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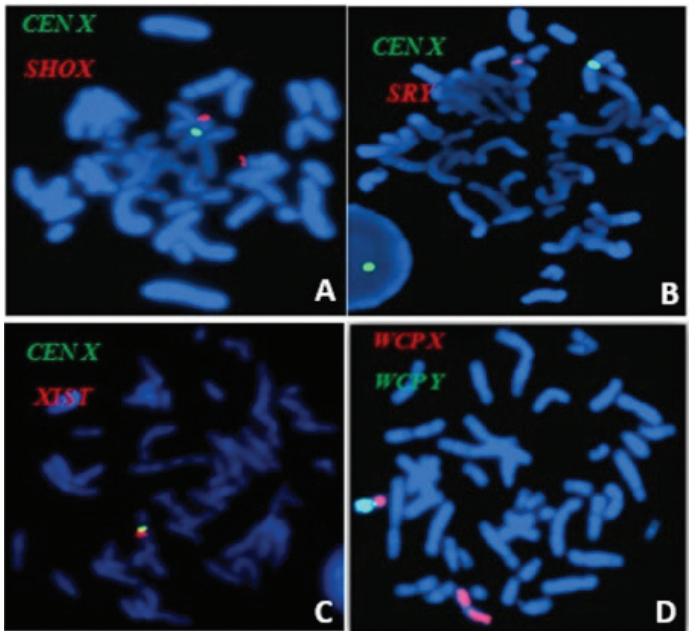
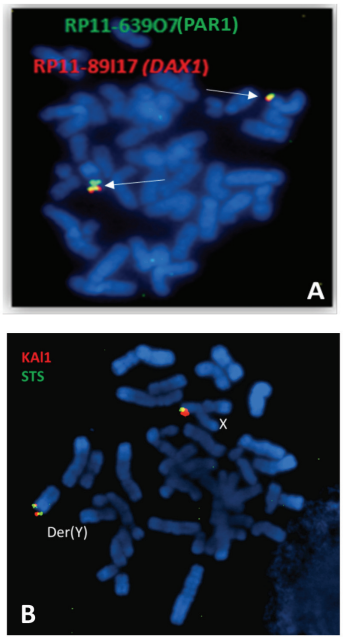



FISH analysis. A) FISH results using SHOX probe, two red spots were detected. B) FISH results using SRY probe, one red spot was detected. C) FISH results using XIST probe, one red spot was detected. D) FISH analysis using WCPX/WCPY showed the presence of a part from chromosome X on the Y chromosome




FISH analysis using specific probes: A) NR0B1 probe showed its presence on both sex chromosomes (white arrows); B) KAL1 and STS probes showed their presence on both sex chromosomes

Anomalies in Human Sex Determination: Usefulness of a Combined Cytogenetic Approach to Characterize an Additional Case with Xp Functional Disomy Associated with 46,XY Gonadal Dysgenesis

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**CONTENTS**

REVIEW

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J Clin Res Pediatr Endocrinol 2023;15(1):1-6

# Specific Functions of Melanocortin 3 Receptor (MC3R)

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## Abstract

Melanocortin 3 receptor (MC3R) is a G-protein coupled receptor which has been defined mostly as a regulator of the appetite/hunger balance mechanisms to date. In addition to its function regarding the weight gain and appetite control mechanisms of MC3R, recent studies have shown that MC3R controls growth, puberty, and circadian rhythms as well. Despite the drastic effects of MC3R deficiency in humans and other mammals, its cellular mechanisms are still under investigation. In this review paper, we aimed to point out the importance of MC3R regulations in three main areas: 1) its impact on weight and appetite control, 2) its role in the control of growth, puberty, and the circadian rhythm, and, 3) its protein-protein interactions and cellular mechanisms.

**Keywords:** Childhood obesity, melanocortin 3 receptor, circadian rhythm, appetite control

## Introduction

Excess amounts of fat consumption and overeating result in disruptions of the energy balance in the body and excess energy storage in adipose tissue. If this storing process continues for too long, due to increased adiposity and inflammations, obesity occurs (1). Along with obesity, there is an increase in the likelihood of diseases, such as cardiovascular diseases, type 2 diabetes, fatty liver, respiratory diseases and cancer, which all can reduce both health and quality of life and lead to early death (2). The cause of obesity is classified under three headings, namely monogenic, syndromic and common obesity (3). According to studies, obesity mutations belonging to the monogenic and syndromic obesity classes may be observed in 20 different genes which clearly cause inherited obesity (4,5). Single gene mutations causing obesity are known as monogenic or syndromic obesity. Monogenic (non-syndromic) obesity is a more severe and uncommon form of obesity in which people have mutations in a single gene which result in an obesity phenotype or inheritance in a Mendelian pattern (6,7). The leptin-melanocortin signaling pathway in the hypothalamus, which is crucial for maintaining energy homeostasis, contains the majority of these genes impacted by monogenic obesity (6). In addition, if mutations and/or chromosomal abnormalities are detected in more than one gene as the cause of obesity, the disease is also classified as syndromic obesity. Similar to other complex traits and disorders, the heritability of syndromic obesity follows a similar trend (7). In addition to the features seen in monogenic obesity patients, dysmorphic features are observed with various physical and mental developmental disorders in patients with syndromic obesity (3). Common obesity is a form of obesity which has an alarming prevalence and life-threatening implications. It is brought on by a combination of genetic and environmental factors, including a high-fat diet and a sedentary lifestyle (7). Differences in weight gain mechanisms observed in the common obesity class are about 40-70 % (8,9).

The creation of physiological activations and the metabolic balance of the organism are ensured by transmitting signals formed as a result of receiving environmental stimuli through the central nervous system (CNS) and evaluating them for metabolic response. In appetite control, glucose and fat metabolisms, the digestion and metabolic rates are regulated by physiological activations (10). The neurons, which are responsible for maintaining this order and balance throughout the life of the organism, maintain their

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plasticity and ensure that the living things have a healthy metabolism for their survival. Chronic over-nutrition may result in changes of the plasticity of neurons and the disruption of their organization in signal transmission (11). For this reason, the hypothalamic and especially the melanocortin pathway neurons within the CNS have been the focus of metabolic studies in recent years (12). As insulin and ghrelin, the hunger hormone leptin from the adipose tissue, are responsible for the control of the melanocortin pathway in the hypothalamus, they play a role in the regulation of the appetite, hunger, and energy mechanisms (13). Leptin modulates the melanocortin pathways, which are also controlled by four neuro-hormones which are synthesized and secreted in the arcuate nucleus (ARC) of the hypothalamus. These peptides for the eating and energy mechanisms are the neuropeptide Y (NPY), the agouti-related peptide (AgRP), proopiomelanocortin (POMC), and cocaine and amphetamine-related transcript (CART) (14). While leptin inhibits NPY and AgRP (which are associated with increasing eating/appetite and reducing energy expenditure), the opposite is true for POMC and CART (15). With the exception of CART (since its receptor has not yet been discovered), the other neuro-hormones carry out signal transmission via G protein-coupled receptors (GPCR) (16).

Among these hormones, POMC is synthesized as a precursor and transformed into eight biologically active hormones with different functions by passing through the regulated secretion pathways in POMC neurons and pituitary cells (17). Five melanocortin receptors have been identified to date, and signal transmission of POMC-derived hormones is provided via these receptors. MC3 and MC4 receptors belong to this family of receptors and interact with hormones involved in the eating and energy mechanisms in the brain (18). Melanocortin 3 receptor (MC3R) has a very high binding capacity for γ-melanocyte stimulating hormone (γ-MSH), while MC4R has a very high binding capacity for α and β-MSH. MC3R is a member of the GPCR family. It regulates pathways associated with nutrient partitioning, weight management, appetite, and hunger (Figure 1) (15,19).

Like most GPCR members, MC3R form dimers/oligomers among themselves and with different GPCR members which activate/deactivate and regulate secondary signaling pathways (20). In fact, 40% of the drugs in the pharmaceutical industry target the GPCR family (21).

Variations of MC3R result in both genetic obesity and delayed entry to sexual maturity in humans. Experimental studies have shown the importance of the MC3R role for the metabolism balance for nutrient partitioning, obesity, circadian rhythm and sexual maturity. Inspite of a broad range of physiological roles of the *MC3R* gene, unfortunately, there is still a lack of knowledge about its intracellular mechanisms in the literature.

## MC3R Impact on Weight and Appetite Control

The most striking phenotype of MC3R loss of function variants and MC3R knock-out (KO) mice models is obesity.

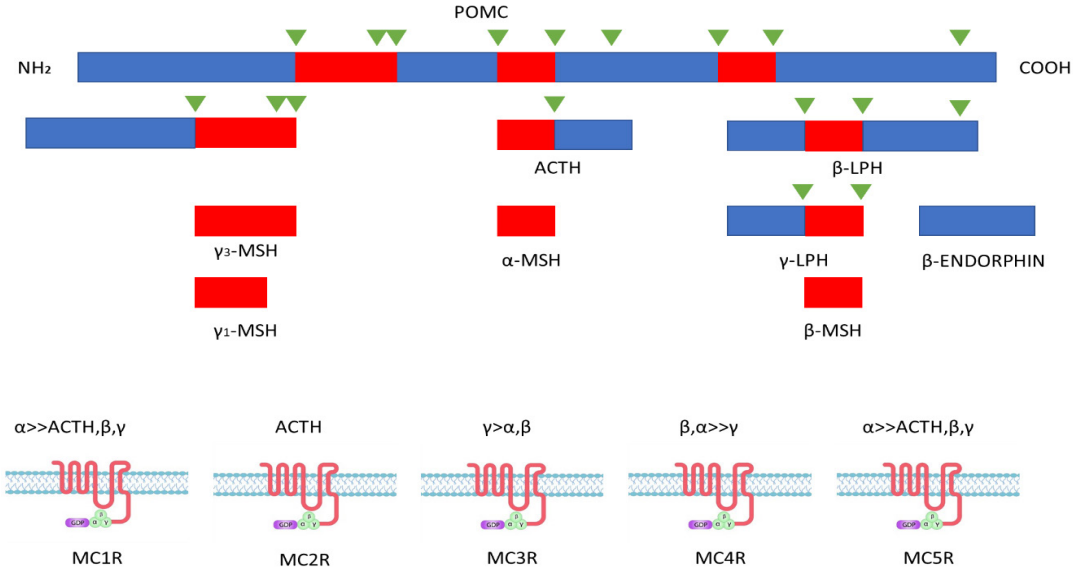


**Figure 1.** Eight separate active peptide hormones obtained from proopiomelanocortin (POMC) hormone after post-translation modifications in the regulated secretory pathway and melanocotin receptors with affinities to their ligands. Green arrows show where prohormone convertases (PCs) perform their proteolytic activity on POMC (19)

*ACTH: Adrenocorticotropic hormone, β-LPH: Beta-lipotropin, γ and α-MSH: Gamma and alpha melanocorticotropin hormone*

The obesity features of MC3R KO mice models were different from the MC4R KO mice obesity phenotype (22). First of all, the ratio of the fat mass over the lean mass was very high for the MC3R KO models. Secondly, MC3R KO mice models display decreased lean mass while increasing fat mass during a high fat diet (HFD), even though they had similar meal amounts vs. wild-type (WT) mice (23). Most interestingly, these mice models had trouble gaining weight after a restriction feeding period since they had trouble balancing their eating amount vs. their caloric requirement and so lost more weight during this period (24). The reason behind this increased fat mass during a regular chow diet causing the obesity phenotype could be a problem with the nutrient partitioning mechanisms. This possibility also helps to explain the mice losing more weight during restriction feeding and having trouble gaining weight afterwards.

The MC3R variants studied were mostly from Caucasian populations in humans. Mencarelli et al. (25) in 2011 investigated MC3R variants in French and Italian populations which revealed S17T, D158Y, V177I, and L249F novel variants which cause the obese phenotype, whereas the I87T and L285V novel variants did not show obese phenotype. In the French population, 1.81% of the obese group had the loss of function MC3R variant, while in the Italian obese patient group, the ratio was 1.16%. Interestingly, the number of point mutation caused variants without the obese phenotype was 7 out of 753 normal weight individuals in the French control group, while this number was only 1 out of 214 people in the Italian control group.

Ghamari-Langroudi et al. (26) in 2018 showed that MC3R signaling in presynaptic AgRP neurons modulates the activity of postsynaptic MC4R paraventricular nucleus neurons through regulation of GABA release. The results of their study revealed the importance of investigating the metabolic roles and intracellular mechanisms of MC3R in weight and appetite control and they emphasized the necessity of the clarification of their intracellular trafficking further, which is lacking in the literature. Unfortunately, the commercial MC3R antibodies used in experimental procedures have many specificities and binding capacities which give false conclusions for intracellular trafficking. To distinguish the intracellular trafficking of MC3R, more specific methodologies are necessary in order to obtain more reliable results.

Feng et al. (27) in 2005 discovered that 8.2% of 355 overweight children were pairwise homozygous for the MC3R Thr6Lys/Val81Ile variant. They transfected HEK293 cells with WT, Thr6Lys/Val81Ile variants together, Thr6Lys and Val81Ile variants of the *MC3R* gene were discovered from child populations of different countries. According to ligand saturation analysis, the Thr6Lys variant and Val81Ile variant of the *MC3R* gene have less ability to bind the ligand, however, the double mutant variant's ability to bind to the ligand decreased more dramatic compared to single mutant variants. The secondary messenger activation capacity of those MC3R variants was also compared using a β-galactosidase activity test in HEK293 cells. Based on their results, Thr6Lys/Val81Ile MC3R variant carrying subjects had the least ability to activate the cAMP dependent secondary messenger system. These results indicated that the combined inhibitory/loss of function mutations in the Thr6Lys/Val81Ile MC3R variant worked together and almost fully inhibited the function of MC3R (27).

Lee et al. (28) (2016) investigated C17A and G241A (Thr6Lys/Val81Ile) MC3R variant's effects on mice models for C57BL/6 mice as WT vs. human MC3R and Thr6Lys/Val81Ile MC3R variant mice. They showed that Thr6Lys/Val81Ile MC3R variant littermates had more body fat with reduced fat-free tissues, even when they were fed with normal rat chow. The mice groups were also compared for their leptin concentrations, their eating amounts and their energy expenditures in both fed and fasting states. The results indicated that Thr6Lys/Val81Ile MC3R variant littermates had higher serum leptin concentrations than the WT controls; however, there was no significant difference between meal amounts or energy expenditure between the two groups.

According to the studies above, the frequency of the Thr6Lys/Val81Ile MC3R variant in the obese population is dramatically high. Many studies have been performed in order to understand the effects of the Thr6Lys/Val81Ile MC3R variant; however, no study has been performed to understand the intracellular trafficking of the Thr6Lys/Val81Ile MC3R variant. The reason behind this situation could be the lack of specific antibodies or the unspecific binding capacity of commercial MC3R antibodies.

## MC3R Has a Role in the Control of Growth, Puberty and the Circadian Rhythm

Over the years, scientific studies have investigated MC3R and its function focusing on the phenotype and metabolic responses. Many KO rat and mice models studies were performed on the "metabolic tuner" and "redundant" MC3R activities.

Numerous phenotypic observations were recorded about MC3R loss of function variants. One of the most interesting phenotype observations was that MC3R loss of function variants cause late growth and late onset of the puberty with irregular menstrual cycles for female patients.

Yung-Seng Lee et al. (29) (2002) performed genetic analyses on a 13-year-old obese Indian girl with irregular menstrual cycle with polycystic ovary syndrome and also on her obese father. They found that the Ile183Asn variant in their MC3R sequence was associated with these phenotypes. Their results clearly showed that MC3R function regulates menstruation cycles, as well as the circadian and ultradian rhythms.

Lam et al. (30) (2021) observed MC3R loss of function variants *in vitro* in a male patient carrying the G240W MC3R variant. The patient was obese with type 2 diabetes and the late onset of puberty phenotype. They investigated MC3R impacts on puberty onset and reproductively by studying MC3R KO mice models *in vitro*. Their findings showed that both male and female mice models delayed the onset of puberty and the female models had a shorter period of ovulation cycle than their WT littermates. The G240W MC3R variant resulted in obese and late onset of puberty phenotypical expressions on a male patient as a result of being a loss of function variant since the ability of the MC3R variant to activate the secondary messenger pathway was lost or significantly reduced. Moreover, the late onset of puberty phenotype with G240W MC3R variant carrier human patients demonstrated the same phenotype as the MC3R KO models. Consistent with the early findings in animal model phenotype investigations, the obese phenotype of the MC3R loss of function variant had a low lean mass with respect to the patient's body mass index with a high body fat. In addition, for female MC3R KO mice, the length of the estrous cycle was prolonged significantly. Thus, the dramatic MC3R effects on the ultradian cycle and neuroendocrinology were validated one more time.

The prolonged and irregular estrous cycles and late onset of puberty phenotypes indicated that MC3R has a regulator role on the circadian rhythm. Sutton et al. (24) (2008) investigated food anticipatory activities (FAA) on MC3R KO models 3 hours before feeding and they showed significantly less FAA with lower X and Z movements. Then, they examined *Bmal1*, *Npas2*, and *Per2* circadian gene expression patterns on *ad libitum* and restricted feeding conditions on MC3R KO mice. Cortical neuronal mRNA expression patterns showed that *Bmal1* mRNA expression was ten-fold lower in MC3R KO mice during the peak expression vs. WT mice. Restricted feeding was also associated with marked differences in the amplitude of the circadian profile of all three genes in the cortex of MC3R KO vs. WT mice. Accordingly, it was put forward that MC3R was required for normal patterns of the clock activity in the cortex. Therefore, the molecular mechanisms of MC3R on circadian rhythm were partially revealed. However, without clear intracellular trafficking of MC3R, the molecular control mechanisms of MC3R are still not fully understood.

### MC3R Protein-protein Interactions and Intracellular Mechanisms

Recent studies have shown that GPCRs are oligomerized in the membrane, paving the way for important studies (31). MC3R and the growth hormone secretagogue receptor (GHSR)-1a belong to the GPCR family and are synthesized in neurons of the hypothalamus (32). Notably, the majority of GHSR-1a-expressing neurons coexpress the MC3R, whereas only a few MC3R-expressing neurons coexpress GHSR-1a in the ARC (31). If α-MSH binds to MC3R in the MC3R signaling pathway, the Gαs protein is activated and increases the cAMP level. Studies have revealed that the dimerization of GHSR-1a and MC3R, as well as basal activation of GHSR-1a, doubled the activity of MC3R stimulated by α-MSH, and the activity of GHSR-1a stimulated with ghrelin decreased by approximately 60%. In this case, MC3R may have different intracellular roles with its ligand-bound and non-ligand forms (31,33). In addition to this interaction, Müller et al. (34) (2016) indicated that GHSR-1a interacts with Gpr83, which regulates the signaling mechanism of GHSR-1a and MC3R heterodimer, and provides ghrelin-dependent and independent energy metabolism control. According to these studies, the molecular regulation of the MC3R signaling could be controlled by GHSR-1a and this control mechanism is dependent on the protein-protein interactions of MC3R.

Subsequently, Müller et al. (34) (2016) revealed the MC3R and MC4R interaction and its intracellular mechanism. According to their study, RING finger protein 11 (RNF11) can make a homodimer and also dimerize with MC3R and MC4R. These heterodimers restricted MC3R and MC4R activation of the secondary messenger pathway system and lowered their activity by about 40%, which may be the reason for decreased MC3R-GHSR-1a heterodimer. In addition, they also specified that the MC3R and MC4R expressions' level remained the same, independent of the RNF11 expression. However, their study lacked confocal microscopy imaging data and the internalization process, which are necessary for a complete understanding of their results. In the literature, most studies consider that MC3R and MC4R may have similar physiological effects and hence called MC3R redundant. However, contrary to what was indicated, they are very different from each other in terms of their phenotypic outcomes, such as more fat mass on HFD and more weight loss in Restriction Feeding Diet in MC3R vs. MC4R, and also the presence of late puberty and poor nutrient partitioning.

Despite MC3R protein-protein interactions, the intracellular trafficking of MC3R is poorly understood. To date, only one publication focused on WT MC3R intracellular trafficking and there are almost no publications studying the loss of function variants intracellular trafficking. The intracellular trafficking of MC3R study published by Wachira et al. (35) in 2007 claimed that MC3R localizes lipid raft regions and endocytic internalization occurs in the presence of γ-MSH. These results are highlighted to understand MC3R intracellular trafficking. However, as a result of the usage of non-specific MC3R antibodies and the lack of quantitative data of the study, MC3R intracellular trafficking is still an unresolved concept and studies with more specific labeling methods are required in order to illuminate MC3R intracellular trafficking.

## Conclusion

The GPCR family member of MC3R is responsible for body weight and appetite control and is often mentioned as "redundant". However, recent studies have revealed that MC3R has crucial functions such as regulating hunger, appetite and body weight. The regulations of MC3R on weight control mechanisms also imply that nutrient partitioning mechanisms are affected by MC3R signaling. In addition, growth and puberty onset are also affected and controlled by the MC3R signaling pathway according to patient and mice model studies. Moreover, protein interaction studies have shown that MC3R interacts with GHSR-1a and crucially controls the secondary messenger pathway signaling of MC3R. Surprisingly, the intracellular trafficking of MC3R has not been fully or reliably revealed because of unreliable methodologies which utilize non-specific commercial antibodies. In order to illuminate the intracellular trafficking of MC3R, more specific and reliable approaches should be used. Fully revealing MC3R intracellular trafficking will open up new areas to develop therapeutic approaches in order to treat obesity.

### Ethics

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Concept: Tulin Yanik, Seyda Tugce Durhan, Design: Tulin Yanik, Seyda Tugce Durhan, Literature Search: Tulin Yanik, Seyda Tugce Durhan, Writing: Tulin Yanik, Seyda Tugce Durhan.

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# Factors Affecting Thyroid Elastography in Healthy Children and Patients with Hashimoto's Thyroiditis

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### What is already known on this topic?

Thyroid elastography is used to distinguish cases with Hashimoto's thyroiditis (HT) from healthy controls in childhood. Although, the relationship between the scores obtained by the shear-wave elastography (SWE) technique and the stage of the disease was investigated, the clinical and biochemical characteristics of the cases at the time of admission were not specifically examined.

### What this study adds?

Our results show that no metabolic factor other than body mass index (BMI) standard deviation score (SDS) has any effect on SWE scores, especially in healthy children. There is a positive correlation between BMI SDS and SWE in healthy children, but not in those patients with HT. Likewise, age is another factor affecting SWE only in healthy children. Since inflammation is the main factor determining thyroid elasticity in patients with HT, the effect of other factors such as age and BMI on SWE seems to be insignificant. We do not recommend routine evaluation of any laboratory parameter other than thyroid functions before thyroid elastography.

## Abstract

**Objective:** Hashimoto's thyroiditis (HT) is the most common form of thyroiditis in childhood. In addition to thyroid ultrasonography, shear-wave elastography (SWE) can evaluate thyroid parenchyma tissue stiffness, and more detailed findings can be obtained with this method. We aimed to evaluate the relationship between SWE values and clinical, biochemical and hormonal parameters of patients with HT and in healthy individuals.

**Methods:** We compared 46 newly diagnosed HT cases with 46 healthy controls. We examined the effect of all metabolic parameters and thyroid-related markers on SWE values.

**Results:** The mean SWE values in those patients with euthyroid HT were $12.5 \pm 5.1$ kilopascal (kPa), whereas it was $8.2 \pm 2.82$ kPa in healthy controls ($p < 0.001$). Although the clinical [age, gender and body mass index (BMI)] and laboratory parameters (such as thyroid function tests, homeostasis model assessment of insulin resistance, insulin-like growth factor-1 values, which we think may affect SWE scores) of those children with HT and the healthy controls were statistically similar ($p > 0.05$), except for their thyroid autoantibodies and thyroglobulin, SWE values and thyroid volume were significantly higher in those individuals with HT ($p < 0.001$). Multiple linear regression analysis was performed to evaluate the direction and degree of the effect of the variables on thyroid elasticity scores. It was observed that age ($p = 0.002$), BMI standard deviation score (SDS) ($p = 0.04$) and anti-thyroid peroxidase ($p = 0.008$) levels were effective on the thyroid elasticity score in the regression model. We detected a SWE cut-off value of 9.68 kPa with 68% sensitivity and 72% specificity, a 70% positive predictive value, and a 69% negative predictive value in thyroid elastography when differentiating between cases with HT and healthy controls.

**Conclusion:** Our results show that no metabolic factor other than BMI SDS has any effect on SWE scores, especially in healthy children. There was a positive correlation between BMI SDS and SWE in healthy children ($r = 0.353$; $p = 0.02$), but not in those patients with HT ($r = 0.196$; $p = 0.19$). Likewise, age is another factor affecting SWE only in healthy children. We do not recommend routine evaluation of any laboratory parameters other than thyroid functions before thyroid elastography.

**Keywords:** Children, Hashimoto's thyroiditis, shear-wave elastography, thyroid

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## Introduction

Hashimoto's thyroiditis (HT) is the most common form of thyroiditis in childhood and the most commonly acquired cause of childhood thyroid disease in regions without iodine deficiency of the world (1). The prevalence of HT is around 1-2 % in childhood (2). It has been observed that its frequency increases with increasing age, reaching around 10-20 % in adulthood. Thyroid antibody positivity can be detected up to 4 times more in women than in men (1,2). Pathologically specific findings are variable degree lymphocytic infiltration and interstitial fibrosis. Ultrasonography has been used for years in the treatment and follow-up of thyroid diseases, with evaluations such as thyroid volume and parenchyma, nodule pseudonodule discrimination, and elasticity. It is possible to evaluate findings, such as fibrosis, nodule and pseudonodule with routine B-mode ultrasonography. In recent years, thyroid parenchymal elasticity has been evaluated in more detail with the shear-wave elastography (SWE) technique (3,4). Although, heterogeneous hypoechoic echogenicity and lobulated contour are specific gray-scale findings of ultrasonography, the sensitivity of these findings is also limited. The SWE technique measures the degree of thyroid parenchymal stiffness and calculates these data as elasticity, i.e. kilopascal (kPa), and velocity, i.e. meters/second (m/s) (5). It is a real-time, non-invasive method which provides quantitative evaluation in this way. There are studies in the literature on the use of the SWE technique in children with HT. In recent years, the use of SWE in children has increased, both in the differentiation of diffuse thyroid diseases and in the differentiation of nodules from benign to malignant (6,7,8,9). In a study investigating the role of the American College of Radiology Thyroid Imaging Reporting and Data System Classification and SWE in differentiating the nature of nodules, it was observed that SWE was more diagnostically accurate in all nodule sizes. However, it was also found that the detection rate decreased as the nodule size increased (10).

Compared to healthy controls, SWE values were higher in those patients with HT. In addition, while comparing the activity and degree of the disease in patients with HT, there are also studies showing that there are significant differences in hypothyroidism, hyperthyroidism and euthyroid (8,11). In these studies, thyroid-specific parameters such as serum thyroid hormones, thyroglobulin (Tg) and thyroid autoantibodies were evaluated in terms of their relationship with SWE. However, the relationship between thyroid and systemic metabolic parameters is undeniable. For example, solid organ stiffness can be affected by serum insulin-like growth factor-1 (IGF-1), which was demonstrated in a study in cases with acromegaly (12). It is known that elasticity scores change in relation to hyperplasia and the fibrosity of the thyroid parenchyma tissue. Hyperinsulinemia and laboratory findings related to insulin resistance are observed in those individuals known to have thyroid nodules. It has also been reported that there is a positive correlation between nodule size and homeostasis model assessment of insulin resistance (HOMA-IR) levels (13). Thyroid stimulating hormone (TSH) and insulin increase the thyroid volume and the formation of hyperplastic thyroid nodules, and there are reports that local growth factors such as IGF-1 are effective independently of TSH. For example, it has been suggested that increased intrathyroid IGF-1 levels in patients with acromegaly have an effect on nodule formation (14). We hypothesized, just as thyroid hormones affect some changes in metabolism, some metabolic markers can affect some changes in thyroid tissue. The number of studies comparing individuals with HT and the healthy groups by means of thyroid elastography is insufficient in the literature. There was a study in Turkey in which shear wave velocity scores were compared. In that study, thyroid gland stiffness was observed more prominently in those patients with HT (15). However, as in the related study, a comparison of the two groups with the aim of being similar in terms of all factors which may affect elastography was not observed in the literature.

Our main purpose in this study was to reveal the underlying reasons why the elasticity cut-off results are very different from one another in the literature, and also to present a new cut-off value to the literature by comparing the elasticity scores of two groups which are very similar in clinical and laboratory terms. We aimed to investigate the relationship with SWE values and anthropometric measurements, and laboratory markers in healthy volunteers and HT cases. In the literature, we could not find any study comparing the SWE scores between the groups in terms of age, gender, puberty stage, thyroid function tests, and glucose and lipid metabolism. Therefore, we considered whether these factors could determine the differences between SWE cut-off values between healthy individuals and individuals with HT. To the best of our knowledge, this is the first study to examine all factors which may affect SWE scores. Considering these factors, we wanted to find an answer to the question of whether a new cut-off value related to SWE scores should be determined.

## Methods

### Participants

All individuals participating in this study were evaluated in the pediatric endocrinology outpatient clinic of University of Health Sciences Turkey, Kayseri City Hospital in 2022.

Among the patients who were referred due to abnormal thyroid function tests (mild TSH elevation, mild fT4 elevation or decrease), thyroid antibody positivity was detected and newly diagnosed patients with HT constituted the case group, and those patients who were found to be within the reference range of thyroid function tests in 2 consecutive check-ups within 3 months during the follow-up and whose thyroid antibodies were found to be negative constituted the control group. The inclusion criteria for this study were determined as not using any medication within the last 3 months, not having a concomitant systemic disease (for example, diabetes mellitus) and being younger than 18 years of age. The exclusion criteria were having any systemic or thyroid disease, and receiving treatment for any condition. The anthropometric measurements and laboratory examinations of all participants were recorded. Thyroid ultrasonography and sonoelastography evaluation were performed by a pediatric radiologist with 12 years of experience. This study was approved by University of Health Sciences Turkey, Kayseri City Hospital Clinical Research Ethics Committee with the number of 591 (date: 24.02.2022). Written informed consent forms were obtained from the legal guardians of all participants. Research ethical principles were conducted in accordance with the Declaration of Helsinki. Sample size calculation was performed using the G*Power version 3.1.9.2 (Kiel University, Kiel, Germany) software program. The sample size was calculated to be 42 patients for each group, with 95% power, 5% significance level, and an effect size value of 0.80. Ninety-two patients were included in this study due to the possibility of patients dropping out. In this way, the actual power of the study was determined to be 0.95.

### Anthropometric Examinations

As an anthropometric evaluation, weight, height, body mass index (BMI) values and age and gender-specific standard deviation (SD) scores (SDSs) of these values were analyzed with an online calculation program (www.childmetrics.org) (16). Age and sex-specific reference values were evaluated according to CDC data.

### Laboratory Measurements

Blood samples were obtained in the early morning after a 10-hour fasting period for biochemical and hormone measurements. Serum glucose, insulin, triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), TSH, free T4 (fT4), free T3, thyroglobulin (Tg), anti-thyroid peroxidase (anti-TPO), anti-Tg, IGF-1 and IGF-binding protein 3 (IGFBP3) levels were measured by the electrochemiluminescence immunological method on the Cobas 8000 e602 analyser (Roche Diagnostics, Mannheim, Germany). The HOMA-IR method [glucose (mmol/L) x insulin (mIU/mL) / 22.5] was used to assess insulin resistance (17). IGF-1 SDS and IGFBP3 SDS were calculated according to age and gender specific reference data (18).

### Thyroid Ultrasonography and Elastography

An Aplio 500 ultrasound system (Toshiba Medical Systems, Tokyo, Japan) with a 14 MHz linear array was used for ultrasonography and 2D SWE examinations of the thyroid gland. Thyroid volume was calculated from the dimensions of each thyroid lobe (length × width × depth × 0.52).

A pediatric radiologists (with 12 years of experience) performed the ultrasound and 2D SWE. At least ten measurements with a round-shaped region of interest (ROI) 2 mm in diameter were obtained from three different sections (upper, middle, and lower parts) of each lobe in a transverse plane. The one-shot method was used and all measurements were recorded as kPa. The average values of the measurements were accepted as the mean stiffness value of gland. Calculations of the mean stiffness value (with SDs) of the thyroid lobes using round ROIs are shown in Figures 1A, 1B and 2A, 2B. SWE scores for each lobe were evaluated for a maximum of 2 minutes.

In both groups, thyroid volumes were evaluated according to age and gender-specific references and SDSs were calculated (19).

### Statistical Analysis

Statistical analyses were calculated with the Statistical Package for the Social Sciences version 24.0 (IBM Corporation, Armonk, NY, USA) software program. The mean and SD values of the numerical variables, the frequency and percentage (%) values of the categorical variables were examined. The Shapiro-Wilk test was used to analyze the normal distribution of variables. In addition, it was accepted that the data with kurtosis and skewness values in the range of -2 to +2 showed a normal distribution. When the means of two independent variables were compared, the Student's t-test was used if the data provided the parametric assumptions, and the Mann-Whitney U test was used if they did not. The chi-squared test was used in the analysis of categorical variables. Pearson and Spearman correlation analyses were used to evaluate the degree and direction of the relationships between variables. The best cut-off value which could be used to differentiate those children with euthyroid HT and healthy controls was calculated by receiver operating characteristic curve analysis. In order to evaluate the direction and degree of the effects of independent variables on thyroid elasticity scores, SDSs were used

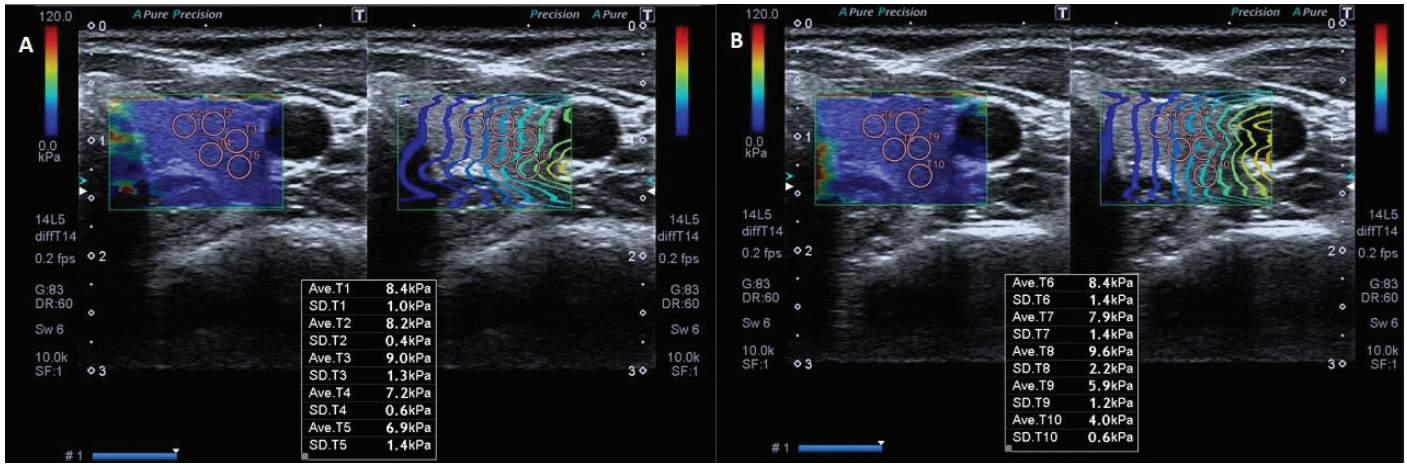


**Figure 1.** A, B) Calculations of the mean stiffness value (with standard deviations) of the left thyroid lobe using round region of interest in a 9-year-old female control

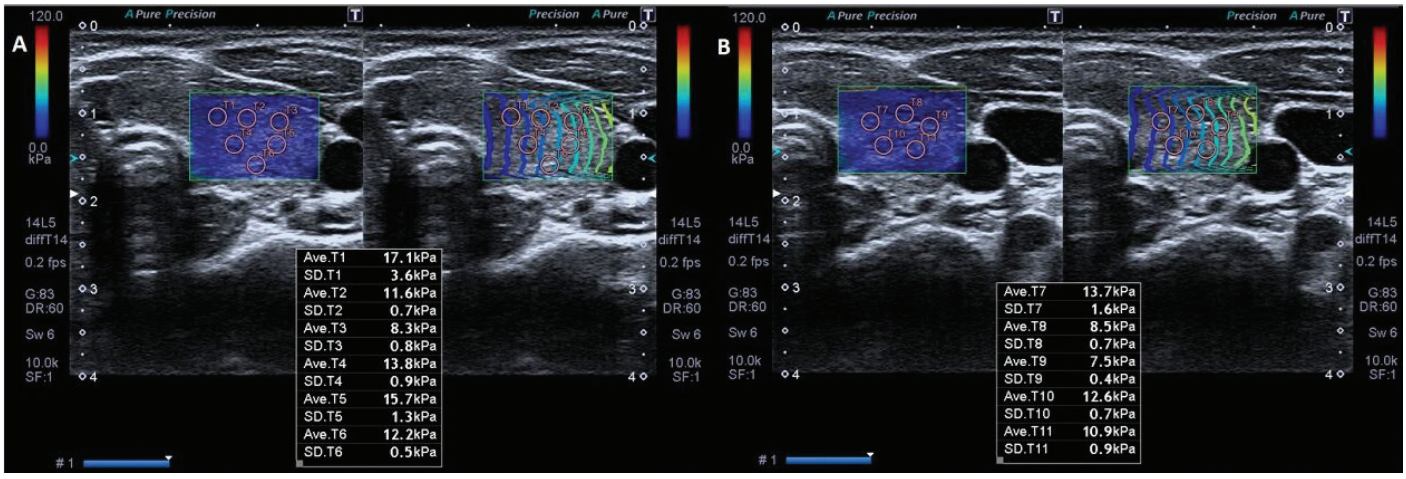


**Figure 2.** A, B) Calculations of the mean stiffness value (with standard deviations) of the left thyroid lobe using round region of interest in an 8-year-old male patient

instead of numerical values from those variables which change with age or sex (for example, IGF-1 SDS instead of IGF-1, BMI SDS instead of body weight and height, HOMA-IR instead of glucose and insulin) in the model and multiple linear regression analysis was performed. A p value less than 0.05 was considered statistically significant.

## Results

A total of 92 children (between the ages of 5.2-17.8 years), 58 (63%) girls and 34 (37%) boys, were included in this study. Those with positive thyroid antibodies (newly diagnosed HT) constituted the case group (46 patient), and those with negative thyroid antibodies formed the healthy control group (46 healthy control). All participants came from the central Anatolian region of Turkey, mostly from the city where we conducted this research. The age, gender, anthropometric, laboratory and radiological measurements of both groups are summarized in Table 1. The average values of the SWE measurements were accepted as the mean stiffness value of the gland. Similar to previous studies, we found that the normal thyroid parenchyma appeared homogeneous. Low elasticity was mapped as blue, and autoimmune thyroiditis appeared heterogeneous with yellow and red areas scattered among the blue (6,20). We took ten measurements of each lobe, and the mean values were calculated for each lobe and for both lobes. Evaluation was carried out in a similar way to the measurement methods recommended in studies in the literature (20,21). Puberty stages were compared according to gender in both groups as they may also affect IGF-1 levels. Girls were compared in the patient and control groups according to their puberty stage. There was no statistical difference in chi-squared analysis ($p = 0.93$). Boys were also compared according to their puberty stage. No statistical difference was found in boys either ($p = 0.23$). When the group with HT and the healthy controls were compared

according to their puberty stages, no significant difference was found again ($p = 0.30$).

Although the clinical (age, gender and BMI) and laboratory parameters (such as thyroid function tests, HOMA-IR, IGF-1 values, which we thought may affect SWE scores) of those children with HT and the healthy controls were statistically similar ($p > 0.05$), except for thyroid autoantibodies and Tg, SWE values and thyroid volume were significantly higher in those individuals with HT ($p < 0.001$). In addition, there were no statistically significant differences between the groups in terms of metabolic markers (such as glucose, insulin, lipids) ($p > 0.05$) (Table 1).

Those patients with HT with normal thyroid function test results (31 euthyroid patient) and healthy controls (46 controls) were also compared. The SWE scores of the patients with euthyroid HT were found to be higher than the healthy controls ($12.5 \pm 5.1$ kPa versus $8.2 \pm 2.8$ kPa, $p < 0.001$).

When the presentation of the patients with HT were examined, 3 of them were hypothyroidism, 3 patient were hyperthyroidism, and 8 patient were mildly elevated TSH (TSH range 5.5-20 mIU/mL) with euthyroidism. When the mean SWE values were examined, although the number of cases were few, the mean SWE values of those cases presenting with hypothyroidism and markedly elevated TSH (TSH > 40 mIU/mL) were significantly higher ($24.8 \pm 6.7$ kPa, versus $8.2 \pm 2.8$ kPa, $p < 0.001$). The mean elasticity scores of those children with hyperthyroidism were high but not statistically different from those of the healthy children ($12 \pm 7.5$ kPa, versus $8.2 \pm 2.8$ kPa, $p = 0.05$).

**Table 1. Anthropometric, laboratory and radiological parameters of the participants**

| | Hashimoto's thyroiditis (mean ± SD) or (median, Q1-Q3) | Healthy controls (mean ± SD) or (median, Q1-Q3) | p value |
|---|---|---|---|
| Age (years) | 13 ± 3.7 | 12.3 ± 3.7 | 0.39 |
| Gender (girl/boy) | 31 (67.4%) / 15(32.6%) | 27 (58.7%) / 19 (41.3%) | $0.39^{\chi^2}$ |
| Weight (kg) | 49.4 ± 19 | 49.5 ± 21 | 0.98 |
| Height (cm) | 151.4 ± 18 | 148.3 ± 18.6 | 0.41 |
| BMI (kg/m$^2$) | 20.2 ± 5.7 | 21.3 ± 5.5 | 0.33 |
| Weight SDS | 0.28 ± 1.18 | 0.47 ± 1.26 | 0.47 |
| Height SDS | 0.08 (-0.60 – 0.45) | 0.04 (-0.96 – 0.81) | $0.94^{\alpha}$ |
| BMI SDS | 0.59 (-0.64 – 1.26) | 0.38 (-0.37 – 1.62) | $0.37^{\alpha}$ |
| FPG (mg/dL) | 85.1 ± 6.2 | 85.2 ± 6.7 | 0.94 |
| Insulin (mIU/mL) | 12.2 ± 7.7 | 13.6 ± 7.4 | 0.38 |
| HOMA-IR | 2.60 ± 1.76 | 2.90 ± 1.64 | 0.40 |
| Triglyceride (mg/dL) | 88 (73 – 115) | 102 (68 – 121) | $0.14^{\alpha}$ |
| Total cholesterol (mg/dL) | 143.7 ± 25.7 | 153.1 ± 26.9 | 0.09 |
| LDL-C (mg/dL) | 85.3 ± 19.5 | 92.6 ± 23.2 | 0.11 |
| HDL-C (mg/dL) | 44 (39 – 57) | 50 (42 – 59) | $0.24^{\alpha}$ |
| TSH (mIU/mL) | 3.31 (2 – 7.08) | 2.56 (1.72 – 4.63) | $0.10^{\alpha}$ |
| Free-T4 (ng/dL) | 12.9 (11.8 – 14.5) | 12.9 (11.7 – 14.4) | $0.96^{\alpha}$ |
| Free-T3 (ng/dL) | 3.99 ± 0.80 | 3.98 ± 0.68 | 0.95 |
| Tg (mg/dL) | 1.20 (0.3 – 16.5) | 13 (7.8 – 25) | **$< 0.001^{\alpha}$** |
| Anti-TPO (U/L) | 187.1 ± 182.8 | 8.3 ± 3.3 | **< 0.001** |
| Anti-Tg (U/L) | 261 (104 – 430) | 15 (13 – 17) | **$< 0.001^{\alpha}$** |
| IGF-1 (ng/mL) | 292 ± 128.6 | 285.7 ± 137.7 | 0.82 |
| IGF-1 SDS | 0.27 ± 1.24 | 0.56 ± 1.31 | 0.27 |
| IGFBP3 (ng/mL) | 5,148.7 ± 1,260.7 | 5,376.1 ± 1,436.7 | 0.43 |
| IGFBP3 SDS | 0.06 ± 0.80 | 0.38 ± 0.70 | 0.05 |
| Thyroid volume (cm$^3$) | 12.02 ± 6.37 | 6.71 ± 3.32 | **< 0.001** |
| Thyroid volume SDS | 3.27 (1.43 – 5.11) | 0.73 (-0.23 – 1.73) | **$< 0.001^{\alpha}$** |
| Mean SWE value (kPa) | 12.94 ± 6.01 | 8.23 ± 2.82 | **< 0.001** |

Data with normal distribution evaluated with Student's t-test. $^{\alpha}$Symbol indicates the Mann-Whitney U test was used. $^{\chi^2}$: chi-squared test.
BMI: body mass index, SDS: standard deviation (SD) score, FPG: fasting plasma glucose, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, Anti-TPO: anti-thyroid peroxidase, Anti-Tg: anti-thyroglobulin, IGF-1: insulin like growth factor-1, IGFBP3: insulin-like growth factor-binding protein 3, SWE: shear wave elastography, kPa: kilopascal, TSH: thyroid stimulating hormone

**Table 2. Relationship of factors affecting SWE values**

| | Whole group | | Hashimoto's thyroiditis group | | Control group | |
|---|---|---|---|---|---|---|
| | r | p value | r | p value | r | p value |
| Age | 0.276 | **0.008** | 0.156 | 0.30 | 0.555 | **< 0.001** |
| Weight | 0.266 | **0.01** | 0.250 | 0.09 | 0.469 | **0.001** |
| Height | 0.227 | **0.03** | 0.141 | 0.35 | 0.402 | **0.006** |
| BMI | 0.153 | 0.15 | 0.122 | 0.42 | 0.491 | **0.001** |
| Weight SDS | 0.088 | 0.40 | 0.117 | 0.44 | 0.212 | 0.16 |
| Height SDS | -0.026 | 0.80* | 0.063 | 0.68* | -0.138 | 0.36 |
| BMI SDS | 0.228 | **0.03*** | 0.196 | 0.19* | 0.353 | **0.02*** |
| FPG | -0.111 | 0.30 | -0.162 | 0.29 | -0.069 | 0.66 |
| Insulin | 0.123 | 0.25 | 0.155 | 0.31 | 0.292 | 0.06 |
| HOMA-IR | 0.096 | 0.37 | 0.120 | 0.43 | 0.259 | 0.09 |
| Triglyceride | 0.181 | 0.09* | 0.395 | **0.007*** | 0.031 | 0.84 |
| Total cholesterol | 0.056 | 0.61 | 0.284 | 0.06 | -0.067 | 0.67 |
| LDL-C | 0.063 | 0.56 | 0.315 | **0.04** | -0.070 | 0.65 |
| HDL-C | -0.230 | **0.03*** | -0.149 | 0.33 | -0.086 | 0.58* |
| TSH | 0.121 | 0.25* | 0.115 | 0.45* | -0.084 | 0.58 |
| Free-T4 | -0.236 | **0.02*** | -0.433 | **0.003*** | -0.060 | 0.69 |
| Free-T3 | -0.202 | 0.06 | -0.262 | 0.08 | -0.171 | 0.27 |
| Tg | -0.255 | **0.01*** | 0.183 | 0.23* | -0.380 | **0.009*** |
| Anti-TPO | 0.354 | **0.001** | 0.142 | 0.35 | 0.098 | 0.52* |
| Anti-Tg | 0.427 | **< 0.001*** | 0.005 | 0.98* | 0.220 | 0.14* |
| IGF-1 | 0.279 | **0.008** | 0.305 | **0.04** | 0.341 | **0.02** |
| IGF-1 SDS | 0.056 | 0.60 | 0.226 | 0.14 | -0.052 | 0.74 |
| IGFBP3 | 0.179 | 0.09 | 0.215 | 0.16 | 0.340 | **0.02** |
| IGFBP3 SDS | -0.017 | 0.87 | 0.118 | 0.44 | 0.025 | 0.87 |
| Thyroid volume | 0.648 | **< 0.001** | 0.542 | **< 0.001** | 0.606 | **< 0.001** |
| Thyroid volume SDS | 0.702 | **< 0.001*** | 0.572 | **< 0.001** | 0.484 | **0.001** |

Pearson correlation analysis was used to examine the relationship between variables. *Indicates Spearman correlation analysis was applied.
BMI: body mass index, SDS: standard deviation score, FPG: fasting plasma glucose, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, Anti-TPO: anti-thyroid peroxidase, Anti-Tg: anti-thyroglobulin, IGF-1: insulin like growth factor-1, IGFBP3: insulin-like growth factor-binding protein 3, TSH: thyroid stimulating hormone

When the relationship between the mean SWE values and other factors were examined; while age, weight, height, BMI SDS, IGF-1 level, thyroid autoantibodies, thyroid volume and thyroid volume SDS were positively correlated, HDL-C, fT4 and Tg were negatively correlated in the whole group. When only those cases with HT were evaluated, the mean SWE scores and triglyceride, LDL-C, IGF-1, thyroid volume and thyroid volume SDS were positively correlated, while fT4 was negatively correlated. The relationships between the mean SWE values and independent variables in the whole group, only in cases with HT and the healthy controls are shown in Table 2.

