was found between HbA1c and urinary microalbumin/creatinine ratios (r=0.442, p=0.003). However, no statistically significant association was found between urinary microalbumin/creatinine ratios and eMg_1 or uMg_1 levels (r=0.068, p=0.663 and r=0.044, p=0.780, respectively).

Discussion

There is a growing interest for studies aiming to clarify the role of trace elements in the etiopathogenesis and complications of diabetes mellitus. The body reserve of trace elements is ideally measured at tissue level (16). Mg tolerance test is a reliable method that can well demonstrate the Mg level in tissue, but it requires short-term hospitalisation (17). In the present study, we found no statistically significant difference in the eMg_1, eZn_1, and eCu_1 levels between patients and control subjects. Multiple factors may lead to Mg deficiency in T1DM patients. Possibly, the most important mechanism is the urinary Mg loss resulting from osmotic diuresis due to the hyperglycemia. Taurine deficiency, changes in vitamin D metabolism, intestinal absorption inadequacy, defects in glutathione metabolism are the other important factors (1,18,19). Recently, numerous studies have

reported lower Mg levels in the plasma and tissue of diabetics (1,2,8). In our study, although serum Mg and eMg_1 levels were similar in both groups, eMg_1 tended to be lower in patients with T1DM than in the controls. Consistent with our study, Rohn et al (19) did not find any significant difference in the eMg_1 levels between patients and controls, but they found a similar tendency for Mg deficiency in patients with T1DM. Sjögren et al (20) reported that eMg_1 measurement was inadequate in evaluating the total body Mg reserve and that intra-leukocyte muscle Mg measurement could be a better method. Resnick et al (21) examined intracellular Mg deficiency among diabetics and found significantly decreased serum ionised Mg levels in these patients. No significant difference was found in the eMg_1 level and urinary Mg excretion between the two groups in our study. This finding may be due to the sufficient dietary Mg intake of the patient group included in this study. In some studies in which normal trace element levels were found among diabetics, this finding was also associated with sufficient dietary Mg intake (22,23).

Table 1. Demographic and anthropometric characteristics in the study and control groups

	Group 1 (n=43)	**Group 2 (n=25)**	**p**
Age (years)*	12.8±3.2	12.4±4.5	0.691
Gender (G/B)	19/24	17/8	0.100
Weight SDS**	0.22 (-4–2.36)	0.41 (-1.24–2.43)	0.294
Height SDS**	-0.09 (-3.48–2.23)	0.41 (-1.57–1.68)	0.079
BMI SDS**	0.32 (-2.97–2.82)	0.53 (-1.33–2.22)	0.824
Systolic BP (mmHg)**	100 (90-120)	100 (60-130)	0.608
Diastolic BP (mmHg)**	60 (50-80)	60 (55-80)	0.242
DM duration (years)	2.5 (0.5-17)	-	
HbA1c (%)	8.8 (6.4-14.4)	-	

*: mean ± standard deviation, **: median (minimum-maximum), G/B: girls/boys, SDS: standard deviation score, BMI: body mass index, BP: blood pressure, DM: diabetes mellitus, HbA1c: glycated hemoglobin

Table 2. Comparison of biochemical parameters by group

	Group 1 (n=43)	**Group 2 (n=25)**	**p**
BUN (mg/dL)*	13.9±3.1	12.1±4.6	0.056
Creatinin (mg/dL)*	0.57±0.13	0.58±0.14	0.728
Glucose (mg/dL)*	224.0±110.9	87.2±10.7	<0.001
Ca (mg/dL)**	9.0 (8.7-9.7)	9.7 (8.9-10.2)	0.903
P (mg/dL)*	4.6±0.7	4.6±0.8	0.946
ALP (IU/L)*	553.2±267.9	341.1±183.4	<0.001
HDL-C (mg/dL)**	52 (23-90)	57 (24-103)	0.990
LDL-C (mg/dL)*	93.5±31.3	86.8±32.3	0.401
TC (mg/dL)*	166.1±31.6	157.9±38.3	0.345
TG (mg/dL)*	91.7±49.4	81.3±31.1	0.061
Serum Mg (mg/dL)*	0.78±0.05	0.79±0.08	0.575
eMg_1 (mg/dL)*	4.16±0.63	4.5±1.0	0.108
eZn_1 (mg/dL)*	836.5±161.9	846.4±156.2	0.807
eCu_1 (mg/dL)*	54.1±9.2	53.9±9.0	0.962
uMg_1 (mg/L)	92 (4-254)	81.6 (20-318)	0.316

*: mean ± standard deviation, **: median (minimum-maximum), BUN: blood urea nitrogen, Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, TC: total cholesterol, TG: triglyceride, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, eMg_1: intra-erythrocyte magnesium, eCu_1: intra-erythrocyte copper, eZn_1: intra-erythrocyte zinc, uMg: urinary magnesium

In some previous studies, Mg deficiency was implicated in the development of micro-and macro-vascular complications of diabetes (8,24,25). Hypomagnesemia is known to be associated with dyslipidemia, inflammatory load, and increased oxidative stress (26). The serum Mg level was found to be low in diabetic patients with concomitant hypertension, ischemic heart disease (27), and severe diabetic retinopathy (28). Mg deficiency accelerated atherosclerosis and vascular damage in experimental animals, and it has been reported that Mg replacement decreased the development of atherosclerotic lesions, thus reducing serum cholesterol and triglyceride levels (29,30). Atabek et al (8) showed that Mg deficiency was related to atherosclerotic changes independent of lipid levels in children with T1DM. We did not find a significant association between urinary microalbumin/creatinine ratios and eMg_1 or urinary microalbumin/creatinine ratios and uMg_1 levels.

In the study of Schnack et al (31), serum and eMg_1 levels were inversely correlated with metabolic control among diabetics. Such a correlation was not found in our study, and there were also no significant differences in eMg_1 levels between the patient and the control groups. We therefore suggest that the measurement of the intra-erythrocyte or the urinary Mg level may be insufficient for evaluating the total body Mg reserve in diabetic patients. The results of Sjögren et al (20) who also did not find any correlation between HbA1c and eMg_1 or urinary Mg excretion, but found a significant correlation between muscle and the mononuclear intracellular Mg and HbA1c, support our suggestion.

In the present study, although the eMg_1 level seems to be unaffected in diabetic patients, the mean urinary Mg retention of 90.2±6.1% and the significant increase in the eMg_1, Zn and Cu levels following iv $MgSO_4$ administration suggest an insufficient total body Mg reserve. Increased retention following the iv $MgSO_4$ tolerance test is superior to other methods in demonstrating Mg deficiency (32,33). Similarly, Simşek et al (15) found >50% Mg retention in 43% of patients with T1DM. By contrast, some studies found that increased urinary Mg excretion was not related to serum Mg concentration (34).

Mg shows multiple effects by acting on bone mineral homeostasis, on stabilisation of the crystal structure as well as on calcium metabolism and ALP efficiency. ALP is known

Table 3. Numbers (ratios) of decreased intra-erythrocyte magnesium and intra-erythrocyte zinc levels by group

		Group 1	Group 2	p
eMg_1	Normal	17 (39.5%)	17 (68%)	0.024
	Low	26 (60.5%)	8 (32%)	
eZn_1	Normal	22 (51.2%)	14 (56%)	0.700
	Low	21 (48.8%)	11 (44%)	

eMg_1: intra-erythrocyte magnesium, eZn_1: intra-erythrocyte zinc

Table 4. Intra-erythrocyte trace element levels before and after iv magnesium infusion in the patient group

	Before iv magnesium	After iv magnesium	p
$Magnesium_{erythrocyte*}$ (mg/dL)*	4.16±0.63	4.32±0.65	<0.001
$Zinc_{erythrocyte*}$ (mg/dL)*	836.5±161.9	855.1±182.0	<0.001
$Copper_{erythrocyte*}$ (mg/dL)*	54.1±9.2	54.3±8.7	<0.001
$Magnesium_{urine*}$ (mg/day)	134.8±73.2	289.1±138.6	<0.001

*: paired sample t-test, iv: intravenous

Table 5. Differences in trace element levels by state of metabolic control

	Well-mediocre controlled (n=24)	Poorly controlled (n=19)	p
$Magnesium_{erythrocyte*}$ (mg/dL)*	4.2±0.5	4.1±0.7	0.633
$Zinc_{erythrocyte*}$ (mg/dL)*	827.9±162.1	847.3±165.4	0.701
$Copper_{erythrocyte*}$ (mg/dL)*	54.6±8.4	53.2±10.3	0.627
$Magnesium_{urine*}$ (mg/day)	140.5±74.8	127.5±72.4	0.572

*: paired sample t-test

to express during the early stage of stiff bone tissue formation in bone and calcified cartilage. Although the effects of ALP on bone mineralisation are not fully understood, ALP is considered to enable the increase in inorganic phosphate (35). In our study, while the intracellular increase in trace elements after iv $MgSO_4$ application indicated total body deficiency, a statistically significant increase in ALP supported the presence of such a deficiency in the diabetic group. ALP may increase to provide adequate functioning during Mg deficiency when the major portion of the Mg is present in the bone tissue. These results suggested that eMg_1 level remained incapable to demonstrate the total body Mg concentration in diabetic patients.

In conclusion, eMg_1 measurement cannot reveal Mg deficiency, but increased retention following iv Mg tolerance test indicates intracellular Mg deficiency in patients with T1DM. Moreover, if Mg deficiency is detected by the Mg retention test, which should be performed at least once a year, then Mg replacement therapy may help to provide glycemic control in poorly controlled T1DM patients.

Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of Osmangazi University Faculty of Medicine, Informed Consent: Informed consent was obtained from all included children and their parents.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Enver Şimşek, Design: Enver Şimşek, Data Collection or Processing: Vahap Uğurlu, Çiğdem Binay, Enver Şimşek, Analysis or Interpretation: Cengiz Bal, Enver Şimşek, Vahap Uğurlu, Çiğdem Binay, Literature Search: Vahap Uğurlu, Çiğdem Binay, Writing: Vahap Uğurlu, Çiğdem Binay, Enver Şimşek.

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Original Article

Neonatal Thyroid-Stimulating Hormone Screening as a Monitoring Tool for Iodine Deficiency in Turkey

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ABSTRACT

Objective: Thyroid-stimulating hormone (TSH) level in neonates is recommended as an indicator for presence of iodine deficiency (ID) at a population level and as a monitoring tool in programs of iodine supplementation. The purpose of this study, based on data from the National Newborn Screening Program (NNSP) for congenital hypothyroidism (CH) in 2014, was to analyze neonatal TSH levels to predict the current status of iodine nutrition in Turkey.

Methods: According to screening methodology, heel-prick blood samples of newborns were collected on filter paper cards usually on day 3-5 after birth (or shortly before discharge). Results of samples collected >48 h after birth were analyzed. The degree of severity of ID was assessed by using the epidemiologic criteria of the World Health Organization (WHO). Elevated TSH levels (>5 mIU/L) were processed and classified according to province, region, birth season, and sampling time.

Results: A total of 1,298531 newborns were registered in the NNSP for the CH database. Of those, 1,270311 newborns had screening results collected >48 h after birth and were included in the statistical analyses. The national prevalence of elevated TSH was 7.2%. While the Gaziantep sub-region had the highest TSH elevation rate (15.9%), the Tekirdağ sub-region had the lowest rate (4.0%; $p<0.001$). Seasonal variations were also significant, and the elevated TSH prevalence rate was highest in winter (7.4%; $p<0.001$).

Conclusion: National CH screening results suggest that Turkey may still be mildly iodine deficient. Nationwide studies should be performed for direct assessment and monitoring of iodine status in vulnerable populations to confirm accuracy of our results.

Keywords: Thyroid-stimulating hormone, screening program, Turkey, newborn

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Iodine deficiency (ID) is one of the most prevalent deficiencies throughout the world and can cause brain damage in newborns. Thyroid-stimulating hormone level in neonates is recommended as an indicator of the degree of ID at a population level and as a monitoring tool in programs of iodine supplementation where a screening program is in force.

WHAT THIS STUDY ADDS?

According to the results of the newborn screening program for congenital hypothyroidism and using the World Health Organization guidelines, Turkey could be classified as mildly iodine deficient and iodine prophylaxis may be insufficient in vulnerable populations.

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Introduction

Iodine deficiency (ID) is one of the most prevalent deficiencies throughout the world and can cause brain damage in newborns; yet, it is easily preventable (1). Serious ID during pregnancy can result in a spectrum of morbidities referred to as ID disorders (IDD) including goiter, hypothyroidism, cretinism, mental retardation and delayed physical development, spontaneous abortion, stillbirth, congenital anomalies, and increased perinatal and infant mortality (1,2).

In order to prevent and treat IDD, universal salt iodization was adopted by the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), and the International Council for the Control of IDD (ICCIDD) in 1993 (3). Four major methods are recommended to assess and monitor the iodine nutritional status of a population: measurement of urinary iodine concentration (UIC), total goiter prevalence by palpation or ultrasonography, and measurement of serum thyroglobulin and thyroid-stimulating hormone (TSH) levels in neonates (4,5,6). TSH level in neonates is recommended as an indicator of the degree of ID at a population level and as a monitoring tool in programs of iodine supplementation where a screening program is in force (5,7,8).

In Turkey, the national IDD control program and mandatory salt iodization began in 1998. Before the initiation of the program, a survey was carried out between 1997 and 1999 and the median UIC in school age children (SAC) was found to be 25.5 µg/L, a finding which indicated presence of moderate ID (9). The main nationwide monitoring method of the program has been UIC in SAC; after implementation of the salt iodization program, the median UIC of SAC increased to 87 µg/L in 2002, to 117 µg/L in 2004, and to 130 µg/L in 2007 (10,11). Although these results suggest that iodine nutrition in the Turkish population has shown a gradual improvement, moderate to severe ID still exists in 27.8% of the Turkish population, mostly in rural areas (11).

The purpose of this study, based on the National Newborn Screening Program (NNSP) for congenital hypothyroidism (CH) 2014 database, was to analyze neonatal TSH levels to predict the current status of iodine nutrition in Turkey.

Methods

In Turkey, the nationwide screening program for CH was initiated in December 2006 by the Public Health Institute (PHI) of the Turkish Ministry of Health, in cooperation with a scientific committee consisting of members of universities and of governmental and non-governmental organizations. Since its initiation, both screening methodology and implementation of the program has gradually improved and the NSSP database has provided an opportunity to evaluate nationwide neonatal TSH levels (12).

Consistent with screening methodology, heel-prick blood samples from newborns were collected on filter paper cards (Whatman 903 filter paper) usually on day 3-5 after birth, or shortly before discharge if earlier discharge was planned. If the first sample was collected <48 h after birth, newborns were referred to a family medicine outpatient clinic for a second sample to be taken on day 3-5. For this manuscript, samples collected >48 h after birth were called timely samples and samples collected <48 h after birth were called early samples.

The filter paper cards were air-dried at room temperature and sent to one of the two laboratories of the PHI (in Ankara and İstanbul) for testing on either of two week days. These cards contained information on ID number, residence, birth province, contact address, date of birth, and date of sampling. The samples were tested within three working days after being received. TSH was detected with Trimaris neonatal TSH FEIA kits using the filter paper blood. Fluorescent enzyme immunoassay based on the TSH-specific two monoclonal antibody sandwich principle was used. The sensitivity of the TSH assay was 0.5-1.1 µIU/mL.

The degree of severity of ID was assessed by using epidemiologic criteria from the WHO (4). These criteria are based on the proportion of newborns with a TSH of >5 mIU/L whole blood: in iodine-sufficient areas <3%; mild 3-19.9%; moderate 20-39.9%, or severe >40% deficiency (4). Elevated TSH levels (>5 mIU/L) were processed and classified according to province, sub-region, birth season, and sampling time.

Database

All personal information and screening data of newborns are registered in the NNSP database. Neonatal TSH screening data and other details were obtained from this system. All data were reviewed and statistical analyses were performed by the working group. Improbable records or those with missing descriptive information were excluded. Early screening results were analyzed separately. Results of samples collected >48 h after birth were used in the main statistical analyses.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS; Version 18.0) and Excel (Microsoft Office Excel 2007) software were used for data processing and statistics. The Kolmogorov-Smirnov test was used to determine normal distribution. Descriptive statistics were presented as mean ± standard deviation (SD) for normally distributed data, and as counts and percentages for categorical data. The relationship between the categorical variables was examined using the chi-square test. Student's t-test was used for the comparison of two groups with normally distributed variables, and the Mann-Whitney U-test was used for abnormally distributed data. For the comparison of three or more groups, one-way analysis of variance (ANOVA) was used for normally distributed variables; otherwise, Kruskal-Wallis variance analysis was used. Results were evaluated with a confidence interval of 95%, and $p<0.05$ was considered statistically significant.

Ethics

The parents of the babies tested were informed about the NNSP and heel-prick blood samples were only collected from live born babies after prior written consent from the parents.

Results

In 2014, 1,298531 newborns were registered with the NNSP for the CH database. Of those, 1,270311 newborns (97.8% of registered newborns; 51.3% boys, 48.7% girls) had timely screening results and were included in statistical analyses and 660946 newborns (50.1% of registered newborns; 51.0% boys, 49.0% girls) had early screening results. Mean sampling time was 7.3 days.

In 2014, the national prevalence of elevated TSH was 7.2%. Elevated TSH prevalence rates of all 26 sub-regions of Turkey were between >3% and 19.9% (in favor of mild ID) (Figure 1). While the Gaziantep sub-region, which is located in southeastern Turkey, had the highest elevated TSH rate (15.9%), the Tekirdağ sub-region, located in the north-western part of Turkey, had the lowest rate (4.0%; Figure 1). The difference between sub-regions was statistically significant (p<0.001). At the provincial level, Gaziantep had the highest (17.7%) and Tekirdağ had the lowest (3.2%) elevated TSH levels. The difference between provinces in terms of elevated TSH level was also significant (p<0.001). The distribution of the number of 81 provinces according to elevated TSH level is presented in Figure 2.

Samples drawn from the NNSP of Turkey were also assessed by birth season. Elevated TSH prevalence rate was highest in winter (7.4%). Seasonal variations were significantly different (p<0.001) and are presented in Figure 3.

Elevated TSH prevalence rates of early samples were also analyzed and compared with the results of timely samples. While the elevated TSH prevalence rate of timely samples was 7.2%, the elevated TSH prevalence rate of early samples was 40.6%; this difference was significant (p<0.001). The cumulative frequency distribution of neonatal blood TSH values according to sampling time is presented in Figure 4.

Figure 1. Elevated neonatal thyroid-stimulating hormone prevalence rates in 26 subregions of Turkey in 2014

Discussion

Historically, after the pioneering studies performed in Zaire and India where ID is endemic, neonatal TSH screening was recommended as a population monitoring tool for ID in addition to its role as a case-detection tool for diagnosing individual neonates with CH (7,8,13,14). This tool has been used to assess the severity of ID and also to monitor the outcome of iodine prophylaxis programs in countries or sub-national regions (7,15,16,17,18,19,20,21,22). Although some of them have provided conflicting results especially in cut off values (15,16), there are many successful country examples (17,18,19,20,21,22). In Thailand, with the application of a geographic information system to their neonatal TSH screening program, it has been possible to identify ID down to the sub-district level. Results of

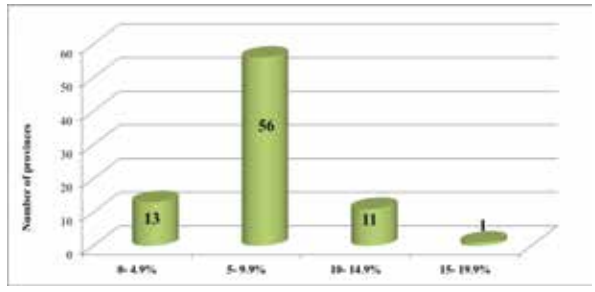


Figure 2. Distribution of the number of provinces according to elevated thyroid-stimulating hormone percentage figures

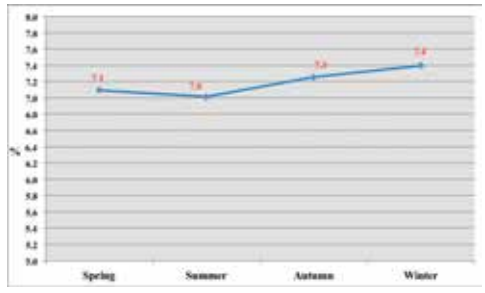
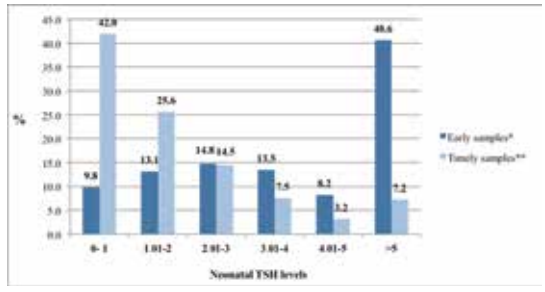


Figure 3. Seasonal variations of elevated neonatal thyroid-stimulating hormone levels



*Early samples: Samples collected <48 h after birth (n=660946)
**Timely samples: Samples collected >48 h after birth (n=1,270311)

Figure 4. Cumulative frequency distribution of neonatal blood thyroid-stimulating hormone values according to sampling time.
TSH: thyroid-stimulating hormone

that study show that all provinces in Thailand suffer from ID at mild to moderate levels and the degree of severity increases year by year (17). In a study from southeast Poland, Tylek-Leman´ska et al (18) demonstrated that with the reintroduction of iodized salt in 1992, the prevalence of neonatal TSH results >5 mIU/L dropped from above 20% in 1991 to just over 5% between 1995 and 2000. The authors concluded that between 1985-2000, a drop in the incidence of IDD in newborns was clearly seen; furthermore, even low-grade iodine supplementation led to a significant decrease in TSH levels in newborns (18). In another study from Switzerland, Zimmermann et al (21) demonstrated that a 25% increase of iodine concentration in iodized salt resulted in a reduction of the neonatal frequency of TSH values >5 mIU/L from 2.9% to 1.7%, and iodine nutrition in children and pregnant women has improved from marginal to clearly sufficient.

Although the results of previous monitoring studies based on UIC in SAC have shown that Turkey is iodine replete, elevated TSH prevalence rates of the national CH screening results were in favor of mild ID (10,12). In Turkey, there are several previous regional studies based on CH screening and neonatal TSH levels (23,24,25,26,27). In a study on incidence of CH in the West Black Sea area (Bolu, Düzce, and Zonguldak provinces), elevated blood TSH (>5 mIU/L) concentrations were 26.7% and recall rate was 1.6% between 2000 and 2002 (24). In another study from Bursa province, between 1995 and 2004, Sağlam et al (25) reported a 5.8% recall rate and a 1/840 CH incidence, which could be explained by existence of ID. Another study on cord blood TSH of newborns showed a high frequency of elevated TSH concentrations (frequency of >10 mIU/L 28%) (26). In another recent study investigating the role of ID in the etiology of CH, ID frequency was 36% in CH patients and 88% in their mothers (27). Although these studies have differences in terms of methodology and study design, data based on regional CH screening also indicate that ID may still be a public health problem in Turkey.

In our study, according to the WHO guidelines, the lowest frequency for elevated TSH levels was detected in the northwest coastal areas of the country (Figure 1). This region is one of the most industrialized and urbanized areas of Turkey. Elevated TSH prevalence rate was highest in the inland areas and the south of the country (Figure 1). Furthermore, elevated TSH prevalence rate was high in winter compared to other seasons (Figure 3). The differences by region and season may be attributable to changes in regional and seasonal food preferences, differences in use of iodized salt, use of rock salt especially in local foods, and different agricultural practices.

The appropriate time of sampling for CH screening is between 48 h to 4 days; early sampling is not recommended due to the neonatal surge in the first 24 h after birth (28). Evidence shows that the mean TSH level in samples taken less than 24 h after birth was significantly higher than the mean TSH level of neonates after the first 24 h (29). In accordance with the recommendations, we found that elevated TSH prevalence rates of early samples were significantly higher than those of timely samples ($p<0.001$). Additionally, the cumulative frequency distribution of neonatal blood TSH values of early samples was considerably different from those of timely samples (Figure 4). In Turkey, both early heel-prick blood samples before discharge and day 3-5 heel blood samples were collected to achieve higher screening coverage by the end of 2014. Eventually, NNSP coverage reached 99% in 2014 and, due to the high costs and increased workload, early samples were stopped at the beginning of 2015.

Our study had some limitations. We had no data on maternal and newborn UIC and we could not correlate them with TSH results. Some factors other than ID (prematurity, birth weight, mode of delivery, maternal or newborn exposure to iodine-containing antiseptics, etc.) can affect newborn TSH levels (30). In this study, it was not possible to demonstrate the effect of these factors.

Finally, results of the national CH screening program suggested that iodine prophylaxis may be insufficient in vulnerable populations and we suggest that the following recommendations be taken into consideration in future work:

1- Nationwide studies should be performed for direct assessment and monitoring of iodine status in pregnant women, nursing mothers, and newborns in addition to systematic monitoring studies assessing the iodine status in SAC.

2- Maternal and newborn UIC and national neonatal TSH screening results should be correlated to determine whether neonatal TSH results can or cannot be used as a monitoring tool for the salt iodization program.

3- Regional and seasonal differences should be investigated.

4- New studies should be planned to determine factors affecting our national neonatal TSH levels other than ID.

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Ethics

Ethics Committee Approval: It was taken, Informed Consent: The parents of the babies tested were informed about the NNSP and heel-prick blood samples were only collected from live born babies after prior written consent from the parents.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Sema Özbaş, Nilgün Çaylan, Bekir Keskinkılıç, Design: Nilgün Çaylan, Başak Tezel, Data Collection and/or Processing: Nuran Şahin, Deniz Acıcan, Analysis and/or Interpretation: Başak Tezel, Nuran Şahin, Nilgün Çaylan, Şirin

Aydın, Literature Research: Nilgün Çaylan, Nuran Şahin, Şirin Aydın, Writing: Nilgün Çaylan, Başak Tezel, Sema Özbaş.

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Original Article

Menstrual Characteristics of Pubertal Girls: A Questionnaire-Based Study in Turkey

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ABSTRACT

Objective: Clinicians should show an awareness on the menstrual characteristics of adolescent girls which may differ from adults in some aspects. To define menstrual cycle features among high school girls residing in a city center in southeastern Turkey.

Methods: A cross-sectional survey was conducted on 1256 girls attending a high school located in the city center of Elazığ, Turkey. Data from 879 girls (median age, 16.2 years; range, 13.6-19.2 years) who agreed to participate in the study and had started to menstruate were evaluated.

Results: Mean age at menarche was 12.7±1.3 years (range, 8.2-17.3 years). The mean cycle duration was 28.7±4.4 days, and the mean menstrual flow lasted 5.9±1.3 days. Severe, moderate, and mild dysmenorrhea was reported in 29%, 43%, and 28% of the girls, respectively, and 52% used analgesics for dysmenorrhea. A total of 34% of the girls defined their menstrual cycle as irregular, and 32% reported school absenteeism due to menstruation-associated complaints (pain and/or heavy bleeding). Menstrual bleeding affected attendance to classes and other school activities, daily work, social, family, and friend relationships, as well as sports/exercise activities in 43%, 49%, 58%, 48%, 44%, and 60% of the participants, respectively. In total, 30% of the responders had a problem with menstruation, and 12% and 17% of these stated that they consulted a primary care physician or specialist, respectively.

Conclusion: Dysmenorrhea was found to be common in adolescent Turkish girls and to affect daily life in approximately half of the girls.

Keywords: Adolescent, menstrual characteristics, dysmenorrhea

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Menstrual-associated complaints are observed commonly in adolescents. In particular, pain accompanying the menstrual period is the most frequently reported complaint.

WHAT THIS STUDY ADDS?

This study determined the menstrual characteristics of pubertal adolescent Turkish girls and the association between menstrual cycle features and interference with life activities.

Introduction

Puberty is the period of human development during which secondary sexual characteristics appear, sexual maturation occurs, and reproductive capacity is attained. Ovulation and menstruation begin in girls during this period (1). Although this period usually

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passes without important problems, menstruation-associated complaints are observed commonly in adolescents. In particular, pain accompanying the menstrual period is the most frequently reported complaint (2,3,4,5,6,7,8). Irregular menstrual cycles are also reported frequently during the first years after menarche, possibly due to immaturation of the hypothalamic-pituitary-gonadal axis in the young adolescent (2,4). Physical, psychological, and emotional symptoms are also observed before and during menstruation in almost all adolescents (4,9,10,11). Mainly pain and also other symptoms occurring during menstruation affect daily life activities, can decrease school performance, and also increase the rate of school absenteeism (2,3,4,6,7,8,12).

In this study, we attempted to define the typical menstrual characteristics of adolescent girls residing in Elazığ city, Turkey and to investigate the effects of menstruation-associated complaints on life activities.

Methods

Subjects and Setting

The study was conducted in 2014 and consisted of a cross-sectional survey on girls attending six high schools randomly selected from different locations in the city center of Elazığ, Turkey. Questionnaires were distributed to 1256 girl students in grades 9-12 to be completed at home and 911 (72.5%) returned the completed questionnaire. Thirty-two students whose menstrual periods had not yet started were excluded from the analysis. Data from 879 pubescent girls who agreed to participate in the study and who had started to menstruate (median age, 16.2 years; range, 13.6-19.2 years) were evaluated.

The answers were transferred to an electronic database.

