# JCRPE

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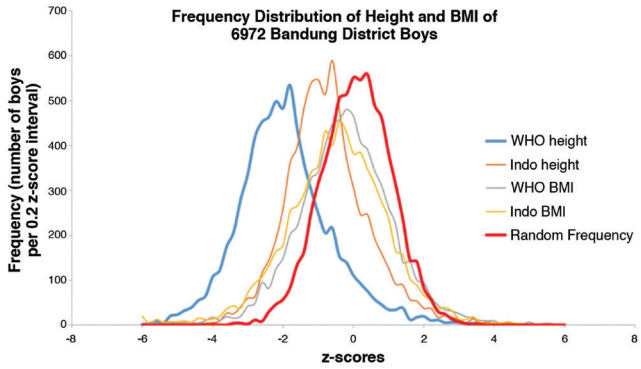
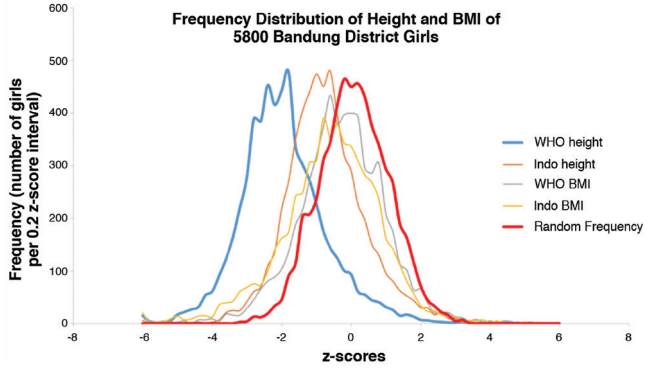



Frequency distribution of height and body mass index of 6,972 Bandung District boys

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Novina N et al.


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## Review



## Original Articles



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REVIEW

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J Clin Res Pediatr Endocrinol 2022;14(1):1-9

# Recommendations for Clinical Decision-making in Children with Type 1 Diabetes and Celiac Disease: Type 1 Diabetes and Celiac Disease Joint Working Group Report

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## Abstract

It is well-known that in children with type 1 diabetes (T1D), the frequency of Celiac disease (CD) is increased due to mechanisms which are not fully elucidated but include autoimmune injury as well as shared genetic predisposition. Although histopathologic examination is the gold standard for diagnosis, avoiding unnecessary endoscopy is crucial. Therefore, for both clinicians and patients' families, the diagnosis of CD remains challenging. In light of this, a joint working group, the Type 1 Diabetes and Celiac Disease Joint Working Group, was convened, with the aim of reporting institutional data and reviewing current international guidelines, in order to provide a framework for clinicians. Several controversial issues were discussed: For CD screening in children with T1D, regardless of age, it is recommended to measure tissue transglutaminase-immunoglobulin A (tTG-IgA) and/or endomysial-IgA antibody due to their high sensitivity and specificity. However, the decision-making process based on tTG-IgA titer in children with T1D is still debated, since tTG-IgA titers may fluctuate in children with T1D. Moreover, seronegativity may occur spontaneously. The authors' own data showed that

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most of the cases who have biopsy-proven CD had tTG-IgA levels 7-10 times above the upper limit. The decision for endoscopy based solely on tTG-IgA levels should be avoided, except in cases where tTG-IgA levels are seven times and above the upper limit. A closer collaboration should be built between divisions of pediatric endocrinology and gastroenterology in terms of screening, diagnosis and follow-up of children with T1D and suspicious CD.

**Keywords:** Children, type 1 diabetes, Celiac disease, anti-tissue transglutaminase-IgA

## Introduction

Among children with type 1 diabetes (T1D), the prevalence of Celiac disease (CD) is higher than in the general population (1.6-16.4% vs 0.7%) since they are susceptible to autoimmune damage to several organ systems (1). Therefore, the screening of children with T1D for CD is critical, and the current screening protocol includes the measurement of tissue transglutaminase-IgA (tTG-IgA) levels regardless of symptom status, followed by endoscopic biopsy in those with positive tTG-IgA titers (2,3). The treatment then involves the institution of a gluten-free diet. Overall, the timing of tTG-IgA measurements has an important role in the decision-making process and the management of the patients.

From the perspective of pediatric endocrinologists, pediatric gastroenterologists and families, avoiding clinically unjustified endoscopies is just as critical as the timely diagnosis of CD. Furthermore, variations exist in the indications for endoscopy and treatment among different countries and centers (4).

This report is intended to convey the most up-to-date information regarding the current diagnostic algorithms, the role of a gluten-free diet, the epidemiologic data in Turkey, recent developments in the literature, and recommendations of international societies in relation to CD in children with T1D. This report was prepared by professionals assigned by the administrative boards of "Pediatric Endocrinology and Diabetes Association" and "Turkish Society of Pediatric Gastroenterology, Hepatology and Nutrition" and was based on data from several centers and discussions that took place at three different meetings.

The goal of this report was to further detail the current recommendations by international societies and to provide a basic framework to be used by practicing physicians.

## Main Questions That were Discussed in the Joint Meetings

- What is the prevalence of CD in children with T1D in Turkey? What are the main issues regarding the screening and diagnosis?

- In general, how long after the diagnosis of T1D would tTG-IgA antibody levels be reliable?

- What is the rate of transient tTG-IgA positivity and how does this affect diagnosis?

- Would making the decision to perform endoscopy according to tTG-IgA titers measured just after a diagnosis of T1D in children with no symptoms and family history of CD lead to unnecessary invasive procedures?

- Does tTG-IgA positivity and incidence of CD in children with T1D vary depending on age and time after the initial diagnosis T1D? Is autoimmune thyroiditis an additional risk factor?

- Would it be beneficial to conduct multidisciplinary meetings for clinical decision-making in caring for children with T1D and a diagnosis of/suspicion of CD? Alternatively, should this process be under the responsibility of pediatric gastroenterologists alone?

- From the perspective of pathologists, what are the basic issues encountered in the diagnosis of CD?

- Why has gluten-free diet become so popular among the public? What do the scientific data suggest?

- What are the issues associated with gluten consumption apart from in the context of CD?

- What do the families experience in terms of the possibility of having a diagnosis of CD, the diagnostic process and the period after the diagnosis? What are their concerns? Does a gluten-free diet have any role in preventing T1D?

- In children who are already diagnosed with T1D, would a gluten-free diet reduce the risk of acquiring CD? Would this diet have any impact on the autoimmune destruction of beta-cells?

- How should we follow children with high tTG-IgA titers but normal endoscopic findings? Should a gluten-free diet be recommended?

- How should the dietary management of a child with T1D and CD be?

## Results and Suggestions

### The Relationship Between Diabetes and Celiac Disease

**1.** The prevalence of positive CD autoimmunity and overt CD was 14.3% [95% confidence interval (CI): 11-17] and

8.5% (95% CI: 5-10), 15- and 8-times higher than the general pediatric population, respectively (5). According to international studies, the prevalence of biopsy-proven CD ranges between 1.6-16.4% among people with T1D (6,7,8,9,10).

**2.** In a recently published study including 52751 children with T1D from the US, Germany, Austria, England and Australia the prevalence of CD was found to be 3.5% (4). In general, the risk of receiving a diagnosis of biopsy-proven CD is higher before the age of 5 years and within the first five years after the diagnosis of T1D. Concomitant autoimmune thyroid disease further increases this risk (3).

**3.** CD is asymptomatic in 85% of children with T1D who have biopsy-proven diagnosis. As a result, it is necessary to screen for CD in this population.

**4.** According to studies from Turkey, the prevalence of biopsy-proven CD in children with T1D is 3.5-12.2% (11,12).

**5.** During the meetings for the preparation of this report, data gathered by the following institutions were presented to seek answers to the previously posed questions: Koç University, Gazi University, Ege University, Ankara University, Dr. Sami Ulus Children's Hospital, Cerrahpaşa Medical Faculty and Elazığ Fırat University:

- In the last five years, 1061 children with T1D were followed-up at Koç University. A total of 401 whose CD screening was conducted in Koç University Hospital were evaluated. tTG-IgA positivity was detected in 61% of CD cases in the first year, 37% between the first and fifth years, and in 2% after the fifth year of T1D diagnosis. The prevalence of biopsy-proven CD was 3.7% in this cohort.

- In the last 10 years, 559 children were diagnosed with T1D at Gazi University. The prevalence of biopsy-proven CD was 3.4% in this cohort. Of these patients, 82.4% were asymptomatic. CD diagnosis was made within the first two years of T1D diagnosis in 50%, and 94% were diagnosed within the first 5 years.

- Data from Ege University encompassed 1300 children with T1D and the prevalence of biopsy-proven CD was 1.9% in this cohort. 72% of the cohort was asymptomatic and 59% were diagnosed within two years following the diagnosis of T1D.

- Data from Ankara University included 158 children with T1D and the prevalence of biopsy-proven CD was 4.4%. 85% of the cases received a diagnosis within the first 5 years following the diagnosis of T1D.

- Data from Dr. Sami Ulus Children's Hospital included a nine-year period, during which 550 children were diagnosed with T1D. In this cohort, 5.2% had biopsy-proven CD. In the first year, 72.4% of the children with CD were diagnosed with CD.

- Data from Cerrahpaşa Medical Faculty included 100 children, among whom 4% were diagnosed with CD based on histopathology.

- Data from Fırat University included a 14-year period, during which 453 children were diagnosed with T1D. Among these, the prevalence of biopsy-proven CD was 5.5%. 76% of the patients were asymptomatic and 64% were diagnosed within the first year following the diagnosis of T1D.

**6.** In the follow-up of children with T1D, the current recommendations for the timing of Celiac serology screening include within first two years and five years after diagnosis of T1D or every year, but the recommendations regarding the frequency of screening after five years following T1D diagnosis are less clear. While the risk of developing CD decreases significantly after this 5-year mark, the possibility of CD should still be kept in mind. In addition to the above recommendations, screening should be conducted in case of any of the below:

- Symptom and laboratory findings suggestive of CD,

- First degree relative with a diagnosis of CD,

- Unexplained frequent hypoglycemia.

## Screening Tests and Their Interpretation

**1.** After checking that the child is consuming normal quantities of gluten, in children with normal serum IgA values for age, tTG-IgA measurements should be used as an initial test regardless of age (2). If serum IgA levels are found to be low for age or $<0.2$ g/L in children older than three years old, IgG based tests (deaminated gliadin peptide, EMA or tTG) should be use. tTG-IgA titers are reported as international unit (IU) or relative unit (RU). The upper limits for IU and RU are 20 and 1, respectively. Threshold tTG-IgA levels that justify performing endoscopy are generally reported as multiples of the upper limit (e.g. 3-fold, 10-fold). In addition, recommendations outlined by the specific testing kits should be considered. On the other hand, EMA-IgA testing should be conducted with techniques involving immunofluorescence.

**2.** International studies have reported that tTG-IgA levels show a fluctuating trend in 10.7-41% of the patients and resolve in 30-40%, despite continued gluten intake (5,13,14,15). Data from Gazi University indicate that 12.7% had a fluctuating course; in this group tTG-IgA levels

were <3x upper limit of normal (ULN) and the rate of spontaneous resolution of antibodies was 97%. Diyarbakır Gazi Yaşargil Hospital and Koç University Hospital have reported spontaneous antibody resolution rates of 23.3% and 22% within five years, respectively (15).

**3.** In recent years, there has been an increasing debate regarding the threshold tTG-IgA level for offering endoscopy. Data presented by Gazi University have been assessed, and the best cut-off was judged to be ≥7-times the ULN in terms of sensitivity, specificity, negative predictive value and positive predictive value. Data from centers that submitted their data to this committee were also congruent in that the majority of the patients with biopsy-proven CD had tTG-IgA levels 7-10x the ULN.

**4.** We therefore recommend that tTG-IgA levels should not be the sole criterion for performing endoscopy, except when tTG-IgA levels ≥7x ULN, there is a family history of CD or the patient has symptoms suggestive of CD. Physicians should keep in mind that during the early stages of T1D, there can be a transient "antibody storm" against not only the pancreatic beta-cells but also other tissues, which may eventually resolve. In children with antibody levels within 3-7 times the ULN and without any of the exceptions highlighted above, antibody test can be repeated at 3-6 months prior to seeking endoscopic evaluation.

**5.** Despite the increasing data on transient and fluctuating antibody positivity, the European Society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) and the International Society of Pediatric and Adolescent Diabetes (ISPAD) currently do not detail any recommendations on this issue in their respective guidelines. Therefore, we believe that it would be clinically beneficial if these societies prepared a joint consensus guideline for the diagnosis and management of CD in children with T1D.

## Decision to Perform Endoscopy and Clinical Management

In the light of the data presented at the meetings and available in the literature, and after taking into consideration the current consensus opinions, the following recommendations regarding the indications for endoscopy and clinical management are set forth below:

**1.** With the exception of frequent attacks of hypoglycemia and symptoms suggestive of CD, invasive procedures, such as endoscopy, should be planned for the most appropriate date given that the diagnosis of CD is not considered urgent, and families require time to get accustomed to the diagnosis of T1D.

**2.** There is no single standard test for measuring antibody levels. Hence, endoscopy should not be undertaken based on tTG-IgA levels measured at another center. Instead a repeat measurement should be conducted prior to deciding on the need for endoscopy.

**3.** Apart from the cases with tTG-IgA levels ≥7x the ULN, tTG-IgA levels measured at the time of diagnosis of T1D should not be used to guide the decision to perform endoscopy.

**4.** Prior to measuring tTG-IgA levels, it should be confirmed that patients have been consuming gluten for at least two weeks.

**5.** If the tTG-IgA levels are ≤3x the ULN there is no indication to perform endoscopy in patients without a family history of CD or symptoms suggestive of CD. Such patients can be followed up by the pediatric endocrinology department through serial antibody testing.

**6.** All patients with a tTG-IgA level >3x the ULN should be referred to the pediatric gastroenterology department as soon as possible.

**7.** For patients with tTG-IgA levels ≥10x the ULN and a second antibody test reveals EMA positivity, the family should be informed that a diagnosis of CD can be made without further endoscopic evaluation. However, making a definitive diagnosis through endoscopic biopsy may improve compliance to dietary management, especially in the setting of our country.

**8.** Patients with fluctuating antibody titers and antibody levels <3x the ULN can be followed up without endoscopy.

**9.** In patients with tTG-IgA levels between 3-7x the ULN, further diagnostic steps may include EMA-IgA testing followed by endoscopy if EMA positive or immediate endoscopy depending on the center's preference.

**10.** There is seldom need to perform testing for HLA DQ2 and HLA DQ8 subgroups for the diagnosis of CD. This testing can be useful in ruling out CD in challenging cases with equivocal biopsy findings.

**11.** All children with biopsy-proven CD should be managed with a gluten-free diet regardless of the presence of symptoms.

The algorithm prepared through these analyses and committee recommendations are presented in the Figure 1.

## Pathology

**1.** All patients scheduled for endoscopic evaluation should be on a gluten-containing diet prior to the endoscopy. The relevant clinical data of the patient, including medical

history, endoscopic findings, laboratory findings, serology, medications, and diet, must be available to the pathologist (16,17).

**2.** In terms of location and the number of biopsy sites and samples, international guidelines should be followed. As per current recommendations of the American College of Gastroenterology and the American Gastroenterological Association, at least two samples from the duodenal bulb and four samples from the distal duodenum should be obtained (18).

**3.** To avoid processing artifacts that can interfere with the histopathological interpretation, endoscopy units and the pathology laboratory should be arranged as required. Biopsies should be reported according to the most recent Marsh-Oberhuber classification. Apart from patients with Marsh 0 grading all patients should be followed by both the pediatric endocrinology and gastroenterology departments (16,19).

**4.** The diagnosis of CD may require a consensus of pathological, clinical and laboratory findings. Findings of the histopathologic examination should be clinically correlated.

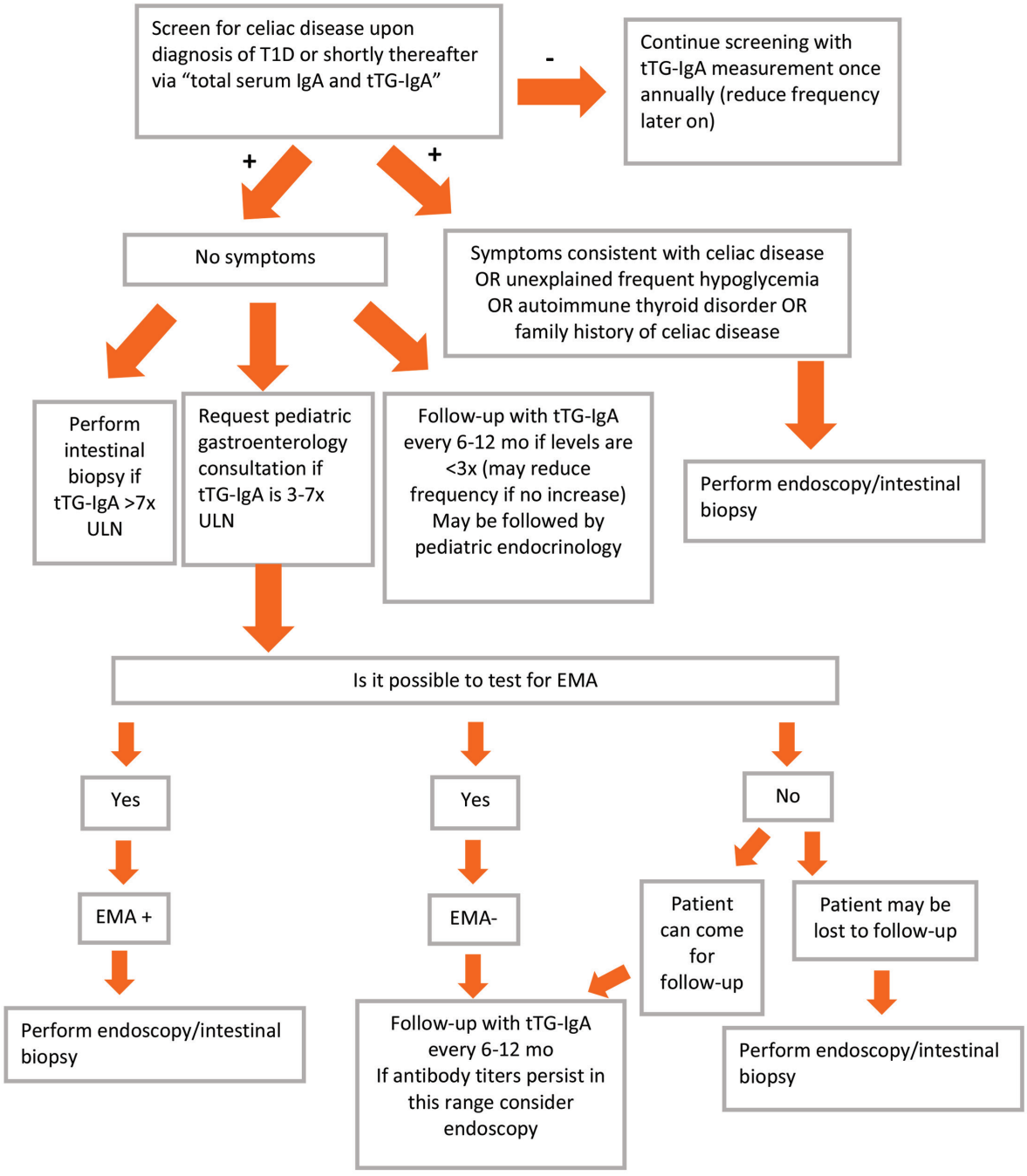


**Figure 1.** An algorithm for the screening and diagnosis of Celiac disease in children with type 1 diabetes

## Dietary Recommendations

**1.** Patients with a diagnosis of CD must follow a strict life-long gluten-free diet (20). Hence, when CD accompanies T1D, patients need closer follow-up along with more intensive counseling and dietary management (21).

**2.** A gluten-free diet involves the complete removal of wheat, barley, rye, and their hybrids/products from the diet (Table 1) (21). Nevertheless, conserved foods, premade salad/pasta sauces, some ice creams, charcuterie products such as sausage and pepperoni, premade jams, sugar cubes, premade meat-chicken broth, fruit jelly, malt drinks and beverage powder may include gluten. Other less conspicuous sources of gluten include toothpaste, mouthwash, the glue on stamps and envelopes (21).

**3.** Patients with CD must inspect whether any orally administered medication, supplement or vitamin includes gluten. Wheat flour and wheat starch are among the products used in drug manufacture. In general, if a medical product does not include wheat flour or wheat starch it is considered to be free of gluten. The amount of gluten contained in a drug is directly related to the amount of wheat flour used in its production. Therefore, if a drug description does not include information about gluten content but mentions wheat flour, that drug should be avoided by patients with CD. Medications may also include other compounds obtained through the processing of wheat flour and starch. Generally, the amount of gluten in a single unit dose of these medications are lower than the gluten amount found in foods labeled as "gluten-free". Oral intake of such medications does not interfere with a gluten-free diet (22).