Multiple linear regression analysis was performed to evaluate the direction and degree of the effects of the variables on the thyroid elasticity scores, since the relationship between SWE scores and variables differed in healthy controls and patients with HT. Age and sex specific SDSs were included in the model as parameters which could change with age. When the independent factors were included in the regression model based on the multicollinearity relationship, it was observed that age, BMI SDS and anti-TPO levels were effective on the thyroid elasticity score in the regression model. The results of the multiple linear regression analysis are shown in Table 3.

We detected a SWE cut-off value of 9.68 kPa with 68% sensitivity and 72% specificity, a 70% positive predictive value, and a 69% negative predictive value in thyroid elastography in order to differentiate cases with HT from healthy controls (Figure 3). The maximum area under curves for mean kPa value was 0.754 (95% confidence interval: 0.65-0.85; $p < 0.001$).

**Table 3. The results of multiple linear regression analysis of variables**

| | Unstandardized coefficients | | Standardized coefficients | | 95% confidence interval for B | | p value |
|---|---|---|---|---|---|---|---|
| | B | Standard error | β | t | Lower limit | Upper limit | |
| Constant | 2.213 | 4.244 | | 0.521 | -6.279 | 10.706 | 0.60 |
| Age | 0.037 | 0.011 | 0.413 | 3.318 | 0.015 | 0.059 | **0.002** |
| BMI SDS | 0.273 | 0.133 | 0.207 | 2.049 | 0.006 | 0.539 | **0.04** |
| HOMA-IR | 0.146 | 0.250 | 0.063 | 0.585 | -0.354 | 0.646 | 0.56 |
| LDL-C | 0.024 | 0.018 | 0.130 | 1.313 | -0.013 | 0.060 | 0.19 |
| HDL-C | -0.046 | 0.027 | -0.177 | -1.711 | -0.099 | 0.008 | 0.09 |
| Triglyceride | 0.004 | 0.009 | 0.046 | 0.429 | -0.015 | 0.023 | 0.67 |
| TSH | 0.231 | 0.275 | 0.088 | 0.838 | -0.320 | 0.781 | 0.41 |
| Free-T4 | -0.004 | 0.190 | -0.002 | -0.020 | -0.384 | 0.377 | 0.98 |
| Tg | -0.023 | 0.029 | -0.093 | -0.806 | -0.080 | 0.034 | 0.42 |
| Anti-TPO | 0.009 | 0.003 | 0.289 | 2.741 | 0.002 | 0.015 | **0.008** |
| Anti-Tg | 0.000 | 0.001 | 0.060 | 0.579 | -0.001 | 0.002 | 0.57 |
| IGF-1 SDS | -0.261 | 0.326 | -0.084 | -0.800 | -0.913 | 0.391 | 0.43 |

As a result of the analysis, it was found that a significant regression model, $F(12, 59) = 4.56$, $p < 0.001$ and 37% of the variance in the dependent variable ($R^2_{adjusted} = 0.37$) was explained by the independent variables.
BMI: body mass index, SDS: standard deviation score, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, Anti-TPO: anti-thyroid peroxidase, Anti-Tg: anti-thyroglobulin, IGF-1: insulin like growth factor-1, TSH: thyroid stimulating hormone

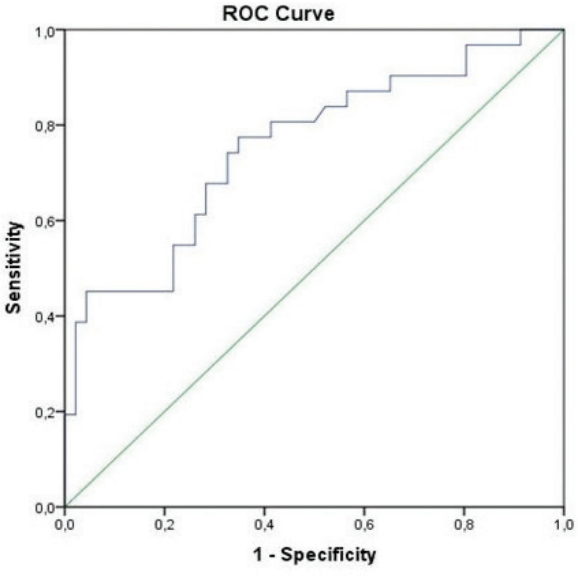


**Figure 3.** ROC curve analysis of the SWE values of euthyroid HT and the best cut-off value for elasticity (kPa)

*ROC: receiver operating characteristic, SWE: shear-wave elastography, HT: Hashimoto's thyroiditis, kPa: kilopascal*

## Discussion

In this study, the degree of thyroid tissue stiffness was evaluated with SWE in both newly diagnosed and untreated patients with HT and in healthy children. In our study, we found a lower cut-off value in differentiating healthy individuals from individuals with HT in terms of SWE scores. In addition, we found that age, BMI SDS and anti-TPO had a positive and significant effect on thyroid elastography scores by means of multiple linear regression analysis.

Thyroid elastography will provide a more comprehensive evaluation of inflammation and fibrosis in the thyroid gland, and it may become more common in the future in the follow-up and treatment of those patients with HT (8). In another study in which HT was classified according to fibrosis in gray-scale ultrasound, the SWE cut-off value with the highest sensitivity was determined to be 12.3 kPa (8). Similar to previous studies, those patients with hypothyroidism had higher elasticity scores (11). SWE values increased as the stage of the disease increased on ultrasound. However, one of the limitations mentioned in that study was that the clinical and biochemical characteristics of the cases diagnosed with HT were not specified. Similarly, in our study, while the mean SWE values in those patients with HT were $12.94 \pm 6.01$ kPa, it was $8.23 \pm 2.82$ kPa in the healthy controls. In this study, we compared two groups with statistically similar clinically, metabolically and laboratory parameters and also examined the relationship of all factors on SWE values. Furthermore, a positive and significant correlation was found between anti-TPO and SWE scores when considering the whole group. Although there are studies with similar results (11), a significant relationship was found with anti-TPO in another study, and it was not found with anti-Tg (8). The positive correlation between SWE scores and age has been previously reported in the literature (11,22). Parameters such as body weight, height,

and BMI increase with age. In addition, as age increases, IGF-1 levels increase with puberty. These variables can be considered in terms of their relationship with age. In the multiple linear regression analysis, we included the SDSs of those parameters which could change with age or gender, independent of the effect of the age factor. In our study, unlike the literature, we found a positive correlation between elasticity scores and BMI SDS. This is striking in terms of the effects of obesity and metabolic syndrome on thyroid elasticity. These data suggest that SWE is a significant but not robust diagnostic test for HT. For example, according to the data of this study, the SWE value of an older, obese (but otherwise healthy) child may be similar to that of a younger, lean child with new-onset HT.

The main factor affecting elasticity scores may be the stage of thyroiditis. In addition, the elasticity scores were found to be significantly higher in those individuals with hypothyroidism in the literature (11). Furthermore, it was observed that the elasticity scores of those who received levothyroxine sodium treatment did not statistically significantly differ from those who did not receive treatment, and the duration of treatment was not effective either (20). These studies show us that receiving treatment for hypothyroidism does not improve thyroiditis in terms of having any effect on SWE scores. In addition, more comprehensive results can be obtained by evaluating the effect of BMI change on SWE scores in individuals with HT in the long term.

In some previous studies in the literature, values such as 12.8 kPa and 12.3 kPa were given as kPa cut-off values for the HT discriminative SWE value. However, some of the main limitations of these studies can be expressed as the fact that the hormonal profiles at the time of application were not compared, that they were not matched for age or gender, or that there were no biochemically and hormonally similar groups (8,23).

In studies conducted with adults, the SWE cut-off values were found to be higher. This situation can be explained by the effects of other factors affecting SWE scores, as we mentioned earlier in our study (6,21,24). We detected a SWE cut-off value of 9.68 kPa with 68% sensitivity and 72% specificity. However, as we know, there are publications stating that thyroid auto-antibody positivity may not be detected in individuals with autoimmune thyroiditis who are not in the hypothyroid stage (25). This condition is referred to as seronegative autoimmune thyroiditis. In these individuals, similar imaging findings observed in chronic autoimmune thyroiditis are detected in conventional ultrasonography. In addition, in recent years, it has been reported that the rate of thyroid autoantibody positivity increases with an increase in TSH measurements together with ultra-sensitive TSH measurements (25,26,27). It has been reported that SWE may also be useful in the differential diagnosis between various types of thyroiditis (28). The fact that our study was conducted in the pediatric age group and it was conducted among similar groups in terms of metabolic factors and factors related to thyroid function tests are the distinguishing features of our study.

### Study Limitations

One of the limitations of our study was the relatively small number of patients and controls in the study group, and secondly, the stages of the cases with HT were not classified according to B-mode ultrasonography.

## Conclusion

SWE scores may be affected by some parameters and if an SWE cut-off value is to be determined between those patients with HT and healthy controls, it should be noted that these factors should be similar between the groups. In our study, we found a lower cut-off value in differentiating healthy individuals from those individuals with HT in terms of SWE scores. As SWE can be a helpful tool in the diagnosis, we recommend that it be used in the follow-up of patients with HT in relation to their thyroiditis stage. Our study shows that although thyroid elastography is not superior to conventional ultrasonography and thyroid auto-antibodies measurements in distinguishing between individuals with HT and healthy individuals, it can be used in the follow-up of the prognosis of the disease as it is useful in evaluating the stage of fibrosis. We think that the lower cut-off value found in our study regarding the elasticity scores in those individuals with HT is due to the evaluation of newly diagnosed individuals and the fact that the inflammation period was still in the early stage. Our results showed that no metabolic factor other than BMI SDS had an effect on SWE scores, especially in healthy children. There was a positive correlation between BMI SDS and SWE in healthy children ($r = 0.353$; $p = 0.02$), but not in those patients with HT ($r = 0.196$; $p = 0.19$). Likewise, age is another factor affecting SWE only in healthy children. Since inflammation is the main factor determining thyroid elasticity in those patients with HT, the effects of other factors such as age and BMI on SWE seems to be insignificant. We do not recommend routine evaluation of any laboratory parameters other than thyroid functions before thyroid elastography.

### Ethics

**Ethics Committee Approval:** This study was approved by University of Health Sciences Turkey, Kayseri City Hospital Clinical Research Ethics Committee with the number of 591 (date: 24.02.2022).

**Informed Consent:** Written informed consent forms were obtained from the legal guardians of all participants.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Serkan Bilge Koca, Concept: Serkan Bilge Koca, Turgut Seber, Design: Serkan Bilge Koca, Turgut Seber, Data Collection or Processing: Serkan Bilge Koca, Turgut Seber, Analysis or Interpretation: Serkan Bilge Koca, Turgut Seber, Literature Search: Serkan Bilge Koca, Writing: Serkan Bilge Koca.

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# Endocrine Abnormalities and Growth Characterization in Colombian Pediatric Patients with 22q11 Deletion Syndrome

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### What is already known on this topic?

It is known that different endocrinopathies occur in 22q11 deletion syndrome, the most common of which are hypoparathyroidism and hypothyroidism. The intervention of a multidisciplinary team is also highlighted within the follow-up of the patients, including the follow-up of their growth pattern, which can be altered in a multifactorial way.

### What this study adds?

This study reinforce the importance of follow-up according to the guidelines and trying to detect endocrinopathies early, such as hypoparathyroidism, which can be detected late and with serious presentations such as convulsive episodes.

## Abstract

**Objective:** Several endocrine manifestations have been described in patients with 22q11 deletion syndrome, including growth retardation, hypoparathyroidism, and thyroid disorders. This study aimed to characterize these abnormalities in a Colombian retrospective cohort of children with this condition.

**Methods:** A retrospective study comprising a cohort of children with 22q11 deletion syndrome in Medellín, Colombia followed up between 2011 and 2017 was conducted.

**Results:** Thirty-seven patients with a confirmed diagnosis of 22q11 deletion syndrome were included. 37.8% had some endocrinopathy, the most frequent being hypoparathyroidism (21.6%), followed by hypothyroidism (13.5%), hyperthyroidism (2.7%) and growth hormone deficiency (2.7%). There was wide heterogeneity in the clinical presentation, with late onset of severe hypocalcemia associated with seizure or precipitated in postoperative cardiac surgery, which highlights the importance of continuous follow-up as indicated by the guidelines. Short stature was mainly related to nutritional factors. Growth monitoring is required with the use of syndrome-specific charts and careful monitoring of the growth rate.

**Conclusion:** As previously reported, a significant proportion of patients with endocrine abnormalities were found in this cohort. This highlights that it is essential to carry out an adequate multidisciplinary follow-up, based on the specific clinical guidelines, in order to avoid serious complications such as convulsions due to hypocalcemia. It is important to track size with curves specific to the syndrome and analyze the growth rate.

**Keywords:** 22q11 deletion syndrome, DiGeorge syndrome, hypoparathyroidism, hypothyroidism, growth disorders, endocrine system diseases

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## Introduction

The 22q11 deletion syndrome, also known as DiGeorge syndrome (DGS), corresponds to the classic triad of thymus aplasia, hypoparathyroidism, and congenital heart disease (1). It has its origin in an alteration of the embryological development of the third and fourth pharyngeal arcs, which is due to a deletion in the chromosome region 22q11, with a size between 0.7 and 3 million base pairs. The variable phenotypic expression of 22q11 deletion syndrome includes, in addition to DGS, other previously known syndromes such as velocardiofacial (VCFS), Cayler, Opitz G/BBB, and facial conotruncal anomaly (CTAFS) (1,2,3). With an estimated prevalence of 1 per 4,000 live newborns and is considered the most common microdeletion syndrome. In addition, it is the main cause of palatal abnormalities of syndromic origin and the second most common cause of congenital heart disease and neurodevelopmental delay. There is no predominance by gender, but it is more prevalent in Hispanics, and in 90-95% of cases, it is due to *de novo* deletions (1,2,3).

The clinical spectrum of this condition is highly variable and includes congenital heart disease, recurrent infections, nasal regurgitation, nasal voice, hypocalcemia, feeding problems, developmental delay mainly on language, laryngotrachoesophageal abnormalities, renal abnormalities, hypothyroidism, low stature, vertebral abnormalities, polydactyly, scoliosis, thrombocytopenia, microcephaly, seizures and hypotonia, and additionally, a history of intrauterine growth restriction is common (1,2,3,4).

Taking into account the diversity of conditions and diseases presented by these patients, in 2011, Bassett and collaborators developed the "practical guidelines for the management of patients with 22q11 deletion syndrome", which stress a required multidisciplinary follow-up and highlight an active search for various conditions, including endocrinopathies (3). Within this last group, hypoparathyroidism, followed by hypothyroidism, short stature, and less frequently hyperthyroidism and growth hormone (GH) deficiency stand out in decreasing order of frequency (1,5).

Hypoparathyroidism occurs frequently with hypocalcemia, usually in the neonatal period; however, cases have been reported of patients with no evidence of hypoparathyroidism in the first years of life, who later may develop hypocalcemia in times of stress in the context of acute diseases, surgeries, or the physiologically difficult periods of adolescence and pregnancy. Additionally, descriptions of *de novo* diagnoses of hypoparathyroidism have been made in adolescent or adult patients who have consulted for episodes of severe hypocalcemia (2,5,6). The frequency of hypocalcemia varies widely depending on the selection criteria applied; an even lower prevalence of hypocalcemia has been reported in patients for whom calcium is not routinely measured, which shows that the wide discrepancy of prevalence between the different reports may be influenced by these selection and follow-up differences. For DGS, the hypocalcemia prevalence is 69-72% (7,8), for VCFS, it is 13-22% (9,10,11), for CTAFS, it is 10% (12), and for 22q11 deletion syndrome, the reported prevalence is 49-60% (13,14).

In 22q11 deletion syndrome, primary hypothyroidism is also present due to a defect in the development of the pharyngeal arches and to the greater frequency of autoimmune alterations. The reported prevalence varies according to the name given to the syndrome: for 22q11 deletion syndrome, it is 0.7-9.5% (14,15), and the presence of Graves' disease has also less frequently been reported, with a prevalence of 1.8% (1,5,15).

Patients with 22q11 deletion syndrome may have short stature and constitutional growth retardation; this is closely related to low weight due to nutritional problems and heart disease (1). However, the probability of GH deficiency, which has also been associated with short stature in these patients, should not be underestimated (2,5,16,17). In VCFS, a prevalence of short stature of 39-41% and constitutional growth and development delay of 30% have been reported (11,18,19). For 22q11 deletion syndrome, the prevalence of short stature is 36-41% and GH deficit is 4% (14,20,21). These differences in the growth of these patients have led to the development of growth curves specific for this condition, which allow early and timely detection of patients who are growing more slowly than is expected for their condition and thus prioritize the search for other causes which may explain this alteration (22,23,24). In addition, follow-up on these specific curves could avoid a possible over-diagnosis of growth retardation in these patients, which could occur if this follow-up is performed with growth patterns for the general population. Unfortunately, there is a lack of broad knowledge of such growth curves in pediatric clinical practice, and we do not have a growth curve of this type developed for the local population, nor validation of those previously stated in our environment, which can lead to diagnostic errors at the time of the interpretation of the growth of these patients.

Endocrinopathies associated with 22q11 deletion syndrome show wide variability in their prevalence, depending on the populations studied. Levy-Shraga et al. (25) described a cohort of 48 patients in Israel with 22q11 deletion syndrome, in whom only 27% had hypoparathyroidism and

10.4% were suffering from hypothyroidism, and in terms of the auxological evaluation, these patients showed a delayed growth pattern, which placed them in the low normal range for height, according to world growth standards. On the regional level, in Latin America, studies by Fomin et al. (26) and Del Carmen Montes et al. (27), which correspond to clinical characterization, stand out. Specifically, in Brazil, 14 patients were described, among whom 35.8% had hypoparathyroidism and 7.1% hypothyroidism. Del Carmen Montes et al. (27) in Argentina found that out of 32 patients with 22q11 deletion, only 3.1% had hypoparathyroidism, without any other endocrinopathies.

On the local level, we do not have clinical characterization studies of endocrinopathies and other comorbidities in patients with 22q11 deletion syndrome. In addition, since the frequency of endocrinopathies reported differ according to the population studied, we cannot extrapolate these data to our population. Therefore, this study aimed to characterize the different endocrinopathies related to 22q11 deletion syndrome, including auxological evaluation in a group of patients who attended the Hospital Universitario de San Vicente Fundación in the city of Medellín during the period from January, 2011 to December, 2017.

## Methods

### Study Setting and Participants

This study was conducted at the Hospital Universitario de San Vicente Fundación, in the city of Medellín, Colombia. For this study, a retrospective review of the medical records of patients treated in the pediatric endocrinology division was performed during the period from January, 2011 to December, 2017. The sample size was determined by convenience sampling. Patients younger than 18 years who had the International Classification of Diseases-10 (ICD-10) code "D821" (DGS) in their medical charts were included. Those patients who had a fluorescent in situ hybridization (FISH) test negative for 22q11 deletion or whose results were not available in the medical charts were excluded. Each of the medical records was reviewed by two of the study researchers in order to decide on the case's entry into this study. For those cases included, the largest amount of information available was collected, taking the first clinical record as the first assessment and the subsequent information as follow-up measurements.

This study was approved by the Ethics Committee of the Hospital Universitario de San Vicente Fundación (no: 11-2018, date: 13.04.2018) and was carried out in compliance with the standards of resolution 8430 of 1993 of the Ministry of Health of the Republic of Colombia, which regulates research with human subjects in the country, and in adherence to the ethical principles set out in the Helsinki Declaration. Confidentiality regarding the identity of patients was maintained throughout this investigation.

### Definitions

The diagnosis of each endocrinopathy was based on the data reported in the medical records, the pathological history or upon the evaluation of the relevant laboratory tests. The auxological evaluation was carried out with weight and height data taken from the clinical history, the body mass index (BMI) was calculated, and the standard deviation was determined for each of these values according to World Health Organization (WHO) growth curves (28).

The diagnosis of hypoparathyroidism was established with decreased serum calcium levels for age, associated with decreased or inappropriately normal parathyroid hormone (PTH) levels accompanied or unaccompanied by hyperphosphatemia or based on physician-based diagnosis written in the medical record. Hypothyroidism was diagnosed by elevated thyroid stimulating hormone (TSH) levels with or without decreased free T4 levels, and hyperthyroidism was defined as the presence of decreased TSH levels with elevated T3 and/or T4 levels. The diagnosis of GH deficit considered the presence of short stature accompanied by low growth rate, decreased serum levels of insulin-like growth factor-1 (IGF-1), and two suboptimal GH challenges (29,30,31). Short stature was defined as a z-score below -2 standard deviations; low weight was catalogued in children under 5 years at a z-score for the weight-to-size ratio of -2 standard deviations and for those over 5 years, a z-score for BMI lower than -2 standard deviations.

### Statistical Analysis

A descriptive analysis was performed using the software Statistical Package for the Social Sciences (version 20 for Mac) and Epidat 4.1. The quantitative variables and their results are presented as mean and standard deviation in cases of normal distribution; otherwise, they are summarized as medians and interquartile intervals. Qualitative variables are shown as frequency and proportions. In addition, an analysis was performed among the subgroups of the patients with short height and normal height, comparing clinical characteristics, and a chi-squared test was applied with a level of statistical significance of $p < 0.05$.

## Results

### Demographics and Clinical Characteristics of the Patients

A total of 125 records of patients diagnosed with DGS were obtained using the ICD-10 code D821, of which 47 patients

were excluded because they had a negative FISH for 22q11 deletion and 41 patients who had not yet been molecularly evaluated during follow-up. Finally, after review, 37 patients met the selection criteria, of which 20 were female (54%) and 17 were male (46%) (Figure 1).

Table 1 summarizes the demographic and clinical characteristics of the patients included; of these, 14 patients (37.8%) had some endocrine abnormality. The most frequent endocrinopathy was hypoparathyroidism, followed by hypothyroidism and, at a lower frequency, hyperthyroidism, GH deficiency, and precocious puberty. Additionally, other characteristics of these patients were reported, such as the presence of heart disease, immunodeficiency, feeding problems, and/or perinatal history.

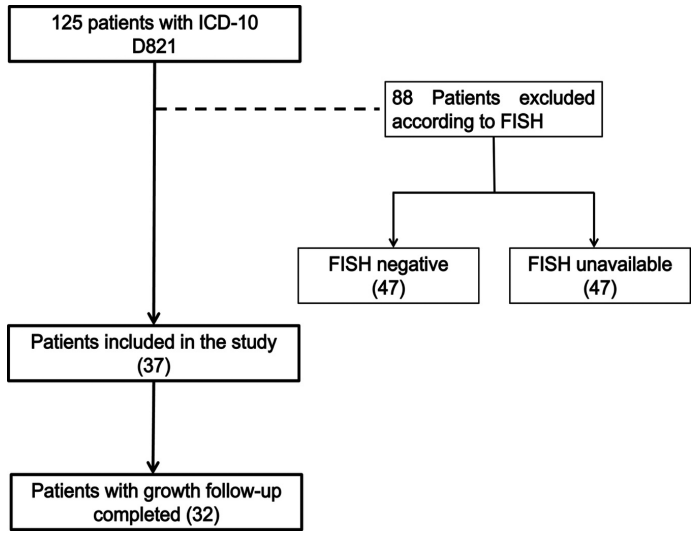


**Figure 1.** Flow diagram for inclusion of patients. There were 125 registries of patients with International Classification of Diseases-10 D821 diagnosis, of which 47 patients were excluded because they had a negative fluorescent in situ hybridization study for 22q11 deletion and 41 patients who did not have a molecular diagnostic study during follow-up. Thirty-seven patients met the selection criteria and of these, in 32 cases, the information was available for auxological follow-up

*ICD-10: International Classification of Diseases-10, FISH: fluorescent in situ hybridization*

## Endocrine Abnormalities

Hypoparathyroidism was the most common endocrine disorder, which was diagnosed in 21.6% of the study subjects. Among the patients with reported data from phosphocalcic metabolism tests at diagnosis, initial PTH levels were found ranging between 6.1 and 53.1 pg/mL (local reference level 10-65 pg/mL), and all of them had low calcium levels, between 7.6 and 8.2 mg/dL (local reference level 8.7-10.1 ng/dL). Some of these PTH values were inappropriately normal in the setting of hypocalcemia. Specifically, in three

**Table 1. General characteristics of the 22q11 deletion syndrome patients included in this study**

| Characteristics | All patients (37) n (%) |
|---|---|
| Male | 17 (46) |
| Female | 20 (54) |
| Age (years) at diagnosis (median, IQR) | 5.33 (0.66-10) |
| Age (years) at first assessment (median, IQR) | 3.25 (1.08-8.2) |
| Age (years) at last assessment (median, IQR) | 6.54 (4.47-9.81) |
| **Clinical data** | |
| Total of patients with endocrine abnormalities | 14 (37.8) |
| Hypoparathyroidism | 8 (21.6) |
| Hypothyroidism | 5 (13.5) |
| Hyperthyroidism | 1 (2.7) |
| GH deficiency | 1 (2.7) |
| Precocious puberty | 1 (2.7) |
| Congenital cardiopathy | 28 (75.6) |
| Immunodeficiency | 4 (10.8) |
| Feeding difficulties | 12 (32.4) |
| **Perinatal history** | |
| Prematurity | 8 (22.2) |
| Low birthweight | 9 (25) |
| Birthweight, g (median, IQR) | 2,700 (2,300-3,000) |
| Length at birth, cm (median, IQR) | 47 (44.75-49) |

IQR: interquartile range, GH: growth hormone
Data are shown as absolute frequencies plus percentages for categorical variables and as median plus interquartile ranges for quantitative traits

of the patients, the diagnosis was reached early, during the first year of life, and one of them during hospitalization for the correction of heart disease at 3 months of age. Two of the patients were diagnosed during adolescence; one of them presented with a seizure secondary to hypocalcemia.

In this cohort, hypothyroidism was the second most frequent endocrine defect, affecting 13.5% of the population studied. All the cases were determined to be acquired hypothyroidism, and of these, it was only possible to demonstrate autoimmunity with positive antibodies in one of the cases. Baseline TSH levels ranging from 6.23 to 14.1 mIU/L were found among patients with reported thyroid test data, but complete free T4 (fT4) data was not found at diagnosis. During follow-up, compensation of thyroid function was achieved.

Within the data of the hormonal studies carried out, it was found that one patient presented with a diagnosis of GH deficiency, having two subnormal challenges with peaks of 2.82 ng/mL and 6.3 ng/mL, through a stimulus test with clonidine and L-DOPA, respectively. The somatomedin C (IGF-1) value was 90.2 ng/mL [-1.1 standard deviation (SD)]. This patient had a growth rate below the 3rd percentile (3.9 cm/year), in the context of poor weight gain associated with loss of appetite and congenital heart disease (atrial and interventricular septal defect plus aortic coarctation). Additionally, this diagnosis was reached during the last visit of this patient and, at this point, GH treatment was not considered.

### Growth Characterization

Regarding the auxological evaluation, according to WHO curves in the first assessment, the average z-score for height was -2.36 SD, for weight, it was -1.89 SD, and for BMI, it was -0.46 SD. In this first assessment, 24 patients (64.8%) were classified as short stature. In those under 5 years of age, 8 patients (38%) had low weight, while in those over 5 years of age, no patient had a BMI less than -2 SD. We had follow-up information from 32 patients, with an average time of 29 months. In the last assessment, the average z-score for height was -1.81 SD, for weight, it was -1.59 SD, and for BMI, it was -0.67. At this last assessment, 19 patients (59.3%) had short stature. In those under 5 years of age, 3 patients (33.3%) were underweight, and in those over 5 years of age, 4 patients (16.6%) had BMI less than -2 SD. The differences between the measurements of the first and the last assessment are also shown (Table 2). In the 24 patients with short stature, a z-score of -3.2 SD was found at the first visit, and it was -2.42 SD for the last evaluation, indicating an improvement of 0.78 SD. The subgroup of 8 underweight patients who had a z-score of -2.73 SD at the first visit was subsequently scored at -3.28 SD at the last evaluation, and so worsening by 0.55 SD.

Additionally, those patients with short stature in the first assessment were compared with those with normal height, and it was found that there was a higher proportion of underweight in those with short stature (33% vs. 0%; $p = 0.01$). It was also observed that there was a higher frequency of endocrinopathies in the group of patients with normal height, as well as a higher frequency of heart disease and feeding problems in those patients with short stature, but these differences were not statistically significant (Table 3).

## Discussion

To the best of our knowledge, this study is the most extensive clinical characterization of patients with 22q11 deletion syndrome reported in Colombia. Although the focus was on endocrinological findings and growth characterization, the frequencies of other conditions typical of the syndrome are also described, such as heart disease which was present in 75.6% of patients, feeding disorders in 32.4% of patients and immunodeficiency in 10.8% of cases, all with prevalences similar to those previously reported in different cohorts (32,33,34). Regarding the endocrinological findings, 37.4% of the patients had some type of endocrine

**Table 2. Growth characterization of the 22q11 deletion syndrome patients included in this study**

| | First visit (n = 37) | | Last visit (n = 32) | |
|---|---|---|---|---|
| Height z-score (SD) | -2.36 (1.46) | | -1.81 (1.25) | |
| Weight z-score (SD) | -1.89 (2.06) | | -1.59 (1.76) | |
| BMI z-score (SD) | -0.46 (1.76) | | -0.67 (1.94) | |
| Short stature (%) | 24 (64.8) | | 18 (56.2) | |
| Underweight | < 5 years old (n = 21) | > 5 years old (n = 16) | < 5 years old (n = 9) | > 5 years old (n = 23) |
| Low weight/height n (%) | 8 (38.09) | | 3 (33.3) | |
| Low BMI n (%) | | 0 (0) | | 4 (16.6) |

SD: standard deviation, BMI: body mass index, WHO: World Health Organization
Data are shown as absolute z-score with its respective standard deviation for anthropometric measurements and as frequencies plus percentages for weight classifications. In order to carry out a more objective progression evaluation, the follow-up was carried out using the WHO growth curves

**Table 3. Comparison of clinical characteristics between subgroups with short height and normal height**

| | Short stature (n = 24) | Normal height (n = 13) | p |
|---|---|---|---|
| M:F | 12:12 | 5:8 | 0.37 |
| **Endocrinopathy, n (%)** | 7 (29.1) | 7 (53.8) | 0.13 |
| **Hypoparathyroidism, n (%)** | 5 (20.8) | 3 (23) | 0.59 |
| **Hypothyroidism, n (%)** | 1 (4.1) | 4 (30.7) | **0.04** |
| **Hyperthyroidism, n (%)** | 0 | 1 (7.6) | 0.35 |
| **GH deficit, n (%)** | 1 (4.1) | 0 | 0.64 |
| **Precocious puberty, n (%)** | 0 | 1 (7.6) | 0.35 |
| **Underweight n (%)** | 8 (33.3) | 0 | **0.01** |
| **Cardiopathy, n (%)** | 20 (83.3) | 8 (61.5) | 0.14 |
| **Immunodeficiency, n (%)** | 3 (12.5) | 1 (7.6) | 0.55 |
| **Feeding disorders, n (%)** | 8 (33.3) | 4 (30.7) | 0.58 |

Data are shown as frequencies plus percentages. The comparisons were carried out using the chi-squared statistic.
M:F male female ratio, GH: growth hormone

alteration; of these, hypoparathyroidism was the most frequent condition, followed by thyroid disorders, GH deficit, and precocious puberty.

Hypoparathyroidism was identified in 8/37 (21.6%) patients with 22q11 deletion syndrome. It should be noted that one of the patients was diagnosed with hypoparathyroidism when debuting with a seizure secondary to hypocalcemia in adolescence, and another presented with hypocalcemia during hospitalization to correct his congenital heart disease. These two cases exemplify the heterogeneity in age and clinical presentation of hypoparathyroidism, so routine follow-up of phosphocalcic metabolism is essential in these patients (2,6,25), especially considering the high risk of presentation with severe forms of hypocalcemia, such as seizures (35). In general terms, the detected prevalence of hypoparathyroidism is in an intermediate range of those previously reported in Latin America, with 3.1% in Argentina (27) and 35.8% in Brazil (26), and very similar to a previous report made in Israel (25).

Different thyroid disorders have been described in patients with 22q11 deletion syndrome. They can be explained by developmental defects of the pharyngeal arches, morphological alterations of the gland (36), and the greater frequency of autoimmune alterations which these patients develop (37). In line with this, our study identified a prevalence of hypothyroidism of 13.5% (5/37), while one patient (2.7%) presented hyperthyroidism, findings similar to those reported by Shugar et al. (15), who documented a 9.5% prevalence of thyroid disorders in 169 patients with 22q11 deletion syndrome, with 7.7% hypothyroidism and 1.8% hyperthyroidism, while they are higher than the rate of thyroid disorders noted by Choi et al. (38) at 3.2% in 61 patients. GH deficit and precocious puberty were each documented in one patient. These findings are consistent with previous anecdotal reports of these conditions in patients with 22q11 deletion syndrome (21,39), and so reaffirms their low prevalence.

We examined the growth of the patients by comparing the auxological parameters recorded during the follow-up visits of approximately 29 months, which were available in 32 out of 37 patients. For this evaluation, WHO growth curves were preferred over those previously published, as they allow for an arithmetic monitoring of growth progression with exact values which facilitate the calculations of deltas. With this approach, it was documented that 89.1% of the patients had negative z-scores for height at their first assessment, compared with 100% described in other cohorts (25). Although follow-up was not possible for the entire sample, at the last evaluation, it was estimated that 87% of the patients had negative z-scores for height. Although there is no significant difference between these two data, it is known that the growth of these patients has a delayed pattern with height, which initially results in negative z-scores, but that in the end, they can approach normal height (5,22,25,26).

When we analyzed the auxological evaluation in detail, 64.8% of the patients had short stature at the initial assessment, which improved over time with an average change of 0.55 standard deviations, and at the last visit, only 56.2% of the patients were of short stature. It is plausible that on continuing with an adequate follow-up of this cohort, their height would continue to improve, and that possibly a good percentage of these patients would not present with a final short stature (5,22,24,25). It is to be noted that of 24 patients with initial short stature, 16 continued with short stature at their last assessment, and 4 presented with improvements in their z-score, so that they were no longer classified as being of short stature. These 4 patients had in common that they did not have any eating disorder, which

further supports the explanation that the affectation of height can be secondary to other factors, such as nutritional factors. It was also seen that 3 patients who previously were classified as normal height with decreased z-scores at their last assessment were classified as short stature, without it being possible to identify any factors related to this finding.

In the subgroup of patients with short stature, when charted on growth curves specific to the syndrome, the prevalence of short height decreased to 16.2% (only 6 patients). This highlights the importance of auxological follow-up of these patients and shows how the frequency of height alterations can vary widely according to the standard used. This proper evaluation, accompanied by a good clinical follow-up of the rest of the auxological parameters, can reduce unnecessary evaluations of these patients (22,24). When comparing subgroups between those patients with short stature and those with normal height according to the WHO curves, at the first assessment, those with short stature were found to have a greater frequency of heart disease and/or feeding problems; in 7 patients, these two conditions occurred simultaneously. In addition, all patients with low weight had associated short stature. Interestingly, the frequency of endocrinopathies was lower in the group of patients with short stature. This is consistent with the fact that the compromise of height of these patients was not exclusively related to endocrine alterations as with feeding problems, low weight, or the presence of heart disease (5,22).

### Study Limitations

As a weakness of this study, it should be considered that this was retrospective and it was based on a review of medical records, so our analysis of these patients depended on the adequate recording of data by the physician who performed the clinical evaluation, as well as the complete recording of some perinatal or personal history data. In addition, the population was not captive, so the multidisciplinary follow-up required by these patients was not performed exclusively in our institution. This may partly explain why the laboratory follow-up reports were not consistent.

## Conclusion

In summary, our results confirm that 22q11 deletion syndrome shows a varied clinical presentation with frequencies of endocrinopathies different from those previously reported in the literature, which is consistent with the known heterogeneity of this syndrome among different populations. The compromise of height of these patients seems to be transitory. In general, improvement during the follow-up was seen in a good percentage of the patients and this was mainly related to nutritional factors. The number of patients who are actually short for age decreases almost by a factor of 3 when using syndrome specific curves for this diagnosis, which shows the importance of follow-up on the growth curves. In addition, it is evident that the auxological follow-up of these patients with the analysis of growth rate is of great importance.

Our results emphasize the importance of adequate multidisciplinary follow-up, based on specific clinical guidelines, in order to avoid complications related to the late diagnosis of any endocrinopathies, as in the case of hypocalcemia due to hypoparathyroidism. Specific studies related to this topic are required with larger sample sizes and preferably prospective evaluation, which will lead to a more accurate knowledge of the clinical conditions of those patients with 22q11 deletion syndrome at a local level.

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### Ethics

**Ethics Committee Approval:** This study was approved by the Ethics Committee of the Hospital Universitario de San Vicente Fundación (no: 11-2018, date: 13.04.2018).

**Informed Consent:** Patient consent was waived due to the retrospective nature of this study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

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# Anomalies in Human Sex Determination: Usefulness of a Combined Cytogenetic Approach to Characterize an Additional Case with Xp Functional Disomy Associated with 46,XY Gonadal Dysgenesis

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### What is already known on this topic?

Xp-Yp translocation, t(X;Y)(p21;p11.3), is a rarely occurring rearrangement resulting in pure functional disomy of Xp, including the dosage sensitive sex (DSS) reversal region and is associated with 46,XY gonadal dysgenesis (GD).

### What this study adds?

We report the fourth case of Xp;Yp translocation with Xp21.2-pter duplication associated with XY GD. Molecular cytogenetic methods are still relevant for the characterization of the exact chromosomal mechanism responsible for severe clinical features including DSD at an early age. This may contribute to understanding the possible genetic cause of syndromic 46,XY DSD cases and provide special and personalized support for these cases.

## Abstract

**Objective:** Disorders of sexual development (DSD) are a heterogeneous group of genital defects affecting chromosomal, gonadal and anatomical sex. 46,XY DSD is a subset of DSD which covers a wide range of phenotypes in which 46,XY gonadal dysgenesis (GD) is the most severe form. In this study, we report on the clinical and molecular cytogenetic findings of a study on a Tunisian girl with the syndromic form of 46,XY DSD.

**Methods:** This case was a phenotypic female patient having several congenital anomalies including growth retardation. Karyotype, fluorescence in situ hybridization and array Comparative Genome Hybridization (array CGH) were performed.

**Results:** The proband exhibited a de-novo 46,X,der(Y) karyotype. Array CGH revealed a pathogenic 27.5Mb gain of an Xp21.2 chromosome segment leading to Xp functional disomy. No deletion was observed in the Y-chromosome. The duplicated region encompassed the NR0B1 (DAX1) and MAGEB genes, located within the dosage sensitive sex (DSS) reversal locus, known as promote genes responsible

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for human sex reversal and testis repression. The extra-dosage and interactions of these genes with different specific genes could result in the impairment of the male sex pathway. Over-dosage of KAL1 and IL1RAPL1 genes fall within the somatic features observed in the patient.

**Conclusion:** To the best of our knowledge, we report on the fourth case of Xp21.2-pter duplication within Xp;Yp translocation associated with XY GD. Our findings suggest that when duplicated, the NR0B1 and MAGEB genes could be a major cause of XY GD. Therefore, we emphasize the usefulness of a combined cytogenetic approach in order to provide an accurate genetic diagnosis for those patients having syndromic XY DSD in a clinical setting.

**Keywords:** Disorders of sexual development, dosage sensitive sex reversal locus, functional disomy Xp, 46,XY gonadal dysgenesis

## Introduction

The type of the gonad of an individual is usually a testis or ovary governed by sex chromosomes. In 46,XY individuals, the presence of the Y chromosome testis-determining gene *SRY* (OMIM#601947) initiates the formation of the testis and inhibits the formation of the ovary. DSD are a set of rare congenital conditions in which the chromosomal, phenotypic, and anatomical sex are discordant and they can occur in isolated or syndromic forms (1).

The subset 46,XY DSD, which includes gonadal dysgenesis (GD), disorders of androgens synthesis or action, or disorders of anti-Müllerian hormone (AMH) synthesis or action in XY GD, is the most prevalent etiology of this condition. It is characterized by an abnormal formation of the testis due to chromosomal imbalances or mutations involving key genes implicated in the formation of the gonad (2).

The emergence of next-generation sequencing technology has allowed for the identification of the genetic etiologies in 50% of DSD cases (3,4). Yet, we cannot ignore the role of banding and molecular cytogenetic techniques in the diagnosis of DSD in which the etiology has been determined in 20% of cases (5).

Remarkably, a large proportion of DSD is caused by copy number variation (CNV) involving critical dosage-sensitive genes with a large spectrum of gonadal phenotypes.

Duplications of chromosomal regions containing Xp21, also termed Xp functional disomy, are known to cause syndromic 46,XY DSD and all reported patients presented with sex reversal as part of a complex phenotype which includes dysmorphic features and/or mental retardation (6,7). Notably, der(Y)t(X;Y)(p21.1;p11.3) is a rarely occurring rearrangement in which the translocation of the duplicated Xp segment to the Y chromosome results in a pure functional disomy of the Xp encompassing the dosage sensitive sex (DSS) locus.

Only three cases with der(Y)t(X;Y)(p21.2;p11.3) have been reported as being raised as females even with the presence of an intact Y chromosome and *SRY* gene (6,7,8).

The duplicated DSS locus contains the melanoma antigen, Family B (*MAGEB)* genes and the nuclear receptor subfamily 0, Group B *NR0B1* gene, the most probable causes of XY GD, if overexpressed (6,7,8,9).

In this paper, we report on an additional case with syndromic 46,XY GD due to Xp functional disomy within Xp;Yp translocation. We underline the complementarity between the different cytogenetic techniques to characterize the duplication and translocation events and their contribution to the management of XY GD cases. Therefore, the Comparative Genome Hybridization (CGH) array can be considered as an efficient tool for the diagnosis of chromosomal aberrations, when investigating syndromic forms of DSD.

## Methods

### Clinical Presentation of the Patient

The patient was a seven-month-old girl with dysmorphic features and profound failure to thrive.

She was referred to our department for genetic diagnosis. Written approval was obtained from the patient's parents in order to perform genetic analyses and complementary studies, as well as to publish this data.

The Local Ethics Board of the University Teaching Hospital Farhat Hached approved the present study (no: IRB00008931, date: 15.03.2022), written consent was taken from the parents for photo publication and consent for the genetic analysis and publication of the case were obtained from the parents.

### Peripheral Blood Karyotype

Reverse Heat Giemsa banded karyotype was performed on the metaphase chromosome preparations obtained from peripheral blood lymphocytes of both the patient and her parents according to standard protocols (450-550 band level). Metaphase chromosome spreads were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Cell cultures were incubated for 72 hours. A minimum of 20 R-banded metaphase chromosomes were

analyzed using Cytovision® Karyotyping software version 4.0. Karyotypes were classified according to the International System of Human Cytogenomic Nomenclature (2020) (10).

### FISH Analysis

Fluorescence *in situ* hybridization (FISH) was carried out on metaphase chromosomes of the patient according to the standard protocol, using commercial probes (Kreatech Diagnostics): Whole chromosomes painting (WCPX,WCPY), centromeric probe for chromosome X (CENX), SHOX (Xp22.33 and Yp11.2), SRY (Yp11.3), XIST probes (Xq13.2) and STS (Xp22.31) were used as telomeric probes. Bac clone RP11-89I17 (*NR0B1* gene), RP11-147O4 (*KAL1* gene) and RP11-639O (primary Pseudoautosomal region) were also used. Probes were applied to metaphase slides and co-denaturized for 7 mins at 75 °C. After 24 hours of hybridization at 37 °C and washing, the chromosomes were counterstained with a 4.6 diamino-2-phenylindole and observed using an Axioskop Zeiss® fluorescent microscope. Images were captured with a CCD camera (Cytovision, Applied Imaging®).