Questionnaire Form

The questionnaire form was prepared in Turkish and was based on a form used previously by Parker et al (4). The questionnaire was tried and assessed in 10 high school girls prior to the study. Based on the feedback, some complex statements were revised, and other questions were excluded. The final questionnaire comprised 82 questions divided into six sections. Students were asked to select from the options yes/no or true/false or to use a 1-10 scale for their answers. The questionnaire took about 20 min or less to complete. Menstrual pattern, duration and intensity of menstruation, school absenteeism due to menstruation, pain during the menstrual cycle, use of painkillers, physical-emotional symptoms during the menstrual cycle, and the effects of menstruation on various life activities were evaluated by the questionnaire. The menstrual period was considered regular if it occurred at intervals of 20-45 days. Pain during menstruation was categorized as severe (8-10 on a 0–10-point scale), moderate (4-7), or mild or no pain (0-3).

Ethical Evaluation and Statistical Analysis

The study protocol was approved by the Non-Interventional Studies Ethics Committee of Fırat University. Written informed consent was obtained from parents and the participating children.

The data were evaluated using the Statistical Package for the Social Sciences 18.0 program (SPSS Inc., Chicago, IL, USA). Incidence data are presented as percentages, and numerical data are expressed as means ± standard deviation. The chi-square test was used to compare categorical data, and the t-test was used to compare the numeric data. Statistical significance was accepted at $p<0.05$.

Results

The subjects had started their menstrual cycles 3.5±1.6 years previously. Mean age at menarche was 12.7±1.3 years (range, 8.2-17.3 years); 22 girls (2.5%) had experienced their first period before age 10 years, and 14 (1.6%) after age 15 years. The mean menstrual period duration was 28.7±4.4 days, and the mean length of menstruation was 5.9±1.3 days (range, 3-10 days) in 581 (66.1%) girls who defined their periods as regular. Approximately 62% (508/818) of the girls stated that their menstrual flow contained clots, whereas 5.2% (43/824) and 8.0% (66/824) stated that they experienced spotting before and in the middle of their menstrual period, respectively. Approximately 24% (213/879) of the girls defined their periods as irregular. Age at menarche and chronological age were comparable in girls with regular periods compared with those with irregular periods ($p>0.05$). Girls with irregular periods had a shorter duration of menstrual flow than did those with regular periods (3.2±1.6 vs. 3.5±1.5 days, respectively) ($p<0.05$). The girls who had reached menarche >2 years before the present study had significantly fewer irregular periods (48/506; 9.6%) compared to girls who had reached menarche ≤2 years before the study (165/373; 44.6%) ($p<0.001$).

The 10 most frequently reported symptoms are listed in Table 1.

Pain During Menstruation (Dysmenorrhea)

Approximately 92% of the responders had dysmenorrhea, and the pain was reported as severe, moderate, and mild or no pain in 28.8% (245/850), 43.3% (368/850), and 27.9% (237/850) of the subjects, respectively. Approximately 52% (412/793) of the girls reported that they used analgesics on their own during their menstrual cycle. Paracetamol was the commonest analgesic used (76%; 290/382), whereas 20% (77/382) and 3% (11/382) reported that they took oral and parenteral nonsteroidal anti-inflammatory drugs (NSAIDs). The data showed that 70% (319/457) of the girls who reported using an analgesic obtained a moderate to good (range, 4-10 on a 10-point scale) analgesic response from the drug. Only 1% (4/382) of the subjects had consulted a healthcare facility

because of menstruation-related pain. Sixty-three (7.5%) girls reported that they never experienced pain during their period.

Menstrual Cycle-Related Problems

Approximately 12.0% (97/811), 16.7% (137/809), and 4.3% (38/807) of the subjects reported that they had consulted a primary-care physician, a specialist, or herbal drug dealer, respectively, for menstrual cycle-related problems. Analgesic tablets were recommended to 59 subjects for use during their menstrual cycle. Five subjects were prescribed oral contraceptive tablets because of a diagnosis of polycystic ovary syndrome.

Effects of Menstruation on Life Activities

Approximately 32% (261/822) of subjects were absent from school due to menstrual cycle-related reasons. Menstruation caused a 1 day absence for most girls (78.1%), but 2- (17.4%) and 3-day absences (4.5%) in 17.4% and 4.5% of the subjects, respectively. The causes for school absenteeism were pain in 88.2%, heavy bleeding in 4.9%, and nausea in 2.3% of the subjects. The effects of menstruation on seven groups of life activities in this group of subjects are shown in Table 2. Complaints related to menstruation and their effects on life activities are given in Table 3.

Self-Perceptions of the Menstruation Cycle

The perceptions of menstruating girls were evaluated in the true/false part of the questionnaire, which consisted of 30 statements. The 10 items most commonly considered to apply to the participant are shown in Table 4.

Discussion

Our results show that the menstrual characteristics of adolescent girls living in Elazığ, Turkey are similar to the typical characteristics reported in other studies (13). The duration of the menstrual cycle varies in adolescent girls, and their periods typically become regular as they age (14). Approximately half of the girls we surveyed who reached menarche <2 years previously reported irregular periods, whereas this number was very low in girls who had started menstruating >2 years ago. The extremes in menstrual cycle length are wider among adolescents as compared to adult women. However, a menstrual cycle beyond the 20-45-day range in young girls is not an expected finding and requires evaluation (13). No subject in our study reported periods more frequent than every 20 days, and a limited number of girls reported that they menstruated less frequently than every 45 days.

Previous studies show that pain usually accompanies menstruation in adolescents, with a reported pain incidence of 39-93% (2,3,4,5,6,7,8,15,16). In our study, the incidence of pain was at the upper limit and is the highest rate reported in Turkey. The frequency of analgesic use during menstruation was similar to rates reported previously (41-80%) (4,5,6). Girls in our cohort used paracetamol most often, which is consistent

Table 1. The 10 most common menstruation-related symptoms reported in the study group

Symptoms	Reported in number of girls	Total number of responders	% (95% CI)
Lower back pain	635	817	78 (75-81)
Fatigue	637	822	77 (75-80)
Malaise	621	814	76 (73-79)
Pelvic pain-moderate	372	560	66 (63-70)
Pelvic pain-stabbing	349	541	65 (60-69)
Abdominal bloating (flatulence)	511	829	62 (58-65)
Altered appetite	476	793	60 (57-63)
Headache	438	807	54 (51-58)
Frequent urge to urinate	370	811	46 (42-49)

CI: confidence interval

Table 2. Effects of menstruation on life activities reported in the study group

	Number of responders	Serious interaction* n (%)	Low interaction** n (%)
Sport and exercise	591	353 (59.7)	238 (40.3)
Social activities	551	318 (57.8)	233 (42.2)
Family relationships	476	293 (49.0)	305 (51.0)
Daily activities	598	230 (48.3)	246 (51.7)
Friendships	482	214 (44.3)	268 (55.7)
School activities	520	224 (43.0)	296 (57.0)
School attendance	371	103 (27.8)	268 (72.2)

*Serious interaction: 5-10 rating on a 10-point scale
**Low interaction: 0-4 rating on a 10-point scale

Table 3. Complaints related to menstruation with serious effects on life activities reported in the study group

Complaint	Number of responders	Serious effect* n (%)	Low or no effect** n (%)
Feeling sick/bad	681	509 (74.3)	172 (25.3)
Malaise	677	501 (74.0)	176 (26.0)
Pain	725	523 (72.1)	202 (27.9)
Exhaustion, fatigue	684	486 (71.1)	198 (28.9)
Heavy bleeding	621	370 (59.6)	251 (40.4)

*Serious effect: 5-10 rating on a 10-point scale
**Low or no effect: 0-4 rating on a 10-point scale

with previous findings, but more recent studies reveal a trend toward use of NSAIDs (4,6,17). A few subjects reported that they suffered from very severe pain during their periods that required a visit to a healthcare facility. These girls should be examined for possible pathologies, such as endometriosis. Parker et al (4) reported that 85% of subjects obtain a moderate-good analgesic response from the painkillers they use. We found a rate of 70%. However, both Parker et al (4) and our studies lacked sufficient data to determine whether the unsatisfactory analgesic responses were caused by too low doses, frequent drug use, or underlying serious pathologies, such as endometriosis.

Approximately one-third of the adolescents in our study reported severe pain during their menstrual cycle, which was similar to rates observed previously (4,6,7,9,15,18). The menstrual cycle-related school absenteeism rate of 32% observed here was also consistent with previous reports (2,3,4,6,7,8,9,12,15,18). Pain was reported to be the most common complaint causing school absenteeism (2,3,4,6,7,8). Similarly, our results showed that severe pain was the most common complaint causing school absenteeism during the menstrual cycle of Turkish adolescent girls. A few girls reported heavy menstrual bleeding as the cause of school absenteeism. We discovered that menstrual pain and symptoms substantially affected daily life activities of these adolescents. Similar results were reported previously in studies conducted in other countries (4,6,7,8).

Puberty is regulated by the hypothalamus through complex genetic mechanisms and is affected by ethnicity, nutritional status, and many other environmental factors. Age at menarche has become younger in developed countries since the mid-20th century, which has been associated with improved nutritional and economic status (19). This trend has stopped, probably because of stabilized socioeconomic status (20). The first study that investigated mean age at menarche in Turkish girls was conducted by Neyzi et al (21) in İstanbul in 1973, who found the mean age at menarche to be 12.4 years. Atay et al (22) conducted a study in İstanbul in 2009 and reported a mean age of menarche of 12.7 years. Our results show a mean age at menarche of 12.7 years, which supports the notion that the trend towards a younger menarche has stopped in Turkey, as reported by Atay et al (22).

To conclude, it may be said that the majority of the adolescent girls evaluated perceived their periods to be normal. However, mild to moderate dysmenorrhea with an impact on school attendance and social life was reported as a common occurrence. More effective and judicious use of painkillers can go a long way in ameliorating these problems. Also increasing awareness and educating adolescent girls and their parents regarding normal periods and related problems would have favorable effects such as a reduction in school absenteeism. Pediatricians interacting with adolescent girls should always discuss the menstrual periods, helping the girls and their families differentiate physiologic from pathologic states.

Ethics

Ethics Committee Approval: This present study was approved by the Ethics Committee of Fırat University in 2013, Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: İhsan Esen, Design: İhsan Esen, Baran Oğuz, Hepsen Mine Serin, Data Collection or Processing: İhsan Esen, Baran Oğuz, Hepsen Mine Serin, Analysis or Interpretation: İhsan Esen, Baran Oğuz, Hepsen Mine Serin, Literature Search: İhsan Esen, Writing: İhsan Esen.

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Table 4. The 10 questionnaire items most commonly considered to apply to the study group

Symptoms	Frequency	Number of responders	% (95% CI)
I usually have my period every month	703	823	85 (83-87)
My periods make me anxious/uncomfortable	547	790	69 (66-72)
I think my periods are usually normal	533	770	69 (66-72)
I talk to a family member about my periods	515	802	64 (61-67)
I think my periods are normal	513	800	64 (61-67)
I get my period every month. I've never missed a month	512	808	63 (60-66)
I become nervous/problematic before and during my period	473	801	59 (56-62)
I want to disappear during my period	411	794	52 (49-55)
I feel suffocated/bored before and during my period and cannot cope	408	802	51 (48-54)
I talk to friends about my period	392	786	50 (47-53)

CI: confidence interval

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Original Article

Clinical and Genetic Characteristics, Management and Long-Term Follow-Up of Turkish Patients with Congenital Hyperinsulinism

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ABSTRACT

Objective: Mutations in the K_{ATP} channel genes is the most common cause of congenital hyperinsulinism (CHI) of infancy. Our aim was to report the clinical and genetic characteristics, treatment modalities, and long-term prognosis of patients with CHI.

Methods: Clinical and biochemical findings, operation procedures, and results of genetic analysis were retrospectively evaluated in 22 CHI patients from two pediatric endocrine centers in Turkey.

Results: Seven of the patients were born large for gestational age. Hypoglycemia was diagnosed within the first 24 hours of life in 9 patients and treatment with diazoxide (n=21) and/or somatostatin (n=8) had been attempted. Seven patients (31.8%) were unresponsive to medical treatment and underwent pancreatectomy. Histological examination of the pancreas confirmed diffuse disease in 6 patients. Diabetes developed in 3 patients following pancreatectomy (10 years, 2.5 years, and immediately after operation). The remaining four patients had neither recurrence of CHI nor of diabetes during the 3.67±0.7 years of follow-up. Sequence analysis identified mutations in 12 out of 19 patients (63%). Mutations in the *ABCC8* gene were the most common finding and were found in 6 out of 7 patients who underwent pancreatectomy. Other mutations included a paternally inherited *KCNJ11* mutation, a homozygous HADH mutation, and a heterozygous GLUD1 mutation.

Conclusion: Mutations in the *ABCC8* gene were the most common cause of CHI in our cohort. These mutations were identified in 85% of patients who underwent pancreatectomy. The development of diabetes mellitus after pancreatectomy may occur at any age and these patients should be screened regularly.

Keywords: Hyperinsulinism, pancreatectomy, diabetes mellitus, ATP-sensitive potassium (K_{ATP}) channel

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

There is no follow-up study in patients with congenital hyperinsulinism (CHI) in Turkey.

WHAT THIS STUDY ADDS?

This study is the longest follow-up in patients with CHI in Turkey.

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Introduction

Congenital hyperinsulinism (CHI) is the most common cause of severe, persistent or recurrent hypoglycemia in the neonatal period and infancy. Mutations in the *ABCC8* and *KCNJ11* genes encoding the ATP-sensitive potassium (K_{ATP}) channel, which regulates the insulin secretion from pancreatic beta cell, are the leading cause of congenital hyperinsulinism. Rarely, mutations in the genes which encode glucokinase (*GCK*), glutamate dehydrogenase (*GLUD1*), 3-hydroxyacyl-coenzyme A dehydrogenase (*HADH*), hepatocyte nuclear factor 4 (*HNF4A*), hepatocyte nuclear factor 1 (*HNF1A*), monocarboxylate transporter 1 (*SLC16A1*), and the mitochondrial inner membrane protein UCP2 (*UCP2*) have been reported to cause hyperinsulinemic hypoglycemia (1).

Clinical signs and symptoms can present at any age from the neonatal period to adulthood and may vary depending on the specific mutation identified (2). Since recurrent severe hypoglycemia has a negative effect on neurocognitive function, especially during the first years of life, early recognition and treatment of this condition can be expected to provide a favorable prognosis. In patients who do not respond to medical therapy, pancreatectomy should be considered. The differentiation between focal and diffuse CHI before surgery would affect the success of surgical treatment (3).

Pancreatectomy is usually performed by removing 95-98% of the pancreatic tissue. This procedure comes with a risk of the patient developing diabetes mellitus and exocrine pancreatic insufficiency. Only a few studies have reported the long-term outcomes of patients following pancreatectomy (4,5,6,7,8,9,10,11).

In this study, we report the clinical features, treatment modalities, and long-term follow-up of our patients with CHI. We aim to contribute further information to the literature by demonstrating the clinical and mutational analyses of all patients at admission and also report the outcomes of patients who underwent pancreatectomy.

Methods

We retrospectively reviewed the medical records of patients diagnosed with CHI at the Göztepe Training and Research Hospital Pediatric Endocrinology Clinic, İstanbul (Centre 1) and at the Derince Training and Research Hospital Pediatric Endocrinology Clinic, Kocaeli (Centre 2).

We identified 24 patients with CHI (Centre 1, n=20; Centre 2, n=4). Two patients were excluded from the study since they were clinically diagnosed as Beckwith-Wiedemann syndrome. Twenty-two patients (7 females, 15 males) were recruited, and clinical data were extracted from the patient files. The diagnosis of CHI was based on detectable insulin levels during spontaneous or provoked hypoglycemia. A fasting provocation test was undertaken in two patients who had a history of hypoglycemia after overnight fasting and in another patient who was $17^{1/12}$ years old. This patient was diagnosed with hypoglycemia at the age of one year, diazoxide treatment was initiated at the age of 3 years, but the patient had missed follow- up for 14 years. He had mental retardation due to recurrent hypoglycemic attacks because of poor compliance with treatment. He suffered from a hypoglycemic attack on admission to our clinic. As severe hypoglycemia was noted during the fasting provocation test, diazoxide therapy was restarted and he did not have any further hypoglycemic attacks (case 10).

For all patients who underwent fasting provocation, the test was stopped when the blood glucose level fell below 45 mg/dL (12) and a blood sample was drawn for measurements of glucose, insulin, ketones, cortisol, growth hormone, ammonia, lactate, and pyruvate. Glucagon was subsequently administered at a dose of 30 µg/kg (max 1 mg) subcutaneously or intramuscularly, and blood glucose was re-measured thirty minutes later.

In case 1, with a history of protein sensitivity, a leucine provocation test was performed to confirm the diagnosis.

All patients were initially treated with intravenous high dose (10-15 mg/kg per min) glucose infusion and diazoxide (5-20 mg/kg per day). With the patient receiving a normal diet and following a 8-12 hr fast, absence of hypoglycemia (>55 mg/dL) with diazoxide treatment in a dose <15 mg/kg/d indicates responsiveness to diazoxide. The patients in whom the hypoglycemia (<55 mg/dL) persisted, despite receiving a maximum diazoxide dose (20 mg/kg/day) for 48 hours, were considered as unresponsive.

In 8 patients who were unresponsive to diazoxide, treatment with octreotide (5-40 mg/kg per day) was initiated. Two patients who underwent pancreatectomy received nifedipine (0.25-2.5 mg/kg per day) prior to pancreatectomy, but nifedipine therapy failed to prevent hypoglycemic episodes.

In case 4, a positron emission tomography scan using fluorine 18 L-3,4- dihydroxyphenylalanine (^{18}F-DOPA PET) was performed in Frankfurt, Germany. Focal involvement was not detected. As ^{18}F-DOPA PET-CT scanning is not available in Turkey, scans were not performed on other patients in our cohort.

In four patients who did not respond to diazoxide and octreotide therapy, 98% pancreatectomy was performed in our hospital. Patients 7, 11, and 12 were operated in other hospitals and subsequently referred to our clinic.

Genetic Analysis

Genetic testing was performed by the University of Exeter Medical School with written informed consent obtained from parents of all patients. Genomic DNA was extracted from peripheral leukocytes of 19 patients using standard procedures and the single exon of *KCNJ11* and 39 exons of *ABCC8* were amplified by polymerase chain reaction (primers available

on request). The amplicons were sequenced using Big Dye Terminator cycler sequencing Kit v3.1 (Applied Biosystems, Warrington, UK). Sequencing reactions were analyzed on an ABI3730 (Applied Biosystems, Warrington, UK) and compared to the reference sequences using Mutation Surveyor software (SoftGenetics, Pa., USA).

The *GLUD1* gene was sequenced in one patient with hyperinsulinism and hyperammonaemia using previously reported methods (13). For all other patients without an ABCC8 or KCNJ11 mutation, sequencing analysis of the *HADH* gene was undertaken (14,15). If no HADH mutation was identified, HNF4A was sequenced in any patient diagnosed with CHI within the first 2 weeks of life (16).

When a mutation was identified and samples were available, the unaffected parents were tested to investigate their carrier status. Microsatellite analysis was undertaken on DNA extracted from the resected pancreatic tissue of one patient to investigate loss of maternal heterozygosity at chromosome 11p15.1.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences 18.0 (SPSS, Chicago, IL, USA) software. Shapiro-Wilk test was used to test the normality of the data. Descriptive data were expressed as mean ± standard deviation values. Skewed data were shown as median and interquartile range (IQR). Spearman's correlation method was used in correlation analysis. For all tests, a p-value of less than 0.05 was accepted as statistically significant.

Results

The clinical characteristics of the patients with CHI are given in Table 1.

Seven patients (33.3%) were born large for gestational age (LGA). Seventeen patients were born at term. Cases 5 and 6 were siblings and 8 parents were known to be consanguineous.

The initial hypoglycemic episode was observed in the first 24 hours of life in nine patients (42.8%), and in the first week of life in seven patients (33.3%).

Thirteen patients (59%) were diazoxide-responsive. Diazoxide treatment was stopped between 15 days and 12 years in seven of these patients (53.8%) and no recurrence of hypoglycemia was observed. There was a history of birth asphyxia in the patient whose diazoxide therapy was stopped after 15 days. In this patient, the insulin level during hypoglycemia (30 mg/dL) was 40 mIU/mL and no genetic analysis was made.

After diazoxide therapy was discontinued, capillary blood glucose was checked before each feeding during minimum period of three days. During their hospitalization, we observed that babies could tolerate fasting for three hours, whereas toddlers and older children could tolerate a six-hour fasting period without hypoglycemia.

Octreotide was started in eight patients (36.3%) who did not respond to diazoxide. Of those, case 3 showed a good response. Overall, 63.6% of the patients were responsive to medical treatment and they did not have any hypoglycemic attacks during diazoxide and/or octreotide therapy with regular food intake. In one patient, normoglycemia was achieved through frequent feeds with a carbohydrate-rich diet.

Four patients who did not respond to medical treatment underwent pancreatectomy in our hospital at a median age of 0.43±0.32 years. Neither hypoglycemia nor hyperglycemia was observed in these patients following surgery. Diabetes did not develop during the 3.67±0.7 years of follow-up.

Case 4, at age $5^{7/12}$ years, was found to have a HbA1c of %6.4, a fasting glucose level of 87 mg/dL, a fasting C- peptide

Table 1. Clinical and biochemical characteristics of the medically and surgically treated patients

	Medically treated patients (n=14) (Mean ± SD, Range)	Surgically treated patients (n=7) (Mean ± SD, Range)	p
Birth weight, g	3059±733 (2060-4100)	3780±751 (2370-4480)	0.031
Birth weight, SDS	-0.71±1.6 (-2.95) -1.59	0.99±1.7 (-2.26) -2.72	0.02
Age at diagnosis, days	2 (329)* (1-820)	2 (6)* (1-82)	0.680
Diazoxide dose, mg/kg/d	8.8±4 (4-15)	18.1±3 (12-20)	<0.001
Age at last visit, years	8.4±6 (1.81-19)	7.4±6 (2.72-16.58)	1.000
Height SDS at last visit	-0.27±1.5 (-3.0) -1.65	0.47±1.1 (-1.54) -1.61	0.852
BMI SDS at last visit	0.76±1.2 (-1.63) -1.97	1.40±1.1 0.07-2.32	0.852
HbA1c at last visit, %	5.0±0.1 4.80-5.30	7±3.7 4.6-14.1	ND
Serum insulin level at last visit, µIU/mL	5.7±3 1.7-10.64	ND	ND
Blood glucose level at last visit, mg/dL	79.3±13 72-105	133.5±103 76-320	ND
Follow-up duration, years	7.1±6.1 1.81-18	5.7±5.1 2.72-16	ND

*median (interquartile range), ND: not determined, BMI: body mass index, SDS: standard deviation score

level of 1.77 ng/mL, and a fasting insulin level of 1.63 mIU/mL. We wanted to perform an intravenous glucose tolerance test because the patient had vomited twice during oral glucose tolerance test. The parents did not consent to this procedure.

Cases 7, 11, and 12 underwent surgery in other centers at a mean age of 0.39±0.44 years. Hypoglycemia was observed in two of these three cases (cases 11 and 12) after subtotal pancreatectomy and they received nifedipine for two and three months, respectively. No hypoglycemic attacks were noted. Diabetes developed immediately after near-total pancreatectomy in case 7, and 2.5 years and 10 years after subtotal pancreatectomy in cases 11 and 12, respectively. All three patients are currently receiving intensive (basal and mealtime bolus) insulin treatment.

Histological examination of the resected pancreatic tissue confirmed diffuse disease in six patients, and a focal lesion was detected in the tail of the pancreas in one patient (case 4). The focal lesion could not be identified prior to the operation (^{18}F DOPA-PET-CT scanning was not available).

Genetic Analysis

Results of the genetic analyses are given in Table 2.

Mutations were detected in 12 out of the 19 patients (63.2%). Nine patients had ABCC8 mutation(s) and KCNJ11, GLUD1 and HADH mutation(s) were each identified in a single patient (Table 2). Three of the mutations were novel. The genetic results for the patient with the homozygous HADH mutation have been reported previously (14). Fourteen unaffected parents were heterozygous for a mutation and are therefore carriers of congenital hyperinsulinism.

A paternally inherited ABCC8 mutation was identified in the patient with a focal lesion (case 4). Analysis of nine microsatellite markers spanning chromosome 11p15.5 to 11p15.1 was performed using DNA extracted from the resected tissue, but no loss of heterozygosity was observed.

Case 3 was heterozygous for two novel KCNJ11 mutations. Family member testing identified both mutations in the unaffected father, confirming that the mutations were on the same allele (in cis). The patient responded well to octreotide treatment. At two years of age, he could tolerate overnight fasting under a very low dose octreotide (9 µg/kg/d). A clinical remission was considered, and octreotide treatment was gradually diminished and stopped. He is currently not requiring medication and is able to keep his glucose levels >70 mg/dL.

Consequently, mutations in the K_{ATP} channel gene were identified in ten patients. Of those, 4 patients had recessively inherited mutations and 85.5% underwent pancreatectomy. However, mutations in the K_{ATP} channel genes were not identified in all patients who underwent pancreatectomy.

In one patient with mildly elevated levels of ammonia (185 µg/dL, normal: 40-80), the genetic analysis identified a heterozygous missense mutation in the *GLUD1* gene. This mutation was not detected in leukocyte DNA from the unaffected parents and it is therefore likely that the mutation was a *de novo* mutation.

The genetic analysis of seven patients who underwent pancreatectomy revealed mutation(s) in the *ABCC8* gene in 6 patients. The mutation analysis results were available in only two patients prior to surgery. In one patient with diffuse pancreatic disease, the screening of the *ABCC8, KCNJ11, HADH,* and *HNF4A* genes did not identify a mutation.

Birth weight was significantly higher in those patients with a *ABCC8* gene mutation compared to those without an *ABCC8* gene mutation (r=0.857, p=<0.001).

Discussion

In this study, we evaluated the clinical and genetic features and long-term follow-up outcomes in a group of Turkish patients with CHI. Severe hypoglycemia occurred in the first days of life in the majority of patients, and this was consistent with the findings of previous studies.

Mutations were identified in previously known CHI genes in more than half (63%) of patients. As in previous studies, the most common genetic etiology among diazoxide-unresponsive patients was ABCC8 mutations.

Snider et al (17), reported in their series of 417 cases that no mutations were found in 3.9% (11/282) of patients with diffuse disease who underwent pancreatectomy. Greer et al (18) did not identify ABCC8 or KCNJ11 mutations in 5 of 21 patients (23%) who underwent pancreatectomy. In another study reporting 175 patients with CHI, 13 of 70 patients (18%) who underwent pancreatectomy had no mutations in the *ABCC8* or *KCNJ11* genes (19). A more recent study indicated that 3% of patients with diffuse CHI had no mutations in the genes encoding the K_{ATP} channel (20). Similarly, we did not identify a mutation in one of our patients with diffuse disease who underwent pancreatectomy. It is possible that this patient has a large deletion and/or a non-coding mutation(s) which was not detected by Sanger sequencing.

Compound heterozygosity is not common in patients with CHI (17,20,21,22,23). Sogno Valin et al (21) identified compound heterozygous K_{ATP} channel mutations in 2 of 33 patients within their cohort. Of those, one patient responded to diazoxide treatment and the other underwent pancreatectomy. Arya et al (24) recently reported 45 patients with CHI who underwent near-total pancreatectomy. They found that one third of patients were compounded heterozygous. One patient in the present study (case 8) had a maternally inherited novel missense mutation and was unresponsive to diazoxide and octreotide therapy. A near-total pancreatectomy was performed and diffuse disease was identified. Sequence analysis also identified a previously reported (25) paternally inherited ABCC8 variant (p.Ala726Thr), but current evidence suggests that this variant is unlikely to be pathogenic.