**4.** Some topical products that are applied to the lips and/or the skin may contain wheat germ oil. The gluten content in highly refined wheat germ oil is infinitesimal and its topical application does not interfere with a gluten-free diet (22).

**5.** Foods with a label that reads "Does not contain gluten" or "Gluten-free" should contain ≤20 ppm of gluten, which can be safely consumed (2).

**6.** When preparing foods at home, communal use of kitchen equipment without adequate cleaning, especially of the oven and bread maker can lead to contamination with gluten. To prevent contamination, ovens and bread makers should be appropriately cleaned after each use to remove any gluten. Kitchen equipment, such as toasters, that cannot be washed should only be used by the patient. In addition, the use of cooking bags in the oven may reduce the risk of contamination. All gluten-free food products that belong to the patient with CD must be labeled and stored separately in their own drawer/cupboard, on higher racks than the products containing gluten (21,23).

**7.** It is safer to use stainless steel pots and pans or glass containers when cooking meals for patients with CD. Equipment such as pots, pans and serving spoons should not be made of materials that may have pores (e.g., wood). Prior to use, all equipment should be thoroughly cleaned and separately provided for the patient (24).

**8.** Children and adolescents with T1D and CD can consume rice, potato, corn (maize), teff, amaranth, buckwheat, quinoa, and sorghum as a source of carbohydrate, since these do not contain gluten (25).

**Table 1. Foods that should be avoided vs are allowed in a gluten-free diet**

| | |
|---|---|
| **Foods to be avoided** | |
| Milk and milk products | Milk products containing malt |
| Meats and meat products | Breaded meats, processed meats (salami, sausage, pepperoni, bacon, etc.), premade meatballs |
| Grains | Barley, rye, wheat and products prepared from them: semolina, bulgur, couscous, noodles, pasta, bread, cereal, baked goods and soup made with these grains |
| Other | Soupmixes, malt products, mustard, mayonnaise, ketchup, soy sauce, tomato paste, sugar cube, powdered sugar, ice cream cone, chocolate, wafer |
| **Foods that are allowed** | |
| Milk and milk products | Milk and milk products that do not contain malt |
| Meat and meat products | Plain meat, chicken, fish and eggs without flour and sauce |
| Grains | Rice, rice flour, maize, corn flour, corn bread (without wheat flour), any flour without gluten, quinoa, buckwheat, soy, teff, amaranth, sorghum, soup made with these grains |
| Vegetables | All fresh, unpackaged, uncanned vegetables |
| Fruits | All fresh, unpackaged, uncanned fruits |
| Legumes | All legumes |
| Fats | All fats and fatty seeds |

The table was adapted from reference 21

**9.** The macronutrient composition of gluten-free products is different from that of their counterparts containing gluten. Most gluten-free foods are poor in protein and fiber, but rich in carbohydrates and fats with a high glycemic index (26,27,28,29,30). Consequently, the glucose peaks in children with T1D and CD may be earlier and higher than those without CD (26). Accordingly, the dose and timing of insulin administration must be determined based on the nutrient content of the gluten-free products. Consumption of soup with meat/vegetables or salad prior to the main source of carbohydrate may improve postprandial glycemic control and dampen potential fluctuations (30,31).

**10.** Increasing the variety of gluten-free foods may help improve the dietary compliance of children and adolescents with T1D and CD and help them achieve a better quality of life and control of their diabetes (26).

**11.** Commonly consumed gluten-free products, such as rice and potatoes, and packaged gluten-free items sold in the supermarkets have a high glycemic index. Instead, including products with a low glycemic index and high fiber content in the diet such as teff, amaranth, buckwheat, quinoa, sorghum, soy, vegetables, fruits with edible skin and legumes helps control the postprandial blood glucose levels (26,27).

**12.** However, some gluten-free products may be poor in carbohydrates and the administration of standard doses of insulin may result in severe hypoglycemia. Labels on food packages must be read and evaluated carefully (28,29,30,31,32).

**13.** To reduce the occurrence of postprandial glucose peaks, meals should include sources of protein such as ayran, kefir, eggs, meat, chicken and fish (33).

**14.** When leaving the home children and adolescents with T1D should carry gluten-free carbohydrates to avoid the intake of products containing gluten to counteract episodes of hypoglycemia occurring outside the house (28,29,30,31).

**15.** In addition to protein and fiber, micronutrients such as iron, calcium and B vitamins should be included in the diet to improve the overall benefit of the gluten-free diet (23,32).

**16.** It may be challenging for children and adolescents with T1D and CD to follow a gluten-free diet. To improve dietary adherence, nutrition-centered education and regular counseling with a dietician specialized in pediatric diabetes management are essential (21).

**17.** In children with T1D, some parents may opt for a prophylactic gluten-free diet in the absence of a diagnosis of CD because of their awareness of this association. Nevertheless, there is no evidence to support that a gluten-free diet can prevent the development of CD in patients with T1D. Such an approach further complicates the management of diabetes in these patients. Moreover, gluten consumption is necessary to avoid false negative results and appropriately diagnose CD should it occur (34,35).

**18.** There is no evidence to support the benefits of a gluten-free diet in individuals without CD or gluten intolerance. Therefore, the gluten-free diet cannot be a medical recommendation for such individuals (34,35).

## Recommendations for Psychological Support

**1.** Parents of children with CD may report higher levels of anxiety, depression, aggression and sleep difficulties in their children, even before the definitive diagnosis is made or positive serology is detected (36). Therefore, pediatric endocrinology and gastroenterology specialists should be aware of the emotional and behavioral signs of CD and consider investigating for CD in children presenting with predominantly psychological and behavioral manifestations.

**2.** Studies show that childhood CD is a risk factor for mood disorders, anxiety disorders, eating disorders, behavioral disorders, attention deficit hyperactivity disorder, autism spectrum disorder and intellectual disability disorder (37). It is recommended for children with CD to be monitored into adulthood in terms of both physical and mental health.

**3.** Dealing with multiple chronic conditions can lead to poorer health outcomes, increasing financial costs and difficulties in the daily management of health. Although studies investigating the experiences of parents of children with T1D and CD are limited, families usually focus on health issues, financial concerns, psychological wellbeing of the child and social situations outside the house (38). Families worry more about the short and long-term complications of diabetes than those of CD. Routinely measuring blood glucose levels, counting carbohydrates and adhering to a strict gluten-free diet are likely to be some of the daily struggles for health management. Gluten-free products are very expensive and both preparing appropriate foods and doctor visits can take significant amounts of time. Children may feel different from their peers and suffer misunderstandings or bullying. False or incomplete information can lead to lack of physical and/or emotional support by society, especially in social settings such as schools.

## General Recommendations

**1.** According to the results of a “questionnaire” conducted prior to the meeting, pediatric endocrinology and

pediatric gastroenterology departments in Turkey exhibit heterogenous practice regarding the screening, diagnosis and management of CD in children with T1D, and 20% do not follow consensus guidelines. As such, greater cooperation between pediatric endocrinology and pediatric gastroenterology departments is necessary, which should be further supported by regular multidisciplinary team meetings that should include pathologists, dieticians and psychologists.

**2.** Standards for screening of CD within the first five years following the diagnosis of T1D should be set and implemented in clinics.

**3.** The main recommendations set forth by this script should be shared with ESPGHAN and ISPAD societies in order to request a joint consensus guideline for the diagnosis and management of children with T1D and CD.

**4.** Efforts should be made to improve the management of children with CD in schools and to garner greater governmental support.

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### Ethics

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

### Authorship Contributions

Concept – Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

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# Efficacy of the Novel Degludec/Aspart Insulin Co-formulation in Children and Adolescents with Type 1 Diabetes: A Real-life Experience with One Year of IDegAsp Therapy in Poorly Controlled and Non-compliant Patients

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### What is already known on this topic?

Achieving optimal metabolic control can be extremely challenging in some children and adolescents with type 1 diabetes (T1DM). Adherence to multiple insulin injections is poor in a subgroup of these children, leading to frequent hospitalization because of diabetic ketoacidosis (DKA). Degludec/aspart co-formulation (70% IDeg + 30% IAsp - IDegAsp) can be beneficial in challenging cases with poor glycemic control and acute complications of diabetes by providing the longer-duration of basal insulin with simplified basal-bolus treatment in 3 injections instead of 4-5 injections.

### What this study adds?

The real-life experience demonstrates that IDegAsp is non-inferior to classic basal-bolus regimen regarding to glycemic control in children with T1DM. Simplified basal-bolus regimen with IDegAsp could be an alternative in patients with frequent hypoglycemia and DKA attacks, who have poor compliance with 4-5 injections per day.

## Abstract

**Objective:** To evaluate the efficacy of degludec/aspart (IDegAsp) insulin co-formulation in children and adolescents with poorly controlled type 1 diabetes (T1DM).

**Methods:** Patients with poorly controlled T1DM on basal-bolus insulin regimes and having compliance problems related to insulin injections were switched to IDegAsp and were included. Data on hemoglobin A1c (HbA1c) levels, hypoglycemic episodes, frequency of diabetic ketoacidosis (DKA) and insulin doses were recorded at baseline and after one year of IDegAsp treatment.

**Results:** Fifty patients (22 girls; 44%) were started on IDegAsp. The mean ± standard deviation (range) age and duration of diabetes were 12.9 ± 3.4 (4-18) and 5.2 ± 3.1 (1.0-13.7) years, respectively. At the end of one year, 38 patients were still on IDegAsp, whereas 12 patients had opted to resume their original treatments. In those who continued on IDegAsp, HbA1c levels did not change, but the number of self-reported mild-moderate hypoglycemic episodes decreased significantly ($p < 0.05$). In the year before switching to IDegAsp, 11 DKA attacks in 9 patients were observed, whereas this decreased to 4 DKA attacks in 4 patients after one year of IDegAsp therapy ($p = 0.06$).

**Conclusion:** IDegAsp regimen may improve clinical management in poorly controlled basal-bolus insulin regimen T1DM patients who have frequent hypoglycemia and DKA attacks, as well as in those with poor compliance with multiple injections. Although a simplified basal-bolus IDegAsp regimen is an attractive option for patients with T1DM, some may not adapt to this treatment due to the fixed IAsp dose of IDegAsp.

**Keywords:** Type 1 diabetes mellitus, hypoglycemia, diabetic ketoacidosis, co-formulation, insulin degludec, insulin aspart, IDegAsp

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## Introduction

Currently, basal-bolus insulin regimes are the most commonly used treatment modality in children and adolescents with type 1 diabetes mellitus (T1DM) worldwide. Insulin pump therapy, although permitting a more physiological insulin delivery, is not available to all patients due to high cost that hinders patient access to treatment (1,2,3).

Optimal glycemic control is considered the key factor in reducing the risk of long term microvascular and macrovascular complications in diabetes patients (1), while only 14-30% of the patients are reported to achieve target hemoglobin A1c (HbA1c) levels (2). Furthermore, achievement of glycemic control is more challenging in an adolescent population due to poor patient compliance with anti-diabetic treatment and insulin injections in particular (2,3). Accordingly, these patients are more prone to acute complications of T1DM such as recurrent episodes of diabetic ketoacidosis (DKA) and hypoglycemia (2,3). Given that a standard basal-bolus insulin regime requires 4-5 injections per day, increasing the likelihood of omitting insulin injections, longer acting basal insulins seem to offer an alternative treatment approach with a potential to enable better glycemic control and reduced risk of recurrent DKA in children and adolescents with T1DM and poor treatment compliance. New long-acting insulin analogue degludec (IDeg), developed by removal of threonine at B30 of human insulin and adding a glutamic acid spacer to a 16-carbon diacid at B29, has a mean half-life of 25 hours, while the half-life of the longest acting insulin (glargine) on the market is 12 hours (4). IDeg is considered to offer stable coverage of basal insulin needs due to its flatter and more consistent pharmacodynamic profile with a duration of action exceeding 42 hours and fourfold less within-subject variability compared to insulin glargine (5,6). Furthermore, IDeg can be mixed with rapid acting insulin analogue-insulin aspart (IAsp) without affecting the pharmacokinetics of either molecule, while other long acting insulin analogues cannot be mixed or co-formulated (5,6).

Insulin degludec/insulin aspart (IDegAsp) combining 70% IDeg and 30% IAsp, is a soluble combination of two individual insulin analogues in one product, designed to provide mealtime glycemic control through the IAsp component and basal glucose-lowering effect through the IDeg component. IDegAsp could provide flat and stable basal insulin coverage (provided by IDeg at steady-state conditions) and bolus mealtime insulin control with reduced injection burden compared to standard basal and bolus therapy (7). In addition, IDegAsp has been approved for use in T1DM patients over two years of age by European Medicine Agency and one year of age and older by the U.S. Food and Drug Administration DA (8,9).

The aim of this study was to investigate the effectiveness of IDegAsp in children and adolescents with T1DM with poor glycemic control and acute complications due to noncompliance with insulin injections over a one year period.

## Subjects, Methods

### Study Population

Children and adolescents with T1DM patients with poor glycemic control [HbA1c >8.5% (69.4 mmol/mol)] on basal-bolus regimen (4-5 injections per day) and frequent omission of insulin injections who were switched to IDegAsp (3 injections per day) were included in this study. Inclusion criteria were: diabetes duration of >1 year; poor glycemic control on basal bolus regimen; and poor treatment compliance.

Written informed consent was obtained from the parent/legal guardian of each patient following a detailed explanation of the objectives and protocol of the study, which was conducted in accordance with the ethical principles stated in the "Declaration of Helsinki" and approved by Marmara University Faculty of Medicine Clinical Research Ethics Committee (protocol no: 70737436-050.06.04, date: 07.02.2014).

### Assessments

Patient demographics, body weight and height measurements and pubertal status were retrieved from the hospital records. Data on HbA1c levels, daily insulin doses, the number of basal and bolus insulin injections per day, the number of total severe hypoglycemic episodes per year, self-reported mild to moderate hypoglycemic episodes per week and the number of DKA episodes requiring hospital care were recorded one year before and after the change of the insulin regimen to IDegAsp (Figure 1).

Severe hypoglycemia was defined as an episode requiring the assistance of another person to actively administer carbohydrate, glucagon, or take other corrective action and neurological recovery following the normalization of plasma glucose levels, or both.

### Switching to IDegAsp

Since, the bolus IAsp dose is fixed in IDegAsp (30%), it was necessary to first determine the main meal which the patient consumed from the dietary history and the relatively fixed and high amount of carbohydrate and the IDegAsp

injection was tailored to that meal. The dose of IDegAsp was calculated based on the IAsp requirement at that meal which was 30 % of the IDegAsp total dose. In the remaining two main meals, the patients received their usual IAsp dose according to insulin carbohydrate ratios. Further dose adjustments of IDegAsp were made based on postprandial glucose after the IDegAsp meal and fasting glucose levels.

### Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). The paired t-test and Mann-Whitney U test were used for analysis of parametric and nonparametric variables, respectively. Data were expressed as mean ± standard deviation (SD), median minimum-maximum (min-max) and percent (%) where appropriate. A $p < 0.05$ was considered statistically significant.

## Results

### Baseline Characteristics

The study population comprised 50 patients with T1DM, of whom 39 were pubertal and 11 were prepubertal. The mean ± SD (range) patient age was 12.9 ± 3.4 (4-18) years and 28/50 (56.0 %) were boys. The mean ± SD (range) duration of diabetes was 5.2 ± 3.1 (1.0-13.7) years. Overall, 34 patients were switched to IDegAsp from glargine and 16 patients from detemir as basal insulins. Twenty-three patients were on two doses of basal (total of five injections/per day) injections with glargine (n = 12) or detemir (n = 11) and 27 patients were on single basal (total of four injections/per day) injections with glargine (n = 22) or detemir (n = 5).

In addition to poor glycemic control and omission of insulin injections, there were frequent episodes of hypoglycemia (n = 11; 22 %), excessive daily glucose variability (n = 10; 20 %) and frequent DKA (n = 9; 18 %) among the subjects.

Twelve (24 %) patients who were switched to IDegAsp did not want to continue on IDegAsp and switched back to their old regimens. The reasons for discontinuation were persistent hyperglycemia (n = 7; 58.3 %), difficulty in making dose adjustment due to fixed dose of IAsp within the IDegAsp (n = 4; 33 %) and transition to insulin pump therapy (n = 1; 8.3 %).

### Overall One Year Treatment Outcome with IDegAsp in Those Who Continued

Overall, 38 (76 %) patients (25 boys and 13 girls) completed one year of IDegAsp therapy.

Mean ± SD and median (min-max) age of this group was 12.8 ± 3.3 years and 13.2 (4.1-17.7) years, respectively.

When values before and after one year of IDegAsp therapy were compared, no significant difference was found in mean body mass index-SD score (BMI-SDS) (0.34 ± 1.01 vs. 0.21 ± 1.07, $p = 0.26$) or in mean HbA1c levels (9.3 ± 1.7 % vs. 9.6 ± 1.9 %, $p > 0.05$) (Table 1). However, one year of IDegAsp therapy was associated with a significant reduction in the number of insulin injections (4.78 ± 0.41 vs. 3.08 ± 0.30, $p < 0.05$), total daily insulin dose (basal + bolus insulin: 1.19 ± 0.34 vs. 1.01 ± 0.22 U/kg/day, $p < 0.05$), long acting/total insulin ratio (0.46 ± 0.09 vs. 0.43 ± 0.05, $p < 0.05$), long acting/rapid acting insulin ratio (0.91 ± 0.31 vs. 0.76 ± 0.17, $p < 0.05$) and long acting insulin dose (28.75 ± 13.73 vs. 23.2 ± 9.09 U/day and 0.55 ± 0.17 vs.

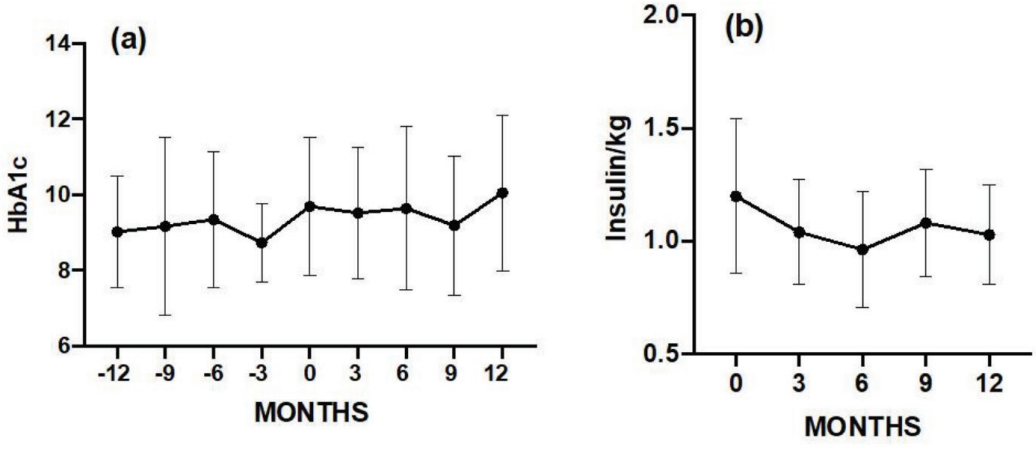


**Figure 1.** HbA1c levels before (previous year) and after the first year of IDegAsp (a) and insulin dose changes under IDegAsp treatment (b)

*HbA1c: hemoglobin A1c, IDegAsp: insulin degludec/insulin aspart*

0.44 ± 0.11 U/kg/day, p < 0.05) when compared to baseline, pre-switch values (Table 1).

After one year of IDegAsp therapy, a significant decrease was noted in the number of blood glucose-confirmed mild-to-moderate hypoglycemic episodes (2.1 ± 1.9 vs. 0.98 ± 1.16 episodes/week, p < 0.05) but not in the total number of severe hypoglycemic episodes (two episodes in two patients before and after therapy, 0.05 ± 0.22 episodes per year in each, p > 0.05) when compared to baseline values (Table 1).

Albeit not reaching significance, there was a tendency for a decrease in total DKA episodes/year after one year of IDegAsp therapy when compared to baseline values (0.28 ± 0.56 vs. 0.10 ± 0.31 episodes per year, p > 0.05). Overall 11 DKA episodes occurred in 9 patients in the year before switching to IDegAsp therapy and 4 DKA episodes were noted in 4 patients after 1-year of IDegAsp therapy (Table 1).

### Insulin Doses at the Third Month of IDegAsp Therapy in Pre-switch Daily Basal Injection Subgroups

Before switching to IDegAsp, 20 patients were on once-daily (OD) and 18 patients were on twice-daily (TD) basal injection. There was a non-significant tendency for higher total daily basal and bolus insulin doses in the twice daily vs. OD group at the time of switch.