### Array CGH

CGH 4x44K micro-array was performed using the agilent platform as previously described (11,12). Agilent® oligonucleotide array was performed according to the manufacturer's instructions (Agilent Human Genome CGH Microarray kit 44K®).

### Statistical Analysis

Percentile study rank level was used to generate the following: baby girl growth chart, infant boy growth chart, height, weight, body mass index and cranial perimeter (https://www.childgrowthcalculator.com/#grafica_longitud).

Percentiles are given according to the World Health Organization data by comparing the growth chart of our patient with most of the children at her age.

## Results

### Clinical Report

The investigated case was the first child of an apparently healthy consanguineous Tunisian couple (second-degree relatives). Her birth weight was 2.350 kg (percentile = 1.8). Her height was 49 cm (percentile = 46), and her head circumference was 33 cm (percentile = 21.2). At the age of seven months, she was referred to our department for exploration of dysmorphic features associated with profound failure to thrive. The anthropometric measurements were below the 3rd percentile. The child's length percentile was 1.4; and her weight and cranial perimeter percentiles were 0.3. She had craniofacial dysmorphic features including long face, exophthalmos, hypertelorism, ogival palate, a relatively short and flat philtrum and strabismus (Figure 1). Moreover, she had exhibited a remarkable weight stagnation since the age of 2 months with marked hypotonia. The family history was unremarkable.

At the age of 11 months, she continued to have mild generalized hypotonia, and was still unable to hold her head up. She had severely retarded psychomotor development. Her anthropometric measurements continued to be below the 3rd percentile. An abdominal ultrasound study was performed and revealed small kidneys, invisible uterine or ovarian structure. Supplementary investigations with pelvic magnetic resonance imaging (MRI) showed an absence of internal female organs.

An echocardiogram showed an inter-atrial communication (IAC) heart defect. When last assessed at the age of 16 months, she was still unable to sit independently with a marked axial hypotonia and nystagmus. Anthropometric measurements continued to be below the 3rd percentile, and the IAC diameter increased (Figure 2).

At the age of eight years, the patient still had severe growth delay and was operated on for a percutaneous closure of IAC. At this age, her hormonal profile was as follows:

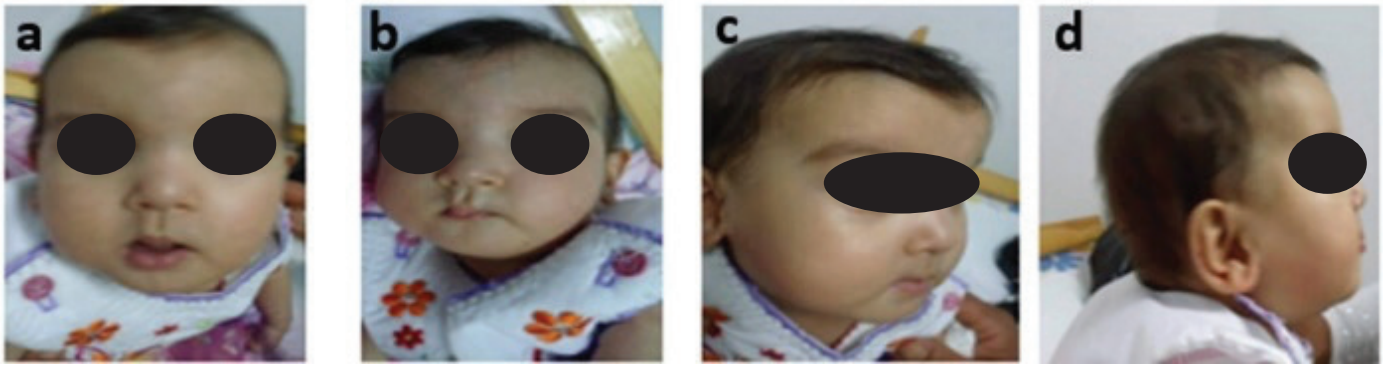


**Figure 1.** Photographs of face (a, b) and profile picture (c, d) of the patient at 7 months

**Figure 2.** Photographs of face of the patient at 16 months

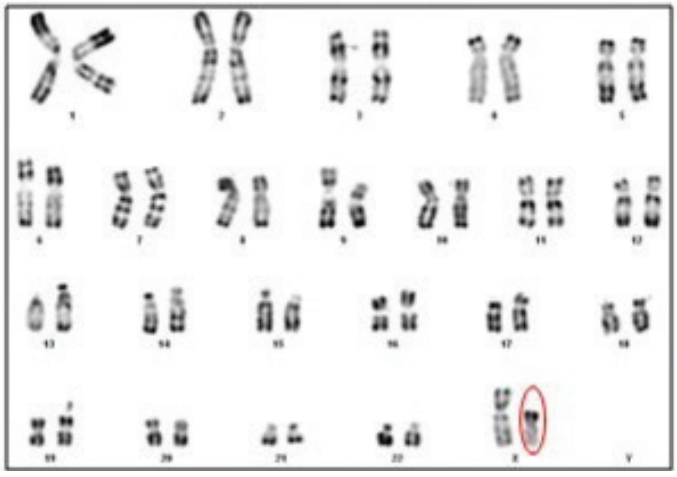

**Figure 3.** RHG banded-karyotype showing a marker chromosome (red circle)

luteinizing hormone (0.1 IU/L; reference range: 1.1-10), follicle-stimulating hormone (1.2 IU/L; reference range: 1.3-11), AMH (0.67 ng/µL; reference range: 1.43-11.6) and estradiol (10 pg/mL; reference range: < 30).

## Genetic Results

Cytogenetic analysis revealed a 46,X,der(Y) karyotype in all metaphase cells from the proband (Figure 3). The parent's karyotypes were normal (data not shown).

The extra material was a *de novo* rearrangement and was identified as a der(Y). FISH using whole-chromosome X and Y painting probes showed labeling along the entire length of the normal X chromosome and on the terminal segment of the short arm of the der(Y) chromosome (Figure 4D).

FISH analysis with specific loci probes showed the presence of the *SRY* gene on the short arm of the Y chromosome and the presence of the *SHOX* gene on each of the sex chromosomes. The XIST probe was present on the long arm of the X chromosome (Figure 4A, 4B, 4C). Specific locus

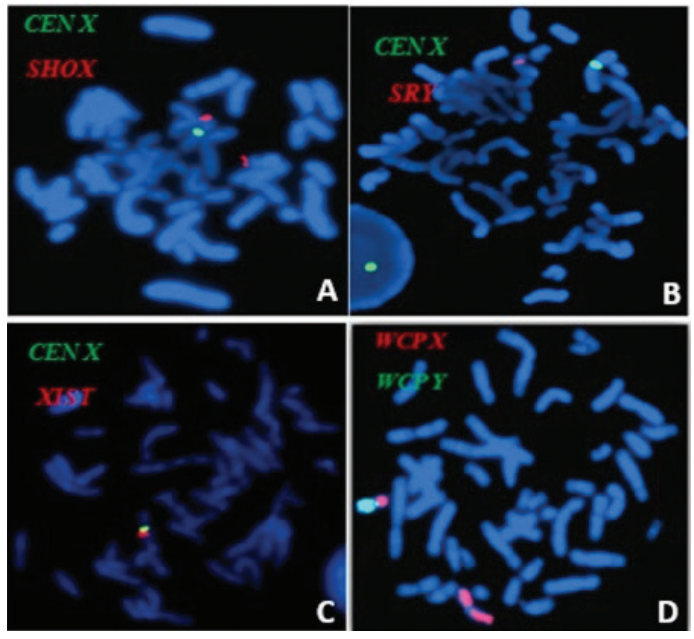


**Figure 4.** FISH analysis. A) FISH results using SHOX probe, two red spots were detected. B) FISH results using SRY probe, one red spot was detected. C) FISH results using XIST probe, one red spot was detected. D) FISH analysis using WCPX/WCPY showed the presence of a part from chromosome X on the Y chromosome

*FISH: fluorescence in situ hybridization*

probes of the X chromosome, *NR0B1* (Xp21.2) and *KAL1* (Xp22.31), were present in double copies on the normal X chromosome and the other on the der(Y) respectively (Figure 5A, 5B). Using the *STS* gene probe, one signal was detected on the X and der(Y) chromosomes telomeres (Figure 5B). Array CGH displayed a gain of genetic material on the short arm of chromosome X encompassing approximately 27.5Mb mapping from 2,710,316 to 30,248,793 according to the Genome reference Consortium Human build 36 assembly (hg18/NCBI36) (Figure 6).

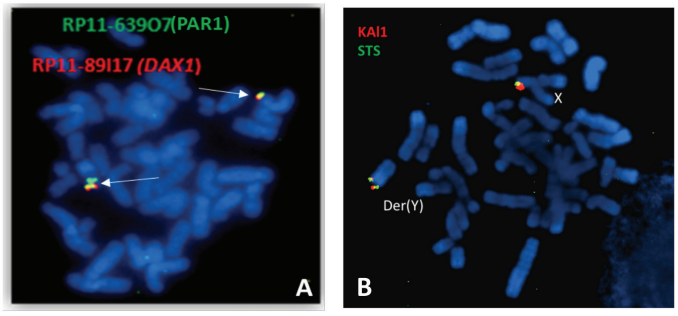


**Figure 5.** FISH analysis using specific probes: A) NR0B1 probe showed its presence on both sex chromosomes (white arrows); B) KAL1 and STS probes showed their presence on both sex chromosomes

*FISH: fluorescence in situ hybridization*

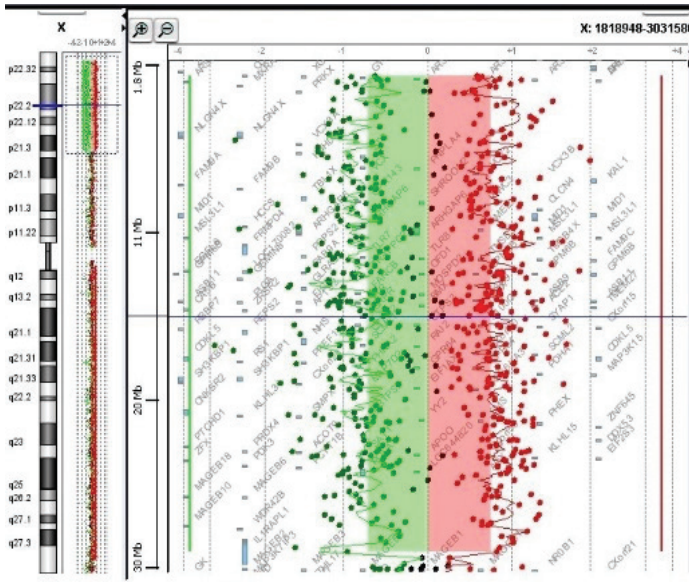

**Figure 6.** 4×44K Agilent Technologies oligonucleotides array profile of our patient showing Xp21.1 duplication of approximately 27.5 Mb

No other chromosomal rearrangement was detected, particularly within the Y chromosome.

Based on these results, the final karyotype of our patient was designated as:

46,X,der(Y)t(X;Y)(p21.2;p11.3).arr[NCBI36]Xp21.2-p22.3 (2,710,316_30,248,793)x2 dn

## Discussion

Sex determination is a complex process that implicates specific genes required for the progressive development of the undifferentiated gonad. An adequate dosage of these genes is required for proper gonadal development. CNV consistent with deletions or duplications of the genomic area including these genes may lead to DSD (13).

In this study, the patient investigated represents one of a small number of reported Xp;Yp translocation cases and the first reported Xp functional disomy case from Tunisia due to a large duplication on the Xp chromosome (Table 1).

The proband presented a 46,X,der(Y) male karyotype. The rearranged Y chromosome was the product of a translocation between sex chromosomes resulting from a non-allelic homologous recombination (NAHR) during paternal meiosis or in the early stages of embryogenesis. The parental karyotypes were normal, indicating a *de novo* origin of the unbalanced chromosome translocation. Array CGH was performed and it showed a gain of nearly 27 Mb on the Xp21 chromosome with a log-ratio equal to 0.58.

Based on the clinical and phenotypical criteria, biochemical assays, and genetic investigations, we can confirm that our patient presents with the syndromic form of 46,XY GD (OMIM≠300018) including a range of extra-gonadal abnormalities (growth delay, mental retardation, hypotonia, dysmorphic features and IAC). Remarkably, our patient had a complete Y-chromosome within an intact *SRY* gene and is a female. This reveals that the male sex development process is relatively complex and further factors are necessary for early testis formation with adequate dosage.

Thus, the duplicated region covers several genes, namely the *NR0B1, MAGEB, KAL1* and *IL1RAPL1* genes, resulting in ectopic expression and causing a disturbance in several developmental systems.

*NR0B1,* also called dosage-sensitive sex reversal gene (*DAX1*;OMIM#300018), is located in the DSS region at Xp21.2. When duplicated, *NR0B1* is considered to be the most likely factor for 46,XY GD (19,20,21,22).

*NR0B1* is an orphan nuclear receptor which acts in a mutually antagonist pathway to ensure testis determination. Its expression has been shown in different tissues (adrenal cortex, gonad, anterior pituitary, and hypothalamus, and also in adult adrenal cortex, Sertoli and Leydig cells in the testis, theca, granulosa, and interstitial cells in the ovary), highlighting its pleiotropic function (20,21,22). The link between *NR0B1* and the sex development process has been established by several studies. Previously, the *NR0B1* gene was known to be a dosage-sensitive ovarian determining gene. It is in fact down-regulated in the developing testis and persists in the ovary in mice (16,21). Conversely, in the last five years, numerous studies have shown that *NR0B1* plays an important role in male gonadogenesis and acts as an anti-testis factor within a critical window of sex development (23,24).

**Table 1. Comparison of clinical and genetic findings in cases with partial disomy Xp21. 1-Xpter and a Y chromosome to Xp disomy with Xp;Yp translocation**

| References | Ogata et al. (6) 1992 | Sanlaville et al. (7) 2004 | Ashton et al. (8) 2013 | Barbaro et al. (14) 2007 | White et al. (15), 2011; case 13 | Bardoni et al. (16) 1994; case 711 | Bardoni et al. (16) 1994; case BG | Ledig et al. (17) 2010; case 9 | Ledig et al. (17) 2010; case 52 | Bernstein et al. (18), 1980 | Present study |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Karyotype** | 46,X,der(Y) t(X;Y) (p21;p11.3) | 46,X,der(Y) t(X;Y) (p21.2;p11.3) | 46,X,der(Y) t(X;Y) (p21.1;p11.3) | 46,XY | 46,XY | 46,Y,dup(X) (p21.2-p22.3) | 46,Y,dup(X) (p21.2-p22.2) | 46,XY | 46,XY | 46,Y,dup(X) (p21-pter) | 46,X,der(Y)t(X;Y) (p21.2;p11.3) |
| **Mechanism** | t(Xp;Yp) | t(Xp;Yp) | t(Xp;Yp) | Duplication | Duplication | Duplication | Duplication | Duplication | Duplication | Duplication | t(Xp;Yp) |
| **Size of the CNV** | NA | NA | NA | 637 Kb | 771 Kb | NA | NA | 16.23 Mb | 729 Kb | NA | 27.5 Mb |
| **Gender** | F | F | F | F | F | F | F | F | F | F | F |
| **Age at diagnosis, years** | 2 years and 3 months | Postnatal | 4 months | 15 years old | NA | NA | NA | NA | NA | 3 year old | 7 months |
| **Cranio-facial dimorphism** | - Frontal bossing<br>- Antimongoloid slant<br>- Large, low set ears with thick auricular folds<br>- Cleft palate | - Bitemporal narrowness<br>- Short nose<br>- Prominent forehead<br>- Scarce hair<br>- Low-set ears<br>- Cleft palate | - A bulbous nasal tip<br>- Pinched nares with protuberant columella<br>- Prominence of the philtral pillars<br>- Small mouth | NA | - | NA | NA | + | NA | - Cleft palate<br>- Prominent forehead<br>- Mild hypertelorism<br>- Short nose<br>- Large ears | - Long face exophthalmos<br>- Hypertelorism<br>- Ogival palate<br>- Short and flat philtrum<br>- Strabismus |
| **Growth retardation** | + | + | + | NA | - | NA | NA | + | - | + | + |
| **Hypotonia** | + | + | + | NA | - | | NA | + | - | + | + |
| **Delayed mental development** | + | + | + | NA | - | NA | NA | + | - | + | + |
| **Delayed motor development** | + | + | + | NA | - | NA | NA | + | - | + | + |
| **External genitalia** | F | F | F | F | F | Hypoplastic female external genitalia | F | F | Clitoris hypertrophy | F | F |
| **Internal genitalia** | | | | Normal Mullerian derivatives | Normal Mullerian derivatives | Normal vagina | Normal Mullerian derivatives | Uterus | NA | Normal vagina and cervix, hypoplastic uterus, and fallopian tubes | No uterus and vagina |

**Table 1. Continued**

| References | Ogata et al. (6) 1992 | Sanlaville et al. (7) 2004 | Ashton et al. (8) 2013 | Barbaro et al. (14) 2007 | White et al. (15), 2011; case 13 | Bardoni et al. (16) 1994; case 711 | Bardoni et al. (16) 1994; case BG | Ledig et al. (17) 2010; case 9 | Ledig et al. (17) 2010; case 52 | Bernstein et al. (18), 1980 | Present study |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gonads** | Streak gonads | - | - | Streak gonads | - | - | Absent left gonad and right streak gonad with primordial sex cords | - | Testicular residues | - | - |
| **Other features** | Autoimmune disease | Partial agenesis of the corpus callosum | Partial agenesis of the corpus callosum | NA | - | NA | NA | NA | - | A ventricular septal defect | Inter-atrial communication heart defect |
| **Clinical diagnosis** | Syndromic XY GD | Syndromic XY GD | Syndromic XY GD | Isolated XY GD | Isolated XY CGD | NA | Isolated XY GD | Syndromic XY GD | Isolated XY GD | Syndromic XY GD | Syndromic XY GD |

F: female, +: present, -absent; NA: not available; GD: gonadal dysgenesis; CGD: complete gonadal dysgenesis, CNV: copy number variation

In normal XY males, a single copy of *NR0B1* is required for normal testis cord formation and testicular hormone synthesis (25,26,27). When *NR0B1* is overexpressed in 46,XY individuals, as in Xp duplication, it inhibits *SOX9* gene expression and antagonizes the synergy between *SF1* and *SOX9* by inactivating the *AMH* gene promoter. The inactivation of the AMH promoter gene blocks the regression of the Müllerian ducts. Thus, testicular formation is disrupted and a female pathway is followed (23,24,28). However, no uterus or gonad were observed in this case. Non-visualized uterus and gonad on MRI do not exclude the possibility of a GD diagnosis, which was confirmed by our genetic finding, since the structures may be too small to be detected at the time of examination. Small-sized structures could lead to suboptimal signal or resolution of the MRIs or suboptimal visualization and subsequently misinterpretation (29).

In addition to the *NR0B1* gene, the duplicated region contained testis expressed genes, called *MAGEB* genes located within the DSS locus (20). Recently, it has been speculated that overexpression of these genes could be involved in male to female sex reversal and may have a role in maintaining fetal testicular identity (14,28). Also, the *MAGEB* (1,2,3) genes seem to be functionally required for X chromosome inactivation mediated by XIST (30). Interestingly, the deletion of the same region containing *NR0B1* and MAGEB has been reported to be responsible for the opposite phenotype in a 46,XX SRY-negative ovotesticular DSD (28). So far, an adequate dosage of both *NR0B1* and *MAGEB* genes is needed for both male and female sex development and most likely these genes belong to overlapping complex molecular cascades in the testicular/ovarian tissue (i.e. from sex determination to sex differentiation).

Hence, a breakage within the DSS region may interfere with the spatiotemporal expression pattern resulting in ectopic expression, and incomplete stimulation/repression of male or female sex development, leading to different stages of sexual ambiguity. All these disorders can be responsible for infertility in adulthood.

Additionally, the duplicated region, in addition to the *NR0B1* gene, included several other genes which may be responsible for the patient's phenotype.

The *KAL1* gene (OMIM#308700) encodes a secreted heparin-binding protein (KAL or anosmin-1) which plays an important role in the embryonic development of the kidneys and human central nervous system. *KAL1* stimulates the signaling activity of the fibroblast growth factor receptor (FGFR1), which is involved in a variety of developmental processes including the formation, growth and shaping of

different tissues and organs (31). The overexpression of *KAL1* may interfere with FGFR1 signaling activity, which may be indirectly responsible for developmental and speech delay, intellectual disability and genital abnormalities (32,33).

The duplicated region also encompassed the gene encoding the IL1 receptor accessory protein-like1 gene (*IL1RAPL1,* #OMIM;300143*)*, a protein with high levels of expression in hippocampal neurons known to be involved in the memory system. Deletions and mutations in this gene were found in patients with mental retardation, which suggests a specific role in the physiological processes underlying memory and learning abilities (14). Within large Xp21 duplications, disruption of this gene could explain mental retardation.

To summarize, cytogenetic techniques are still as important as ever in the detection of chromosomal rearrangements, especially when the clinical manifestations are highly evocative of a known syndrome.

In fact, in a review of 116 patients with idiopathic DSD, array CGH was able to detect clinically relevant CNV in 21.5% of the patients (34). In another study of a cohort of 87 patients, array CGH identified CNV in 31.25% of syndromic DSD cases and in 29.57% of non-syndromic DSD cases (17). This justifies its relative contribution to the identification of CNV related to different DSD phenotypes by characterizing the exact size, and breakpoints as well as the expansion of the pool of candidate genes in disease pathogenesis in a single step. The discovery of new genomic analysis tools such as Hi-C technology may provide new insights into the physical genomic interactions and support the hypothesis that a common genomic region can be bound by both pro-testis and pro-ovarian transcription factors and genes (35,36).

### Study Limitations

A possible limitation of the present study may be that we reported on a single case which may not be that conclusive. The number of patients presenting with GD resulting from Xp;Yp translocation is also limited due to the rarity of this rearrangement.

## Conclusion

The results presented in this study illustrate the first Tunisian case having 46,XY GD due to a large duplication within the Xp21.2 DSS locus and associated with an X;Y translocation event.

Such DSD cases are very rare and require a careful, systematic, and sensitive approach to diagnose. Together, the karyotype, FISH and array CGH can prove useful in delivering a conclusive genetic diagnosis for those patients with the syndromic form of DSD by identifying chromosome abnormalities associated with dosage changes in genes, such as *NR0B1*, which play a pivotal role in human sex development.

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### Ethics

**Ethics Committee Approval:** The Local Ethics Board of the University Teaching Hospital Farhat Hached approved the present study (no: IRB00008931, date: 15.03.2022).

**Informed Consent:** Written consent was taken from the parents for photo publication and consent for the genetic analysis and publication of the case were obtained from the parents.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Ikbel Hadj Hassine, Afef Jelloul, Ali Saad, Soumaya Mougou-Zerelli, Concept: Khouloud Rjiba, Fethi El Amri, Monia Zaouali, Kenneth Mcelreavey, Design: Khouloud Rjiba, Kenneth Mcelreavey, Data Collection or Processing: Wafa Slimani, Meriem Gaddas, Hela Ben Khelifa, Analysis or Interpretation: Khouloud Rjiba, Wafa Slimani, Literature Search: Khouloud Rjiba, Writing: Khouloud Rjiba.

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# Clinical Utility and Outcome Prediction of Early ZnT8-IgG Testing and Titer in Type 1 Diabetes

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### What is already known on this topic?

Type 1 diabetes mellitus occurs due to the autoimmune destruction of pancreatic beta cells. Autoantibodies to components of these beta cells are markers for this disease. It is known that autoantibodies against Zinc transporter 8 (ZnT8) are more commonly seen in children with diabetes than in adults. Several studies have shown conflicting results regarding the clinical disease presentation in the presence or absence of ZnT8 autoantibodies, some showing that they are associated with older age, higher body mass index (BMI) and a more aggressive disease onset (more diabetic ketoacidosis), other studies have contradicted these results. One study has follow-up data on the clinical course of diabetes post-diagnosis (2 years) suggesting that those with ZnT8 autoantibodies have a more aggressive disease course (higher insulin requirements).

### What this study adds?

Our study adds to the very limited literature on ZnT8 autoantibody positivity in pediatric diabetes. We looked at disease onset and the subsequent disease course and showed that at disease onset, there were no differences in age, BMI or severity of the disease between those children with and those without ZnT8 autoantibodies. We had a longer longitudinal follow-up than other studies and observed no statistically significant differences in the development of other autoimmune conditions, macrovascular or microvascular complications between ZnT8 positive and negative groups. We also studied ZnT8 antibody titers and found that the only difference seen in follow-up between the low and high titer groups was a slightly higher cumulative excess glucose in the low titer group compared to the high titer and ZnT8 antibody negative groups.

## Abstract

**Objective:** Type 1 diabetes autoantibodies are directed against multiple antigens including: glutamic acid decarboxylase, protein tyrosine phosphatase-like islet antigen 2 (IA2), insulin (IAA), and Zinc transporter 8 protein (ZnT8). The aim of our study was to determine if the presence or titer of ZnT8 antibodies (Ab) was predictive for clinical presentation at diagnosis or for the subsequent disease course.
**Methods:** Between January, 2003 and May, 2019, 105 patients aged ≤21 years with a clinical diagnosis of type 1 diabetes mellitus had at least 1 autoantibody measured. A retrospective chart review was completed. At diagnosis, we evaluated the body mass index z-score, hemoglobin (HbA1c), and the presence of diabetic ketoacidosis (DKA). Complications analyzed post-diagnosis included episodes of DKA, the diagnosis of autoimmune disease, and the presence of vascular complications. We evaluated cumulative lifetime excess glucose as HbA1c area under the curve (AUC) >6%.

**Conflict of interest:** Sean Pittock reports grants, personal fees and non-financial support from Alexion Pharmaceuticals, Inc.; grants from Grifols, Autoimmune Encephalitis Alliance; grants, personal fees, non-financial support and other from MedImmune, Inc.; Dr. Pittock has a patent #9,891,219 (Application#12-573942) "Methods for Treating Neuromyelitis Optica (NMO) by Administration of Eculizumab to an individual that is Aquaporin-4 (AQP4)-IgG Autoantibody positive". Dr Pittock also has patents pending for the following IgGs as biomarkers of autoimmune neurological disorders (septin-5, Kelch-like protein 11, GFAP, PDE10A and MAP1B).

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**Results:** Seventy-one patients were ZnT8-Ab(+) (68%), with 19 having low titer ZnT8-Ab and 52 with high titer ZnT8-Ab. Follow-up ranged from 10 days to 15.7 years (median 2.08 years). There were no differences in the characteristics at disease onset or in the subsequent follow-up between those with and those without ZnT8-Ab or those with high or low titers of ZnT8 Ab, except for a small but statistically significant difference in cumulative excess glucose (HbA1c AUC >6%) between those with low and high titers ($p = 0.0095$).
**Conclusion:** Our study adds to the limited literature on the effect of the presence and titer of ZnT8-Ab in pediatric diabetes. The small effect of ZnT8-Ab titer on glucose excess as measured by HbA1c AUC warrants further study.
**Keywords:** Type 1 diabetes mellitus, autoantibodies, GAD65, IA2, IAA, ZnT8

## Introduction

Type 1 diabetes mellitus (T1DM) is a common chronic disease which affects 187,000 children and adolescents younger than 20 years of age in the United States (1). T1DM is an autoimmune (AI) condition which results in beta cell destruction leading to insulin deprivation. Due to the current obesity epidemic, distinguishing between type 1 and type 2 diabetes in children can be difficult. Diabetes-associated autoantibodies can help delineate the diabetes classification. Autoantibodies to pancreatic beta cell components are currently the best indicator of ongoing beta cell destruction in humans leading to AI T1DM. The diabetes specific autoantibodies are directed against glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase-like islet antigen 2 (IA2), insulin (IAA), and Zinc transporter 8 protein (ZnT8) (2,3,4,5,6,7). Of the four autoantibodies, ZnT8-Ab is the newest diabetes specific autoantibody in clinical use and is more commonly present in children than in adults (2,8). Additionally, the presence of multiple autoantibodies (2 or more) is more common in children (8).

T1DM can present with a severe, potentially life-threatening form known as diabetic ketoacidosis (DKA), defined as hyperglycemia, acidosis, and the presence of ketones (9,10). Between 15% and 67% of patients with newly diagnosed T1DM present with DKA (11). The severity of DKA is dependent on the degree of acidosis. DKA can occur not only at the time of initial diagnosis, but also any time post-diagnosis when there is inter-current illness, trauma, or stress in conjunction with inadequate insulin delivery.

Due to the chronic nature of T1DM, patients are at risk of diabetes related complications from long standing hyperglycemia. These include both microvascular (peripheral neuropathy, diabetic kidney disease, retinopathy, and gastroparesis) and macrovascular (cerebral infarction and myocardial infarction) complications (12,13). Patients with T1DM are also at a higher risk of other AI diseases, such as hypothyroidism, celiac disease, and Addison's disease, which can lead to additional burdens and can impact their quality of life (14,15,16,17).

The aim of our study was to determine if the presence and titer of ZnT8 antibodies (Ab) was predictive of clinical presentation at diagnosis or in the subsequent disease course. The novel aspect of our study was that we had up to 15 years of follow-up available in order to analyze the subsequent disease course.

### Research Design and Methods

This clinical-serological cohort study was approved by the Institutional Review Board at Mayo Clinic with a waiver of consent for the clinical data obtained as part of serological test validation (study: 08-006647, date: 15.07.2020). All Mayo Clinic patients whose medical charts were analyzed provided written consent for medical research.

### Patients

Mayo Clinic Laboratories have been analyzing diabetes specific autoantibodies since 1997. GAD65-Ab testing became available in 1997, IAA (the IAA includes insulin autoantibody) in September 2009, IA2-Ab in March 2011, and ZnT8-Ab in May 2017. Since October 2017, a diabetes mellitus evaluation antibody panel, which includes all 4 autoantibodies, has been available for clinical use.

Between January, 1998 and May, 2019, we had 324 patients ≤21 years of age with a clinical diagnosis of T1DM, who had at least 1 of these autoantibodies tested. Of these patients, residual serum samples were available for 230 patients. We included those with autoantibody testing which was completed within 1 year from the date of diagnosis of T1DM. This resulted in a final analysis of 105 patients.

Residual serum samples are stored in our Neuro-Immunology Laboratory. Since the diabetes specific autoantibodies became available for analysis at different times, the ability to retrieve the residual serum samples allowed for complete analysis of all 4 Ab. Residual serum samples also allowed for the potential of a longer follow-up time period. For those patients in whom all 4 T1DM autoantibodies were not tested initially, stored serum was retrieved, thawed, and the remaining autoantibodies were tested.

Retrospective chart review was completed. Data retrieved included: date and age at diagnosis, ethnicity, length of follow-up, symptoms at diagnosis, body mass index (BMI), glycosylated hemoglobin (HbA1c), additional laboratory results to identify if DKA was present and its severity [central glucose, bicarbonate ($HCO_3$), pH, beta-hydroxybutyrate]. The severity of DKA was classified according to the initial laboratory evaluation in accordance with American Diabetes Association guidelines: mild = pH 7.25-7.3, $HCO_3$ 15-18, moderate = pH 7-7.24, $HCO_3$ 10-14, severe = pH < 7, $HCO_3$ < 10 (18). Post diabetes diagnosis analysis included evaluation for the number of admissions for DKA, and diagnoses of AI disease (hypothyroidism, hyperthyroidism, celiac disease, AI adrenal insufficiency, and other), and vascular complications (microvascular – peripheral neuropathy, diabetic kidney disease, retinopathy gastroparesis; macrovascular – cerebral infarction, and myocardial infarction).

BMI z-scores were determined for all ages according to Center for Disease Control guidelines (19). The equation for determining BMI z-scores was (patient BMI – population mean BMI/population standard deviation BMI, using age-specific population means and standard deviations). It was assumed that the population mean and standard deviation for BMI was constant for age ≥20 years.

All available HbA1c results since the diagnosis of diabetes were collected, and a life-long measure of dysglycemia was calculated using an HbA1c index [HbA1c area under the curve (AUC) > 6% which computed total AUC > 6.0% using the trapezoidal rule, divided by the time between diagnoses and study assessment] (20,21). HbA1c results ≥2 months from diagnosis were used to calculate HbA1c AUC > 6%.

### Laboratory Data

All assays were clinically validated laboratory developed tests or Food and Drug Administration approved assays performed in accordance with CLIA, CAP, and New York State regulatory guidelines. GAD65-Ab, IA2-Ab, and IAA were measured by radioimmunoassay (normal reference range ≤0.02 nmol/L). ZnT8-Ab was detected by enzyme-linked immunosorbent assay (normal reference range ≤15 U/mL).

### Statistical Analysis

Patient characteristics were summarized with frequencies and percentages, or medians and ranges, as appropriate. Comparisons by ZnT8-Ab status [negative vs. positive; negative vs. low positive titer (< 100 U/mL) vs. high positive titer (≥100 U/mL)] were assessed with chi-squared or Fisher's exact tests (categorical data) or Wilcoxon rank-sum or Kruskal-Wallis tests (ordinal or continuous data). The incidence rates (along with 95% confidence intervals) for total diabetes complications, DKA admissions, and AI diseases were summarized per 100 person years, and were estimated and compared between ZnT8-Ab groups with Poisson regression models. P values less than 0.01 were considered statistically significant. All analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

## Results

Between January, 2003 and May, 2019, we had a total of 105 T1DM patients, ≤21 years old, with a clinical diagnosis of type 1 diabetes and autoantibody results within 1 year of diagnosis. Males made up 67% of the total cohort (Table 1).

When looking at ZnT8-Ab status, 34/105 were ZnT8-Ab negative, 71 were ZnT8-Ab positive (68%). Of the ZnT8-Ab positive patients, 27% had a low ZnT8-Ab titer (< 100 U/mL) and 73% had a high ZnT8-Ab titer of ≥100 U/mL (Table 1). There was no significant difference found between genders with respect to ZnT8-Ab positivity (p = 0.04) or titer concentration (p = 0.12). Additionally, there was no significant difference in ZnT8-Ab positivity or titer by ethnicity (p = 0.71 and p = 0.49 respectively).

### At Diabetes Diagnosis Analysis

At diabetes diagnosis, we did not find a difference in age (p = 0.94), BMI z-score (p = 0.83), rate of DKA (p = 0.26), or HbA1c (p = 0.38) relative to ZnT8-Ab positivity. There remained no significant difference when evaluating ZnT8-Ab titer concentrations between age at diagnosis (p = 0.87), BMI z-score (p = 0.96), or rate of DKA (p = 0.03).

Although the age at diagnosis was similar between the ZnT8-Ab negative and positive groups (median 12.8 for each group, p = 0.94), we found that the low titer positive patients had a slightly (non-significantly) higher age than the high titer patients (median 14.8 vs. 12.5, p = 0.58).

When evaluating rates of DKA at diagnosis, within the low titer ZnT8-Ab group, there were no episodes of DKA. Although this might be clinically significant, it did not reach statistical significance. Of the 21 patients who presented with DKA, 9 of them were ZnT8-Ab negative (43%), none had low titer ZnT8-Ab, and 12 had high titer ZnT8-Ab (57%).

The median BMI z-score was similar between the ZnT8-Ab negative, low titer ZnT8-Ab positive, and high titer ZnT8-Ab groups at 0.31, 0.36 and 0.23 respectively (p = 0.96).

### Post Diabetes Analysis

We evaluated cumulative post diagnosis excess glucose as HbA1c AUC >6% divided by years of available follow-up following diabetes diagnosis. We found a statistically significant difference when comparing low positive titer ZnT8-Ab to high titer ZnT8-Ab HbA1c AUC >6%, with the low positive titer group having a higher AUC (p=0.0095). The HbA1c AUC >6% was not stepwise as the AUC for HbA1c >6% was similar between the ZnT8 Ab negative and high titer groups (p=0.23) (Table 1).

When we evaluated for the rate of post diabetes complications and ZnT8-Ab status along with ZnT8-Ab titer concentration, we did not find a statistical significance in diabetes complications (retinopathy, peripheral nephropathy, diabetes kidney disease, and gastroparesis), subsequent DKA admissions, or concomitant AI disease (Table 2).

There were 10 patients in total who experienced at least one diabetes complication following diagnosis. Although the incidence rate of complications was highest for the ZnT8-Ab negative patients (incidence rate per 100 person years: 4.46) as compared to the low positive titer (1.76) and high titer (3.15) patients, this was not statistically significant.

Only 18 subjects had one or more DKA admission following their initial diagnosis. The incidence rates of DKA were 14.64, 15.80, and 10.73 per 100 person years, for the negative, low positive titer, and high titer groups, respectively (not significant).

**Table 1. Characteristics at diagnosis stratified by ZnT8-Ab status and titer**

| | ZnT8-Ab(-) (n=34) | ZnT8-Ab(+) <100 (n=19) | ZnT8-Ab(+) ≥100 (n=52) | Overall p value |
|---|---|---|---|---|
| Sex | | | | |
| F | 16 (47.1%) | 5 (26.3%) | 14 (26.9%) | 0.12 |
| M | 18 (52.9%) | 14 (73.7%) | 38 (73.1%) | |
| Age at diagnosis | | | | |
| Median | 12.8 | 14.8 | 12.5 | 0.87 |
| Range | (2.2-21.2) | (4.4-21.7) | (2.1-21.9) | |
| Age at diagnosis (categorized) | | | | |
| <6 yrs | 4 (11.8%) | 3 (15.8%) | 8 (15.4%) | 0.72 |
| 6-<12 yrs | 11 (32.4%) | 3 (15.8%) | 16 (30.8%) | |
| 12-21 yrs | 19 (55.9%) | 13 (68.4%) | 28 (53.8%) | |
| BMI z-score | | | | |
| n | 26 | 15 | 43 | 0.96 |
| Median | 0.3 | 0.4 | 0.2 | |
| Range | (-5.8-2.5) | (-2.3-1.9) | (-2.6-2.8) | |
| Ethnicity | | | | |
| n | 30 | 18 | 46 | 0.49 |
| Hispanic or Latino | 3 (10.0%) | 0 | 5 (10.9%) | |
| Not Hispanic or Latino | 27 (90.0%) | 18 (100%) | 41 (89.1%) | |
| DKA | | | | |
| n | 32 | 18 | 48 | **0.03** |
| No | 23 (71.9%) | 18 (100%) | 36 (75.0%) | |
| Yes | 9 (28.1%) | 0 | 12 (25.0%) | |
| DKA severity at diagnosis (excludes unknowns) | | | | |
| n | 8 | 0 | 10 | |
| Mild | 4 (50.0%) | | 6 (60.0%) | 1 |
| Moderate | 2 (25.0%) | | 2 (20.0%) | |
| Severity | 2 (25.0%) | | 2 (20.0%) | |
| HbA1c at diagnosis (%) | | | | |
| n | 29 | 18 | 48 | 0.66 |
| Median | 12.0 | 10.9 | 11.2 | |
| Range | (5.6-19.0) | (5.7-16.1) | (5.8-17.1) | |
| Any thyroid disease at or prior to baseline diabetes diagnosis | 1 (2.9%) | 0 | 3 (5.8%) | 0.82 |
| Any other* type of AI disease at or prior to baseline diabetes diagnosis | 2 (5.9%) | 1 (5.3%) | 0 | 0.17 |
| Days between diagnosis and specimen collection | | | | |
| Median | 19.5 | 15.0 | 25.0 | 0.58 |
| Range | (4-358) | (0-290) | (0-309) | |

*Other type of AI disease classified as Vitiligo (2) and juvenile idiopathic arthritis.
AI: autoimmune, yrs: years, DKA: diabetic ketoacidosis, F: female, M: male, ZnT8-Ab: Zinc transporter 8 protein-antibodies

There were 13 patients who had one or more AI disease following their diagnosis. Although the incidence rate of AI disease was slightly higher for the high titer ZnT8-Ab (5.67 per 100 person years) compared to the low positive titer (1.76) and negative (1.91) patients, this did not reach statistical significance (Table 2).

## Discussion

ZnT8-Ab is the newest diabetes specific autoantibody used in clinical settings. Zinc is essential for the structural stabilization of insulin and the pancreas is one of the tissues with the highest Zinc concentration (2,5). Studies have shown that ZnT8-Ab is a valuable biological marker in the diagnosis of T1DM for both children and adults when obtained in addition to the classic diabetes autoantibodies (GAD, IA2, and insulin). ZnT8-Ab testing has led to significant improvements in the positive predictive value of autoantibody measurements at the time of diagnosis of T1DM (4). However, there is a paucity of data evaluating the predictive value of ZnT8-Ab titer concentrations.

Our study analyzed if ZnT8-Ab status positivity and ZnT8-Ab titer concentration resulted in a difference at diabetes diagnosis and in the post diabetes diagnosis course. Our rates of ZnT8-Ab positivity were similar to previous studies. Elmaoğulları et al. (2) evaluated 84 patients < 18 years of age, and they noted 58% prevalence at diabetes onset.

Our study differs from others in that we did not find a difference in age at diagnosis, BMI z-score, rates of DKA, or HbA1c based on ZnT8-Ab status. Juusola et al. (18) evaluated 723 patients < 15 years of age at diagnosis and followed up for 2 years thereafter. They concluded that positivity for ZnT8-Ab at diagnosis seemed to reflect a more aggressive disease process both before (more frequent episodes of DKA, older age at diagnosis), and after (higher insulin doses required) diagnosis (22). Our overall median time of follow-up was similar at 2.08 years (range: 10 days to 15.7 years) from the date of T1DM diagnosis. We have the added benefit of 55 subjects with follow-up times of ≥2 years up to 15.7 years (22 subjects with 2 to < 5 years of follow-up, 18 subjects with 5 to < 10 years of follow-up, and 15 subjects with ≥10 years of follow-up). This follow-up time allowed us to evaluate for the development of complications and rates of concomitant AI disease in conjunction with the autoantibody status, and there was no significant difference in the available follow-up times between the negative and ZnT8-Ab positive patients.

The association of ZnT8-Ab and DKA at presentation has been analyzed before but with conflicting results. Niechciał et al. (8) evaluated 218 pediatric patients, median age 9 years, and they found that ZnT8-Ab positive children had higher rates of DKA at diagnosis (p = 0.002) and that these children were found to have high ZnT8-Ab titers (range: 35.5-524.5 U/mL), p < 0.0001. They also noted that ZnT8-Ab positive subjects were more likely to be older than 5

**Table 2. Characteristics post-diagnosis stratified by ZnT8-Ab status**

| | Incidence rate per 100 person years (95% CI) | | | p values | | |
|---|---|---|---|---|---|---|
| **Outcome[1]** | **ZnT8-Ab (-) (n = 34)** | **ZnT8-Ab (+) < 100 (n = 19)** | **ZnT8-Ab (+) ≥100 (n = 52)** | **Low pos. vs. neg.** | **High pos. vs. neg.** | **High pos. vs. low pos.** |
| Vascular complication[2] | 4.46 (2.64, 7.51) | 1.76 (0.66, 4.66) | 3.15 (1.70, 5.84) | 0.10 | 0.40 | 0.32 |
| DKA admission[3] | 14.64 (8.58, 24.98) | 15.80 (8.64, 28.91) | 10.73 (5.76, 19.98) | 0.85 | 0.46 | 0.38 |
| AI disease[4] | 1.91 (0.75, 4.88) | 1.76 (0.56, 5.54) | 5.67 (3.30, 9.75) | 0.91 | 0.049 | 0.07 |
| | **Other follow-up characteristics, median (range)** | | | | | |
| Total years from diagnosis to last follow up | | | | | | |
| Median | 2.4 | 2.7 | 1.8 | 0.29 | 0.20 | 0.05 |
| Range | (0.03-12.9) | (0.1-14.8) | (0.05-15.7) | | | |
| AUC for HbA1c > 6%, divided by total years of available data | | | | | | |
| n | 23 | 17 | 39 | 0.27 | 0.23 | **0.0095** |
| Median | 1.3 | 1.9 | 1.2 | | | |
| Range | (0-4.8) | (0.5-6.9) | (0-4.9) | | | |

[1]Considering the total of each type of event per patient over total available follow-up time per patient.
[2]10 patients had at least one type of diabetes complication (6 negative, 2 low positive, and 2 high positive patients). The most common complication was retinopathy (n = 5 patients), followed by peripheral neuropathy (n = 4), nephropathy (n = 4), and gastroparesis (n = 1), with some patients having multiple types.
[3]18 patients had at least one DKA admission (6 negative, 5 low positive, and 7 high positive patients).
[4]13 patients had at least one type of autoimmune disease (3 negative, 2 low positive, and 8 high positive patients). The most common type was thyroid disease (n = 8 patients), followed by celiac disease (n = 4), and other type (n = 2). Only 1 patient had multiple additional autoimmune diseases (thyroid disease and celiac disease).
AI: autoimmune, DKA: diabetic ketoacidosis, CI: confidence interval, pos.: positive, neg.: negative

years of age with a gradual decrease in rates after 10 years of age, which was in agreement with two other studies (8,22,23). In contrast, Salonen et al. (19) and Elmaoğulları et al. (2) found that rates of DKA at diagnosis were lower in ZnT8-Ab positive patients (23). We did not find a significant difference in the rate of DKA at diagnosis when comparing our 3 groups (negative, low titer or high titer ZnT8-Ab).

It has been well established that patients with poor glycemic control are at a higher risk of episodes of DKA and longstanding hyperglycemia can lead to vascular complications (24). The frequency of vascular complications in our cohort was low, which likely contributed to the lack of statistical significance. Of the 10 patients with vascular complications, 6 were in the ZnT8-Ab negative group (60%), 2 in the low ZnT8-Ab titer group (20%), and 2 in the high ZnT8-Ab titer group (20%), possibly suggesting a trend towards more vascular complications in the ZnT8 Ab negative group (Table 2, footnote 2).

We found some differences in the clinical course between the different ZnT8-Ab groups, although the clinical significance of these differences is unclear. There was a statistically higher AUC HbA1c >6% in those with lower ZnT8-Ab titers compared to those with higher titers. However, there was no difference between the low titer vs. negative groups and high titer vs. negative groups. There was a trend towards higher rates of additional AI disease in the high titer ZnT8-Ab group, but no difference between the negative and low titer groups.