Autosomal dominant inheritance of hyperinsulinism with a variable response to diazoxide has been reported

Table 2. Genetic and clinical characteristics of 12 patients with mutation-positive congenital hyperinsulinism

No	Current age (years)	Gene	Mutation	Consequence	Zygosity	Maternal mutation	Paternal mutation	Diazoxide-responsive	Pancreatectomy age/histology	Follow-up duration, years	Outcome
1	13.8	*HADH*	p.? (c.636+471G>T)	Aberrant splicing	Homozygous	p.? (c.636+471G>T)	p.? (c.636+471G>T)	Yes	-	5.2	On diazoxide treatment
2	13.56	*GLUD1*	p.Ser445Leu (c.1334C>T)	Missense	Heterozygous	N/N	N/N	Yes		12.3	On diazoxide treatment, mental retardation
3	3.24	*KCNJ11*	p.Leu270Met; p.Glu288Lys (c.808C>A; c.862G>A)	Two novel missense	Heterozygous	N/N	p.Leu270Met; p.Glu288Lys (c.808C>A; c.862G>A)	Yes		3.2	Mental retardation, no treatment
4	5.08	*ABCC8*	p.Cys26X (c.78C>A)	Nonsense	Heterozygous	N/N	p.Cys26X (c.78C>A)	No	7 months old/ focal	4.7	No treatment
5	4.64	*ABCC8*	p.Asn493Lys (c.1479T>A)	Novel missense	Heterozygous	p.Asn493Lys (c.1479T>A)	N/N	Yes		4.6	On diazoxide treatment
6	1.8	*ABCC8*	p.Asn493Lys (c.1479T>A)	Novel missense	Heterozygous	p.Asn493Lys (c.1479T>A)	N/N	Yes		1.8	On diazoxide treatment
7	3.48	*ABCC8*	p.? (c.3992-9G>A)	Aberrant splicing	Homozygous	p.? (c.3992-9G>A)	p.? (c.3992-9G>A)	No	25 days old/ diffuse	3.5	On basal and bolus insulin treatment
8	3.88	*ABCC8*	p.Gly1485Ala (c.4454G>A)	Novel missense	Heterozygous	p.Gly1485Ala (c.4454G>A)	N/N	No	3 months old/ diffuse	3.4	No treatment
9	3.48	*ABCC8*	p.Pro1563Thr (c.4687C>A)	Novel missense	Homozygous	p.Pro1563Thr (c.4687C>A)	p.Pro1563Thr (c.4687C>A)	No	10 months old/ diffuse	3.5	No treatment
10	19	*ABCC8*	p.? (c.1817+1G>C)	Aberrant splicing	Homozygous	(c.1817+1G>C)	Not available for testing (exitus)	Yes		18	Mental retardation, on diazoxide treatment
11	16.24	*ABCC8*	p.Gly52fs (c.155delG)	Frameshift	Homozygous	p.Gly52fs (c.155delG)	p.Gly52fs (c.155delG)	No	35 days old/ diffuse	16.2	On basal and bolus insulin treatment
12	16.58	*ABCC8*	p.Leu434X (c.1301T>A)	Nonsense	Homozygous	p.Leu434X (c.1301T>A)	p.Leu434X (c.1301T>A)	No	2.5 months old/ diffuse	16.5	Mental retardation, on basal and bolus insulin treatment

Patients 5 and 6 are siblings

(26,27,28,29,30,31). MacMullen et al (30) reported patients with dominantly inherited CHI with some affected parents carrying the same mutation whilst other parents were asymptomatic. In that study which included 17 diazoxide-unresponsive and 13 diazoxide-responsive patients, the mutation was maternally inherited in five patients from each group. In our study, two siblings with diazoxide-responsive HI were heterozygous for a novel ABCC8 missense mutation. Both children had inherited this mutation from their unaffected mother.

Preoperative diagnosis of focal HI is of great importance for determining the extent of pancreatectomy. ^{18}F-DOPA PET CT scanning remains the only accurate method for localizing a focal lesion prior to surgery. Almost all patients with focal CHI have a paternally inherited recessively acting mutation in a K_{ATP} channel gene (17). In our series, two patients had a paternally inherited K_{ATP} channel gene mutation (*ABCC8*, n=1; *KCNJ11*, n=1). Since it is unavailable in our country, ^{18}F-DOPA PET CT could not be performed in these patients. While CHI showed a spontaneous remission in the patient with *KCNJ11* gene mutation at the age of 2.5 years, the other patient with an ABCC8 mutation was unresponsive to medical therapy and underwent near-total pancreatectomy.

The most common surgical method is near-total pancreatectomy in patients who are unresponsive to medical treatment. Post-pancreatectomy hyperglycemia may require either temporary or permanent insulin treatment. In long-term follow-up studies of patients with CHI who underwent pancreatectomy, diabetes has been reported in a limited number of patients (4,11,24,32,33). In 1984, Greene et al (4) reported five patients with CHI whose hypoglycemia could not be managed with medical therapy and who underwent pancreatectomy twice. Diabetes developed in all patients and insulin was required to achieve normoglycemia. Diabetes often occurs when patients undergo ≥95% pancreatectomy and then need a second operation (8,9,32,33). The interval between the surgery and the development of diabetes varies from immediately after (5,6,7,8,9,10,24) to several years after the (>18 years) surgery. Nevertheless, insulin-dependent diabetes frequently develops during adolescence (8,10,24,32). In one study, diabetes developed in 100% of patients by the 11th year following surgery (24). In the present study, diabetes developed immediately after the operation in one patient, and 2.5 years and 10 years after surgery in two patients. At their recent evaluation, all of these patients were on insulin treatment. The remaining four patients who underwent near-total pancreatectomy did not develop diabetes or had no evidence of exocrine pancreatic insufficiency during follow-up. However, this does not eliminate the risk of developing diabetes in the future. Therefore, these patients need to be evaluated periodically for diabetes.

It is not known whether there is a relation between diabetes development after pancreatectomy and the type of mutation causing CHI. It has been suggested that K_{ATP} channel gene mutations lead to an increase in the apoptosis of the beta cells of the pancreas (34). Leibowitz et al (35) demonstrated that insulin response to glucose stimulation was diminished in patients with CHI who underwent pancreatectomy. Impaired glucose tolerance and diabetes may present in these patients particularly during puberty when insulin response to hyperglycemia is blunted. However, ketoacidosis was not reported in the patients with diabetes, so one may consider that the residual pancreatic tissue secretes some insulin. The deterioration in glucose homeostasis is progressive in these patients and frank diabetes develops after many years.

Hypoglycemia requiring medical treatment in patients with CHI who underwent pancreatectomy may be explained by the regeneration of the residual pancreatic tissue (36). In our study group, all three patients who had diabetes subsequent to pancreatectomy had homozygous ABCC8 mutations. Two of the remaining four patients who underwent near-total pancreatectomy also had homozygous ABCC8 mutations and they still have no symptoms. The patient with no identifiable mutations and diffuse pancreatic disease and the patient with a paternally inherited ABCC8 mutation and a focal lesion have not yet developed diabetes. The limitation of our study is its retrospective design and having performed an ^{18}F-DOPA PET-CT scan in only one patient prior to surgery.

Previous studies from our country regarding congenital hyperinsulinism did not report long-term follow-up data (37,38). Besides, in one of these reports, the diagnosis is based only on clinical and laboratory findings (37). In another multicenter study, clinical findings and genetic analyses of patients with CHI treated in four different centers were demonstrated, yet long-term follow-up was not reported (38).

Based on the information above, this present study stands out as the longest follow-up study of the patients with CHI from Turkey. Our study confirms that K_{ATP} channel gene mutations are the most common mutations causing CHI in Turkish patients. These mutations were identified in 85% of patients who underwent pancreatectomy. The development of diabetes mellitus after pancreatectomy may occur at any age, for that reason, patients should be screened regularly. We could not establish a relation between the type of mutation and the development of diabetes. Since ^{18}F-DOPA PET-CT is not widely available, genetic analysis might be useful to identify focal CHI in those patients who cannot be subjected to ^{18}F-DOPA PET-CT.

Ethics

Ethics Committee Approval: Yes, Informed Consent: Genetic testing was performed by the University of Exeter Medical School with written informed consent obtained from parents of all patients.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Ayla Güven, Design: Ayla Güven, Data Collection and/or Processing: Ayla Güven, Ayşe Nurcan Cebeci, Analysis and/or Interpretation: Ayla Güven, Sian Ellard, Sarah E. Flanagan,

Literature Research: Ayla Güven, Writing: Ayla Güven, Ayşe Nurcan Cebeci.

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Original Article

A Novel Null Mutation in P450 Aromatase Gene (CYP19A1) Associated with Development of Hypoplastic Ovaries in Humans

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ABSTRACT

Objective: The *CYP19A1* gene product aromatase is responsible for estrogen synthesis and androgen/estrogen equilibrium in many tissues, particularly in the placenta and gonads. Aromatase deficiency can cause various clinical phenotypes resulting from excessive androgen accumulation and insufficient estrogen synthesis during the pre- and postnatal periods. In this study, our aim was to determine the clinical characteristics and *CYP19A1* mutations in three patients from a large Turkish pedigree.

Methods: The cases were the newborns referred to our clinic for clitoromegaly and labial fusion. Virilizing signs such as severe acne formation, voice deepening, and clitoromegaly were noted in the mothers during pregnancy. Preliminary diagnosis was aromatase deficiency. Therefore, direct DNA sequencing of *CYP19A1* was performed in samples from parents (n=5) and patients (n=3).

Results: In all patients, a novel homozygous insertion mutation in the fifth exon (568insC) was found to cause a frameshift in the open reading frame and to truncate the protein prior to the heme-binding region which is crucial for enzymatic activity. The parents were found to be heterozygous for this mutation. Additionally, all patients had hypoplastic ovaries instead of cystic and enlarged ovaries.

Conclusion: A novel 568C insertion mutation in *CYP19A1* can lead to severe aromatase deficiency. Homozygosity for this mutation is associated with the development of hypoplastic ovaries. This finding provides an important genetic marker for understanding the physiological function of aromatase in fetal ovarian development.

Keywords: Aromatase, *CYP19A1* gene, ovarian development

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Aromatase deficiency can cause various clinical phenotypes resulting from excessive androgen accumulation and insufficient estrogen synthesis. Aromatase deficiency has been reported to cause delayed puberty in adolescent girls, minimal or absent breast development, primary amenorrhea, hypergonadotropic hypogonadism, tall stature, delayed bone age, and enlarged and multicystic ovaries.

WHAT THIS STUDY ADDS?

A novel insertion mutation in the aromatase gene (*CYP19A1*) was found which caused a frameshift in the open reading frame and a truncation of the protein prior to the heme-binding region. Homozygosity for this mutation was associated with the development of hypoplastic ovaries. This finding provides an important genetic marker for understanding the physiological function of aromatase in fetal ovarian development.

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Introduction

Aromatase *(CYP19A1)* catalyzes the conversion of androgens to estrogens, which is a key step in estrogen biosynthesis (1,2). The enzyme is mainly located in the endoplasmic reticulum of estrogen-producing cells in the ovary, placenta, testis, brain, adipose tissue, liver, muscle, and hair follicles (3,4). During pregnancy, dehydroepiandrosterone sulfate (DHEAS) and 16OH-DHEAS, arising from the fetal adrenal gland and liver, respectively, become important sources for the synthesis of placental estrogens (4,5). To date, more than eleven genetic mutations in *CYP19A1* gene have been reported in multiple studies with *in vivo* phenotype information (6,7,8,9,10,11,12,13,14). Placental aromatization of androgens has been suggested to be essential for protecting both the mother and any female fetus against the virilizing action of fetal androgens, particularly during differentiation of external genitalia (4). Autosomal recessive mutations in the *CYP19A1* gene have been reported to cause disorders of sex development (DSD) and virilization of the mother during pregnancy (11,12,13,15). Here, we report the clinical and genetic features of three cousins with a novel homozygous mutation (568insC) in *CYP19A1* that caused severe aromatase deficiency. The present study suggests that severe aromatase deficiency appears to be associated with hypoplastic rather than enlarged and multicystic ovaries.

Methods

Study Subjects

Case 1 (index case, IV.3) was a Turkish female presenting with clitoromegaly and partial labial fusion at birth (Prader II), the third child of consanguineous parents (Figure 1). A history of voice changes and hirsutism in the mother during pregnancy was reported. The mother was also reported to demonstrate symptoms of hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome, and she died during labor. The infant was then hospitalized due to hypoxic-ischemic encephalopathy (HIE) and neonatal sepsis. Laboratory evaluation revealed

Table 1. Laboratory values at diagnosis[a] and at last visit[b]

	Case 1		Case 2		Case 3	
	a	b	a	b	a	b
Age (months)	<1	84	<1	60	<1	26
Na (mmol/L) (Normal range: 133-145)	140	136	139	138	137	138
K (mmol/L) (Normal range: 3.3-5.1)	5	3.5	4	4.4	5.9	4.6
LH (mIU/mL) (Normal range: 0.02-0.3)	ND	0.1	1.9	1.9	ND	2.7
FSH (mIU/mL) (Normal range: 1.1-4.2)	ND	2.6	9.8	43.3	ND	49.1
Estradiol (pg/mL) (Normal range: 10-114)	ND	<5	16.4	<5	ND	15.4
Total testosterone (ng/mL) (Normal range: 0.03-0.68)	2.0	0.03	2.6	0.02	1.63	0.02
ACTH (pg/mL) (Normal range: 7.2-68)	25.1	4.1	16.9	23.2	28	60.1
Cortisol (µg/dL) (Normal range: 4.3-22.4)	12.3	7.7	9.2	10.7	9.5	12.5
17-OH Progesterone (ng/mL) (Normal range: 0.07-1.53)	7.5	0.45	25.0	0.87	9.8	0.15
Androstenedione (ng/mL) (Normal range: 0.1-0.9)	1.10	0.28	5.72	0.3	1.28	0.30
DHEA-S (µg/dL) (Normal range: 11-255)	59.6	78.8	93.1	17.7	61.9	11

Na: sodium, K: potassium, LH: luteinizing hormone, FSH: follicle-stimulating hormone, ACTH: adrenocorticotropic hormone, DHEA-S: dehydroepiandrosterone sulfate, ND: not determined

hyperandrogenism without adrenal insufficiency. The karyotype was 46,XX. After discharge from the hospital, she was again admitted at 7 years of age. At this time, the girl was found to have severe cerebral palsy and failure to thrive [height: 103 cm, -3.6 standard deviation score (SDS); weight: 13 kg, -1.7 SDS]. She had no breast or pubic hair development but was noted to have clitoromegaly (1.5 cm). Pelvic ultrasonography (US) revealed a 26-mm uterus with hypoplastic ovaries (0.02 mL). Laboratory evaluation revealed normal androgen and basal gonadotropin levels with undetectable estradiol (Table 1).

Case 2 (IV.6) was a Turkish female presenting with clitoromegaly, complete labial fusion, and single urogenital sinus at birth (Prader III). She was a cousin of case 1 and the third child of consanguineous parents (Figure 1). A history of maternal voice changes, acne formation, and clitoromegaly during pregnancy was reported. Laboratory evaluation revealed hyperandrogenism without adrenal insufficiency. The karyotype was 46,XX. The patient underwent vaginoplasty. At her last visit, at 5 years of age, she had mild clitoromegaly (1 cm) without any other clinical signs of hyperandrogenism. Pelvic US revealed a 23-mm uterus with hypoplastic ovaries (0.06 mL). Laboratory evaluation revealed normal androgen and high basal gonadotropin levels with undetectable estradiol (Table 1).

Case 3 (IV.1) was a cousin of case 2 and presented with clitoromegaly, complete labial fusion, and single urogenital sinus at birth (Prader III) (Figure 1). A history of maternal voice changes and acne formation during pregnancy was reported. Laboratory evaluation revealed hyperandrogenism without adrenal insufficiency. The karyotype was 46,XX. Vaginoplasty was performed. At her last visit, at 2.2 years of age, she had clitoromegaly (1.5 cm) without any other clinical sign of hyperandrogenism. Pelvic magnetic resonance imaging revealed a rudimentary uterus (11 mm) with hypoplastic ovaries (0.04 mL). Laboratory evaluation revealed normal androgen and high basal gonadotropin levels with normal estradiol (Table 1).

The subjects were interviewed carefully to determine their lineage and birth and were considered to belong to a pedigree. After explanation of the study, written informed consent was obtained from all participants before obtaining blood samples. DNA could not be isolated from other siblings due to socio-economic reasons and parental non-compliance. However, according to the parents, those female siblings were healthy and had no clitoromegaly.

Sequencing and Identification of *CYP19A1* Variants

Genomic DNA was isolated from peripheral whole blood using a QiAamp blood kit (Qiagen, Valencia, CA, USA). The research protocol for the use of human DNA was approved by the institutional review board of Busan Paik Hospital, Busan, Korea, and conformed to institutional guidelines (16). The exons, intron-exon junctions, promoter region, and 3'-untranslated region of *CYP19A1* were polymerase chain reaction (PCR)-amplified and directly sequenced. Primers for PCR amplification and DNA sequencing were identical to those used previously (16).

Genetic Analysis of *CYP19A1* Variants

Haploview 4.2 population genetic analysis software (http://www.broad.mit.edu/mpg/haploview/) was used to analyze linkage disequilibrium (LD) and haplotype diversity. The sequence analysis programs NNSPLICE 0.9 (www.fruitfly.org/seq_tools/splice.html) and TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) were used to predict alternative splice sites and transcription factor-binding changes introduced by mutations, respectively.

Results

Direct DNA sequencing analysis of the *CYP19A1* gene in all subjects (n=8) revealed a total of 12 genetic variants. A summary of the identified variants is presented in Table 2. Among these 12 variations, a 568Cins in exon 5 was previously unidentified. The 568Cins was found as a heterozygous mutation in parents and a homozygous mutation in the three probands (Figure 2A). This insertion mutation caused a change of amino acid 190Leu to 190Pro and the subsequent frameshift was predicted to generate a stop codon, resulting in a truncated protein of 199 amino acids rather than the full functional 503 amino acids of the *CYP19A1* protein (Figure 2B). No particular linkage was found with this novel mutation. None of variants were predicted to create or disrupt splice sites or transcription factor-binding elements.

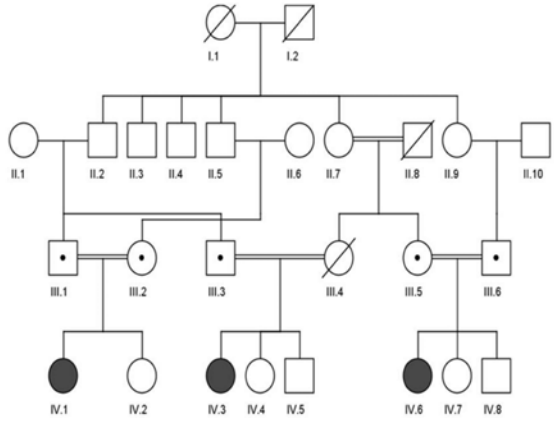

Figure 1. Pedigree of the family covering four generations. The symptomatic subjects with homozygous *CYP19A1* mutation of a novel mutation (568insC) in exon 5 of *CYP19A1* gene are shown as solid symbols. The slashed symbols represent deceased family members. Dots in circles refer to subjects carrying the heterozygous mutation. Circles represent female family members and squares male family members

Table 2. *CYP19A1* single-nucleotide polymorphisms in the subjects (n=8)

Site	Position[a]	Amino acid change	Reference	Subject (n)			Frequency (%)
				wt/wt (n)	wt/mt (n)	mt/mt (n)	
5′-UTR Exon 1.6	-196A>C		rs10459592	0	0	8	100
5′-UTR Exon 1.6	-77G>A		rs4775936	0	0	8	100
Intron 2	IVS2-59 A>G		rs3759811	0	1	7	93.8
Exon 3	240A>G	V80V	rs700518	0	1	7	93.8
Intron 4	IVS4+77 (TTTA)n		rs57921193				
	n=7			0	1	0	6.3
	n=8			0	4	4	75
	n=9			0	3	0	18.8
Exon 5	568Cins[b]	Frame shift		0	5	3	68.8
Intron 5	IVS5-16 T>G		rs4324076	0	1	7	93.8
Intron 6	IVS6+36 A>T		rs1143704	0	1	7	93.8
Intron 6	IVS6-106 T>G		rs2304463	0	1	7	93.8
Exon 7	790C>T	R264C	rs700519	7	1	0	6.3
Intron 7	IVS7-79 A>G		rs2289105	0	1	7	93.8
3′-UTR	1531 C>T		rs10046	0	1	7	93.8

[a]The reference sequence used was GenBank accession no. NC_000015. Position is indicated with respect to the start codon ATG in *CYP19A1* gene; the A in ATG is +1 and the next base toward to 5′ is -1.
[b]New variant allele was identified in the present study. 3′-UTR, 3′ untranslated region. 5′-UTR, 5′ untranslated region.

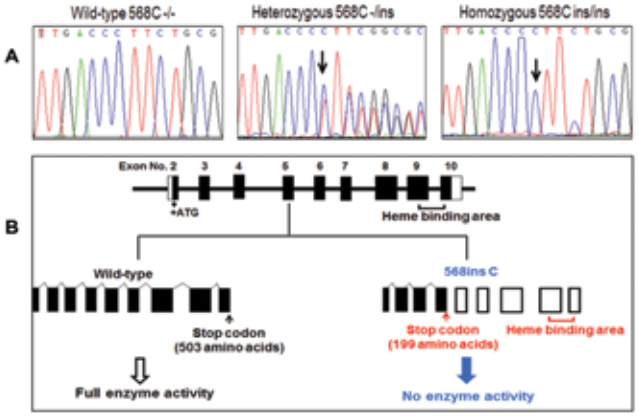

Figure 2. Aromatase deficiency resulting from a novel null mutation in the *CYP19A1* gene. (A) DNA sequence of the *CYP19A1* gene around the site of mutation in exon 5. The C *CYP19A1* is indicated by the arrow. (B) Schematic representation of *CYP19A1* gene structure and the location of the mutation of 568insC in the map. Black bars represent exons. Truncated *CYP19A1* protein caused by the 568insC is indicated by the arrow, resulting in 199 amino acids

Discussion

Aromatase deficiency causes virilization of the mother during pregnancy and ambiguous genitalia of the female fetus due to prenatal exposure to adrenal androgens. In the postpartum period, some clinical features of androgen excess regress and elevated androgen concentrations return to normal levels. Aromatase deficiency has been reported to cause delayed puberty in adolescent girls, minimal or absent breast development, primary amenorrhea, hypergonadotropic hypogonadism, tall stature, delayed bone age, decreased bone mineral density, and multicystic ovaries (8,9). Additionally, it has been speculated that in aromatase-deficient prepubertal girls, an amplification of follicle-stimulating hormone (FSH) signaling might occur in the presence of high intraovarian androgen production and be responsible for the development of ovarian follicular cysts (4). However, affected male infants have normal internal and external genital development. Affected males have usually been diagnosed after puberty with tall stature,

delayed bone age, decreased bone mineral density, and infertility (14). Interestingly, in our cases, the ovaries were hypoplastic without cyst formation. Luteinizing hormone (LH) and especially FSH levels were high at the final visits in cases 2 and 3 but not in case 1. Prepubertal levels of LH and FSH in case 1 may have been due to hypogonadotropic hypogonadism resulting from severe HIE during the neonatal period. Hypoplastic ovaries rather than enlarged ovaries in aromatase-deficient females have rarely been reported. There are only two cases with similar phenotype reported in the literature. Lin et al (12) reported a case of aromatase deficiency with hypoplastic ovaries and uterus. Karyotype was 46,XX. The patient had a severe mutation (exon5del) in exon 5 of the *CYP19A1* gene, generating a truncated protein without the heme-binding region crucial for enzymatic activity. Recently, another case of severe aromatase deficiency due to a 27-base duplication in exon 8 has been reported (17). The case was a 25-year-old woman with delayed puberty and osteoporosis. She had a hypoplastic uterus and bilateral streak ovaries with 46,XX karyotype. The authors concluded that the streak ovaries may be an inherent manifestation of *CYP19A1* deficiency. These two cases, similar to the present cases, exhibited a severe phenotype with clitoromegaly, labial fusion, and/or single urogenital sinus at birth.

Indeed, as the studies of *CYP19A1* knockout (ArKO) mouse model have shown, follicular development in ovaries of ArKO is abnormal in an age-dependent manner, with an early block in follicular development at the antral stage with absent corpora lutea. Then, haemorrhagic cysts with absent secondary and antral follicles and atresia of primary follicles with increased collagen deposition are observed. Additionally, in the ArKO mouse, uterine weight was found to be very low compared to that of wild-type, possibly because of hypoestrogenism (18). These findings are consistent with those reported in the *CYP19A1* knockout mouse.

Taking into consideration all these reports, we speculate that severe aromatase deficiency in intrauterine life can cause insufficient production of fetal estrogens in the human fetus as well as testosterone excess which in turn might result in maldevelopment of the fetal ovaries. However, it is obvious that not all patients with severe aromatase deficiency have hypoplastic ovaries. This can be explained by the fact that gonadal development is a multifactorial process and involves many genes that interact with each other. Further *in vitro* and *in vivo* studies are necessary to understand this complex process.

In conclusion, a novel genetic variant in *CYP19A1* gene was found to generate a null mutation. We suggest that severe aromatase deficiency caused by 568insC mutation can result in maldevelopment of ovaries in female fetuses. Further studies in large numbers of subjects would be necessary to understand the effect of this mutation on cancer, osteoporosis, and ovarian development.

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Ethics

Ethics Committee Approval: Institutional Review Board of Busan Paik Hospital, Informed Consent: It was taken.

Peer-review: Internal peer-reviewed.

Authorship Contributions

Concept: Sema Akçurin, Doğa Türkkahraman, Design: Sema Akçurin, Doğa Türkkahraman, Su-Jun Lee, Data Collection and/or Processing: Doğa Türkkahraman, Erdem Durmaz, Analysis and/or Interpretation: Woo-Young Kim, Erdem Durmaz, Jae-Gook Shin, Su-Jun Lee, Literature Research: Doğa Türkkahraman, Erdem Durmaz, Writing: Doğa Türkkahraman, Su-Jun Lee.

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Original Article

Gonadotropin-Releasing Hormone Analogue Treatment in Females with Moderately Early Puberty: No Effect on Final Height

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ABSTRACT

Objective: To investigate the effects of treatment with gonadotropin-releasing hormone analog (GnRHa) on final height in girls who experienced moderately early puberty with symptoms beginning at 7-8.5 years of age.

Methods: Female cases diagnosed with moderately early puberty which had started between ages 7 to 8.5 years were included in the study. In the treatment groups, all cases with a bone age ≤10.5 years constituted group 1 (n=18) and those with a bone age >10.5 years constituted group 2 (n=23). The 8 patients for which treatment approval could not be obtained constituted group 3. The 49 cases in all three groups were observed until they reached their final height.

Results: Target height, target height standard deviation score (SDS), final height, and final height SDS values were similar in all 3 groups. Final height showed a significant positive correlation with target height (p=0.000, r=0.54) and height at diagnosis (p=0.003, r=0.467) in all groups. Linear regression analysis revealed that a 1 cm longer height at diagnosis increased the final height 0.213 fold, and a 1 cm longer target height at diagnosis increased the final height 0.459 fold.

Conclusion: We found that GnRHa did not make a positive contribution to final height in cases of moderately early puberty.

Keywords: Early puberty, gonadotropin-releasing hormone analog, final height

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

There are only a few studies on the effects of gonadotropin-releasing hormone analog (GnRHa) treatment on final height in cases with borderline early puberty.

WHAT THIS STUDY ADDS?

GnRHa therapy did not make a positive contribution to final height in cases aged 7-8.5 years with early puberty in this study.

Introduction

Early puberty is defined as puberty that begins at an earlier age than the age accepted as "normal". Moderately early puberty is not a rare condition (1). There is no single age range which defines moderately early puberty; in girls, studies have reported age ranges of 8-10 years (2,3), 7.5-8.5 years (4), 8-9 years (5), and 6-8 years (6).

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Moderately early puberty is a paraphysiological condition, in reference to the earlier appearance of pubertal signs (4). Such children can reach Tanner stage 4 before 10 years of age, rather than the normal age of 11.9±1 years. This may lead to psychosocial problems and has also been reported to stunt growth (2,4,7,8). Data on the effectiveness of treatment in this group are very limited. Most relevant studies were not randomized and did not include a control group (9).

We investigated the effects of treatment with gonadotropin-releasing hormone analog (GnRHa) on final height in girls who experienced early puberty with symptoms beginning at 7-8.5 years of age, and determined whether the group that received early treatment, when their bone age (BA) was younger, showed a better effect regarding final height than the group that received treatment when bones were more mature. We followed the participants until they reached their final height.

Methods

Female cases who presented to the Pediatric Endocrinology Outpatient Clinic with symptoms of puberty starting at 7-8.5 years of age were included in the study. The Tanner and Marshall criteria were used for puberty staging. Patients who had at least Tanner stage 2 breast development were assessed as cases of early puberty (10). Height and body weight measurements were taken in the morning using a SECA® 767 height and weight meter (Carson City, NV, USA). Body mass index (BMI) was calculated as kg/m^2. Height and BMI were compared to standard curves for Turkish children. Height standard deviation score (SDS) and BMI SDS were calculated according to calendar age. Cases with a BMI SDS >2 were considered obese and those with a BMI SDS of 1-2 were considered overweight (11,12).

A left hand wrist graph was obtained from all cases and BA was determined according to the Greulich and Pyle method (13). The predicted final height (PFH) based on BA was calculated according to the Bayley-Pinneau method (14). PFH SDS was determined for each case. Height achieved when the epiphyses were closed and when the growth rate within the last 1 year was <1 cm were considered to represent final height (15). Maternal and paternal heights were measured at the outpatient follow-ups, and target height (TH) and SDS were determined (16).

Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and 17β-estradiol (E2) were measured using a morning blood sample in all cases. A basal serum LH level ≥0.3 IU/L (as long as it was consistent with the findings) was accepted as activation of the hypothalamic-pituitary-gonadal (HPG) axis (17). Cases with a basal LH level <0.3 IU/L underwent the standard stimulation test of 100 µg GnRH (Ferring Pharmaceuticals Inc., Parsippany, NJ, USA) with an intravenous injection between 8.00 and 8.30 AM to assess the patient for early puberty, and blood samples were taken at 0, 40, 60, 90, and 120 min to measure serum LH and FSH levels. Peak LH ≥5 IU/L was accepted as the diagnostic criterion for activation of the HPG axis (17).

Cranial magnetic resonance imaging was performed to exclude an organic lesion in cases diagnosed with early puberty using basal or peak LH level at the time of the GnRH stimulation test (18). Cases with such lesions were excluded from the study. Cases with problems that could affect growth and puberty, such as growth hormone deficiency, thyroid pathology, adrenal and gonadal pathology, dysmorphic syndrome, skeletal dysplasia, chronic illness, learning disability, cerebral palsy, hydrocephalus, and those with a history of chronic drug use were also excluded.