At the third month of IDegAsp therapy, total (1.13 ± 0.27 vs. 1.03 ± 0.19 U/kg/day in pre-switch OD group and 1.26 ± 0.41 vs. 1.07 ± 0.27 U/kg/day in pre-switch TD group, p < 0.05 for each) and basal (0.49 ± 0.15 vs. 0.44 ± 0.09 U/kg/day in OD group and 0.57 ± 0.21 vs. 0.47 ± 0.14 U/kg/day in TD group, p < 0.05 for each) daily insulin doses decreased significantly from baseline, similarly in both the pre-switch OD and pre-switch TD subgroups (Table 2).

No significant difference was noted between pre-switch OD and pre-switch TD subgroups in terms of bolus insulin dose (U/kg/day) at baseline vs. the third month of IDegAsp therapy as well as in IDegAsp doses (U/kg/day) at onset and the third month of therapy (Table 2).

## Discussion

IDegAsp is the fixed-ratio co-formulation of two different insulin analogues, which provides an option for long-lasting basal insulin coverage and rapid acting post-prandial control in a single injection. It may provide an opportunity to decrease the number of insulin injections and could therefore be preferable in noncompliant patients frequently missing injections. IDegAsp has been confirmed to be non-inferior to IDet + IAsp in terms of HbA1c reduction, together with similar hypoglycemia rates in children (10). Representing the first real-life study evaluating the efficacy of IDegAsp in noncompliant patients, our findings have shown that IDegAsp is non-inferior to basal bolus regimens regarding glycemic control despite fewer injections. Additionally, IDegAsp therapy was associated with lesser likelihood of non-severe hypoglycemia and DKA episodes in the current study. Albeit not statistically significant, a tendency for a lower frequency of DKA episodes was noted after switching to IDegAsp, from 11 DKA episodes in nine patients at baseline to 4 DKA episodes in four patients after the first year of IDegAsp therapy. These data demonstrate the potential of IDegAsp to reduce the rate of metabolic decompensation and DKA in children with T1DM who are

**Table 1. Study parameters at baseline and one year after IDegAsp therapy in those completing the full year (n = 38)**

| | | IDegAsp continuers (n = 38) | | |
|---|---|---|---|---|
| **Mean ± SD** | | **Baseline (pre-switch)** | **One year after IDegAsp** | **p value** |
| HbA1c* | (%) | 9.3 ± 1.7 | 9.6 ± 1.9 | 0.75 |
| BMI-SDS | | 0.34 ± 1.01 | 0.21 ± 1.07 | 0.26 |
| Number of insulin injections | | 4.78 ± 0.41 | 3.08 ± 0.30 | **0.001** |
| Insulin dose (U/kg/day) | | 1.19 ± 0.34 | 1.01 ± 0.22 | **0.033** |
| Long acting/total insulin ratio | | 0.46 ± 0.09 | 0.43 ± 0.05 | **0.047** |
| Long acting/rapid acting insulin ratio | | 0.91 ± 0.31 | 0.76 ± 0.17 | **0.009** |
| Rapid acting insulin dose | U/day | 32.83 ± 14.63 | 31.79 ± 13.3 | 0.56 |
| | U/kg/day | 0.65 ± 0.25 | 0.60 ± 0.17 | 0.19 |
| Long acting insulin dose | U/day | 28.75 ± 13.73 | 23.2 ± 9.09 | **0.0015** |
| | U/kg/day | 0.55 ± 0.17 | 0.44 ± 0.11 | **< 0.0001** |
| Mild-to-moderate hypoglycemia (total episodes/week) | | 2.1 ± 1.9 | 0.98 ± 1.16 | **0.001** |
| Severe hypoglycemia (total episodes/year) | | 0.05 ± 0.22 | 0.05 ± 0.22 | 1.00 |
| DKA (total episodes/year) | | 0.28 ± 0.56 | 0.10 ± 0.31 | 0.06 |

*Mean HbA1c levels of the previous year before changing to IDegAsp and first year of IDegAsp treatment.
BMI-SDS: body mass index-standard deviation (SD) score, DKA: diabetic ketoacidosis, IDegAsp: insulin degludec/insulin aspart

**Table 2. Insulin doses at baseline vs. the third month of IDegAsp therapy in pre-switch once-daily and twice daily basal injection subgroups**

| | Baseline (pre-switch) | Third month after IDegAsp | p value |
|---|---|---|---|
| **Total daily doses (Unit/kg/day)** | | | |
| Once-daily basal injection[a] (n = 20) | 1.13 ± 0.27 | 1.03 ± 0.19 | 0.028 |
| Twice daily basal injection[b] (n = 18) | 1.26 ± 0.41 | 1.07 ± 0.27 | 0.012 |
| **Basal insulin dose (Unit/kg/day)** | | | |
| Once-daily basal injection[a] (n = 20) | 0.49 ± 0.15 | 0.44 ± 0.09 | 0.046 |
| Twice daily basal injection[b] (n = 18) | 0.57 ± 0.21 | 0.47 ± 0.14 | 0.037 |
| **Bolus insulin dose (Unit/kg/day)** | | | |
| Once-daily basal injection[a] (n = 20) | 0.62 ± 0.19 | 0.60 ± 0.14 | 0.66 |
| Twice daily basal injection[b] (n = 18) | 0.70 ± 0.3 | 0.68 ± 0.18 | 0.78 |
| **IDegAsp dose (Unit/kg/day)** | | | |
| Once-daily basal injection[a] (n = 20) | 0.58 ± 0.13[c] | 0.63 ± 0.13 | 0.34 |
| Twice daily basal injection[b] (n = 18) | 0.61 ± 0.19[c] | 0.65 ± 0.16 | 0.18 |

[a]Total 4 injections/day, [b]total 5 injections/day, [c]calculated IDegAsp dose at the initiation of treatment.
IDegAsp: insulin degludec/insulin aspart

not compliant with insulin injections on a regular basal-bolus regime. This effect seems to be related to the longer duration of action of IDeg, which provides better and durable basal insulin coverage and prevents ketone production unless the patient omits the IDegAsp injection. Similarly, Thalange et al (11) also reported that in children with T1DM, IDegAsp as compared with basal bolus regimen with detemir was associated with a decrease in the rate of ketosis. IDegAsp permits a reduction in the number of injections, which could be additional motivation for the patients and might increase compliance.

Studies of the effectiveness of IDegAsp in children and adolescents with T1DM are scarce (10,11,12). In adult patients with T1DM, OD treatment with IDegAsp and IAsp as bolus insulin for the remaining meals was reported to be associated with significantly lower risk of nocturnal hypoglycemia, improved glycemic control and showed non-inferiority compared with IDet + IAsp (13). Although we could not evaluate nocturnal hypoglycemia, real-life experience with IDegAsp in our noncompliant T1DM patients resulted in a decrease in the total number of non-severe hypoglycemic episodes. The achievement of intensive insulin therapy goals with three injections under IDegAsp + IAsp instead of a minimum of four injections under a conventional basal-bolus insulin regimen was considered to be the actual value of this therapy, enabling a reduced injection burden and thereby potentially improving patient adherence and quality of life. Additionally, no significant change in BMI-SDS was observed after switching to IDegAsp in our study, nor in previous studies (12,14,15,16).

Only a few studies in children with IDegAsp have been published and there are no standard protocols for switching doses in children. The switching protocol used in the current study was based on the decreased basal insulin doses with optimal IAsp dose for injection at the meal when IDegAsp was injected to avoid postprandial hypoglycemia. At the end of the third month after initial dose adjustment our patients' insulin doses decreased 11% in total and 16% in basal dose. In a pediatric study, dose reduction of 15% in total daily insulin and 26% in basal insulin was also reported (10). Thus, when deciding the initial dose of IDegAsp, a 20-30% reduction in basal insulin dose may be a reasonable and safe starting dose.

In addition, at the end of the third month after initial dose adjustment, insulin doses decreased by 9% for patients who were on OD basal insulin and by 15% for those on twice daily basal insulin prior to switching. IDegAsp doses became 0.63 ± 0.13 and 0.65 ± 0.16 U/kg/day, respectively. Thus, when deciding the initial dose of IDegAsp, previous basal daily injection and doses should be taking into account, together with IAsp dose. Nonetheless, starting with 0.5-0.6 U/kg/day or half of the daily total insulin doses seems to be a good strategy.

Although simplified basal-bolus regimen with IDegAsp in T1DM is an attractive option for some patients, a significant number of the patients could not adapt to the treatment and returned to their old regimens. The main reasons for discontinuing IDegAsp therapy were difficulty of dose adjustment and inflexibility of fixed IAsp dose within IDegAsp, which requires a fixed amount of carbohydrate consumption in the meal when IDegAsp is injected. For these reasons, IDegAsp may not be suitable for every patient with T1DM and regimen change should be attempted only after careful evaluation and after a full

explanation, including advantages and disadvantages, is given to each patient.

### Study Limitations

Although, our study did not have a non-IDegAsp treated control group, treatment with a different regimen in the previous year was used to compare the effectiveness of IDegAsp treatment in the same group of patients. Another limitation of the study was that glucose monitoring and frequency of hypoglycemic episodes were obtained from self-determined blood sugar measurements and self reported by patients/families. Utilizing a continuous glucose monitoring system could have provided a more accurate picture of glycemic control.

## Conclusion

In conclusion, this real-life experience study indicated that IDegAsp was non-inferior to basal-bolus regimen in terms of glycemic control, which was accompanied by a significant reduction in the number of daily injections and frequency of mild-to-moderate hypoglycemic episodes, along with likelihood of lower risk of DKA, in children with T1DM and selected for having poor therapy compliance. Accordingly, use of a simplified basal-bolus regimen with IDegAsp could be an alternative in T1DM patients with frequent hypoglycemia and recurrent DKA, who have poor compliance when having 4-5 injections per day and in whom insulin pump treatment is not available.

### Ethics

**Ethics Committee Approval:** The study were approved by the Marmara University Faculty of Medicine Clinical Research Ethics Committee (protocol number: 70737436-050.06.04, date: 07.02.2014).

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

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

### Authorship Contributions

Medical Practices: Tarık Kırkgöz, Mehmet Eltan, Sare Betül Kaygusuz, Zehra Yavaş Abalı, Didem Helvacıoğlu, Tuba Seven Menevşe, Büşra Gürpınar Tosun, Concept: Serap Turan, Design: Tarık Kırkgöz, Tülay Güran, Abdullah Bereket, Serap Turan, Data Collection or Processing: Tarık Kırkgöz, Mehmet Eltan, Sare Betül Kaygusuz, Zehra Yavaş Abalı, Didem Helvacıoğlu, Tuba Seven Menevşe, Büşra Gürpınar Tosun, Tülay Güran, Abdullah Bereket, Serap Turan, Analysis or Interpretation: Tarık Kırkgöz, Tülay Güran, Abdullah Bereket, Serap Turan, Literature Search: Tarık Kırkgöz, Serap Turan, Writing: Tarık Kırkgöz, Abdullah Bereket, Serap Turan.

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# Feminizing Adrenocortical Tumors as a Rare Etiology of Isosexual/Contrasexual Pseudopuberty

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### What is already known on this topic?

Feminizing adrenocortical tumors (FATs) are extremely rare tumors that are most commonly seen in men and boys presenting with gynecomastia.While boys present with contrasexual pseudopuberty signs, girls present with isosexual pseudopuberty.

### What this study adds?

FATs are more common in children ≤8 years of age, with a median age at diagnosis of six years. FATs are usually malignant in adults whereas in children approximately half of the FATs are benign. In children the assessment of malignant potential depends on clinical behavior of the tumor. Although complete surgical resection of benign FATs is thought to be curative, long-term follow-up is required because of the unpredictability of these tumors. FATs occurring in childhood may carry a better prognosis than in adult males because most of the FATs in children did not recur during follow-up since diagnosis is made early, as typical presenting signs are more obvious before puberty.

## Abstract

**Objective:** Estrogen-secreting adrenocortical tumors (ACTs) are quite rare with feminizing adrenocortical tumors (FATs) accounting for 0.37-2% of all ACTs. The aim was to evaluate clinical and hormonal characteristics of FATS as well as treatment options and follow-up in the pediatric age group.

**Methods:** Medical records of children with ACTs presenting to a single center in the last two decades were reviewed. Literature review within Pubmed revealed 34 pediatric patients (22 boys) with FAT among 192 articles.

**Results:** Among the 25 children presenting with ACTs in the last two decades, two new pediatric cases of FAT were identified, one benign and the other malignant, in two genders with different clinical presentations. Literature review showed that FATs are extremely rare tumors that are most commonly seen in men and boys presenting with gynecomastia. FATs are more common in children ≤8 years of age, with a median age at diagnosis of six years. While boys present with contrasexual pseudopuberty signs, girls present with isosexual pseudopuberty. A high estrogen level strongly supports diagnosis, while elevations in other adrenal hormones may be seen. FATs are usually malignant in adults and prognosis is generally very poor. However, in children approximately half are benign although assessment of malignant potential depends on clinical behavior of the tumor. FATs are very unpredictable so even after surgery long-term follow-up is required. FATs presenting in childhood may have a better prognosis than adult presentation tumors as most FATs in children are followed without recurrence of tumor.

**Conclusion:** FATs are more common in children ≤8 years of age, with a median age at diagnosis of six years. FATs in childhood may have a better prognosis than in adult males.

**Keywords:** Adrenal adenoma, adrenal tumor, adrenocortical carcinoma, adrenocortical tumors, childhood, children, children and adolescents, estrogen

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## Introduction

Adrenocortical tumors (ACTs) are rare in childhood, accounting for less than 0.2 % of childhood malignancies, but nearly 6 % of adrenal tumors (1). Childhood ACTs are usually functional tumors; the rate of being hormonally active is 90 % in children compared to 50 % in adult cases (2,3). While adults usually have single hormone secretion, children often have multiple hormone secretion. Complete hormonal workup of ACTs revealed the predominance of mixed hormone-secreting types of tumor although some cases lack the appropriate clinical findings. Hormonal work-up of these tumors showed that half of the patient population had tumors secreting a combination of androgens and cortisol, whereas almost one third had ACTs secreting androgens alone (4). The clinical presentation depends on the hormones secreted by the tumor. The most common presenting symptoms are virilization due to androgen secretion, followed by Cushing's syndrome due to cortisol secretion and then hyperaldosteronism (3,4,5,6). Estrogen-secreting ACTs are quite rare, feminizing adrenocortical tumors (FATs) accounting for 0.37-2 % of all ACTs (7). FATs may secrete estrogen (estrone and estradiol) alone or in combination with other adrenal hormones and are more prevalent in adult males, are usually malignant and have a poor prognosis (7).

The clinical manifestations of FATs include feminizing symptoms of excessive estrogen production, symptoms due to compression by the tumor mass, or constitutional symptoms such as weight loss and fatigue. Girls usually present with precocious puberty (isosexual pseudopuberty), while boys present with bilateral gynecomastia (contrasexual pseudopuberty) and delayed puberty. In boys gynecomastia is the most common symptom of a FAT with a rate of 98 % (8). Diagnosis of FATs depends on the clinical signs and hormonal analysis. Hormone profiles show overproduction of estrogen alone or in combination with other adrenocortical hormones, alongside normal or low gonadotropin levels (9,10). Radiological and histological findings are similar to those of other ACTs. Histopathology examination normally shows positive immunostaining for aromatase in the tumor tissue (11).

In this study the aim was to evaluate the clinical and hormonal characteristics, treatment options and outcome in FATs occurring in the pediatric age group. For this purpose the medical records of children with an ACT followed in our unit in the last two decades were reviewed with the intention of detailed evaluation of the clinical and hormonal characteristics, treatment methods and outcomes of the patients who were diagnosed with FAT during this period. A systematic literature review was performed and the features of similar published pediatric cases were analyzed. We wished to highlight this rare tumor and draw attention to the variety of FAT characteristics by presenting the cases with FAT followed-up in our unit and by reviewing the published cases with FAT in childhood period.

## Methods

The medical records of children with ACT who were followed up between 1999 and 2020 in the pediatric endocrinology unit at Hacettepe University İhsan Doğramacı Children's Hospital, Ankara, were reviewed retrospectively. Data regarding age, sex, presenting symptoms, clinical characteristics, laboratory investigations including hormonal analysis, imaging techniques including ultrasonography (USG), computed tomography (CT), and magnetic resonance imaging, histopathological reports, treatment and outcomes were extracted from the medical files. Cases with FAT were identified from amongst all the ACTs followed up in our unit. Cases with FAT were described in detail. A systematic literature review was also performed. A search was made using PubMed/MEDLINE using the following keywords: "Feminizing adrenocortical tumor" and "Feminizing adrenal tumor" and was filtered for articles published in English and Turkish. The literature was queried from inception to December 1, 2020. We included all articles that reported children who had FATs. The following parameters were collected from included studies: age at diagnosis, gender, clinical presentation, hormonal evaluation, treatment options, clinical course of the tumor, and survival/follow-up period.

### Statistical Analysis

Data analyses were performed by using Statistical Package for the Social Sciences (SPSS) for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Continuous data were described as mean ± standard deviation (SD) and categorical data were described as the number of cases (%). Differences between two independent groups were compared by Student's t-test while differences between more than two independent groups were analyzed by one-way ANOVA. A $p < 0.05$ was considered to indicate statistical significance.

## Results

A total of 25 patients with ACT had been followed up in our pediatric endocrinology unit during the study period. Since histological criteria to differentiate malignant behavior in childhood ACTS are not reliable, all lesions were categorized as ACT instead of adenoma or carcinoma. By this classification two patients had FAT and the other twenty-three patients had ACT. The clinical, hormonal and pathological characteristics of the cases with ACT are given

in a recently written article by Ardicli et al (12). Clinical, hormonal, pathological evaluations as well as management and outcome of the patients with FAT are given below in detail.

## Case 1

A 13.5-year-old boy presented with bilateral breast enlargement and weight gain for the last four months. No discharge from the breasts was noted. He did not have headache, nausea or vomiting, flushing, palpitation, sweating or diarrhea. He was not using any medication. His family history was unremarkable. On physical examination body weight was 61.4 kg [1.1 SD score (SDS)], height was 156.4 cm (-0.5 SDS), body mass index was 25.1 kg/m$^2$ (1.6 SDS), heart rate was 90/min and blood pressure was 140/85 mmHg. He had a cushingoid appearance with purple striae, buffalo hump, moon face and centipetal fat accumulation. He had bilateral, Tanner stage 4 gynecomastia with Tanner stage 4 pubic hair, testis volumes of 6/8 mL, and stretched penile length of 7.5 cm. Laboratory examination showed that he had hyperestrogenism, hyperandrogenism and hypercortisolism (Table 1). Abdominal USG showed a 102x94 mm, round, hypoechoic mass with echogenic septates and increased blood flow at the right adrenal lodge. A hypoechoic, thrombotic mass markedly occluded the lumen of the inferior vena cava (IVC). Abdominal CT showed the mass was exerting pressure on the right kidney and the right lobe of the liver. A chest CT revealed pulmonary metastasis. Antithrombotic treatment (enoxaparin sodium) was administered for the thrombus in the IVC. Amlodipine was started for hypertension. The mass with right adrenal gland and tumor thrombus were resected under steroid coverage. Histopathological examination confirmed the diagnosis of adrenocortical carcinoma with high mitoses, sinusoidal and venal invasions and penetration of the capsule of the adrenal gland. Hormone levels declined in the postoperative period. The patient was administered a chemotherapy protocol, including cisplatin, etoposide and doxorubicin. Hydrocortisone replacement at maintenance dose was given along with mitotane treatment, and the dose was adjusted with respect to the patient's clinical findings. The patient was monitored for blood pressure, serum potassium, plasma renin and aldosterone levels to check for a possible mineralocorticoid deficiency, though no such deficiency was found during follow-up. As the patient received mitotane, thyroid function tests were closely monitored and remained normal.