We had 7 subjects whose diagnosis of additional AI disease preceded their diagnosis of T1DM. The prevalence of pre-existing AI conditions was no different between the ZnT8-Ab(+) and ZnT8-Ab(-) groups. Thirteen patients developed another AI disease following their diagnosis. Thyroid disease was the most common, developing in 8 patients, 5 of whom had high ZnT8-Ab titers (62.5%), 2 low titers and 1 was ZnT8-Ab negative. Rydzewska et al. (25) evaluated the status of diabetes associated autoantibodies in children and adolescents with AI thyroid disease. When looking at AI thyroid disease only (without a diagnosis of diabetes), they found that 9.1% of patients with Grave's disease and 9.2% of patients with Hashimoto's thyroiditis were found to have positive ZnT8-Ab. When they evaluated patients with T1DM ± AI thyroid disease, they found that 53% of those with T1DM + AI thyroid disease had ZnT8-Ab and 67% of those with T1DM without AI thyroid disease had ZnT8-Ab. Even though our findings were not statistically significant, there appeared to be a tendency towards a higher occurrence of thyroid disease in those with higher ZnT8-Ab titers, however, larger numbers are needed to validate this finding.

### Study Limitations

Limitations exist due to the nature of this study being a retrospective analysis. In the past, it was not common practice to obtain a type 1 diabetes autoantibody panel at the time of diagnosis. Patients with T1DM may seroconvert to negative status over time. Fabris et al. (4) reported a significant seroconversion rate for ZnT8-Ab over a 5 year time period. They showed that the percentage of patients with positive ZnT8-Ab was 61.1% at diabetes diagnosis, but only 33.8% of patients were found to have positive ZnT8-Ab if it was analyzed ≥5 years from diagnosis. Wenzlau et al. (26) found that only 6.7% remained positive for ZnT8-Ab after 25 years.

We only included patients in this study whose autoantibody profiles were obtained within 1 year from diabetes diagnosis to mitigate this issue. There was no difference in time from diagnosis to antibody testing between any of the groups (antibody negative, low titer or high titer).

## Conclusion

Our study analyzed if ZnT8-Ab status positivity and ZnT8-Ab titer concentrations resulted in a difference in key patient characteristics at diabetes diagnosis and in the subsequent disease course. Our study is novel as we evaluated the predictive value of ZnT8-Ab titer concentrations along with having a longer follow-up period than previously cited in the literature. At diabetes diagnosis, we did not find a difference in ZnT8-Ab status by age at diagnosis, BMI z-score, rate of DKA, or HbA1c.

During diabetes follow-up, there were no statistically significant differences in admissions for DKA, glycemic control, vascular complications or the development of additional AI disease between those with and those without ZnT8-Ab. Some slight differences were seen between those with high vs. low titer and those with lower titer having a higher AUC for HbA1c >6%.

Our findings add further to the limited literature on the predictive value of the presence of ZnT8-Ab in patients with diabetes and further studies with larger numbers and longer follow-ups are warranted.

### Ethics

**Ethics Committee Approval:** The study was approved by the Mayo Clinic of Institutional Review Board (study: 08-006647, date: 15.07.2020).

**Informed Consent:** Retrospective study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Amanda R. Dahl, Siobhan T. Pittock, Concept: Amanda R. Dahl, Siobhan T. Pittock, Design: Amanda R. Dahl, Siobhan T. Pittock, Sean J. Pittock, Data Collection or Processing: Amanda R. Dahl, Sean J. Pittock, Analysis or Interpretation: Amanda R. Dahl, Sarah Jenkins M., Siobhan T. Pittock, Sean J. Pittock, Literature Search: Amanda R. Dahl, Siobhan T. Pittock, Writing: Amanda R. Dahl, Sarah Jenkins M., Siobhan T. Pittock, Sean J. Pittock.

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# Comparison of Commonly Used Methods to Predict the Final Height in Constitutional Tall Stature

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**What is already known on this topic?**

The literature on predicting the adult height of young children with tall stature is scant.

**What this study adds?**

Proves that adult height can be predicted in prepubertal children with constitutional tall stature.

## Abstract

**Objective:** To determine the accuracy of adult height prediction in children with constitutional tall stature.

**Methods:** The medical records of 138 non-syndromatic prepubertal and early pubertal children (52 male, 86 female) with a height of ≥90$^{th}$ percentile born between the years 1975 and 1988 were included in this study. Using the Bayley-Pinneau (BP) and Tanner-Whitehouse I (TWI) prediction methods, their height standard deviation score (SDS) at referral was compared with their height SDS at age 17 years when measured at the IDF conscription center.

**Results:** While remaining tall, the height SDS at age 17 years was lower than that at referral decreasing from $2.13 \pm 1$ to $1.65 \pm 1.21$ in boys and from $2.48 \pm 1$ to $2.15 \pm 1$ in girls.

**Conclusion:** The prediction by the BP and TWI methods can be useful for estimating adult height in constitutional tall stature even in the prepubertal and early pubertal period. However, the fallibility of these methods should be kept in mind during clinical practice. We think that this study will shed light on these issues.

**Keywords:** Tall stature, height prediction, growth, adult height, familiar tall stature

## Introduction

The social impact of tall stature has caused controversial opinions. Tall stature may have advantages in adult life according to some authors (1); by others there are reports that tall children often suffer from social unattractiveness and may have difficulties in finding partners in their adult age. Additionally, it has also been reported that tall adolescent girls have a higher prevalence of depression (2,3,4,5).

Similar to the parents of children with growth retardation, also parents of children with early tall stature are concerned about their adult height (6). To answer their queries, a series of height prediction methods have been developed (7,8,9,10,11,12). The most frequently used methods are that of Bayley-Pinneau (BP) based on skeletal age (7) and that devised by Tanner and Whitehouse which includes mid-parental height in their formula (9).

The majority of height prediction studies were performed in untreated children of short stature of various etiologies (10,11,12,13,14,15,16,17) and in those treated by growth hormone in order to estimate the success of their treatment

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(18,19). We found only 2 reports of height prediction for children diagnosed with constitutionally tall stature (20,21). Both parents of short and tall statured children are concerned whether their children will reach adult height within normal limits (5,22).

In the present study, we compared two methods of height prediction in a large group of prepubertal or early pubertal children with constitutional tall stature, with the actual height measured at age 17 years.

### Subjects

The clinical data of children with constitutional tall stature (height $> 90^{th}$ percentile) referred between the years 1975-1998 was retrieved from the medical records of the Pediatric Endocrine Clinic at the Beilinson Hospital and Schneider Children Medical Center. Children with syndromic tall stature and endocrine disorders were excluded. One hundred seventy-three individuals (62 boys and 111 girls) fitted the diagnosis of constitutional tall stature. For 138 records (52 males, 86 females), measurements were available at the Israel Defense Forces (IDF) conscription center. All eligible Israeli adolescents, both boys and girls at age 17 years, a year before their conscription, undergo medical assessment in order to determine their medical fitness for military service.

## Methods

The BP method (7) of adult height prediction was compared to that of Tanner-Whitehouse 1 (TWI) (9).

This study was approved by the Ethics Committees of the Rabin Medical Center and that of the IDF Medical Corps.

### Statistical Analysis

The data were analyzed using BMDP software (23). Analysis of variance (ANOVA) and covariance with repeated measures was used to determine changes over time. The t-test was used to compare differences between groups. A p value of ≤0.05 was considered significant.

## Results

Previous medical history was reported and height and weight parameters were measured. In this study, records of height and weight at age of 17 were obtained for those who were early constitutionally tall children i.e $> 90^{th}$ percentile.

The mean age at referral was 9.54 ± 3.6 years for boys and 8.86 ± 3.2 years for girls. The majority of boys and girls (n = 80) were prepubertal, while others had early stage puberty. The mean birth weight was 3,607 ± 496 gr for boys (n = 52) and 3,313 ± 539 gr for girls (n = 84). The mean birth lengths of 19 boys was 51.5 ± 3 cm and it was 51.3 ± 2.8 cm for 42 girls.

Table 1 presents height standard deviation score (SDS) and body mass index (BMI) at referral compared to that at age 17 years. It can be seen that in both boys and girls there is a decrease in height SDS, at the same time that BMI increased in both genders.

The comparisons between the predicted age at referral and the height SDS at age 17 by either the BP or TWI methods (Table 2) show that the TWI method, which includes mid-parental calculation, underestimates the adult height by a mean of 5 cm in both sexes, whereas the BP method provides closer results to the actual height in girls, but with an overestimation by a mean of 5 cm for boys.

Comparing the height SDS of the boys at age 17 years with that of their fathers and that of the girls with their mothers (Table 3), it is evident that boys and girls are taller by one height SDS than their parent.

**Table 1. Comparison between height SDS and BMI at referral and at age 17 years**

| | Height SDS m ± SD | | p | BMI m ± SD | | p |
|---|---|---|---|---|---|---|
| | Referral | Age 17 | | Referral | Age 17 | |
| Males (n = 52) | 2.13 ± 1.00 | 1.65 ± 1.21 | 0.001 | 20.47 ± 3.75 | 25.1 ± 5.79 | < 0.001 |
| Females (n = 84) | 2.48 ± 1.00 | 2.15 ± 1.05 | 0.005 | 18.47 ± 3.15 | 22.79 ± 3.85 | < 0.001 |

SDS: standard deviation (SD) score, BMI: body mass index

**Table 2. Comparison between the actual height at age 17 years and those predicted by the Bayley-Pinneau and Tanner-Whitehouse I**

| | Height at age 17 years (cm) | BP prediction (cm) | p | TW prediction (cm) | p |
|---|---|---|---|---|---|
| Males (n = 52) | 185.9 ± 8.06 | 191.2 ± 6.35 | 0.001 | 180.1 ± 5.37 | < 0.001 |
| Females (n = 84) | 173.6 ± 5.31 | 172.5 ± 5.12 | 0.035 | 168.6 ± 6.17 | < 0.001 |

BP: Bayley and Pinneau, TW: Tanner Whitehouse I

**Table 3. Comparison between the height SDS at age 17 years of the males with that of their fathers, and that of the females with that of their mothers**

| | Boys | Fathers | Δ SDS | p |
|---|---|---|---|---|
| Height SDS | 1.65 ± 1.20 | 0.74 ± 1.17 | 0.93 ± 1.30 | **< 0.001** |
| | Girls<br>2.15 ± 1.05 | Mothers<br>1.30 ± 1.29 | 1.10 ± 1.31 | **< 0.001** |

Δ: delta, SDS: standard deviation (SD) score

## Discussion

The prospective height of tall children is of great concern to parents both for boys as well as for girls. For boys, the hope is that they will remain tall, as tall men have been described as being more successful (1,2,3,4,5). For parents of tall girls, the hope is that they will not be too tall, so as to avoid social problems (4) often leading to requests to enhance puberty to limit adult height by pharmacologic intervention, which is a treatment with risks (24).

The number of follow-up studies of the growth of children with constitutional tall stature are rare. Dickerman et al. (25), in a retrospective study of 36 boys and 29 girls, found that their birth length was increased: 53.5 ± 1 cm for boys (norm: 50.5 ± 1.53) and 52 ± 2.3 cm for girls (norm: 49.8 ± 1.5 cm) followed by a progressive growth acceleration, with both sexes reaching at age 9 a mean height corresponding to 2.75 SD above the 50$^{th}$ percentile for age.

In our study comparing the height SDS heights at referral with those at age 17 years, we found that both sexes showed a decrease. Whereas the height SDS of the boys decreased to a normal percentile, that of the girls remained above the 90-97 height percentile.

Testing the adult height prediction using 2 of the mostly used methods, that of BP (7) and that of TWI (9), the first based on skeletal age, and the second incorporating mid-parental height in the calculation, we found the BP method (7) was closer to the actual height, whereas the TW1 underestimated the actual height. Even Tanner et al. (10) in a later study and Wright and Cheetham (12) found that it was unwise to make an allowance for parental height. This is supported by our findings showing that both boys and girls ended up taller than their parents.

A study by de Waal et al. (21) using another method for the prediction of adult height of pubertal children with constitutional tall stature also found that the BP predication method was preferable (7). The limitation of our study was the use of only two prediction methods.

### Study Limitations

Compared to studies on the prediction of height in short children, publications on the early prediction of tall children are scant.

## Conclusion

The prediction by the BP and TWI methods can be useful for estimating adult height in constitutional tall stature even in the prepubertal and early pubertal period. However, the fallibility of these two methods should be kept in mind during clinical practice. We think that this study will shed light on these issues.

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### Ethics

**Ethics Committee Approval:** This study was approved by the Ethics Committees of the Rabin Medical Center and that of the IDF Medical Corps (0572 – 20RMC – 07.03.2020).

**Informed Consent:** Retrospective study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Concept: Zvi Laron, Design: Zvi Laron, Data Collection or Processing: Alma Kamar Matias, Analysis or Interpretation: Evgenia Muginshtein-Simkovitch, Lilos Pearl, Literature Search: Alma Kamar Matias, Writing: Alma Kamar Matias, Gilad Twig, Zvi Laron.

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# Frequency, Clinical Characteristics and Predictors of Ketoacidosis at Diagnosis of Type One Diabetes Mellitus in Children and Adolescents from Jordan

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### What is already known on this topic?

Rates and predictors of diabetic ketoacidosis (DKA) at onset of type one diabetes (T1D) vary worldwide. Data from developing countries are scarce.

### What this study adds?

The frequency of DKA at diagnosis of T1D in Jordan is relatively high at 31.7%. In this study, being aged less than two years and lower paternal education and employment levels were associated with DKA at diagnosis of T1D. A family history of T1D was protective against presenting with DKA at onset of T1D.

## Abstract

**Objective:** Data regarding diabetic ketoacidosis (DKA) at diagnosis of type one diabetes (T1D) in developing countries are scarce. The aim of this study was to describe the frequency of DKA at the onset of T1D in children and adolescents in Jordan and to compare the clinical and biochemical characteristics between the group that presented with DKA and the group that did not.

**Methods:** The records of 341 children and adolescents, less than sixteen years of age, who were diagnosed with T1D between 2015 and 2019 were evaluated retrospectively.

**Results:** Of all the children diagnosed with T1D, 108 (31.7%) presented with DKA. The majority had mild or moderate DKA (38% and 33.3% respectively). Higher paternal education levels were associated with a lower probability of presenting with DKA ($p = 0.043$). A family history of T1D had a protective effect on the occurrence of DKA (Odds ratio = 2.138; 95% confidence interval = 1.167-3.917, $p = 0.014$). Patients with celiac disease and higher HbA1c levels were more likely to experience recurrent episodes of DKA, ($p = 0.004$ and 0.011, respectively).

**Conclusion:** In Jordan, the rate of DKA at presentation of T1D remains high. Prevention campaigns are needed to increase diabetes awareness among the public and healthcare providers.

**Keywords:** Type one diabetes, diabetic ketoacidosis, Jordan

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## Introduction

Type one diabetes (T1D) is one of the most common chronic endocrine disorders which affects children and adolescents worldwide (1,2). Diabetic ketoacidosis (DKA) is a known acute complication of T1D which can be present at time of diagnosis or occur afterwards. It results from a deficiency of circulating insulin and increased levels of the counter regulatory hormones: catecholamines, glucagon, cortisol, and growth hormone (3). Despite the recent reported decrease in all-cause mortality in some populations with T1D, DKA remains the most common cause of death in children and adolescents with T1D (4,5). Moreover, DKA results in significant morbidity and is considered as a predictor of poor glycemic control (6,7). Overall mortality for children with DKA varies from 0.15 to 0.35% in developed countries (8,9) and from 3.4 to 13.4% in developing countries (10,11). In addition, there are parts of the world, such as some countries in Africa, where mortality rates at onset of T1D are under-reported and might be much higher. This could be due to the inability of families to promptly reach medical care for reasons related to unavailability or remote access. Globally, reported DKA rates at the time of T1D diagnosis vary from 14.7% to 79.8% (12). Data from the middle eastern region are scarce and a systematic review by Zayed (13) showed DKA rates between 17% and 100% at the time of T1D diagnosis in various middle eastern countries. In Jordan, we previously reported a DKA rate of 40.7% at the time of T1D diagnosis (14).

The aim of our study was to describe the frequency of DKA at the onset of T1D in children in Jordan in comparison with earlier data, to compare the clinical and biochemical characteristics of children and adolescents who presented with DKA at diagnosis of T1D and those who did not, to compare children who had recurrent episodes of DKA after diagnosis with the rest of the cohort, and to identify the risk factors associated with DKA development.

## Methods

### Subjects and Study Design

This was a retrospective cohort study of all children and adolescents who were less than 16 years of age at diagnosis of T1D at Jordan University Hospital from January, 2015 to December, 2019. The electronic medical records of 341 children who presented to our service were reviewed and their data were retrieved after obtaining approval from Institutional Ethics Committee of Jordan University Hospital, Amman, Jordan (approval no.: 99/2021, dated: 14/03/2021). Any type of diabetes other than T1D was excluded from this study.

Socio-demographic data included: birth date, sex, date of T1D diagnosis, presenting symptoms, family history of T1D and type 2 diabetes (T2D) in first and second-degree relatives and the levels of education and the occupations of both parents. Families with missing data were contacted by phone. Laboratory investigations at diagnosis were collected including: venous blood gas results, electrolytes, creatinine, blood glucose levels, glycosylated hemoglobin (HbA1c), glutamic acid decarboxylase antibodies (GAD Ab), islet cell antibodies, thyroid peroxidase antibodies (TPO), thyroglobulin antibodies, tissue transglutaminase IgA antibodies, thyroid stimulating hormone and free thyroxine.

DKA with its various levels of severity were defined according to the International Society for Pediatric and Adolescent Diabetes guidelines 2018 as follows: hyperglycemia [blood glucose $>11$ mmol/L (200 mg/dL)], venous pH $<7.3$ or serum bicarbonate $<15$ mmol/L with ketonemia or ketonuria. Mild DKA was defined as venous pH $<7.3$ or serum bicarbonate $<15$ mmol/L, moderate DKA as pH $<7.2$ or serum bicarbonate $<10$ mmol/L and severe DKA as pH $<7.1$ or serum bicarbonate $<5$ mmol/L (15).

The cohort was divided into two groups: the *DKA at onset of T1D group* and the *no DKA at onset of T1D group*. Both groups were compared with each other in terms of age at diagnosis, sex, season of presentation, presenting signs and symptoms, family history of T1D and/or T2D, the education levels and the occupations of both parents and their laboratory investigations. The group of patients who developed two or more DKA episodes excluding the one at presentation were termed the recurrent DKA group and they were also compared with the rest of the cohort.

### Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). Continuous data were presented as mean $\pm$ standard deviation, and categorical data as frequency (%). Associations between categorical variables were evaluated using chi-squared analysis. Associations between continuous variables were evaluated using the independent samples t-test. Univariate and multivariate logistic regression was used to assess possible predictors of dichotomous dependent variables. Statistical significance was assumed for p values less than 0.05.

## Results

A total of 341 children were enrolled in this study, 161 (47.2%) were males. The average age of the children was 11.03 ± 3.88 years and the average duration of T1D was 2.75 ± 1.47 years. Almost one third of the children had DKA at time of diagnosis, 108 (31.7%). Several characteristics and symptoms were compared between the group which presented with DKA at T1D diagnosis and the group that did not; age at diagnosis, sex, polyurea, polydipsia, enuresis, and weight loss were not significantly different in the participants of both groups. However, abdominal pain, vomiting, and rapid breathing were significantly higher in the DKA at onset group, p = 0.015, 0.017, 0.008 respectively.

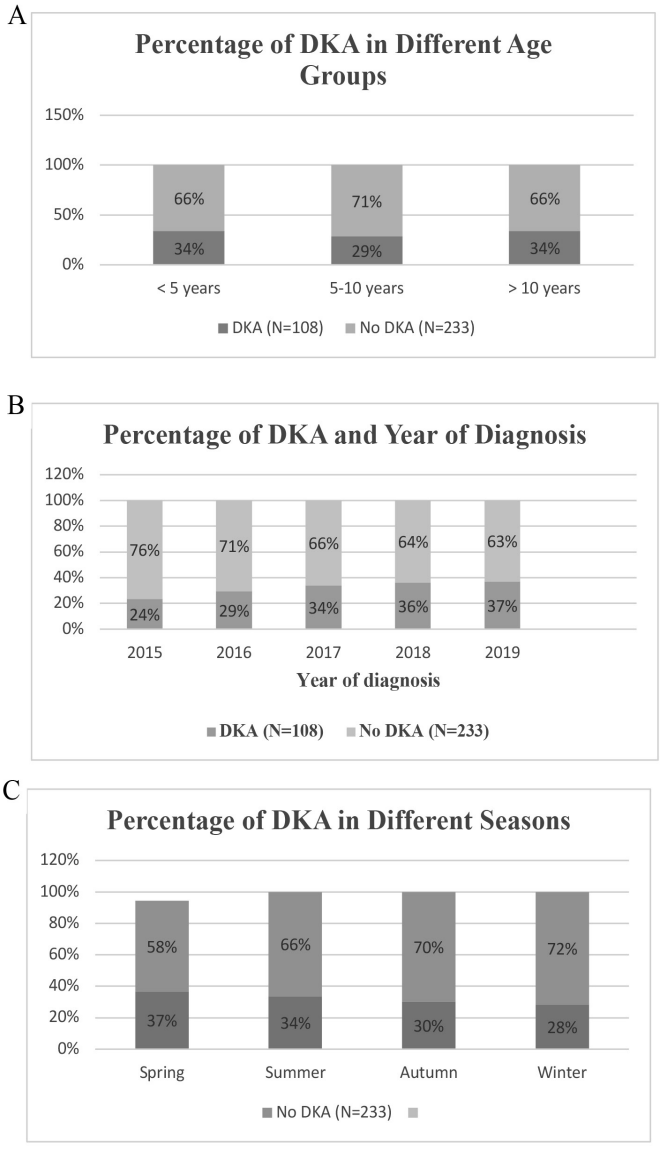


**Figure 1.** Frequency of DKA according to age at diagnosis (A), year of diagnosis (B), and different seasons (C). All were statistically non-significant

*DKA: diabetic ketoacidosis*

The frequency of DKA in different age groups, different years of diagnosis, and different seasons of the year were all statistically non-significant, p = 0.563, 0.578, and 0.654, respectively, Figure 1.

Further analysis of the years of diagnosis showed that the age at diagnosis, presences of celiac disease, recurrent DKA and HbA1c at diagnosis were all statistically non-significant, p = 0.424, 0.325, 0.372, 0.955, respectively.

When the children were categorized into two groups according to age, as two years or younger and older than two years, the difference in the frequency of DKA neared significance with a p value of 0.056. Results of the analysis showed that 50.0% of the children who were two years or younger presented with DKA compared to 30.4% in those children older than two years.

Different laboratory tests were evaluated, and values among children who had DKA at time of diagnosis and those who did not were compared. Creatinine levels, glucose levels, and HbA1c at diagnosis were significantly higher in those children who had DKA at time of diagnosis, Table 1.

The socioeconomic status of both groups (with/without DKA) was compared. Variables included paternal and maternal occupations and education levels, Table 2.

Among the 108 children who had DKA at the time of T1D diagnosis; 41 (38.0%) had mild DKA, 36 (33.3%) had moderate DKA and 31 (28.7%) had severe DKA. Further analysis revealed that the severity of DKA was not associated with sex or age. Degrees of severity of DKA in males and females and in the different age groups were compared, and there were no statistically significant differences, p values = 0.857 and 0.998, respectively.

Possible predictors of DKA at diagnosis were analyzed, and family history of T1D was the only statistically significant predictor of DKA. Children with no family history of T1D were two times more likely to present with DKA than those with a family history of T1D (OR = 2.138, p = 0.014), Table 3.

Thirty-eight children (11.1%) had recurrent episodes of DKA. Those with celiac disease had a significantly higher percentage of recurrent DKA, 23.7%, p = 0.004. In addition, those children with recurrent DKA had a significantly higher HbA1c than those without recurrent DKA, 8.85% and 7.97%, respectively p = 0.011, Table 4.

## Discussion

The results from this study identified an association between presentation with DKA at T1D onset and lower paternal education and employment levels. In addition, having a positive family history of T1D was protective against the development of DKA at T1D diagnosis. Furthermore, higher levels of HbA1c and having celiac disease as a comorbidity were associated with recurrent episodes of DKA.

### Rate of DKA at Diagnosis of T1D with Regional and International Comparison

In our analysis, the DKA rate at manifestation of T1D in children and adolescents under 16 years of age was 31.7%. This rate is almost 10% less than our previously reported rate of 40.7% (14). This could be due to the fact that we are reporting from a tertiary hospital with an established pediatric diabetes practice which resulted in good awareness and the prompt recognition of diabetes symptoms. This is supported by a study from Kuwait which showed that DKA at onset of T1D was significantly more common in hospitals lacking a structured diabetes team ($p < 0.002$) (16). Studies from the Middle East region showed almost similar rates of DKA at onset of T1D ranging between 33.6% in Kuwait, 31% in Oman and 37.7% in Saudi Arabia (17,18,19). In Sudan, however, a recent study reported a DKA rate of 17.6% at diagnosis of T1D (20). This variation in the rates of DKA at diagnosis of T1D was also seen in developed countries ranging from 19.5% and 19.8% in Sweden and Germany to 41.2% and 43.8% in Italy and Luxembourg respectively (21,22). In the USA and the UK, these rates were 36.9% and 25% respectively (22). Countries with higher incidence of T1D and hence more awareness of this disease were reported to have lower rates of DKA at T1D diagnosis due to prompt diagnosis and early treatment initiation (23,24). Unfortunately, many Middle Eastern countries, including Jordan, lack T1D registries and both the incidence and prevalence rates are unknown. Many other factors were studied as contributors to presentation with DKA at the time of T1D diagnosis, such as age, sex, family history of T1D, ethnic background and the socioeconomic status of the families (25).

**Table 1. Laboratory characteristics of children in both groups**

| | DKA at presentation n = 108 n (%) | No DKA at presentation n = 233 n (%) | p value[Ω] |
|---|---|---|---|
| **TTG IgA, n = 245** | | | 0.393 |
| Positive | 17/88 (19.3) | 33/157 (21.0) | |
| Negative | 71/88 (80.7) | 124/157 (79.0) | |
| **TPO Ab, n = 224** | | | 0.979 |
| Positive | 12/81 (14.8) | 21/143 (14.7) | |
| Negative | 69/81 (85.2) | 122/143 (85.3) | |
| **TG Ab, n = 197** | | | 0.308 |
| Positive | 17/72 (23.6) | 22/125 (17.6) | |
| Negative | 55/72 (76.4) | 103/125 (82.4) | |
| **GAD Ab, n = 222** | | | 0.598 |
| Positive | 51/78 (65.4) | 89/144 (61.8) | |
| Negative | 27/78 (34.6) | 55/144 (38.2) | |
| **Islet cells Ab, n = 218** | | | 0.08 |
| Positive | 15/78 (19.2) | 15/140 (10.7) | |
| Negative | 63/78 (80.8) | 125/140 (89.3) | |
| | **DKA at presentation (mean ± SD)** | **No DKA at presentation (mean ± SD)** | **p value[α]** |
| Creatinine mg/dL | 0.64 ± 0.315 | 0.51 ± 0.23 | **0.005** |
| HbA1c % | 11.43 ± 1.915 | 10.78 ± 2.01 | **0.019** |
| Glucose mg/dL | 506.5 ± 172.3 | 440.3 ± 207.9 | **0.032** |
| Na at diagnosis mmol/L | 132.44 ± 5.85 | 133.07 ± 5.22 | 0.466 |
| K at diagnosis mmol/L | 4.89 ± 4.06 | 5.69 ± 12.59 | 0.631 |

[Ω]: chi-squared; [α]: independent sample t-test.
TTG IgA: anti-tissue transglutaminase IgA antibodies, TPO Ab: thyroid peroxidase antibodies, TG Ab: thyroglobulin antibodies, GAD Ab: glutamic acid decarboxylase antibodies, SD: stardard deviation, DKA: diabetic ketoacidosis

**Table 2. Socio-economic characteristics of children in both groups**

| | DKA at presentation n = 108 | No DKA at presentation n = 233 | p value[Ω] |
|---|---|---|---|
| **Paternal occupation, n = 326** | | | **0.010** |
| Professional | 46/106 (43.4) | 133/220 (60.5) | |
| Manual | 46/106 (43.4) | 75/220 (34.1) | |
| Unemployed | 9/106 (8.5) | 9/220 (4.1) | |
| Deceased | 5/106 (4.7) | 3/220 (1.4) | |
| **Paternal education level, n = 326** | | | **0.043** |
| No school/elementary/high school | 69/106 (65.1) | 125/220 (56.8) | |
| Higher than high school | 33/106 (31.1) | 93/220 (42.3) | |
| Death | 4/106 (3.8) | 2/220 (0.9) | |
| **Maternal occupation, n = 328** | | | 0.944 |
| Professional | 25/106 (23.6) | 54/222 (24.3) | |
| Manual | 3/106 (2.8) | 5/222 (2.3) | |
| Unemployed | 78/106 (73.6) | 163/222 (73.4) | |
| **Maternal education level, n = 328** | | | 0.199 |
| No school/elementary/high school | 79/106 (74.5) | 150/222 (67.6) | |
| Higher than high school | 27/106 (25.5) | 72/222 (32.4) | |
| Deceased | 0/106 (0) | 0/222 (0) | |
| **Parent marital status, n = 341** | | | 0.130 |
| Married | 99/108 (91.7) | 223/233 (95.7) | |
| Single parent | 9/108 (8.3) | 10/233 (4.3) | |
| **Family history of T1D, n = 341** | | | **0.022** |
| Yes | 17/108 (15.7) | 63/233 (27.0) | |
| No | 91/108 (84.3) | 170/233 (73.0) | |
| **Family history of T2D, n = 341** | | | 0.424 |
| Yes | 49/108 (45.4) | 95/233 (40.8) | |
| No | 59/108 (54.6) | 138/233 (59.2) | |

[Ω]: chi-squared.
DKA: diabetic ketoacidosis, T1D: type one diabetes, T2D: type 2 diabetes

## Factors Associated with the Development of DKA at Diagnosis of T1D

Several studies investigated the effect of the level of education and parental employment on the possibility of having DKA at T1D diagnosis with variable findings. In our study, we found that having a father with a higher educational level and/or working in a professional job was associated with a lower probability of presenting with DKA at onset of T1D. This is in support of other studies which linked higher educational and employment levels of at least one of the parents to a decreased likelihood of presenting with DKA at diagnosis of T1D (25). A study which was conducted in Italy showed significantly higher DKA frequencies (both overall and severe) in children of 0.5-4 years of age, with both a low level of mother's education and parents' occupation (26). An explanation to this could be that having a higher educational level might prompt the family to seek medical advice earlier upon recognition of symptoms suggestive of diabetes. In addition, having a professional job is usually linked to being medically insured.

As for the age at T1D diagnosis, we found an association between young age (below two years) and presentation with DKA with a near statistically significant p value of 0.056. This association has been reported by many other studies from different parts of the world (25,27). This could be due to many factors such as a lower index of suspicion of diabetes in this age group where the classical symptoms are not very clear to clinicians. In addition, this may be due to these children having a stronger humoral autoimmunity and aggressive destruction of beta cells compared to older age groups (28). This highlights the importance of raising awareness among healthcare professionals on the different patterns of presentation of diabetes in different age groups.

**Table 3. Predictors of diabetic ketoacidosis at diagnosis of type one diabetes**

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| **Sex** | **OR** | **95% CI** | **p value** | **OR** | **95% CI** | **p value** |
| Male (reference) | | | | | | |
| Female | 1.467 | 0.924-2.328 | 0.104 | 1.444 | 0.893-2.336 | 0.134 |
| **Age** | 0.990 | 0.931-1.053 | 0.748 | 0.983 | 0.921-1.049 | 0.596 |
| **Parents marital status** | | | | | | |
| Married (reference) | | | | | | |
| Single parent | 2.027 | 0.799-5.144 | 0.137 | 1.448 | 0.483-4.342 | 0.509 |
| **Paternal education level** | | | | | | |
| No school/elementary/high school (reference) | | | | | | |
| Higher than high school | 0.643 | 0.392-1.054 | 0.080 | 0.704 | 0.388-1.279 | 0.249 |
| Deceased | 3.623 | 0.647-20.287 | 0.143 | 2.932 | 0.416-20.663 | 0.280 |
| **Maternal education level** | | | | | | |
| No school/elementary/high school (reference) | | | | | | |
| Higher than high school | 0.712 | 0.424-1.197 | 0.200 | 0.832 | 0.442-1.567 | 0.569 |
| Deceased | | | | | | |
| **Family history of T1D** | | | | | | |
| Yes (reference) | | | | | | |
| No | 1.984 | 1.096-3.590 | 0.024 | 2.138 | 1.167-3.917 | **0.014** |

OR: odds ratio, CI: confidence interval, T1D: type one diabetes

In our cohort, having a family history of T1D was the only protective factor against presenting with DKA at diagnosis of T1D. This is probably due to increased awareness among families with prior experience of diabetes or having a family history of diabetes alerting clinicians to an increased possibility of T1D (16,25). This fact emphasizes the importance of awareness among parents and clinicians about the early symptoms of diabetes in preventing delays in diagnosis and hence the development of DKA. Studies have demonstrated that awareness campaigns were successful in reducing the percentage of children presenting with DKA at diagnosis of T1D. In Parma, Italy, during the 8 years of their campaign, the cumulative frequency of DKA dropped from 78% to 12.5% (29). This was replicated with a more modest impact in Saudi Arabia where, after launching a diabetes awareness campaign, DKA rates at diagnosis of T1D dropped from 48% in 2010 to 39% in 2014 (30).

### Factors Associated with the Development of Recurrent DKA

Recurrent episodes of DKA were associated with an increase in mortality rates up to 23.3% in people who have had more than five episodes of DKA compared to 5.2% in people with one episode (31). Many modifiable and non-modifiable risk factors for recurrent DKA have been identified and it is of high importance to recognize patients at risk and work on preventing further episodes of DKA (32). During the follow-up of our cohort, recurrent episodes of DKA were seen in those patients with higher HbA1c concentrations. There is strong evidence in the literature that elevated HbA1c level is a risk factor for recurrent DKA in children and adolescents with T1D (33,34). It is a marker of poor metabolic control which might be due to general problems with diabetes management, such as non-adherence or lack of knowledge. Hence, addressing these issues might contribute to a reduction in further episodes of DKA in this group of patients. The second association with recurrent DKA in our cohort was the diagnosis of celiac disease as a comorbidity with T1D. In a study from the DPV registry, 608 patients with biopsy proven celiac disease and T1D were followed for three years, and their celiac disease specific antibody status was observed. The group which reached antibody negative status had better HbA1c and lesser rates of DKA in comparison to the group which continued to have positive antibodies and in comparison to the general T1D group which did not have celiac disease. The authors suggested that the gluten free diet-adherent, antibody-negative patients were more compliant to therapy, in general, and hence, had better metabolic control (35). This might indicate worse adherence to management in our patients who have celiac disease in addition to T1D.

### Study Limitations

Limitations to our study are its retrospective design and the fact that it might not represent the whole country as it is reporting from a single tertiary center.

**Table 4. Characteristics of children with and without recurrent DKA**

| | **Recurrent DKA n = 38 n (%)** | **No recurrent DKA n = 303 n (%)** | **p value[α]** |
|---|---|---|---|
| **Sex** | | | 0.478 |
| Male | 20/38 (52.6) | 141/303 (46.5) | |
| Female | 18/38 (47.4) | 162/303 (53.5) | |
| **DKA at onset** | | | **0.003** |
| Yes | 20/38 (52.6%) | 88/303 (29.0%) | |
| No | 18/38 (47.4%) | 215/308 (71.0%) | |
| **Thyroid disease, n = 341** | | | 0.163 |
| Yes | 3/38 (7.9) | 10/303 (3.3) | |
| No | 35/38 (92.1) | 293/303 (96.7) | |
| **Celiac disease, n = 341** | | | **0.004** |
| Yes | 9/38 (23.7) | 26/303 (8.6) | |
| No | 29/38 (76.3) | 277/303 (91.4) | |
| **Paternal occupation, n = 326** | | | 0.805 |
| Professional | 20/38 (52.6) | 159/288 (55.2) | |
| Manual | 16/38 (42.1) | 105/288 (36.5) | |
| Unemployed | 1/38 (2.6) | 17/288 (5.9) | |
| Deceased | 1/38 (2.6) | 7/288 (2.4) | |
| **Paternal education level, n = 326** | | | 0.382 |
| No school/elementary/high school | 26/38 (68.4) | 168/288 (58.3) | |
| Higher than high school | 12/38 (31.6) | 114/288 (39.6) | |
| Deceased | 0/38 (0) | 6/288 (2.1) | |
| **Maternal occupation, n = 328** | | | 0.066 |
| Professional | 8/38 (21.1) | 71/290 (24.5) | |
| Manual | 3 (7.9) | 5 (1.7) | |
| Unemployed | 27 (71.1) | 214 (73.8) | |
| **Maternal education level, n = 328** | | | 0.860 |
| No school/elementary/high school | 27/38 (71.1) | 202/290 (69.7) | |
| Higher than high school | 11/38 (28.9) | 88/290 (30.3) | |
| Deceased | 0/38 (0) | 0/290 (0) | |
| **Parents marital status, n = 341** | | | 0.158 |
| Married | 34/38 (89.5) | 288/303 (95.0) | |
| Single parent | 4/38 (10.5) | 15/303 (5.0) | |
| | **Recurrent DKA (mean ± SD)** | **No recurrent DKA (mean ± SD)** | **p value[α]** |
| Current age | 10.59 ± 3.37 | 11.08 ± 3.94 | 0.464 |
| Age at diagnosis | 7.48 ± 3.53 | 8.30 ± 3.71 | 0.197 |
| HbA1c % (last year of follow up) | 8.85 ± 1.99 | 7.97 ± 1.78 | **0.011** |

[Ω]: chi-squared; [α]: independent sample t-test.
DKA: diabetic ketoacidosis, SD: stardard deviation

## Conclusion

Despite the reduction in our reported rate of DKA at T1D diagnosis, it is still considered high. Awareness campaigns for the public and the health care professionals should be implemented and continued in an effort to reduce the rates of DKA whether at T1D diagnosis or thereafter.

### Ethics

**Ethics Committee Approval:** The study was approved by the Institutional Ethics Committee of Jordan University Hospital, Amman, Jordan (approval no.: 99/2021, dated: 14/03/2021).

**Informed Consent:** Retrospective cohort study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Concept: Rasha Odeh, Abeer Alassaf, Design: Rasha Odeh, Abeer Alassaf, Data Collection or Processing: Lobna Gharaibeh, Bahaa Ashour, Fatima Al Barakat, Dina Dahabreh, Hiba Hadadin, Tala Melhem, Analysis or Interpretation: Rasha Odeh, Lobna Gharaibeh, Amirah Daher, Jumana Albaramki, Abeer Alassaf, Literature Search: Rasha Odeh, Amirah Daher, Jumana Albaramki, Bahaa Ashour, Fatima al Barakat, Dina Dahabreh, Hiba Hadadin, Tala Melhem, Writing: Rasha Odeh, Lobna Gharaibeh, Amirah Daher, Jumana Albaramki, Bahaa Ashour, Fatima al Barakat, Dina Dahabreh, Hiba Hadadin, Tala Melhem, Abeer Alassaf.

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# The Relationship Between Premature Adrenarche and Platelet Aggregation

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### What is already known on this topic?

Premature adrenarche (PA) has been associated with an increase in adrenal androgens. A hyperandrogenic hormonal environment is known to lead to increased platelet (PLT) aggregation.

### What this study adds?

We have shown increased collagen-induced PLT aggregation in girls with PA. This is significant as PA may cause increased cardiovascular event risks later in life due to increased PLT aggregation.

## Abstract

**Objective:** Premature adrenarche (PA) has been associated with an increase in adrenal androgens, and the hyperandrogenic hormonal environment is known to lead to increased platelet (PLT) aggregation. Here, we evaluated the effects of PA on PLT aggregation in PLT-rich plasma samples from female patients.

**Methods:** The study included 40 female patients diagnosed with PA between February, 2014 and June, 2018 and 30 healthy female individuals as a control group. Adenosine diphosphate (ADP) and collagen-induced PLT aggregation were studied via the photometric aggregometry method.

**Results:** There were no significant differences in the PLT count or volume values between those participants with PA and the control group. Additionally, the ADP-induced maximum aggregation time, value, and slope values did not significantly differ between the patient and control groups ($p > 0.05$). However, the collagen-induced maximum aggregation time, value, and slope values were significantly higher in the studygroup ($p < 0.001$).

**Conclusion:** Increased collagen-induced PLT aggregation was detected in female patients with PA. As PA is associated with a higher risk of cardiovascular events later in life, close follow-up of PA in this respect may be beneficial.

**Keywords:** Premature adrenarche, cardiovascular diseases, hyperandrogenism, platelet aggregation, ADP, collagen

## Introduction

Premature adrenarche (PA) has been associated with an increase in adrenal androgens due to the premature maturation of the zona reticularis layer of the adrenal cortex before the age of 8 in girls and 9 in boys (1). The most secreted androgens from the adrenal gland are dehydroepiandrosterone (DHEA) and androstenedione, which are weak androgens. DHEA undergoes sulfation in the liver and becomes DHEA-sulfate (DHEAS), and it is considered a marker of adrenal androgenic activity (2).

Girls with PA are at higher risk of developing symptoms of metabolic syndrome, including obesity and type 2 diabetes, and cardiovascular disease later in life (3). The mechanisms

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underlying these relationships remain unclear but have been partially associated with excess adipose tissue in adulthood (4). Indeed, earlier puberty is predictive of a higher adult body mass index (BMI) and a greater risk of obesity in women (5).

Cardiovascular diseases cause significant morbidity and mortality worldwide. Risk scoring systems have been developed to identify people at high risk of developing an adverse cardiovascular event by evaluating known risk factors (6). However, many patients whose risk assessments for cardiovascular diseases are determined to be at low or moderate levels may experience a cardiovascular event. Platelet (PLT) activity, which is not routinely found in current risk score algorithms, may also be a factor increasing cardiovascular risk (7).

The relationship between PLT aggregation and cardiovascular events has been evaluated in several studies (8). Different measures of PLT separation and purification methods and PLT aggregation measurements with different agonists at varying concentrations have been used in most of these studies (8). Although the data on increased PLT aggregation leading to cardiovascular events are far from conclusive, some significant results have been reported (8). A moderate increase in spontaneous PLT aggregation was detected in vascular events (8,9). It was recently shown that having an increased PLT aggregation response is associated with future arterial thrombosis, and the incidence of coronary heart disease-related mortality may increase significantly in these individuals (10).

Although there are various studies in children and adolescents regarding PLT counts and coagulation factors, as well as various PLT aggregation studies regarding non-hematological diseases (11,12,13,14), there are no studies on PLT counts and PLT aggregation in girls with PA.

In this case-control study, we investigated how PLT counts and aggregation are affected by adenosine diphosphate (ADP) and collagen agonists in girls with PA.

## Methods

The patient group of this case-control study included 40 female patients diagnosed with PA between February, 2012 and June, 2018 (Group 1) at the pediatric outpatient clinic of Gülhane Training and Research Hospital (Ankara, Turkey). After the cases were diagnosed with PA, the relevant laboratory studies were conducted prospectively, and the cases and controls were followed up over a 6-year period. Girls with PA who had at least one clinical sign of adrenal androgen action (i.e., adult-type body odor, greasiness of hair and skin, comedones/acne, and axillary or pubic hair) together with increased DHEAS secretion before the age of 8 years, and other sources of hyperandrogenism (including central puberty, congenital adrenal hyperplasia, and androgen-producing tumors) were excluded. DHEAS concentrations of >40 µg/dL were considered adrenarche (15). Thirty healthy female individuals were included as the control group (Group 2). All girls in the control group were healthy, did not use any medication, and did not have any premature signs of androgen action.

Written informed consent from the families of the patients and approval of the Gülhane Training and Research Hospital Local Ethics Committee (date: 30.06.2009, no: 135) were obtained.

The inclusion criteria for both groups of patients were as follows: no use of antiplatelet drugs within the last 30 days, and no hematological diseases, chronic heart, kidney, and/or liver diseases (excluding PA in the patient group).

Complete blood count results, DHEAS, DHEA, luteinizing hormone (LH), 17-hydroxyprogesterone (17-OH progesterone), 11-deoxycortisol, adrenocorticotropic hormone (ACTH) and cortisol hormone levels, ferritin levels, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen levels, and PLT aggregation were analyzed in both groups. Hormone levels were measured with the chemiluminescence method using the Beckman Coulter DxI® 600 analyzer.

Erythrocyte indices, PLT counts, and mean PLT volume (MPV) values were obtained using an automated device (Technicon H-1 System, Technicon Co, Tournai, Belgium).

Venous blood samples were obtained from the antecubital vein citrate between 8:00 and 9:30 in the morning after 8-12 hours of night fasting and collected into plastic syringes containing 3.8% trisodium by 1/10 volume. PLT-rich and PLT-poor plasma were prepared by centrifugation (16). PLT aggregation was assessed by photometric aggregometry using a complete blood aggregometer (Model 560; Chrono-Log Corporation, Havertown, PA, USA).

Collagen (5 µg/mL, Chrono Par No: 385; Chrono-Log Corporation) and ADP (10 µmol, Chrono Par No: 384; Chrono-Log Corporation) were used as agonists. The maximum aggregation time (s), value (%), and slope (%/min) were determined from the aggregation curves. The effects of ADP and collagen on aggregation were evaluated in both the control and patient groups considering the effect of iron deficiency on aggregation (17,18).

### Statistical Analysis

The statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) software (version 22; SPSS Inc., Chicago, IL, USA). The data are presented as mean and standard deviation values. The normally distributed data were compared by independent samples t-test. We analyzed the correlations of DHEAS and DHEA levels with aggregation using the Pearson correlation test, and we used regression analysis for the aggregation values. Differences were considered statistically significant at $p < 0.05$.

## Results

Forty female patients diagnosed with PA were included in the patient group (Group 1), and thirty healthy female individuals constituted the control group (Group 2). The demographic characteristics of Groups 1 and 2 are given in Table 1. There were no significant differences between the groups in terms of age, height, weight, or BMI ($p > 0.05$).

The complete blood count parameters of Groups 1 and 2 are given in Table 2. There were no significant differences between the groups in terms of their white blood cell count, red blood cell count, hemoglobin (HGB) level, hematocrit level, mean erythrocyte volume, mean erythrocyte HGB level, mean erythrocyte HGB concentration, red cell distribution width, PLT count, or MPV values ($p > 0.05$).