All cases underwent pelvic ultrasonography at the time of diagnosis to evaluate the consistency of the pubertal findings with the pelvic ultrasonography findings. Uterus length >3.5 cm, ovary volume >1.5 cm^3, and a visible endometrial echo were accepted as criteria supportive of early puberty (19).

Forty-nine cases were diagnosed with idiopathic central early puberty (CEP). A family information booklet was provided to all families. The 41 patients whose families consented to the treatment schedule were administered a standard 3.75 mg dose of depot leuprolide acetate subcutaneously once every 28 days. These 41 patients were divided into two groups based on BA. All cases with a BA ≤10.5 years constituted group 1 (n=18) and those with a BA >10.5 years constituted group 2 (n=23). Eight patients for whom treatment approval could not be obtained constituted group 3 (control).

The patients were evaluated as followed in our outpatient clinic at 3-month intervals during treatment. Their compliance with the treatment and pubertal findings were recorded on observation forms. Morning height and body weight measurements were taken at each evaluation. BMI, height SDS, and BMI SDS were calculated. A hormonal evaluation was also performed once every 3 months. The cases underwent a standard intravenous GnRH stimulation test within 3-4 days after administration of the third dose of depot leuprolide. Peak LH <3 IU/L on this test was accepted as a suppressed HPG axis (20). The 3.75 mg depot leuprolide injections were continued once every 28 days in suppressed cases. Follow-up was continued with LH measurements in blood samples taken before the GnRHa injection and at minute 120 after the GnRHa injection at 3-month intervals. A peak LH response <4 IU/L on the GnRH analogue test was accepted as a criterion for a suppressed HPG axis (21) and the treatment was stopped in these cases. Regressing breast development and achieving prepubertal basal and GnRH- or GnRHa-stimulated LH levels were also accepted as HPG axis suppression criteria and a cause for cessation of treatment. HPG axis suppression was ensured in all treated cases.

BA was evaluated annually during treatment. The bone maturation ratio was calculated as the ΔBA/Δ chronological age (ΔBA/ΔCA) ratio using the annual change in the years following

treatment compared to that at treatment initiation. PFH and PFH SDS at the start of the treatment and during years 1 and 2 of treatment were used to evaluate the short-term effectiveness of the treatment on height.

Treatment was terminated based on an individual evaluation of each patient at the earliest CA of 10 years after considering Tanner stage at the start of the treatment, BA, growth rate during the last course of treatment, and the request of the child and the family. The follow-up continued after terminating treatment until cases reached their final height. Final height and final height SDS were determined.

Gonadotropin levels in serum were measured with an Access DXI 800 (Beckman Coulter, Brea, CA, USA) device using the immune chemiluminescence method in all cases. The detection limit for LH and FSH was 0.2 mIU/mL. Serum E2 levels were measured with the Modular E170 Immunological analyzer system (Roche Diagnostics, Manheim, Germany) using the electro-chemiluminescence method. The detection limit for E2 was 5 pg/mL.

All data were evaluated using the Statistical Package for the Social Sciences 15.0 statistics program (SPSS, Inc., Chicago, IL, USA) at our biostatistics department. The chi-square test was used to compare percentage values between the groups. Mean values were compared using the t-test when the distribution was normal, the Mann-Whitney U-test when the data were not normally distributed and when comparing just two groups, and analysis of variance (ANOVA) or the Kruskal-Wallis test when the three groups were compared. The intra-group time comparisons were made with the paired t-test when the distribution was normal, the Wilcoxon test when the data were not normally distributed, and repeated-measures ANOVA and the Friedman's analysis when the number of times was more than two. A p-value <0.05 was considered significant for all tests. Values are presented as means ± two standard deviations or median (range). Spearman's rho correlation was used for non-parametric correlation statistical analyses.

Results

Mean age at diagnosis of the 49 female cases with early puberty was 8.65±0.81 years and the median pubertal stage was Tanner 3. The mean symptom duration was 11 months (1-72 months). Puberty was reported to have started with premature pubarche in three cases (6.1%). Only 3 of the 49 cases had a history of preterm birth. Mean birth weight was 3100 g. Retardation of intrauterine growth was present in three cases (6.1%), and all three were term births. Mean age of diagnosis of the total group was 8.65±0.81 years, pubertal stage 3±0.81, height SDS 1.39±0.92, BMI SDS 1.02±0.91, BA 10.5±1.5 years, BA/CA 1.12±0.12, THSDS -0.22±0.94, predicted height standard deviation score (PHSDS) -0.11±1.00. Mean age of diagnosis was 8.09±0.42 years, pubertal stage 3±0.57, height SDS 0.96±0.73, BMI SDS 0.81±0.81, BA 9.42±0.87, BA/CA 1.15±0.08, THSDS -0.26±0.83, PHSDS 0.08±0.92 in group 1, while mean age of diagnosis in group 2 was 9.03±0.74 years, pubertal stage 4±0.73, height SDS 1.78±0.83, BMI SDS 1.28±0.86, BA 11.63±0.78, BA/CA 1.28±0.09, THSDS -0.4±0.9, PHSDS -0.45±1.08. In group

Table 1. Characteristics of the three groups at the time of diagnosis

	Group 1	Group 2	Group 3	All groups
	n=18	n=23	n=8	n=49
Age (years)	8.09±0.42	9.03±0.74	8.83±1.04	8.65±0.81
Pubertal stage	3±0.57	4±0.73	2±1.03	3±0.81
HSDS	0.96±0.73	1.78±0.83	1.22±1.18	1.39±0.92
BMI SDS	0.81±0.81	1.28±0.86	0.79±0.72	1.02±0.91
BA (years)	9.42±0.87	11.63±0.78	9.98±2.05	10.5±1.5
BA/CA	1.15±0.08	1.28±0.09	1.1±0.12	1.12±0.12
Maternal height (cm)	157.1±5.86	156.84±5.93	159.25±4.4	157.35±5.63
Paternal height (cm)	173.57±7.4	171.36±5.88	171.6±3.53	172.14±6.07
Target height (cm)	158.46±4.98	157.78±5.27	158.9±3.65	158.2±4.86
TH SDS	-0.26±0.83	-0.4±0.9	0.38±1.12	-0.22±0.94
PFH (cm)	160.55±5.53	157.56±6.49	161.6±4.06	159.32±5.95
PFH SDS	0.08±0.92	-0.45±1.08	0.39±0.59	-0.11±1.00

HSDS: height standard deviation score, BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age, TH: target height, PFH: predicted final height

3, mean age of diagnosis was 8.83±1.04 years, pubertal stage 2±1.03, height SDS 1.22±1.18, BMI SDS 0.79±0.72, BA 9.98±2.05, BA/CA 1.1±0.12, THSDS 0.38±1.12, PHSDS 0.39±0.59 (Table 1).

The demographic data and anthropometric characteristics of the study groups at the time of diagnosis are compared in Table 2. Duration of symptoms, maternal menarche age, maternal height, paternal height, TH, and BMI SDS were similar in the three groups. Age, pubertal stage, height SDS, BA, and BA/CA were significantly higher in group 2 compared to group 1 at diagnosis. The BA/CA ratio was similar in groups 1 and 3 and it was significantly higher in group 2 compared to group 3.

The first and second year follow-up data of the treatment groups are compared in Table 3. Height SDS, BMI SDS, growth velocity (GV), and GV SDS at the first and second year follow-up were similar in groups 1 and 2, while ΔBA/ΔCA in group 2 during the first and second years of follow-up was significantly lower than that in group 1 (p=0.009).

PFH and SDS at the time of diagnosis, PFH and SDS during the first year of follow-up, final height, final height SDS, TH, and TH SDSs were similar among the three groups (Table 4). PFH and SDS at the second year follow-up were lower in group 1 than in group 2 (p=0.021).

Final heights were similar among the groups (p=0.403) (Figure 1) and were positively correlated with TH (p=0.000, r=0.54) and height at the time of diagnosis (p=0.003, r=0.467). A linear regression analysis showed that for each 1 cm of height at the time of diagnosis, the final height increased by 0.213 times and for each 1 cm of TH, the final height increased by 0.459 times.

Discussion

Whether there is a height benefit of GnRHa administration when early puberty is diagnosed at 6-8 years of age is a controversial issue. Very few studies have been conducted with GnRHa in cases with advanced puberty starting at 8-10 years of age (8,22,23). The lack of control groups in these studies has raised unanswered questions regarding the effectiveness of treatment in these age groups (2). We compared the effects of GnRHa treatment on final height in early puberty cases whose symptoms started at 7-8.5 years of age with a control group and found that GnRHa did not make a positive contribution to final height. The group with advanced BA was taller at the time of diagnosis and their increase in BA slowed

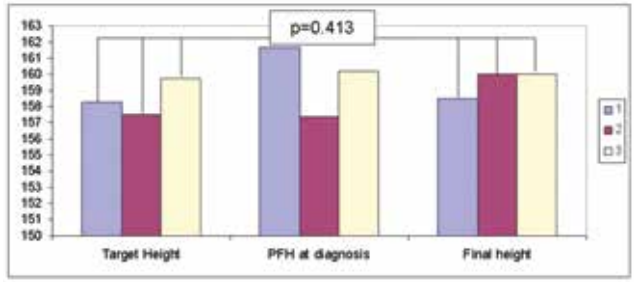

Figure 1. Target height, predicted final height at diagnosis and final height comparison

Table 2. Comparison of demographic characteristics and anthropometric data of the study groups at the time of diagnosis

	Group 1 (n=18)	Group 2 (n=23)	Group 3 (n=8)	Group 1-2 p-value	Group 1-3 p-value	Group 2-3 p-value
Age at diagnosis (years)	8.09±0.42	9.03±0.74	8.83±1.04	**0.001**	0.107	1.000
Duration of symptoms (years)	7.74±5.42	12±7.2	15.8±19	0.134	0.134	0.134
Tanner stage	3±0.57	4±0.73	2±1.03	**0.004**	1.000	0.077
Maternal menarche age (years)	12.2±1.03	11.9±0.89	12.6±1.14	0.377	0.377	0.377
Mother's height (cm)	157.1±5.86	156.84±5.93	159.25±4.4	0.528	0.528	0.528
Father's height (cm)	173.57±7.4	171.36±5.88	171.6±3.53	0.729	0.729	0.729
Target height (cm)	158.46±4.98	157.78±5.27	158.9±3.65	0.747	0.747	0.747
HSDS	0.96±0.73	1.78±0.83	1.22±1.18	**0.017**	0.92	0.874
BMI SDS	0.75±0.87	1.32±0.95	0.79±0.72	0.129	0.129	0.129
BA (years)	9.42±0.87	11.63±0.78	9.98±2.05	**0.000**	0.322	0.054
BA/CA	1.15 ±0.08	1.28±0.09	1.1±0.12	**0.000**	1.000	**0.003**

HSDS: height standard deviation score, BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age

with treatment, leading to no regression in final height with treatment. Administering GnRHa to cases according to BA did not improve final height; final height in this age group was positively influenced only by TH and height at the time of diagnosis.

Lazar et al (24) observed that the rate of growth decreased after treatment in cases with early puberty diagnosed after 6 years of age and interpreted this as a negative effect of the intrinsic changes at the growth plate before the treatment. However, Brito et al (25) did not find a significant relationship between the CA at the start of treatment and linear growth after treatment. Although age at the start of treatment was negatively associated with final adult height in that study, GnRHa treatment was started after the age of 6 years in most girls who reached a normal final height. This led to the conclusion that genetic TH potential in girls >6 years can be preserved with GnRHa treatment. Similarly, Carel et al (26) reported a significant increase in adult height and predicted adult final height with treatment in 42 patients when puberty started at 6-8 years of age.

The final height of 75% of the cases was consistent with TH in a meta-analysis in which more than 637 female cases treated with GnRH analogues were evaluated (27). When final height was compared to PFH the start of treatment, the best results were obtained in patients treated earlier. However, no positive effect was found in PFH after GnRHa treatment of girls whose puberty started at 8-10 years of age. Cassio et al (4) reported results similar to ours in which mean final height was similar in their groups and not different than the TH in their study; they treated 23 of 46 patients whose puberty started at 7.5-8.5 years of age and followed the other half without treatment. Final height was equal to or greater than TH in 14 of the 20 patients who reached their final height in the treated group and in 12 of 18 patients who reached their final height in the untreated group. They reported that BA more advanced than the CA and a height age/BA ratio <0.9 could be prognostic criteria for a poor initial height prognosis. The final height of these patients was significantly lower than that of patients with a good initial height prognosis; however, treating cases with a poor initial height prognosis did not contribute to final height. The authors reported that treatment had no positive effect on final height in cases of moderately early puberty and that administering treatment to cases with a good or poor prognosis according to the height prognosis did not contribute to final height. The authors concluded that final height is only affected by height at the beginning of

Table 3. Comparison of the 1st and 2nd year follow-up values of the study groups

	Group 1 (n=18)	Group 2 (n=23)	Group 1-2 p-value
HSDS (first year)	1.08±0.71	1.58±0.85	0.247
HSDS (second year)	0.77±0.7	1.14±1.39	0.179
BMI SDS (first year)	1.05±0.8	1.51±0.81	0.089
BMI SDS (second year)	1.15±0.81	2.15±0.64	0.281
GV (first year)	6.08±1.41	5.58±1.55	0.265
GV (second year)	5.08±1.14	4.9±1	0.773
GV SDS (first year)	0.77±1.62	-0.07±1.76	0.216
GV SDS (second year)	-0.32±1.54	-0.91±0.95	0.451
ΔBA/ΔCA (first year)	1.11±0.54	0.5±0.7	0.009
ΔBA/ΔCA (second year)	1.04±0.74	0.4±0.41	0.009

HSDS: height standard deviation score, BMI: body mass index, SDS: standard deviation score, BA: bone age, CA: chronological age, GV: growth velocity

Table 4. Comparison of target height, predicted final height, final height, and the standard deviation score values of these parameters between the groups

	Group 1 n=18	Group 2 n=23	Group 3 n=8	p-value
Target height (cm)	158.4±4.98	157.7±5.27	158.9±3.65	0.747
PFH at diagnosis (cm)	160.5±5.53	157.5±6.49	161.6±4.06	0.112
PFH at first year (cm)	159.7±5.53	160.3±5.68	159.7±5.6	0.171
PFH at second year (cm)	157.8±4.07	159.5±7.53		0.021
Final height (cm)	159±4.32	159.6±5.5	158.3±5.6	0.812
TH SDS	-0.26±0.83	-0.4±0.9	0.38±1.12	0.230
PFH SDS at diagnosis	0.08±0.92	-0.45±1.08	0.39±0.59	0.074
PFH SDS at first year	0.05±0.92	0.1±0.95	-1.1±0.04	0.186
PFH SDS at second year	-0.39±0.67	-0.12±1.26		0.017
Final height SDS	-0.21±0.72	-0.1±0.92	-0.32±0.94	0.813

PFH: predicted final height, TH: target height, SDS: standard deviation score

puberty and TH, and that height prognosis before treatment is not corrected by treatment. Our results are very similar to these findings. However, Cassio et al (4) emphasized that their data may have been influenced by the number of patients with a poor starting height prognosis being included in the study. We found a lower BA progression rate with treatment in the group with more advanced BA compared to the group with a younger BA. The similar results in the groups with and without advanced BA indicate that the treatment does not have an effect on final height independent of BA in this age group. Magiakou et al (28) similarly reported that final height in cases with idiopathic CEP starting at age of 7.9 years and treated with GnRHa was not different than that in the untreated group. Tanaka et al (29) monitored children with CEP in two prospective clinical studies (phase 2 and phase 3 studies) where they studied the effects of leuprorelin acetate on CEP. They were able to follow 76 (63 girls and 13 males) of these children until they reached their final height. These authors reported that 90% of the female cases with a CA of 7.7±2.2 years and a BA of 10.2±1.5 years at the start of the treatment achieved a final height in the TH range.

We found that GnRHa administration did not contribute to final height in cases aged 7-8.5 years with early puberty. Height at the time of diagnosis in the group with advanced BA was greater and BA progression was slower with treatment, leading to no change in final height with treatment. Administering GnRHa to cases according to BA did not improve final height, and the final height in this age group was positively influenced only by TH and height at the time of diagnosis. In our study, the number of girls in the control group was low and this state has caused a weakness of the study power.

In conclusion, factors such as age at menarche and the psychological condition of the child should be considered rather than height when deciding whether to start GnRHa treatment in girls showing signs of moderately early puberty.

Ethics

Ethics Committee Approval: It was taken from Ankara University Faculty of Medicine, Informed Consent: It was taken.

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Authorship Contributions

Surgical and Medical Practices: Şenay Savaş Erdeve, Merih Berberoğlu, Concept: Şenay Savaş Erdeve, Merih Berberoğlu, Gönül Öcal, Design: Şenay Savaş Erdeve, Merih Berberoğlu, Data Collection or Processing: Şenay Savaş Erdeve, Zeynep Şıklar, Bülent Hacıhamdioğlu, Pınar Kocaay, Emine Çamtosun, Merih Berberoğlu, Gönül Öcal, Analysis or Interpretation: Şenay Savaş Erdeve, Merih Berberoğlu, Literature Search: Şenay Savaş Erdeve, Writing: Şenay Savaş Erdeve, Merih Berberoğlu.

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Original Article

Homozygous Ala65Pro Mutation with V89L Polymorphism in SRD5A2 Deficiency

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ABSTRACT

Objective: Deficiency of steroid 5-alpha reductase type 2 (5αRD2) is a rare autosomal recessive disorder caused by mutations in the *SRD5A2* gene. A defect in the 5-alpha reductase enzyme, which ensures conversion of testosterone into dihydrotestosterone, leads to disorders of sex development. This study presents the clinical and genetic results of patients with 5αRD2 deficiency.

Methods: 5αRD2 deficiency was detected in 6 different patients from 3 unrelated families. All patients were reared as girls. Two of the patients presented with primary amenorrhea, one with primary amenorrhea and rejection of female gender, and the others with masses in their inguinal canals. Chromosome and sex-determining region Y (SRY) gene analyses were performed in all patients. Additionally, five exons of the *SRD5A2* gene were amplified with polymerase chain reaction in the obtained DNA samples and evaluated.

Results: While 46,XY was identified in 5 patients, 47,XXY was detected in one patient. The *SRY* gene was positive in all patients. The p.Ala65Pro (c193G>C) mutation and V89L polymorphism were observed in exon 1 of the *SRD5A2* gene in all patients.

Conclusion: Identification of this mutation and polymorphism is a significant indicator of presence of 5αRD2 deficiency in Southeastern Turkey, a geographical region where consanguineous marriages are also highly common.

Keywords: 46,XY disorders of sex development, 5-alpha-reductase, testosterone, mutation, polymorphism

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

5-alpha reductase type 2 deficiency can cause disorders of sex development. p.Ala65Pro (c193G>C) mutation has been reported before in Turkey.

WHAT THIS STUDY ADDS?

The p.Ala65Pro (c193G>C) mutation with V89L polymorphism is reported first time. This association can be found in Southeast region of Turkey.

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Introduction

Steroid 5-alpha reductase type 2 (5αRD2) deficiency is a rare autosomal recessive disorder caused by mutations in the *SRD5A2* gene. A defect in the 5-alpha reductase enzyme, which ensures conversion of testosterone (T) into dihydrotestosterone (DHT), leads to disorders of sex development (DSD) (1). The 5-alpha reductase enzyme has two isoenzymes (namely, *SRD5A1* and *SRD5A2*), and *SRD5A2* is found in genital skin tissue. Depending on the level of enzyme deficiency caused by the changes in this gene, patients may develop various phenotypic characteristics, including perineal hypospadias, bifid scrotum, micropenis, and complete female phenotype (2). It has been indicated that enzyme activity is affected by the polymorphisms in the *SRD5A2* gene. The most commonly known of these are the TA repeat polymorphism in the 3'-untranslated region and the polymorphisms ensuring mutation of tyrosine to alanine in codon 49', as well as the mutation of alanine to leucine codon 89 (3).

This study presents six patients who were diagnosed with 5αRD2 deficiency and showed mutation and polymorphism association.

Methods

Patient Studies

Patient 1, Family 1

Patient 1, a 12-year-old patient who presented to our endocrine clinic with complaints of amenorrhea and gender dysphoria had not been examined by another physician before. There was a consanguineous marriage between the parents. The patient's height was 176.4 cm and she weighed 63.3 kg. Thelarche was stage 1 and pubarche was stage 4 according to Tanner's classification. Gonads were palpable in both inguinal canals, and genital ambiguity was stage 3 according to Sinnecker's classification. The two masses, 10x24 mm and 15x25 mm, visualized in right and left inguinal canals in a pelvic magnetic resonance imaging (MRI), were consistent with testicles. Laboratory findings were as follows: follicle-stimulating hormone (FSH) 4.97 IU/L, luteinizing hormone (LH) 4.26 IU/L, total T 2350 pg/mL, and DHT 70 pg/mL. Serum T to DHT ratio was found to be 33.5, and genetic evaluation was performed regarding 5αRD2 deficiency. Examinations revealed a 46,XY karyotype and a positive sex-determining region Y (SRY). Additionally, the patient was found to a have a homozygous mutation of p.Ala65Pro (c193G>C) in exon 1 of the *SRD5A2* gene, as well as a p.Leu89Val (V89L) polymorphism. The patient was asked to come back for a follow-up evaluation regarding gonadectomy.

Patient 2, Family 2

A 15-month-old patient was referred to the pediatric endocrinology outpatient clinic due to a mass in the inguinal region. Her parents were third-degree cousins. She was 77 cm in length and weighed 9650 g. There was a palpable mass in the right inguinal canal and a gonad was observed in the left labium majus. Cliteromegaly was not detected (Figure 1). Internal genitalia were consistent with male structures in ultrasonography. Laboratory results were as follows: FSH 1.25 IU/L, LH 0.00 IU/L, and T <200 pg/mL. An human chorionic gonadotropin (hCG) test was not performed. Karyotype was 47, and both XXY and SRY were positive. A V89L polymorphism and a homozygous mutation of p.Ala65Pro (c193G>C) nucleotide substitution in exon 1 of *SRD5A2* gene were detected.

Patients 3, 4, 5, 6, Family 3

Patients 3, 4, 5, and 6 were all members of the same family. Patient 3 was a nine-year-old who presented with palpable bilateral masses in the inguinal area. Her parents were first-degree cousins. The patient was 137 cm [0.48 standard deviation score (SDS)] in height and 32.5 kg (0.45 SDS) in weight. Clitoromegaly was not detected. Hormone levels were as follows: FSH 1.57 IU/L, LH 0.15 IU/L, and T 110 pg/mL. After hCG simulation, was 1800 pg/mL, DHT was 53 pg/mL, and the T/DHT ratio was 33.9. As the patient's all 3 siblings had histories of DSD, they were also evaluated within the scope of the study.

Patient 4 was 3 years old. Her height was 93 cm (-0.31 SDS) and weight was 15 kg (0.59 SDS). Gonads were palpable in both inguinal areas. After hCG simulation, T was 1020 pg/mL, DHT <20 pg/mL, and T/DHT ratio was >51.

Patient 5 was a 15-year-old and was admitted to the clinic with complaints of primary amenorrhea and masses in the inguinal region. She was 163 cm (0.24 SDS) tall and weighed 54.1 kg (0.3 SDS). Gonads were palpable bilaterally in the inguinal region. Breast development was at stage 1 and pubic hair growth was at stage 4 according to Tanner's classification. Laboratory results were as follows: FSH 4.59 IU/L, LH 2.89 IU/L, T 1740 pg/mL, DHT 35 pg/mL, and the T/DHT ratio was 49.7.

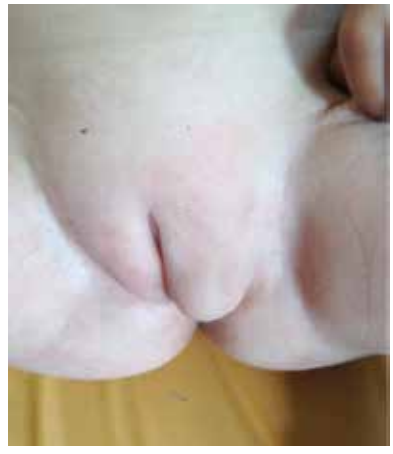

Figure 1. Female external genitalia and prolapse of the left labium majus due to the presence of a gonad (patient 2)

Patient 6 was a 24-year-old with a history of inguinal hernia repair at the age of 10. Additionally, she was being followed-up for DSD and receiving hormone replacement therapy. Her height was 174.6 cm (2.9 SDS) and weight was 63.3 kg (1.8 SDS). Her thelarche was stage 4. The T/DHT ratio could not be analyzed in this patient.

Uterus and ovary were not present in three patients: patients 3, 4, and 5. Their karyotype was 46,XY. Homozygous mutation of p.Ala65Pro (c193G>C) nucleotide substitution and V89L polymorphism in exon 1 of the *SRD5A2* gene were detected. Figure 2 shows the pedigree of these probands.

Genetic Analysis

The six patients from Family 1, 2, and 3 were examined at Harran University Faculty of Medicine Hospital. Detailed clinical findings are presented in Table 1. Informed consent was obtained from the parents of all patients. Blood samples from the five patients were available for genetic analysis, and DNA was extracted from whole blood using a salting out procedure. Primers were designed for polymerase chain reaction amplification of five exons of the *SRD5A2* gene (Table 2) (4), and the amplification products were sequenced on an Applied Biosystems 3730xl automated sequencer. The *SRD5A2* gene sequence analyses of Patients 1-6 are shown in Figure 3. Based on the sequencing results, the *SRD5A2* gene c.193G>C (p.Ala65Pro) nucleotide substitution in exon 1 was determined. This nucleotide variation identified at position 193 has caused the alteration of amino acid (Alanin-Prolin) in codon 65. Also, we have observed the *SRY* gene in all patients. In addition, we have found p.Leu89Val polymorphisms in all patients with 5αRD2 deficiency.

Hormonal Evaluation

In all patients, blood FSH, LH, and total testosterone levels were determined using the electrochemiluminescence immunometric assay method with the Roche Elecsys E170 immuno-analyzer (Roche Diagnostics, Burgess Hill, UK). Serum DHT levels were measured with RIA.

Discussion

In humans, the *SRD5A2* gene is located on chromosome 2p23 and contains 5 exons and 4 introns. 5αRD2 deficiency was first described in 1974 and genetic mutations were identified for nearly 20 years after that (5,6,7). More than 50 mutations have been identified to date (8,9). It has been reported that the incidence of the syndrome is high in the Dominican Republic, in some regions of New Guinea, and in Turkey. These findings are possibly related with "founder effect" and consanguineous marriages (10). Detection of the same mutation in different

Table 1. Clinical, laboratory, and genetic characteristics of the patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	12.47	1.21	9.21	3.03	14.64	24
Reared as	Female	Female	Female	Female	Female	Female
Affected sibling	No	No	Yes	Yes	Yes	Yes
Complaint	PA	IM	IM	IM	PA, IM	IM
Sinnecker classification	3	4	5	5	4	5
Basal/stimulated T (pg/mL)	2350	<200	1800	1020	1740	ND
Basal/stimulated DHT (pg/mL)	70	ND	53	<20	35	ND
T/DHT ratio (n<12)	33.5	ND	33.9	>51	49.7	ND
Chromosome analysis	46,XY	47,XXY	46,XY	46,XY	46,XY	46,XY
Mutation Polymorphism	*SRD5A2* gene, exon 1, p.Ala65Pro (c193G>C) p.Leu89Val					

PA: primary amenorrhea, IM: inguinal mass, T: testosterone, DHT: dihydrotestosterone, ND: not determined

Table 2. Primers and polymerase chain reaction products for exons and promoter regions of the *SRD5A2* gene (23)

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
1	GCAGCGGCCACCGGCGAGG	AGCAGGGCAGTGCGCTGCACT	358
2	TGAATCCTAACCTTTCCTCCC	AGCTGGGAAGTAGGTGAGAA	235
3	TGTGAAAAAAGCACCACAATCT	CAGGGAAGAGTGAGATCTGG	208
4	TGATTGACCTTCCGATTCTT	TGGAGAAGAAGAAAGCTACGT	232
5	TCAGCCACTGCTCCATTATAT	CAGTTTTCATCAGCATTGTGG	166

families in Iran has also been explained by the "founder effect" (11). The mutation identified in this study was detected in two patients who were the subjects of another study. The first patient was nine years old and reared as a girl. She presented to the clinic with the complaint of masses in both inguinal canals. There was a first-degree consanguinity between her parents. Genetic analysis revealed a mutation resulting from proline-to-alanine substitutions in exon 1, codon 65 (12). The second patient was seven years old and reared as a girl. She presented to the clinic with bilateral inguinal masses as well. Her parents were third-degree cousins. Genetic analysis revealed the same pAla65Pro mutation as in the first patient (13). We also detected the same mutations in three unrelated families from the same ethnic group. This finding has given rise to the thought that the existence of a common ancestor or a founder effect may be responsible for the spread of that genetic abnormality. This mutation, as far as we know, has not been detected in other ethnic populations.