While a successful decline was observed in the hormonal secretion and gynecomastia post-surgery, the patient again developed gynecomastia accompanied by a huge elevation of testosterone (T), adrenal androgen (androstenedione) and estradiol levels following commencement of mitotane. Since the imaging studies did not show a relapse or recurrence, elevated T was attributed to the inhibition of 5-α-reductase enzyme activity due to mitotane therapy. This suggestion was confirmed by an elevated T/dihidrotestosterone (DHT) ratio of 38.5 (T: 1374.17 ng/dL, DHT: 357.12 pg/mL) (13). Serial measurements of T and androstenedione revealed a gradual decline in the hormone levels after the cessation of mitotane treatment. During the three-and-a-half year follow

**Table 1. Pre-op and post-op laboratory examinations of the cases**

| | Case 1 | | Case 2 | | Normal values |
|---|---|---|---|---|---|
| Name of the hormone | Pre-op | Post-op | Pre-op | Post-op | |
| FSH (IU/L) | < 0.3 | 2.94 | 0.66 | 4.6 | 0.7-18 |
| LH (IU/L) | 0.27 | 1.74 | < 0.2 | 0.3 | 2.4-10 |
| Testosterone (ng/dL) | 151.01 | 14.8 | 116 | < 20 | < 20 (prepubertal), 39-631 (pubertal in boys) |
| $E_2$ (pg/mL) | 129.37 | 11.82 | 275 | < 20 | < 10 |
| 08.00 ACTH (pg/mL) | < 5.0 | 22.3 | 19 | 25 | 0-46 |
| 08.00 cortisol (µg/dL) | 10.47 | 10.26 | 6.5 | 11.6 | 6.7-22.6 |
| 23.00 ACTH (pg/mL) | < 5.0 | | | | 0-30 |
| 23.00 cortisol (µg/dL) | 10.2 | | | | 3-16.6 |
| 24 hour urinary cortisol (µg/day) | N/A* | | | | 2.6-37 |
| 17-OH progesterone (ng/mL) | 5.66 | 1.11 | 1.2 | 0.42 | 0.59-3.44 |
| 11-deoxycortisol (ng/mL) | 116.16 | 4.55 | 3.4 | 3.2 | < 7.2 |
| Androstenedione (ng/mL) | > 10.0 | 0.66 | 4.1 | 0.36 | < 0.51 (prepubertal), 0.31-2.4 (pubertal) |
| DHEA-$SO_4$ (µg/dL) | 415.26 | 47.1 | > 1000 | < 30 | < 35 (prepubertal), 35-430 (pubertal) |
| Na (mEq/L) | 139 | 136 | 138 | 140 | 135-145 |
| K (mEq/L) | 4.2 | 4.3 | 4.3 | 4.4 | 3.4-4.7 |
| Cl (mEq/L) | 103 | 101 | 105 | 107 | 101-109 |
| Renin (pg/mL) | 33.2 | 18.6 | 28.1 | N/A | 2.7-16.5 (supine), 5.41-34.53 (standing) |
| Aldosterone (pg/mL) | 77.0 | 82.4 | 82.6 | N/A | 10-160 (supine), 35-300 (standing) |

*N/A: not available, FSH: follicle-stimulating hormone, LH: luteinizing hormone, ACTH: adrenocorticotropic hormone, DHEA: dehydroepiandrosterone

up there were no further clinical symptoms and radiological finding did not indicate recurrence of the tumor.

### Case 2

A 7-year-old girl presented with bilateral breast development and appearance of pubic hair which had been present for the last six months. She was not using any medication and her family history was unremarkable. On physical examination she had bilateral Tanner stage 3 breast development with Tanner stage 3 pubic hair. Laboratory examination revealed elevated estradiol and androgen levels with suppressed luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Table 1). Abdominal ultrasound showed a 65x53 mm, round, hypoechoic, heterogeneous mass with clear boundaries at the left adrenal lodge with a uterus of pubertal size and pre-pubertal sized ovaries. An abdominal CT indicated mass pressure affecting the left kidney. The mass, together with the left adrenal gland was resected. Histopathological examination confirmed the diagnosis of ACT. Hormone levels declined in the postoperative period. The patient was followed until the age of 20 years and during this time she had neither clinical symptoms nor radiological findings indicating recurrence of the tumor. Thus there was 13 years of disease-free survival.

### Literature Review

There were 34 pediatric FAT cases that have been described among 192 articles identified from the PubMed database (Table 2). The first pediatric case was a 4.6-year-old male described in 1948 (14). Twenty-two of the cases (65%) were

**Table 2. Feminizing adrenocortical tumors in the pediatric age group**

| First author (reference number) | Age (years) | Gender | Clinical findings at presentation | Hormonal evaluation (Increased hormone levels) | Treatment | Clinical course of the tumor | Survival/Follow-up |
|---|---|---|---|---|---|---|---|
| Wilkins (14) | 4.7 | Boy | Bilateral gynecomastia | Estrogens, 17 ketosteroids | Surgery | Benign | No recurrence in 4 year of follow-up, alive after 14 years |
| Fontaine et al (30) | 5 | Boy | Bilateral gynecomastia | Estrogens, 17 ketosteroids | Surgery | Malignant with vascular spread | No recurrence 7 years later, alive after 10 years |
| Snaith (25) | 5.5 | Girl | Breast development, pubic hair, vaginal bleeding | Urinary estrone, estradiol, estriol, 17 ketosteroids | Surgery | Anaplastic changes (malignant?) | Alive and well after 8 years |
| Mosier and Goodwin (31) | 7 | Boy | Gynecomastia, accelerated linear growth | Estrogens, 17 ketosteroids | Surgery | Benign | 2 year follow-up, no recurrence, alive after 4 years |
| Peluffo (17) | 6.7 | Girl | Breast development, axillary and pubic hair, vaginal bleeding, painful abdominal mass | N/A | Surgery | Malignant | N/A |
| Ferrante et al (65) | 2.2 | Girl | Breast enlargement, pubic hair, hypertrophy of labia minora, advanced bone age | N/A | Surgery | Malignant | N/A |
| Wilkins (66) | 3 | Girl | Vaginal bleeding, breast development, accelerated bone age, pubic hair | Estrogens, 17 ketosteroids in 24 hour urine | Surgery | Benign | 3 years no recurrence |
| Bacon and Lowrey (18) | 6 | Boy | Gynecomastia; acne, pubic hair<br>Palpable tumor | Estrogens, 17-ketosteroids | Surgery | Originally benign, at follow up malignant (adenoma with neoplastic thrombus) | He was alive, well and free of metastases for 7.5 years, then died of disseminated metastasis 8 yrs after operation; 9 yrs. after disease onset. |
| Halmi and Lascari (20) | 4 | Girl | Signs of virilization, hypercortisolism, hyperestrogenism | Urinary 17-ketosteroids, 17-hydroxycorticoids, Urinary DHEA | Surgery op'DDD | Malignant | Recurrence of tumor and metastases to lungs and liver<br>Died 3 years after the onset of disease |

**Table 2. Continued**

| First author (reference number) | Age (years) | Gender | Clinical findings at presentation | Hormonal evaluation (Increased hormone levels) | Treatment | Clinical course of the tumor | Survival/Follow-up |
|---|---|---|---|---|---|---|---|
| Castleman et al (27) | 6.8 | Boy | Gynecomastia, pubic and axillary hair deveopment, grow rapidly | Elevated urinary 17-ketosteroid | Surgery | Malignant with pulmonary metastases | Died on operating table |
| Leditschke and Arden (19) | 5 | Boy | Bilateral gynaecomastia (no pain, no discharge from the nipples), advanced bone age, increased appetite, pubic and axillary hair development, palpable tumor | Elevated urinary 17-ketosteroid and 17-hydroxyketosteroid, and urinary estrogens | Surgery | Benign (From the pathological evidence it was unable to predict how the tumor will behave) | No recurrence 1 year post excision |
| Bhettay and Bonnici (10) | 6 | Boy | Bilateral gynecomastia | Urinary estrogens | Surgery | Benign | 3 years later no signs of recurrence |
| Howard et al (32) | N/A | Boy | Bilateral gynecomastia | Elevated estrogen levels but normal levels of 17-ketosteroids | Surgery | Benign | N/A |
| Visconti et al (16) | 3.7 | Girl | Left abdominal mass, weight loss, irritability, high blood pressure | 24 hour urinary 17-hydroxy- and 21-oxosteroids | Surgery, Chemotherapy | Malignant | No recurrences in 39 months |
| Sultan et al (33) | 15 | Boy | Bilateral gynecomastia,prepubertal sized testes, pubic hair was present | Estradiol (urine and plasma) urinary 17-hydroxysteroids slightly increased | Surgery | Benign | 2 year follow-up no recurrence |
| Wohltmann et al (24) | 1.8 | Girl | Breast development, clitoral enlargement, and an estrogenized vagina, pubic hair | Estradiol, DHEA-$SO_4$ | Surgery | Capsular invasion (malignant?) | Patient is healthy over 10 years later |
| Drop et al (34) | 6.5 | Girl | Breast development, pubic and axillary hair | Estradiol, testosterone, 11 deoxycortisol, 17-OH-progesterone, DHEA, DHEA-$SO_4$, androstenedione | Surgery, Radiotherapy | Malignant | No recurrence after 1 year |
| Itami et al (35) | 6 | Boy | Bilateral gynecomastia, axillary and pubic hair, penile size enlargement | Estrone, estradiol, testosterone, androstenedione, 11-deoxycortisol | Surgery | Benign | N/A |
| Comite et al (23) | 2.8 | Girl | Breast and pubic hair development | Estradiol, estrone, DHEA, DHEA-$SO_4$, androsteneione, testosterone | Surgery | Potentially malignant (Extension of the tumor into the capsule) | No recurrence 1 year after surgery |
| Telander et al (36) | N/A | Boy | Bilateral gynecomastia | Estradiol | Surgery | Benign | N/A |
| Desai and Kapadia (42) | 8 | Boy | Bilateral gynecomastia | Estradiol | Surgery | Benign | N/A |
| McKenna et al (15) | 6 | Girl | Virilization | Estradiol, DHEA-$SO_4$, testosterone, androstenedione | Surgery | Malignant | Recurrence and died 16 months after presentation |
| Bass and Sochett (37) | 7 | Boy | Bilateral gynecomastia, and rapid growth over the preceding six months | Estradiol elevated DHEA-S, androstenedione, 11-deoxycortisol mildly increased | Surgery | Benign | No recurrence in 6 months |
| Ghazi et al (21) | 14 | Boy | Gynecomastia, facial edema, striae, hypertension, delirium, features of Cushing syndrome | Estrogen, cortisol, 24 hour urinary 17-hydroxysteroids | Surgery Bilateral mastectomy | Malignant? (tumor > 100 gr and 6 cm in size) | No recurrence after one year |

**Table 2. Continued**

| First author (reference number) | Age (years) | Gender | Clinical findings at presentation | Hormonal evaluation (Increased hormone levels) | Treatment | Clinical course of the tumor | Survival/Follow-up |
|---|---|---|---|---|---|---|---|
| Watanabe et al (38) | 1.5 | Boy | Bilateral gynecomastia, pubic hair, penile size enlargement | Estradiol, testosterone DHEA-S | Surgery, mitotane | Malignant | No recurrence in 6 months |
| Phornphutkul et al (57) | 7.8 | Girl | Isosexual precocious puberty, breast development at 7.75 years old, menarche at 8.75 years old, pubic hair development | Estradiol, estrone, DHEA-S, increased 17-ketosteroids, estrone, estradiol, and estriol in 24 hour urine analysis, No suppression in cortisol levels in dexamethasone suppression tests | Surgery | Benign | No residue or recurrence in the first year following surgery |
| Hsiao et al (29) | 2.2 | Boy | Bilateral gynecomastia, pubic hair development | Estradiol, testosterone | Surgery | Benign | 5 years later no recurrence |
| Bouyahia et al (28) | 5 | Girl | Isosexual precocious puberty (breast development, menarche, pubic hair deveopment | Estradiol, testosterone | Surgery | Benign | After a follow-up of 6 years, patient has not had any relapse or metastasis |
| Bawri et al (22) | 18 | Boy | Bilateral gynecomastia, striae over axilla, and breast, thigh, and facial puffiness for 4 years, Cushingoid appearance Dull, vague abdominal pain localized to the right upper quadrant | Cortisol, DHEA-$SO_4$ | Surgery | Malignant | The patient is alive with no metastases 1 year after surgery |
| Sindgikar et al (39) | 6 | Boy | Bilateral gynecomastia | Estradiol | Surgery, chemotherapy (cisplatin, etoposide) | Malignant with micrometastasis | N/A |
| Angotti et al (26) | 7 | Boy | Bilateral gynecomastia, increased somatic growth | Estradiol | Surgery | Low risk of malignancy | N/A (1 month following surgery patient was well) |
| Soliman et al (67) | 6 | Boy | Bilateral gynecomastia and breast tenderness | Estradiol | Surgery | Benign | N/A |
| Guidoni et al (68) | 7.5 | Boy | Bilateral gynecomastia | Estradiol | Surgery | Benign | No recurrence after 1 year |
| Takeuchi et al (40) | 4.7 | Boy | Bilateral gynecomastia, growth spurt, no sign of virilization | Low LH/FSH levels and elevated estradiol/ testosterone levels, Elevated estradiol, testosterone, DHEA-$SO_4$, androstenedione high percentage of urinary estrogen metabolites | Surgery | Malignant | No relapse after 2 years An investigation for *TP53* gene aberrations revealed the presence of a germline point mutation in exon 4 [c.215C > G (p.Pro72Arg)] |
| Patients presented in this report | 13.5<br>7 | Boy<br>Girl | Bilateral gynecomastia, cushingoid appearance, hypertension<br>Breast development, vaginal bleeding | Estradiol, androgens, cortisol<br>Estradiol, DHEA-$SO_4$ | Surgery<br>Surgery | Malignant (metastatic)<br>Benign | Alive with no recurrence for 3.5 years<br>Alive with no recurrence for 13 years |

N/A: not available, FSH: follicle-stimulating hormone, LH: luteinizing hormone, DHEA: dehydroepiandrosterone, yrs: years

boys and 12 of them were girls, giving a male to female ratio of 1.8:1 (22/12). The age at diagnosis of the cases ranged between 1.5 and 18 years with a median age of six years old. The age of diagnosis of two cases was not specified. While the age of diagnosis of 21.9% (7/32) of the remaining 32 cases was <4 years, 65.6% (21/32) of them were 4-8 years old, and 12.5% (4/32) were ≥8 years of age at diagnosis. All 22 boys had bilateral gynecomastia at the time of diagnosis. Penile enlargement with pre-pubertal volume testes were also reported in boys. In girls, breast development was present in all except two cases, while vaginal bleeding was reported in five cases. In the two girls without breast development, one exhibited virilization (15), and the other case was admitted at the age of 3.7 due to weight loss, irritability, and high blood pressure while a left abdominal mass was detectable by palpation on physical examination (16). In addition, abdominal mass was palpated during examination of two boys of five and six years old and a girl of 6.7 years old (17,18,19). Besides typical findings of virilization (pubic and axillary hair), acne, accelerated linear growth and advanced bone age were also observed in both genders. Features of Cushing syndrome, including facial edema, striae over the body, hypertension, and delirium were reported in two girls aged 3.7 and four years and two boys aged 14 and 18 years (16,20,21,22). Isolated estrogen secretion was reported in eight boys (23.5%, 8/34) aged between 6-8 years. All other cases had mixed hormone secretion. While androgen and estrogen secretions were seen together most frequently (61.8%, 21/34), in five cases these secretions were accompanied by excess cortisol (14.7%, 5/34).

Masses were surgically removed in all cases. Of the 34 cases, 16 (47.1%) were benign, and 12 (35.3%) were malignant, based on histological reports. In five cases (14.7%), a distinction between benign/malignant could not be made histologically (21,23,24,25,26). In addition there was a tumor that was initially considered benign by the histopathologist but later metastasized and so was subsequently considered to have been malignant (18). Including these six tumor cases, the total number of malignant tumors becomes eighteen of 34 and the benign to malign ratio in the literature was 47% to 53%.

It was stated that mitotane was used in two cases with malignant pathology, while two further cases were given chemotherapy, and the final two received radiotherapy. No other treatment methods were mentioned in the other cases. All sixteen of the patients with histologically benign tumors were alive without recurrence of the tumor between one to fourteen years after surgical excision of the tumors. Fourteen out of eighteen patients with malignant tumors were alive without recurrence of the tumor for varying periods of six months to ten years after removal of the tumors. One of the malignant cases died within sixteen months after disease onset, the other one in the third year, and another died during the resection operation (15,20,27). A case who was considered to be benign on histological examination was followed up with free of metastasis for 7.5 years, but died 8 years after the operation due to disseminated metastasis (18).

## Discussion

FATs are exceedingly rare, accounting for 0.37% of 801 adrenalectomies performed between 1970 and 2003 (7). They are usually seen in adult males, and are extremely rarely reported in children. Due to this rarity, FATs are usually reported as single case reports (10,16,19,20,28-40). The larger published series usually include only two or three cases (7,8,15,41,42). There are two exceptions; reviews performed by Gabrilove et al (43) in 1965, which included 52 cases reported before 1965 and another by Chentli et al (44) in 2015 which contains 50 cases reported between 1979 and 2014. These two large reviews mainly involve adult males. To the best of our knowledge, there is no previous study that has summarized pediatric cases of FAT. In this study, we reported two cases of FAT in childhood period and managed at our center, one benign and the other malignant, in two genders with different clinical presentations. We also performed a systematic literature search (Table 2) and reviewed 34 pediatric patients with FAT. Thus, this report focusses on the clinical and hormonal characteristics, as well as treatment options and the follow-up of this rare tumor in the pediatric age group.

FATs were more frequent in boys, accounting for nearly two thirds of all pediatric cases. They are more common in younger children and 85% of cases were under eight years old with most between the ages of 4 and 8. The diagnosis of FATs are based on clinical findings and hormonal evaluation, and estrogen secretion is vital. FATs present with different clinical findings in boys and girls. The two new cases reported in this study highlight the divergence of the presentation of FAT in different genders in children. While boys present with contrasexual pseudopuberty, girls present with isosexual pseudopuberty. All of the boys in the literature, including our first case, had bilateral gynecomastia, which was also painful. Most cases of gynecomastia, including physiological gynecomastia seen during puberty, are caused by an imbalance between estrogen and androgens (45,46). Physiological pubertal gynecomastia is most commonly seen in mid-puberty with Tanner stage 3-4 pubic hair and bilateral testicular volumes

of 5-10 mL. Most adolescents during this period have normal estrogen levels, but a few studies have shown high levels in some cases (47,48,49). Pathological gynecomastia may rarely be seen in adolescents and prepubertal boys. It is related to conditions where there is absolute or relative estrogen excess. Pathological gynecomastia may be seen in cases of exogenous intake of estrogen-containing drugs, the presence of endogenous estrogen-producing tumors, increased peripheral conversion of androgens to estrogens secondary to increased aromatase activity, androgen deficiency or androgen insensitivity (50). Gynecomastia presenting at pre-pubertal ages or with a progressive increase in size at pubertal ages in boys should be raise suspicion of a hormone secreting tumor, especially FAT. In contrast to physiological pubertal gynecomastia in the adolescent period, androgen levels inconsistent with the pubertal stage should be carefully investigated, and tumors need to be excluded. In girls, the main signs associated with FATs were early breast development and/or menstruation. Other signs of precocious puberty, such as increased growth velocity and advanced bone age were observed in both genders. Children rarely presented with the neurological complications of Cushing syndrome, such as delirium and aggressive behavior and signs of high blood pressure (21,51). Although it was very rare in childhood, in advanced stages, some patients also presented with abdominal masses or metastases (16,18,19).

From a hormonal perspective, FATs secrete estrogen alone or in combination with other adrenocortical hormones (mixed secretions). In adults, estrogen secretion is usually isolated but sometimes overt or subclinical hypercortisolism may also be found while other hormone secretions, such as androgen precursors, aldosterone or inhibin, were rarely reported (52,53). Among the previously reported pediatric patients, mixed secretion was often observed and hyperandrogenism due to secretion of adrenal androgen precursors, including 17-hydroxyprogesterone, deoxycorticosterone, 11-deoxycortisol, androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate were more common than overt or subclinical hypercortisolism. While isolated estrogen secretion was reported in a quarter of the cases, 60% had high levels of both estrogen and androgen, and the remaining 15% had cortisol hypersecretion in addition to hyperestrogenism and hyperandrogenism. The two new cases described in this study also had mixed hormone secretion. The male case was found to cosecrete estrogen, cortisol, and androgens whereas in the second case both estrogen and androgen hypersecretion was observed. Contrary to pediatric cases, adult patients with FAT generally have low T levels, attributable to several mechanisms. The first is the hypothalamic inhibition of FSH and LH secretion by estrogen; the second is the inhibition of Leydig cells by high estrogen levels; and the third is an increase in sex hormone binding globulin (SHBG) levels, secondary to excess estrogen (7,52). As SHBG has a high affinity for T, free T levels would fall, leading to findings of hypogonadism (54).

The mechanisms by which FATs cause hyperestrogenism are excessive direct estrogen production by the tumor and enhanced conversion of peripheral androgens to estrogen in adipose tissue (55). Studies have also shown that FATs produces aromatase mRNA, and increased aromatase activity has been reported in patients with FATs and demonstrated *in vitro* (7,56,57). Hypogonadotropic hypogonadism due to inhibition of the hypothalamus-pituitary-gonadal axis and thereby suppressed LH and FSH secretion usually accompany hyperestrogenism (7,56,57). This is important because the gonadotropin level is the only way to differentiate between peripheral, and central precocious puberty as well as normal puberty in children presenting with pubertal findings. In addition, estrogen secretion seen in patients with FATs may remain dependent on pituitary adrenocorticotropic hormone activity and estrogen release from the tumor can be reduced by dexamethasone therapy (54).