There was no significant difference between Groups 1 and 2 in terms of their plasma ferritin level, PT, aPTT, fibrinogen level, LH, 17-OH progesterone, 11-deoxycortisol, ACTH, or cortisol hormone level values ($p > 0.05$); however, in the patient group, DHEAS and DHEA levels were significantly higher than of those in the control group ($p < 0.05$; Table 3).

The mean maximum aggregation time, value, and slope induced by 10 µmol ADP and 5 µg/mL collagen in Groups 1 and 2 are shown in Table 4. In the patient group, at 10 µmol ADP, the mean maximum aggregation time, value, and slope did not significantly differ from the values in the control group ($p > 0.05$). However, in the patient group, at a collagen concentration of 5 µg/mL, the mean maximum aggregation time, value, and slope were significantly higher than of those in the control group ($p = 0.001$, 0.002, and 0.04, respectively). DHEAS was positively correlated with the maximum aggregation time ($r = 0.446$, $p = 0.013$), maximum aggregation value ($r = 0.397$, $p = 0.018$), and slope ($r = 0.263$, $p = 0.034$) values in collagen-induced PLT aggregation in the patients with PA. Similarly, DHEA was positively correlated with the maximum aggregation time ($r = 0.356$, $p = 0.024$), maximum aggregation value ($r = 0.308$, $p = 0.029$), and slope ($r = 0.217$, $p = 0.039$) values in collagen-induced PLT aggregation (Table 5). The results of the multivariate logistic regression analysis revealed that the maximum aggregation time [Odds ratio (OR); 95% confidence interval (CI) 1.42 (1.05–2.11);

**Table 1. Demographic characteristics of the study and control groups**

| | Study group | Control group | |
|---|---|---|---|
| **Patient characteristics** | **n = 40** | **n = 30** | **p value** |
| Age (year) | 7.38 ± 0.08 | 7.50 ± 0.09 | 0.321 |
| Height SDS | 0.79 ± 0.22 | 0.75 ± 0.20 | 0.944 |
| Weight SDS | 0.51 ± 0.12 | 0.56 ± 0.13 | 0.476 |
| BMI SDS | 0.22 ± 0.04 | 0.24 ± 0.03 | 0.216 |

Data are given as mean ± SD.
BMI: body mass index, SDS: standard deviation (SD) score

**Table 2. Complete blood parameters in the study and control groups**

| | Study group | Control group | |
|---|---|---|---|
| **Parameters** | **n = 40** | **n = 30** | **p value** |
| WBC (/µL) | 6,370 ± 188 | 6,433 ± 269 | 1.00 |
| RBC ($\times 10^6$/µL) | 4.85 ± 0.05 | 4.93 ± 0.06 | 0.117 |
| HGB (g/dL) | 13.07 ± 0.10 | 12.93 ± 0.13 | 0.376 |
| HCT (%) | 38.84 ± 0.44 | 39.19 ± 0.54 | 0.714 |
| MCV (fL) | 80.86 ± 0.49 | 80.88 ± 0.50 | 0.733 |
| MCH (pg) | 27.23 ± 0.17 | 27.02 ± 0.26 | 0.737 |
| MCHC (g/dL) | 33.69 ± 0.21 | 33.30 ± 0.19 | 0.13 |
| RDW (%) | 13.64 ± 0.19 | 13.50 ± 0.14 | 0.81 |
| PLT (/µL) | 308621 ± 932 | 282100 ± 131 | 0.58 |
| MPV (fL) | 8.97 ± 0.86 | 9.21 ± 1.01 | 0.17 |

Data are given as mean ± standard deviation.
WBC: white blood cell, RBC: red blood cell, HGB: hemoglobin, HCT: hematocrit, MCV: mean erythrocyte volume, MCH: mean erythrocyte hemoglobin, MCHC: mean erythrocyte hemoglobin concentration, RDW: red cell distribution width, PLT: platelet, MPV: mean platelet volume

p = 0.002], maximum aggregation value [OR; 95% CI 1.33 (1.07–1.76); p = 0.003], and slope [OR; 95% CI 1.06 (1.01–1.13); p = 0.03] in collagen-induced PLT aggregation were associated with PA in the patient group (Table 6).

## Discussion

To the best of our knowledge, there is no study in the literature concerning PLT counts and PLT aggregation in girls with PA. In this study, we provide evidence of increased collagen-induced PLT aggregation in girls with PA.

Girls with a history of PA display a hyperandrogenic hormonal environment which may lead to increased cardiovascular risk (19,20,21,22,23,24,25). Çelik et al. (20) detected early atherosclerotic changes and subclinical deterioration in cardiac functions in children with PA. However, it was not fully explained why children with PA tend to show early cardiovascular changes. The authors showed that PA increased the risk of coronary heart disease, and this result was attributed to increased carotid intima-media thickness and epicardial adipose tissue measurements in PA patients. The increased risk of coronary heart disease in girls with PA in general was partly associated with the excess

**Table 3. DHEAS, DHEA, LH, 17-OH progesterone, 11-deoxycortisol, ferritin level, PT, aPTT, and fibrinogen level in the study and control groups**

| | Study group | Control group | |
|---|---|---|---|
| **Parameters (± SD)** | **n = 40** | **n = 30** | **p value** |
| Ferritin (ng/mL) | 29.18 ± 1.71 | 36.97 ± 4.73 | 0.61 |
| PT (s) | 13.66 ± 0.16 | 13.71 ± 0.22 | 0.808 |
| aPTT (s) | 30.00 ± 0.27 | 30.48 ± 0.29 | 0.222 |
| Fibrinogen (mg/dL) | 290 ± 6.40 | 293 ± 7.88 | 0.980 |
| DHEAS (µg/dL) | 69.1 ± 13.20 | 26 ± 8.30 | ***0.002*** |
| DHEA (ng/dL) | 149.2 ± 12.57 | 106.6 ± 9.45 | ***0.003*** |
| LH (mlU/mL) | 0.1 ± 0.03 | 0.1 ± 0.02 | 0.79 |
| 17-OH progesterone (ng/mL) | 0.79 ± 0.07 | 0.51 ± 0.05 | 0.09 |
| 11-deoxycortisol (ng/mL) | 3.1 ± 0.3 | 2.2 ± 0.4 | 0.11 |
| Cortisol (µg/dL) | 12.1 ± 3.12 | 14.3 ± 3.25 | 0.35 |

Data are given as mean ± SD.
PT: prothrombin time, aPTT: activated partial thromboplastin time, DHEAS: dehydroepiandrosterone-sulfate, DHEA: dehydroepiandrosterone, LH: luteinizing hormone, 17-OH progesterone: 17-hydroxyprogesterone, SD: standard deviation

**Table 4. Platelet aggregation parameters of the study and control groups**

| | Study group | Control group | |
|---|---|---|---|
| **Agonist** | **n = 40** | **n = 30** | **p value** |
| **ADP (10 µmol)** | | | |
| Maximum aggregation time (s) | 342.50 ± 15.49 | 339.75 ± 12.89 | 0.975 |
| Maximum aggregation value (%) | 74.73 ± 2.07 | 70.63 ± 2.35 | 0.16 |
| Slope (%/min) | 114.30 ± 7.36 | 98.98 ± 3.98 | 0.09 |
| **Collagen (5 µg/mL)** | | | |
| Maximum aggregation time (s) | 405.00 ± 14.64 | 337.83 ± 10.54 | ***0.001*** |
| Maximum aggregation value (%) | 74.95 ± 1.27 | 66.20 ± 2.33 | ***0.002*** |
| Slope (%/min) | 127.46 ± 4.69 | 111.30 ± 6.44 | ***0.04*** |

Data are given as mean ± standard deviation.
ADP: adenosine diphosphate

**Table 5. Correlation analysis of androgen levels and platelet aggregation parameters in patients with PA**

| | DHEAS | DHEA |
|---|---|---|
| **Agonist** | **r (p)** | **r (p)** |
| **ADP** | | |
| Maximum aggregation time | 0.044 (0.65) | 0.036 (0.71) |
| Maximum aggregation value | 0.087 (0.22) | 0.073 (0.31) |
| Slope | 0.151 (0.08) | 0.136 (0.09) |
| **Collagen** | | |
| Maximum aggregation time | ***0.446 (0.013)*** | ***0.356 (0.024)*** |
| Maximum aggregation value | ***0.397 (0.018)*** | ***0.308 (0.029)*** |
| Slope | ***0.263 (0.034)*** | ***0.217 (0.039)*** |

ADP: adenosine diphosphate, DHEAS: dehydroepiandrosterone-sulfate, DHEA: dehydroepiandrosterone, PA: premature adrenarche

**Table 6. Multivariable logistic regression analysis for PA in the study group**

| Collagen | OR | 95% CI | p value |
|---|---|---|---|
| Maximum aggregation time | **1.42** | **1.04-2.11** | **0.002** |
| Maximum aggregation value | **1.33** | **1.07-1.76** | **0.003** |
| Slope | **1.06** | **1.01-1.13** | **0.03** |
| **ADP** | | | |
| Maximum aggregation time | 1.01 | 0.92-1.13 | 0.39 |
| Maximum aggregation value | 1.09 | 0.96-1.22 | 0.27 |
| Slope | 1.04 | 0.93-1.16 | 0.15 |

ADP: adenosine diphosphate, OR: Odds ratio, CI: confidence interval, PA: premature adrenarche

adipose tissue found in these patients in adulthood (5). It is thought that a process beginning with childhood obesity may ultimately lead to cardiovascular diseases in later life, in association with PA and adult obesity (21,22,23). The relationship of an atherogenic abnormal lipid profile such as increased serum triglyceride and low-density lipoprotein cholesterol with PA has also been demonstrated (24,25). Moreover, Topaktaş et al. (26) showed that cholesterol and triglyceride-related arterial involvement caused by obesity and metabolic syndrome are effective in the pathogenesis of arterial stiffness in PA, rather than increasing androgens.

Along with obesity and abnormal lipid status, it was found that a hyperandrogenic hormonal environment is also associated with PLT aggregation. It is known that sex steroids are absorbed at PLT membranes, and they modify the surface properties of these membranes. These modifications induce permeability changes (27). Sex steroids may also interact with fibrinogen, plasminogen, or fibrinolytic inhibitors (28). For instance, it was reported that testosterone increases the concentration of plasma prostaglandins (29). Furthermore, it was demonstrated that PLT aggregation induced by arachidonic acid (30) is enhanced after androgens are added under *in vitro* conditions (31). This effect of testosterone on PLT aggregation leads to an increase in thrombus formation and mortality. In a rat study, arterial thrombosis development was observed after the administration of testosterone (32). Human studies have also demonstrated that increased testosterone levels affect the induction of PLT aggregation. It was speculated that this effect might have resulted via testosterone receptors in PLTs (33). The decrease in the incidence of acute coronary syndrome in men with prostate cancer due to flutamide use was associated with these results (34).

Although sexual maturity has been shown to affect PLT aggregation in pigs, no study has investigated the role of PLT aggregation in the increased risk of cardiovascular disease in later life, either in girls or older women with a history of PA (35). In our study, we demonstrated a relationship between PA and collagen-induced PLT aggregation. In PA patients, mainly DHEA and DHEAS levels are increased, and these androgens are known as weak androgens. However, it is known that the most potent androgens have the greatest aggregation-increasing effect (33). This effect was more significant in collagen-induced PLT aggregation than ADP-induced PLT aggregation (36). In studies with weak androgens, inhibition, rather than an increase in PLT aggregation, has been observed. In a previous study, the *in vitro* administration of DHEAS showed dose- and time-dependent inhibition in arachidonate-induced PLT aggregation (37). In another study, the administration of DHEAS at physiological doses with thrombin or supraphysiological doses with collagen, thrombin, and TxA2 analog U-46619 also inhibited PLT aggregation (38). The difference in the results of our study may be due to the different potencies of DHEA and DHEAS in children.

In our study, we showed that collagen-induced PLT aggregation was increased in those girls with PA, and this may be due to increased DHEAS levels. Collagen is a strong agonist, but ADP is a weak one (39). In our study, ADP was used at a concentration of 10 µmol, which is sufficient for PLT aggregation (39). We did not detect any changes in aggregation at this level of ADP, but there was an increase in collagen, suggesting selectivity for collagen-induced PLT activation pathways. Similar to our findings, Leng et al. (40) achieved an increase in collagen-induced PLT aggregation, but not thrombin-induced PLT aggregation, by giving different estrogen derivatives to ovariectomized mice, which was done to examine the effects of estrogens on arterial thrombosis. In their study, estrogens acted in an agonist-specific fashion which changed collagen-induced PLT surface glycoprotein-VI expression, which then initiated adhesion followed by aggregation. Collagen, unlike ADP, also stimulates the release of thromboxane A2 (TxA2), a strong aggregation agent (41). Several studies have shown that adrenal androgens increase the number of TxA2 receptors (42,43). This may indicate that the increased collagen-induced PLT aggregation in our study may be due to increased adrenal androgens via glycoprotein-VI and/or TxA2. In order to explain this, studies showing the effects

of androgens on PLT surface glycoprotein-VI expression are needed.

There are several studies which revealed normal and increased PLT counts in healthy people using anabolic androgenic steroids (43,44,45). In a study of 25-month-old Sprague-Dawley rats, a slight increase in PLT numbers was found with DHEAS treatment (46). In our study, we found no significant difference between the patient and control groups in terms of PLT counts and volumes.

Although an increased risk of coronary heart disease in girls with PA history is partly associated with the excess adipose tissue and the increased atherogenic lipid profile found in adulthood in these individuals, the increased PLT aggregation we found in girls with PA may ultimately lead to cardiovascular disease.

### Study Limitations

Some limitations of our study were 1) the small sample size of our study may have been insufficient in the evaluation of PLT aggregation; 2) other high-risk factors for cardiovascular events were not discussed in detail in our study, and 3) it is not known how long the increase in PLT aggregation detected in these children with PA would last and whether this increase in aggregation would continue when they reach the normal age of puberty as we did not study the results of this test again in these children.

## Conclusion

In conclusion, increased collagen-induced PLT aggregation was detected in girls with PA. As PA is associated with a higher risk of cardiovascular events later in life, close follow-up of PA may be beneficial. Repeated studies of PLT aggregation in patients with PA are needed in order to demonstrate whether the increase in collagen-induced PLT aggregation persists later in life.

### Ethics

**Ethics Committee Approval:** The study was approved by the Gülhane Training and Research Hospital Local Ethics Committee (date: 30.06.2009, no: 135).

**Informed Consent:** Written informed consent from the families of the patients.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Onur Akın, Concept: Orhan Gürsel, Mehmet Emre Taşçılar, Design: Ahmet Bolat, Cengiz Zeybek, Data Collection or Processing: Ahmet Bolat, Analysis or Interpretation: Ahmet Bolat, Literature Search: Ahmet Bolat, Cengiz Zeybek, Onur Akın, Writing: Ahmet Bolat, Cengiz Zeybek.

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# Factors Associated with Low Bone Mineral Density at the Time of Diagnosis in Children with Celiac Disease

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**What is already known on this topic?**

It has been reported in many studies that some children diagnosed with Celiac disease (CD) have low bone mineral density (BMD). However, which subgroup of children with CD is at risk for low BMD is still controversial.

**What this study adds?**

This study showed that in children newly diagnosed with CD, age at diagnosis, gender, body size, Celiac symptoms, biochemical parameters, tissue transglutaminase antibody-IgA level, human leukocyte antigen type and histopathological stage have no predictive values for low BMD.

## Abstract

**Objective:** It has been reported that bone mineral density (BMD) is decreased in children with Celiac disease (CD) compared to their healthy peers. The aim of this study was to reveal possible risk factors for low BMD in Turkish children newly diagnosed with CD.

**Methods:** Eighty-six patients (2-18 years old) with CD were included in this retrospective study. The relationship between their lumbar BMD z-scores calculated according to their chronological age (CA) and height age (HA) and their clinical, laboratory [biochemical parameters, tissue transglutaminase antibody-IgA (TTGA) levels, human leukocyte antigen (HLA) types] and histopathological parameters were evaluated.

**Results:** The mean age of the patients at diagnosis was 8.06 ± 4.08 years. The BMD z-score CA was ≤-2 standard deviation (SD) in 26.7% of the patients. The BMD z-score HA was ≤-2 SD in 12.8% of the patients. The BMD z-score HA only correlated with their age at diagnosis of CD ($r_s$ value 0.269). However, there was no statistically difference between the BMD z-score HA >-2 SD and ≤-2 SD subgroups regarding their clinical, laboratory and histopathological parameters.

**Conclusion:** Low BMD is common in children with newly diagnosed CD. Age at diagnosis, gender, body size, Celiac symptoms, biochemical parameters, TTGA level, HLA type, and histopathological stage had no predictive values in terms of low BMD in this patient group.

**Keywords:** Low bone mineral density, Celiac disease, children, risk factors

## Introduction

Celiac disease (CD) is a chronic disease characterized by inflammation of the proximal small intestine triggered by gluten in wheat, barley and rye in genetically predisposed individuals (1,2). Patients may present with typical findings such as abdominal distension, inability to gain weight, loss of appetite, vomiting, and diarrhea, or with atypical findings such as short stature, treatment-resistant iron deficiency anemia, and/or delayed puberty (3). One of the most common complications of CD is metabolic bone disease (4). It has been reported in many studies that some of the children diagnosed with CD have low bone mineral density (BMD) (5,6,7). However, the necessity of screening

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for low BMD with dual energy X-ray absorptiometry (DEXA) method at diagnosis or during follow-up in pediatric CD is still controversial. In order to decide who should be screened in a cost-effective way, we aimed to investigate which subgroups of children with CD are at risk of low BMD. In the literature, several factors including hypocalcemia due to malabsorption, vitamin D deficiency, secondary hyperparathyroidism, low physical activity due to fatigue, autoimmune factors, and inflammation have been suggested to be associated with low BMD. However, in most of these studies, the number of cases was limited and results were varied (4,8,9,10,11,12). In a recent retrospective cohort study, the only difference between the group with the BMD z-score of <-2 standard deviation (SD) and the rest of the cases was the low (<-0.4) standard deviation of the body mass index (BMI) (13).

In this study, it was aimed to determine the frequency of low BMD at the time of diagnosis and to investigate those parameters which can predict low BMD in Turkish children diagnosed with CD.

## Methods

In this retrospective study, a total of 99 patients, aged 2-18 years, who were diagnosed with CD histopathologically and whose BMD was measured at admission to İnönü University Pediatric Gastroenterology, Hepatology and Nutrition Clinic, between 2010 and 2019, were determined. Following this, a total of 13 patients with an additional diagnosis of type 1 diabetes mellitus, hypothyroidism, hyperthyroidism, Turner syndrome and/or precocious puberty (which could affect BMD) were excluded from this study in order to avoid interpretation difficulties. For the remaining 86 patients, the demographic and clinical features of the patients at diagnosis (age, gender, complaints at presentation, height, weight and BMI), laboratory data [serum calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), 25-OH vitamin D, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), ferritin, vitamin B12, folate, zinc, tissue transglutaminase antibody-IgA (TTGA), human leukocyte antigen (HLA)-DQ2, and HLA-DQ8], histopathological results of endoscopic biopsies, and lumbal (L1-L4) BMD levels measured by the DEXA method were examined from the hospital information system, retrospectively.

The diagnosis of CD was made according to the revised criteria of the European Committee of Pediatric Gastroenterology, Hepatology and Nutrition (14). TTGA titer was considered positive if it was increased three times or more above the laboratory upper limit of 18 AU/mL. The modified Marsh-Oberhuber classification was used for histopathological staging (15) and patients with stage ≥2 were diagnosed with CD. Type of admission was classified as typical in patients presenting with classical gastrointestinal symptoms such as chronic diarrhea and it was classified as atypical in those who presented with other symptoms, such as short stature or anemia (14). The SD scores of the weight, height, and BMI values of the patients [weight SD (WSD), height SD (HSD), BMI SD] and height age (HA) were calculated according to the Turkish percentile charts (16,17). BMD measurements were performed using a Hologic 4500w (Bedford, MA, USA) bone densitometer device. BMD z-scores were calculated according to chronological age (CA) and HA using lumbar 1-4 BMD (g/cm$^2$) values. BMD z-scores were calculated by reference to the BMD data of healthy Turkish children in the study of Goksen et al. (17,18). Since the z-score under the age of two could not be calculated by these data, only those cases whose CA and/or HA were equal to or above two years of age were included in this study. The correlations between the BMD z-scores and the clinical, laboratory, and histopathological parameters according to CA and HA were examined. A BMD z-score ≤-2 SD was considered as low BMD (19). The patients were divided into two groups; namely, those with a BMD z-score >-2 SD and ≤-2 SD according to CA and HA, and the groups were compared in terms of their clinical, laboratory and histopathological parameters.

Ethical approval for the study was obtained from İnönü University Scientific Research Ethics Committee (decision no: 2021/1536, date: 01.06.2021). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Since this study was retrospective, informed consent was not obtained from the patients.

### Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences version 20.0 software. Normality of distribution for the data was tested using visual (histogram and probability charts) and analytical methods (Kolmogorov-Smirnov and Shapiro-Wilk tests). Descriptive analyses are presented as percentile, mean and standard deviation. Correlation analysis was performed to determine whether there was a relationship between two numerical variables, and if so, the direction and severity of this relationship. Spearman rank correlation was preferred for correlation analysis as some numerical data were not normally distributed. Spearman rank difference correlation coefficient was expressed as $r_s$. Normally distributed numerical data were compared using the independent samples t-test and non-normally distributed numerical data were compared using the Mann-Whitney U test. Pearson's chi-squared test was used to compare the frequency rates

of categorical variables. A value of $p < 0.05$ was considered statistically significant. Logistic regression analysis was performed to evaluate whether weight and height z-scores pose a risk for low BMD.

## Results

The mean age at diagnosis of the patients (67 girls, 19 boys) was 8.06 ± 4.08 (2.25-17.71) years. The mean WSD was -1.59 ± 1.20 SD, mean HSD was -1.66 ± 1.29 SD, and HSD was < -2 SD in 33.7% of the patients at the time of diagnosis.

BMD z-score according to CA was found to be ≤-2 SD in 26.7% of the patients. BMD z-score according to HA was found to be ≤-2 SD in 12.8%.

When the correlations between BMD z-scores (according to CA and HA) and the clinical and laboratory parameters were examined, a positive correlation was found between the BMD z-score CA and WSD and HSD ($r_s$: 0.373 and 0.380 respectively), a negative correlation was found between the BMD z-score CA and serum ALT and AST levels ($r_s$: -0.246 and -0.296 respectively). In addition, a positive correlation was found between the BMD z-score HA and age at diagnosis ($r_s$: 0.269) (Table 1).

**Comparison between the BMD z-score CA groups:** When the BMD z-score CA groups (> -2 SD and ≤-2 SD groups) were compared in terms of their clinical features; it was found that the mean WSD and HSD values were different between the groups (Table 2) and it was significantly lower in the group with BMD z-score CA ≤-2 SD. Logistic regression revealed that WSD between -2 SD and -3 SD had 10.5 Odds ratio (OR) [1.12-97.91, 95% confidence interval (CI)], WSD < -3 SD had 36.75 OR (3.50-386.30, 95% CI), and HSD < -2 SD had 4.62 OR (1.65-12.93, 95% CI) for BMD z-scores CA ≤-2 SD.

There were no differences between the groups in terms of age at diagnosis, gender, BMI SD, and type of admission (Table 2).

When the BMD z-score CA groups were compared in terms of their laboratory findings; the AST level was significantly higher, and the vitamin B12 and P levels were significantly lower in the BMD z-score CA ≤-2 SD group than the > -2 SD group. There was no difference between the groups in terms of the other biochemical parameters, HLA DQ2 or DQ8 positivity rates, and the distribution of histopathological stages.

**Comparison between the BMD z-score HA groups:** There were no differences between the groups in terms of their clinical parameters, laboratory parameters, genotype characteristics or the distribution of their histopathological stages (Table 3).

**Table 1. The relationship of BMD z-score with clinical and laboratory parameters at the time of diagnosis**

| | BMD z-score CA | | BMD z-score HA | |
|---|---|---|---|---|
| | p | $r_s$ | p | $r_s$ |
| **Age at diagnosis** | 0.529 | -0.069 | **0.015** | **0.269** |
| **Weight SDS** | **0.000** | **0.373** | 0.959 | 0.006 |
| **Height SDS** | **0.000** | **0.380** | 0.404 | -0.094 |
| **BMI SD** | 0.393 | 0.094 | 0.337 | 0.108 |
| **Serum calcium mg/dL** | 0.345 | 0.104 | 0.179 | -0.153 |
| **Serum phosphorus mg/dL** | 0.131 | 0.168 | 0.876 | -0.018 |
| **Serum alkaline phosphatase U/L** | 0.192 | 0.156 | 0.506 | 0.081 |
| **Serum 25-OH vitamin D ng/mL** | 0.262 | -0.128 | 0.872 | -0.019 |
| **Serum albumin g/dL** | 0.162 | 0.157 | 0.387 | 0.100 |
| **Serum alanine aminotransferase U/L** | **0.025** | **-0.246** | 0.616 | -0.057 |
| **Serum aspartate aminotransferase U/L** | **0.007** | **-0.296** | 0.130 | -0.172 |
| **Serum ferritin ng/mL** | 0.989 | -0.003 | 0.871 | -0.031 |
| **Serum vitamin B12 pg/mL** | 0.403 | 0.102 | 0.563 | 0.073 |
| **Serum folate ng/mL** | 0.275 | 0.154 | 0.401 | 0.121 |
| **Serum zinc mg/dL** | 0.974 | 0.004 | 0.232 | -0.142 |
| **TTGA AU/mL** | 0.817 | -0.025 | 0.344 | 0.106 |

Spearman rank correlation test. $P < 0.05$ is significant. $r_s$: Spearman rank difference correlation coefficient.
SDS: standard deviation score, BMI SD: body mass index standard deviation, HA: height age, BMD: bone mineral density, CA: chronological age, TTGA: tissue transglutaminase antibody

**Table 2. Comparison of BMD z-score CA subgroups according to clinical, laboratory and histopathological features at diagnosis**

| | | BMD z-score by chronological age | | |
|---|---|---|---|---|
| **Variable** | **All patients n = 86** | **> -2 SD n = 63** | **≤-2 SD n = 23** | **p** |
| **Age at diagnosis** | 8.06 ± 4.08 | 7.79 ± 3.90 | 8.80 ± 4.58 | NS* |
| **Female**<br>**Male** | 67 (77.9%)<br>19 (22.1%) | 47 (74.6%)<br>16 (25.4%) | 20 (87%)<br>3 (13%) | NS** |
| **Typical presentation** | 25 (29.1%) | 18 (28.6%) | 7 (30.4%) | NS** |
| **Weight SDS** | -1.59 ± 1.20 | -1.27 ± 1.05 | -2.46 ± 1.18 | **< 0.001*** |
| **Height SDS** | -1.66 ± 1.29 | -1.32 ± 1.16 | -2.64 ± 1.16 | **< 0.001*** |
| **BMI SD** | -0.84 ± 1.09 | -0.73 ± 0.98 | -1.16 ± 1.32 | NS* |
| **Serum calcium mg/dL** | 9.41 ± 0.50 | 9.46 ± 4.22 | 9.30 ± 0.67 | NS* |
| **Serum phosphorus mg/dL** | 4.89 ± 0.70 | 4.99 ± 0.65 | 4.62 ± 0.75 | **0.032*** |
| **Serum alkaline phosphatase U/L** | 208.13 ± 69.60 | 209.19 ± 67.53 | 205.16 ± 76.93 | NS* |
| **Serum 25-OH vitamin D ng/mL** | 21.56 ± 13.40 | 20.40 ± 11.33 | 24.74 ± 17.90 | NS* |
| **Serum albumin g/dL** | 3.89 ± 0.40 | 3.93 ± 0.34 | 3.76 ± 0.55 | NS* |
| **Serum alanine aminotransferase U/L** | 20.61 ± 10.38 | 19.65 ± 9.83 | 23.27 ± 11.61 | NS* |
| **Serum aspartate aminotransferase U/L** | 31.48 ± 10.35 | 29.60 ± 8.45 | 36.68 ± 13.26 | **0.027*** |
| **Serum ferritin ng/mL** | 13.27 ± 13.38 | 14.07 ± 14.62 | 10.40 ± 7.52 | NS* |
| **Serum vitamin B12 pg/mL** | 349.65 ± 181.95 | 378.64 ± 193.44 | 253.62 ± 86.88 | **0.001*** |
| **Serum folate ng/mL** | 8.89 ± 4.25 | 9.35 ± 3.97 | 7.36 ± 4.94 | NS* |
| **Serum zinc mg/dL** | 64.71 ± 13.39 | 64.48 ± 13.15 | 65.33 ± 14.35 | NS* |
| **TTGA AU/mL** | 118.73 ± 55.86 | 116.36 ± 51.75 | 125.20 ± 66.71 | NS* |
| **HLA DQ2 positive** | 66 (76.7%) | 47 (74.6%) | 19 (82.6%) | NS** |
| **HLA DQ8 positive** | 12 (14.0%) | 10 (15.9%) | 2 (8.2%) | NS** |
| **Histopathological stages**<br>**2**<br>**3A**<br>**3B**<br>**3C** | <br>6 (7.0%)<br>21 (24.4%)<br>38 (44.2%)<br>21 (24.4%) | <br>5 (7.9%)<br>19 (30.2%)<br>26 (41.3%)<br>13 (20.6%) | <br>1 (4.3%)<br>2 (8.7%)<br>12 (52.2%)<br>8 (34.8%) | NS** |

P < 0.05 is significant. P > 0.05 is not significant (NS). *Independent Student's t-test. **Crosstab, chi-squared tests.
SDS: standard deviation score, SD: body mass index standard deviation score, TTGA: tissue transglutaminase antibody-IgA, HLA: human leukocyte antigen, CA: chronological age, BMD: bone mineral density

## Discussion

In the literature, it has been reported that BMD was found to be lower in those children with CD compared to healthy children (5,6,7,12).

In various studies, it has been reported that the rate of children with a BMD z-score CA <-2 SD was 10.8-16% at the time of diagnosis (11,12). Kalayci et al. (4), in an earlier study on 16 pediatric patients diagnosed with CD, reported this rate to be much higher (50%). However, in a recent large-series study conducted in the USA, the rate of cases with a BMD z-score <-2 SD was reported to be lower than in other studies (6.8%) (13). As can be seen, varying rates of low BMD in children with CD have been reported in different societies or at different centers or different times even in the same society. In our study, BMD z-scores CA were found to be ≤-2 SD in 26.7% of the children with CD at the time of diagnosis.

Classically used areal BMD measurements are closely related to age and height. Since BMD increases with age in childhood, it is evaluated by calculating the z-score according to age and gender. Since the BMD z-score calculated according to CA is found to be misleadingly lower in children with short-stature, it is recommended to correct the BMD z-score according to the height or HA in these children (20). Short stature is common in children diagnosed with CD at the time of admission. In our study, short stature was found in 33.7% of the cases in our study, and therefore, the BMD z-scores were calculated according to both CA and HA. In most of the studies evaluating BMD in children with CD, the BMD z-score was calculated according to CA (4,11,13). Tuna Kırsaçlıoğlu et al. (12), found short stature in 37.8% of 37 pediatric patients diagnosed with CD, and when they calculated the BMD z-score according to HA at the time of diagnosis, they found <-2 SD in 2.7%. In our study, BMD z-score HA was found to be ≤-2 SD in 12.8% of the cases.

**Table 3. Comparison of BMD z-score HA subgroups according to clinical, laboratory and histopathological features at diagnosis**

| | | BMD z-score by height age | | |
|---|---|---|---|---|
| **Variable** | **All patients n = 81** | **> -2 SD n = 70** | **≤-2 SD n = 11** | **p** |
| **Age at diagnosis** | 8.40 ± 3.98 | 8.56 ± 4.02 | 7.35 ± 3.68 | NS* |
| **Female** | 62 (76.5%) | 54 (77.1%) | 8 (72.7%) | NS** |
| **Male** | 19 (23.5%) | 16 (22.9%) | 3 (27.3%) | |
| **Typical presentation** | 23 (28.4%) | 19 (27.1%) | 4 (36.4%) | NS** |
| **Weight SDS** | -1.59 ± 1.22 | -1.55 ± 1.28 | -1.81 ± 0.83 | NS* |
| **Height SDS** | -1.64 ± 1.31 | -1.64 ± 1.36 | -1.61 ± 1.03 | NS* |
| **BMI SD** | -0.86 ± 1.10 | -0.79 ± 1.07 | -1.32 ± 1.18 | NS* |
| **Serum calcium mg/dL** | 9.42 ± 0.48 | 9.39 ± 0.43 | 9.56 ± 0.74 | NS* |
| **Serum phosphorus mg/dL** | 4.87 ± 0.68 | 4.87 ± 0.66 | 4.85 ± 0.85 | NS* |
| **Serum alkaline phosphatase U/L** | 210.39 ± 70.01 | 212.64 ± 70.66 | 197.10 ± 68.04 | NS* |
| **Serum 25-OH vitamin D ng/mL** | 21.70 ± 13.38 | 21.80 ± 13.97 | 21.11 ± 9.72 | NS* |
| **Serum albumin g/dL** | 3.89 ± 0.39 | 3.89 ± 0.39 | 3.90 ± 0.43 | NS* |
| **Serum alanine aminotransferase U/L** | 20.49 ± 10.61 | 19.69 ± 10.00 | 26.00 ± 13.45 | NS* |
| **Serum aspartate aminotransferase U/L** | 30.73 ± 9.63 | 30.03 ± 9.55 | 35.60 ± 9.19 | NS* |
| **Serum ferritin ng/mL** | 13.49 ± 13.69 | 13.49 ± 13.69 | - | NS* |
| **Serum vitamin B12 pg/mL** | 351.37 ± 181.30 | 347.83 ± 186.30 | 380.71 ± 140.47 | NS* |
| **Serum folate ng/mL** | 8.69 ± 4.18 | 8.70 ± 3.88 | 8.57 ± 6.97 | NS* |
| **Serum zinc mg/dL** | 65.21 ± 13.17 | 64.69 ± 12.93 | 68.09 ± 14.75 | NS* |
| **TTGA AU/mL** | 120.34 ± 56.73 | 122.63 ± 59.47 | 105.77 ± 32.68 | NS* |
| **HLA DQ2 positive** | 62 (76.5%) | 52 (74.3%) | 10 (90.9%) | NS** |
| **HLA DQ8 positive** | 10 (12.3%) | 10 (14.3%) | 0 (0%) | NS** |
| **Histopathological stages** | | | | |
| **2** | 6 (7.4%) | 5 (7.1%) | 1 (9.1%) | |
| **3A** | 19 (23.5%) | 17 (24.3%) | 2 (18.2%) | NS** |
| **3B** | 36 (44.4%) | 31 (44.3%) | 5 (45.5%) | |
| **3C** | 20 (24.7%) | 17 (24.3%) | 3 (27.3%) | |

P < 0.05 is significant. P > 0.05 is not significant (NS). *Independent Student's t-test. **Crosstab, chi-squared tests.
SDS: standard deviation score, BMI SD: body mass index standard deviation, TTGA: tissue transglutaminase antibody-IgA, HLA: human leukocyte antigen, BMD: bone mineral density, HA: height age

In the literature, there are varying results regarding the relationship between age at the time of CD diagnosis and BMD z-score CA. Turner et al. (11) found an inverse correlation between age at diagnosis and BMD z-score. In other studies, no relationship was found between the age at diagnosis and BMD z-score CA (9,10,13). In our study, no correlation was found between BMD z-score CA and age at diagnosis either. Although we detected a weak positive correlation between the age at diagnosis and BMD z-score HA, there was no statistically significant difference between the BMD z-score HA > -2 SD subgroup and the ≤-2 SD subgroup.

The studies evaluating the relationship between BMD z-score and gender or presenting features at the time of admission have failed to show the effect of these parameters on the BMD z-score (11,12,13). Similarly, in our study, no significant difference was found between the BMD z-score subgroups regarding these parameters. Puberty has positive effects on BMD in children, and delayed puberty is associated with low BMD. However, Tuna Kırsaçlıoğlu et al. (12) reported that 27 (72.9%) pediatric Celiac patients were prepubertal at presentation, but their BMD z-scores did not differ from those of pubertal patients. Since pubertal staging information could not be found in the medical records of most of our patients, the effect of puberty on BMD z-scores could not be interpreted in our study. Prospective studies which include pubertal evaluation are needed. There was a positive correlation between BMD z-score CA and WSD and HSD. It is known that the BMD z-score CA shows a decrease as body size decreases . When the BMD z-score was calculated according to HA, no difference was found between the groups in terms of WSD and HSD. In the study of Webster et al. (13), BMI SD was found to be significantly lower in cases with low BMD z-scores (< -2 SD). In our study, it was observed that the BMI SD values were lower in the BMD z-score ≤-2 SD groups for CA and HA. However, there was no statistically significant difference between the

groups in terms of BMI SD. The limited number of cases in our study may have affected this result.

In some studies, when evaluating the relationship between biochemical parameters and BMD at the time of diagnosis of CD, a negative correlation was found between PTH level and BMD and it was suggested that low BMD in CD was associated with vitamin D and/or Ca deficiency (4,8). However, in other studies, no correlation was found between the BMD z-scores at diagnosis and serum Ca, P, ALP, PTH, or vitamin D levels (9,10,13). Similarly, in our study, no correlation was found between BMD z-scores for either CA and HA and Ca, P, ALP, or vitamin D levels. There was no difference between the BMD z-score subgroups in terms of these parameters either. This indicates that there may be other factors affecting BMD in patients with CD, and that normal findings of Ca metabolism parameters do not exclude low BMD. Due to the retrospective design of our study, serum PTH could not evaluated since serum PTH levels had not been measured in most of our cases.

One of the extra intestinal findings which can be seen in the course of CD is liver involvement. While isolated elevation of transaminases is frequently observed, severe liver pathology or other accompanying chronic liver diseases (autoimmune, viral or metabolic) can be detected less frequently (21). In the literature, no study was found evaluating the relationship between BMD z-score and liver functions in patients with CD. In our study, the mean ALT levels were within normal ranges at the time of diagnosis, and there was no statistically significant difference between the BMD z-score subgroups for CA and HA. The mean AST level was found to be higher (slightly above the upper limit), in those patients with BMD z-score ≤-2 SD according to CA. Serum AST levels were not statistically different between the BMD z-scores for the HA subgroups. In our study, no correlation was found between BMD z-score and serum albumin, ferritin, vitamin B12, folic acid, zinc, or TTGA levels. Similarly, in the study of Webster et al. (13), no correlation was found between low BMD and serum albumin or TTGA levels.

HLA DQ2 alleles are detected in more than 90% of Celiac patients, and HLA DQ8 alleles are found in others (21). There was no difference between the BMD z-score CA and HA subgroups in terms of HLA DQ2 or DQ8 positivity rates. It was thought that this lack of difference was due to the high rates of these alleles in our cases. We could not find any other study evaluating this issue.

The relationship between BMD and histopathological staging at the time of diagnosis was evaluated in several studies and various results have been obtained. Lewis and Scott (22) did not report any relationship between BMD and the degree of villous atrophy (mild-severe) in 43 adults diagnosed with CD. In two different studies, it was shown that BMD is lower in those patients with histopathological stage Marsh 3 compared to those with Marsh 1-2 (23,24). In our study, no correlation was found between BMD z-scores (both CA and HA) and histopathological stage. In the studies which found a relationship between BMD and histopathological stage, BMD was reported to vary between the patient groups with Marsh stage 1-2 and Marsh stage 3. However, nowadays, the diagnosis of CD requires the presence of at least Marsh stage 2 histopathological findings. In our study, the cases with histopathological Marsh stage 2 or above were included, and Marsh stage 3 (a, b, c) was detected in more than 90% of the cases. Therefore, it was thought that we have a similar patient profile and similar results with the studies which evaluated the relationship between BMD z-scores and Marsh stage 3 subgroups (22). Based on these findings, we suggest that BMD is affected more negatively as histopathological findings become more severe, while the degree of low BMD does not differ between subgroups when the pathology is compatible with stage 3 disease.

### Study Limitations

The strengths of our study include that it was conducted on a higher number of patients compared to most studies in the literature (especially from our country) and the relationships of more parameters with BMD were evaluated in our study, compared to previous studies. In addition, the BMD z-scores were calculated according to both CA and to HA for all parameters. Also, patients with additional diseases which could have affected BMD were excluded from this study. The limitations include; firstly, the fact that some parameters (pubertal stage, PTH levels) could not be evaluated due to the retrospective nature of this study, and the second is that the patients were not homogeneously distributed in terms of histopathological stage.

## Conclusion

In conclusion, low BMD is common in children with CD at the time of diagnosis. When the BMD z-scores are calculated only according to CA, the frequency of low BMD seems misleadingly higher. However, when the BMD z-scores are corrected for HA, the frequency is slightly lower, though still high. Age at diagnosis, gender, body size, Celiac symptoms, biochemical parameters, TTGA level, HLA type and histopathological stage have no predictive value for low BMD. Further studies are needed to determine consistent parameters which predict low BMD in patients with CD. We recommend evaluating BMD at diagnosis in newly diagnosed pediatric Celiac patients until consistent factors

which can predict low BMD are identified. If these risk factors associated with low BMD can be found, switching to the assessment of only high risk groups will reduce costs.

### Ethics

**Ethics Committee Approval:** Ethical approval for the study was obtained from İnönü University Scientific Research Ethics Committee (decision no: 2021/1536, date: 01.06.2021).

**Informed Consent:** Retrospective study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Fatma İlknur Varol, Şükrü Güngör, Mukadder Ayşe Selimoğlu, Concept: Emine Çamtosun, Fatma İlknur Varol, Design: Emine Çamtosun, Data Collection or Processing: Emine Çamtosun, Fatma İlknur Varol, Şükrü Güngör, Mukadder Ayşe Selimoğlu, Analysis or Interpretation: Emine Çamtosun, Fatma İlknur Varol, Şükrü Güngör, Mukadder Ayşe Selimoğlu, Literature Search: Emine Çamtosun, Fatma İlknur Varol, Writing: Emine Çamtosun, Fatma İlknur Varol, Şükrü Güngör, Mukadder Ayşe Selimoğlu.

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# Increased Carotid Intima-media Thickness and Its Association with Carbohydrate Metabolism and Adipocytokines in Children Treated with Recombinant Growth Hormone

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### What is already known on this topic?

There is an ongoing discussion as to whether treatment with recombinant human growth hormone (rhGH) may increase the risk of cardiovascular events, including ischemic heart disease, stroke, cardiomyopathy, and aneurysm in adulthood. In addition, knowledge about possible early diagnostic markers and risk factors for cardiovascular events associated with rhGH therapy in childhood is limited.

### What this study adds?

The present study showed that carotid intima-media thickness (cIMT) is increased in growth hormone deficiency children treated with rhGH when compared to healthy matched controls. Notably, the level of serum ghrelin was higher in those with high cIMT-standard deviation scores, which suggests that ghrelin may play a role in changes of vascular endothelium.

## Abstract

**Objective:** Reports on the association between growth hormone (GH) therapy and cardiovascular risk factors in children are limited. This study aimed to evaluate carotid intima-media thickness (cIMT) in children treated with recombinant human GH (rhGH) and assess the effects of rhGH therapy and changes in serum carbohydrate metabolism, lipid profile and adipocytokines on cIMT.

**Methods:** Seventy-one isolated idiopathic GH deficiency (GHD) children and 44 age- and sex-matched healthy controls were enrolled in this study. The study group was divided into two subgroups according to insulin resistance (IR) on oral glucose tolerance tests. Insulin secretion [homeostatic model assessment (HOMA) B, total insulin] and sensitivity (HOMA-IR, QUICKI, Matsuda) indices were calculated. cIMT was measured and the standard deviation scores (SDS) were calculated. Associations between cIMT-SDS and insulin secretion and sensitivity indices, serum lipid levels, adipocytokines (leptin, resistin, ghrelin), and other rhGH treatment-related factors were evaluated.

**Results:** cIMT-SDS was increased in GHD children treated with rhGH compared to the controls [0.02 (2.27) vs. -1.01 (1.63), $p = 0.003$]. cIMT-SDS did not differ between those children on rhGH treatment with or without IR. High cIMT-SDS was significantly associated with higher serum ghrelin levels and lower serum high density lipoprotein (HDL) levels ($\beta = 0.491$, $p = 0.001$ and $\beta = -0.027$, $p = 0.017$), but not with BMI-SDS, blood pressure, insulin secretion and sensitivity indices, or the dose and duration of rhGH therapy.

**Conclusion:** Our findings showed that GHD children treated with rhGH have increased cIMT. Alterations in carbohydrate metabolism were not associated with cIMT in children treated with rhGH. GH therapy *per se* appears to be associated with this increased cIMT but causality should be elucidated in further studies. cIMT also appears to be associated with higher ghrelin and lower HDL levels.

**Keywords:** Recombinant growth hormone, carotis intima-media thickness, adipocytokines, carbohydrate metabolism

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## Introduction

There are ongoing concerns about the long-term effects of recombinant growth hormone (rhGH) treatment on cardiovascular morbidity and mortality (1,2). Both growth hormone (GH) deficiency and GH overproduction in acromegaly are known to be associated with higher mortality from cardiovascular disease (CVD) (3,4,5). However, discussion of the cardiovascular safety of rhGH treatment was sparked in 2012 by a study in a French cohort of children, which reported an increased risk of cardiovascular mortality (6). Further studies also uncovered an increased risk of cerebrovascular morbidity (7). Although the overall cardiovascular safety profile of rhGH treatment has been considered favorable in several reviews and consensus statements (8,9,10), a nationwide, population-based cohort study from Sweden showed that treatment with rhGH in childhood was associated with an increased risk of cardiovascular events (2). However, the absolute risk of cardiovascular events was low and evidence of causality is still limited.

Carotid intima-media thickness (cIMT) is a noninvasive biomarker of atherosclerosis (11). The prodromal stage of atheromatous plaque begins very early in life (12,13) and it predicts CVD (14,15). Excessive GH secretion leading to acromegaly is a known cause of increased cIMT (4), but the results of the various studies which have examined cIMT in GH-deficient children treated with rhGH are controversial (16,17,18,19). Furthermore, none of these studies used sex- and height-specific normative cIMT values to calculate standard deviation (SD) scores (SDS) to compare with healthy children.