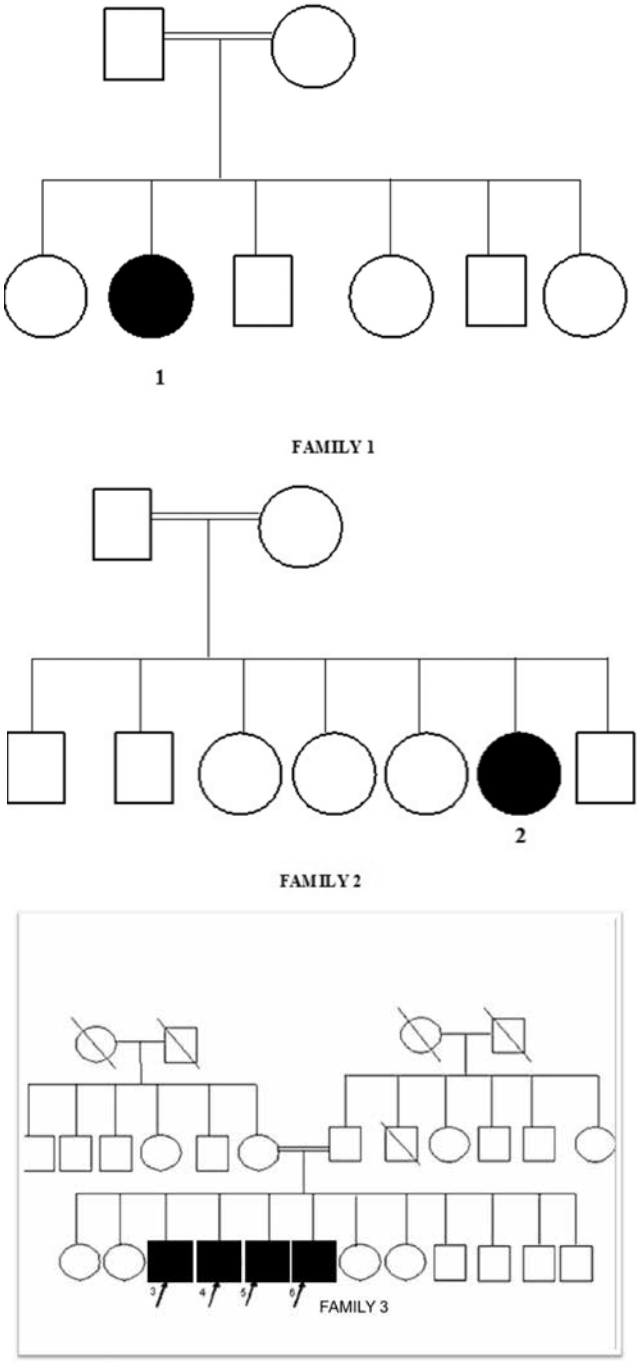

Figure 2. Pedigree of probands in Families 1, 2, 3: It was known that there was 5αRD2 deficiency in all patients

Some disorders such as prostate cancer or hypospadias may be related with *SRD5A2* gene polymorphisms. In the *SRD5A2* gene, the V and L polymorphisms have been associated with 5αRD2 activity; while the V allele is considered to be related to high activity, the L allele is related to low activity. It has been found that having the V allele of the *SRD5A2* gene doubles the risk of prostate cancer development (14). Specifically, these polymorphisms have been reported to increase the risk of prostate cancer in an Ecuadorian population (15). However, a meta-analysis involving 45 studies and a total of 15 562 patients was presented in 2013 and it was reported that there is no correlation between V89L polymorphism and prostate cancer although the A49T polymorphism may play a role in the etiology of prostate cancer in the Caucasian race (8). Another study revealed a strong correlation between V89L polymorphism and breast cancer (9).

A negative correlation has been noted between the V89 allele and hypospadias, implying that having the V allele may protect against hypospadias (14). It has been indicated that the individuals with the LL genotype of *SRD5A2* in India are at a 3.6 times higher risk for hypospadias development. Additionally, as the individuals at risk for hypospadias are generally from agricultural regions, the probability of a correlation between pesticide exposure and risk of hypospadias has been highlighted (16). Various publications have reported the presence of V89L polymorphism in patients

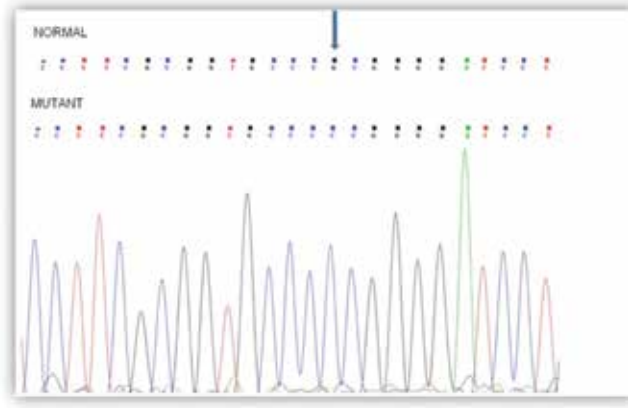

Figure 3. Mutation analyses of patients' 5RD genes c.193G>C nucleotide substitution in exon 1

with 5αRD2 deficiency. The first patient, who appeared in 2005, was an eight-year-old who was reared as a boy and had a heterozygous A207D mutation and V89L polymorphism (17). A multi-center international study published in 2011 analyzed 55 patients with 5αRD2 deficiency and found heterozygous mutations in 69.1%, compound heterozygous mutations in 25.5%, and compound heterozygous mutations characterized by V89L polymorphism in 5.4% (n=3) of the patients (18). In India, a patient with perineoscrotal hypospadias and micropenis was observed to have a novel heterozygous missense mutation Q56H and V89L homozygous polymorphism (19). Maimoun et al (20) reported three newborns diagnosed with DSD and new mutations. It is remarkable that one of those three newborns was a Turkish patient with a micropenis, hypospadias, and bifid scrotum who had an S12R mutation in exon 1 as well as V89L polymorphism. All our patients were detected to have this polymorphism. It may be inferred that the V89L polymorphism is particularly common in our country. However, this is the first study that indicates the association of this mutation with V89L polymorphism. Our results confirm that V89L polymorphism affects the development of external genitalia.

Klinefelter's syndrome is considered to be the most common chromosomal abnormality among males. DSD is not very common in individuals with Klinefelter's syndrome. However, it was reported in 1994 that in 22 patients with ambiguous genitalia one had a 47,XXY genotype and another one had 46,XX/47,XXY. Another study carried out on 30 patients with DSD in 2010 reported 4 patients as having a 45,X/47,XXY pattern (21,22). Akcay and Ulucan (23) performed genetic analyses in three unvirilized patients with the 47,XXY genotype and found a p.g196s homozygous mutation in the *SRD5A2* gene in patient one, 23 repeat polymorphisms in exon 1 of the androgen receptor gene in patient two, and heterozygous p.f891l mutations in androgen receptors, along with repeat polymorphisms, in patient three. In the present study, however, we reported a novel mutation of the *SRD5A2* gene in combination with p.Ala65Pro in unvirilized patients with 47,XXY genotype. It should be kept in mind that in rare cases, Klinefelter patients may have ambiguous genitalia.

As a concluding remark, we could state that p.Ala65Pro mutation in the *SRD5A2* gene causes 5αRD2 deficiency, especially in Turkey. V89L polymorphism may also be an important factor in the development of external genitalia.

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Ethics

Ethics Committee Approval: The present study was approved by local ethic committee (Harran University Faculty of Medicine), Informed Consent: It was taken.

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Authorship Contributions

Concept: Erdal Eren, Tuba Edgünlü, Design: Erdal Eren, Tuba Edgünlü, Sevim Karakaş Çelik, Data Collection and/or Processing: Erdal Eren, Tuba Edgünlü, Sevim Karakaş Çelik, Analysis and/or Interpretation: Erdal Eren, Tuba Edgünlü, Sevim Karakaş Çelik, Literature Research: Erdal Eren, Emre Asut, Writing: Erdal Eren, Emre Asut.

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Case Report

A Novel c.554+5C>T Mutation in the DUOXA2 Gene Combined with p.R885Q Mutation in the DUOX2 Gene Causing Congenital Hypothyroidism

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ABSTRACT

The coexistence of mutations in the dual oxidase maturation factor 2 (*DUOXA2*) and dual oxidase 2 (*DUOX2*) genes is rarely identified in congenital hypothyroidism (CH). This study reports a boy with CH due to a novel splice-site mutation in the *DUOXA2* gene and a missense mutation in the *DUOX2* gene. A four-year-old boy was diagnosed with CH at neonatal screening and was enrolled in this study. The *DUOXA2*, *DUOX2*, thyroid peroxidase (*TPO*), and thyrotropin receptor (*TSHR*) genes were considered for genetic defects screening. Genomic DNA was extracted from peripheral blood leukocytes, and Sanger sequencing was used to screen the mutations in the exon fragments. Family members of the patient and the controls were also enrolled and evaluated. The boy harbored compound heterozygous mutations including a novel splice-site mutation c.554+5C>T in the maternal *DUOXA2* allele and c.2654G>A (p.R885Q) in the paternal *DUOX2* allele. The germline mutations from his parents were consistent with an autosomal recessive inheritance pattern. No mutations in the *TPO* and *TSHR* genes were detected. A novel splice-site mutation c.554+5C>T in the *DUOXA2* gene and a mutation p.R885Q in the *DUOX2* gene were identified in a 4-year-old patient with goitrous CH.

Keywords: Congenital hypothyroidism, dual oxidase maturation factor 2, dual oxidase 2, mutation

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The dual oxidase maturation factor 2 (*DUOXA2*) and dual oxidase 2 (*DUOX2*) genes are rarely identified in congenital hypothyroidism (CH).

WHAT THIS STUDY ADDS?

We detected a novel splice-site mutation in the *DUOXA2* gene and a missense mutation in the *DUOX2* gene in a boy with CH.

Introduction

It is generally known that congenital hypothyroidism (CH) is the most common neonatal endocrine disorder and occurs in approximately 1:2000-1:4000 of newborns. CH cases are caused by various defects including thyroid dysgenesis and thyroid hormone synthesis defects (1,2). Previous studies revealed

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inactivating mutations in a specific subtype of CH. Of the known genes, mutations in dual oxidase maturation factor 2 (*DUOXA2*), dual oxidase 2 (*DUOX2*), thyroid peroxidase (*TPO*), and thyrotropin receptor (*TSHR*), that are all known to be related to thyroid dysgenesis or dyshormonogenesis and that are all inherited in an autosomal recessive pattern, have been reported (3,4,5,6).

The existing data suggest that inactivating mutations in the TSHR gene are responsible for thyrotropin (TSH) resistance and thyroid dysgenesis (1,2,6,7). Mutations in the *DUOXA2*, *DUOX2*, and *TPO* genes are responsible for thyroid dyshormonogenesis and goitrous congenital hypothyroidism (GCH) (3,4,5). Defective thyroid hormone synthesis represents most cases of GCH. Mutations in the *DUOXA2, DUOX2, TPO*, and *TSHR* genes are more common than those in thyroglobulin (TG) and paired box 8 (PAX8) genes in CH (1,2).

Currently, it is believed that H_2O_2 generation needs the catalytic core of *DUOX2*. Oxidation reaction is crucial for the iodination of TG during thyroid hormone synthesis. *DUOXA2* is required for normal *DUOX2* enzymatic activity. It has been identified that *DUOXA2* is crucial for *DUOX2* maturation, and genetic defects in *DUOX2* cause CH and subclinical another important candidate gene for CH and SCH (3,4,8,9,10).

To date, the genetic defects in CH have not been fully understood. In this study, the *DUOXA2*, *DUOX2*, *TPO*, and *TSHR* genes were considered for screening genetic defects in a male patient with GCH reported below.

Case Report

A four-year-old boy came from the city of Suqian in Jiangsu Province, China. He had been diagnosed as CH at the neonatal screening and treatment was initiated. He was recruited by our team for investigation of a possible mutation. The patient was born to non-consanguineous parents without thyroid disease. CH was diagnosed on the basis of serum TSH, free thyroxine (fT_4), and free triiodothyronine (fT_3) levels. Daily L-thyroxine was administered to the patient at diagnosis. Thyroid gland examinations were performed with ^{99m}Tc thyroid scan and ultrasound at age four years. A total of 105 unrelated healthy controls were enrolled in this study. This study was approved by the ethics committee of the hospital. Written informed consent was obtained. Blood samples were collected from the participants.

At the beginning of the study, venous blood samples were obtained from the boy. DNA was extracted from peripheral blood leukocytes. Primers were designed to target the flanking intron regions of the exons. All exons of the *DUOXA2* (MIM# 612772, GenBank NM_207581.3), *DUOX2* (MIM# 606759, GenBank NM_014080.4), *TPO* (MIM# 606765, GenBank NM_000547.5), and *TSHR* (MIM# 603372, GenBank NM_000369.2) genes were amplified by polymerase chain reaction (PCR). The amplified PCR products were Sanger sequenced directly for variance analysis. All exons of the above genes were first amplified in the patient. If a mutation was identified, the target fragment was also amplified in the patient's parents and in 105 control individuals. Novel mutations were analyzed by bioinformatic tools.

The clinical summary and thyroid function of the boy and his parents are shown in Table 1. The proband had overt CH at neonatal screening. L-thyroxine was the treatment of choice at diagnosis, with a starting dose of 10 μ was the treatment of choice at diagnosis, with a starting dose of 10s are shown in Table 1. The proband had. Thyroid function tests showed that the parents had normal thyroid function. Thyroid ultrasound examination demonstrated enlarged thyroid lobes in our patient. Thyroid ^{99m}Tc scan revealed that the boy's thyroid appeared normally located but enlarged (Figure 1, Panel A).

As shown in Figure 2, the genetic analysis demonstrated two heterozygous mutations, a novel maternal allele splicing site variant (c.554+5C>T) (C to T substitution at position +5 of the donor site of intron 4) in the *DUOXA2* gene and another paternal allele missense mutation c.2654G>A (p.R885Q) in the exon 20 of the *DUOX2* gene, which has been reported previously (3). No mutations in the *TPO* and *TSHR* genes were detected in this study. None of the controls showed the same pathogenic variants.

The splicing site variant c.554+5C>T at the exon 4/intron 4 junction of the *DUOXA2* was not present in the Human Gene Mutation Database, nor in the dbSNP database, 1000 Genomes Project database, or PubMed. The splicing variant prediction was carried out using Human Splicing Finder, Alternative Splice Site Predictor, and SplicePort. The prediction results showed that the variant might alter gene splicing by removing the normal splice donor at the abnormal site (potential splice site: ATGgtaagc, consensus value: 92.12) or (constitutive donor: TAAAGTTCCTgtaagtatta, score: 13.255; constitutive acceptor: tgtctcccagGAATCTCCCT, score: 9.038) or (donor short sequence: ttcctgtaagta, score: 1.59992; donor short sequence: ttcctgtattaa, score: -0.995081), respectively. We concluded that the c.554+5C>T might lead to intron 4 splicing loss and altered *DUOXA2* messenger ribonucleic acid (RNA) sequence and the protein primary structure.

Table 1. Clinical and biochemical data of the family in May 2015

Variables	Normal range	Patient$^{\phi}$	Mother	Father
Age (years)	/	4	28	30
Height (cm)	/	108	160	169
Weight (kg)	/	12	55	65
Vision	/	Normal	Normal	Normal
Thyrotropin (mIU/mL)	0.34-5.44	>100.00	1.61	2.80
Free triiodothyronine (pmol/L)	2.92-5.93	2.84	3.77	4.00
Free thyroxine (pmol/L)	7.91-20.59	4.47	15.20	17.52
Thyroid size	/	Goiter	Normal	Normal

$^{\phi}$: thyroid function tests were performed at screening in the neonatal period

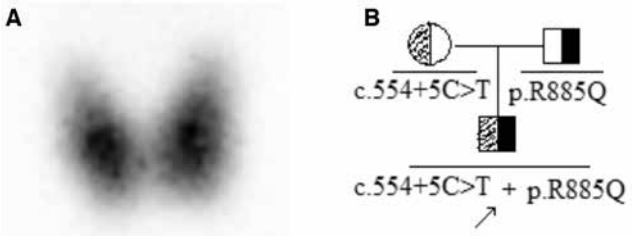

Figure 1. Thyroid ^{99m}Tc scan revealed the enlarged thyroid lobes of the boy (Panel A, anterior view). The arrow indicating the proband in the pedigree with the compound heterozygous mutations (Panel B)

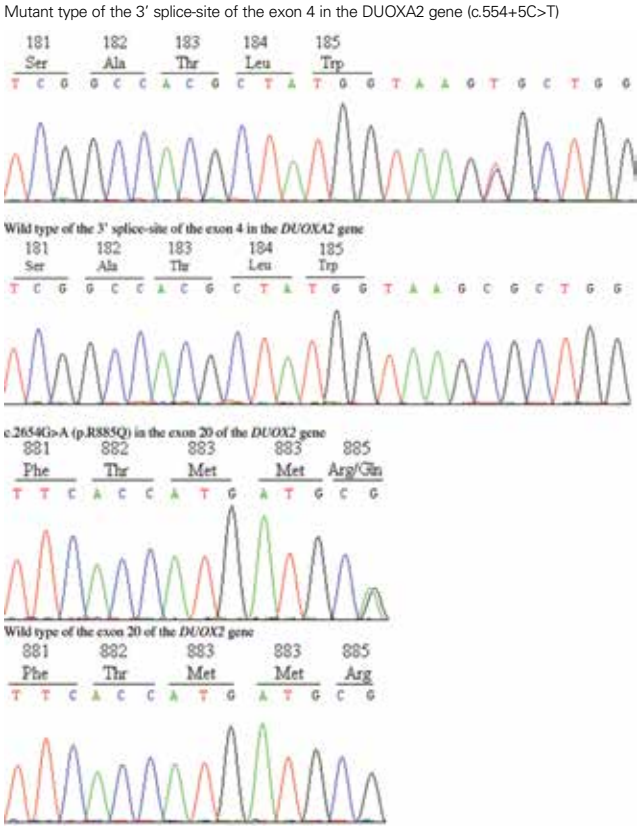

Figure 2. The genotypes revealing the heterozygous mutations in the *DUOXA2* gene (c.554+5C>T) and in the *DUOX2* gene (c.2654G>A, p.R885Q)

Discussion

The present study demonstrated compound heterozygous mutations, c.554+5C>T in the *DUOXA2* gene and c.2654G>A (p.R885Q) in the *DUOX2* gene in a pedigree with one four-year-old boy with GCH. H_2O_2 is a key element in iodine organification. *DUOXA2/DUOX2* is the main enzyme for the H_2O_2-generating system. Defects in the *DUOX2/DUOXA2* heterodimer lead to hypothyroidism and goiter. Since the first report in 2002 of *DUOX2* mutations causing CH (10,11), over 40 mutations in the *DUOX2* gene have been described correlated with CH, while only four mutations have been identified in the *DUOXA2* gene (3,8,9,10). Thus far, our splice site mutation, as far as we know, is identified for the first time as being causative of CH.

The patients with *DUOX2* or *DUOXA2* mutation show a great genotype-phenotype variability (10,11). Maruo et al (3) firstly reported the p.R885Q mutation in the *DUOX2* gene exhibiting transient hypothyroidism, which is not similar to our patient. The patient in this study had permanent CH and needed L-thyroxine replacement therapy. Heterozygous *DUOX2* gene mutations result in different phenotypes, such as transient CH, subclinical hypothyroidism, and euthyroidism. However, the coexistence of heterozygous *TSHR* and *DUOXA2* mutations causes overt hypothyroid condition (12).

Four mutations in the *DUOXA2* (p.I26M, p.Y138X, p.C189R and p.Y246X) were found to be associated with CH (3,4,8,9,10). The patient with the p.I26M, p.C189R, and p.Y138X heterozygous missense mutation in *DUOXA2* gene presented as a mild transient CH case. A homozygous nonsense mutation (p.Y246X) in patients with mild permanent CH and goiter was also identified. These patients are all of Chinese origin, indicating that this specific variant may occur at a high frequency in Chinese cohorts with CH.

Splice-site mutations are important disease-causing defects. It is estimated that approximately 10% of human genetic diseases are caused by mutations at splice sites (13). Analysis of the c.554+5C>T variation in the *DUOXA2* gene revealed that it is capable of causing disease. Possibly this is the first report of a c.554+5C>T mutation in the *DUOXA2* gene. The proband in this study presented with a normally located but enlarged thyroid gland. His parents, each with a single heterozygous mutation, both exhibited normal thyroid positioning and normal serum thyroid hormone levels. Our patient demonstrated no physical or cognitive developmental defects, primarily due to the timely and effective treatment.

Additionally, the c.554+5C>T mutation may affect the RNA transcription process and lead to genetic instability of the *DUOXA2* gene. Further comprehensive functional assessments of the detected mutation will reveal its exact mechanism in the pathogenesis of CH. The R434X mutation in the *DUOXA2* was detected by a two-stage strategy of genetic linkage studies and targeted sequencing of the candidate genes, suggesting a new testing strategy which uses next-generation sequencing in CH cases (14).

In conclusion, the present study reports a novel splicing site variant (c.554+5C>T) in the *DUOXA2* gene and another missense mutation c.2654G>A (p.R885Q) in the *DUOX2* gene. The findings indicate the importance of molecular genetic studies for the accurate diagnosis and classification of CH.

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Ethics

Ethics Committee Approval: Huai'an Second People's Hospital Ethics Committee (Approval number: 05-23-2014), Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Shao-Gang Ma, Design: Shao-Gang Ma, Data Collection or Processing: Xiao Zheng, Ya-Li Qiu, Analysis or Interpretation: Man-Li Guo, Xiao-Juan Shao, Literature Search: Man-Li Guo, and Xiao-Juan Shao, Writing: Xiao Zheng and Shao-Gang Ma.

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Case Report

Early Presentation of Hyperinsulinism/Hyperammonemia Syndrome in Three Serbian Patients

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ABSTRACT

Hyperinsulinism/hyperammonemia (HI/HA) syndrome is considered as the second most common type of hereditary HI. Correlation of genotype and phenotype in HI/HA syndrome has been described in several studies. We present three Serbian patients with HI/HA syndrome with emphasis on a possible correlation between genotype and clinical manifestations. Patient 1 was heterozygous for a *de novo* mutation p.S445L in the *GLUD1* gene, while patients 2 and 3 (son and mother) both carry the p.R221C mutation. Early onset of hypoglycaemia with generalized seizures was recorded in infancy in all three patients. The two male patients had mild developmental delay, while the female patient presented with epilepsy. Analysis of Serbian patients with HI/HA syndrome confirms the association of p.S445L and p.R221C mutations with hypoglycaemic seizures noted within the first three months of life and with subsequent risk for cognitive impairment and/or epilepsy.

Keywords: Hyperinsulinism/hyperammonemia syndrome, genotype, phenotype

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Hyperinsulinism/hyperammonemia (HI/HA) syndrome is considered as the second most common type of hereditary HI. Dominantly expressed, activating mutations in *GLUD1* gene encoding glutamate-dehydrogenase are responsible for occurrence of HI/HA syndrome. Mutations in exons 6 and 7 tend to be associated with increased risk of epilepsy, but genotype-phenotype correlation was also noted for other characteristics of HI/HA syndrome patients (e.g. age of onset, birth weight).

WHAT THIS STUDY ADDS?

Patients carrying p.R221C mutation may present with hypoglycaemic seizures earlier than previously reported with increased risk for cognitive impairment and/or epilepsy at later age. The carriers of p.S445P mutation could be prone to early hypoglycaemic seizures and mild developmental delay.

Introduction

Hyperinsulinism/hyperammonemia (HI/HA) syndrome is considered as the second most common type of hereditary HI (1). Dominantly expressed, activating mutations in *GLUD1* gene encoding glutamate-dehydrogenase (GDH) are responsible for occurrence of HI/HA syndrome (2). It was estimated that nearly 80% of disease-causing mutations arise *de novo* in *GLUD1* gene (3). First clinical manifestations are usually hypoglycaemic

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seizures with median age of onset ranging from 4 to 11 months according to different studies (1). During the course of the disease, recurrent symptomatic hypoglycaemia, both during fasting and after protein-rich meals, remains the main clinical feature. Plasma ammonia levels are commonly elevated 2-5 fold the upper limit, but HA is mostly considered as benign due to the specific pathophysiology of the HI/HA syndrome (1). On the other hand, a high prevalence of epilepsy and delayed mental development are reported in these patients (4,5,6,7). The risk for epilepsy is not in correlation with HA but is speculated to be the consequence of altered GDH activity in the brain (8). Mutations in exons 6 and 7 tend to be associated with increased risk of epilepsy (4,5,9). Genotype-phenotype correlation was also noted for other characteristics of HI/HA syndrome patients such as age of onset and birth weight (4,5). Diazoxide treatment of hypoglycaemia in patients with HI/HA is usually successful (3).

We present three Serbian patients with HI/HA syndrome with emphasis on possible correlation between genotype and clinical manifestations.

Case Reports

Patient 1 is the second male child of healthy non-consanguineous parents. He was born at term with a birth weight of 3850 g, birth length of 51 cm, and an Apgar score of 10 at one minute. At the age of nine weeks, the infant had generalized tonic-clonic seizures. Upon admission, the blood glucose level was 2.0 mmol/L with a simultaneous serum insulin level of 32.4 mIU/L. The plasma ammonia level was of 277 μmol/L (reference range 11-50 μmol/L). During the following days, the infant had recurrent hypoglycaemia and persistently high ammonia levels ranging from 219 to 315.5 μmol/L. There were no abnormalities in plasma amino acid levels nor in urinary organic acid concentrations. Mild hypotonia was observed at 9 weeks of age. At this time, the infant's weight had increased to 6.9 kg (92nd centile) and length to 62 cm (75th centile). Electroencephalography (EEG) pattern was normal. Family history for hypoglycaemia was negative.

Genetic analysis was performed in the Molecular Genetics Laboratory of Exeter and Plymouth Universities (UK), and the patient was found to be heterozygous for *de novo* missense mutation p.S445L (c.1334C'T) in exon 11 of *GLUD1* gene. Initial treatment included diazoxide (5 mg/kg/day) and hydrochlorothiazide (1 mg/kg/day) with advice for avoiding protein-rich meals. During the following four months, hypoglycaemic episodes were verified once in two months, usually after increased protein intake. Diazoxide dose was increased to 7 mg/kg/day, while the patient was weaned off hydrochlorothiazide therapy. At 5 months of age, the boy was overweight with 8.6 kg (93rd centile), and at 8 months, he weighed 9.85 kg (88th centile). However, at age 30 months, his body mass index was within normal limits (17.49 kg/m^2, 82nd centile). The boy walked independently at 14 months of age. A borderline delay in mental development was verified at 30 months with a developmental quotient (DQ) of 85 according to Brunet-Lézine scale. After introduction of diazoxide, the boy remained seizure-free.

Patient 2 was admitted to a local hospital due to generalized seizures at the age of 10 weeks. Hypoglycaemia (2.4 mmol/L) was verified, but no further investigations were done at the time. This male infant was the first child of non-consanguineous parents, born at term, with a birth weight of 3050 g, a birth length of 49 cm, and an Apgar score 9 at one minute. At the age of six months, seizures reoccurred with hypoglycaemia (1.4 mmol/L), and the patient was referred to our Institute. On admission, the infant had normal physical findings with a weight of 8200 g (62nd centile) and length of 68 cm (50th centile). During the first day of hospitalization, the lowest value of glycaemia was 2.8 mmol/L with a concomitant insulinemia of 19.82 μmol/L and HA of 159.2 μmol/L. EEG pattern was described as normal. Treatment was started with diazoxide (5 mg/kg/day) and hydrochlorothiazide (2 mg/kg/day) with advice for avoiding protein-rich meals. Since the patient missed regular check-ups, the next examination was performed at 20 months of age and speech delay with mild hyperactivity was noted. The parents acknowledged several hypoglycaemic crises (non-seizure) early after treatment introduction. At the age of 3.5 years, physical status was normal while neuropsychological assessment confirmed borderline mental delay (DQ 90 at Brunet-Lézine scale) associated with signs of attention-deficit/hyperactivity disorder.

Patient 3 (the mother of patient 2) was discovered by the family history which revealed that she had presented to a hospital at age 12 weeks with generalized tonic-clonic seizures. The blood glucose level was not measured during the initial investigation in this local hospital. Her perinatal history was uneventful. Her birth weight was 2800 g. At the age of six months, this patient was referred to our Institute due to recurrent seizures and at admission, severe hypoglycaemia of 0.6 mmol/L was verified. Further testing revealed HI (an insulin level of 59.8 pmol/L associated with hypoglycaemia of 1.0 mmol/L). Diazoxide was initiated (10 mg/kg/day) and very good control of glycaemia was achieved in the following years. From the second year of life on, the patient's seizures were mostly episodes of absence with eyelid myoclonia and occasional generalized tonic-clonic seizures. The epileptic state worsened at the age of 15 years requiring administration of two antiepileptic drugs but without achieving complete seizure control. Currently, she experiences several generalized tonic-clonic seizures per year (usually after protein-rich meals) and continues to have eyelid myoclonic seizures at least once weekly. She became increasingly non-compliant to diazoxide treatment during her late teenage period and stopped taking this medication at 18 years of age without consulting her physician. Ammonia was tested for the first time in her adult

age, and values ranged from 100 to 120 µmol/L. The patient is a housewife who graduated from a regular high school, however, her intellectual level was not clinically estimated at adult age.