The main treatment for FATs, as with other ACTs, is surgical removal of the tumor. It is recommended that at least 90% of the tumor be excised, even in the presence of metastasis (54), as there is a high risk of relapse if not removed. In the literature review, surgical removal of the tumor was attempted in all cases. If complete excision of the tumor is not possible, it is necessary to destroy the remaining tumor tissue and reduce the excess hormones secreted from the tumor. The medical agent frequently used for this purpose is mitotane (ortho paraprime dichloro diphenyl dichloroethane), given its inhibition of hormonal secretion and its cytolytic and adrenolytic properties. Mitotane also induces the formation of free radicals, which in turn inhibit 11-β-hydroxylase and block steroidogenesis (58,59). The male case presented here received mitotane and hydrocortisone 10 mg/m$^2$/d cover to counter the adrenolytic effect of the mitotane. He was monitored for mineralocorticoid deficiency, although no such findings developed. Mitotane also strongly inhibits 5-α-reductase enzyme activity. In our case this produced an exaggerated elevation of T and its precursors and even estrogen, due to aromatization of excessive androgens. This presented a clinical challenge in the assessment of relapse. Since, the abdominal and thoracal imaging did not reveal any sign of relapse, high levels of T, estradiol and androstenedione were attributed to the inhibition of 5-α-reductase enzyme activity.

This was confirmed by the elevated T/dihydrotestosterone ratio. Subsequently to cessation of mitotane therapy, T and other elevated hormones gradually declined and reached normal values. This phenomenon should be considered in case of unexpectedly elevated androgen with no sign of relapse in conjunction with a history of mitotane treatment. This inhibition may be persistent since mitotane is lipophilic and is therefore metabolized slowly.

Most of the reported cases of FAT in adult patients are malignant, whereas in children benign FATs represented around half of the reported cases. Unfortunately, there are no accepted clinical, radiological or even histological criteria to differentiate benign from malignant tumors, other than its behaviour in terms of recurrence and metastasis. Thus the differentiation between adenoma and carcinoma is usually difficult (8,60). Although the presence of certain histological predictive criteria involving mitotic activity, atypical mitosis, high nuclear grade, low percentage of clear cells, necrosis, diffuse tumor architecture, capsular invasion, sinusoidal invasion, and vascular embolism leans more to the diagnosis of carcinoma, the assessment of malignant potential still depends on clinical behavior of the tumor in children (8). In adult tumors histopathological markers, such as positive immunostaining for aromatase, can help assessment of malignant potential (11). Pediatric cases have been reported in whom a differentiation between benign and malignant could not be made and in one case the tumor was initially considered to be benign, based on histopathological examination, but the tumor turned out to be malignant when metastasis was later detected (18,23,24,25). Therefore, it has been suggested that even in the case of benign histopathological findings, clinicians should have a high degree of suspicion for recurrence/metastasis when there is a tumor secreting estrogen (9). In the first case from our center, the patient already had metastasis at diagnosis and in this case the FAT was malignant which required medical treatment with mitotane as well as chemotherapy. In the second case presented, there was no metastasis or recurrence of the tumor at follow-up, total resection of the primary tumor leading to cure and this tumor had a benign course.

Given the unpredictable nature of these tumors clinical, hormonal, and radiological follow-up after surgical treatment is necessary. As FATs secrete estrogen, postoperative estrogen levels may be used to confirm whether a complete resection of the tumor has been achieved. In the presence of other hormone secretions, the postoperative monitoring of their levels may provide information on any residual tumor. In both presented patients, as well as in the previously reported cases, postoperative hormone levels declined. However, re-elevation following mitotane therapy, perhaps due to inhibition of 5-α-reductase activity, was observed in the first case. It is recommended to check the hormone profile every three months to screen for recurrence. Imaging with chest and abdomen CT every three months for the first two years, and then every six months for three years, is also recommended in malignant tumors. Annual imaging is recommended after the fifth year (61,62).

In the earlier reports, all but one of the cases classified histologically as a benign tumor were alive without recurrence of the tumor. Although complete surgical resection of benign FATs is thought to be curative, long-term follow-up is required because of the unpredictability of these lesions. Approximately 75-80 % of the malignant cases were alive for a period of six months to 10 years after removal of tumor. Five-year survival for these tumors was not calculable since the follow-up periods of all tumors were uncertain. FAT has a better prognosis in children compared to adults, since most diagnoses are made in the early period before puberty. As with other adrenal tumors, FATs in the childhood period tend to occur in pre-pubertal ages, so excess secretion of adrenal hormones is easily noticed before puberty (4,63). Early diagnosis of FAT is important, as tumor size is associated with prognosis. Although there is not much data in the literature on long-term survival rates of pediatric FATs, they are thought to behave like other adrenocortical carcinomas. In general, the survival rate with adrenocortical carcinomas varies according to tumor stage, and the prognosis changes dramatically in the case of metastatic disease. Five-year survival rate was 90 % in patients with small, totally resectable tumors, but this fell to 10 % in patients with distant metastases (64). Mixed hormone secretion, very high estradiol levels at baseline and large tumor size are all reported to be poor prognostic factors in FATs (7). In the first presented case there were mixed hormone secretion, elevated basal estradiol level, large tumor size, pulmonary metastasis and thrombus in IVC which were all criteria for poor prognosis.

### Study Limitations

One of the limitations of the study was that it was conducted retrospectively. The number of patients with FATs were also limited due to rarity of this tumor.

## Conclusion

FATs are extremely rare tumors that are most commonly seen in men and boys presenting with gynecomastia. FATs are more common in children ≤8 years of age, with a median age at diagnosis of six years. While boys present with contrasexual pseudopuberty signs, girls present with

isosexual pseudopuberty. A high estrogen level strongly supports the diagnosis, while elevations in other adrenal hormones may also been seen. FATs are usually malignant in adults whereas in children approximately half of the FATs are benign. The assessment of malignant potential depends on clinical behavior of the tumor in children. Although complete surgical resection of benign FATs is thought to be curative, the long-term follow-up is required because of the unpredictability of these tumors. For treatment of malignant FATs, mitotane or aromatase inhibitors, with or without standard or targeting chemotherapy, in addition to surgical removal, is recommended. The prognosis depends on the stage of the tumor, although it is generally very poor in adult males. FATs occurring in childhood period may carry a better prognosis than in adult males with most pediatric FATs being followed without recurrence since the diagnosis is made early, as typical presenting signs are more obvious before puberty.

### Ethics

**Ethics Committee Approval:** The study was approved by Hacettepe University Ethics Committee (approval number: GO 20/401).

**Informed Consent:** The requirement for informed consent was waived due to the retrospective nature of the study.

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

### Authorship Contributions

Surgical and Medical Practices: Doğuş Vurallı, Nazlı Gönç, Alev Özön, Saniye Ekinci, H. Serkan Doğan, Serdar Tekgül, Ayfer Alikaşifoğlu, Concept: Doğuş Vurallı, Nazlı Gönç, Ayfer Alikaşifoğlu, Design: Doğuş Vurallı, Nazlı Gönç, Ayfer Alikaşifoğlu, Data Collection or Processing: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nazlı Gönç, Alev Özön, Analysis or Interpretation: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nazlı Gönç, Alev Özön, Literature Search: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nazlı Gönç, Alev Ozon, Writing: Doğuş Vurallı, Ayfer Alikaşifoğlu, Alev Özön, Nazlı Gönç.

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# Diagnostic Value of Bilateral Petrosal Sinus Sampling in Children with Cushing Disease: A Multi-center Study

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### What is already known on this topic?

Although the sensitivity and specificity of bilateral inferior petrosal sinus sampling (BIPSS) were shown to be high in adult patients, studies in children are limited in number and have conflicting results since it is much less common in this population.

### What this study adds?

Our study supports that BIPSS is a superior diagnostic work-up than MRI to confirm the diagnosis of Cushing disease. Moreover, BIPSS was shown to provide better information about adenoma localization.

## Abstract

**Objective:** Although the sensitivity and specificity of bilateral inferior petrosal sinus sampling (BIPSS) were shown to be quite high in adult patients, pediatric studies are limited in number and have conflicting results, since BIPSS is much less commonly performed in children. The aim of this study was to assess the role of BIPSS in the detection and accuracy of lateralization of pituitary adenomas in pediatric patients with Cushing disease (CD) and its possible advantage over other diagnostic methods.

**Methods:** This was a multicenter, nationwide, web-based study. The diagnostic value of BIPSS in 16 patients, aged between four and 16.5 years with a confirmed diagnosis of CD, was evaluated retrospectively. The sensitivity and specificity of BIPSS and magnetic resonance imaging (MRI) were calculated, and compared statistically.

**Results:** Standard tests, except for morning cortisol level, were effective in proving the presence of Cushing syndrome. While MRI findings were consistent with microadenoma in eight cases (50%), CD presence and lateralization was successfully predicted in 14 of 16 patients using BIPSS. BIPSS compared with MRI examination was significantly more accurate, both in pre-stimulation and post-stimulation results ($p = 0.047$ and $p = 0.041$, respectively). BIPSS showed a significantly higher sensitivity (92.8%) than MRI in detecting the pituitary source of adrenocorticotropic hormone secretion.

**Conclusion:** These results suggest that BIPSS is superior to MRI for diagnostic work-up to confirm the diagnosis of CD. Moreover, in line with previous studies, BIPSS was shown to provide better information about adenoma location, which is vital for possible surgical intervention.

**Keywords:** Cushing's disease, pituitary adenoma, petrosal sinus sampling, sensitivity, lateralization

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## Introduction

Cushing syndrome (CS) arises from chronic exposure to excess amount of exogenous or endogenous glucocorticoids. Exogenous administration of steroids is the most common cause of CS in children. The incidence of endogenous CS is 0.7-2.4/1,000,000 people/year, and approximately 10% of cases are children (1). Endogenous causes of CS are rare and can be either adrenocorticotropic hormone (ACTH)-dependent (75-90%) or ACTH-independent (15-20%). ACTH-dependent CS results from overproduction of ACTH from the pituitary by ectopic secretion of ACTH or corticotropin-releasing hormone (CRH) (2).

Once CS is suspected based upon clinical manifestations, diagnostic evaluation requires the administration of several tests (3). Loss of the cortisol circadian rhythm is the earliest biochemical marker of endogenous hypercortisolism (4). The sensitivity of 24-hour urinary cortisol measurement in children was found to be 88%, and serial measurements were recommended in pediatric practice (5). A spot morning plasma ACTH level may be an alternative, which has a sensitivity of 70%, with a cut-off value above 29 pg/mL in identifying children with ACTH dependent syndrome (6,7). Moreover, some patients with pituitary disease may present with ACTH levels in a low-to-normal range, and conversely, some patients with adrenal forms can present with ACTH levels that are not fully suppressed (8). A low-dose dexamethasone suppression test can be used to evaluate the lack of negative feedback of cortisol on the hypothalamic-pituitary-adrenal axis. A late-night serum cortisol value above 1.8 µg/dL is considered to be abnormal, with a sensitivity higher than 95% and a specificity of 80% (9). The standard high-dose dexamethasone suppression test is used to differentiate Cushing disease (CD) from ectopic ACTH secretion and adrenal causes of CS. Batista et al (7) reported that in a pediatric population, cortisol suppression of 20% from baseline had a sensitivity and specificity of 97.5% and 100%, respectively. Several tests have been used in the differential diagnosis of CS, but none of them can precisely differentiate the source of ACTH. Bilateral inferior petrosal sinus (IPS) sampling (BIPSS) is the gold standard for determining the source of ACTH to reveal whether the source is pituitary or ectopic. Although the sensitivity and specificity of BIPSS were shown to be quite high in adult patients (10,11), studies in children are limited in number and have conflicting results, since BIPSS is much less commonly performed in this population (11,12,13,14,15,16).

Many studies have shown that CRH increases the sensitivity of BIPSS by stimulating the secretion of ACTH from pituitary adenomas in adults and children (10,11,17,18). Desmopressin is administered in adult patients as a cheaper and feasible alternative to CRH (19).

In addition to the differentiation of CD from ectopic ACTH sources, the ACTH ratio between left and right veins is also useful in determining the location/lateralization of pituitary microadenomas (15,20), and thus guiding the neurosurgeon during surgery. A pituitary magnetic resonance imaging (MRI) should be performed in all patients with a suspicion of ACTH-dependent CS, but this does not identify pituitary adenoma in 36-78% of the cases in series (21,22,23). Sensitivity in determining the lateralization of tumor by BIPSS was reported to be up to 60-90% before and after CRH stimulation in children, despite limited data (24,25,26).

In the hands of an experienced interventional radiologist, BIPSS is a safe procedure with few but significant complications, such as inguinal hematoma or brainstem hemorrhage, or non-hemorrhagic brainstem infarctions or thromboembolic events (27).

Our study aimed to assess the role of BIPSS in the detection and accuracy of lateralization of pituitary adenomas and compare this to other diagnostic methods.

## Methods

This retrospective study was conducted as a multicenter, nationwide, web-based exercise, and an electronic recording form (ERF) to collect the demographic data and clinical and laboratory findings of the patients with CD was used. All centers providing data to this study were university hospitals. Users reached the ERF from CEDD.net Web Registration System website (https://cedd.saglik-network.org/).

Data of 32 patients with ACTH-dependent CS were recorded (Figure 1). Of these 32 patients, 16 had BIPSS performed. Fourteen were confirmed to have CD histopathologically, and the remaining two cases responded to medical therapy with regression of clinical and laboratory findings.

In the overnight dexamethasone suppression test procedure, dexamethasone (1 mg, orally) was administered at 11:00 p.m., and blood samples for ACTH and cortisol measurement were obtained in the following morning. For the low-dose dexamethasone suppression test, 0.5 mg of dexamethasone was given at six-hour intervals for two days, with the cortisol level measured six hours after the final dose was given. For the high-dose dexamethasone suppression test, 2 mg of dexamethasone was given at six-hour intervals for two days, with the cortisol level measured six hours after the final dose was given. Gadolinium-enhanced MRI of the pituitary gland was performed on a 1.5 Tesla (15 patients) or a 3 Tesla (1 patient) MRI system

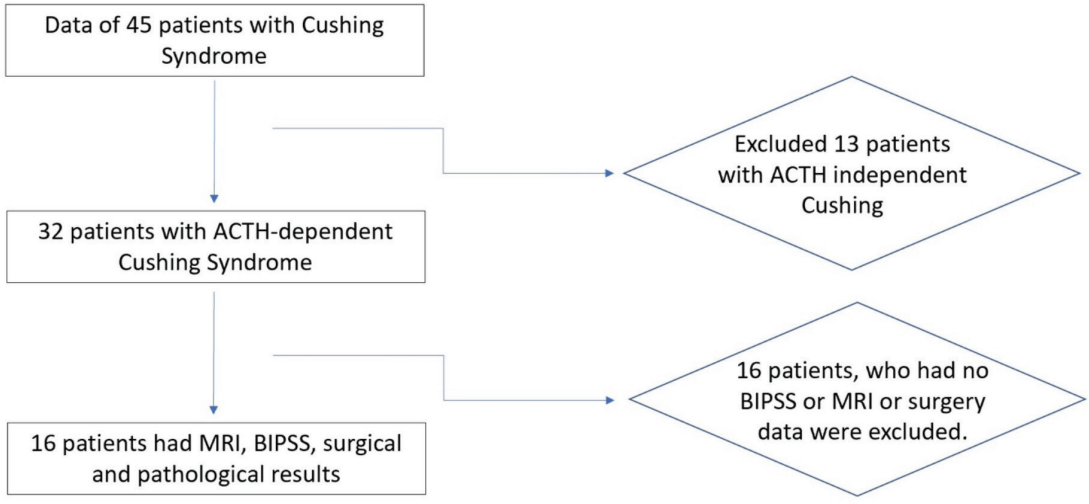


**Figure 1.** Flow chart

*MRI: magnetic resonance imaging, BIPSS: bilateral inferior petrosal sinus sampling, ACTH: adrenocorticotropic hormone*

in imaging studies. A microadenoma was described as a pituitary tumor of less than 1 cm in diameter and a macroadenoma was described as a tumor above 1 cm in diameter. Patients were sent for BIPSS, especially when pituitary MRI was negative or suspicious for adenoma, or when clinical and imaging results were inconsistent. The BIPSS procedures were performed by radiologists based on the technique described by Doppman et al (28). Blood samples were collected from the peripheral veins and left and right IPS. CRH stimulation test was conducted with 100 µg of CRH intravenously after catheterization. Sampling lateralization was used to determine which side of the pituitary gland involved a tumor responsible for the overproduction of ACTH.

IPS to peripheric vein ACTH ratio of >2 before CRH stimulation and IPS to peripheric vein ACTH ratio >3 post-stimulation were accepted as diagnostic for CD (29). The lateralization of the adenoma was considered in patients with the intergradient difference of the right and left petrosal sinuses of more than 1.4 (29).

All patients underwent transsphenoidal surgery. The lateralization of the tumor was also recorded during surgery. Suspicious tumor tissue was resected for histological assessment, including immunohistological staining for ACTH. The diagnosis of CD was confirmed with positive immunohistological staining for ACTH in histopathological studies in 14 patients.

The exclusion criteria of the study were: cases with findings of CS but not with a definitive diagnosis of CS; or cases with insufficient or missing data.

The study protocol was approved by the Ethical Committee of İzmir Tepecik Training and Research Hospital (decision no: 10, date: 04.02.2015). Informed consent was obtained from all patients and/or parents at admission to hospital.

### Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences v.21 for Windows (IBM Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation for parametric data and median (minimum-maximum) for non-parametric data. The sensitivity and specificity of tests were calculated according to standard statistical formulae. Descriptive statistics were used to analyze the data. Mann-Whitney U or Kruskal-Wallis tests were conducted to compare the parameters.

## Results

MRI findings, laboratory tests, histopathological evaluation, and treatment results of 16 patients who underwent BIPSS with a pre-diagnosis of Cushing's disease at eight centers were assessed. There were eight boys and eight girls with a mean age of 12.1 ± 3.76 years, ranging from 4.23 to 16.5 years.

Baseline early morning cortisol levels of the cohort ranged between 12.3 and 59.8 mg/dL and seven were in the normal range. All patients had high late-night cortisol levels (>7.5 mg/dL). Twenty-four-hour urinary free cortisol excretion of 14/16 was increased. All 16 patients had ACTH levels above 20 pg/dL. Baseline characteristics of these 16 children and adolescents with CD are shown in Table 1. In 13 patients,

**Table 1. Baseline characteristics of 16 children and adolescents with Cushing disease**

| No | Gender | Age at the time of diagnosis (years) | Cortisol early morning mcg/dL | Cortisol midnight mcg/dL | ACTH pg/mL | 24 hours UFC µg/24 h |
|---|---|---|---|---|---|---|
| **1** | F | 14.9 | 29.8 | 18.9 | 66.7 | 2331 |
| **2** | M | 13.4 | 16.3 | 19.7 | 91.5 | 264 |
| **3** | F | 16.2 | 20 | 35 | 73 | 260 |
| **4** | F | 8.1 | 31 | 26 | 46.7 | 160 |
| **5** | F | 10.2 | 19 | 15.2 | 34.9 | 77.9 |
| **6** | M | 8.9 | 23.6 | 10.4 | 39.2 | 431 |
| **7** | M | 16.5 | 20.3 | 10.9 | 49.9 | 130 |
| **8** | M | 8.82 | 13.4 | 16.2 | 40.3 | 136.8 |
| **9** | F | 15.2 | 18 | | 48 | 164.8 |
| **10** | M | 14.2 | 14 | | 49 | 285 |
| **11** | M | 11.1 | 41.8 | 27.9 | 63.6 | 1713 |
| **12** | M | 15.8 | 40.8 | 21.6 | 150 | 237 |
| **13** | M | 4.23 | 59.8 | 19 | 53.6 | > 1000 |
| **14** | M | 7.54 | 23.14 | 23 | 33.8 | 450 |
| **15** | F | 15.7 | 23.12 | 19.34 | 49.9 | Ø |
| **16** | F | 13.4 | 20.6 | 18.4 | 33 | Ø |
| **Reference range** | | | 3-21 mcg/dL | < 1.8 mcg/dL | 10-50 pg/mL | 30-90 µg/24 h |

F: female, M: male, ACTH: adrenocorticotropic hormone, UFC: urinary free cortisol

1 mg dexamethasone suppression test was performed overnight; none of them were suppressed. A low dose dexamethasone suppression test was performed in 10 of 16 patients and none of them were suppressed. A high-dose dexamethasone suppression test was performed in 13 of 16 patients, and suppression of serum cortisol level over 50% was achieved in all 13 patients. On MRI, findings compatible with microadenoma were detected in 8 (50%), while no finding supporting CD was detected in the remaining eight cases (Table 2).

Severe adverse effects were not observed in any of 16 patients who underwent BIPPS protocol during or after the procedure. The percentage of predicting CD was 81% (13/16) pre-stimulation and 87.5% (14/16) after stimulation with CRH (Table 2). There was no statistically significant difference between before and after stimulation ($p = 0.106$). When compared accuracy of BIPSS to MRI examination, a statistical significance was obtained both in pre-stimulation and post-stimulation results ($p = 0.047$ and $p = 0.041$, respectively). BIPSS showed a significantly greater sensitivity 92.8% than MRI (sensitivity, 53.3%; specificity, 100%) in detecting the pituitary source of ACTH secretion. Overall, lateralization of ACTH levels by a baseline inter-petrosal sinus gradient (IPSG) ≥1.4 was compatible with the surgical location of the pituitary corticotropinoma in nine of the cases in which a tumor was located by surgery. After CRH stimulation, an IPSG of ≥1.4 predicted the site of the pituitary lesion in 14 of the cases.