It has been suggested that the direct action of GH contributes to the inflammatory process in atherogenesis (20). In addition, GH and its polypeptide mediator, insulin-like growth factor 1 (IGF-1), alter lipid and carbohydrate metabolism (21), which may increase the risk of CVD. Regarding carbohydrate metabolism, studies have postulated a six-fold increase in the incidence of type 2 diabetes in children treated with rhGH (22). Before overt glucose abnormalities occur, rhGH increases insulin secretion and decreases insulin sensitivity (23,24). In recent years, a variety of different parameters and indices derived from the oral glucose tolerance test (OGTT) have been used to diagnose glucose abnormalities, but few reports have shown their potential application in studying the effects of rhGH treatment on glucose and insulin homeostasis (25,26,27). To the best of our knowledge, no study has evaluated the relationship between cIMT, glucose sensitivity and insulin secretion indices to date.

The aim of this study was to measure cIMT in a group of children with isolated GH deficiency (GHD) treated with rhGH and to compare their cIMT-SDS with healthy children. We also hypothesized that the direct effects of GH and its indirect effects via IGF-1 on carbohydrate metabolism, lipid profile, and adipocytokines might be associated with cIMT in children treated with rhGH. Therefore, we measured cIMT and analyzed its association with the carbohydrate metabolism indices derived from OGTT, adipocytokines (leptin, resistin, ghrelin), and other cardiovascular risk factors, such as hypertension, obesity, and dyslipidemia.

## Methods

### Study Design

This cross-sectional observational study included 71 children and adolescents with isolated idiopathic GHD [female, n = 17 (24%), mean age 13.7 ± 2.6 years] who were consulted and treated at İstanbul University, İstanbul Faculty of Medicine, Department of Pediatric Endocrinology (28). GHD was identified using clinical, auxological and biochemical criteria from the GH Research Society. The inclusion criteria were: (i) GHD, defined as an absence of GH (peak GH levels below 10 µg/L) in response to two stimuli (clonidine and L-Dopa test); and (ii) treatment with rhGH for at least one year. The exclusion criteria were: (i) multiple pituitary hormone deficiency, except for hypothyroidism; (ii) any cardiovascular, respiratory, renal, or liver diseases; (iii) personal or family history of lipid disorders; and (iv) bioinactive GH syndrome. All children with GHD were treated with biosynthetic rhGH once daily before bedtime, for a total of seven injections per week. The initial subcutaneous dose was 30.2 ± 4.1 mcg/kg/day which was gradually adjusted during follow-up based on growth velocity and IGF-1 concentration. The demographic, clinical, and radiologic information, including magnetic resonance imaging (MRI) findings, were obtained from the patient records.

The control group included 44 healthy, age- and sex-matched children [female, n = 15 (34.1%), mean age 13.4 ± 2.9 years]. Organic diseases were excluded, based on physical examination in our hospital. The control group was selected from children referred to our hospital for well-child care visits.

The procedure was performed with the written and informed consent of the parents or guardians of the minors and in accordance with all applicable ethical and legal rules for medical research involving human subjects, according to the Declaration of Helsinki ethical statement. The study protocol and this consent procedure were approved by the

İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (date: 20.06.2014, no: 2014/990).

### Assessment of Anthropometric Measures and Blood Pressure

Height was measured to the nearest millimeter using a wall-mounted calibrated Harpenden rigid stadiometer (Holtain Ltd., Crymych, UK). Weight was measured in underwear to the nearest 0.1 kg using a calibrated balance scale (Seca, Hamburg, GER). Waist circumference (WC) was measured using a non-elastic tape with the subject in a standing position. The abdominal circumference midway between the lowest rib and the top of the iliac crest at the end of expiration was measured to obtain WC. Height, weight, body mass index (BMI) (kg/m$^2$) and WC were expressed as SDS, based on Turkish reference growth charts (29,30). Growth velocity SDS was determined (31). Pubertal status was assessed using Tanner staging (32).

Blood pressure (BP) was measured on the right arm after a 15-minute rest with an oscillatory device. Three repeated measurements were taken with five minute intervals between each, and the lowest values of systolic and diastolic BP were documented. Office BP values for patients younger than 16 years were assessed according to the age-, sex-, and height-specific normative values of the Fifth Report (33).

### Assessment of Glucose Metabolism

All assessments were made after an overnight fast. Fasting glucose, fasting insulin, and hemoglobin A1c (HbA1c) were determined and an OGTT was performed (glucose load of 1.75 g/kg, maximum of 75 g). Blood samples were collected every 30 minutes for two hours for glucose and insulin measurements (34).

Altered glucose metabolism was defined according to the American Diabetes Association criteria for prediabetes and included impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or impaired HbA1c (5.7-6.4%) (35). Diabetes was diagnosed if fasting glucose was ≥126 mg/dL or glucose at the 120-minute measurement was ≥200 mg/dL or HbA1c ≥6.5%. If there was no clear hyperglycemia, the results were confirmed by repeated testing (35).

Fasting insulin levels, homeostatic model assessment (HOMA)-insulin resistance (IR), QUICKI, and Matsuda index values were used to assess insulin sensitivity (36,37,38). Upper limits for HOMA-IR were 2.67 for prepubertal boys, 2.22 for prepubertal girls, 5.22 for pubertal boys, and 3.82 for pubertal girls (36). Fasting insulin, total insulin levels, and HOMA B were used to assess insulin secretory capacity (37). All formulae used for the calculation of indices are given in Supplementary Table 1.

The GHD children treated with rhGH were divided into two subgroups according to hyperinsulinemia: a) IR and b) without IR. Hyperinsulinemia was diagnosed when the total insulin level measured throughout OGTT (0, 30, 60, 90, and 120 minutes) was above 300 µU/mL or the 120-minute insulin level was above 75 µU/mL or the peak insulin level was above 150 µU/mL (39).

### Assessment of Serum Lipid Profile and Adipocytokines

After an overnight fast, blood samples were collected from both the GHD and control groups to analyze serum lipid levels, including triglycerides (TG), total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and adipocytokines including leptin, resistin and ghrelin. The lipid profile was analyzed using routine laboratory methods. Serum samples were stored at -80 °C. Leptin was measured with a kit from Labsystems Diagnostics Oy (Vantaa, Finland), and resistin and ghrelin with kits from USCN Life Science Inc (Wuhan, PRC) using the enzyme-linked immunosorbent assay (ELISA) method. The sensitivities of the ELISA kits (detection limits) were 0.04 ng/mL, 0.2 ng/mL, and 21 pg/mL for leptin (Cat. No. 140101/A), resistin (Cat. No. L140718502), and ghrelin (Cat. No. L140718468), respectively. Intra-assay variability was 3.5-13.3%, <5.8% and 3.3-10.0% for leptin, resistin, and ghrelin, respectively. Inter-assay variability was 10.2%, 14.8% and 14.7% for these markers, respectively.

### Assessment of cIMT

Images of the carotid artery were obtained by a single and experienced cardiologist (M.K.) from the children with GHD and the control group using a colored Doppler ultrasonographic device (Aplio SSA 770, Toshiba, Tokyo, Japan), with the linear probe set at 7.5 mHz according to the Mannheim cIMT consensus (40). Measurements were performed in the supine position with the neck slightly hyperextended. Three manual measurements were obtained in both carotid arteries, 1 to 2 cm proximal to the bifurcation at the far wall longitudinally, after a 10 minute rest, and then these measurements were averaged for each individual (11). The SDSs for cIMT were calculated using the LMS method and height-specific normative values (41).

### Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 21 (SPSS Inc., Chicago, IL., USA). Normality of distribution for the variables was confirmed by a Shapiro-Wilk test. Continuous variables are presented as mean (± SD) or median and interquartile range according to the distribution of data. Student's t-test or the Mann-Whitney U test were used to compare

differences between two independent groups according to the distribution of data. The paired t-test and the Wilcoxon signed rank test were used to compare differences between two paired groups. Categorical variables are expressed as number (percentage). Fisher's exact test was used to compare categorical variables. Correlations between biomarkers and other clinical and laboratory variables were analyzed using Spearman's test. To identify independent factor(s) influencing cIMT, all variables which had a p value ≤0.1 on univariate analysis and were known clinical risk factors were tested in a stepwise multivariable linear regression analysis. Statistical significance was defined as a two-tailed p value $< 0.05$.

## Results

### Patient-related Characteristics

The mean age (minimum-maximum) of the children at the start of rhGH treatment was 11.2 (2.3-16.2) years, and the male to female ratio was 3.2:1.0. The frequency of consanguinity was 8% in the study group. The proportions of the study group with a family history of obesity, hypertension, diabetes, hyperlipidemia, and coronary heart disease were 7%, 4%, 14%, 1%, and 1%, respectively.

All the cranial MRI findings were normal, except in seven children (10%) who had Arnold Chiari malformation, small hypophysis (n = 3), microadenoma (n = 1), ectopic neurohypophysis (n = 1) and hypophyseal cyst (n = 1). Except for two term-born children with intrauterine growth retardation, all the rest of the GHD group was born with a gestational age-appropriate weight. Two of the children with GHD were treated with testosterone propionate before starting GH treatment because of micropenis. Hypothyroidism was diagnosed in twelve children (primary hypothyroidism in four children and secondary hypothyroidism in eight children) before starting rhGH treatment. All those children were under treatment and euthyroid at the time of this study.

The mean height-SDS (H-SDS) of the children with GHD (-2.42 ± 0.87) was 1.04 SD away from target H-SDS (-1.38 ± 0.78). GHD was supported by the absence of GH response in two stimulation tests (clonidine and L-Dopa test) with peak GH levels of 3.3 ± 2.3 µg/L and 4.2 ± 2.2 µg/L, respectively. The anthropometric findings and characteristics of the study group at the start of rhGH treatment are shown in Table 1.

At the time of investigation, the duration of rhGH therapy for the children with GHD was 2.5 ± 1.4 years. The percentage of children treated for 1-3 years and for more than 3 years were 73% (n = 52) and 27% (n = 19), respectively. The mean H-SDS increased significantly during the study period with rhGH treatment (-2.42 ± 0.87 vs. -1.65 ± 0.88, $p < 0.001$). The mean rhGH dose was 31.1 ± 4.0 mcg/kg/day. A consistent increase in IGF-1 SDS and IGF-binding protein 3 (IGFBP-3) SDS was noticed over the study period ($p < 0.001$) (Table 1). Eleven (15%) of the children with GHD were underweight

**Table 1. Anthropometric and clinical features of growth hormone deficient children group (evaluation at diagnosis and at the time of study) and the control group**

| | GHD children at diagnosis (n = 71) | GHD children at time of study (n = 71) | Control group (n = 44) | $p^1$ | $p^2$ |
|---|---|---|---|---|---|
| Age (years) | 12.0 ± 1.9 | 13.7 ± 2.6 | 13.4 ± 2.9 | - | 0.55 |
| Pubertal children, n (%) | 36 (51%) | 60 (84%) | 37 (84%) | - | 0.20 |
| H-SDS | -2.42 ± 0.87 | -1.65 ± 0.88 | -0.09 ± 0.98 | **< 0.001** | **< 0.001** |
| Weight SDS | -1.34 (1.08) | -1.25 (0.96) | -0.39 (1.41) | 0.059 | **< 0.001** |
| BMI-SDS | -0.33 (1.23) | -0.68 (1.08) | -0.47 (1.22) | 0.184 | 0.619 |
| WC-SDS | - | 0.36 (1.40) | 0.49 (0.90) | - | 0.90 |
| Systolic BP-SDS | -0.12 ± 0.88 | -0.11 ± 0.85 | -0.29 ± 0.86 | 0.97 | 0.26 |
| Diastolic BP-SDS | 0.69 ± 0.69 | 0.70 ± 0.68 | 0.60 ± 0.77 | 0.97 | 0.44 |
| Bone age/age | 0.81 ± 0.13 | 0.87 ± 0.12 | - | **< 0.001** | |
| Growth velocity-SDS | -0.96 ± 1.60 | 4.77 ± 3.89 | - | **< 0.001** | |
| Target H-SDS | -1.32 ± 0.78 | | - | | |
| Predicted H-SDS | 2.28 (3.68) | 0.96 (2.93) | - | **< 0.001** | |
| IGF-1 SDS | -2.13 (1.97) | 1.15 (3.23) | 0.37 (3.10) | **< 0.001** | 0.056 |
| IGFBP-3 SDS | -0.97 (2.22) | 0.66 (1.65) | -0.45 (1.52) | **< 0.001** | **< 0.001** |

$p^1$: comparison between GHD children at the start of the treatment and the time of study, $p^2$: comparison between GHD children at the time of study and control group. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).
BMI: body mass index, BP: blood pressure, SDS: standard deviation (SD) score, IGF-1: insulin-like growth factor-1, IGFBP-3: IGF-binding protein 3, GHD: growth hormone deficiency, WC: waist circumference, H-SDS: height SDS

and four (5%) had obesity at the start of the treatment. At the time of this investigation, these frequencies were 10% and 7%, respectively, but weight SDS and BMI-SDS did not change significantly over the study period (Table 1). None of the controls were obese or underweight. At the time of this study, the BMI-SDS and WC-SDS of the children with GHD and the control group showed no statistical difference. Table 1 shows the anthropometric measurements, demographics, pubertal status, IGF-1 SDS, IGFBP-3 SDS and office BP of the children with GHD at the start of rhGH treatment and at the time of investigation, and their comparisons with the control group.

### Carbohydrate Metabolism

Sixty-six children from the GHD group completed OGTT, while five children could not complete the test because of nausea and vomiting. None of the children had evidence of type 2 diabetes or IGT. Alterations in glucose metabolism were detected in three patients, one presenting with IFG and impaired HbA1c, and two presenting only with impaired HbA1c. In total, 30 (45.45%) had hyperinsulinemia and were grouped as IR.

Comparison of those children with GHD and those without IR did not show any difference in the frequency of family history of obesity, hypertension, diabetes, dyslipidemia or coronary heart disease. Furthermore, the proportion of patients with pathological MRI findings was not different between the GHD children with or without IR [1/30 (3.3%) vs. 6/36 (16.7%), respectively].

Age at start of rhGH treatment, peak GH levels of the two stimulation tests, SDSs of anthropometric measurements before treatment and growth velocities did not show any difference between the GHD children with or without IR (Table 2). Although the treatment times were similar between the groups, those children with IR were significantly older

**Table 2. Anthropometric and clinical features of growth hormone deficient children with or those without insulin resistance and the control group**

| | GHD + IR (n = 30) | GHD without IR (n = 36) | Control (n = 44) | p |
|---|---|---|---|---|
| Female n (%) | 5 (16.6) | 12 (33.3) | 15 (34.1) | - |
| **At diagnosis** | | | | |
| Age at diagnosis (years) | 11.8 ± 2.0 | 10.5 ± 2.8 | | **0.030** |
| Puberty at diagnosis, n (%) | 20 (66.6) | 13 (36.1) | - | **0.013** |
| Bone age/age | 0.82 ± 0.14 | 0.80 ± 0.12 | - | 0.55 |
| Weight SDS | -1.24 (1.09) | -1.49 (0.95) | - | 0.17 |
| H-SDS | -2.38 ± 0.84 | -2.78 ± 1.00 | - | 0.10 |
| BMI-SDS | -0.55 (1.55) | -0.17 (0.90) | - | 0.50 |
| Growth velocity SDS | -0.77 ± 1.59 | -1.16 ± 1.63 | - | 0.33 |
| Predicted H-SDS | 2.21 (3.50) | 2.07 (3.85) | - | 0.63 |
| **At study time** | | | | |
| Age at study (years) | 14.5 ± 1.7 | 13.0 ± 2.8 | 13.4 ± 2.9 | 0.06 |
| Duration of rhGH treatment (years) | 2.6 ± 1.4 | 2.5 ± 1.3 | | 0.72 |
| Pubertal children, n (%) | 27 (90.0) | 28 (77.7) | - | 0.24 |
| Bone age/age | 0.86 ± 0.10** | 0.87 ± 0.14** | 0.97 ± 0.10 | 0.96 |
| Weight SDS | -1.22 (0.77)** | -1.37 (1.31)** | -0.40 (1.41) | 0.84 |
| H-SDS | -1.50 ± 0.74** | -1.89 ± 0.96** | -0.09 ± 0.96 | 0.18 |
| BMI-SDS | -0.68 (0.94) | -0.72 (1.14) | -0.47 (1.22) | 0.88 |
| Growth velocity SDS | 5.55 ± 4.75 | 4.44 ± 3.13 | | 0.26 |
| Target H-SDS | -1.24 ± 0.72* | -1.42 ± 0.84** | | 0.64 |
| Predicted H-SDS | 1.29 (2.53) | 0.26 (3.68) | | 0.12 |
| WC-SDS | 0.40 (0.93) | 0.26 (1.49) | 0.49 (0.90) | 0.91 |
| ΔWeight-SDS | 0.02 (0.82) | 0.09 (0.92) | - | 0.38 |
| ΔH-SDS | 0.83 (1.07) | 0.83 (0.69) | - | 0.88 |
| ΔBMI-SDS | 0.72 (0.95) | 0.79 (0.88) | - | 0.70 |

p: comparison between GHD-IR and GHD without IR subgroups, *p < 0.05 for comparison with control group, **p < 0.01 for comparison with control group. Δ = change between the time of study and the time of diagnosis. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).
BMI: body mass index, SDS: standard deviation (SD) score, rhGH: recombinant growth hormone, WC: waist circumference, H-SDS: height SDS, GHD: growth hormone deficiency, IR: insulin resistance

than the children without IR (p = 0.003). The proportion of pubertal children at onset of therapy was higher in the IR subgroup (n = 27; 90%) compared to the non-IR-subgroup (28; 78%; p = 0.013). Anthropometric measurements during the study of those GHD children with and those without IR are summarized in Table 2. Neither the BMI-SDS at study time nor the ΔBMI-SDS showed any difference between these subgroups.

The insulin sensitivity and secretion indices are summarized in Table 3. The children with GHD treated with rhGH showed significantly lower levels of fasting glucose and higher fasting insulin levels compared to the control group [81.0 ± 8.7 vs. 88.8 ± 9.1 mg/dL, p < 0.0001 and 11.5 (8.8) vs. 7.9 (5.6), p = 0.014]. Both HOMA-IR and HOMA-B indices were significantly higher in the GHD group compared to the controls [2.3 (1.9) vs. 1.7 (1.4), p = 0.039 and 160.6 (79.0) vs. 100.6 (49.0), p < 0.001] (Table 3).

Fasting insulin, HOMA-IR, total insulin level at OGTT, 120-minute glucose levels, HOMA-B, and Matsuda indices were significantly higher in the GHD children with IR than in those without IR (p = 0.001, p = 0.001, p < 0.001, p = 0.004, p = 0.044, and p < 0.001, respectively). Among the insulin

**Table 3. Comparison of cardiovascular risk factors and carotid intima-media thickness measurement between growth hormone deficient children groups with or those without insulin resistance and the control group**

| | All children with GHD (n = 71) | Control (n = 44) | p[1] | GHD + IR (n = 30) | GHD without IR (n = 36) | p[2] |
|---|---|---|---|---|---|---|
| IGF-1 SDS (study time) | 1.15 (3.23) | 0.37 (3.10) | **0.019** | 1.68 (3.32) | 0.92 (3.09) | 0.89 |
| IGFBP-3 SDS (study time) | 0.66 (1.65) | -0.45 (1.52) | **< 0.001** | 0.65 (1.61)** | 0.66 (1.74)** | 0.68 |
| **Blood pressure** | | | | | | |
| Systolic BP-SDS | -0.11 ± 0.85 | -0.29 ± 0.86 | 0.263 | -0.05 ± 0.88 | -0.17 ± 0.88 | 0.57 |
| Diastolic BP-SDS | 0.70 ± 0.68 | 0.59 ± 0.77 | 0.441 | 0.81 ± 0.69 | 0.57 ± 0.68 | 0.16 |
| **Glucose metabolism indices** | | | | | | |
| Fasting glucose (mg/dL) | 80.1 ± 8.7 | 88.8 ± 9.1 | **< 0.001** | 82.8 ± 7.6* | 78.8 ± 8.4** | 0.050 |
| 2-h glucose (mg/dL) | 105.4 ± 19.8 | - | - | 112.7 ± 19.5 | 98.8 ± 18.1 | **0.004** |
| HbA1c (%) | 5.1 ± 0.3 | - | - | 5.2 ± 0.3 | 5.1 ± 0.3 | 0.37 |
| **Insulin secretion indices** | | | | | | |
| Fasting insulin (μU/mL) | 11.5 (8.8) | 7.9 (5.6) | **0.014** | 15.1 (11.9)** | 10.0 (6.9) | **0.001** |
| HOMA-B | 160.6 (79.0) | 100.6 (49.0) | **< 0.001** | 178.6 (90.1)** | 148.9 (69.3)** | **0.044** |
| Total insulin (μU/mL) | 253.1 (255.7) | - | - | 432.5 (210.3) | 180.8 (81.8) | **< 0.001** |
| **Insulin sensitivity indices** | | | | | | |
| HOMA-IR | 2.3 (1.9) | 1.7 (1.4) | **0.039** | 2.9 (2.3)** | 1.9 (1.4) | **0.001** |
| QUICKI | 2.8 (0.3) | 3.1 (0.4) | **< 0.001** | 2.8 (0.2)** | 2.9 (0.3) * | **< 0.001** |
| MATSUDA | 3.9 (3.1) | - | - | 2.8 (1.4) | 5.6 (3.3) | **< 0.001** |
| **Adipocytokines** | | | | | | |
| Leptin (ng/mL) | 0.26 (2.09) | 0.78 (2.84) | 0.728 | 0.38 (2.47) | 0.24 (1.75) | 0.80 |
| Resistin (ng/mL) | 1.36 (0.41) | 1.51 (0.66) | **0.023** | 1.28 (0.51) ** | 1.37 (0.37) | 0.39 |
| Ghrelin (pg/mL) | 62.38 (52.49) | 45.60 (31.26) | **0.001** | 69.84 (67.46) * | 60.82 (47.33) | 0.65 |
| **Lipid profile at the time of the study** | | | | | | |
| Cholesterol (mg/dL) | 166.3 ± 27.5 | 152.9 ± 34.2 | **0.044** | 168.9 ± 29.2 | 163.1 ± 26.2 | 0.77 |
| Triglyceride (mg/dL) | 86.4 ± 53.7 | 74.8 ± 30.1 | 0.256 | 83.0 ± 32.0 | 88.9 ± 67.2 | 0.90 |
| LDL (mg/dL) | 88.0 ± 24.0 | 83.6 ± 26.8 | 0.437 | 85.7 ± 22.9 | 88.6 ± 23.8 | 0.90 |
| HDL (mg/dL) | 60.0 ± 18.9 | 56.6 ± 17.0 | 0.395 | 64.0 ± 22.2 | 57.0 ± 16.3 | 0.35 |
| VLDL (mg/dL) | 15.5 ± 6.1 | 16.1 ± 6.0 | 0.781 | 15.3 ± 6.3 | 15.3 ± 5.4 | 0.97 |
| **Carotid intima-media thickness** | | | | | | |
| cIMT (mm) | 0.50 (0.16) | 0.42 (0.07) | **0.003** | 0.50 (0.17) * | 0.50 (0.14) * | 0.52 |
| cIMT-SDS | 0.02 (2.27) | -1.01 (1.63) | **0.003** | 0.02 (2.45) | 0.01 (2.01) | 0.86 |

p[1]: comparison between all GHD children and control group, p[2]: comparison between GHD-IR and without IR groups, *p < 0.05 for comparison with control group, **p < 0.01 for comparison with control group. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).

BMI: body mass index, BP: blood pressure, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor-binding protein-3, cIMT: carotid intima-media thickness, HbA1c: hemoglobin A1c, HOMA: homeostatic model assessment, LDL: low-density lipoprotein, VLDL: very LDL, HDL: high-density lipoprotein, SDS: standard deviation (SD) score, IR: insulin resistance, GHD: growth hormone deficiency

sensitivity indices, QUICKI was significantly lower in the GHD children with IR than in those without IR ($p < 0.001$) (Table 3).

### Lipid Profile and Adipocytokines

Fourteen (20%) of the children in the GHD group had elevated serum lipid levels (Table 3). Seven children had hypercholesterolemia, four children had hypertriglyceridemia, and three had high LDL levels. The proportion of children with elevated serum lipids did not differ between the GHD group and the control group. Except for the total cholesterol level, which was significantly higher in GHD children treated with rhGH than in the controls ($p = 0.044$), none of the other serum lipid profile parameters differed between the groups. The serum lipid profile parameters and the number of children with elevated serum lipids did not differ between the GHD subgroups.

Whereas serum leptin levels in the GHD group did not differ from the control group, serum resistin levels were lower and ghrelin levels were higher in the GHD group than in the control group [1.36 (0.41) vs. 1.51 (0.66), $p = 0.023$ and 62.38 (52.49) vs. 45.60 (31.26), $p = 0.001$, respectively]. Although serum resistin levels tended to be lower and ghrelin levels tended to be higher in the GHD IR-subgroup compared to the GHD without IR subgroup, this was not significant. Resistin levels were lower in GHD children with IR than in the controls ($p = 0.008$), and ghrelin levels were higher than in the controls ($p = 0.014$). A comparison of adipocytokine levels between the groups is summarized in Table 3.

### cIMT and Associated Factors

Both cIMT and cIMT-SDS were increased in the GHD children treated with rhGH compared to the controls [0.50 (0.16) vs. 0.42 (0.07) mm, $p = 0.003$ and 0.02 (2.27) vs. -1.01 (1.63), $p = 0.003$] (Figure 1). Both subgroups of GHD children with and those without IR showed increased cIMT and cIMT-SDS levels compared to the control group (Table 3) but there were no differences between the IR subgroups.

Higher CIMT-SDS only correlated significantly with higher ghrelin ($r = 0.338$, $p = 0.010$) but not with other adipocytokines (leptin, resistin), anthropometric measures (BMI-SDS, H-SDS, W-SDS, WC-SDS), BP-SDS, rhGH treatment related factors (dose of rhGH, IGF-1, growth velocity SDS, ΔH-SDS), or glucose metabolism indices (HOMA-IR, HOMA-B, Matsuda, QUICKI) (Table 4).

In multivariate linear regression analysis, a high cIMT-SDS was significantly associated with higher serum ghrelin levels [b-coefficient 0.491, 95% confidence interval (CI) = 0.007-0.028, $p = 0.001$] and lower serum HDL levels (b-coefficient -0.027, 95% CI = -0.049 – -0.005, $p = 0.017$). The cIMT and cIMT-SDS were not significantly associated with any other parameters (anthropometric measures, BP SDS, rhGH treatment related factors, or glucose metabolism indices) in multiple linear regression analyses.

## Discussion

The present study showed that cIMT is increased in GHD children treated with rhGH when compared to healthy

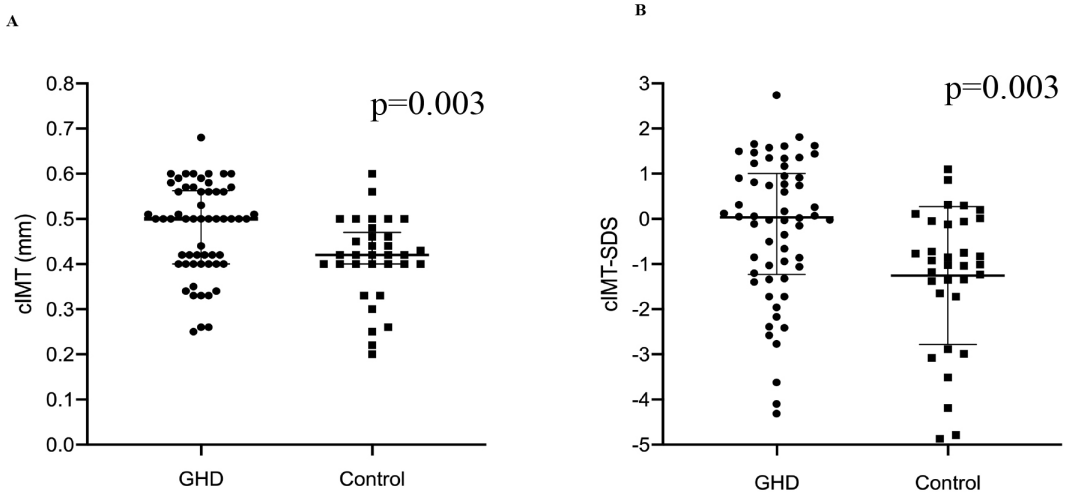


**Figure 1.** This figure shows the comparisons of cIMT and cIMT-SDS between healthy controls (n = 44) and GHD children treated with rhGH (n = 71). Original levels [median (interquartile range)] of cIMT and cIMT-SDS in healthy controls and GHD children treated with rhGH, A) cIMT levels: 0.50 (0.16) vs. 0.42 (0.07) mm, B) cIMT-SDS: 0.02 (2.27) vs. -1.01 (1.63)

*cIMT: carotid intima-media thickness, SDS: standard deviation score, GHD: growth hormone deficiency*

**Table 4. Spearman's rank correlation between cIMT or cIMT-SDS and cardiovascular risk factors, age, IGF-1, IGFBP-3**

| | cIMT | | cIMT-SDS | |
|---|---|---|---|---|
| | r | p | r | p |
| Age at diagnosis (years) | 0.147 | 0.27 | 0.001 | 0.99 |
| Age at study (years) | 0.060 | 0.65 | -0.107 | 0.42 |
| Duration of rhGH treatment (years) | -0.101 | 0.45 | -0.136 | 0.30 |
| rhGH dose (mcg/kg/day) | -0.073 | 0.58 | -0.077 | 0.56 |
| H-SDS | -0.128 | 0.23 | -0.171 | 0.11 |
| BMI-SDS | -0.09 | 0.39 | -0.069 | 0.52 |
| WC-SDS | 0.135 | 0.31 | 0.140 | 0.29 |
| ΔH-SDS | -0.003 | 0.98 | -0.013 | 0.92 |
| ΔBMI-SDS | -0.233 | 0.08 | -0.241 | 0.07 |
| Mean growth velocity SDS | 0.109 | 0.41 | 0.065 | 0.63 |
| IGF-1 SDS | 0.041 | 0.76 | 0.073 | 0.59 |
| IGFBP-3 SDS | -0.105 | 0.44 | -0.113 | 0.40 |
| Systolic BP-SDS | 0.002 | 0.99 | -0.024 | 0.86 |
| Diastolic BP-SDS | 0.131 | 0.32 | 0.015 | 0.91 |
| Fasting glucose (mg/dL) | 0.138 | 0.30 | 0.069 | 0.60 |
| 120-minute glucose (mg/dL) | 0.113 | 0.40 | 0.110 | 0.41 |
| HbA1c (%) | -0.040 | 0.81 | -0.077 | 0.64 |
| Fasting insulin (μU/mL) | 0.119 | 0.38 | 0.071 | 0.60 |
| HOMA-B | 0.111 | 0.41 | 0.137 | 0.31 |
| Total insulin (μU/mL) | -0.003 | 0.98 | -0.056 | 0.68 |
| HOMA-IR | 0.137 | 0.30 | 0.086 | 0.52 |
| QUICKI | -0.097 | 0.47 | -0.063 | 0.64 |
| MATSUDA | -0.078 | 0.56 | -0.019 | 0.89 |
| Leptin (ng/mL) | -0.037 | 0.79 | -0.013 | 0.92 |
| Resistin (ng/mL) | -0.072 | 0.59 | -0.086 | 0.52 |
| Ghrelin (pg/mL) | 0.306 | **0.019** | 0.338 | **0.010** |
| Cholesterol (mg/dL) | -0.162 | 0.27 | -0.069 | 0.64 |
| Triglyceride (mg/dL) | 0.120 | 0.41 | 0.103 | 0.48 |
| LDL (mg/dL) | -0.063 | 0.67 | 0.023 | 0.87 |
| HDL (mg/dL) | -0.259 | 0.07 | -0.248 | 0.09 |

BMI: body mass index, BP: blood pressure, WC: waist circumference, rhGH: recombinant growth hormone, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor-binding protein-3, SDS: standard deviation score, H-SDS: height SDS, cIMT: carotid intima-media thickness, HbA1c: hemoglobin A1c, HOMA: homeostatic model assessment, LDL: low-density lipoprotein, HDL: high-density lipoprotein

matched controls. This study also shows that the prevalence of alterations in glucose metabolism is low (5%) despite an increased risk of IR in children and adolescents treated with rhGH. Moreover, rhGH-treated children without IR exhibited increased insulin secretion capacity compared to the control group. However, there was no association between cIMT and loss of carbohydrate metabolism homeostasis, insulin secretion or sensitivity indices in these children. Furthermore, none of the factors related to the direct effects of rhGH treatment showed an association with changes in vascular endothelium. Adipocytokine levels and elements of the lipid profile also showed changes in GHD children treated with rhGH compared to the control group. Notably, serum HDL levels, which were lower, and ghrelin levels, which were higher in those with high cIMT-SDS scores, suggests that these adipocytokines and lipid profiles may play a role in changes of vascular endothelium.

We evaluated cIMT as an early marker of vascular changes in our cohort. Our results revealed that cIMT and cIMT-SDS were increased in GHD children treated with rhGH compared to the control group. In our study, all of the children were diagnosed as isolated GHD and treated with physiological rhGH dose and almost one third of the cohort were treated for more than three years. The results of the various studies which have examined cIMT in GH-deficient adults and children treated with rhGH are controversial. In a cohort of children and adults consisting of 105 subjects

over eight generations with GHD, there was no association between increased cIMT and GHD, but treatment with rhGH led to the appearance of carotid plaques (42). Rothermel et al. (16) demonstrated cIMT increase in children treated for three years with a supraphysiological rhGH dose but not in those treated with a physiological dose. Another study did not demonstrate any change after four years of rhGH treatment in cIMT levels in a cohort consisting of children and adolescents receiving rhGH for various indications (17). Two studies showed improvement in cIMT parameters in children and adolescents after one year of treatment with rhGH (18,19).

Atherosclerosis is a complex and multifactorial disease commonly associated with traditional cardiovascular risk factors; however, both GH and IGF-1 may influence vascular wall changes (42,43,44). The final effect of both of them in this process result from an interplay between their atherosclerotic and anti-atherosclerotic functions. On one hand, they promote atherogenesis, by stimulating vascular smooth muscle proliferation and inflammation, but on the other hand, they induce vasodilatation by stimulating nitric oxide production (42,43,44). It has been postulated that the direct action of GH contributes to the inflammatory process in atherogenesis (20). The longer the duration of rhGH treatment and the higher the cumulative dose in childhood, the stronger the association with CVD risk (2). A persistent very low IGF-1 level might have a protective role, whereas a milder decrease, such as in adult-onset GH-deficiency, might be predisposing to CVD risk (45). Rothermel et al. (16) showed a correlation between cIMT and changes in IGF-1 SDS after rhGH treatment in GHD children, but not with the dose of the rhGH treatment. In our study, there was no association between IGF-1 SDS and cIMT-SDS. Also, none of the parameters due to the direct effect of rhGH treatment (growth velocities, dose of rhGH and the duration of the treatment) were associated with vascular changes.

Alterations in body composition, carbohydrate metabolism and lipid profile have long been suspected of being risk factors for CVD in children with GHD (46), and in children treated with rhGH (16,17,25). In GHD children, rhGH therapy reduces insulin sensitivity and causes a compensatory hyperinsulinemia with normal glucose levels (26). Our current results are in line with these data. We found an increase in insulin values in half of the cohort, with a concomitant decrease in the insulin sensitivity indices QUICKI and Matsuda, after almost three years of treatment. In addition, GHD children without IR also showed an increased HOMA-B index compared to the control group, which indicates an increased insulin secretion capacity in these children. Although Cutfield et al. (22) reported an increased incidence of diabetes mellitus in children and adolescents receiving rhGH treatment, there was no individual with type 2 diabetes in our cohort. To the best of our knowledge, this is the first study evaluating the relationship between cIMT and parameters of carbohydrate metabolism in children with GHD treated with rhGH, but we could not find any association between the alterations in carbohydrate metabolism and changes in cardiovascular endothelium.

Studies have shown that rhGH treatment has a beneficial effect on body composition in GHD children (46,47). However, studies assessing the effect of rhGH treatment on lipid profile in children with GHD have reported conflicting results with some studies demonstrating a favorable effect on lipid profile with rhGH treatment (47,48), while others do not (49). The differences in severity and duration of GHD, and the variable presence of other hormone deficits might be the reason for these conflicting results. Due to the cross-sectional design of our study, we were able to compare the body composition and lipid profile of GHD children treated with rhGH and healthy controls, and there was no difference between the groups. Furthermore, there was no difference between the subgroups stratified by IR. Limited studies evaluating the effect of body composition and serum lipid profile on cIMT in children have shown a positive association between cIMT and BMI-SDS (17) and baseline LDL-cholesterol (16). In our study, there were no associations between cIMT-SDS and body composition parameters (BMI-SDS at diagnosis and study time, ΔBMI-SDS, and waist-SDS), but lower HDL-cholesterol levels showed an independent association with higher cIMT-SDS. However, longitudinal research is needed to evaluate the conflicting relationship between blood lipid profile and cIMT-SDS in GHD children treated with rhGH.

Adipocytokines, including leptin, resistin and ghrelin, are involved in the processes of utilizing and storing energy in a tight interaction with GH. GHD children treated with rhGH showed decreased levels of leptin and des-acyl ghrelin and increased acyl-ghrelin and serum resistin levels (50,51). The changes in leptin and ghrelin are thought to cause the metabolic effects of rhGH on lipid mobilization and promote fat loss (52). Ghrelin is especially known for its cardiovascular effects. Low circulating des-acyl ghrelin levels increase cardiovascular risk (53). However, Oliveira-Santos et al. (54) argued that there are favorable vascular and metabolic outcomes of reduced postprandial ghrelin secretion in GHD individuals. In our cohort, GHD children treated with rhGH showed higher total ghrelin and lower resistin levels than the control group.

Although some studies showed an increase in resistin levels after rhGH treatment in GHD children (50,55), others showed a decline in resistin after treatment (56,57). It has been shown that ghrelin decreases after rhGH treatment compared to the GHD period (56). Because of the lack of baseline results, we were unable to make any conclusions about the changes of these adipocytokines after rhGH treatment. Previously, no study had evaluated the relationship between ghrelin and cIMT in GHD children treated with rhGH. In our study, cIMT and cIMT-SDS showed a positive association with total ghrelin levels. High endogenous ghrelin levels are associated with increased carotid artery stenosis in the adult population, and they related with high IL-1β and vascular smooth muscle cell dysfunction (58,59). Our study showed that ghrelin should be considered as a potential early marker for vascular changes in GHD children treated with rhGH, but further studies are needed.

### Study Limitations

The changes in carbohydrate metabolism in rhGH treated children and cardiovascular evaluation have been investigated in different studies. Our study attempted to elucidate the association between vascular and metabolic changes with a broad perspective. A major limitation of our study was that we did not use a glucose clamp test, which is the gold standard for the evaluation of IR. Secondly, our study protocol did not include pre-rhGH treatment evaluations of the lipid, carbohydrate or cardiovascular metabolism. Thirdly, our cohort was followed for a relatively short period of time and, as adulthood risks for CVD in this group of children are important, long-term follow-up studies are needed to evaluate the exact role of IR in CVD in GHD children treated with rhGH. Fourthly, we did not demonstrate any change between basal and post-treatment cIMT results in our cohort, due to the lack of evaluation at the start of the rhGH treatment. Lastly, our control group consisted of healthy children, but not of GHD children without rhGH treatment. There are several studies in children and adolescents showing improvements in cIMT after rhGH treatment. As we compared GHD children treated with rhGH with healthy controls, the difference of cIMT levels between the groups may also be effected by GHD itself.

## Conclusion

In conclusion, this study found increased cIMT-SDS in GHD children treated with rhGH compared to a healthy control group. Alterations in carbohydrate metabolism were not directly associated with cIMT-SDS. Changes in ghrelin may be associated with early vascular changes. rhGH therapy *per se* appears to be associated with this increased cIMT but causality should be elucidated in future studies.

### Ethics

**Ethics Committee Approval:** The study protocol and this consent procedure were approved by the İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (date: 20.06.2014, no: 2014/990).

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

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Seha Saygılı, Mehmet Kocaağa, Mine Şükür, Concept: Seha Saygılı, Feyza Darendeliler, Design: Seha Saygılı, Feyza Darendeliler, Data Collection or Processing: Seha Saygılı, Mehmet Kocaağa, Gamze Kaya, Mine Şükür, Firdevs Baş, Şükran Poyrazoğlu, Analysis or Interpretation: Seha Saygılı, Firdevs Baş, Şükran Poyrazoğlu, Rüveyde Bundak, Feyza Darendeliler, Literature Search: Seha Saygılı, Feyza Darendeliler, Writing: Seha Saygılı, Feyza Darendeliler.

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**Click for Supplementary Table 1. Formulas for the assessment of insulin sensitivity and secretory capacity**

**Access link: http://glns.co/wgb0p**

CASE REPORT

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# Weight Loss During Topiramate Treatment in a Severely Obese Adolescent with Congenital Adrenal Hyperplasia and Migraine

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### What is already known on this topic?

Youth with classical congenital adrenal hyperplasia (CAH) exhibit earlier adiposity rebound, increased obesity and abdominal adiposity compared to unaffected youth. There is evidence that topiramate therapy is effective in appetite suppression resulting in body mass index reduction in obese adults and adolescents. Little is known about the efficacy of topiramate in treating severe obesity associated with CAH.

### What this study adds?

Topiramate produced clinically meaningful and significant weight loss and reduced central adiposity in an adolescent with classical CAH and severe obesity. Topiramate was used safely without an increase in the frequency of adrenal crises or glucocorticoid requirement. Topiramate therapy may be especially effective in patients with CAH, given their increased visceral adiposity.

## Abstract

Youth with classical congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency exhibit an increased prevalence of obesity, early adiposity rebound, and increased abdominal adiposity compared to unaffected youth. Current obesity management in CAH largely focuses on lifestyle modifications. There is evidence that topiramate therapy is effective in reducing body mass index (BMI), as well as visceral adipose tissue (VAT), in unaffected adolescents with exogenous obesity. However, little is known about the efficacy of topiramate in patients with classical CAH. We report on a 17-year-old female with severe obesity and salt-wasting CAH due to 21-hydroxylase deficiency, who demonstrated reductions in BMI, as well as abdominal visceral and subcutaneous adipose tissue (SAT) while on topiramate therapy. The patient was diagnosed with classical CAH as a newborn with a 17-hydroxyprogesterone 11,000 ng/dL. She had a BMI over the 95$^{th}$ percentile at 3 years of age, followed by unremitting obesity. At 17 years old, she was started on topiramate to treat chronic migraines. Following three years of topiramate therapy, her BMI z-score decreased from +2.6 to +2.1. After four years of therapy, her waist circumference decreased from 110 to 101 cm, abdominal VAT decreased substantially by 34.2%, and abdominal SAT decreased by 25.6%. Topiramate therapy was associated with effective weight loss and reduced central adiposity in an adolescent with classical CAH and severe obesity, without any side effects. Further study is warranted regarding topiramate therapy in obese youth with classical CAH and increased central adiposity, who are at higher risk for significant morbidity.

**Keywords:** Congenital adrenal hyperplasia, topiramate, obesity, body composition, body fat percentage, adolescent, 21-hydroxylase deficiency

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## Introduction

Youth with classical congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency are at increased risk of earlier adiposity rebound, obesity, and increased abdominal adiposity compared to unaffected youth (1). These factors can lead to increased risks of cardiovascular disease, hypertension, and diabetes (2). However, current obesity management in CAH largely focuses on lifestyle modifications. Management guidelines for children with exogenous obesity recommend an intensive, in-clinic, multidisciplinary intervention, involving 26 contact hours over a six-month period (3). This may not be feasible in all clinical settings, and weight loss can be difficult to achieve through these modifications alone. Pediatric guidelines also suggest early, intensive intervention with lifestyle modifications, with consideration of anti-obesity pharmacotherapy after failure of lifestyle modifications (4). Youth with CAH and concomitant severe obesity are a high-risk cohort and require earlier consideration of anti-obesity pharmacotherapies. In addition, adolescents and young adults with classical CAH can have increased abdominal visceral adipose tissue (VAT) which is associated with inflammation, cardiovascular disease, and risk for metabolic syndrome (1). Thus, patients with CAH may also benefit from therapeutic options which specifically reduce VAT.

Topiramate is an Food and Drug Administration (FDA)-approved drug utilized for the treatment of epilepsy and migraines in children. It is a GABA receptor modulator which reduces glutamate release by blocking voltage-gated Na channels. There is also evidence that topiramate therapy is effective in appetite suppression resulting in body mass index (BMI) reduction in obese adolescents and adults (5), with a side effect profile which can include paresthesias, cognitive slowing, and taste impairment (6). Little is known about the efficacy of topiramate in the treatment of severe obesity associated with chronic conditions such as CAH. However, as topiramate has been shown to reduce VAT more than placebo in randomized control trials, it may be particularly helpful in conditions such as CAH with fat distributions which involve increased VAT (7,8).

We report on an adolescent case of severe obesity associated with classical CAH due to 21-hydroxylase deficiency in which sustained BMI reduction and decreased central adiposity were achieved via topiramate therapy.

## Case Report

Our female patient was diagnosed with classical, salt-wasting CAH shortly after birth. She presented with virilized external genitalia at birth, and an elevated serum 17-hydroxyprogesterone of 11,000 ng/dL (normal < 78 ng/dL). She was treated with hydrocortisone, fludrocortisone, and salt supplementation. Molecular genetic assessment of *CYP21A2* showed the presence of compound heterozygous mutations (G110del8nt and Q318X) consistent with 21-hydroxylase deficiency. She also had metopic craniosynostosis and developed generalized tonic-clonic seizures at 3 months old, requiring phenobarbital treatment for 4 years.