Genetic analysis performed in the Molecular Genetics Laboratory of Exeter and Plymouth Universities (UK) showed that the boy and his mother (patients 2 and 3) are heterozygous for mutation p.R221C (c.833C>T) in exon 6 of *GLUD1* gene.

Discussion

We have identified three patients with HI/HA syndrome from two Serbian families. These are the first cases of HI/HA syndrome reported from Serbia. Patient 1 manifested with hypoglycaemic generalized seizures at the 10th week of life, a finding which is in accordance with reports that p.S445P is associated with early onset of HI/HA syndrome. In other words, generalized tonic-clonic seizures occurred between the 1st week and 5th month of life in all patients with p.S445P reported by Kapoor et al (4), Bahi-Buisson et al (5), Raizen et al (6), and Diao et al (7).

In one study, it was observed that p.S445P mutation was associated with epilepsy in one third of patients and also with borderline to mild development delay in all patients. Thus, our patient showed a neurologic pattern showing similarities to previous reports (5). However, the follow-up period for patient 1 is still relatively short since he is only 3 11/12 years old at the time of this report.

Patient 1 harboring a p.S445P mutation exhibited higher peak plasma ammonia concentration than our patients with p.R221C mutation. This observation is also in accordance with previous reports (4). As expected, the level of the HA did not correlate with neurological outcome in our HI/HA patients.

Early onset of generalized tonic-clonic seizures associated with frank hypoglycaemia was noted in patient 2. In patient 3, carrying the same p.R221C mutation, generalized seizures also occurred as early as three months of age. Carriers of this mutation have been previously reported to have neonatal disease onset (4). However, patients with p.R221C mutation usually have somewhat later disease onset (7-23 months) or stay asymptomatic until adulthood as suggested by Bahi-Buisson et al (5).

Our patient 2 has a borderline developmental delay noted during the second year of life, while epilepsy did not occur during the follow-up period of 3 years and 10 months. His mother (patient 3), who had very similar initial presentation, developed absence epilepsy with eyelid myoclonia during her second year of life. Bahi-Buisson et al (5) reported a strong association of p.R221C mutation with absence and eyelid myoclonia epilepsy starting between 2 and 6 years of age, refractory to treatment in 40% of cases. A similar course of epilepsy was also noted in our patient 3. The same study reported hypoglycaemic seizures starting at 7-23 months as a first manifestation of HI/HA syndrome caused by p.R221C mutation, with borderline to moderate learning disability in the majority of these patients. An earlier study described one family with five members affected by HI/HA syndrome due to p.R221C mutation (10). Similar to other studies, in this family, first symptoms are reported to occur between 6 and 15 months of age, and all patients who survived until adulthood had mental retardation, while 50% of them had epilepsy. Our findings also suggest a risk for mild cognitive impairment, attention deficit, hyperactivity, and refractory epilepsy for carriers of p.R221C mutation. However, our patients had an earlier onset than the majority of previously reported cases.

Apart from studies of HI/HA syndrome, investigations of cohorts with congenital HI (CHI) showed a substantial prevalence of mental retardation (26-44%) and epilepsy (17.7-25%) in this population (11,12). Interestingly, these researchers reported conflicting results regarding age of onset as a risk factor for mental retardation in CHI patients. A case series study of HI/HA syndrome patients suggested that neonatal onset of the disease could be a risk factor for occurrence of infantile spasms (13). The largest study on neurological outcome in patients with HI/HA syndrome showed that onset within the first 3 months of life was associated with borderline to mild developmental delay in all patients and with epilepsy in more than 70% (5).

Incompliance to diazoxide treatment has led to worsening of epilepsy in our patient 3. One recent report pointed out the success of diazoxide treatment in inducing remission of epileptic seizures in both pediatric and adult patient from the same family (9). All three of our patients had a good response to diazoxide treatment, a finding which is in agreement with the findings of others (4,5,6,7). We did not perform a protein loading test, so the recommendation to avoid protein-rich meals was given on the basis of previous studies of HI/HA syndrome (1,14). Japanese authors reported a patient with mutation in exon 7 of *GLUD1* gene who presented with absence epilepsy and eyelid myoclonia refractory to treatment and whose seizures intensified after protein-rich meals (15). Patient 3 from our series also experienced seizures after higher intake of proteins, in absence of hypoglycaemia. Avoidance of protein-rich meals appears to be necessary through adulthood, especially after the cessation of diazoxide therapy.

Proposed pathogenic mechanisms for epilepsy and developmental delay in HI/HA syndrome include effects of chronic HA, hypoglycaemic brain injury, and decreased brain tissue concentration of glutamate and gamma-aminobutyric (GABA) acid due to GDH hyperactivity (4,16). So far, there is no evidence for correlation between serum concentration of ammonia and the risk for epilepsy and/or intellectual impairment in patients with HI/HA syndrome (5). A typical pattern of ammonia toxicity to the brain is not present in HI/HA syndrome since hyperactivity of GDH prevents brain oedema by decreasing intracellular concentration of glutamine. Similarly, correlation of frequency and severity of hypoglycaemia

to neurological outcome has not been proven in previous studies of this disorder (6,16). On the other hand, hyperactive GDH causes depletion of glutamate in brain cells leading to disturbed synthesis of GABA which acts as the key inhibitory neurotransmitter (16). It seems that pathogenetic mechanisms involved in occurrence of epilepsy and developmental delay in HI/HA syndrome are somewhat different than in patients with other types of CHI. Higher prevalence and earlier occurrence of neurological complications in patients with HI/HA syndrome when compared to other causes of CHI syndrome prompts a need for further elucidation of its pathophysiology in future studies.

In summary, we believe that an analysis of Serbian patients with HI/HA syndrome could contribute to a better understanding of genotype-phenotype correlations in this rare disease. Namely, we have shown that patients carrying p.R221C mutation may present with hypoglycaemic seizures earlier than previously reported with increased risk for epilepsy and/or cognitive impairment. Data from previous studies as well as from our case series suggest that neurologic outcome in patients with HI/HA syndrome depends on a multitude of factors, including genotype, age of onset, and compliance to dietary and drug treatment.

Ethics

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

Authorship Contributions

Concept: Adrijan Sarajlija, Tatjana Milenkovic, Khalid Hussain, Design: Adrijan Sarajlija, Data Collection and/or Processing: Adrijan Sarajlija, Tatjana Milenkovic, Maja Djordjevic, Katarina Mitrovic, Sladjana Todorovic, Bozica Kecman, Analysis and/or Interpretation: Adrijan Sarajlija, Tatjana Milenkovic, Maja Djordjevic, Khalid Hussain, Literature Research: Adrijan Sarajlija, Tatjana Milenkovic, Maja Djordjevic, Khalid Hussain, Writing: Adrijan Sarajlija, Tatjana Milenkovic.

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Case Report

A Novel Mutation in Human Androgen Receptor Gene Causing Partial Androgen Insensitivity Syndrome in a Patient Presenting with Gynecomastia at Puberty

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ABSTRACT

Partial androgen insensitivity syndrome (PAIS) typically presents with micropenis, perineoscrotal hypospadias, and a bifid scrotum with descending or undescending testes and gynecomastia at puberty. It is an X-linked recessive disorder resulting from mutations in the *androgen receptor (AR)* gene. However, *AR* gene mutations are found in less than a third of PAIS cases. A 16-year-old boy was admitted with complaints of gynecomastia and sparse facial hair. Family history revealed male relatives from maternal side with similar clinical phenotype. His external genitalia were phenotypically male with pubic hair Tanner stage IV, penoscrotal hypospadias, and a bifid scrotum with bilateral atrophic testes. He had elevated gonadotropins with a normal testosterone level. Chromosome analysis revealed a 46,XY karyotype. Due to the family history suggesting a disorder of X-linked trait, PAIS was considered and molecular analysis of *AR* gene was performed. DNA sequence analysis revealed a novel hemizygous mutation p.T576I (c.1727C>T) in the *AR* gene. The diagnosis of PAIS is based upon clinical phenotype and laboratory findings and can be confirmed by detection of a defect in the *AR* gene. An accurate approach including a detailed family history suggesting an X-linked trait is an important clue for a quick diagnosis.

Keywords: Partial androgen insensitivity, gynecomastia, androgen receptor gene

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Partial androgen insensitivity syndrome typically presents with micropenis, perineoscrotal hypospadias, and a bifid scrotum with descending or undescending testes and gynecomastia at puberty. It is an X-linked recessive disorder resulting from mutations in *androgen receptor (AR)* gene. In approximately 50% of cases, a mutation in *AR* gene cannot be detected.

WHAT THIS STUDY ADDS?

DNA sequence analysis revealed a novel hemizygous mutation p.T576I (c.1727C>T) in the *AR* gene.

Introduction

Androgen insensitivity syndrome (AIS) is the commonest cause of 46,XY disorders of sex development (DSD) and is characterized with defective masculinization of external genitalia in 46,XY individuals despite normal androgen production and

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metabolism (1). The estimated prevalence of complete AIS (CAIS) ranges from 1:20.400 to 1:99.100 genetic males on the basis of proven molecular diagnosis (2), while partial AIS (PAIS) is at least as common as CAIS (1/130.000) (3). According to the masculinization degree of external genitalia, AIS is divided into three clinical forms including mild AIS (MAIS), PAIS, and CAIS (4).

Patients with CAIS usually present with normal female external genitalia and bilateral inguinal hernias. Individuals who are not diagnosed during childhood are detected after puberty because of primary amenorrhea with a blind vagina and absent uterus. On the other hand, depending on the degree of responsiveness of the external genitalia to androgens, findings in PAIS will show a wide spectrum varying from perineoscrotal hypospadias, bifid scrotum, cryptorchidism and pubertal gynecomastia to extreme inadequate virilization appearing as cliteromegaly and labial fusion (5).

This report describes an adolescent boy with PAIS who presented with gynecomastia at puberty and was found to have a novel mutation in the *androgen receptor (AR)* gene.

Case Report

A 16-year-old boy was admitted to our clinic with complaints of gynecomastia and sparse facial hair. He was born at term with a birth weight of 3000 grams. A detailed history revealed operations for bilateral cryptorchidism and penoscrotal hypospadias at ages one and two years, respectively. There was no parental consanguinity. Family history revealed male relatives from the maternal side with similar clinical phenotype, including gynecomastia, hypospadias, sparse facial hair, and infertility (pedigree shown in Figure 1). On physical examination, the patient weighed 94 kg [2.84 standard deviation (SD)] and was 170 cm (-0.56 SD) tall. His external genitalia was phenotypically male with pubic hair Tanner stage IV, penis size 8 cm in length and 2.5 cm in diameter, penoscrotal hypospadias, and a bifid scrotum in which both testes were palpable as 2 mL. He had normal axillary hair and gynecomastia compatible with breast development of Tanner's stage III. The rest of the physical examination was normal. Hormone levels were as follows: follicle-stimulating hormone (FSH) 42.8 mIU/mL (normal: 1.5-12.4 mIU/mL), luteinizing hormone (LH) 37.4 mIU/mL (normal: 1.7-8.6 mIU/mL), total testosterone (T) 419 ng/dL (normal: 180-763 ng/dL), estradiol 30.5 pg/mL (normal: 7.6-42 pg/mL), beta-human chorionic gonadotropin (β-hCG) 0.73 mIU/mL (normal: 0-2 mIU/mL), and alpha-fetoprotein 2.78 ng/mL (normal: 0-7 ng/mL). Scrotal ultrasound revealed that both testes were atrophic and were 28x10x17 mm (left) and 12x14x27 mm (right) in size. Chromosome analysis revealed a 46,XY karyotype. Due to the family history suggesting a disorder of X-linked trait, PAIS was considered and molecular analysis of *AR* gene was performed. DNA sequence analysis revealed a novel mutation hemizygous p.T576I (c.1727C>T) in the *AR* gene. Due to the presence of atrophic testes and increased risk of germ cell tumor development, bilateral gonadal biopsy was recommended. However, the patient and his family did not accept the procedure. Therefore, the patient is still being followed-up by physical examination and testis ultrasonography on a six-monthly basis.

Molecular Analysis

To investigate the etiology of proband's PAIS, after getting informed consent from the parents, genomic DNA was extracted from peripheral EDTA anticoagulant whole blood using the MagNA Pure LC automated system (Roche Applied Science, Manheim, Germany) following the manufacturer's instructions. The polymerase chain reaction fragments were sequenced by Illumina MiSeq system using V2 chemistry (Illumina, Ca, USA). Sequencing results were analyzed using IGV software (http://www.broadinstitute.org/igv/).

A hemizygous mutation p.T576I (c.1727C>T), which had not previously been reported, was identified in the *AR* gene (Figure 2). Analysis of this novel mutation by bioinformatic tools that examine functional effects of single nucleotide variants in humans [Mutation Taster (http://www.mutationtaster.org)] predicted the variant p.T576I (c.1727C>T) to be disease causing.

Discussion

The AIS describes a spectrum of disorders where the degree of receptor insensitivity may vary from minimal to complete

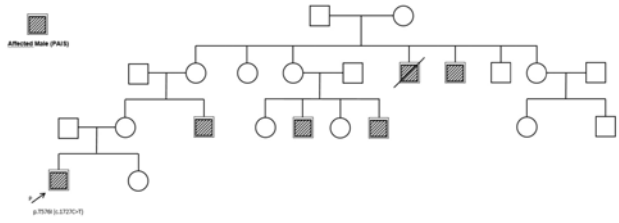

Figure 1. Pedigree

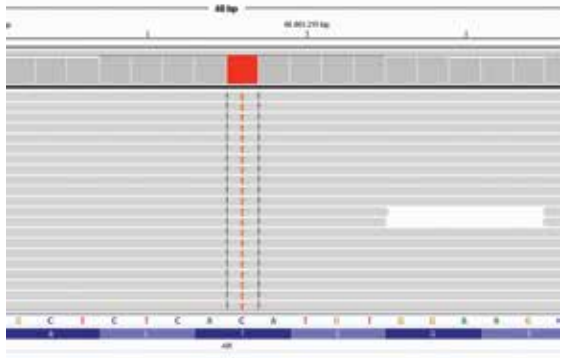

Figure 2. Hemizygous p.T576I (c.1727C>T) mutation detected with MiSeq NGS system

insensitivity. In case of MAIS, the individual is phenotypically male with sterility, azoospermia, and gynecomastia without genital abnormalities. The other end of the spectrum comprises XY individuals with CAIS who phenotypically appear as tall females with well-developed breasts, blind vagina, and absent or scanty pubic and axillary hair (6). The typical phenotype in PAIS is micropenis, perineoscrotal hypospadias, and a bifid scrotum with descending or undescending testes, and gynecomastia at puberty. In the present patient, penoscrotal hypospadias and bilateral cryptorchidism and gynecomastia at puberty were considered as the clinical findings of PAIS.

In the differential diagnosis of PAIS, 5α-reductase deficiency, partial gonadal dysgenesis (due to mutations in *SF1, SRY, WT1* etc.), and testosterone biosynthesis defects need to be considered. If the karyotype is 46,XY, then serum testosterone and dihydrotestosterone levels are helpful for the differential diagnosis. Normal or elevated levels of testosterone and dihydrotestosterone are essential to exclude androgen biosynthesis defects. Nevertheless, hCG stimulation test may be necessary to differantiate partial gonadal dysgenesis in prepubertal children (5). While an elevated or normal testosterone level suggests PAIS or 5hile an elevated ordeficiency, a low testosterone level is indicative for partial or complete gonadal dysgenesis (7). Serum anti-Müllerian hormone (AMH) level is another useful tool in the differential diagnosis of DSD. AMH is synthesized from Sertoli cells and in healthy males, its level decreases at puberty due to the stimulation of *AR* on Sertoli cells. Serum AMH levels are low in gonadal disgenesis due to aberrant Sertoli cell development and tend to be abnormally high in CAIS because of the lack of intact *ARs* on Sertoli cells (8,9,10). In our case, due to financial difficulties, we were unable to measure serum AMH level.

In cases with AIS, high levels of testosterone, a substrate for aromatase activity, result in substantial amounts of estrogens, which are responsible for breast development at puberty. However, due to functional *AR* activity, gynecomastia has not been reported in cases with 5α-reductase type 2 deficiency and partial gonadal dysgenesis (5,7). In the present patient, normal testosterone level and the presence of gynecomastia made us consider PAIS. Although the size of the testes is usually normal in PAIS, PAIS cases with atrophic testes have also been reported (11,12). Similar to the previous reports, in our patient, delayed orchiopexy might have led to atrophy in the testes.

The hormonal profile is similar in individuals with CAIS and PAIS. At birth, levels of testosterone and LH remain high or slightly above the normal range for males. During puberty, individuals with PAIS maintain normal or slightly elevated testosterone and LH levels (7). Estradiol, which is derived from peripheral conversion of testosterone and from testicular secretion, tends to be in the normal female range (8). Serum FSH levels are reported to be normal in AIS (13). However, serum FSH level is high in our patient, reflecting the damage to the seminiferous tubules possibly due to delayed orchiopexy of intraabdominal testes.

AIS is an X-linked recessive disorder resulting from mutations in the *AR* gene (14). While the vast majority of CAIS cases (90-95%) are attributable to AR mutations, less than a third of cases with a phenotype consistent with PAIS are associated with AR mutations (15), To date, more than 800 mutations have been identified in the *AR* gene (7,16,17). In the current patient, detailed family history revealed male relatives from the maternal side with similar clinical phenotype suggesting an X-linked trait, which was an important clue for the diagnosis of PAIS. PAIS has a broad heterogeneity in phenotypic expression, which is partly explained by different *AR* defects. However, individuals with same mutations may exhibit widely variable phenotypes both within and between affected families (16). As a result, there is no definite relationship between phenotype and genotype in PAIS, suggesting that other factors are contributing to the degree of masculinization (18). The present patient was identified to have a novel hemizygous mutation p.T576I (c.1727C>T) in the human *AR* gene. However, a limitation of this report is that we could not perform molecular analysis of other affected family members and therefore cannot comment on the relationship between genotype and phenotype.

Gender decision, genitoplasty, timing of gonadectomy (due to cancer risk), hormone replacement therapy, genetic counseling, and psychological support comprise the basis of management in PAIS. The majority of the patients with PAIS are reared as males (19). When the external genitalia is female, treatment is similar to that for CAIS, except that gonadectomy is recommended before puberty to avoid the physical and emotional discomfort of pubertal virilization. On the other hand, if the patient had a penis, albeit small, a male sex is assigned, and the individual may have to wait until puberty for the clinical picture to manifest more clearly by a lack of male secondary characteristics and the development of gynecomastia (7). The present patient had a phenotypically male external genitalia with a penis size 8 cm in length and 2.5 cm in diameter and penoscrotal hypospadias. He had been reared as male and psychiatric evaluation revealed a male gender identity at the age of sixteen.

Patients with PAIS are under the risk of malignancy which mostly are gonadoblastoma or dysgerminoma. Gonadal tumor risk is 0.8-2% during the prepubertal period and rises up to 30% during late adulthood. Risk of malignancy is low before the age of 25 years and more frequent between 30 and 50 years (20). The risk of type 2 germ cell tumors is higher in PAIS than in CAIS, with a suggested incidence of 15% and even higher (~50%) if the testes are not scrotal in position (19,21). Due to the high risk of malignancy, gonadectomy at the time of diagnosis is the current recommendation for PAIS if presenting with undescended testes (non-scrotal). However, it was also reported that gonadectomy might be delayed until a stable gender identity has been established (21). Many authors

recommend gonadectomy after puberty to achieve adequate bone mineralization and body maturation. However, some researchers recommend biopsy at the end of the pubertal age (17-24 years) and gonadectomy if a premalignant lesion and/or carcinoma *in situ* are detected (7,22,23). Our patient was under the risk of a gonadal tumor as he had a history of cryptorchidism and delayed orchiopexy. A testicular biopsy was recommended but could not be performed since the parents and the patient refused the procedure.

In conclusion, PAIS constitutes one of the most common causes of 46,XY DSD. The diagnosis is based upon clinical phenotype and laboratory findings, and can be confirmed by detection of a defect in the *AR* gene. An accurate approach including a detailed family history suggesting an X-linked trait is an important clue for a quick diagnosis.

Ethics

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

Authorship Contributions

Concept: Bumin Nuri Dündar, Design: Gönül Çatlı, Data Collection and/or Processing: Cemil Koçyiğit, Analysis and/or Interpretation: Hüseyin Onay, Literature Research: Serdar Sarıtaş, Writing: Serdar Sarıtaş.

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Case Report

Phenotype, Sex of Rearing, Gender Re-Assignment, and Response to Medical Treatment in Extended Family Members with a Novel Mutation in the SRD5A2 Gene

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ABSTRACT

Deficiency of steroid 5-alpha reductase-2 (*5ARD2*) is an inborn error of metabolism causing a disorder of sexual differentiation. It is caused by a mutation in the *SRD5A2* gene in which various mutation types have been reported. Affected individuals have a broad spectrum of presentation ranging from normal female-appearing genitalia, cliteromegaly, microphallus, hypospadias, to completely male-appearing genitalia. We report an extended Emirati family with 11 affected members. The family displayed various phenotypes on presentation leading to different sex of rearing. Some family members were reassigned gender at various stages of life. The index case was born with severe undervirilization with bilaterally palpable gonads and was raised as male from birth. He had a 46,XY karyotype and a high testosterone/dihydrotestosterone ratio. Genetic investigation revealed a novel homozygous deletion of exon 2 of the *SRD5A2* gene. Both parents were found to be carriers for the gene deletion. The patient had masculinizing surgery and a course of topical dihydrotestosterone. No beneficial effect of the hormone application was noted over 3 months and the treatment was discontinued. The findings on this kindred indicate that deletion of exon 2 in the *SRD5A2* gene causes various degrees of genital ambiguity leading to different sex of rearing in affected family members. Gender reassignment may be done at various ages even in conservative communities like the Gulf region.

Keywords: 5-alpha reductase-2, reductase deficiency, ambiguous genitalia, gender re-assignment

Conflict of interest: None declared
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WHAT IS ALREADY KNOWN ON THIS TOPIC?

5-alpha reductase deficiency is a known cause of male undervirilization. Virilization at puberty is a common presentation and might cause patients to re-assign to male gender if they were raised as females at birth. Over 61 mutations have been reported in the *SRD5A2* gene.

WHAT THIS STUDY ADDS?

The study is the first extended family research to be reported from the Gulf region with genetically-confirmed diagnosis of 5-alpha reductase deficiency. A novel mutation is described in the reported family. Male gender re-assignment is not uncommon even in conservative communities like Arabs in the Gulf area.

Introduction

Deficiency of steroid 5-alpha reductase-2 (*5ARD2*) is an inborn error of metabolism inherited in an autosomal recessive pattern. The enzyme defect results in a disorder of sexual differentiation (DSD) wherein patients with 46,XY genotype

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have impaired virilization during embryogenesis (1). The defect results in impaired conversion of testosterone to dihydrotestosterone (DHT). Affected individuals have a broad spectrum of presentation which may include normal female-appearing genitalia, cliteromegaly, microphallus, hypospadias, or completely male-appearing genitalia (2). This syndrome was first described clinically and biochemically in 1974 in studies of 24 affected subjects from 13 families in a large Dominican kindred (3). To date, more than 61 mutations, in the gene which codes for this isoenzyme of 5-alpha reductase called *SRD5A2*, have been identified to be on chromosome 2p23 (4).

There is uncertainty with regard to sex of rearing in children born with genital ambiguity in this disorder. Similar to other disorders of sexual differentiation, multiple factors beside the external genital phenotype come to play (5). Sex reassignment has been widely reported in this condition and many others. Money et al (6) reported better adjustment to sex reassignment if it was done prior to 27 months of age. Eleven of 14 children adjusted to the change without complications when the reassignment occurred prior to age 27 months, in contrast to only 1 of 4 children who adjusted to the change without complications if it occurred after 27 months.

Genital surgery is widely performed for children with genital ambiguity. Penile construction remains a challenging task for surgeons. However, some newer techniques offer improvement in males with severe micropenis and aphalia (7). Medical treatment has been tried on this enzyme defect by using topical DHT with variable rates of success (8,9). Encouraging results with topical DHT in preparation for genital surgery have been reported (10).

In this paper, we report a large Emirati kindred with high consanguinity rate and many affected members. We highlight the variable phenotypes that led to different gender assignment and re-assignment. We also report our experience in using topical DHT in the severe form of undervirilization.

Case Report

Our index case was a male infant, a baby born as the 2nd twin at 37 weeks with an elective cesarean section. His birth weight was 2.6 kg. It was the mother's first pregnancy during which she had not received any medications. A postnatal examination revealed that the patient had an apparently normal external female genitalia but was also noted to have bilaterally palpable gonads in the labial folds (Figure 1). Phallus/clitoral length was around 0.5 cm. Twin 1 had normal male external genitalia with bilateral palpable gonads in the scrotum. The index case is an Emirati baby who was born to a first degree cousin Emirati parents.

Ultrasound scan of the indexd case showed absent mullerian duct structures, adrenal glands of normal size, and presence of gonads in the labial folds. Initial investigations showed normal adrenal androgen levels, and normal levels for random cortisol, adrenocorticotropic hormone, and gonadotropin. Basal serum testosterone was 11.4 nmol/L [normal range (NR): 0.5-3.0] while DHT was 0.34 nmol/L. Testosterone/ DHT ratio was 33.6. Fluorescence *in situ* hybridization analysis on metaphases and interphase of 300 cells showed a normal male pattern of hybridization. Karyotype was 46,XY with positive *SRY* gene marker. Genetic studies showed a homozygous deletion of exon2 of *SRD5A2* gene. Both parents were carriers. The parents decided to raise the baby as a male from birth.

The patient had genital surgery at the age of 6 months when he had construction of the scrotal sac. By the age of 14 months, he received a course of percutaneous 2.5% DHT once nightly for 3 months. No response in terms of improving phallus size was noted. The child developed pubic hair at the age of 16 months and the parents stopped the medication (consent was obtained from parents to show the genital pictures) (Figure 2). Although the parents were instructed on the proper way of applying the DHT, appearance of pubic hair on the scrotal sac might indicate that the application was mainly over the genital skin rather than the phallus. Nonetheless, as the phallus was extremely small, it was believed that lack of response was possibly due to the severity of the defect rather than the imperfect application. The parents confirmed their compliance to the treatment and following the exact instruction given by the physicians on the use of DHT.

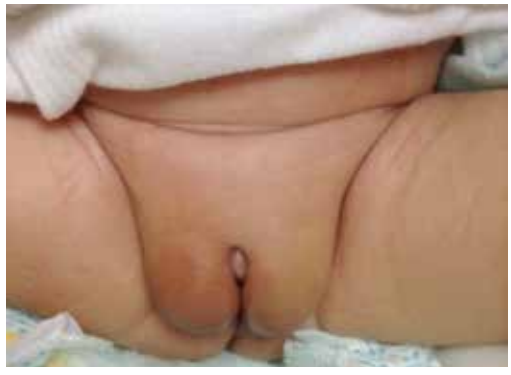

Figure 1. Genitalia of index case at birth (twin 2)

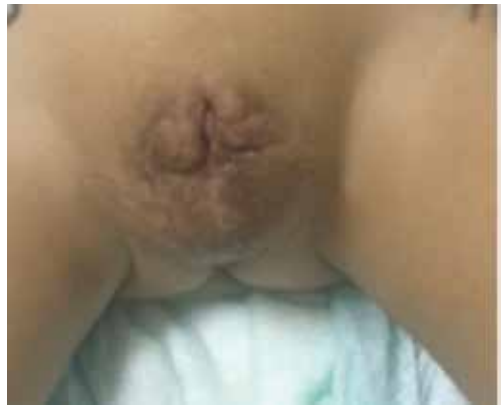

Figure 2. Genitalia of index case at 18 months after surgery and treatment with dihydrotestosterone cream

In addition to our index patient, 7 affected family members were born with varying degree of genital ambiguity (Table 1, Figure 3). In addition to the index case, 3 members (cases 4, 5, and 9) presented with apparently normal female genitalia with palpable gonads at labial folds. All patients (except for patients 7 and 8) were raised as females. 5 patients had virilization at puberty (patients 1, 2, 3, 9, 10). Three of them reassigned their gender as males (1,2,9), while 2 (patients 3, 10) kept the female gender. Patient 10 expressed a male gender identity but was satisfied with her gender role as female in the community. Patients 4 and 5 were raised as females and are currently pre-pubertal. Parents of patients 6, 7, 8 witnessed the abnormalities in the older extended relatives and were aware of the gender re-assignment at puberty in some family members. They raised patients 6 and 7 as males and re-assigned patient 8 into a male sex at the age of 2. Patient 2 fathered 3 healthy girls after a non-consanguineous marriage. Patients 9 married twice and fathered 5 healthy boys from one marriage and a healthy girl from another.