All patients underwent surgical intervention (Table 2). After the operation, empty sella was detected in one of the two patients whose clinical findings did not regress. Thus, curettage was performed, but clinical findings regressed only after gamma-knife application. Clinical findings of the other patient with failed surgery regressed with cabergoline treatment. Except for these two patients, the diagnosis of CD was confirmed by histopathological studies in 14 patients, and clinically with the regression of CD/CS findings in all patients.

## Discussion

Different studies have reported the results of children and adolescents with CD undergoing BIPPS from different countries, and the role of BIPSS in diagnosis and lateralization has been evaluated and a significant difference between MRI and BIPSS in terms of success in detecting CD was found (3,14,16,17,30,31). Herein, we present the first report of a series of children and adolescent with CD who underwent BIPSS from Turkey. The results suggest that BIPSS is a superior diagnostic tool compared to MRI for diagnosing CD and determining lateralization.

Since cortisol is secreted in a circadian rhythm, basal cortisol value is not used in the diagnosis of hypercortisolemia, and morning cortisol level is also not recommended to be useful for the diagnosis of CS because it may cause false-positive results due to the increase in morning cortisol

values (32,33,34). In addition, 80% of cortisol is bound to cortisol-binding globulin, and since assays measure total protein-bound globulin, serum cortisol levels change as serum protein decreases. In our study, the finding that 50% of 16 patients with confirmed CD diagnosis had normal morning cortisol levels support the view that it cannot be used in diagnosis. However, in our study, it was also found that standard tests, except for the morning cortisol level, were useful for proving the presence of CS.

MRI is commonly used to investigate CD and to identify pituitary adenomas non-invasively (21). ACTH-secreting microadenomas are commonly not visible on MRI in patients with CD. This may be in part related to their small sizes, or it could be related to the fact that these lesions have a signal and enhancing characteristics similar to those seen in the normal pituitary gland. In a case series of 200 patients, Lonser et al (3) detected adenoma in 97 patients (50%) on MRI, and lateralization was achieved in 96 patients with MRI. Chen et al (30) reported that lateralization by MRI was found to be consistent with operation in 80% of patients. In our study in 8 (57%) of 14 surgically proven CD cases, findings consistent with microadenoma were detected and lateralization could be achieved in 7 of these 14 cases (50%) on MRI. When BIPSS was compared with MRI examination, statistical significance was obtained for both pre-stimulation and post-stimulation results in terms of CD diagnosis. BIPSS is a highly specialized and invasive technique and is routinely used in adults, to distinguish CD from ectopic ACTH syndrome (EAS), and for lateralization of the pituitary microadenoma. Although BIPSS is a routinely used technique to distinguish CD from EAS, in our study, there were no cases of EAS. To date, a total of 453 children, who underwent BIPSS, have been reported by different studies, but only 12 were found to have EAS (3,14,35).

In our cases, the accuracy of BIPSS for predicting CD was 81% (13/16) pre-stimulation and was 87.5% (14/16) after stimulation with CRH ($p = 0.106$). BIPSS showed a sensitivity of 92.8% and specificity of 100% in detecting the pituitary source. In a series with a large number of adult cases, the sensitivity of CRH stimulation was reported as >90% (36). In 2019, Chen et al (30) reported the sensitivity of BIPSS without stimulation as only 64.7% for the diagnosis of CD in children and adolescents, while the sensitivity with desmopressin stimulation was 83.3%. The difference between this study and our study was the use of desmopressin for stimulation, rather than CRH.

Since EAS is very rare in children, the main use of BIPSS in the pediatric age group is for accurate location of the

**Table 2. MRI, BIPSS and histopathological examination data of 16 patients**

| No | Tumor lateralization by MRI | Tumor size (mm) | Tumor lateralization by BIPSS before CRH stimulation | Tumor lateralization by BIPSS after CRH stimulation | Surgery | Histopathological examination | Medications |
|---|---|---|---|---|---|---|---|
| 1 | L | 7*5 | L | L | Total hypophysectomy | ACTH + Adenoma | |
| 2 | R | 7*7 | Not determined | R | Total hypophysectomy | ACTH + Adenoma | |
| 3 | Not seen[1] | | R | R | Total hypophysectomy | ACTH + Adenoma | |
| 4 | Middle | 3*4 mm | Not determined | Not shown | Adenomectomy | Adenoma ACTH + | |
| 5 | R | 6*4 mm | R | R | Adenomectomy | Adenoma ACTH + | |
| 6 | Not seen | | Not determined | R | Adenomectomy | Normal | |
| 7 | Not seen | | R | R | Hemihypophysectomy | ACTH + Adenoma | |
| 8 | L | 5*5 mm | Not determined | L | Hemihypophysectomy | "Olfactory neuroblastoma" ACTH + | |
| 9 | Not seen | | L | L | Adenomectomy | ACTH + Adenoma | |
| 10 | Middle | 5*5 mm | L | L | Adenomectomy | ACTH + Adenoma | |
| 11 | L | 4*4 mm | L | L | Adenomectomy | ACTH + Adenoma | |
| 12 | Not seen | Empty sella | Not determined | Not determined | Curettage | Empty sella | Gamma knife |
| 13 | Not seen[2] | | Not determined | L | Adenomectomy | ACTH + Adenoma | |
| 14 | Not seen | | Not determined | L | Hemihypophysectomy | ACTH + Adenoma | |
| 15 | Not seen | | R | R | Hemihypophysectomy | ACTH + Adenoma | Cabergoline |
| 16 | R | 6*4 mm | R | R | Hemihypophysectomy | ACTH + Adenoma | |

[1]Not seen with 1.5 Tesla; 6*3 mm contrast enhancing lesion on the right with 3 Tesla.
[2]No adenoma but less contrasted millimetric area on the left.
MRI: magnetic resonance imaging, R: right, L: left, CRH: corticotropin-releasing hormone, BIPSS: bilateral inferior petrosal sinus sampling, ACTH: adrenocorticotropic hormone

pituitary microadenoma. However, few studies have investigated the usefulness of BIPSS in predicting the location of pituitary adenoma in the pediatric population (14,15,17,31). As reported previously in children and adolescents, rates of identifying the laterality of the tumor by BIPSS ranged from 73.7 to 100%, and the consistency of these predictions with the actual tumor lateralization was 58.7-100% (3,13,18,26,30,37). In Lonser's series (3), BIPSS accurately predicted the lateralization of the adenoma in 57 of the 82 patients (70%) in whom an adenoma located off midline was found at surgery. In 2006, Batista et al (17) published the second largest series in the literature and evaluated the results of 43 patients, reporting that BIPSS was a poor predictor of the site of a microadenoma in children. Lateralization estimation (confirmed by surgery) in Magiakou et al's (14) series of 50 patients before and after stimulation with CRH was 67% and 76%, respectively. In a study published by the same center in 2013, the lateralization rate was reported as 88% in the evaluation of 140 pediatric patients (3). In the current study, while the lateralization rate before CRH stimulation was 56.25%, it increased to 87.5% after CRH. These percentages are comparable to those previously reported (3,14,26,30). It was suggested that in the previous studies, the centers being tertiary referral centers may have caused a selection bias, i.e., referral of the cases with no signs on MRI, with a history of an unsuccessful surgery or mildly affected cases to those centers may have underestimated the lateralization rates. The centers our patients were referred to were also reference centers. In addition, different results are most likely to occur due to the different number of cases. Lienhardt et al (13) evaluated seven patients, reporting a lateralization rate of 91%, but commented that this rate would decrease as the number of cases increased. Another factor affecting the results is that when calculating the lateralization rate in the studies, some of them only included tumors with lateralization, while others included midline tumors. Different results may be obtained with smaller age groups and depending on the experience of the surgeon. It was reported that anatomical variations of the IPS were reported to influence the venous drainage of the pituitary or hinder the correct positioning of the catheter, which might lead to misleading results from BIPSS (38,39). Such variations are reported to be common (40). In a quarter of cases, the IPS was plexiform (40), but in most cases, this did not cause diagnostic errors (36).

### Study Limitations

The limitation of our study was that MRI reports sent from different centers, taken with different quality devices, and interpreted by different radiologists, may have affected our false-negative results.

## Conclusion

In our study, we found that standard tests, except for morning cortisol level, were effective in proving the presence of CS. The reliability of the high-dose dexamethasone suppression test was confirmed in our cases, since CD was found in all cases with a positive response to this test. These results support that BIPSS is a superior diagnostic work-up compared with MRI to confirm the diagnosis of CD. Moreover, in line with previous studies, BIPSS was shown to provide better information about adenoma location, which is vital for possible surgical intervention.

### Ethics

**Ethics Committee Approval:** The study were approved by the Tepecik Training and Research Hospital of Local Ethics Committee (decision no: 10, date: 04.05.2015).

**Informed Consent:** Retrospective study.

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

### Authorship Contributions

Concept: Oya Ercan, Bumin Dündar, Gönül Çatlı, Design: Oya Ercan, Bumin Dündar, Gönül Çatlı, Hande Turan, Data Collection or Processing: Hande Turan, Gönül Çatlı, Aslı Derya Kardelen, Ece Böber, Ayşehan Akıncı, Semra Çetinkaya, Özgecan Demirbaş, Eren Er, Saadet Olcay Evliyaoğlu, Bumin Dündar, Oya Ercan, Analysis or Interpretation: Hande Turan, Oya Ercan, Literature Search: Oya Ercan, Bumin Dündar, Gönül Çatlı, Hande Turan, Writing: Hande Turan, Gönül Çatlı, Bumin Dündar, Oya Ercan.

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# First Evaluation of P Dispersion and Tp-e Parameters in Electrocardiograms of Children with Diabetic Ketoacidosis

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

Diabetic ketoacidosis (DKA) is one of the leading causes of morbidity and mortality in children with type 1 diabetes mellitus. Atrial and ventricular arrhythmias are commonly seen during DKA. On electrocardiography (ECG), P-wave dispersion (Pd) has been associated with risk of atrial arrhythmias. QT dispersion (QTd), corrected QTd (QTcd), Tp-e duration, Tp-e/QT and Tp-e/QTc indicate the risk of ventricular arrhythmias.

**What this study adds?**

To the best of our knowledge, this is the first article evaluating Pd, Tp-e, Tp-e/QT and Tp-e/QTc parameters in children with DKA. In this study, cardiac arrhythmia risk markers, including Pd, QTd, QTcd and Tp-e, Tp-e/QT, were found to be increased in children with DKA.

## Abstract

**Objective:** Diabetic ketoacidosis (DKA) is an important complication of type 1 diabetes mellitus. We aimed to evaluate the effect of metabolic disorders of DKA on electrocardiography (ECG) parameters in children.

**Methods:** This study was performed between December 2018 and March 2020 and included 39 children with DKA and 40 healthy children. Three ECGs (one before and two after treatment) were obtained from the patient group. P-wave dispersion (Pd), QT dispersion (QTd), QTc dispersion (QTcd), Tp-e intervals, and the ratios of Tp-e/QT and Tp-e/QTc were measured electrocardiographically. ECG parameters from children with DKA and healthy controls were compared statistically.

**Results:** The mean age of the patient group was $10.50 \pm 4.12$ years. There was no significant difference in terms of age, gender, weight, height and body mass index between patients and controls. In the patient group, a statistically significant increase was found in Pd, QTd and QTcd in the initial ECG compared to the second and third ECGs. Also, when the first and third ECGs were compared, a significant increase in Tp-e and Tp-e/QT was evident in the first ECG. There was a significant difference in the values of Pd, QTd, QTcd, Tp-e and Tp-e/QT in the first ECGs, obtained before DKA treatment, and those values obtained from the control group.

**Conclusion:** This is the first article evaluating Pd and Tp-e parameters in children with DKA. Cardiac arrhythmia risk markers were increased in children with DKA compared to controls. Therefore, clinicians should be aware of the possibility of developing new arrhythmias during DKA treatment.

**Keywords:** Cardiac arrhythmia, children, diabetic ketoacidosis, electrocardiography

## Introduction

Type 1 diabetes mellitus (T1DM) accounts for about 10% of all diabetic cases, and more than 3 million people in the United States and 15 million people worldwide are affected (1). Diabetic ketoacidosis (DKA) is one of the leading causes of morbidity and mortality in children with T1DM (2). In diabetic individuals, morbidity and mortality of cardiovascular diseases were found to be increased when compared to healthy individuals. The incidence of diseases such as myocardial infarction and ischemic stroke has also increased (3).

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Malignant cardiac arrhythmias resulting in sudden cardiac death may be present in individuals who are presumably healthy or with medical problems (4). There are some electrocardiographical (ECG) markers that may be associated with cardiac arrhythmias (4,5). On ECG, P-wave dispersion (Pd) has been associated with a risk of atrial arrhythmias. Increased Pd represents non-homogeneous propagation of sinus impulses and atrial depolarization abnormalities associated with atrial arrhythmia (5). QT dispersion (QTd), corrected QT (QTc), and corrected QTd (QTcd) indicate heterogeneity of ventricular repolarization. An increase in ventricular heterogeneity increases myocardial electrical sensitivity and predisposes to ventricular arrhythmias. Tp-e duration, the interval between the peak and the end of the T wave,is also a useful parameter for predicting cardiac arrhythmias and Tp-e/QT and Tp-e/QTc are considered more beneficial than QTd (4).

In the light of recent evidence, we aimed to evaluate atrial (Pd) and ventricular arrhythmia risk markers (QTd, QTcd, Tp-e, Tp-e/QT and Tp-e/QTc) on ECG during and after DKA in children with T1DM.

## Methods

### Selection of Study Populations

This study was conducted prospectively inchildren diagnosed with DKA between December 2018 and March 2020 in a tertiary child health care center. In the patient group, cases with pre-existing heart disease or dysrhythmia, or syndromes and sequences associated with cardiac components, or chronic disease other than diabetes mellitus (DM) were excluded from the study. Exclusion criteria for the control group were the presence of known heart disease or dysrhythmia, syndromes and sequences associated with cardiac components, and other chronic diseases.

The diagnostic criteria for DKA were hyperglycemia (blood glucose > 200 mg/dL), ketosis (ketone positivity in blood or urine) and metabolic acidosis (pH < 7.3 in venous blood sample or plasma bicarbonate < 15 mEq/L), in accordance with literature (2).

In the patient group, a history of being previously or newly diagnosed with T1DM, age at diagnosis and follow-up duration, age at the occurrence of DKA, gender, weight, height and body mass index (BMI), blood pressure and biochemical values, including blood glucose levels at the time of DKA, hemoglobin A1c (HbA1c) and lipid panel taken in the last three months were examined. The clinical severity of DKA in patients was classified into: mild (pH: 7.2-7.3, bicarbonate: 10-15 mEq/L); moderate (pH: 7.1-7.2, bicarbonate: 5-9 mEq/L); and severe (pH: < 7.1, bicarbonate: < 5 mEq/L) (6). No blood tests were performed in the control group.

### Electrocardiography

ECG examination was performed in all cases. Recordings were obtained with a speed of 25 mm/s and amplitude of 10 mm/mV using SeaMed ECG 1200G (Qinhuangdao, China) 12-channel/12-lead ECG device. All ECGs were scanned at a resolution of 300 DPI and transferred to electronic medium. Images were analyzed with "Adobe Photoshop CS2 version 9.0" program at a resolution of 1500 DPI and accuracy of four milliseconds. A total of three ECG recordings were obtained from the patient group; the first at the time of DKA, the second shortly after recovery from the DKA (3-7 days later), and the third approximately 1-2 weeks later after discharge from hospital. Only one ECG record was performed in each subject in the control group. Standard measurements, such as heart rate (HR), PR interval, P-wave duration, QT interval and QTc interval were performed on all ECGs, and then Pd, QTd, QTcd, Tp-e, and the ratios of Tp-e/QT and Tp-e/QTc were measured electrocardiographically. P-wave duration was evaluated as the duration between initial deflection and its return junction to the isoelectric baseline. QT interval was calculated as the duration between the beginning of the QRS complex and the end of T-wave in isoelectric baseline. QTc was measured using Bazett's formula ($QTc = QT/\sqrt{RR}$). Tp-e interval was evaluated as the duration between the peak and the end of the T wave on isoelectric baseline. If the beginning and end of the T waves were not clearly seen, it was determined according to the tangent method defined by Lepeschkin and Surawicz (7). In this study, Pd, QTd and QTcd were measured from at least nine leads, and the Tp-e interval was evaluated primarily using V5, and if not possible, then preferentially by V4 or V6 derivation. Measurements were made in three consecutive heartbeats and the average was calculated. The U wave, if present, was not included in the Tp-e range. While calculating Tp-e/QT and Tp-e/QTc ratios, QT and QTc were measured from the same derivation where Tp-e interval was measured.

### Echocardiography

Echocardiographic (ECHO) examination of the patient and control groups were also compared during this study. Evaluations were performed by an experienced pediatric cardiologist using a Vivid S5 N (General Electric, Horten, Norway) ECHO device and 3S (2-4 MHz) probe. ECHO studies were performed using standard imaging techniques recommended by the American Society of Echocardiography (8). Left atrial diameter, aortic root, left ventricular end-systolic dimension, left ventricular end-diastolic dimension,

end-diastolic interventricular septal thickness, end-diastolic left ventricular posterior wall thickness, left ventricular ejection fraction (EF) and left ventricular fractional shortening were measured echocardiographically.

All procedures performed in the current study were in accordance with the 1964 Helsinki Declaration and ethical approval for the study was obtained from Necmettin Erbakan University Ethical Committee with a decision no. 2018/1321, dated 03.05.2018.

Informed consent was obtained from all individual participants and/or their legal guardians included in the study.

### Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences statistics, version 22 (IBM Inc., Armonk, NY, USA). Continuous variables are expressed as mean ± standard deviation when normal distribution is observed. Descriptive analysis was used in the analysis of the distribution and frequency of data. Chi-square test was used to evaluate categorical data in independent groups. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine whether the continuous variables fit the normal distribution or not. When homogeneous distribution was observed, an independent sample t-test (Student's t-test) was applied in independent groups and a dependent samples t-test (paired t) and two-way ANOVA tests were applied in dependent groups. In cases where normal distribution was not observed, Mann-Whitney U tests in independent groups, and Wilcoxon and Friedman tests in dependent groups were used. For correlation analysis, Pearson's correlation analysis was performed when the continuous variables were parametrically distributed and, if not, Spearman correlation analysis was used. During correlation examinations, the r-value was rated as: negligible between 0.00-0.29; weak between 0.30-0.49; moderate between 0.50-0.69; strong between 0.70-0.89; and very strong between 0.90-1.0 (9). A $p < 0.05$ was considered statistically significant.

## Results

### Patient Demographic and Clinical Characteristics

Forty children, aged 0-18 years with DKA as patient group and 40 age- and gender-matched healthy children as control group were included. However, one case from the patient group was excluded due to incomplete data. In the patient group 59.0% (n = 23) were female (F) and 41.0% (n = 16) were male (M) in patient group with a female to male (F/M) ratio of 1.44:1 in all patients with DKA (n = 39). While the F/M ratio was 3.25:1 in previously diagnosed T1DM patients, it was 0.83:1 in newly diagnosed patients. Mean ages of the patient and control groups were 10.50 ± 4.12 and 10.47 ± 4.11 years, respectively. There was no statistically significant difference between the patient and control groups in terms of gender, age, weight, height and BMI. General characteristics of the patients and controls are presented in Table 1.

The patient groups consisted of 43.6% (n = 17) previously diagnosed patients and 56.4% (n = 22) newly diagnosed patients. The mean duration of DM in previously diagnosed patients was 5.18 ± 3.32 years (range: 1-12 years). Of the patients, 33.3% (n = 13) presented with mild, 20.5% (n = 8) moderate and 46.2% (n = 18) severe DKA. Between these severity groups, there was no significant difference in terms of gender, history of being previously or newly diagnosed for T1DM and mean duration of diabetes ($p > 0.05$ for all).