By 3 years of age, her BMI was 110 percent of the 95$^{th}$ percentile, and remained > 95$^{th}$ percentile thereafter. She developed chronic migraine headaches and was started on a topiramate dose of 50 mg daily by her neurologist at 17 years old. The topiramate dose was titrated to 100 mg daily over 2 years. At the time of topiramate initiation, lifestyle modifications had already been recommended as treatment for obesity, including routine exercise and nutrition counseling (*e.g.,* a reduction of sugar-sweetened beverage intake and an increase in fruit and vegetable consumption) during clinical visits. She had only implemented participation in physical exercise at school and her neighborhood team soccer and these efforts did not result in any notable weight loss. She was on 15 mg/m$^2$/day of glucocorticoid treatment and had good hormonal control with 17-hydroxyprogesterone 59 ng/dL, androstenedione 91 ng/dL, and testosterone 28 ng/dL. She was not receiving other medications which would have had an impact on her weight. Her growth plates were fused. During the 4-year course of topiramate treatment, she continued her previous lifestyle interventions and did not start any intensive lifestyle modification programs, new therapies, prescriptive dietary regimens or major lifestyle changes. At the time of the 4-year assessment, her total daily dose of glucocorticoid had remained unchanged. Hormone analytes showed: 17-hydroxyprogesterone 221 ng/dL, androstenedione 84 ng/dL, and testosterone 50 ng/dL. She did not have any adrenal crises while on topiramate.

### Anthropometric Measures

All measurements were obtained at clinical visits and during two research studies [protocols were approved by the Children's Hospital Los Angeles (CHLA) Institutional Review Board (CCI-09-00261, CHLA-14-00191)]. Written informed consent and assent were obtained from one of the parents and the patient, respectively. BMI was calculated, and the BMI z-score was reported up to 20 years of age. Waist circumference was measured, and waist-to-height ratio was calculated.

At initiation of topiramate treatment, the patient's BMI was 48.5 kg/m$^2$ (weight 100.2 kg). Following 2.4 years of

treatment, her BMI z-score decreased from +2.6 to +2.1 (Figure 1). She had the greatest BMI reduction on topiramate 100 mg daily, with a nadir of 35.1 kg/m$^2$. Although she had a slight rebound increase in BMI of 12% during the third year of treatment, she experienced a total BMI reduction of 23% after 4.2 years of topiramate treatment. She also had a decrease in waist circumference [110 cm (90-95$^{th}$ percentile) to 101 cm (50-75$^{th}$ percentile)] and in her waist-to-height ratio (0.76 to 0.70).

### Body Composition and Adiposity

Body fat was measured using multiple modalities over time as part of a research protocol examining body composition and adiposity distribution in patients with classical CAH. The subject had study visits at 12 and 21 years of age.

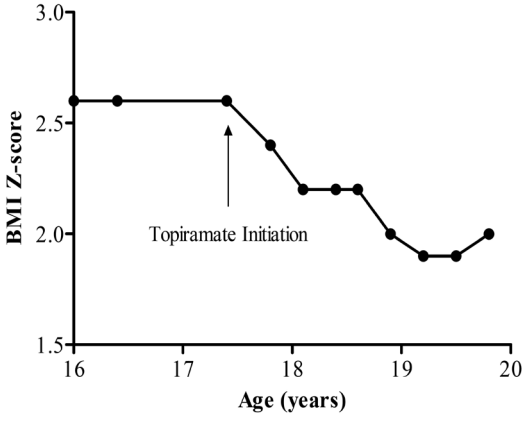


**Figure 1.** BMI z-score pre- and post-topiramate initiation in a female adolescent with classical congenital adrenal hyperplasia and severe obesity. Over the course of 2.4 years from the initiation of topiramate, the BMI z-score decreased from 2.6 to 1.9 at its lowest point, suggesting that topiramate therapy resulted in a substantial and sustained weight loss over a short period of time

*BMI: body mass index*

Whole body dual energy X-ray absorptiometry (DEXA; Hologic Delphi®/Horizon®, Marlborough, MA) was utilized to examine body composition by measuring total body fat and trunk fat. Prior to topiramate treatment, the subject exhibited a high total fat mass of 48.8 kg, a total fat percentage of 51.9%, and trunk fat of 45.8%. After 4 years of treatment with topiramate, she exhibited a decrease in total fat mass (48.8 to 36.7 kg), total fat percent (51.9% to 45.9%), and trunk fat (45.8% to 40%).

In order to analyze abdominal adiposity, including VAT and subcutaneous adipose tissue (SAT), single-slice computed tomography (CT) imaging (HiLight Advantage CT, GE, Chicago, IL) was utilized at the time of the patient's initial study visit, prior to topiramate treatment. The CT abdominal axial slice corresponded to the level of the umbilicus and the L4 vertebra. The patient exhibited high amounts of abdominal adipose tissue, as can be seen in adolescents and young adults with classical CAH (9): VAT was 84.5 cm$^2$ and SAT was 654.5 cm$^2$. The imaging modality subsequently changed to magnetic resonance imaging (MRI) on a 3-Tesla human platform (Achieva, R5.3, Phillips Healthcare, Cleveland, OH) employing a chemical-shift sequence, at the time of her next study visit four years post-initiation of topiramate therapy. For comparison across pre- and post-treatment time points, an MRI abdominal axial slice at the level of the L4 vertebra using anatomic landmarks of the musculature and orientation of the intestines to best match the initial CT single-slice taken at the second study visit was selected (Figure 2). Adipose tissue had segmented (Synapse 3D Fujifilm, Stamford, CT) on quantitative fat fraction images (9). VAT was observed to be substantially

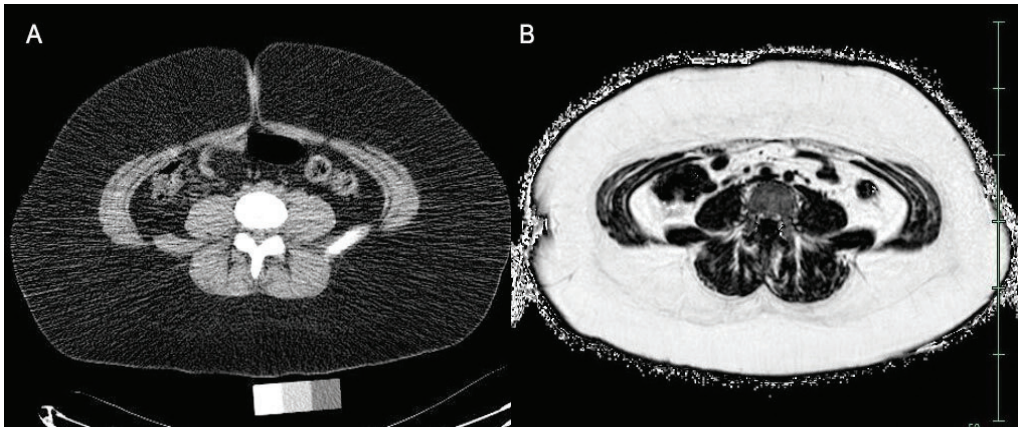


**Figure 2.** Abdominal adipose tissue imaging of a female adolescent with classical congenital adrenal hyperplasia to quantify visceral and subcutaneous adipose tissue. Single-slice images in the axial view, at the L4 vertebra. A) Single-slice computed tomography image was acquired at 12 years of age, prior to the initiation of topiramate treatment. B) Magnetic resonance image was acquired at 21 years of age, four years following the initiation of topiramate treatment

decreased by 34.2% (55.6 $cm^2$) and SAT had decreased by 25.6% (486.8 $cm^2$) on the MRI. Although ideally, the comparison would have been made using images from the same modality, given the accuracy of both single-slice CT and MRI (mDixon) techniques to quantify adipose tissue, our method of comparison incorporating these modalities is supported by the current standards of imaging analysis (10).

## Discussion

In our patient with classical CAH and severe obesity, topiramate therapy initiated for migraines produced a reasonably sustained BMI reduction over 4 years, along with reductions in total body fat and central adiposity. The BMI z-score reduction of 0.5 (23%) which she experienced has been associated with improvements in systolic blood pressure, high-density lipoprotein cholesterol, and triglycerides in youth (11). Her dramatic reduction in VAT by over 50%, along with improvements in markers of abdominal adiposity, carry additional implications for the lowering of ectopic fat accumulation, inflammation, insulin resistance, and cardiovascular disease risk (1,12).

Topiramate has been demonstrated in some studies to be associated with BMI reduction in adolescents and adults with obesity which is not associated with CAH (5,13). However, data to support a new indication (i.e. from the U.S. FDA) for pediatric obesity, or guidelines supporting its off-label use, are lacking. Nonetheless, its clinical utilization by pediatric obesity specialists has continued to increase (14). However, more research is needed to understand topiramate stand-alone therapy vs. combination therapy with phentermine (15) and the off-label use of this medication.

There are several limitations to our report. Firstly, our patient was prescribed topiramate for chronic migraines, with BMI reduction being an incidental outcome. Secondly, an expanded study of topiramate use is needed in a larger number of patients with classical CAH and severe obesity. Importantly, topiramate did not produce adverse events in our patient, including any increase in the frequency of adrenal crises or glucocorticoid requirements, even though a report of topiramate-induced hypoadrenalism exists in an adult with CAH (16). Our case suggests that topiramate could be a safe pharmacological option for adolescents with CAH and obesity and this merits further study.

## Conclusion

We conclude that topiramate treatment was associated with substantial weight loss and reduced central adiposity in an adolescent with classical CAH and severe obesity. Topiramate may be especially effective in patients with CAH given their increased visceral adiposity. It is evident from our case and other wider applications of topiramate therapy that more research is needed in obese youth with and those without CAH. Further study is warranted on the safety and efficacy of topiramate as an adjunctive treatment in obese youth with classical CAH and increased central adiposity, who are at higher risk for significant morbidity.

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### Ethics

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

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Amy Seagroves, Alaina P. Vidmar, Mitchell E. Geffner, William S. Kim, Darryl Hwang, Concept: Amy Seagroves, Darryl Hwang, Design: Amy Seagroves, Darryl Hwang, Data Collection or Processing: Amy Seagroves, Heather M. Ross, Darryl Hwang, Claudia Borzutzky, Nicole R. Fraga, Mimi S. Kim, Analysis or Interpretation: Amy Seagroves, Heather M. Ross, Alaina P. Vidmar, Mitchell E. Geffner, William S. Kim, Darryl Hwang, Claudia Borzutzky, Nicole R. Fraga, Mimi S. Kim, Literature Search: Amy Seagroves, Alaina P. Vidmar, Darryl Hwang, Writing: Amy Seagroves, Heather M. Ross, Alaina P. Vidmar, Mitchell E. Geffner, William S. Kim, Darryl Hwang, Claudia Borzutzky, Nicole R. Fraga, Mimi S. Kim.

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CASE REPORT

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# Rare Coexistence of Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency and Turner Syndrome: A Case Report and Brief Literature Review

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**What is already known on this topic?**

The combination of Turner syndrome and congenital adrenal hyperplasia (CAH) is rarely reported in the literature.

**What this study adds?**

We report a new case of the coexistence of mosaic Turner syndrome and the non-classical form of CAH due to 21-hydroxylase deficiency, associated with a *de novo* mutation in the *CYP21A2* gene. This case did not present with short stature.

## Abstract

The coexistence of congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency and Turner syndrome (TS) is rare. We report on a 6-year-old Portuguese girl with mosaic TS [45,XO(39)/47,XXX(21)] presenting with premature pubarche at the age of 5 years. Laboratory findings showed elevated 17-hydroxyprogesterone, dehydroepiandrosterone sulfate, androstenedione and total testosterone, and her sex-determining region Y (SRY) was negative. *CYP21A2* gene analysis revealed two mutations (c.[844G>T]; [*CYP21A2*del]), consistent with the non-classical form of CAH. Complete deletion of *CYP21A2* allele occurred *de novo*. At 6 years and 4 months, she presented with accelerated growth velocity and hydrocortisone at a dose of 5 mg/m$^2$/day was initiated. This case highlights the need to perform global examinations looking for virilization signs in TS patients' follow-ups. It also supports the reported genetic combination of TS and CAH. Therefore, CAH should be kept in mind in TS patients with SRY negative and virilization signs, even in the absence of short stature.

**Keywords:** Adrenal hyperplasia, congenital, Turner syndrome, virilism, karyotyping

## Introduction

Turner syndrome (TS) is a common genetic disorder among young females and it is characterized by infertility, premature ovarian deficiency, short stature and other abnormalities (1). Some patients have the classical monosomy X (45,X) and others have various 45,X mosaicism, including mosaic monosomy X with a Y-bearing cell line. Virilization occurring in TS patients should prompt a search for the Y chromosome-bearing cell line, as these individuals are at risk of developing malignant gonadal tumors and they can present with ambiguous genitalia, as with congenital adrenal hyperplasia (CAH) (2,3).

CAH secondary to 21-hydroxylase (21-OH) deficiency is one of the most common causes for virilization in females. There are three forms: the classic salt-wasting, simple virilising and the non-classical or late-onset, the latter being the most prevalent type (4).

CAH and TS are not very rare diseases, but their combination is rare and may be confounding (4,5). We report on a case of

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TS with coexisting 21-OH deficiency. The second condition was only recognized during follow-up with the evaluation of the patient's puberty signs.

Written informed consent was obtained from the mother.

## Case Report

The patient, known to be a mosaic for TS [45,XO(39)/47,XXX(21)], was diagnosed during amniocentesis and confirmed by postnatal karyotype. She was referred to the Pediatric Endocrinology Department at 20 months of age. She was born at term from the second gestation of a 35-year-old mother. Her birth weight was 2,565 g, her length was 45 cm and her head circumference was 32.5 cm. The parents were non-consanguineous. On physical examination, she presented with good general appearance, low posterior hairline, micrognathia, and Tanner stage 1. During follow-up, she had recurrent otitis media. Echocardiography, performed as part of routine investigations in TS patients, revealed no pathology. At 5 years and 8 months, she presented with premature pubarche with three dark thick pubic hairs on the labia majora (Tanner stage 2 pubic hair and Tanner stage 1 breasts). Her height was 109.2 cm [-0.62 standard deviation (SD)] and her weight was 19.4 kg (0.09 SD). Initial laboratory findings showed 17-hydroxyprogesterone (17-OHP) 18 (0.03-0.9) ng/mL, dehydroepiandrosterone sulfate 2.76 (< 0.05-0.57) ug/mL, androstenedione 1.4 (0.08-0.5) ng/mL, total testosterone 0.3 (< 0.03-0.1) ng/ml, LH < 0.1 (0.02-0.3) mUI/mL and FSH 2.6 (1.0-4.2) mUI/mL (Table 1). Repeated laboratory work-up confirmed these results (Table 1). Renal and pelvic ultrasonography demonstrated normal kidneys without renal anomalies, and a uterus with dimensions of 2.3x0.7x1.1 cm. Both ovaries were 1.2x0.6 cm. Analysis of the sex-determining region Y (SRY) gene was negative. Analysis of the *CYP21A2* gene revealed the presence of the mild variant c.844G > T [p.(Val282Leuc)] in hemizygote associated with enzymatic activity of 21-OH of 50%, and the presence of non-functional allele, complete deletion of *CYP21A2* (*CYP21A2*del), associated with null enzymatic activity of 21-OH. These results were consistent with a partial deficiency of 21-OH compatible with the non-classical form of CAH.

Her mother did not present with any of the genetic alterations and her father was a carrier of the mild variant c.844G > T. Her 13-year-old sister had recurrent otitis media and premature pubarche starting at the age of six. Her genetic testing also identified mild variant c.844G > T, associated with the enzymatic activity of 50% 21-OH, in heterozygosity, in the *CYP21A2* gene, although this was not sufficient for a diagnosis of CAH. The sister's last laboratory evaluation showed sodium 137 (136-146) mmol/L, potassium 4.6 (3.5-5.1) mmol/L, 17-OHP 4.19 (0.18-2.3) ng/mL, androstenedione 3.4 (0.77-2.25) ng/mL, total

**Table 1. Laboratory analysis of the patient**

| Age | 5 years and 8 months | 5 years and 10 months | 6 years and 7 months |
|---|---|---|---|
| Sodium, mmol/L | 139 (136-146) | | 141 (136-146) |
| Potassium, mmol/L | 4.9 (3.5-5.1) | | 4.3 (3.5-5.1) |
| ACTH, pg/mL | | 22 (10-60) | 20 (10-60) |
| Cortisol, ug/dL | | 10 (3-21) | 7.1 (3-21) |
| Glucose, mg/dL | 82 (60-100) | | 89 (60-100) |
| Creatinine, mg/dL | 0.56 (0.44-0.64) | | |
| FSH, mUI/mL | 2.6 (1.0-4.2) | 2.7 (1.0-4.2) | |
| LH, mUI/mL | < 0.1 (0.02-0.3) | 0.1 (0.02-0.3) | |
| Estradiol, pg/mL | < 13 (5-20) | < 13 (5-20) | |
| AMH, ng/mL | | 0.28 | |
| 17-OH progesterone, ng/mL | 18 (0.03-0.9) | 17 (0.03-0.9) | 19 (0.03-0.9) |
| Total testosterone, ng/ml | 0.3 (< 0.03-0.1) | 0.2 (< 0.03-0.1) | |
| Androstenedione, ng/mL | 1.4 (0.08- 0.5) | 1.4 (0.08- 0.5) | |
| DHEA-$SO_4$, ug/mL | 2.76 (< 0.05-0.57) | 3.72 (< 0.05-0.57) | 3.30 (< 0.05-0.57) |
| IGF-1, ng/mL | 150 (35-232) | 142 (35-232) | |
| TSH, uUI/mL | | 2.7 (0.70-4.17) | |
| FT4, ng/dL | | 0.90 (0.89-1.37) | |
| Aldosterone, pg/mL | | 185 (30-350) | 155.0 (30-350) |
| Active renin, uU/mL | | 55 (7-76) | 84 (7-76) |

17-OH: 17-hydroxyprogesterone, ACTH: adrenocorticotropic hormone, AMH: Anti-Mullerian hormone, DHEA-$SO_4$: dehydroepiandrosterone sulfate, FSH: follicle-stimulating hormone, FT4: free thyroxine, IGF-1: insulin like growth factor-1, LH: luteinizing hormone, TSH: thyroid stimulating hormone

testosterone 0.3 (0.13-0.32) ng/mL, LH 5.0 (< 12.0) mUI/mL and FSH 4.1 (< 9.6) mUI/mL.

At 6 years and 4 months, the weight of the patient was 24.3 kg (0.98 SD) and her height was 119.8 cm (0.54 SD), with accelerated growth velocity (10.6 cm in 10 months). Hydrocortisone treatment at a dose of 5 mg/m$^2$/day was initiated. During her last visit at 6 years and 7 months, her weight was 25.3 kg (1.03 SD) and her height 121.3 cm (0.51 SD). Laboratory work-up (Table 1) was performed, under hydrocortisone at a dose of 5 mg/m$^2$/day, although with irregular compliance. The need for treatment was reinforced in order to avoid complications.

## Discussion

We described a new case of CAH due to 21-OH deficiency in a 6-year-old Portuguese girl with a mosaic form of Turner karyotype.

The first sign of virilization in our patient was premature pubarche at the age of 5 years. She was known to have a mosaicism TS, but only had a few TS stigmas and did not present with short stature. Laboratory investigation revealed elevated levels of 17-OHP and androgens, with normal sodium, potassium, FSH, LH, IGF1, cortisol, adrenocorticotropic hormone, active renin and aldosterone levels. As is strongly recommended, an SRY gene analysis was performed and this was negative. Continuing the investigation, the rare occurrence of coexisting CAH was investigated (2). Her elevated basal 17-OHP level and the *CYP21A2* gene analysis (*CYP21A2* genotype: c.[844G > T]; [*CYP21A2*del]) established a diagnosis of the non-classical form of 21-OH deficiency. As her mother did not present with any genetic alterations, it is possible to infer that the complete deletion of *CYP21A2* allele occurred *de novo*. The occurrence rate of *de novo* mutations in *CYP21A2* alleles in affected patients with 21-OH deficiency has been assessed to be 1-2%. Her sister's genetic testing did not confirm non-classical CAH, although it also did not allow it to be ruled out completely.

This rare combination of TS and CAH was first described by del Arbol et al. (6) in 1983. So far, ten cases with both TS and CAH due to 21-OH deficiency have been reported in the literature (1,2,3,5,6,7,8,9,10,11). Unlike most of the previously reported cases which were diagnosed as TS during the investigation of ambiguous genitalia or presented with concomitant diagnosis (2,3,6,7,8,9,10), in our case, the diagnosis of TS was made initially. Only three cases known to have TS were later diagnosed as CAH (1,5,11).

As in our patient, most of the previous cases had different degrees of virilism (2). Only one case of a 28-year-old woman who had decreased endometrial receptivity during IVF did not show virilism (1). Likewise, all cases described to date, except for one, had a mosaic Turner karyotype (2,8).

The diagnosis of coexisting CAH, particularly the non-classical type, is difficult in patients with TS, as typical signs such as short stature, amenorrhea and hirsutism may be present in both diseases (2,11). Furthermore, at an early age as in our case, it is even more difficult to detect coexisting CAH, because some of these signs of both diseases, including short stature, have not yet manifested. Therefore, it is important to include genital examinations for virilization signs in routine visits in patients with TS (4), and to measure 17-OHP levels, especially in the presence of moderate-to-severe virilization (2).

The final heights of patients with concomitant TS and CAH tend to deteriorate due to both diseases (3). Unopposed hyperandrogenism caused by CAH may lead to initial skeletal maturation. However, it can mask the growth disorder, because premature closure of the growth plates leads to short final heights (2). In addition, insufficient hormone replacement therapy or overtreatment of CAH also causes final short stature (7). At the same time, TS can cause short stature. However, the prevalence of short stature in rare 45,X/47,XXX mosaicism individuals is only 64.3%, that is, much less frequent than in pure 45,X monosomy (over 95%) (12). Therefore, we think the patient's karyotype may lead to better growth. It has been speculated that this may be related to the presence of 47,XXX cell lines, because the triple-X syndrome often presents with taller stature (12). Nevertheless, the final adult height is not guaranteed without growth hormone (GH) treatment (13).

While it is possible to achieve good results in CAH patients with regular follow-up and treatment, in TS, GH treatment initiated at supraphysiological doses and at an early age (before the age of 4 years) can lead to a considerable height gain, despite the absence of GH deficiency in TS (7,11). In a previously case of a one-year-old patient with TS and CAH, in addition to treatment with appropriate doses of glucocorticoids and mineralocorticoids, GH treatment was initiated when a slowing in growth was later observed (7). Our patient did not have short stature, probably due to accelerated skeletal maturation and some partial protection provided by her karyotype, as discussed previously. However, due to the coexistence of the two pathologies and irregular therapeutic compliance, her growth potential may be compromised. In our country, Portugal, GH treatment

is approved for TS when there is a diagnosis confirmed by chromosomal analysis, chronological age >2 years, bone age <12 years before puberty, and height <-2 SD and z-score of the height velocity <10$^{th}$ percentile for 1 year. After improving our patient's compliance and adequately controlling her CAH, we can question whether our patient will benefit from the earliest possible GH treatment, because her height may never be <-2 SD, but growth may end too soon.

## Conclusion

In conclusion, we presented a patient with the non-classical form of CAH due to 21-OH deficiency and mosaic TS, who presented with premature pubarche. To the best of our knowledge, this is the first report of this rare combination in a Portuguese patient. A review of the literature showed that this is the fourth case where the diagnosis of CAH was later than the diagnosis of TS. If signs of virilism are detected in patients with TS, rare coexisting CAH should be suspected in the absence of SRY.

### Ethics

**Informed Consent:** Written informed consent was obtained from the mother.

**Peer-review:** Externally peer-reviewed.

### Author Contributions

Surgical and Medical Practices: Joana Serra-Caetano, Rita Cardoso, Isabel Dinis, Alice Mirante, Concept: Isabel Inácio, Joana Serra-Caetano, Alice Mirante, Design: Isabel Inácio, Joana Serra-Caetano, Rita Cardoso, Alice Mirante, Data Collection or Processing: Isabel Inácio, Joana Serra-Caetano, Rita Cardoso, Analysis or Interpretation: Isabel Inácio, Joana Serra-Caetano, Rita Cardoso, Literature Search: Isabel Inácio, Writing: Isabel Inácio, Joana Serra-Caetano, Rita Cardoso, Isabel Dinis, Alice Mirante.

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# Expanding the Phenotype of *TRMT10A* Mutations: Case Report and a Review of the Existing Cases

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**What is already known on this topic?**

The mutation of the tRNA methyltransferase 10 homologue A *(TRMT10A)* gene causes a novel recessive syndrome of abnormal glucose homeostasis associated with distinctive features. A few cases have been reported to date.

**What this study adds?**

Ovarian failure with small ovaries, high gonadotropins and low anti-Mullerian hormone levels can lead to pubertal delay in these patients. Growth hormone deficiency can be an additional finding of this syndrome.

## Abstract

The tRNA methyltransferase 10 homologue A *(TRMT10A)* gene encodes tRNA methyl transferase, and biallelic loss of function mutations cause a recessive syndrome of intellectual disability, microcephaly, short stature and diabetes. A case with intellectual disability and distinctive features including microcephaly was admitted. She was diagnosed with epilepsy at 2.5 years old. At 3.6 years of age, severe short stature related to growth hormone (GH) deficiency was detected. She had an incidental diagnosis of diabetes at age 11.4 years which was negative for diabetes antibodies with persistent C-peptide level and she was treated with metformin. Spontaneous puberty did not begin until 15.7 years of age and she was found to have primary ovarian failure. A homozygous p.Arg127* mutation in *TRMT10A* was detected. In addition to the typical clinical features which characterize *TRMT10A* syndrome, we observed an unusual form of impaired glucose metabolism which presented in early childhood with hypoglycemia followed by diabetes in late childhood. GH deficiency and primary ovarian failure may also be additional findings of this syndrome. Patients with slow onset diabetes who are negative for auto-antibodies and have extra-pancreatic features should be tested for all known subtypes of monogenic diabetes.

**Keywords:** *TRMT10A*, monogenic diabetes, ovarian failure

## Introduction

Human glucose metabolism can be disrupted by pathogenic mutations in numerous genes with some of them associated with distinctive clinical and laboratory features (1). Recently, a novel syndrome has been reported characterized by abnormal glucose homeostasis or non-autoimmune diabetes associated with microcephaly, epilepsy, intellectual disability, failure to thrive and delayed puberty due to biallelic pathogenic mutations in the tRNA methyltransferase 10 homologue A *(TRMT10A)* gene (2,3,4,5,6).

tRNAs are non-coding RNA molecules essential for protein synthesis. They are crucial for cellular function and can undergo modifications of their bases and sugar moieties. Reduced modifications may lead to tRNA degradation or fragmentation. TRMT10A is a tRNA modifying nuclear enzyme with methyl transferase activity and it is localized in the nucleolus where tRNA modifications occur. TRMT10A

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deficiency induces oxidative stress and initiates the apoptosis of beta cells (7).

Although short stature and delayed puberty in addition to disturbed glucose metabolism have been reported in those patients with TRMT10A deficiency, growth hormone (GH)-insulin-like growth factor-1 (IGF-1) axis has not been evaluated in the patients reported; except for one (8). We report on a patient with GH deficiency, ovarian failure, non-autoimmune diabetes and a homozygous mutation in the *TRMT10A* gene. Additionally, a review was undertaken of any existing cases for further evaluation.

## Case Report

The patient was the second child born to non-consanguineous parents. The pregnancy was uneventful with the child being born at term without immediate postnatal problems. The child's birth weight was 2,100 g (z-score: -3.03) and she had microcephaly (head circumference: 30 cm, $< 3^{rd}$ centile). She was found to have difficulty in feeding during the first two years of life and was underweight (her weight was 8.8 kg at 2 years old, her z-score was -2.39). Later, she was diagnosed with delayed neuromotor development with mild intellectual disability (24 months walking, 36 months speech). This was followed by epilepsy diagnosed at 2.5 years old which was being treated with valproic acid at the time of writing. On cranial magnetic resonance imaging (MRI), a small pituitary (3 mm in length) with normal structure and an anterior arachnoid cyst (3.5x2 cm) of the left temporal lobe were detected.

Informed consent both for publication of this case report and for molecular analyses were given by the parents of the patient.

**Growth and puberty of the patient:** She was referred to the pediatric endocrinology clinic at 3.6 years of age for short stature. Her height was 84 cm [standard deviation score (SDS): -3.7], her weight was 11.4 kg (SDS: -2.22), her head circumference was 45 cm (SDS: -3.2) and her bone age was 2.5 years. Her mid-parental height was 153.2 cm (-1.14 SDS). Clinical examination revealed distinctive features including microcephaly, a small face and deep-set eyes.

On laboratory examination; complete blood counts, electrolytes, liver enzymes, renal function tests, thyroid function tests [thyroid stimulating hormone: 4.75 µmIU/mL (N: 0.4-5.3), free T4: 12,1 pmol/L (N: 7-16 pmol/L), prolactin level [14.2 ng/mL (N: 4-20 ng/mL)], urine and blood amino acid level results were normal. Polyuria was not observed, and urine specific gravity was 1.018. Celiac antibodies were negative and the karyotype was 46, XX. Her brainstem auditory evoked response test was also normal.

Serum IGF-1 and IGF-binding protein 3 levels were low (40.9 ng/mL, SDS: -1.44 and 2,550 ng/mL, SDS: -2.31, respectively). GH stimulation tests (L-Dopa and insulin tolerance tests) were consistent with GH deficiency (peak GH levels were 6.1 ng/mL in the L-Dopa test and 3.3 ng/mL in the insulin tolerance test) (9). Growth velocity was low during follow-up. At 5.4 years of age, her height SDS decreased to -4.38.

At that time, recombinant human GH (rhGH) therapy was initiated at a dose of 0.28 mg/kg/week. Growth velocity increased from 4 cm/year to 8 cm/year after the first year of GH therapy. At the second year of treatment, growth velocity decreased, and the dose of GH was increased to 0.35 mg/kg/week (Figure 1). IGF-1 levels often remained within normal ranges with GH treatment (annual IGF-1 levels were 117.1, 223, 254, 441, 327, 425, 254 and 234.6 ng/mL). GH could be maintained until the age of 14.7 years. Her compliance with GH treatment was low and the parents of the patient did not accept further GH treatment. At the cessation of hGH therapy, her achieved height was 142 cm, height-SDS: -3.3 (mid-parent height 153.2 cm) (Figure 1).

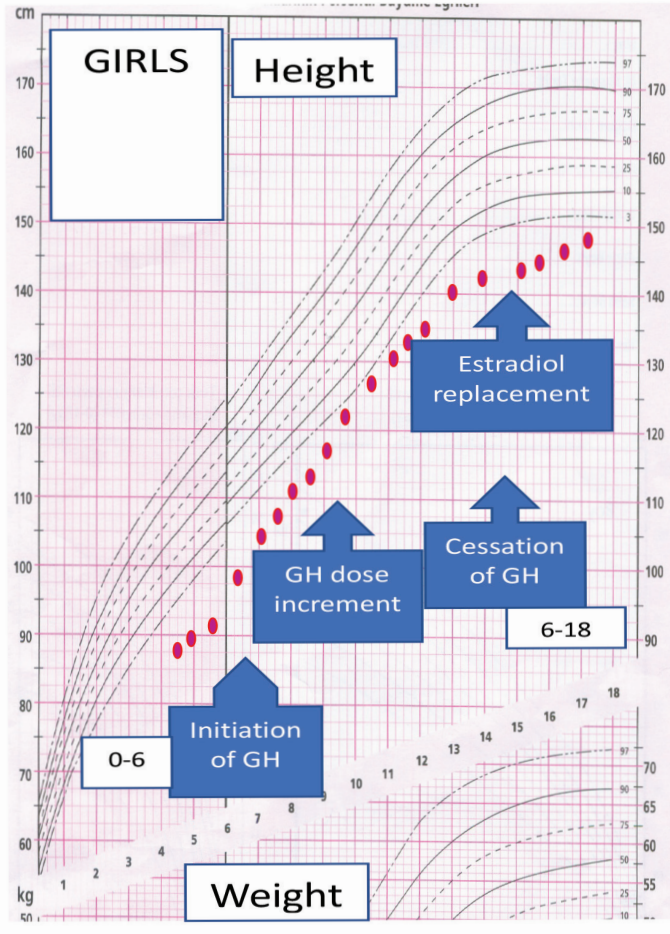


**Figure 1.** Growth chart of the patient

At age 13 years, she was still pre-pubertal and her bone age was 12 years. Basal luteinizing hormone (LH) levels (0.12 mIU/mL), estradiol (0.12 pg/mL) and LH response to LH-releasing hormone stimulation test (4.42 mIU/mL) were pre-pubertal. However, basal and stimulated follicle stimulating hormone (FSH) were 6.48 mIU/mL and 24.85 mIU/mL, respectively. A transabdominal pelvic ultrasound study showed that her small uterine volume (1.6 $cm^3$) and ovarian volumes were as low as 0.12 mL (right) and 0.06 mL (left). In both ovaries, follicles were not detected. Although it was decided to start pubertal induction, the parents did not give estrogen replacement therapy until 15.7 years of age. At this time, the following results were obtained: height: 145 cm (SDS: -2.95), weight: 35.8 kg (z-sore: -3.87), body mass index (BMI): 17.3 kg/$m^2$ (z-score: -2.2), relative BMI: 80%, puberty stage: still Tanner 1 and bone age: 13.5 years. Low estradiol (< 10 pg/mL), high basal LH (9.5 mIU/mL) and FSH (18.4 mIU/mL) were consistent with hypergonadotropic hypogonadism. Her serum anti-Mullerian hormone (AMH) level was found to be 0.07 pmol/L (N: 3.1-17.8 pmol/L). The small ovaries, high gonadotropins and low AMH levels were consistent with ovarian failure. After convincing the family, the patient was started with estradiol (Estrofem)® 2 mg tablets, 1/8 of a tablet once a day. It was aimed to switch to cyclic treatment within 2 years by increasing the dose at 6-month intervals. At the age of 17.5, she reached Tanner stage 3 puberty and her height was 149.8 cm (Table 1).

**Glucose metabolism of the patient:** At 4 years of age, on routine blood testing during a check-up at a pediatric neurology clinic, fasting blood glucose (FBG) was detected as 34 and 39 mg/dL, while fasting insulin was 2.1 IU/mL at a second blood glucose (BG) measurement. FBG was also low at GH stimulation test (35 mg/dL). Adrenocorticotropic hormone and cortisol levels were normal and ketones were negative when hypoglycemia occurred. The patient was asymptomatic during the test and remained so thereafter with no symptomatic episodes of hypoglycaemia.

She had an incidental diagnosis of diabetes at age 11.4 years. On routine examination, FBG levels were found to be 136 mg/dL. She was asymptomatic, without polyuria or polydipsia. Her oral glucose tolerance test (OGTT) results indicated diabetes (Table 1).

Hemoglobin A1c (HbA1c) at diagnosis was 7.4%. She tested negative for islet cell, anti-GAD and anti-insulin antibodies, while her C-peptide level was 1.29 ng/mL (N: 1.1-4.4), her insulin level was 7.2 mIU/mL (N: 4-16), and her BG level was 240 mg/dL. Her BMI z-score was -2.1. She was treated with metformin from the time of her diagnosis. As her C-peptide level was not low, metformin treatment was started at 2x500 mg daily. In the follow-up, it was increased to 1,500 mg/day. No side effects were observed. Her last HbA1c at age 17.1 years was 6.4% on 1.500 mg/day metformin.

**Genetic analysis of our case:** Sequencing analysis of all known monogenic diabetes genes by targeted next generation sequencing was undertaken as part of the Genetics of Early Onset Diabetes Study (GOOD study). The GOOD study is a cross sectional multi-center clinic-based study which aims to identify novel genetic subtypes of monogenic diabetes by excluding type 1 diabetes using a polygenic risk scores. The recruited patients undergo sequencing analysis of the known monogenic diabetes genes first, and those patients with negative tests are further investigated by whole-genome-sequencing in order to find novel genetic etiologies.

A homozygous nonsense mutation in *TRMT10A* (c.379C > T, p.Arg127*) was identified. This mutation has been reported previously in a patient with TRMT10A syndrome (2) and,

**Table 1. Anthropometric and laboratory values of patients**

| | On admission | On GH initiation | On metformin initiation | On estrogen initiation | On last examination |
|---|---|---|---|---|---|
| Age (year) | 3.6 | 5.4 | 11.4 | 15.7 | 17.5 |
| Height (cm) | 84 | 91 | 132.6 | 145 | 149.8 |
| Height SDS | -3.6 | -4.38 | -2.4 | -2.95 | -2.23 |
| Weight (kg) | 11.4 | 13.7 | 25.2 | 35.8 | 41 |
| Weight SDS | -2.22 | -2.45 | -2.56 | -3.87 | -3.06 |
| Tanner stage | Telarche 1<br>Pubarche 1 | Telarche 1<br>Pubarche 1 | Telarche 1<br>Pubarche 1 | Telarche 1<br>Pubarche 1 | Telarche 3<br>Pubarche 2 |
| Laboratory values | Hemogram, thyroid functions, electrolytes, renal functions and liver function tests were normal | IGF-1: 18.4 ng/mL<br>Peak GH: 6.1 ng/mL (L-Dopa) and 3.3 ng/mL (ITT) | FBG: 136 mg/dL<br>Fasting C-peptide: 1.29 ng/mL<br>Fasting insulin: 7.2 mIU/mL<br>OGTT: BG at 120': 242 mg/dL, insulin at 120': 47 mIU/mL) | E2: < 10 pg/mL<br>LH: 9.5 mIU/mL<br>FSH: 18.4 mIU/mL<br>AMH: 0.07 pmol/L | |

LH: luteinizing hormone, FSH: follicle stimulating hormone, AMH: anti-Mullerian hormone, OGTT: oral glucose tolerance test, SDS: standard deviation score, GH: growth hormone, IGF-1: insulin-like growth factor-1, ITT: insulin tolerance test, FBG: fasting blood glucose, BG: blood glucose

based on American College of Medical Genetics and Genomics variant classification guidelines, was considered to be a pathogenic variant. The parents could not be studied for any possible genetic mutations, and therefore, their carrier status could not be confirmed.

## Discussion

There have been a total of 19 reported cases of TRMT10A syndrome within 12 families, with the first case reported by Igoillo-Esteve et al. (2) in 2013 (Table 2) (3,4,5,6,8,10,11,12,13,14). All patients, except for those cases reported by Zung et al. (4), had homozygous or compound heterozygous loss of function mutations within the *TRMT10A* gene. The cases of Zung et al. (4) also had a large deletion within the chromosomal region 4q23 which included nine genes, one of them being *TRMT10A*.

The current case was the first case in our country with TRMT10A syndrome. She was born SGA with distinctive features including microcephaly, intellectual impairment and epilepsy. At 3.65 years of age, hypoglycemia and severe short stature related to GH deficiency were detected. At 11.41 years of age, she had an incidental diagnosis of non-autoimmune diabetes. Before the development of diabetes, fasting hypoglycemia occurred. A pubertal disorder resembling primary ovarian failure was also detected at the age of 15.7 years.

Microcephaly and intellectual disability were observed in all cases. Epileptic seizures were also observed frequently in some cases. Eight out of 11 cases with cranial imaging results were found to be normal (2,3,4,5,6,10,12,13). Some minor clinical findings such as low anterior hair line, deep-set eyes with mild hypotelorism, shortened forehead and buffalo hump were also reported, which are similar to our case. In addition to findings of central nervous system involvement, in our patient, an arachnoid cyst was detected, which we believe to be an incidental finding as they are common in the population (2.6%) (15).

Growth was retarded in most of the cases. However, detailed information about the height SDS, body proportions and GH axis evaluations of patients was not fully available. Igoillo-Esteve et al. (2) reported the final adult height of their patients as being short (Table 2). The height of those cases by reported Gillis et al. (3) were below the third percentile with no given height z-scores. On the other hand, similar to our case, the height z-score of the case reported by Zung et al. (4) indicated serious short stature (-4.24). In addition, only one case had an evaluation of GH secretion (8). This case which was reported by Stern et al. (8) had short stature, low growth velocity and delayed bone age. After GH stimulations tests were performed, peak GH was found to be 7.3 mcg/l on clonidine and 1.8 mcg/l on Arginine stimulations. The MRI of the patient was found to be normal. GH replacement therapy could not be given. Our case was diagnosed with GH deficiency before the *TRMT10A* mutation was identified. Low growth velocity, inadequate responses to GH stimulation tests, retarded bone age and small hypophysis were all consistent with the diagnosis of GH deficiency. Moreover, the patient responded well to rhGH treatment as her growth velocity increased. These features suggest that the GH deficiency in our patient was caused by the *TRMT10A* mutation, although it is difficult to conclude precisely and more cases are needed to confirm if this is indeed a feature of TRMT10A syndrome. As tRNAs are crucial for cellular function and TRMT10A deficiency causes tRNA modification, all cells expressing TRMT10A would be affected. It is known that many cells in the brain including the hypothalamus and pituitary glands express TRMT10A protein. So hypothalamohypophysial functions in terms of GH secretion could be affected by TRMT10A deficiency.

Our case displayed the characteristics of hypergonadotropic hypogonadism. Puberty had not begun spontaneously when the patient was seen at 15.7 years of age. Laboratory values and pelvic ultrasound were compatible with ovarian failure. Even though pubertal delay in TRMT10A deficiency has been mentioned before, no detailed description of this was explained except by Zung et al. (3,4,5). Zung et al. (4) described the unusual pubertal progression of a female patient who showed intermittent progression with high gonadotropin levels during pubertal arrest and normal/low gonadotropin levels with estradiol elevation following pubertal progression periods. They concluded that this intermittent progression of puberty was related to the occurrence of transient episodes of gonadal failure and successive episodes of gonadal recovery. There were no detailed descriptions of pubertal progress in the other two cases.

Although gonadal expression of *TRMT10A* has not been demonstrated to date, this syndrome may lead to primary gonadal failure. We cannot be certain that primary ovarian failure is caused by the loss of TRMT10A and further validation in future cases will be required in order to be certain.

The most interesting characteristics of these cases could be the abnormalities in glucose metabolism. Eight of the reported cases with TRMT10A syndrome had diabetes. The age at diagnosis for diabetes varied between 9 and 28 years. C-peptide was detectable in all diabetic patients. Five patients were treated with insulin, three with metformin, and one case with insulin plus metformin (2,3,4,5,8,14).

**Table 2. Characteristics of patients with *TRMT10A* mutations reported in the literature**

| | Igoillo-Esteve et al. (2), 2013 | | | Gillis et al. (3), 2014 | | | Zung et al. (4), 2015 | Yew et al. (5), 2016 |
|---|---|---|---|---|---|---|---|---|
| **Case no** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| **TRMT10A Mutation (based on RefSeq NM_152292.5)** | c.379G > A, p.Arg127* | c.379G > A, p.Arg127* | c.379G > A, p.Arg127* | c.616G > A, p.Gly206Arg | c.616G > A, p.Gly206Arg | c.616G > A, p.Gly206Arg | 4q23 deletion | c.79G > T, p.Glu27* |
| **Sex** | **F** | **F** | **M** | **F** | **M** | **M** | **F** | **F** |
| **Short stature** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **Yes** | **No** |
| **Delayed puberty** | **NR*** | **NR** | **NR** | **Yes** | **NR** | **NR** | **Yes** | **No** |
| **Diabetes mellitus/age of diagnosis** | **Yes/ 22 y** | **Yes/ 19 y** | **Yes/ 14 y** | **Yes/ 9 y** | **-** | **-** | **Yes/ 15 y** | **Yes/ 24y** |
| **Diabetes treatment** | **Ins**** | **Ins** | **Ins** | **Diet** | **-** | **-** | **Ins** | **Ins + Met** |
| **Hypoglycemia** | **NR** | **NR** | **NR** | **Yes** | **Yes** | **Yes** | **Yes** | **No** |
| **Other clinical features** | Short neck, wide nose, low hairline, buffalo hump, retraction of right 5th toe, scoliosis, joint laxity, microcephaly, epilepsy | Microcephaly | Microcephaly | Microcephaly, epilepsy | Microcephaly, epilepsy | Microcephaly, epilepsy | Small face, clinodactyly, sensorineural hearing impairment, microcephaly | Buffalo hump, Microcephaly, epilepsy |

NR*: not reported, Ins**: insulin, Met***: metformin, F: female, M: male, y: year

TRMT10A syndrome patients have persistent insulin secretion similar to Maturity Onset Diabetes of the Young (MODY) and contrary to type 1 diabetes. The clinical picture of diabetes was usually slow onset as seen in our case. Since diabetes appeared at 11.4 years of age, the patient responded well to metformin and subsequently HbA1c decreased. This suggested that there was a persistence of endogenous insulin. Our case highlighted that *TMRT10A* mutations are an important cause of non-obesity related and non-insulin dependent diabetes in childhood. Genetic testing for *TRMT10A* along with common MODY genes should be undertaken in patients with non-insulin treated diabetes, particularly in the presence of other extra-pancreatic features.

Interestingly, loss of function mutations in *TRMT10A* can cause hyperinsulinism (HI) as well as diabetes in the same subject (3,4,14). Fasting hypoglycemia developed before diabetes in two cases (3,4). Another two cases within the family reported by Gillis et al. (3) only had fasting hypoglycemia and high fasting insulin was also detected in those cases (4). Lin et al. (14) reported a diabetic child with high insulin and C-peptide levels to OGTT. They suggested that according to the OGTT results, insulin resistance appeared to be the dominant pathophysiological mechanism in their patient. In their case, spontaneous mild hypoglycemia was also detected.

Childhood onset hypoglycemia prior to developing diabetes has been reported in HNF4A MODY. The exact mechanism of this bidirectional glucose variability is unknown (16,17). Our patient had an incidental finding of low BG with no clear symptoms. The previously reported case by Zung et al. (4) had HI between 2.5 and 7.4 years of age and developed diabetes at 15.2 years of age.

TRMT10A deficiency leads to oxidative stress mediated apoptosis in the pancreatic beta cells. In an elegant study, Cosentino et al. (7) showed that tRNA guanosin 9 hypomethylation induces tRNAGln fragmentation. These fragments mediated TRMT10A-deficient beta cell death. The clinical hallmark of *TRMT10A* related diabetes is slow onset with non-autoimmunity.