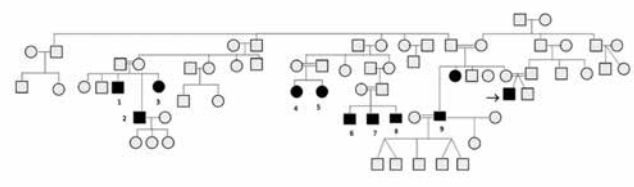

Figure 3. Affected family members are indicated with black squares or circles. All affected members are 46,XY. Squares indicate current male gender assignment, while circles indicate female assignment

Discussion

5-alpha reductase is an enzyme which exhibits 2 isoforms: types 1 and 2, out of which the type 2 coded by *SRD5A2* on 2p23 is the predominant isoenzyme which is required for full masculinization of the fetal external genitalia (11). Type 1, which is coded by *SRD5A1* gene on 5p15, expressed from the time of puberty, is responsible for virilization in type-2 isoenzyme deficient individuals (12). The *5ARD2* enzyme is responsible for the conversion of testosterone to DHT. In the fetus, both testosterone and DHT bind to the same androgen receptor protein inside the nucleus of the cell. However, they exert different physiological stimuli; testosterone has a major role in stimulation of the Wolffian ducts during sexual differentiation and control over spermatogenesis, while DHT is required for the development of normal male external genitalia (12). It is also to be noted that DHT is considered as the essential androgen as it also facilitates most of the changes of male puberty including facial, body, and genital hair appearance and prostate growth (12).

SRD5A2 deficiency is often suspected in infants with ambiguous genitalia or when adolescents, who have

Table 1. Clinical description of genital appearance at birth, sex of rearing, and gender reassignment of the 10 affected members belonging to the same family as the index patient

Patient number	Karyotype	Appearance of the genitalia at birth	Sex of rearing at birth	Gender re-assignment	Fertility
1	46,XY	Ambiguous genitalia	Female (virilization at puberty)	Changed into male gender at puberty	
2	46,XY	Ambiguous genitalia	Female (virilization at puberty)	Changed into male gender at puberty	Fathered 3 healthy girls
3	46,XY	Ambiguous genitalia	Female (virilization at puberty)	Remained as female	
4	46,XY	Female external genitalia with palpable gonads	Female (currently pre-pubertal)	Remained as female	
5	46,XY	Female external genitalia with palpable gonads	Female (currently pre-pubertal)	Remained as female	
6	46,XY	Ambiguous genitalia	Male	Remained as male	
7	46,XY	Ambiguous genitalia	Male	Remained as male	
8	46,XY	Ambiguous genitalia	Female	Changed into male gender at 2 years of age	
9	46,XY	Female external genitalia with palpable gonads	Female (virilization at puberty)	Changed into male gender at puberty	Fathered 5 healthy boys and 1 girl
10	46,XY	Ambiguous genitalia	Female (virilization at puberty)	Remained as female	

previously ascribed the female gender, present with marked masculinization and/or phallic growth at puberty (13). The clinical features of this disease are highly variable owing to different mutations within the same gene. It is also known for patients with the same mutations to show a wide spectrum of phenotypes (14). Often the external genitalia are female at birth. However, external genitalia may also present as complete male with microphallus and varying degrees of hypospadias, female genitalia with clitoromegaly, or normal female genitalia. The position of the testes also varies although most of the time they are found outside of the abdominal cavity in the inguinal canals or the labia majora or the scrotum (2). Our index case presented with severe undervirilization and was thought to be a female newborn at birth due to the complete appearance of female genitalia until the postnatal examination when gonads were palpated in the labial folds. The other 10 affected family members had a different phenotype of genital abnormalities. Four of them presented with apparently normal female external genitalia with palpable gonads in the labial fold and others had a varying degree of genital ambiguity.

Biochemical analysis in infants with ambiguous genitalia usually reveals a normal serum testosterone level with an elevated serum T:DHT (testosterone:dihydrotestosterone) ratio of more than 20 (2). The cut-off for this ratio is debatable for different age groups. Walter et al (11) recommend a cut-off value of 8.5 for a stimulated T:DHT estimation in young infants to avoid a false exclusion. Urinalysis may also reveal excretion patterns of 5-alpha to 5-beta reduced steroids. Occasionally, the disease can be confirmed by detecting a mutation in the *SRD5A2* gene in the presence of a normal T:DHT ratio (14). In our patient, the T:DHT ratio was very high at 33. It was considered sufficient for the biochemical diagnosis and human chorionic gonadotropin test was not required to test the stimulated levels of the different androgens. Subsequently, the diagnosis was confirmed by detecting the *SRD5A2* gene mutation.

SRD5A2 deficiency is more prevalent than expected in the adult female 46,XY DSD population (15). It is not uncommon for XY individuals with 5-alpha reductase deficiency reared as female to reverse gender assignment at puberty (16,17). Imperato-McGinley et al (18) interviewed affected 46,XY subjects and reported that 17 of 18 subjects with this disorder had, successfully, changed gender identity from female to male. In our kindred, 3 patients were reassigned to male sex at puberty. On interviewing a 4th individual at 42 years of age, she declared her tendency to a male gender identity and role but was not able to reverse gender as she found herself unable to acknowledge maleness. Also, she felt quite satisfied with her role as a successful female manager in the community. As to patients 6, 7, and 8, their parents decided on male sex of rearing for 2 of their affected children at birth and they re-assigned the 3rd child to male sex at the age of 2. Their decision was based on their experience on progress of other affected members in the extended family.

Male gender reassignment has been encouraged in this disorder due to the normal psychosexual development and normal genital virilization in those who converted to the male sex. In addition, fertility has been reported to be normal in this group of patients (10). In our kindred, 2 men fathered children. Patient number 9 married twice and had 6 healthy children. The other patient (number 2) also fathered 3 healthy children.

The *SRD5A2* gene has over 61 known mutations reported to date. Inherited in an autosomal recessive fashion, many affected individuals are homozygotes associated with high degree of consanguinity, however, this disorder is known to exist in compound heterozygotes as well (19). Some mutations are more common in certain ethnic groups, which could be due to a founder effect (20). The *SRD5A2* gene consists of 5 exons separated by 4 introns (12). Mutations of all the exons have been reported so far (14). Exons 1 and 4 were the most frequently encountered sites for mutation in a cohort of 55 patients (12). In our family, the *SRD5A2* sequence analysis revealed a homozygous deletion of exon 2 in the index case. Both parents were carriers of the mutation. As of now, this mutation has not been reported. Various deletions have been reported in the *5SRD2* gene among the three largest kindred with *5ARD2* deficiency in the world: the Dominican, New Guinean, and Turkish kindred. In 2 related patients diagnosed with *SRD5A2* deficiency in the Highlands of Papua New Guinea, deletion of most of the *SRD5A2* gene was detected (21). In addition, Boudon et al (22) reported a trinucleotide deletion straddling codons 156 and 157, responsible for a methionine residue deletion at position 157 of the protein in a Turkish patient. Mutations in exon 2 have been reported. An adenine (GAC) for guanine (GGC) change in exon 2 causing a substitution of aspartic acid for glycine at amino acid 115 (G115D) was detected in 1 family (19).

Topical DHT treatment has been tried in various forms of undervirilization with variable degree of success (8,9). Our index case has also received topical treatment for a few weeks, but the parents stopped it due to lack of phallus growth and appearance of pubic hair. Destruction of the phallus tissue due to surgery was also thought of as a possible reason for non-responsiveness to the DHT. However, the main surgical work was done for construction of the scrotal sac and correction of the hypospadias. There was no attempts for phalloplasty at this stage. Moreover, as the family was highly consanguineous, the digenic inheritance was considered as another possible reason for the poor response to DHT (23) particularly in combination with a defect like androgen insensitivity syndrome. However, as the detected mutation clearly explained the various phenotype seen in the family members, no further genetic analysis was sought.

In conclusion, we believe that this is the first report on an extended family with 5-alpha reductase deficiency caused by a novel deletion of exon 2 of the *SRD5A2*. Our kindred displayed a variable genital appearance at birth among affected members.

Male sex re-assignment was chosen in half of the members presenting with virilization at puberty. Familial and cultural issues are crucial in the decision of sex of rearing at birth and on the sex re-assignment at puberty.

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Ethics

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

Authorship Contributions

Concept: Asma Deeb, Hana Al Suwaidi, Design: Asma Deeb, Fakunle Ibukunoluwa, Data Collection or Processing: Asma Deeb, Salima Attia, Hana Al Suwaidi, Analysis or Interpretation: Asma Deeb, Hana Al Suwaidi, Literature Search: Asma Deeb, Fakunle Ibukunoluwa, Writing: Asma Deeb, Salima Attia, Fakunle Ibukunoluwa.

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Case Report

A Novel Mutation in Thyroid Peroxidase Gene Causing Congenital Goitrous Hypothyroidism in a German-Thai Patient

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ABSTRACT

Thyroid dyshormonogenesis is responsible for 10-15% of all cases of congenital hypothyroidism and is usually inherited. We report a 26-year-old German-Thai male with congenital hypothyroidism caused by a compound heterozygous mutation in the *thyroid peroxidase* (*TPO*) gene. He was diagnosed with congenital goitrous hypothyroidism at 4 months of age and had been treated with levothyroxine replacement therapy. His goiter size had increased due to poor compliance to treatment. Ultrasonography of the thyroid gland showed a pattern suspicious for malignancy. The patient later underwent near-total thyroidectomy. Pathologic examination results were consistent with a multinodular goiter and no malignancy. Genetic analyses by direct sequencing of the entire exons and flanking regions of the *TPO* gene were performed in the index case and family members. The analyses revealed a compound heterozygote of novel TPO mutation of c.1727C>T in exon 10 resulting in amino acid substitution (p.Ala576Val) and c.2268_2269insT in exon 13 causing a frameshift mutation which introduced a stop codon after the insertion site. The latter has been reported in Chinese subjects. However, there is no previous report of c.1727C>T mutation in the literature. We found the allele contained a novel exon 10 mutation inherited from the patient's German mother and an exon 13 mutation from his Thai father. Analysis using two bioinformatic software programs indicated that this variant was likely to cause damage in the resulting protein molecule. The present report emphasizes the importance of regular follow-up and patient compliance to levothyroxine replacement in patients with goitrous congenital hypothyroidism to avoid prolonged stimulation of thyroid tissue by thyroid-stimulating hormone.

Keywords: Thyroid dyshormonogenesis, goiter, thyroid peroxidase, mutation

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The most common defect in thyroid dyshormonogenesis resides in thyroid peroxidase (*TPO*) gene. The incidence of congenital hypothyroidism due to homozygous *TPO* defects has been estimated at 1:66,000 for a Dutch population. The salient clinical manifestations of *TPO* gene mutation are permanent congenital hypothyroidism and goiter, with a variable degree of hypothyroidism and thyroid gland enlargement depending on the severity of the defect.

WHAT THIS STUDY ADDS?

We report on a novel *TPO* gene mutation in a German-Thai patient who presented with congenital hypothyroidism and large multinodular goiter. The present report emphasizes the importance of regular follow-up and patient compliance with adequate levothyroxine replacement to avoid prolonged stimulation of thyroid tissue by thyroid-stimulating hormone.

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Introduction

Congenital hypothyroidism is considered the most common congenital endocrine disorder and causes preventable mental retardation in children with a prevalence of 1 in 2000-4000 live births (1). Thyroid dysgenesis, including agenesis, ectopy and hypoplasia of the gland, is the most frequent cause of congenital hypothyroidism (80-85%); defects in thyroid hormone synthesis (i.e. thyroid dyshormonogenesis) constitute the etiology in the remaining patients. Some clinicians believe that determination of the cause of congenital hypothyroidism is not obligatory due to similar management regardless of etiology. However, to unravel this genetically heterogeneous entity could lead to new possibilities for more specific molecular diagnoses and the discovery of new targets for molecular therapies in the future. Moreover, this knowledge is also useful for providing reliable parental genetic counseling.

Thyroid peroxidase (TPO), an important enzyme in the steps of thyroid hormone synthesis, is located at the apical membrane of thyroid follicular cells. It catalyzes the iodination of tyrosyl residues in thyroglobulin and the coupling of iodotyrosines to produce iodothyronines. Defects in the *TPO* gene are the cause of the majority of cases of thyroid dyshormonogenesis with permanent congenital hypothyroidism (2,3). Although *TPO* mutations have been characterized in subjects of various populations in Asia (4,5,6,7,8,9,10,11) including Japanese, Chinese, Malaysian, and Indian, none have been reported in the Thai population to date. Herein, we report on a novel compound heterozygous *TPO* mutation in a German-Thai patient with permanent congenital hypothyroidism who presented with a huge multinodular goiter necessitating surgical removal.

Case Report

A 26-year-old man presented with a gradually enlarging multinodular goiter. Previously, he had been a patient in another hospital, but was lost to follow-up in the past 5 years. He had initially presented with delayed bone growth and muscular hypotonia at 4 months of age and was diagnosed to have congenital goitrous hypothyroidism. Levothyroxine (LT4) therapy was started. He was born to non-consanguineous parents. His father is Thai and had no thyroid disorder; however, his mother is German, and in her teens was diagnosed with primary hypothyroidism without goiter and began receiving LT4 replacement. She had neither a history of neck surgery nor radiation. No other family member was reported to have a thyroid disorder.

During childhood, the patient had been regularly followed up by a pediatric endocrinologist. His growth and development were normal except for moderate impairment in fine motor skills and coordination. Ultrasonography of the thyroid gland had revealed that its size was in the upper normal range and LT4 therapy could not be withdrawn. The patient was born and lived in Germany, but later the family moved to Thailand. He graduated with a bachelor's degree and currently works in the family business. He reported that his goiter size had gradually increased over the past 5 years. He had received LT4 replacement irregularly at a dose of 125 µg/day before he came to us with a concern about the enlargement of his goiter.

At presentation, his body weight was 66 kg and his height was 180 cm. A large multinodular goiter without signs of compression was noted. Thyroid function tests revealed that serum thyroid-stimulating hormone (TSH) level was higher than 100 mIU/L (normal range 0.27-4.2 mIU/L) and serum free thyroxine (fT_4) was less than 0.40 ng/dL (normal range 0.93-1.70 ng/dL). Ultrasonography of the thyroid gland showed an enlarged goiter (9.5x8 cm) with multiple solid/cystic nodules in both lobes, ranging in size from 0.8 to 3.6 cm. Microcalcification was also detected in the left lobe of the thyroid gland. At that time, surgery was advised due to a concern about possible thyroid carcinoma. No perchlorate discharge test was done to establish the cause of congenital hypothyroidism. After adequate LT4 replacement, the patient underwent a near-total thyroidectomy and had no complications. Pathology revealed a benign multinodular goiter without any evidence of cancer. Thyroid hormone replacement was given at a dose of 200 µg/day to maintain his thyroid function.

Total triiodothyronine (TT_3), fT_4, TSH, antibody to TPO (anti-TPO), and antibody to thyroglobulin (anti-Tg) were measured using electrochemiluminescent immunoassays (Abbott Diagnostics, Illinois, USA). Written consent was obtained from the patient and family members. The study was approved by the Ethics Committee of Theptarin Hospital and the Faculty of Medicine Ramathibodi Hospital, Mahidol University.

Genetic analysis was performed in the proband and in all available family members after obtaining informed consent. Because *TPO* gene mutation is the most frequent cause of thyroid dyshormonogenesis, the entire exons 1-17 and flanking regions of *TPO* gene were sequenced directly from genomic DNA. To identify a novel mutation, a co-segregation study and human genetic bioinformatics analysis were performed.

The pedigree is shown in Figure 1. We identified a compound heterozygous mutation on the *TPO* gene in the index case (II-3). The paternal allele had a frameshift mutation due to an insertion of one nucleotide (c.2268_2269insT) in exon 13. This T insertion caused a stop codon after the insertion point, resulting in a truncated polypeptide of 756 amino acids. The maternal allele had a novel missense mutation (c.1727C>T) in exon 10 resulting in an amino acid substitution from alanine to valine at codon 576 (p.Ala576Val). Based on analysis using two bioinformatic software programs, this variant is likely causing protein damage (SIFT score=0 and Polyphen-2 score=1.0).

These findings were also confirmed by their absence in 100 ethnically matched normal control subjects (courtesy of Professor Joachim Pohlenz).

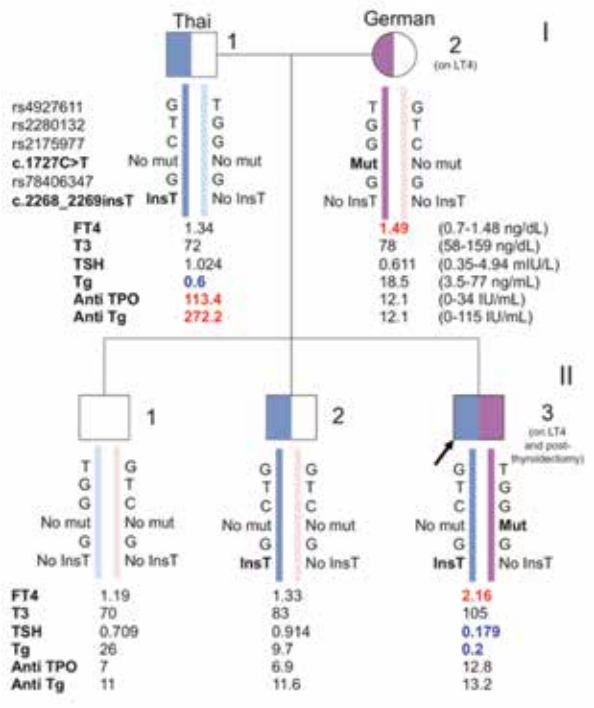

Figure 1. A pedigree of the index patient (II-3) which revealed a compound heterozygote of novel *thyroid peroxidase* mutation of c.1727C>T in exon 10 resulting in p.Ala576Val and c.2268_2269insT in exon13 causing a frameshift mutation which introduced a stop codon after the insertion site. Exon 10 mutation is maternally-derived, while exon 13 mutation is paternally-derived. The patient's oldest brother (II-1) had no mutation. Square symbols indicate males, circles females, Roman numerals to the right of the pedigree indicate the generation, and numerals to the right of each symbol indicate individual family members. TSH: thyroid-stimulating hormone, fT_4: free thyroxine, T_3: triiodothyronine, Tg: thyroglobulin, anti-Tg: antibody thyroglobulin, anti-TPO: antibody thyroid peroxidase

The patient's father (I-1) and older brother (II-2) had a heterozygous c.2268_2269insT mutation and normal thyroid function. The father has autoimmune thyroid disease (AITD) as evidenced by high levels of anti-TPO 113 IU/mL (normal range 0-34 IU/mL) and anti-Tg 272 IU/mL (normal range 0-115 IU/mL). The mother (I-2) with primary hypothyroidism was receiving LT4 therapy and had a heterozygous c.1727C>T mutation. She had no positivity of anti-TPO and anti-Tg.

Discussion

The most common defect in thyroid dyshormonogenesis resides in *TPO* gene. The incidence of congenital hypothyroidism due to homozygous *TPO* defects has been estimated at 1:66.000 for a Dutch population (12). The salient clinical manifestations of *TPO* gene mutation are permanent congenital hypothyroidism and goiter, with a variable degree of hypothyroidism and thyroid gland enlargement depending on the severity of the defect. A severe phenotype resulting in mental retardation and a large goiter has been reported in untreated patients with a complete defect of *TPO* gene (13). However, some patients who received treatment immediately after birth had normal development without goiter. Also, goiter has been reported to resolve after initiation of LT4 treatment in some patients. In previous studies, enlargement of the thyroid gland was shown in 60-80% of patients, mostly with multinodular appearance, and in some cases with huge goiter or retrosternal invasion necessitating surgical intervention (14). In rare cases, the presence of a thyroid nodule or a goiter in thyroid dyshormonogenesis has been reported to lead to the development of thyroid cancer (15,16). Therefore, in standard practice, all suspected nodules should be evaluated in cases of thyroid dyshormonogenesis.

A delay in treatment of congenital hypothyroidism could partly explain the development of large multinodular goiter in these patients. The diminished thyroid hormone feedback on the pituitary thyrotroph leads to an increase in TSH secretion, stimulating the thyroid gland. Unknown additional factors might also be involved in the development of multinodular goiter, as some patients develop multinodular goiter despite early and adequate LT4 treatment. Organic iodo-compounds have been shown to inhibit thyroid epithelial cell proliferation; therefore, TPO mutations might increase the risk for multinodular goiter due to the lack of these compounds (17). In the present case, the huge goiter most likely resulted from delayed diagnosis and treatment after birth. In addition, poor compliance might have further contributed to goiter enlargement later in life.

Defects in the *TPO* gene are commonly inherited in an autosomal recessive pattern; therefore, differentiating the genetic basis of congenital hypothyroidism from other causes of hypothyroidism has important implications in terms of genetic counseling. Clinically, a perchlorate discharge test in most *TPO* gene mutation patients will demonstrate the pattern of total iodide organification defect (18). Unfortunately, our patient did not undergo this test before surgical intervention. In our patient, a compound heterozygous condition of a frameshift mutation from insertion of one nucleotide (c.2268_2269insT) in exon 13 and a novel missense mutation (c.1727C>T) in exon 10 from his mother was confirmed, explaining the molecular basis of the *TPO* gene mutation. The c.2268_2269insT has been reported in Chinese subjects (5,6). Haplotype analysis revealed that the high prevalence of c.2268insT mutation among Taiwanese is due to a founder effect (6). The ancestors of Taiwanese families have their origins in mainland China. Thailand is home to the largest overseas Chinese community in the world. This could explain why the father of the patient harbored this mutation. However, there is no previous report of a c.1727C>T mutation in the literature. Although functional analysis of missense mutations is important, it is usually not feasible. There are several in silico possibilities to evaluate

functional effects of missense mutations. In the present study, analysis using two bioinformatic software programs (SIFT and Polyphen-2) (19,20) demonstrated that this novel mutation is likely to cause protein damage. However, further molecular studies on messenger ribonucleic acid expression might be necessary to help provide a more comprehensive understanding of the exact effect of this novel mutation on the structure and function of the resulting protein. In the largest series of patients with *TPO* gene mutations (17), a study conducted in Israel, no significant correlation was observed between the specific type of mutation and the severity of clinical presentation. Further case reports for specific mutations should be accumulated in order to gain more detailed insights into the broad phenotypic variations in this entity (21,22).

In summary, we report on a novel *TPO* gene mutation in a German-Thai patient who presented with congenital hypothyroidism and a large multinodular goiter. The present report emphasizes the importance of regular follow-up and patient compliance to adequate LT4 replacement treatment in patients with goitrous congenital hypothyroidism to avoid prolonged stimulation of thyroid tissue by TSH. There are a small number of previously reported cases of thyroid carcinoma in *TPO* gene mutation patients who harbored multinodular goiter. Therefore, long-term follow-up of patients with *TPO* gene mutations is warranted also for early detection of thyroid carcinoma arising in multinodular goiter.

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Ethics

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

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Chutintorn Sriphrapradang, Yotsapon Thewjitcharoen, Thep Himathongkam, Design: Chutintorn Sriphrapradang, Yotsapon Thewjitcharoen, Data Collection and/or Processing: Chutintorn Sriphrapradang, Yotsapon Thewjitcharoen, Suwannee Chanprasertyothin, Soontaree Nakasatien, Thep Himathongkam, Analysis and/or Interpretation: Chutintorn Sriphrapradang, Suwannee Chanprasertyothin, Objoon Trachoo, Literature Research: Chutintorn Sriphrapradang, Yotsapon Thewjitcharoen, Writing: Chutintorn Sriphrapradang, Yotsapon Thewjitcharoen.

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Case Report

A Newly-Discovered Mutation in the RFX6 Gene of the Rare Mitchell-Riley Syndrome

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ABSTRACT

Mitchell-Riley syndrome is a genetic disorder characterized by neonatal diabetes, pancreatic hypoplasia, intestinal atresia and/or malrotation, biliary atresia, and gallbladder aplasia or hypoplasia. It was considered a variant of the Martinez-Frias syndrome with similar phenotypic characteristics, except for neonatal diabetes and tracheoesophageal fistula. However, the genetic mutation in (regulatory factor X on chromosome 6) RFX6 was only detected in babies who had diabetes, making it different from the previously known mutations for the disease. This is the first reported case of a classical Mitchell-Riley syndrome in the Arab peninsula along with additional features and novel mutations in the *RFX6* gene.

Keywords: Mitchell-Riley syndrome, diabetes, pancreatic hypoplasia

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

The Mitchell Riley Syndrome is a recently diagnosed genetic disorder characterised by neonatal diabetes, pancreatic hypoplasia, intestinal atresia, malrotation, biliary atresia, and gallbladder aplasia or hypoplasia. A novel genetic mutation in the RFX6 gene (regulatory factor X on chromosome 6) was detected in babies with neonatal diabetes.

WHAT THIS STUDY ADDS?

We report a case with neonatal diabetes, pancreatic hypoplasia gall bladder agenesis, duodenal atresia, haemochromatosis, hypospadias and intrauterine growth restriction with some additional features along with a different mutation in the RFX6 gene which has not been reported before.

Introduction

The Mitchell-Riley syndrome (1) is a recently diagnosed genetic disorder characterized by neonatal diabetes, pancreatic hypoplasia, intestinal atresia and/or malrotation, biliary atresia, and gallbladder aplasia or hypoplasia (2). It was initially considered as a variant of Martinez-Frias syndrome, diagnosed in 1992, with similar phenotypic characteristics (3). However, the two syndromes differ in that neonatal diabetes is present in Mitchell-Riley, while tracheoesophageal fistula is found in the Martinez-Frias syndrome (4). Over the years, many cases were reported with the same phenotypes, but additional features have also been discovered such as haemochromatosis, thyroid dysfunction, auditory canal defects, hypospadias in males and anteriorly-placed anus in females (5,6). Infants with neonatal diabetes have also been investigated for gene defects for diabetes, such as *PLAGL-1 (ZAC)*, glucokinase and *PDX-1 (IPF-1)* genes, with negative results

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(6). In 2010, Smith et al (7) detected a novel genetic mutation in the *RFX6* gene (regulatory factor X on chromosome 6) in 6 babies, all of whom had neonatal diabetes. This defect could not be found in babies who had same phenotypic features but did not have neonatal diabetes (8). After that, two further cases were reported, an Israeli Arab patient reported by Spiegel et al (9) and another patient from a Vietnamese family in 2014 reported by Concepcion et al (10). Sansbury et al (11) studied a Turkish family in which 2 double first cousins had intestinal atresia consistent with a diagnosis of Mitchell-Riley syndrome, but did not develop diabetes until the ages of 3 years and 6 years.

Here, we report a case with neonatal diabetes, pancreatic hypoplasia, gall bladder agenesis, duodenal atresia, haemochromatosis, hypospadias, and intrauterine growth retardation (IUGR) with some additional features along with a different mutation in the *RFX6* gene which has not been reported before.

Case Report

This male baby was born, at term, by normal delivery to a consanguineous (third degree) couple from the United Arabian Emirates (UAE) with a prenatal diagnosis of duodenal atresia. The infant had severe IUGR with a birth weight of 1.3 kilograms and practically no subcutaneous fat. Hypospadias was also present. No facial dysmorphism was noted. During the first week of life, the patient developed hyperbilirubinemia with mildly elevated liver enzymes, hyperammonemia, and hyperglycaemia which required insulin. He also tested positive for the direct agglutination test (DAT positive), had renal dysfunction, microangiopathic haemolytic anaemia, and coagulopathy with prolonged activated partial thromboplastin time (APTT). With these problems, he was initially diagnosed and managed as a patient with an inborn error of metabolism. Although the surgery for duodenal atresia was delayed because of thrombocytopenia and severe coagulopathy, these issues were later found to be related to the factor IX deficiency. At the same time, the Doppler test showed a congenital large portosystemic shunt in the liver with a ratio less than 30%. An echocardiogram revealed multiple echogenic masses in the ventricles which were consistent with cardiac rhabdomyoma. An ultrasound of the brain revealed periventricular calcification. TORCH infections and tuberous sclerosis were ruled out by viral and genetic studies.

Once the clotting profile and anaemia had been stabilised by multiple transfusions of fresh frozen plasma (FFP), cryoprecipitate, packed red-blood cells (PRBC), platelets and Factor IX, a laparotomy was performed for duodenal atresia and for a duodenostomy when the baby was 4 weeks of age. The surgery also revealed absence of the gall bladder. A liver biopsy and an endoscopic retrograde cholangiopancreatography (ERCP) could not be performed due to the patient's poor condition. He had developed severe peritonitis and sepsis, requiring antibiotic and ventilator support for a prolonged period of time. Later on, a hepatobiliary scan (HIDA) revealed poor uptake by the liver and delayed excretion of bile with no visualisation of the gall bladder.

The patient had persistent hyperglycemia, requiring insulin in high doses, followed by hypoglycaemia at intervals. These findings were labelled as neonatal diabetes. Thyroid function tests were slightly abnormal on multiple occasions so a small dose of levothyroxine was started. The ferritin level was also checked and was found to be >6000 µg/L, suggesting hemochromatosis. At the age of nine weeks, severe hypertension developed, which was controlled by antihypertensive medication. Renal Doppler and renal functions were normal at this time.

The baby started tolerating small amounts of expressed breast milk (EBM) two weeks after the surgery. Ingested amounts increased slowly, but soon, he started passing loose, sticky and green-coloured stool. The work-up for malabsorption showed minute levels of stool elastase (<50 µg/g of stool) and low serum lipase which was consistent with severe exocrine insufficiency of the pancreas and possible pancreatic hypoplasia.

Magnetic resonance imaging (MRI) of the abdomen confirmed aplasia of the gall bladder and hypoplasia of pancreas, while the brain MRI showed periventricular calcification. The genetic studies done for Mitchell-Riley syndrome (*RFX6* gene locus) confirmed presence of a homozygous mutation in the *RFX6* gene (c.1153C>T p.Arg385*), a previously unreported homozygous mutation in exon 11 of the *RFX6* gene. Therefore, this was a confirmed case of Mitchell-Riley syndrome with additional features. The parents were not studied for a carrier state.