There was a statistically significant decrease in both systolic and diastolic blood pressure in the patient group compared to the control group ($p = 0.006$ and $p = 0.04$, respectively) (Table 1). As expected, leukocyte count and blood glucose values obtained at the time of DKA were above and sodium, pH and bicarbonate were below the laboratory reference ranges. Other data were within normal range according to laboratory references. In previously diagnosed cases the mean sodium level was 132.47 ± 4.00 mmol/L, mean potassium level was 4.54 ± 0.96 mmol/L, mean calcium level was 9.37 ± 0.93 mg/dL, mean phosphorus level was 4.21 ± 1.37 mg/dL, and mean magnesium level was 1.95 ± 0.22 mg/dL. Similarly, in newly diagnosed cases

**Table 1. General characteristics of the cases**

| | Patient group (n = 39) | Control group (n = 40) | p |
|---|---|---|---|
| Gender (female/male) | 23/16 | 24/16 | 0.926 |
| Age (year) | 10.50 ± 4.12 | 10.47 ± 4.11 | 0.948 |
| Weight (kg) | 34.86 ± 14.92 | 38.51 ± 18.49 | 0.341 |
| Height (cm) | 138.54 ± 23.53 | 143.19 ± 24.16 | 0.398 |
| Body mass index (kg/m²) | 17.34 ± 4.60 | 17.88 ± 3.74 | 0.301 |
| Systolic blood pressure (mm/Hg) | 101.89 ± 13.69 | 108.79 ± 10.83 | **0.006** |
| Diastolic blood pressure (mm/Hg) | 63.46 ± 6.60 | 68.18 ± 9.51 | **0.040** |

the mean sodium level was 134.86 ± 4.86 mmol/L, mean potassium level was 4.36 ± 0.72 mmol/L, mean calcium level was 9.30 ± 0.69 mg/dL, mean phosphorus level was 3.79 ± 1.33 mg/dL and mean magnesium level was 2.01 ± 0.40 mg/dL. In the patient group some laboratory abnormalities were seen, such as hyponatremia (< 136 mmol/L) in 61.54% (n = 24), hypernatremia (> 145 mmol/L) in 2.56% (n = 1), hypokalemia (< 3.5 mmol/L) in 15.38% (n = 6), hyperkalemia (> 5.1 mmol/L) in 15.38% (n = 6), hypophosphatemia (< 2.9 mg/dL) in 17.95% (n = 7), hyperphosphatemia (> 5.1 mg/dL) in 17.95% (n = 7), hypocalcemia (< 8.4 mg/dL) in 10.26% (n = 4), and hypercalcemia (> 10.2 mg/dL) in 15.38% (n = 6). There was no statistically significant difference between the previously and newly diagnosed T1DM patients in terms of mean electrolyte levels of the patient group ($p > 0.05$ for all). The mean lipid values of the patient group were in the normal range, except for mild mean triglyceride elevation of 216.27 ± 183.43 mg/dL (0-150 mg/dL). The mean HbA1c values were markedly above the reference range at 12.65 ± 2.92% (normal range 4-6%). There was no significant difference between the previously and newly diagnosed T1DM cases in terms of mean HbA1c values of the patient group ($p = 0.975$). No blood test was performed inthe control group and therefore no comparison was made with patient group. Laboratory findings of the patient group are summarized in Table 2.

### Electrocardiographic Findings

In the patient group there was a statistically significant increase in terms of HR, Pd, QTd and QTcd in the first ECGs, obtained at the time of DKA, when compared to both the second and third (after treatment) ECGs (Table 3). A significant increase in Tp-e and Tp-e/QT was found when the first ECGs were compared to third ECGs ($p = 0.045$ and $p < 0.001$, respectively) and there was a significant increase in Tp-e/QT in the first and second ECGs compared to the third ECGs ($p < 0.001$ and $p = 0.013$, respectively). There was no significant difference in Tp-e/QTc in any of the inter-group comparisons ($p > 0.05$). Significant differences were found in the parameters HR, Pd, QTd, QTcd and Tp-e/QT between the three ECGs obtained at different times. In terms of Tp-e/QT, a significant difference was found when comparing the third ECGs with the first and second ECGs ($p = 0.013$). The maximum QTc value of nine patients was found to be increased (> 450 ms) in the first ECG. This persisted in two of these patients in the second ECGs but all of them had recovered by the third ECG.

**Table 2. Laboratory findings of the patient group**

| Blood samples | Mean ± SD | Minimum | Maximum | Laboratory reference range |
|---|---|---|---|---|
| Leukocyte count (/mm$^3$) | **16857 ± 11825** | 3900 | 57300 | 4000-10000 |
| Hemoglobin (g/dL) | 14.18 ± 1.40 | 10.2 | 18.0 | 12.1-17.2 |
| Platelet count (/mm$^3$) | 331143 ± 123929 | 33.000 | 700.000 | 150000-400000 |
| Blood glucose (mg/dL) | **446.17 ± 133.83** | 225 | 947 | Fasting: 70-105/<br>Postprandial: 80-140 |
| Urea (mg/dL) | 33.94 ± 21.28 | 12.5 | 125.6 | 16.6-48.8 |
| Creatinine (mg/dL) | 0.84 ± 0.30 | 0.40 | 1.91 | 0.39-0.87 |
| Sodium (mmol/L) | **133.82 ± 4.61** | 125 | 147 | 136-145 |
| Potassium (mmol/L) | 4.44 ± 0.82 | 2.6 | 6.4 | 3.5-5.1 |
| Chlorine (mmol/L) | 99.03 ± 6.79 | 82 | 115 | 98-107 |
| Calcium (mg/dL) | 9.33 ± 0.80 | 6.88 | 10.71 | 8.4-10.2 |
| Phosphorus (mg/dL) | 3.97 ± 1.35 | 1.71 | 7.82 | 2.9-5.1 |
| Magnesium (mg/dL) | 1.98 ± 0.33 | 1.56 | 3.37 | 1.7-2.2 |
| Albumin (g/dL) | 4.56 ± 0.56 | 3.10 | 5.56 | 3.2-4.5 |
| pH | **7.12 ± 0.12** | 6.88 | 7.29 | 7.35-7.45 |
| $pCO_2$ (mmHg) | 22.53 ± 6.80 | 10.2 | 41.2 | 35-45 |
| $HCO_3$ (mEq/L) | **7.70 ± 3.88** | 3 | 15 | 21-27 |
| Ketone (serum) | **2.08 ± 0.77** | 1 | 3 | 0 |
| Total cholesterol (mg/dL) | 167.30 ± 47.09 | 70 | 277 | 0-200 |
| Triglyceride (mg/dL) | **216.27 ± 183.43** | 42 | 979 | 0-150 |
| HDL (mg/dL) | 40.21 ± 13.55 | 10 | 66 | 35-70 |
| LDL (mg/dL) | 90.33 ± 35.19 | 32 | 163 | 0-100 |
| HbA1c (%) | **12.65 ± 2.92** | 5.5 | 18.0 | 4-6 |

HbA1c: hemoglobin A1c, $HCO_3$: bicarbonate, HDL: high density lipoprotein, LDL: low density lipoprotein, $pCO_2$: carbon dioxide partial pressure, SD: standard deviation

The parameters HR, Pd, PR, QTd, QTcd, Tp-e and Tp-e/QT all differed significantly between the first ECG performed in the patient group and the values obtained from the control group. There was a statistically significant increase in PR interval when the second and third ECGs were compared to the control ECGs ($p = 0.005$ and $p = 0.032$, respectively). Thus, PR interval was found to be significantly increased compared to controls in all patient group ECGs, while Tp-e/QTc was similar between all the groups. ECG findings of the groups were summarized in Table 3 and illustrated in Figure 1 and Figure 2.

The EF value in the patient group was slightly but significantly greater than in the control group ($p = 0.035$). Nevertheless, the EF values of both groups were within the normal range. Other parameters were similar between the groups. Comparative results of the ECHO examinations of the cases are given in Table 4.

## Discussion

DM is a complex metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion and/or insulin sensitivity (10). In T1DM, women and men are affected almost equally. Some populations, including Western Europe and the USA, have a slight male dominance and some societies, such as Japan, have a female dominance (1). It has been reported that the incidence of DKA in children does not change with gender (11). However, the incidence of having DKA at first presentation leading to diagnosis of T1DM was found to be higher in boys (female/male ratio 0.51:1) (12). In our study, male gender was more predominant in newly diagnosed patients, consistent with the literature, while the female to male ratio was determined to be higher in all patients with DKA compared to previous studies. Thus the rate of DKA events in our previously diagnosed patients was greater in girls.

Lipid disturbances, including hypertriglyceridemia and low high density lipoprotein cholesterol, in patients with T1DM have been recognized for some time (13). Dyslipidemia has been associated with poor glycemic control (14). However, studies showing hyperlipidemia in DKA are rarely seen in the literature (15,16). In our patient group, only mild mean triglyceride elevation was seen, although it should be noted that the lipid profiles in our patient group was not taken at the time of DKA. In a multi-center study conducted by Turton et al (17), the HbA1c level in patients with T1DM ranged between 6.8 and 11.1%. In another study in children with T1DM, mean HbA1c level was found to be $7.59 \pm 1.34$% between 0-5 years of age, $7.61 \pm 1.32$% between 6-12 years of age, and $8.46 \pm 1.85$% between 13-18 years of age (18). DKA was more frequent in children with T1DM and poor glycemic control (2). Since all our cases, by definition,were children with DKA and more than 50% of them were newly diagnosed for T1DM, the mean HbA1c value in our cohort was much higher than previous studies.

In a previous study, the rate of DKA in children with T1DM was found to be inversely proportional to age. The

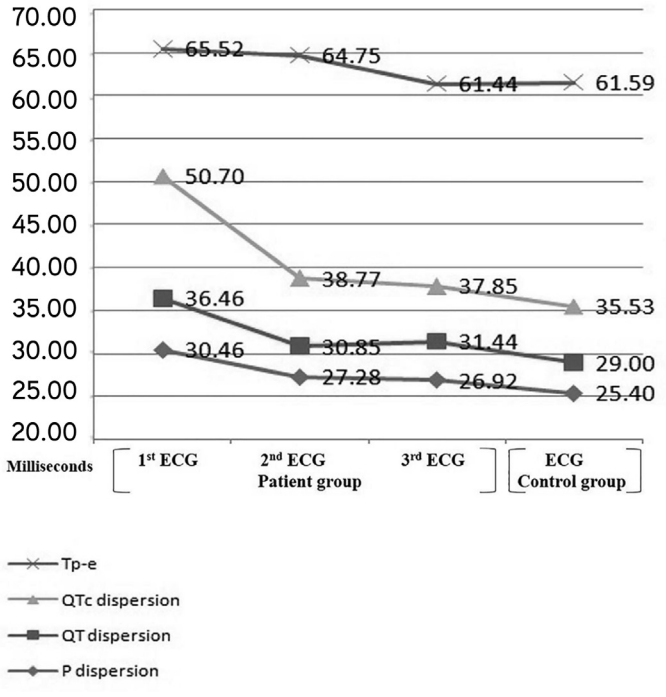


**Figure 1.** Mean values of P-wave dispersion, QT dispersion, corrected QT dispersion and Tp-e in patient and control groups

*ECG: electrocardiography*

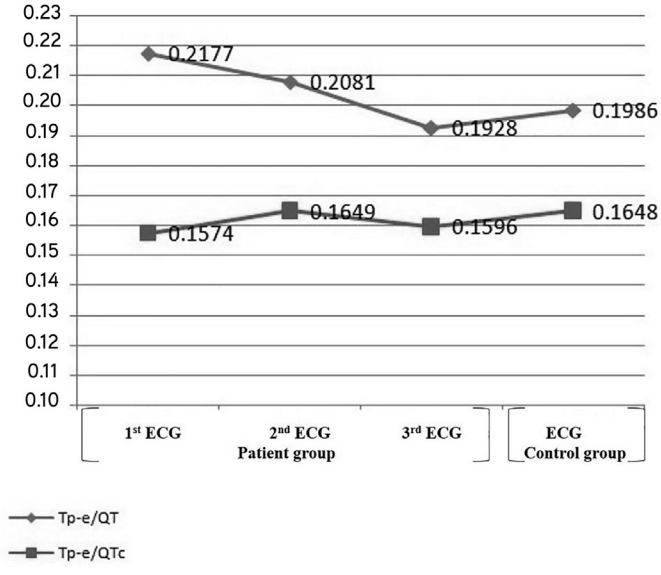


**Figure 2.** Mean values of Tp-e/QT and Tp-e/QTc ratios in patient and control groups

*ECG: electrocardiography*

**Table 3. Electrocardiographic findings incases and controls**

| | Mean ± SD | | | | p values | | |
|---|---|---|---|---|---|---|---|
| | Patient group 1st ECG | Patient group 2nd ECG | Patient group 3rd ECG | Control group ECG | p1<br>p4 | p2<br>p5 | p3<br>p6 |
| **HR (/min)** | 118.12 ± 23.91 | 97.52 ± 25.15 | 89.48 ± 18.48 | 92.20 ± 27.78 | **< 0.001**<br>**< 0.001** | 0.067<br>0.133 | **< 0.001**<br>0.992 |
| **P-min (ms)** | 126.20 ± 22.09 | 128.74 ± 20.38 | 125.13 ± 17.84 | 116.09 ± 18.15 | 0.455<br>0.218 | 0.498<br>**0.041** | **0.006**<br>**0.007** |
| **P-max (ms)** | 55.08 ± 8.81 | 56.46 ± 8.82 | 57.53 ± 7.88 | 52.70 ± 7.24 | 0.286<br>**0.001** | 0.535<br>**0.008** | 0.893<br>**0.002** |
| **Pd (ms)** | 85.64 ± 10.53 | 83.74 ± 10.38 | 84.44 ± 9.69 | 78.10 ± 7.58 | **0.035**<br>**0.003** | 0.927<br>0.398 | **0.017**<br>0.272 |
| **PR (ms)** | 30.46 ± 7.56 | 27.28 ± 7.16 | 26.92 ± 5.89 | 25.40 ± 4.40 | 0.363<br>**0.029** | 0.179<br>**0.005** | 0.854<br>**0.032** |
| **QT-min (ms)** | 280.31 ± 28.41 | 308.72 ± 38.23 | 314.39 ± 29.26 | 303.10 ± 36.05 | **< 0.001**<br>**< 0.001** | 0.127<br>1.000 | **< 0.001**<br>0.370 |
| **QT-max (ms)** | 316.77 ± 27.26 | 339.56 ± 39.17 | 345.83 ± 30.92 | 332.10 ± 36.83 | **< 0.001**<br>**0.015** | 0.145<br>0.821 | **< 0.001**<br>0.193 |
| **QTd (ms)** | 36.46 ± 7.87 | 30.85 ± 9.61 | 31.44 ± 9.89 | 29.00 ± 5.26 | **0.002**<br>**< 0.001** | 0.969<br>1.000 | **0.005**<br>0.613 |
| **QTc-min (ms)** | 387.89 ± 22.00 | 384.92 ± 20.67 | 379.09 ± 23.33 | 367.01 ± 23.58 | 0.533<br>**< 0.001** | 0.352<br>**0.001** | 0.127<br>**0.028** |
| **QTc-max (ms)** | 438.60 ± 17.77 | 423.69 ± 21.28 | 416.94 ± 22.45 | 402.54 ± 25.09 | **0.001**<br>**< 0.001** | 0.176<br>**< 0.001** | **< 0.001**<br>**0.015** |
| **QTcd (ms)** | 50.70 ± 12.01 | 38.77 ± 12.38 | 37.85 ± 11.38 | 35.53 ± 8.15 | **< 0.001**<br>**< 0.001** | 0.585<br>0.462 | **< 0.001**<br>0.603 |
| **Tp-e (ms)** | 65.52 ± 8.48 | 64.75 ± 10.05 | 61.44 ± 8.04 | 61.59 ± 8.29 | 0.691<br>**0.041** | 0.054<br>0.132 | **0.045**<br>0.934 |
| **Tp-e/QT** | 0.21 ± 0.03 | 0.21 ± 0.04 | 0.19 ± 0.03 | 0.20 ± 0.02 | 0.143<br>**0.001** | **0.013**<br>0.195 | **< 0.001**<br>0.292 |
| **Tp-e/QTc** | 0.16 ± 0.02 | 0.16 ± 0.03 | 0.16 ± 0.02 | 0.16 ± 0.02 | 0.134<br>0.120 | 0.090<br>0.979 | 0.596<br>0.319 |

HR: heart rate, P-min: minimum P wave duration, P-max: maximum P wave duration, Pd: P-wave dispersion, QT-min: minimum QT duration, QT-max: maximum QT duration, QTd: QT dispersion, QTc-min: minimum QTc duration, QTc-max: maximum QTc duration, QTcd: QTc dispersion, p1: p value comparing 1st and 2nd ECG, p2: p value comparing 2nd and 3rd ECG, p3: p value comparing 1st and 3rd ECG, p4: p value comparing 1st case and control ECGs, p5: p value comparing 2nd case and control ECGs, p6: p value comparing 3rd case and control ECGs, SD: standard deviation, ECG: electrocardiography

**Table 4. Echocardiographic findings inpatient and control groups**

| Mean ± SD | Patient group (n = 39) | Control group (n = 40) | p values |
|---|---|---|---|
| LA (mm) | 24.38 ± 4.10 | 24.47 ± 4.15 | 0.949 |
| Ao (mm) | 20.38 ± 3.59 | 20.43 ± 2.98 | 0.960 |
| LA/Ao | 1.20 ± 0.10 | 1.20 ± 0.13 | 0.986 |
| LVESD (mm) | 21.95 ± 3.88 | 23.63 ± 4.51 | 0.081 |
| LVEDD (mm) | 37.42 ± 6.22 | 39.46 ± 6.69 | 0.159 |
| IVSd (mm) | 6.66 ± 1.10 | 7.03 ± 1.18 | 0.127 |
| LVPWd (mm) | 6.74 ± 1.09 | 6.94 ± 1.30 | 0.473 |
| EF (%) | 72.79 ± 3.82 | 71.00 ± 3.60 | **0.035** |
| FS (%) | 40.72 ± 4.62 | 40.03 ± 3.44 | 0.113 |

Ao: aortic root, EF: left ventricular ejection fraction, FS: left ventricular fractional shortening, IVSd: end-diastolic interventricular septal thickness, LA: left atrial diameter, LVEDD: left ventricular end-diastolic dimension, LVESD: left ventricular end-systolic dimension, LVPWd: end-diastolic left ventricular posterior wall thickness, SD: standard deviation

prevalence of DKA was 36% under 5 years of age, where as it was 16% in those older than 14 years old (11). The incidence of having DKA at the onset of diagnosis of T1DM varies widely between 14.7% and 79.8% in various countries (19). Große et al (19) reported that the rate of DKA at the time of T1DM diagnosis increased in countries closer to the equator or there was a higher sensitivity to diabetic symptoms. The same article reported the rate of

concurrent DKA and T1DM diagnosis as 65.9% for Turkey, in accordance with our data.

The use of insulin in diabetic patients has greatly reduced the risk of mortality due to DKA, but cardiovascular dysfunction continues to be an important cause of mortality in the chronic disease process (20). Diabetes is recognized as a strong and independent risk factor for cardiovascular morbidity and mortality, which in turn are frequently associated with rhythm disturbances, such as atrial fibrillation and ventricular arrhythmia (21). Autoimmune mechanisms, which are also important in the etiology of T1DM, may be involved in the pathogenesis of cardiac autoimmunity (22). Lipid disorders have also been shown to contribute to this process by increasing atherosclerotic risk (23). It has been reported that cardiovascular risk was increased tenfold in individuals with T1DM compared to the healthy population (24).

The ECG parameters Pd, QTd, QTcd,Tp-e, Tp-e/QT, and Tp-e/QTc have been shown to berisk markers and are used to predict the risk of cardiac arrhythmias (4,5). Yet, there is limited data on arrhythmia risk markers in children with DKA. In studies performed in adult (25) and pediatric patients with T1DM (5,26), Pd was reported to be significantly increased compared to controls. In a study conducted in adult T2DM patients with DKA, it was observed that Pd was increased significantly before DKA treatment compared to after treatment (27). In another study evaluating children with DKA, it was reported that the mean P wave duration before DKA treatment was significantly increased compared to after treatment, however Pd assessment was not performed in that study (28). To the best of our knowledge, there has been no study reporting the relationship between Pd and DKA in a pediatric population. In our study, Pd in the first, pre-DKA treatment, ECG was found to be significantly higher than both the second and third ECGs, which were obtained after treatment, and the Pd value concurrent with DKA was also greater than in the control group ECGs. Our findings suggest that there may be an increased risk of atrial arrhythmia at the time of DKA in children with T1DM.

QTd and QTcd have been associated with an increased risk of malignant ventricular arrhythmia (29). QTd values above 58 ms in healthy individuals increase the cardiovascular mortality risk by 3.2 times and if QTd is above 80 ms, it has been reported to increase four times (30). QTd and QTcd were associated with a risk of cardiac arrhythmia in studies conducted in adults (31,32) and children (4,33) with T1DM. In an adult study of T1DM patients with DKA, QTc was found to be prolonged (>450 ms) in 38 (62.3%) of 61 patients, regardless of potassium level (34). It was also reported that the QTd and QTcd values obtained during DKA in children with T1DM were increased compared to the healthy population (35,36). In our study, QTd and QTcd in the first patient group ECG were found to be significantly prolonged compared to both second and third ECGs, as well as the ECGs of the control group. This also suggests that there is an increased risk of ventricular arrhythmia in children with T1DM especially during episodes of DKA.