Our knowledge and understanding of TRMT10A syndrome is increasing rapidly, despite it being recently described. However, some questions remain unanswered with regards to early hypoglycemic events and its characteristics of pubertal delay.

| | Narayanan et al. (6), 2015 | | Boonsawat et al. (10), 2019 | Duerinckx et al. (11), 2020 | Hu et al. (12), 2019 | Reuter et al. (13), 2017 | Lin et al (14), 2020 | Stern et al. (8), 2021 | Presented case |
|---|---|---|---|---|---|---|---|---|---|
| 9 | 10 | 11 | 12 | 13 | 14/15 | 16/ 17 | 18 | 19 | 20 |
| c.79G > T, p.Glu27* | c.277C > T (p.Arg93*) c.397C > T (p.Arg133*) | c.277C > T (p.Arg93*) c.397C > T (p.Arg133*) | c.379C > T, p.Arg127* | c.379C > T, p.R127* | c.370 > A p.Q124K | c.348G > C p.K116N | c.496–1G > A) | c.616G > A, p.G206R | c.379C > T, p.(Arg127*) |
| M | F | M | NR | NR | NR/NR | M/M | NR | F | F |
| No | NR | Yes | Yes | NR | NR/NR | Yes /yes | Yes | Yes | Yes |
| Yes | NR | NR | NR | NR | NR/NR | NR/NR | NR | No | Yes |
| Yes/ 28 y | - | - | NR | NR | NR/NR | NR/NR | Yes/ NR | Yes/ 11 y | Yes/ 11.4 y |
| Met*** | - | - | - | - | - | - | Met | Ins | Met |
| No | NR | NR | NR | NR | NR/NR | NR/NR | NR | NR | Yes |
| Microcephaly, epilepsy | low anterior hair line, deep set eyes with mild hypotelorism, shortened forehead, microcephaly | low anterior hair line, deep set eyes with mild hypotelorism, microcephaly, epilepsy | | | | Hypotonia, uvula bifida, mild turuncal adiposit, microcephaly, epilepsy | Microcephaly | Microcephaly in both | Small face and deeply located eyes, microcephaly, epilepsy |

## Conclusion

*TRMT10A* gene mutations cause a syndrome of intellectual disability, microcephaly and delayed puberty resulting from ovarian failure, such as in our case. These characteristics are associated with non-autoimmune diabetes with persistent insulin secretion. GH deficiency can be an additional finding of this syndrome. *TRMT10A* should be tested in children from populations with higher rates of consanguinity when the patient has slow onset, auto-antibody negative diabetes and extra-pancreatic features.

### Ethics

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

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Zeynep Şıklar, Tuğba Kontbay, Kevin Colclough, Kashyap A. Patel, Merih Berberoğlu, Concept: Zeynep Şıklar, Merih Berberoğlu, Design: Zeynep Şıklar, Merih Berberoğlu, Data Collection or Processing: Zeynep Şıklar, Tuğba Kontbay, Kevin Colclough, Kashyap A. Patel, Merih Berberoğlu, Analysis or Interpretation: Zeynep Şıklar, Kevin Colclough, Kashyap A. Patel, Merih Berberoğlu, Literature Search: Zeynep Şıklar, Tuğba Kontbay, Writing: Zeynep Şıklar, Kevin Colclough, Kashyap A. Patel, Merih Berberoğlu.

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CASE REPORT

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# Compound Heterozygous Variants in *FAM111A* Cause Autosomal Recessive Kenny-Caffey Syndrome Type 2

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### What is already known on this topic?

Kenny-Caffey syndrome (KCS) is characterized by hypoparathyroidism, dwarfism, and dysmorphism. Osteocraniostenosis (OCS) is another syndrome similar to KCS. The autosomal dominant (AD) *FAM111A* mutation causes KCS2 and OCS syndrome; the perinatal and lethal forms should be kept in mind in the differential diagnosis. *FAM111A* is essential for parathyroid and bone formation; it might also be an essential factor in male genital development.

### What this study adds?

To the best of our knowledge, this case is the first patient with genetically confirmed KCS2 or OCS in Turkey. It is known that the *FAM111A* mutation is transmitted in an AD manner. We present a case who showed autosomal recessive transmission. Unlike the known AD features of these syndromes, the cause of the patient's phenotype may be the identified compound heterozygous mutations of the *FAM111A* gene. The present patient probably has OCS, which is a severe form of KCS2.

## Abstract

Kenny-Caffey syndrome (KCS) is a rare autosomal recessive (AR)/dominant disease characterized by hypoparathyroidism, skeletal dysplasia, dwarfism, and dysmorphism. *FAM111A* or *TBCE* gene mutations are responsible for this syndrome. Osteocraniostenosis (OCS) is a lethal syndrome with similar features to KCS, and it can be a severe form of KCS type 2 which results from the *FAM111A* gene mutation. The *FAM111A* mutation is generally characterized by the autosomal dominant transition. We present a male case having compound heterozygous variants (c.976T > A and c.1714_1716del) in the *FAM111A* gene with an AR inheritance pattern. Hypocalcemia developed on the second day of life. The patient and his older sister had a dysmorphic face, skeletal dysplasia, and they were diagnosed with hypoparathyroidism. Both siblings died due to septicemia. He is the first reported patient with the *FAM111A* mutation in Turkey. The phenotype of the patient is compatible with OCS, and the detected variants may explain the disease genetically.
**Keywords:** Hypoparathyroidism, skeletal dysplasia, osteocraniostenosis, short stature, dysmorphism, *FAM111A* gene, autosomal recessive

## Introduction

Kenny-Caffey syndrome (KCS) is a rare autosomal recessive (AR) or dominant disease characterized by short stature, cortical thickening, medullary stenosis of the tubular bones, delayed closure of the anterior fontanel, eye abnormalities, and hypoparathyroidism (1). There are two inherent forms of KCS. While the AR form is caused by *TBCE* gene mutation, the autosomal dominant (AD) form results from *FAM111A* gene mutation. These gene mutations cause hypoparathyroidism, short stature, bone problems, and dysmorphic features (2,3). Although knowledge about the *FAM111A* gene is limited, many cases presented in the literature show that *FAM111A* is an essential molecule for

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normal bone and parathyroid gland development. In this paper, we identified and present a patient with the *FAM111A* mutation using whole-exome sequencing (WES).

## Case Report

A male patient was born as the third child of healthy, consanguineous (3rd degree) parents at 38 weeks gestation, and skeletal dysplasia was suspected during the prenatal period. The grandfathers of the patient's mother and father are siblings. Except for the present patient and his sister, there was no family history with a similar disease. The patient's sister passed away due to hypocalcemia and sepsis at the age of 2 months.

The patient was admitted to hospital experiencing respiratory distress after birth. On physical examination, the child's weight was 2,770 grams [-1.4 standard deviation score (SDS)], his length was 44 cm (-2.73 SDS), and his head circumference was 34 cm (-0.64 SDS). Dysmorphic face (deep-set eyes, low set ear, microphthalmia, depressed nasal bridge), large anterior fontanel (5x6 cm), short arm span, increased upper/lower ratio (2.2, the normal ratio is about 1.7), and micromelia were noted (Figure 1A). External genitalia was male, but micropenis (stretched penile length of 2.2 cm) and low testicular volume (2 mL/2 mL) were notable. There was no renal abnormality, and neurological examination was normal. The patient was admitted to hospital due to respiratory distress. Hypocalcemia and hypoparathyroidism were detected [Ca 7.8 mg/dL (N: 8.5-11), P 9.3 mg/dL (N: 5.6-10.5), PTH <3 pg/mL (N: 10-65), 25-OH vitamin D 37.8 mcg/l (N: 20-50)] on the second day, and oral calcium and calcitriol therapies were initiated. His calcium level was normalized two days after the start of treatment. The respiratory problems continued up to the 50th day of life. Except for a tibial fracture noticed on the 38th day, no further fracture was observed during follow-up.

Calcium administration was discontinued due to hypercalcemia on the 65th day. The calcium level was not stable, and oral calcium treatment was continued intermittently. Low-dose calcitriol therapy was continued, but this treatment was ceased due to hypercalcemia. The patient was discharged on the 76th day. In follow-up, hypoparathyroidism became evident again, and oral calcium and calcitriol therapies were commenced on the 85th day. The patient was reluctant to feed and did not gain weight. He was readmitted to the intensive care unit with septicemia at the age of 3.5 months and died from respiratory failure after 30 days.

A skeletal survey showed incomplete ossification of the calvaria, short and thin ribs, hypoplastic thorax, and long thin bones (Figure 1B). He was diagnosed with KCS or Sanjad-Sakati syndrome (SSS) based on his clinical and radiological findings. Karyotype analysis was 46, XY and

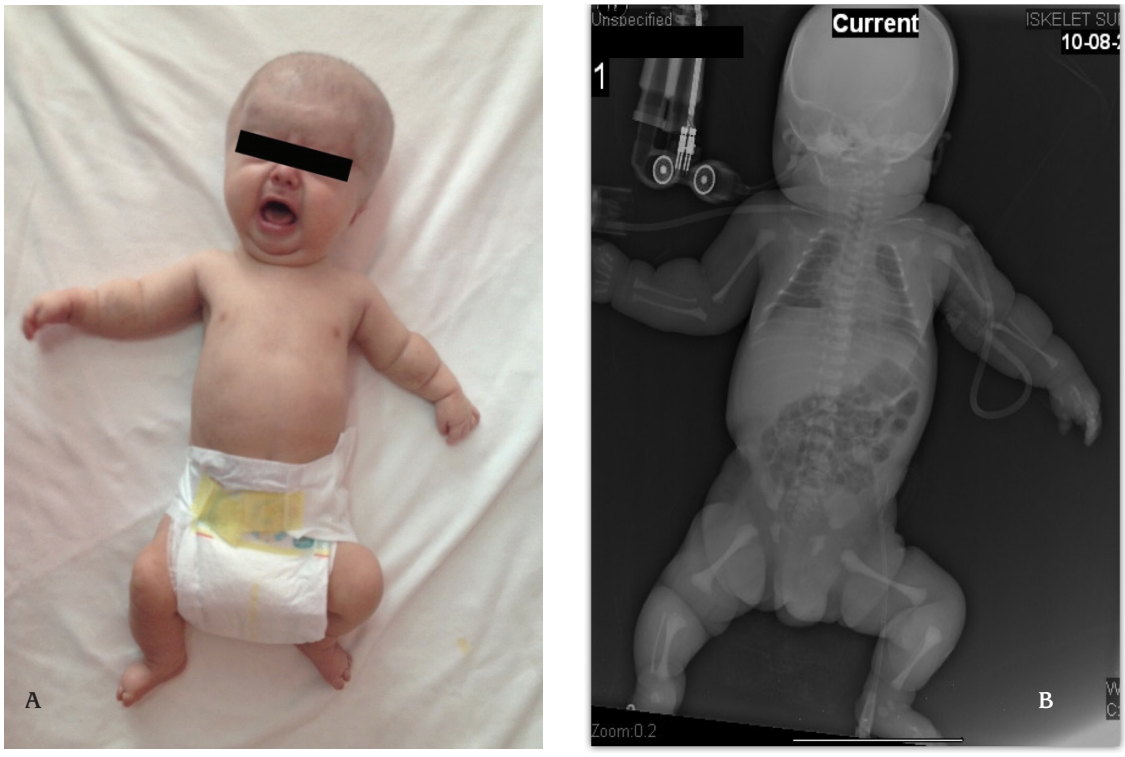


**Figure 1.** A) The general appearance of the patient (a relatively large head, small eyes, and inappropriate body size were noted) and B) Skeletal radiogram of the patient (narrowing, long, thin bones, and thin ribs were noted)

FISH analysis for 22q11.2 deletion showed no abnormality. The patient's clinical features supported the initial diagnosis of SSS; however, *TBCE* exons Sanger sequencing revealed no abnormalities. WES was performed using peripheral blood genomic DNA from the patient and his parents. WES was performed by the Intergen Genetic Diagnosis Center in Ankara on a Miseq sequencing platform (Illumina, San Diego, CA, USA). After detecting the potential variants of the family, data analysis and bioinformatics processing were performed after receiving the primary sequencing data. The family history of the probands identified as carrying variants in the *FAM111A* is shown in the pedigree of Figure 2. The patient pedigree demonstrates that the inheritance pattern reveals AR transmission. After the data analysis, we identified compound heterozygous mutations of the *FAM111A* gene in the patient, including a paternal heterozygous missense variant c.976T > A (p.L326I) and a maternal heterozygous in-frame deletion variant c.1714_1716del (p.Ile572del, rs779963813) (Figure 3).

In this study, two variants were identified and examined in public databases (Ensembl, MutationTaster, Franklin, and Clinvar). Briefly, amino acid substitutions were identified with PROVEAN (http://provean.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and SIFT (http://sift.jcvi.org/) tools to determine the potential consequences of missense variants on protein function. The mutation taster (http://www.mutationtaster.org/) program was used to evaluate protein stability. The transcript number of the *FAM111A* gene is ENST00000528737.5. The c.1714_1716del mutation caused the FAM111A protein to lack only one amino acid, and the *FAM111A*: c.976T > A led to an amino acid change from leucine (Leu) to isoleucine (Ile).

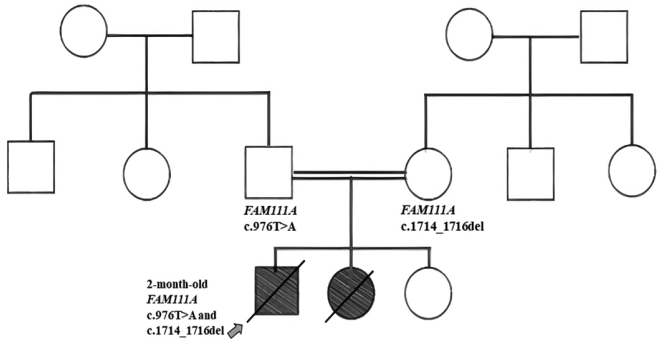


**Figure 2.** Family pedigrees of probands found to carry c.976T > A and c.1714_1716del compound heterozygous variants. Circles are females; squares are males. Filled symbol are affected with Kenny-Caffey syndrome

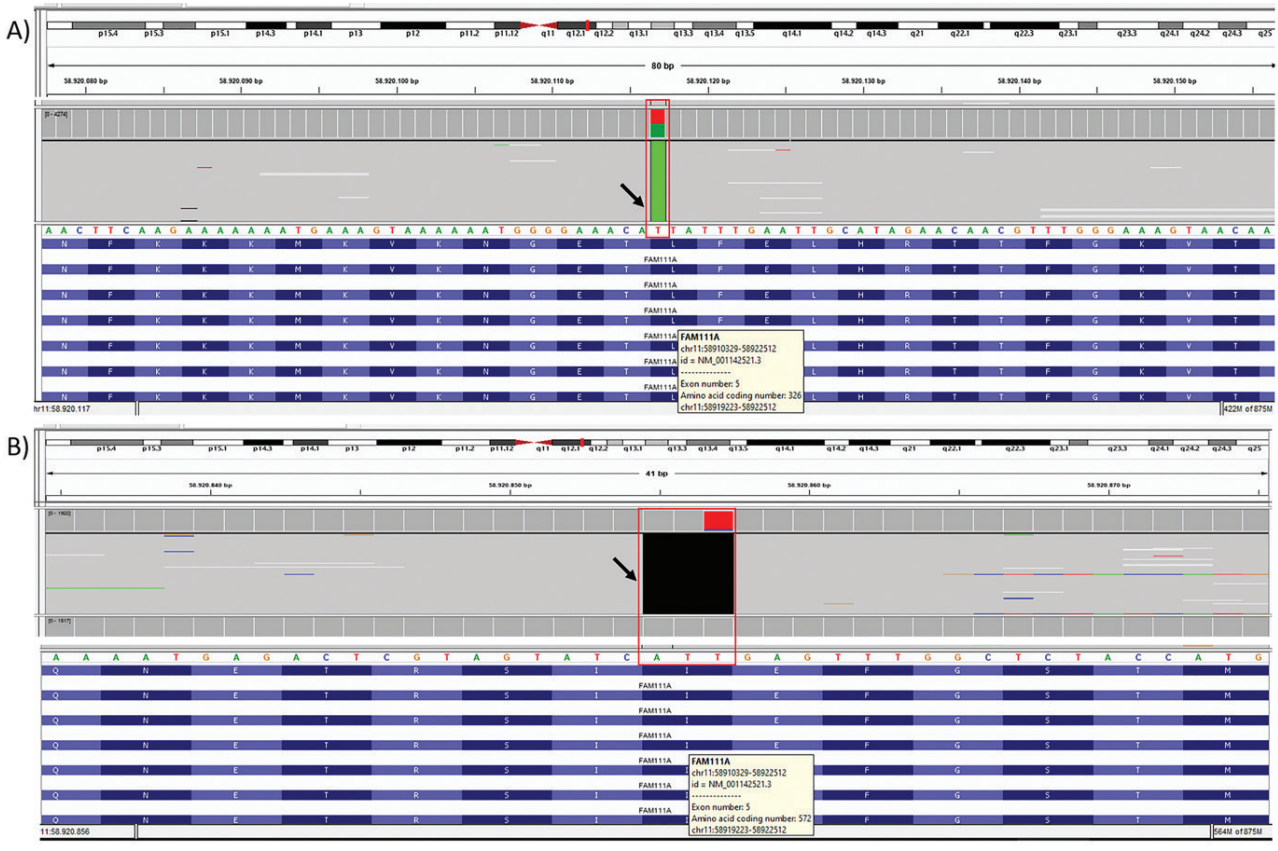


**Figure 3.** A schematic diagram of identified variants in the *FAM111A* gene, A) c.976T > A and B) c.1714_1716del in the index case

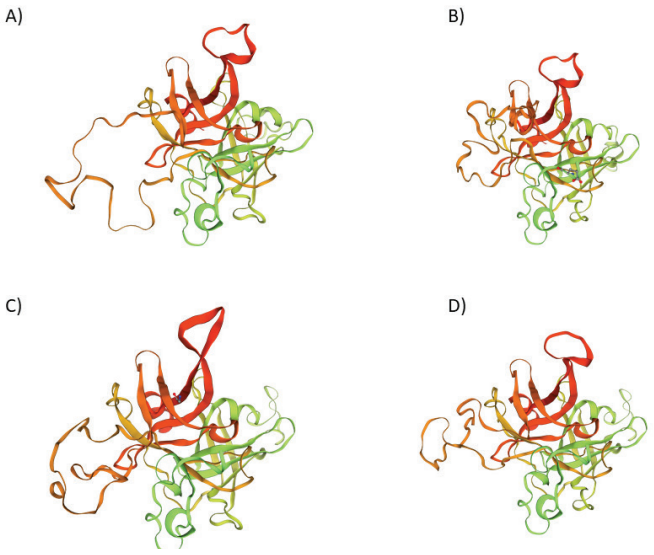


**Figure 4.** Figure shows the comparative 3D protein structure modeling (template 1.dua1., A) of *FAM111A*. The effect of c.976T > A (B), c.1714_1716del (C), c.976T > A, and c.1714_1716del together (D) variants on protein structure by the Swiss model, respectively. A wild-type, B, C, and D affected protein

The SWISS-MODEL used for tertiary structure prediction showed a marked variation in the structure of c.976T > A and c.1714_1716del (Figure 4). Additionally, the p.Ile572del in the *FAM111A* gene was categorized as deleterious (score = -11.79) by PROVEAN Prediction and was presumed to be 'disease-causing' by Mutation Taster. The allele frequency of this variant is 9/251416 in the GnomAD exome database. In silico prediction tools other than Mutation Taster predicted this variant to be benign. Some other missense mutations are given very close to this mutation. Furthermore, the missense variant c.976T > A (p.L326I) in the *FAM111A* gene has not previously been reported in the literature, Ensembl genome databases, or Clinvar. The c.976T > A (p.Leu326Ile), a novel variant, was not found in the GnomAD exomes and GnomAD genomes database despite good coverage. This variant was classified as a variant of uncertain significance (VUS) (PM2, PP2, BP4). However, the results of bioinformatic prediction by PolyPhen2 and SIFT confirmed that the amino acid substitution p.L326I in protein FAM111A was possibly damaging by PolyPhen (score = 0.635), tolerated by SIFT (score = 0.064), and neutral by PROVEAN Prediction (score = -1.05). Moreover, the variant was presumed to be a polymorphism by Mutation Taster; it was not found in ExAC and 1000G.

Additionally, the c.976T > A and c.1714_1716del variants were both stated to have "splice site changes" by Mutation Taster. However, these variants were classified as VUS by the Franklin variant classification tool (https://franklin.genoox.com). As in the present case with a clinically clear picture, VUS and new/uncharacterized variants are notable because these variants can reveal unpredictable genetic and protein alterations involved in biochemical processes.

## Discussion

We present a patient having a *FAM111A* variant which has not been previously reported as causing congenital hypoparathyroidism, dysmorphism, and/or skeletal dysplasia. KCS is classified into two types according to its clinical features and inheritance. Whereas the *TBCE* gene mutations are responsible for KCS type 1 (KCS1), the *FAM111A* gene mutation causes KCS type 2 (KCS2). SSS and KCS1 are AR syndromes characterized by hypoparathyroidism, mental retardation, facial dysmorphism, and extreme growth retardation.

The family with sequence similarity 111, member A (*FAM111A* or *KIAA1895*) gene is mapped on 11q12.1 and was first cloned by Nagase et al. (4) in 2001. The highest *FAM111A* expression is found in the adult spleen, followed by the adult kidney, lung, ovary, liver, pancreas, and fetal liver. The functions of *FAM111A* and *FAM111B* are still unknown, and knowledge of them is insufficient in the literature. The Swiss group firstly reported the *FAM111A* gene mutation in KCS2 patients in 2013 (2). They reported the R569H mutation in 4 out of the five patients who were from different countries. Isojima et al. (3) reported the R659H mutation in the *FAM111A* gene in three unrelated patients with KCS2.

It is stated that osteocraniostenosis (OCS) or gracile bone dysplasia can be considered a differential diagnosis of KCS2. OCS is characterized by thin, fragile, narrowing diaphyses, micromelic dwarfism, brachydactyly, facial dysmorphism, microphthalmia, and hypoparathyroidism. Unger et al. (2) reported on five KCS and five OCS patients. The authors speculated that KCS2 and OCS might both be allelic disorders of differing severity. OCS is a lethal perinatal condition and was named by Verloes et al. (5) in 1994. Thomas et al. (6) reported a lethal dysplasia in male and female siblings with severe pulmonary hypoplasia. These cases had pulmonary hypoplasia and required pulmonary support in the early period of their life. The patients (one who was genetically confirmed) died at 2 and 3.5 months. Most OCS patients died in the newborn period - only one survived to the age of 21 months in Unger et al.'s (2) series. Our case and his female sibling passed away due to pulmonary failure and septicemia at the age of 3.5 months and 2 months, respectively. The present case also had long, thin bones and ribs, as well as a tibial fracture. The long, thin bones may be related to hypoparathyroidism or insufficient

bone development commensurate with the genetic defect's severity.

It has been postulated that *FAM111A* variants do not affect neural development, and therefore the affected patients would not present with developmental delay (2). In contrast, Cavole et al. (7) presented a patient with KCS2 having an intellectual disability and microcephaly. Our case had no neurological developmental delay and microcephaly, but the disease developed in the early prenatal period. OCS is a severe and life-threatening form, more so than KCS2, and so these cases might be labeled as OCS.

It is stated that KCS1 and SSS might be allelic disorders of differing severities (7). KCS2 and OCS may cause allelic disorders and have an overlapping phenotype. The differential diagnoses of some syndromes caused by *TBCE* and *FAM111A* mutation are shown in Table 1. KCS2 generally presents later in life, but OCS presents early. The clinical data suggest that our case had OCS because of a dysmorphic face, early-onset presentation, micromelia, long bone fracture, and disease severity. Our case had a micropenis. Unger et al. (2) stated that all OCS patients were male, and a micropenis was seen in 4 out of 5 cases. *FAM111A* might be an essential factor in male genital development, especially in OCS.

The c.976T>A and c.1714_1716del variants are reported as benign or VUS in prediction tools. In a large Chinese cohort of childhood-onset hypoparathyroidism (173 cases), 15 candidate genes were screened by NGS. They found a c.1706G>A (R569H) hotspot mutation in the *FAM111A* gene in a patient showing the pattern of KCS2. A novel heterozygous variant c.A881G/c.1714_1716del was reported in the same study (8). We found compound heterozygous variants (c.976T>A and c.1714_1716del) in our case. To date, almost 14 genetically confirmed cases

**Table 1. Differential diagnosis of some syndromes caused by *TBCE* and *FAM111A* gene mutations**

| | KCS1 | KCS2 | SSS | Gracile bone dysplasia | Index case |
|---|---|---|---|---|---|
| Other name | | | HRD | OCS | |
| Inheritance | AR | AD | AR | AD | AR |
| Gene | *TBCE* | *FAM111A* | *TBCE* | *FAM111A* | *FAM111A* |
| OMIM number | #244460 | #127000 | #241410 | #602361 | |
| Growth and height | Short stature<br>Birth length <3rd percentile | Short stature, severe | Severe intrauterine growth retardation<br>Postnatal growth retardation | Short stature, severe<br>Failure to thrive | Short stature, severe |
| Head, neck, skull | Delayed anterior fontanel closure<br>Hypertelorism<br>Dental caries<br>Poorly ossified skull bones<br>Absent diploic space<br>Calvarial osteosclerosis | Delayed anterior fontanel closure<br>Macrocephaly<br>Prominent forehead<br>Hyperopia, microphthalmia, papilledema<br>Defective dentition<br>Osteosclerosis | Microcephaly<br>Micrognathia<br>Prominent forehead, long philtrum<br>Deep-set eyes<br>Low-set ears, posteriorly rotated ears<br>Delayed bone age Patchy osteosclerosis | Prominent forehead<br>Microphthalmia<br>Cloverleaf-shaped skull<br>Hypoplastic cranial bones<br>Decreased mineralization of skull (in some patients) | Microphthalmia<br>Decreased mineralization of skull |
| Limbs | Medullary stenosis of tubular bones<br>Thin long bones<br>Internal cortical thickening<br>Small hands | Thickened cortex of long bones<br>Dense tubular bones and narrow marrow cavities | Small hands | Micromelic short limbs<br>Flared metaphyses<br>Long bone fractures prenatally<br>Brachydactyly | Micromelic short limbs<br>Long bone fracture |
| Genitourinary | | Microorchidism | Microorchidism | Micropenis | Micropenis |
| Endocrine | Neonatal hypoparathyroidism, low parathyroid hormone | Low parathyroid hormone, low calcitonin<br>Small to absent parathyroid glands | Low parathyroid hormone<br>Congenital hypoparathyroidism | Hypocalcemia<br>Low parathyroid hormone | Hypocalcemia<br>Low parathyroid hormone |
| Immune, hematological | Recurrent bacterial infections<br>Anemia | Anemia | Recurrent bacterial infections | Recurrent bacterial infections | Anemia |
| Neurological deficit | Yes, usually | No, usually | Yes, usually | Yes | No |

AR: autosomal recessive, AD: autosomal dominant, *FAM111A*: Family with Sequence Similarity 111 Member A, KCS1: Kenny-Caffey syndrome type 1, KCS2: Kenny-Caffey syndrome type 2, OCS: osteocraniostenosis, SSS: Sanjad-Sakati Syndrome, *TBCE*: Tubulin-Specific Chaperone E, HRD: hypoparathyroidism, retardation, dysmorphism

have been presented, but knowledge about KCS2 or OCS is still limited. Detected variants in the *FAM111A* gene should be published in the literature in order to reveal genotype-phenotype correlations. The phenotype of the present case was compatible with OCS, and the variants presented may explain the disease genetically. The relationship between genotype and phenotype will be further enlightened with more cases presented in the literature.

## Conclusion

There is limited knowledge about the *FAM111A* gene. Some genetically unconfirmed KCS patients can have the *FAM111A* gene mutation. The *FAM111A* mutation is generally characterized by AD transmission. Our study patient had compound heterozygous variants (c.976T > A and c.1714_1716del) in the *FAM111A* gene with an AR inheritance pattern. Our study case probably had OCS, which is a severe form of KCS2. OCS or KCS2 should be suspected in a hypocalcemic neonatal/infantile patient with short stature and facial dysmorphism.

### Ethics

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

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Erdal Eren, Ömer Tarım, Concept: Erdal Eren, Havva Tezcan Ünlü, Data Collection or Processing: Erdal Eren, Havva Tezcan Ünlü, Serdar Ceylaner, Analysis or Interpretation: Serdar Ceylaner, Erdal Eren, Havva Tezcan Ünlü, Literature Search: Erdal Eren, Ömer Tarım, Writing: Erdal Eren, Ömer Tarım.

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CASE REPORT

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# Maltodextrin May Be a Promising Treatment Modality After Near-total Pancreatectomy in Infants Younger Than Six Months with Persistent Hyperinsulinism: A Case Report

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**What is already known on this topic?**

Congenital hyperinsulinism (CHI) is the most common cause of persistent hypoglycemia in newborns and infants. While several medical treatment agents are used to treat infants with CHI, hypoglycemia management has been quite difficult to date.

**What this study adds?**

In those cases with CHI, maltodextrin addition in the early period enables us to achieve more stable serum glucose. Maltodextrin addition also shortens the patient's discharge period, prevents complications, and protects the patient from treatment side effects. Continuous glucose monitoring systems help to manage the patients' follow-ups more efficiently in this group.

## Abstract

Persistent hypoglycemia in infants with congenital hyperinsulinism (CHI) can be challenging in approximately half of these cases, even after undergoing a near-total pancreatectomy. While maltodextrin has been recommended in the nutritional management of CHI cases younger than six months, information regarding its efficacy in managing hypoglycemia are not yet clear. Here, we present a male infant with CHI who experienced persistent hypoglycemia even after undergoing a near-total pancreatectomy and despite multiple medical treatments. The infant's hypoglycemic episodes were successfully controlled by adding maltodextrin to his diet.

**Keywords:** Congenital hyperinsulinism, *ABCC8* gene, maltodextrin, near-total pancreatectomy, continue glucose monitoring systems

## Introduction

Congenital hyperinsulinism (CHI) is the most common cause of persistent hypoglycemia in newborns and infants (1). While several medical treatment agents are used to treat these infants, the management of hypoglycemia has been quite difficult (2). It has been reported that persistent hypoglycemia can be seen in approximately 50% of cases with diffuse forms where a near-total pancreatectomy is performed (3).

Adzick et al. (4) reported that of the CHI cases who underwent a near-total pancreatectomy in their series, 31% had euglycemia, 20% had hyperglycemia, and 49% had hypoglycemia requiring treatment. Moreover, it has been emphasized that surgery for the diffuse form is not a cure, but only helps hypoglycemia control.

Nutritional support is a critical factor, alongside medical treatment, in cases with CHI. Frequent feeding with breast milk or formula is recommended for those who do not have oral intake problems (5). Uncooked cornstarch helps with

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the stabilization of serum glucose in cases above six months, but it is not recommended for cases under six months because of its side effects. A small number of studies have reported on the use of maltodextrin (Malt Extract, Wakodo, Fantomalt Nutricia) added to breast milk or formula in the nutritional management of cases younger than six months. However, none of these studies clearly demonstrated the effectiveness of maltodextrin in controlling hypoglycemia (6).

In this article, we present a male infant with CHI who was experiencing persistent hypoglycemia even after undergoing a near-total pancreatectomy and despite multiple medical treatments and whose hypoglycemic episodes were successfully controlled by adding maltodextrin to his diet.

## Case Report

A male patient was born with a weight of 4,020 grams in the 37$^{th}$ week of pregnancy from a twenty-seven-year-old mother's first pregnancy. Critical blood samples taken due to seizure at the 3$^{rd}$ hour of life revealed a serum glucose level of 22 mg/dL, and a serum insulin level of 288 µU/mL. An intravenous (IV) dextrose infusion at a rate of 6 mg/kg/min was initiated along with frequent breastfeeding. Due to persistent severe hypoglycemia in the follow-up, the IV infusion rate was gradually increased to 14 mg/kg/min. Due to the recurrence of hypoglycemia and the persistence of hyperinsulinism during hypoglycemia, CHI was considered a factor and diazoxide treatment was started at a dose of 10 mg/kg/day. Subsequently, his hypoglycemic episodes persisted and he was transported to our clinic on the 30$^{th}$ day of life.

Preparation for surgery immediately began, diazoxide treatment was increased to 15 mg/kg/day, and octreotide was started at a dose of 5 mcg/kg/day and was gradually increased to 40 mcg/kg/day. Hypoglycemic episodes persisted during this treatment, and an IV infusion of glucagon was added. Facilities to carry out $^{18}$F-L-DOPA PET imaging were not available and so could not be performed on our patient and transfer to another center was considered inappropriate. A near-total pancreatectomy (95-98% resection) was performed on the 35$^{th}$ day of life. Histopathological samples showed diffuse nesidioblastosis. Genetic analysis revealed a previously reported heterozygous c.2113 C>T mutation in the *ABCC8* gene which is known to be associated with diazoxide unresponsiveness.

After the surgery, octreotide and glucagon were continued, nifedipine was added, and the dextrose infusion was continued at a dose of 14 mg/kg/min. Despite full enteral nutrition and other parenteral treatments, the hypoglycemic episodes continued. Since the IV dextrose infusion could not be reduced, oral maltodextrin (1 measuring spoon of Fantomalt Nutricia® contains 5 grams of CHO) was added to each meal (12 times per day) at a total dose of 5 gram/kg/day. After this, the addition of maltodextrin significantly controlled the patient's hypoglycemic episodes, and dextrose support was gradually decreased. Dextrose treatment was discontinued on the 7$^{th}$ day following the addition of maltodextrin, and the patient was discharged.

Serum glucose monitoring was enabled using a continuous glucose monitoring system (CGMS) (Medtronic Guardian Connect CGM, Ca, USA) during both the inpatient and outpatient periods. This system was used off-label after receiving informed consent from the parents to monitor glucose variability in order to prevent hypoglycemia using trend arrows, and to improve the efficacy of treatments. CGMS of the patient revealed that the hypoglycemic episodes decreased significantly after the addition of maltodextrin (Figures 1 and 2). It was noticed that the percentage of serum glucose, which was below 70 mg/dL per day, decreased significantly after the addition of maltodextrin.

Glucagon and nifedipine treatments were discontinued after maltodextrin treatment, in the first and the fourth months, respectively. The octreotide dose was reduced to 14 mcg/kg/day.

The patient's neurological examination was comparable to his peers, while his body weight was 14 kg [standard deviation score (SDS): 2.63], his height was 84 cm (SDS: 2.31), his body mass index was 19.8 (SDS: 1.5) and his head circumference was 47 cm (SDS: -0.01) at the age of 12 months. He is still receiving octreotide and maltodextrin treatment while not experiencing any hypoglycemic episodes.

## Discussion

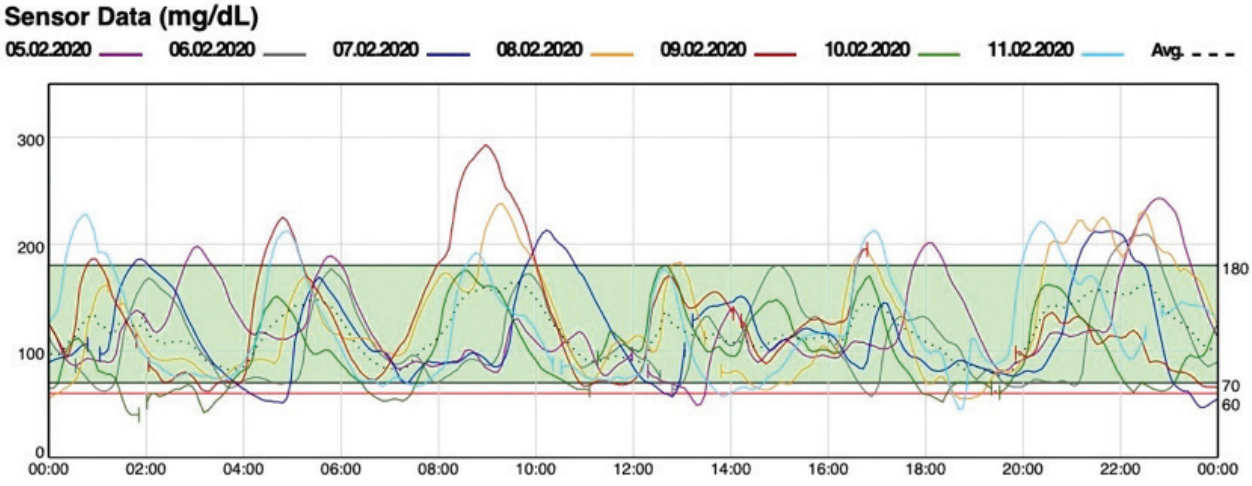
CHI is a rare glucose metabolism disease which most frequently causes persistent hypoglycemia in the neonatal period. Early diagnosis is essential in order to prevent neurological damage due to hypoglycemia (7). It is emphasized that frequent feeding with high-caloric carbohydrates can reduce hypoglycemia attacks (5). Xu et al. (6) stated that maltodextrin, a glucose polymer, can be used in the first six months of life. In contrast, uncooked corn starch is not used for the first six months due to its side effects. Cappella et al. (8) reported that adding maltodextrin to the diet instead of increasing IV glucose infusion is an effective procedure in cases of CHI. De Cosio and Thornton

(9) recommended that patients with CHI should be supported with maltodextrin.

In the literature, a limited number of articles regarding maltodextrin use in CHI were found. In one report, it was stated that maltodextrin was given to 4 CHI patients without any explanation regarding its efficacy (10).

Although maltodextrin is one of the most commonly known high-calorie formulae already being used in the feeding plans of CHI patients, the efficacy of maltodextrin on the hypoglycemic control of patients with CHI has not yet been studied in detail. There is no data on how adding maltodextrin to the diet effects the course of hypoglycemic episodes, the dosage, the feeding intervals or CGMS reports. Meanwhile, the possible side effects of maltodextrin are weight gain, gas, bloating and allergic reactions; however, none of these side effects were observed in our case (11).

In our case, the maltodextrin addition enabled us to achieve more stable serum glucose, to change the treatment modalities and to shorten the discharge period of the patient. CGMS also helped us to manage the patient's in/outpatient follow-ups more efficiently. The importance and efficacy of adding maltodextrin in the early period of hypoglycemia management was demonstrated in this CHI case by CGMS.




| | Wed 5 Feb | Thu 6 Feb | Fri 7 Feb | Sat 8 Feb | Sun 9 Feb | Mon 10 Feb | Tue 11 Feb | Average / Total |
|---|---|---|---|---|---|---|---|---|
| # Sensor Values | 285 | 284 | 285 | 283 | 249 | 287 | 284 | 1.957 |
| High SG (mg/dL) | 243 | 209 | 213 | 238 | 293 | 180 | 228 | 293 |
| Low SG (mg/dL) | 49 | 57 | 47 | 55 | 62 | 40 | 45 | 40 |
| Average SG (mg/dL) | 122 | 111 | 116 | 128 | 130 | 99 | 121 | 118 |
| Standard Dev. | 41 | 38 | 43 | 50 | 54 | 36 | 47 | 45 |
| MAD % | 3,8 | 1,8 | 3,3 | 9,9 | 8,4 | 20,9 | 6,3 | 8,9 |
| # Valid Calibrations | 1 | 2 | 2 | 2 | 3 | 4 | 2 | 16 |

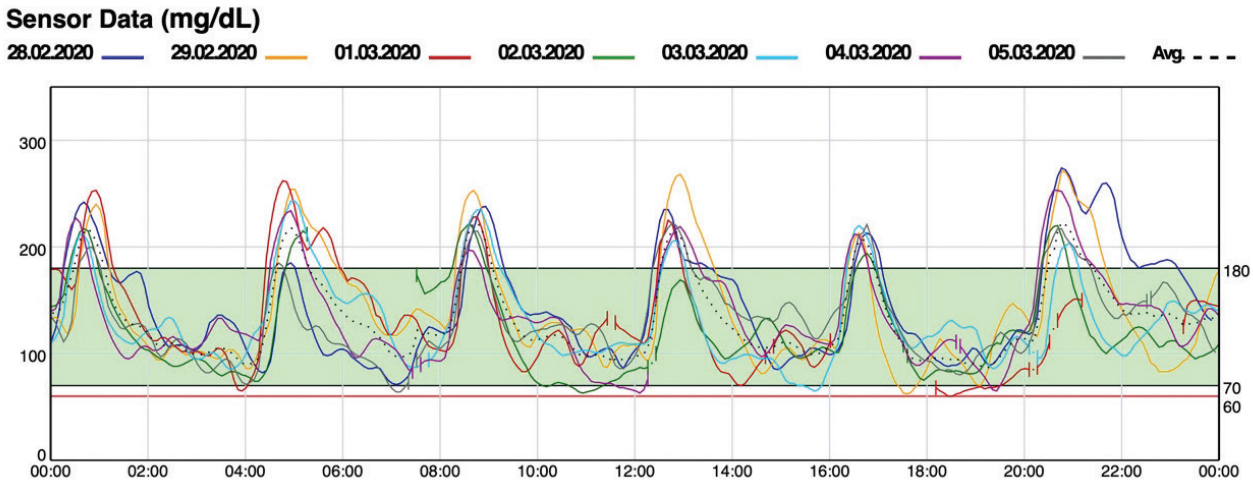
**Excursion Summary**

| | Wed 5 Feb | Thu 6 Feb | Fri 7 Feb | Sat 8 Feb | Sun 9 Feb | Mon 10 Feb | Tue 11 Feb | Average / Total |
|---|---|---|---|---|---|---|---|---|
| # Excursions | 5 | 2 | 5 | 5 | 4 | 4 | 7 | 32 |
| # High Excursions | 4 | 1 | 3 | 4 | 4 | 0 | 5 | 21 |
| # Hypo Excursions | 1 | 1 | 2 | 1 | 0 | 4 | 2 | 11 |
| AUC Above Limit | 3,1 | 1,0 | 2,1 | 5,0 | 7,7 | 0,0 | 3,5 | 3,1 |
| AUC Below Limit | 0,3 | 0,4 | 1,3 | 0,7 | 0,2 | 2,8 | 0,6 | 0,9 |

**Duration Distribution (hh:mm)**

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Above 180 | 3:00 | 13% | 1:05 | 5% | 2:40 | 11% | 4:40 | 20% | 3:20 | 16% | 0:00 | 0% | 3:40 | 15% | 18:25 | 11% |
| Within (70 - 180) | 20:00 | 84% | 20:10 | 85% | 18:30 | 78% | 16:40 | 70% | 16:10 | 78% | 17:15 | 72% | 17:55 | 76% | 126:40 | 78% |
| Below 70 | 0:45 | 3% | 2:25 | 10% | 2:35 | 11% | 2:15 | 10% | 1:15 | 6% | 6:40 | 28% | 2:05 | 9% | 18:00 | 11% |

**Figure 1.** Continuous glucose monitoring system report: Before the addition of maltodextrin (it is noted that time in below range was 11 %, the ratio of below < 70 mg/dL)

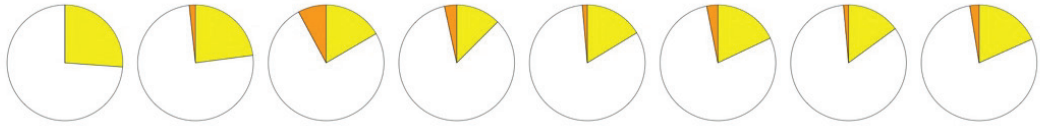



| | Fri 28 Feb | Sat 29 Feb | Sun 1 Mar | Mon 2 Mar | Tue 3 Mar | Wed 4 Mar | Thu 5 Mar | Average / Total |
|---|---|---|---|---|---|---|---|---|
| # Sensor Values | 287 | 288 | 241 | 264 | 288 | 289 | 288 | 1.945 |
| High SG (mg/dL) | 274 | 271 | 262 | 222 | 243 | 253 | 222 | 274 |
| Low SG (mg/dL) | 71 | 62 | 60 | 63 | 65 | 63 | 64 | 60 |
| Average SG (mg/dL) | 145 | 141 | 130 | 121 | 132 | 135 | 133 | 134 |
| Standard Dev. | 50 | 53 | 49 | 42 | 41 | 44 | 38 | 46 |
| MAD % | 8,1 | 6,0 | 2,0 | 8,7 | 1,6 | 5,0 | 2,8 | 4,7 |
| # Valid Calibrations | 2 | 2 | 3 | 2 | 2 | 2 | 2 | 15 |

**Excursion Summary**

| | Fri 28 Feb | Sat 29 Feb | Sun 1 Mar | Mon 2 Mar | Tue 3 Mar | Wed 4 Mar | Thu 5 Mar | Average / Total |
|---|---|---|---|---|---|---|---|---|
| # Excursions | 2 | 2 | 1 | 4 | 4 | 4 | 5 | 22 |
| # High Excursions | 2 | 2 | 1 | 4 | 4 | 4 | 5 | 22 |
| # Hypo Excursions | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AUC Above Limit | 9,2 | 10,3 | 6,5 | 2,9 | 4,4 | 5,3 | 3,1 | 6,0 |
| AUC Below Limit | 0,0 | 0,1 | 0,3 | 0,1 | 0,0 | 0,1 | 0,1 | 0,1 |

**Duration Distribution (hh:mm)**



| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Above 180 | 6:15 | 26% | 5:30 | 23% | 3:20 | 17% | 2:45 | 12% | 3:55 | 16% | 4:20 | 18% | 3:35 | 15% | 29:40 | 18% |
| Within (70 - 180) | 17:40 | 74% | 18:05 | 75% | 15:10 | 75% | 18:30 | 85% | 19:45 | 83% | 19:00 | 79% | 20:05 | 84% | 128:15 | 79% |
| Below 70 | 0:00 | 0% | 0:25 | 2% | 1:35 | 8% | 0:45 | 3% | 0:20 | 1% | 0:45 | 3% | 0:20 | 1% | 4:10 | 3% |

**Figure 2.** Continuous glucose monitoring system report: After the addition of maltodextrin (it is noted that time in below range was 3 %, the ratio of below < 70 mg/dL)

## Conclusion

The management of CHI requires a multidisciplinary approach. Hypoglycemia can persist, even after a near-total pancreatectomy, especially in patients with diffuse form and potassium channel mutations. Our case suggests that the addition of maltodextrin in the early pre-op or post-op period may shorten hospitalizations, prevent complications, and even protect the patient from the side effects of treatments. However, more case series or case-control studies are needed in order to determine the efficacy of maltodextrin supplementation in the management of infants with CHI.

### Ethics

**Informed Consent:** The consent form was filled out by the parent.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Yasemin Denkboy Öngen, Erdal Eren, Halil Sağlam, Concept: Yasemin Denkboy Öngen,

Design: Halil Sağlam, Data Collection or Processing: Yasemin Denkboy Öngen, Erdal Eren, Analysis or Interpretation: Erdal Eren, Literature Search: Yasemin Denkboy Öngen, Erdal Eren, Halil Sağlam, Writing: Yasemin Denkboy Öngen, Erdal Eren, Halil Sağlam.

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