Discussion

This is the second case of Mitchell-Riley syndrome diagnosed in a population of Arab ethnicity, the first case ever reported from the Arab peninsula and the ninth case overall (9,10). Although this infant had the classical features of the Mitchell-Riley syndrome including neonatal diabetes, pancreatic hypoplasia, duodenal atresia, gall bladder aplasia, he did not have malrotation or biliary atresia. The infant also had chronic diarrhoea/malabsorption due to severe exocrine pancreatic insufficiency and cholestatic jaundice, findings which have been reported in most of the published cases (5,6,9,10,12). He even had hemochromatosis, reported only by Martinovici et al (5). However, similar to other patients, this infant also had several features overlapping with the Martinez-Frias syndrome such as hypothyroidism (4,12,13,14), severe IUGR, and hypospadias (4,15). In most of the previously known patients, severe hypoplasia or aplasia of the gall bladder and biliary atresia with acholia were the main features and the Kasai procedure was successfully carried out in one of these patients (2,10,12,13,14). Although the gall bladder could not be visualized on the HIDA scan, in the MRI scan nor per-operatively, our patient never had acholic stools. He had mild direct hyperbilirubinemia and elevated liver enzymes and unfortunately, we could not carry out an ERCP or a liver biopsy to confirm a diagnosis of biliary atresia. Our patient also had anaemia during his first week and tested DAT positive; since he had thrombocytopenia, microangiopathic anaemia was considered. Anaemia was also found in one of

Table 1. Reported cases of Mitchell-Riley syndrome

No.	BW (g)	GA (weeks)	Diabetes onset (age)	GI atresia/ malrotation	Hepatobiliary defects	Pancreas	Other	RFX6 mutation-nucleotide	RFX6 mutation-protein	Reference
1	1540	36	1 day	DA, JA	GBA	AP	Malabsorption unresponsive to pancreatic supplements/bile acids; cholestasis	c.380+2T4C homozygous	p.?	Mitchell et al (2)
2	1310	34	2 days	DA, JA	GBA	AP	Duodenal biopsy: partial villous atrophy. Intrahepatic cholestasis.	c.380+2T4C homozygous	p.?	Mitchell et al (2)
3	2295	39	2 days	Duodenal web and malrotation	GBA	Small Pancreas	Intrahepatic cholestasis; bilateral inguinal hernias	c.672+2T4G/ c.224-12A4G compound heterozygote	p.?/p.?	Mitchell et al (2)
4	1700	35	8 days	DA, malrotation	GBA	Undetectable faecal elastase	Intrahepatic cholestasis; anteriorly placed anus	c.649T4C homozygous	p.Ser217Pro	Chappel et al (6)
5	1340	38	Soon after birth	DA, JA (apple peel type), intestinal malrotation	GBA	Pancreatic hypoplasia	Intrahepatic cholestasis; malabsorption unresponsive to pancreatic supplements/ bile acids; neonatal haemochromatosis	c.542G4A homozygous	pArg181Gly	Martinovici et al (5)
6	<10th centile	35	2 days	DA	No anomaly reported	No anomaly reported	Ascites, sepsis, gastro-intestinal haemorrhage	c.776_780 +8del13 homozygous	p.?	Smith et al (7)
7	1490	38	1 day	DA, JA, intestinal malrotation	GBA	AP	Intrahepatic cholestasis; red cell aplasia confirmed on bone marrow biopsy; malabsoprtion unresponsive to pancreatic supplements/bile acids	c.781-2_787delAGGTT-GATAinsG homozygous	p.?	Spiegel et al (9)
8	1375	34	1 day	DA, intestinal malrotation	GBA	AP	Intrahepatic cholestasis; malabsoprtion unresponsive to pancreatic supplements/bile acids	c.779A4C homozygous	p.Lys260Thr	Concepcion et al (10)
9	1650	32	3 years	DA, jejunal web, Meckel's diverticulum	GBA	No anomaly reported	No abnormality reported	c.2176C4T homozygous	p.Arg726X	Sansbury et al (11)
10	1700	34	6 years	DA, mid-gut malrotation	No anomaly reported	No anomaly reported	No abnormality reported	c.2176C4T homozygous	p.Arg726X	Sansbury et al (11)
11	1300	38	2 days	DA without malrotation	GBA	Pancreatic hypoplasia	Malabsorption, unresponsive to pancreatic supplements/ bile acids; cholestasis, neonatal haemochromatosis, sepsis, DAT+ive, Factor IX, periventricular calcification, cardiac rhabdomyomas, hypertension, hypothyroidism, hypospadias	c.1153C>T homozygous	p.Arg385	This report

BW: body weight, GA: gestational age, GI: gastro intestinal, DA: duodenal atresia, JA: jejunal atresia, AP: aplasia of pancreas, GBA: gall bladder aplasia

the previously reported cases and a blood transfusion was given. However, a cause for anaemia was not mentioned and thrombocytopenia was never reported in any patient (10).

This patient had various other previously unreported features such as cerebral calcification and cardiac rhabdomyomas. A cardiac lesion was reported previously, but that patient had a septal defect. Cerebral lesions were most likely never detected, due to most of the infants dying in their first few days of life with no reports of post-mortem findings. Our patient had coagulopathy with factor IX deficiency which has also not been reported in any other patient. Gastrointestinal bleeding was noted in some patients, but the cause had not been identified. The portosystemic shunt and hyperammonemia in our patient can be considered an incidental finding or possibly a developmental defect along with the intestinal atresia. The shunt in our baby closed spontaneously and the ammonia level returned to normal. A minimal shunt was shown afterwards. The systemic hypertension requiring antihypertensive medicine was another finding not reported before and unfortunately, its cause could not be identified; either it was a part of the syndrome due to the distinctive genetic mutation or just an additional finding. The genetic mutation was also different from previously reported patterns, which consisted of c.649T4C homozygous (6), c.781-2_787delAGGTT-GATAinsG homozygous (9) and c.779A4C homozygous (10), while this patient had a previously unreported mutation: c.1153C>T p.Arg385*.

In conclusion, we have reported a confirmed case of Mitchell-Riley syndrome with a previously unreported homozygous mutation in exon 11 of the *RFX6* gene. To our knowledge, this is the first ever reported case from the Arab peninsula. Furthermore, the patient had atypical additional features which could be related to a new mutation which needs to be further explored.

Ethics

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

Authorship Contributions

Concept: Nusrat Khan, Waleed Dandan, Design: Nusrat Khan, Data Collection and/or Processing: Nusrat Khan, Suha Hadi, Noura Al Hassani, Waleed Dandan, Analysis and/or Interpretation: Suha Hadi, Noura Al Hassani, Literature Research: Nusrat Khan, Suha Hadi, Noura Al Hassani, Writing: Nusrat Khan, Waleed Dandan.

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Case Report

Long-term Outcome after Robotic-assisted Gastroplication in Adolescents: Hunger Hormone and Food Preference Changes Two Case Reports

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ABSTRACT

Weight loss surgery (WLS) is efficacious for long-term weight reduction and decreases overall mortality in severely obese patients. The mechanisms implicated in long-term weight loss are not fully understood. Proposed mechanisms include changes in gut hormones and brain regulation of appetite and satiety. We aimed to investigate the long-term ghrelin and leptin profiles and changes in food preference and eating behavior after WLS in adolescent patients. Two obese females aged 15 years and 14 4/12 years, who did not respond to lifestyle changes, including dietary intervention and physical exercise in combination with medical therapy, underwent robotic-assisted gastroplication. Anthropometric measurements, food habits and eating behavior, as well as metabolic and hormonal changes during long-term post-surgical follow-up were monitored. Long-term weight reduction was obtained in both patients, with a significant decrease in waist circumference. Resting energy expenditure showed a decrease over time, with a respiratory quotient that increased showing a shift from oxidation of a high-fat diet before surgery to oxidation of a mixed diet two and three years later. Both subjects improved their eating habits and lifestyle. Co-morbidity resolution was also noted. Increased pre-prandial ghrelin levels as well as higher post-prandial ghrelin and a leptin drop compared with pre-surgery values were observed in both patients. Persistent weight loss after gastroplication is associated with a favorable change in gut hormones and food preferences. The role of hormonal and sensory components in long-term results seems crucial. Particularly in adolescent patients, a multidisciplinary approach and continuous nutritional care is mandatory for weight maintenance and consolidation of changes.

Keywords: Robotic surgery, gastroplication, ghrelin, leptin, adolescent, food choices, eating behavior

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WHAT IS ALREADY KNOWN ON THIS TOPIC?

Weight loss surgery is efficacious for long-term weight reduction and decreases overall mortality in severely obese patients. The mechanisms implicated in long-term weight loss are not fully understood. Changes in gut hormones and brain regulation of appetite and satiety are proposed.

WHAT THIS STUDY ADDS?

We reported long-term follow-up after gastroplication in two adolescents. Weight loss is associated to a favorable change in hunger hormone and food preferences. Hormonal and sensory components in the long-term results seems to be crucial.

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Introduction

Weight loss surgery (WLS) is efficacious for long-term weight reduction and decreases overall mortality in severely obese patients (1,2,3,4). The effect of WLS is probably not only due to restriction of food intake and/or malabsorption of ingested food, however, the mechanisms implicated in long-term weight loss are not fully understood. Proposed mechanisms include changes in gut hormones and brain regulation of appetite and satiety (5,6,7). Hormones such as ghrelin, leptin, peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and cholecystokinin (CCK), secreted by the gastrointestinal (GI) tract, the pancreas, and by the adipose tissue, are released into the periphery in response to increased or decreased intake of nutrients and are able to act peripherally on the vagus nerve and centrally on target areas in the hypothalamus (8,9). In addition, crosstalk between the adipose tissue and the gut may also be relevant in the context of regulating energy homeostasis, satiety, and body weight. Leptin is released continuously from the adipose tissue into the circulation and acts mainly on the hypothalamus, regulating the long-term energy storage. In addition, exocrine-secreted gastric leptin is proposed to ensure proper food processing and food intake in the short term independently of adipose-derived leptin (10).

Modifications in the perception of food and hence eating behavior changes are also considered crucial in weight loss with long-term maintenance. Patients after WLS, particularly post Roux-En-Y Gastric Bypass (RYGB), report feeling less hungry, reaching satiety earlier, thus reporting a change in their taste and food choices. These changes have been strongly attributed to variations in taste processes and food reward (11,12,13). Reports on neuro-hormonal assessment and shifts in food habits after WLS of subjects in the pediatric age group are scarce (14,15).

In this paper, we report long-term ghrelin and leptin profiles and changes in food choices and eating behavior after robotic-assisted gastroplication in two adolescent patients.

Case Reports

Two adolescents, who did not respond to lifestyle changes, including dietary intervention and physical exercise in combination with medical therapy, underwent robotic-assisted gastroplication.

Patient 1, a 15-year-old obese female with a body mass index (BMI) of 38.8 kg/m^2, was submitted to an eighteen-month organized and supervised lifestyle modification intervention, including family involvement and medical treatment (6 months of metformin) with no significant improvement. She had developed hyperinsulinism, hyperandrogenism, amenorrhea, ultrasound signs of Polycystic ovarian syndrome (PCOS), and hypertension with left ventricular hypertrophy.

Patient 2, a 14-year, 4-month-old obese female with a BMI of 41.2 kg/m^2, had an unsuccessful outcome after 20 months of supervised lifestyle changes, including family involvement. She was noncompliant with the medical treatment prescribed (metformin). Hyperinsulinism, dyslipidemia, moderate hepatic steatosis, gastroesophageal reflux (GER), obstructive sleep apnea (OSA) were reported before surgery.

Both girls had attained skeletal and developmental maturity (final height and pubertal stage) before the surgical treatment. No major contraindications for WLS were found, including eating disorders and psychopathologies. A multidisciplinary intervention with specific nutritional, psychological, and training sessions was started two months before surgery and continued post-surgery. Both girls were prescribed moderate-low energy diets balanced in macro- and micronutrients according to our national recommendations specific for age and sex (16) and were invited to weekly nutrition education sessions intended to be informative and interactive. For both patients, the capability to commit to comprehensive medical and psychological evaluation before and after surgery as well as patient and family willingness to participate in a postoperative multidisciplinary treatment program were documented. Written informed consent was obtained prior to enrollment. Gastroplication was performed using the Robotic surgery Da Vinci system® (Intuitive Surgical, Inc., Sunnyvale, California, USA) with an 8.5 mm scope and two 5-mm operative trocars. Two rows of non-absorbable interrupted sutures (2-0 Ethibond™) were placed along the greater dissected curvature starting 1 cm below the angle of His, narrowing the stomach (80-100 mL of volume). No intra- or postoperative complications occurred. Pain control medication was only necessary for 2 days postoperatively and then stopped. The hospital stay lasted 96 hours. Proton pump inhibitors and anticoagulation were prescribed for 14 days.

During follow-up, metabolic and hormonal changes were documented. A complete nutritional assessment with anthropometric measurements, bioimpedance analysis, and indirect calorimetry (IC) was performed under standard conditions: overnight fasting, abstaining from caffeine, alcohol, nicotine, and strenuous physical activity starting the day before, as abstaining from moderate physical activity for at least 2 hours (17,18).

Food intake and eating habits were documented by the subjects keeping a daily food diary and a validated self-administered food frequency questionnaire (19,20) starting two months before surgery (T0) and repeated yearly post treatment.

Ghrelin and leptin were evaluated, before and after surgery, using a commercial enzyme immunometric assay (Human Unacylated Ghrelin, BioVendor R&D, Brno, Czech Republic) and an enzyme-linked immunosorbent assay kit (Human Leptin Immunoassay, R&D Systems, Minneapolis, MN) respectively, following the manufacturer's instructions. The results were expressed as pg/mL.

The study was performed according to the Declaration of Helsinki. The Ethics Committee of the Fondazione IRCCS Policlinico San Matteo and Department of Internal Medicine, University of Pavia, approved the study protocol. All procedures were carried out with adequate understanding and written consent of the patients and their parents.

Nutritional assessment as well as metabolic and hormonal changes during long-term post-surgical follow-up are reported in Tables 1, 2, 3.

Anthropometric Measurements and Food Habits and Eating Behavior

Long-term weight reduction was obtained in both patients with a significant decrease in waist circumference (Table 1). Resting energy expenditure (REE) measured by IC also showed a significant decrease over time, with a respiratory quotient (RQ) that increased, showing a shift from oxidation of a high-fat diet before surgery to oxidation of a mixed diet two and three years later.

Coupling the measurement of body composition to that of REE expands the diagnostic potential of IC (18). Once the lean and fat compartments had been measured by bioelectrical impedance analysis, it was possible to establish on the basis of REE whether the two subjects were becoming hyper- or hypo-metabolic. The results show, as expected, a slight decrease in metabolic efficiency during the first period (1 year after surgery) and values similar to initial ones despite weight loss during the following recovery period (Table 1).

Table 2 reports the main changes after WLS for food consumption, eating behavior, and lifestyle behavior.

Table 1. Anthropometric, metabolic, and endocrine profile of the two patients, during long-term post-surgical follow-up

	Patient 1				**Patient 2**			
	Before surgery	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**	**Before surgery**	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**
Social isolation	Yes	No	No	No	Yes	No	No	No
BMI kg/m^2	42.2	34.6	26.9	29.3	41.2	38.3	34.4	34.2
WC cm	122	98	94	92	123	108.5	105	105
PA°	5.5	5.7	4.8	5.0	5.7	6.0	5.7	5.6
FM kg (%)	53.6 (42.4)	36.6 (34.9)	32.4 (40.2)	36.6 (41.7)	44.2 (43.6)	38.9 (41.4)	34.3 (40.6)	44.2 (49.9)
IC (Kcal/day)	2090	1544	1354	1347	2012	1761	ND	1641
RQ (VCO_2/VO_2)	0.79	0.72	0.82	0.8	0.84	0.73	ND	0.97
REE/kg FFM (kcal/ kg FFM)	28.8	22.8	28	26.3	35	32	ND	33
Fasting insulin (microIU/mL)	66	13	7.8	4.3	36.1	19.7	14.1	10.2
Fasting blood glucose (mg/dL)	90	77	77	73	92	88	80	83
HOMA-IR	14.7	2.4	1.5	0.8	8.2	4.2	2.8	2.1
HbA1C	5.6	5.1	5.1	5	5.9	5.5	5.4	5.4
Total cholesterol (mg/dL)	149	134	157	160	207	192	172	179
HDL-cholesterol (mg/dL)	53	48	67	73	34	37	32	45
Triglycerides (mg/dL)	55	70	63	41	96	82	79	60
Ghrelin (pg/mL)*								
- Pre-prandial	38.23	75.54	127.68	57.35	21.14	76.68	110.13	42.97
- Post-prandial	28.28	68.83	62.04	21.11	18.50	43.38	53.88	20.37
Leptin (pg/mL)**								
- Pre-prandial	8121	13265	26321	14610	56312	32497	18391	23487
- Post-prandial	10508	20850	33264	26389	58200	64414	52353	39950

BMI: body mass index, WC: waist circumference, PA: phase angle value, FM: fat mass estimated by bioelectrical analysis, IC: indirect calorimetry, RQ: respiratory quotient, REE/kg FFM: resting energy expenditure per kg of fat-free mass estimated by bioelectrical impedance analysis, HDL: high density lipoprotein, HOMA-IR: homeostatic model assessment-insulin resistance, ND: not determined

*Ghrelin normal values: 98-389 pg/mL

**Leptin normal values: 3877-77273 pg/mL

Both subjects improved their eating habits and changed their lifestyle behavior (Table 1).

Dietary intake was assessed using a prospective 7-day food diary. Total energy intake (kcal/day) was estimated based on the food diary and showed, though not equal, significant reductions in both subjects. Major changes were evident in particular the intake of proteins, compared to baseline (before surgery). Lipid and carbohydrate intake changed until reaching the national reference recommended dietary intake (16). Alternatively, fiber intake slowly increased by a more than expected rate, over the years (Table 3).

Metabolic Changes and Co-Morbidities

Patient 1 had a significant reduction in blood insulin level and a decrease in homeostatic model assessment-insulin resistance (Table 1). Resolution of hormonal and ultrasonographic features of PCOS was observed, and the girl regained normal menstrual cycles 5 months after surgery. Blood pressure also decreased to normal levels.

Patient 2 showed resolution of insulin resistance, of dysfunctional lipid metabolism and RGE (Table 1). Improvement in steatosis and OSA were also reported.

Improved social and emotional well-being and self-esteem were reported in both girls.

Ghrelin and Leptin Profile

Increased pre-prandial ghrelin levels were observed in both patients as well as a higher post-prandial ghrelin drop and leptin increase compared with pre-operative levels (Table 1, Figure 1).

Discussion

Recent evidence highlighting the prevalence of severe obesity in the pediatric population, coupled with disappointing outcomes related to medical weight loss interventions, has led to increased interest in WLS (2,3,4). It is reported that the number of surgeries being performed in adolescents has increased 5-fold from 1997

Table 2. Lifestyle and food habits of the two patients, before and after surgery

	Patient 1				Patient 2			
	Before surgery	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**	**Before surgery**	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**
Number of meals/day	4	4	5	5	3	5	4	4
Breakfast	No	Yes	Yes	Yes	No	Yes	Yes	Yes
Food avoidance	Yes	No	No	No	Yes	No	No	No
Portion size*	Medium	Small	Small	Medium	Big	Medium	Medium	Medium
Fruit and vegetable portions/day	3	3	2	2	0	2	2	2
Soft drinks or junk food consumption	Everyday	0	0-1 time per week	1-2 times per week	Everyday	Never	Never	0-1 time per week
Physical activity	None	3 times per week	2 times per week	None	None	2 times per week	None	3 times per week

*Turconi et al (19,20)

Table 3. Daily energy intake and diet composition of the two patients during long-term post-surgical follow-up

	Patient 1				Patient 2			
	Before surgery	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**	**Before surgery**	**1 year after surgery**	**2 years after surgery**	**3 years after surgery**
Energy intake (kcal/day)	3000	1300	1000	1150	2200	1400	1800	1900
Diet composition								
Proteins % (g)	18 (134)	21 (69)	20 (49)	14 (41)	16 (91)	19 (68)	15 (68)	13 (64)
Lipids %	43	40	29	23	27	34	29	32
Saturated fats %	16	13	10	8	9	11	8	9
Carbohydrates %	39	39	52	63	57	47	56	50
Simple sugars %	18	10	12	16	18	12	11	11
Fiber (g)	24	16	12	20	14	10	17	18

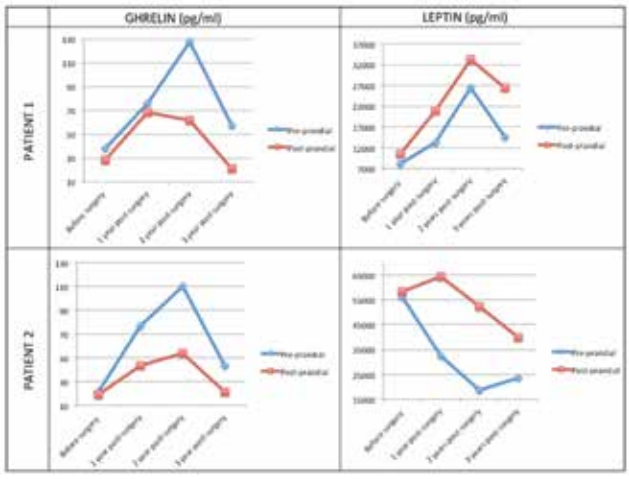

Figure 1. Ghrelin and leptin profiles of the two patients, before surgery and during long-term post-surgical follow-up

to 2003 and tripled in 2000-2003. The number of procedures performed yearly is rising, and WLS is currently the most effective treatment for morbid obesity and improvement of comorbidities in adolescents. While pharmacological and behavioral treatments are usually associated with weight loss followed by weight regain, WLS provides weight loss for at least 15 years in obese patients (2,3,4,16).

We describe long-term weight reduction associated with resolution of comorbidities and a relevant change in neuroendocrine profile and food preferences as well as eating behaviors in two adolescents, after robotic-assisted gastroplication. This surgical approach in adolescence has the added advantages of being reversible, not requiring the use of foreign materials, and conforming to the physiological development of the individual. The clinical, metabolic, and hormonal improvements observed following a long-term follow-up confirm the effectiveness of this technique in young patients.

Gut hormones such as ghrelin, PYY, GLP-1, pancreatic polypeptide, oxyntomodulin are implicated in the short-term regulation of ingestion and adiposity signals such as insulin and leptin are involved in long-term energy homeostasis (8,10). Our data confirmed that modifications in the milieu of gut hormones is implicated in the sustained weight loss observed following WLS. After food is ingested, nutrients pass through the GI tract, stimulating the release of a range of peptide hormones. In the context of their local, central, and peripheral actions, these hormones also mediate satiety.

Ghrelin is the only known orexigenic gut hormone and it is principally secreted from X/A-like cells within gastric oxyntic glands. Ghrelin mediates its orexigenic action via stimulation of neuropeptide Y (NPY)/agouti-related peptide (AgRP), coexpressing neurons within the arcuate nucleus (ARC) of the hypothalamus. The brainstem and vagus nerve may also contribute to the effects of ghrelin on food intake.

Insulin is synthesized in the ß cells of the pancreas and is secreted rapidly after a meal, with well-characterized hypoglycemic effects. However, insulin also acts as an anorectic signal within the central nervous system (CNS); insulin receptors are widely expressed in the brain, particularly in hypothalamic nuclei, such as the ARC, dorsomedial nucleus (DMN), and paraventricular nucleus, which are involved in the control of food intake.

Leptin is predominantly secreted by adipocytes with circulating levels proportional to fat mass. It exerts its anorectic effect via the ARC, where both NPY/AgRP and pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons express leptin receptors. Leptin inhibits NPY/AgRP neurons and activates POMC/CART neurons, resulting in reduced food intake and increased energy expenditure (8,10,21,22).

Obesity in children is associated with leptin and insulin resistance, manifested by reduced serum levels of ghrelin and increased leptin and insulin levels. Preprandial levels of circulating ghrelin rise, then fall rapidly in the postprandial period. Obese subjects show a less marked drop in plasma ghrelin after meal ingestion (23,24,25).

In our girls, ghrelin, leptin, and insulin levels were abnormal before surgery and a pronounced change in orexigenic and anorectic hormones was observed following WLS (4,5,6,7). The analysis showed a significant post-prandial decrease in ghrelin and an increase in leptin levels compared with pre-operative levels and confirmed the favorable impact of this surgical procedure on hunger hormonal changes. However, changes in hormones does not fully explain the magnitude of weight loss after WLS. Following WLS, body weight decreases, but body composition improves without relevant changes in the phase angle. It has been shown that phase angles are sensitive to differences in water distribution and can be used to predict % body fat mass (26). The phase angle deviates little from initial values suggesting the maintenance of metabolically active mass. Besides, the phase angle is not just an indicator of adequate nutritional status preservation but more of an overall measure of function and general health (27). Further studies are necessary to show how phase angle differs between different populations, according to age, ethnicity, and body composition (27).

Both our patients improved their eating habits by eating fruits and vegetables which they never used to eat before surgery, although their fiber intake increased more slowly than expected over the years, highlighting the obstacle of adjusting to adequate consumption of fruits and vegetables, typical of teenager habits and food dislikes.

The patients also stopped skipping breakfast in the morning, increased the number of their meals per day decreasing the food portion size and avoided spontaneous soft drinks and junk food intake. Their diet improved with changes not only in energy intake but also in macronutrient composition with

an impact not only on food choice and preference but also on metabolism with a better use of energy substrates reflected in a notable increase in RQ and metabolic efficiency (18,28).

The mechanisms responsible for suppression of appetite and changes in food preferences are not well understood. Although a number of changes in food choice, taste functions, hedonic evaluation, motivation, and self-control have been documented in both humans and rodents after surgery, their importance and relative contribution to diminished appetite is still under investigation (4,5,6,7). Hedonic and sensory components like olfactory and gustatory stimuli significantly affect the appetite and taste. Recent studies have increased our knowledge on the expression of receptors being targeted by metabolic hormones and peptides governing cellular processes underlying hunger (ghrelin, NPY) and satiety (insulin, leptin) in the olfactory mucosa, the olfactory bulb, and olfactory-related brain areas (29). This chemical nutritional information alters the olfactory message and adapts the function of the olfactory system. Obesity-related changes influence the olfactory function (30). About one quarter of morbidly obese patients are hyposmic with significantly decreased discrimination and identification ability and limited gustatory function. The olfactory function can change metabolism and feeding-related behavior thus affecting the energy balance and body weight.

After laparoscopic WLS, the discrimination ability of the olfactory and gustatory functions improves (13). In our girls, no data on olfactory function were available. However, after gastroplication a shift in food preferences and development of food dislikes were observed supporting the sensory component role in long-term WLS outcomes.

It must be added that the inevitable changes due to the modifications in body weight, body shape, and body image perception create new stimuli that affect lifestyle changes and sociality.

Both of our two girl patients became more physically active and more engaging in their social relationships. Both showed a notable decrease in social isolation and reported an improvement in self-esteem and quality of life (3).

Major behavioral changes occur postoperatively. However, it is recommended to have a preoperative program to educate the subject on food choices, implement nutritional changes, and to prepare them for post-surgery modifications. The ultimate goal is to encourage the subjects to independently choose a healthier diet, which involves long-term preparation. In our patients, a very satisfactory post-operative weight loss was reported in the first and second year of follow-up, while a steady state in BMI as well as some other parameters from the second year to the third year were observed. A moderate decrease in the patients' compliance may have contributed to this trend. The surgical procedure is only one part of the treatment plan if the goal is to obtain effective and long-lasting results leading to persistent changes.

Substantial nutrition guidance is required before and after the surgical procedure. A multidisciplinary continuous approach is required to support these patients, helping them to change their food habits, meet their protein and fluid requirements, increase their physical activity, and learn to listen to their bodies recognizing the signals for hunger and satiety. Post-surgery weight loss and food choices can be monitored by a registered dietitian or a nutritionist, while the clinician may assess the state of health and well-being and also monitor the hormonal changes. In the follow-up, micronutrients should also be evaluated and specific supplementation should be prescribed if necessary.

In conclusion, the findings in our two patients show that persistent weight loss after gastroplication is associated with a favorable change in gut hormones as well as in food preferences, eating behavior, and lifestyle. The role of hormonal and sensory components in the long-term WLS results seems crucial. We should also emphasize the need for a multidisciplinary approach and continuous nutritional education in the long-term weight maintenance and consolidation of changes, particularly in adolescent patients.

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Ethics

Ethics Committee Approval: Fondazione IRCCS Policlinico San Matteo Committee (Approval number: 02-15-12), Informed Consent: It was taken.

Peer-review: External peer-reviewed.

Authorship Contributions

Concept: Gloria Pelizzo, Valeria Calcaterra, Hellas Cena, Design: Gloria Pelizzo, Valeria Calcaterra, Hellas Cena, Data Collection or Processing: Maria Luisa Fonte, Mara De Amici, Matteo Vandoni, Michela Albanesi, Analysis or Interpretation: Gloria Pelizzo, Valeria Calcaterra, Hellas Cena, Literature Search: Valeria Calcaterra, Hellas Cena, Writing: Gloria Pelizzo, Valeria Calcaterra, Hellas Cena.

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