The results of studies on Tp-e, Tp-e/QT and Tp-e/QTc values in adult T1DM patients are controversial. Inanır et al (32) reported these parameters to be increased in adults with T1DM compared to their control group, whereas in another study, these ECG parameters were found to be similar between T1DM patients and controls (37). In the study of Güney et al (26), conducted in children with T1DM, Tp-e was increased compared to the control group, but Tp-e/QT and Tp-e/QTc ratios were similar with healthy controls. We could not find any data in the literature about Tp-e, Tp-e/QT or Tp-e/QTc in a pediatric population with DKA. In our study, a statistically significant increase was found in pre-treatment ECGs in Tp-e and Tp-e/QT values compared to the control group. However, these parameters were similar between the values obtained at the third ECG and controls. In the study of Güney et al (26), all patients with T1DM were previously diagnosed, whereas our patient group was composed of more than half newly diagnosed T1DM. Additionally, there was no significant difference in regard to Tp-e/QTc in our study. This may be attributed to the fact that QTc values increases much more than QT as the HR increases. So the rise in Tp-e and of its variations in children diagnosed with T1DM related DKA remains controversial.

### Study Limitations

Due to the limited number of cases, the previously and newly diagnosed T1DM patients could not be differentiated during the evaluation. ECG examinations were not standardized during the episode of DKA, since the focus of this study was mostly ECG markers related to cardiac arrhythmia risk.In addition, 24-hour holter monitoring couldn't get recorded due to technical incompetence.

## Conclusion

In conclusion, the current study was a prospective study conducted in children with T1DM and experiencing an episode of DKA. To the best of our knowledge, this is the first article evaluating Pd, Tp-e, Tp-e/QT and Tp-e/QTc parameters in children with DKA. In this study, cardiac arrhythmia risk markers were increased in children with DKA. While T1DM is already considered a risk factor for cardiac arrhythmias, DKA has been observed to contribute

to this process. We found that the increase in cardiac arrhythmia risk markers during DKA persisted for a while immediately after the DKA symptoms and/or signs recovered, at the time of the second ECG, but they were similar to the control group 2-3 weeks after the episode of DKA. Thus, we suggest that it is important to consider the possibility of atrial and/or ventricular arrhythmias that may develop during treatment and immediate follow-up period in children with T1DM who have an episode of DKA in order to avoid adverse cardiac events. However, further prospective studies, including larger numbers of patients and longer follow-up periods, are needed to better understand the risks.

### Ethics

**Ethics Committee Approval:** Ethical approval for the study was obtained from Necmettin Erbakan University Ethical Committee with a decision no. 2018/1321, dated 03.05.2018.

**Informed Consent:** Informed consent was obtained from all individual participants and/or their legal guardians included in the study.

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

### Authorship Contributions

Surgical and Medical Practices: Oğuz Eğil, Fatih Şap, Beray Selver Eklioğlu, Mehmet Burhan Oflaz, Mehmet Emre Atabek, Tamer Baysal, Concept: Fatih Şap, Beray Selver Eklioğlu, Mehmet Burhan Oflaz, Design: Fatih Şap, Beray Selver Eklioğlu, Mehmet Burhan Oflaz, Data Collection or Processing: Oğuz Eğil, Fatih Şap, Beray Selver Eklioğlu, Analysis or Interpretation: Mehmet Emre Atabek, Tamer Baysal, Literature Search: Oğuz Eğil, Mehmet Burhan Oflaz, Writing: Oğuz Eğil, Fatih Şap.

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# Mutation Screening and Functional Study of *SLC26A4* in Chinese Patients with Congenital Hypothyroidism

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### What is already known on this topic?

Defects in the human *SLC26A4* gene are reported to be one of the causes of congenital hypothyroidism (CH). *SLC26A4* encodes the apical iodide transporter protein, pendrin.

### What this study adds?

We identified seven distinct variants of *SLC26A4*, including one novel mutation, in a cohort of Chinese patients with CH. Functional studies showed that five out of six missense mutations had different effects on gene function, including ion transport and/or membrane location of the SLC26A4 protein, Pendrin. These results provide an important basis for future mechanism research.

## Abstract

**Objective:** Defects in the human solute carrier family 26 member 4 (*SLC26A4*) gene are reported to be one of the causes of congenital hypothyroidism (CH). We aimed to identify *SLC26A4* mutations in Chinese patients with CH and analyze the function of the mutations.

**Methods:** Patients with primary CH were screened for 21 CH candidate genes mutations by targeted next-generation sequencing. All the exons and exon-intron boundaries of *SLC26A4* were identified and analyzed. The function of six missense mutation in *SLC26A4* were further investigated *in vitro*.

**Results:** Among 273 patients with CH, seven distinct *SLC26A4* heterozygous mutations (p.S49R, p.I363L, p.R409H, p.T485M, p.D661E, p.H723R, c.919-2A>G) were identified in 10 patients (3.66%, 10/273). *In vitro* experiments showed that mutation p.I363L, p.R409H, p.H723R affect the membrane location and ion transport of *SLC26A4*, while p.S49R did not. Mutation p.T485M and p.D661E only affected ion transport, but had no effect on the membrane location.

**Conclusion:** The prevalence of *SLC26A4* mutations was 3.66% in Chinese patients with CH. Five mutations (p.I363L, p.R409H, p.T485M, p.D661E and p.H723R) impaired the membrane location or ion transport function of *SLC26A4*, suggesting important roles for Ile363, Arg409, Thr485, Asp661, and His723 residues in *SLC26A4* function. As all variants identified were heterozygous, the pathogenesis of these patients cannot be explained, and the pathogenesis of these patients needs further study.

**Keywords:** Congenital hypothyroidism, next-generation sequencing, *SLC26A4*, cell location, ion transport

## Introduction

Congenital hypothyroidism (CH) is a common neonatal endocrine disorder. Unless treated in the first few months of life, severe CH can lead to growth retardation and permanent intellectual disability (1). The incidence of CH was reported to be about 1:4000 in 1970, and the incidence of the disease has increased to 1:2000 in the past decades (2). About 85% of cases of CH are caused by abnormal thyroid development (thyroid dysgenesis), but genetic associations with thyroid dysgenesis have only been identified in 2-5% of cases. These pathogenic genes leading to dysgenesis include

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thyroid stimulating hormone (TSH) receptor *(TSHR), PAX8, GLIS3, NKX2.1* and *FOXE1* (3). The remaining 15% of cases of CH are due to defects of thyroid hormone biosynthesis (dyshormonogenesis). Dyshormonogenesis is often caused by mutations in genes that are involved in the pathway of thyroid hormone synthesis, such as thyroperoxidase (TPO), dual oxidase 2 (DUOX2), sodium-iodide symporter (NIS; SLC5A5) and the apical iodide transporter, pendrin (PDS; *SLC26A4*) (4).

*SLC26A4* encodes pendrin, a multi transmembrane (TM) protein composed of 780 amino acids, consisting of 12-14 TM segments and a segment of intracellular STAS (Sulfate Transporter and Anti-Sigma factor antagonist) functional domain (5,6,7,8,9). Pendrin is an anion exchanger that is highly expressed in thyroid, inner ear and kidneys. In thyroid, pendrin is expressed at the apical membrane of thyroid follicular cells. It acts as a chloride-iodide exchanger, transporting iodide from the cell to the follicular lumen, where thyroid hormone is synthesized (10). Although previous studies have shown that *SLC26A4* biallelic mutation may result in CH (11), biallelic mutation of *SLC26A4* have not been found in Chinese patients with CH. In order to evaluate the role of *SLC26A4* in the pathogenesis of the Chinese CH patients, our study identified *SLC26A4* mutations in a cohort of Chinese patients with CH and analyzed the function of any identified mutations *in vitro*.

## Methods

### Clinical Subjects

We enrolled Chinese CH patients through newborn screening. Newborn screening was done with filter-paper blood spots between 3 and 5 days after birth. Blood samples were collected from the heel and TSH level was measured by time-resolved fluorescence assay (PerkinElmer, USA). Subjects with increased TSH (TSH ≥10 mU/L) levels observed during neonatal screening were recalled for further evaluation. The levels of TSH, total triiodothyronine (T3), total thyroxine (T4), free T3, and free T4 (fT4) in serum were determined by performing an immuno-chemiluminometric assay (UniCel DxI 800, Beckman, USA). The details of the diagnostic criteria to establish permanent CH in patients were from our previous study (12); briefly these included i) elevated TSH levels, ii) T4 or fT4 levels less than the reference range, and iii) restoration of normal thyroid parameters after receiving replacement therapy with L-thyroxine, but, after stopping treatment, a rise in TSH and a drop in fT4 were observed again. In addition, some patients were recruited from outpatient clinics who were on L-thyroxine replacement therapy. Although these patients lack initial diagnostic data, they have a definite history of CH. A written consent was obtained from the parents of the CH patients, and the study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine (decision no: 2016-76-T33, date: 2016-08-03). Informed consent was obtained from all patients or their legal guardians, and all unaffected family members who participated in the study.

### Next-generation Sequencing

Genomic DNA was extracted from the peripheral blood using the Quick Gene DNA Whole Blood Kit L (Kurabo, Japan) according to the manufacturer's protocol (13). Twenty-one previously reported possible causative genes for CH, including TPO (GenBank reference sequence: NM_000547), SLC5A5 (NM_000453), thyroglobulin (TG) (NM_003235), TSHR (NM_000369), DUOX2 (NM_014080), DUOXA2 (NM_207581), *SLC26A4* (NM_000441), FOXE1 (NM_004473), PAX8 (NM_013952), NKX2-1 (NM_001079668), NKX2-5 (NM_004387), IYD (NM_001164694), DIO1 (NM_000792), DIO2 (NM_000793), THRA (NM_001190918), THRB (NM_00125263), DUOX1 (NM_017434), DUOXA1 (NM_001276268), GNAS (NM_016592), SLC16A2 (NM_006517) and HHEX (NM_002729) were analyzed in this study (16). All the exons and exon-intron boundaries of these genes were amplified by performing multiplex polymerase chain reaction (PCR) using a 48×48 Access Array™ microfluidic platform (Fluidigm, USA) according to the manufacturer's protocol. The primers were designed using iPLEX Assay Design software (Sequenom, USA). The HiSeq 3000 platform (Illumina, San Diego, CA, USA) was used to perform deep sequencing of these amplicon libraries. The target sequences were amplified and deep sequenced in duplicate for each sample to avoid base pair (bp) variants caused by multiplex PCR.

### Calling of *SLC26A4* Variants from Next-generation Sequencing Data and Verification Using Sanger Sequencing

Raw sequence data was analyzed in fastq format and the quality scores were obtained, as previously described (14,15). Credible variants were selected according to the following criteria: (i) the quality scores of variants with ≥30 bps; (ii) mapping the quality scores of variants with ≥50 bps; (iii) sequencing to estimate the depth of variants with ≥20 bps; (iv) variant allele frequency ≥30%; (v) variants with read depth ≥5; and (vi) the presence of mutation on both the DNA strands (16). Variants with frequencies >1% in the dbSNP 135 and ESP6500 v2 databases were filtered out and the focus of the study was on the functional (protein altering) variants after removal of intergenic and 3'/5' UTR variants, nonsplice related intronic variants, synonymous

variants identified in duplicate samples. Then the remaining variants were selected for validation by Sanger sequencing.

### Construction of Plasmid

Human wild-type (WT) cDNA of *SLC26A4* was cloned into p-enhanced green fluorescent protein (EGFP)-N2 plasmid (TransGen Biotech, China). Identified missense mutations were introduced into the *SLC26A4*-pEGFP-N2 WT plasmid by Fast Mutagenesis System kit (TransGen Biotech, China) according to the manufacturer's protocol. Meanwhile, human NIS cDNA was cloned into a eukaryotic expression vector pcDNA3.1. All the plasmid constructs were validated by Sanger sequencing.

### Cell Culture and Transfections

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, USA) at 37 °C in a humidified atmosphere containing 5% $CO_2$. Transfections were performed on cells by Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, USA) following the manufacturer's instructions. Cells were plated in 20 mm glass bottom cell culture dish (NEST), transfected with 1 μg plasmid DNA to detected the cell localization of the WT or mutants of *SLC26A4* plasmids. Iodide efflux assays were performed on 293T cells, cultured in 12 well plates, co-transfected with 0.5 μg pcDNA3.1-*NIS* and 0.5 μg WT or mutant *SLC26A4*-pEGFP-N2 plasmids.

### The Assays for the Cell Localization of WT or Mutants of *SLC26A4*

Forty-eight hours after transfecting with WT or mutants of *SLC26A4*-pEGFP-N2 plasmids, 293T cells were washed twice in PBS (1X). Then cells were fixed in 4% paraformaldehyde for 30 minutes. After washing with PBS (1X), cells were stained with the membrane probe DiI (Beyotime, Haimen, China) at 37 °C for 5-10 min, then nucleii were stained with DAPI (Beyotime Biotech, Haimen, China) at room temperature for 5 minutes. Confocal imaging for cells was carried out on Nikon A1 confocal microscope using the 40x objective (Nikon A1 Microsystems, Japan).

### Iodide Efflux Assay for WT or Mutants of *SLC26A4* in 293T Cells

The iodide efflux assay was performed as described previously (17). In brief, forty-eight hours after co-transfecting with 0.5 μg pcDNA3.1-*NIS* and 0.5 μg WT or mutant *SLC26A4*-pEGFP-N2 plasmids, 293T cells were washed once in serum-free DMEM medium and incubated for 1 hour in 1 mL serum-free medium containing $^{131}I$ at 5 KBq/mL as the only source of iodide. The cells were then washed briefly in HBSS buffer and then incubated with 1 mL HBSS for 5 minutes after which HBSS was removed. The cells were solubilized by the addition of 1 mL 1 N NaOH and the radioactivity measured using a γ counter (GC1200, Anhui, China). All experiments were carried out three times on triplicate cultures. Statistical significance of the iodide efflux assay results was determined by use of t-test.

### Statistical Analysis

All data are expressed as mean ± standard deviation. Data analysis is mainly processed by Excel, elisacalc and Statistical Package for the Social Sciences 19.0 statistical software. $P < 0.05$ is considered to be statistically significant. The images in this paper are mainly processed and produced by Photoshop software, image J and Graphpad prism 6. The gene sequence retrieval website National Center for Biotechnology Information used in this paper: http://www.ncbi.nlm.nih.gov/; University of California, Santa Cruz: http://genome.ucsc.edu/

## Results

### Clinical Characteristics of Patients with CH

The cohort of CH patients enrolled consisted of 273 patients, including 141 (51.6%) females. The median value of serum TSH and serum fT4 level were 54.075 uIU/mL and 0.718 ng/dL, respectively. All of them had normal hearing. Whether these children had enlarged vestibular aqueduct (EVA) is unknown because the examination was unnecessary and the patient's family refused.

### Screening the Missense Mutations of *SLC26A4* in the Chinese Patients with CH

All the exons and exon-intron boundaries were amplified by performing multiplex PCR using customized primers designed to generate 200-250bp amplicons. After the quality control assessment, the average coverage of *SLC26A4* with sequencing depth ≥20x was 89.04%. Seven heterozygous mutations in *SLC26A4* were identified in 10 patients, including one novel mutation (p.I363L). Interestingly, 8 of these 10 patients also carried mutations in other candidate gene for CH (Table 1). All mutation sites were verified by Sanger sequencing (Figure 1), with the exception of one patient. In patient 190 it was not possible to obtain a Sanger sequencing result because of DNA sample damage and patient refusal to provide a repeat sample. The frequency of *SLC26A4* mutation in Chinese patients diagnosed with CH was 3.66% (10/273). Among the seven mutations, p.S49R was located in the N-terminal intracellular region, p.D661E and p.H723R were located in the STAS domain of the C-terminal intracellular region which plays a key role in the membrane location of *SLC26A4*. The remaining mutation

**Table 1. The clinical data and genetic characteristics of the 10 congenital hypothyroidism patients with mutation of *SLC26A4***

| ID | Gender | At diagnosis | | | | Mutation information | | | | The frequency observed in public databases | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Age (day) | Thyroid ultrasound | fT4 (0.58-1.64) ng/dL | TSH (0.34-5.6) uIU/mL | Mutated gene | Annotation | Zygosity | Classify sequence variants according ACMG/AMP guideline | ExAC_ ALL | ExAC_ EAS |
| 6 | Male | 30 | Thyroid ectopy | NA | 43 | *SLC26A4* | NM_000441:c.A2168G p.H723R | Heterozygous | Pathogenic | 1.24E-04 | 0.0006 |
| 31 | Female | 30 | Normal | NA | > 150 | *SLC26A4* | NM_000441:c.A1087C p.I363L | Heterozygous | Uncertain significance | 9.10E-05 | 4.40E-04 |
| | | | | | | *TG* | NM_003235:c.G5486C p.R1829P | Heterozygous | Uncertain significance | NA | NA |
| 42 | Male | 30 | Absence | NA | 150 | *SLC26A4* | NM_000441:c.C147G p.S49R | Heterozygous | Likely benign | 0 | 0 |
| | | | | | | *TG* | NM_003235:c.A2276G p.Y759C | Heterozygous | Uncertain significance | 1.70E-05 | 8.00E-05 |
| | | | | | | *TG* | NM_003235:c.C4859T p.T1620M | Heterozygous | Likely benign | 0.0005 | 0.0068 |
| 51 | Male | 40 | Goiter | > 0.4 | > 100 | *SLC26A4* | NM_000441:c.919-2A > G | Heterozygous- | NA | 8.24E-06 | 0 |
| | | | | | | *DUOX2* | NM_014080:c.C4027T p.L1343F | Heterozygous | Pathogenic | 7.42E-05 | 0.0003 |
| | | | | | | *DUOX2* | NM_014080:c.G2794A p.D932N | Heterozygous | Uncertain significance | 2.49E-05 | 0 |
| 125 | Male | 15 | NA | 0.8 | 63.71 | *SLC26A4* | NM_000441:c.C1983A p.D661E | Heterozygous | Likely benign | 0.0001 | 0.0016 |
| | | | | | | *DUOX2* | NM_014080:c.C227T p.P76L | Heterozygous | Uncertain significance | 3.43E-05 | 0.0005 |
| 190 | Male | NA | NA | NA | NA | *SLC26A4* | NM_000441:c.C1454T p.T485M | Heterozygous | Uncertain significance | 5.77E-05 | 0.0002 |
| 241 | Female | 30 | Normal | 0.65 | > 150 | *SLC26A4* | NM_000441:c.G1226A p.R409H | Heterozygous | Pathogenic | 1.24E-04 | 0 |
| | | | | | | *TSHR* | NM_003235:c.T1574C p.F525S | Heterozygous | Uncertain significance | 1.32E-04 | 1.74E-03 |
| 245 | Male | 20 | Normal | 1.41 | 20.48 | *SLC26A4* | NM_000441:c.919-2A > G | Heterozygous | NA | 8.24E-06 | 0 |
| | | | | | | *DUOX2* | NM_014080:c.A2033G p.H678R | Heterozygous | Benign | 0.1020 | 0.055 |
| | | | | | | *IYD* | NM_001164694:c.793_794delTGinsCA p.C265H | Heterozygous | NA | NA | NA |
| 247 | Male | 21 | Normal | 0.22 | > 100 | *SLC26A4* | NM_000441:c.919-2A > G | Heterozygous- | NA | 8.24E-06 | 0 |
| | | | | | | *DUOX2* | NM_014080:c.G3616A p.A1206T | Homozygous | Pathogenic | 7.41E-05 | 0 |
| | | | | | | *IYD* | NM_001164694:c.793_794delTGinsCA p.C265H | Heterozygous | NA | NA | NA |
| | | | | | | *TSHR* | NM_003235:c.G1349A p.R450H | Heterozygous | Pathogenic | 0.0003 | 0.0044 |
| 259 | Female | 40 | Normal | 0.53 | 51.676 | *SLC26A4* | NM_000441:c.A1087C p.I363L | Heterozygous | Uncertain significance | 9.10E-05 | 4.40E-04 |
| | | | | | | *DUOXA2* | NM_207581:c.554 + 6T > C | Homozygous | NA | 0.8764 | 0.9352 |

**Table 1. Continued**

| ID | Gender | At diagnosis | | | | Mutation information | | | | The frequency observed in public databases | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Age (day) | Thyroid ultrasound | fT4 (0.58-1.64) ng/dL | TSH (0.34-5.6) uIU/mL | Mutated gene | Annotation | Zygosity | Classify sequence variants according ACMG/AMP guideline | ExAC_ ALL | ExAC_ EAS |
| | | | | | | *TG* | NM_003235:c.G7318A p.V2440I | Heterozygous | Uncertain significance | 5.80E-05 | 1.20E-04 |
| | | | | | | *TG* | NM_003235:c.A5791G p.I1931V | Heterozygous | Likely benign | 0.0002 | 0.0021 |
| | | | | | | *DUOX2* | NM_014080:c.G2654T p.R885L | Heterozygous | Pathogenic | 3.38E-04 | 4.39E-03 |

fT4: free thyroxine, TSH: thyroid-stimulating hormone, NA: not available

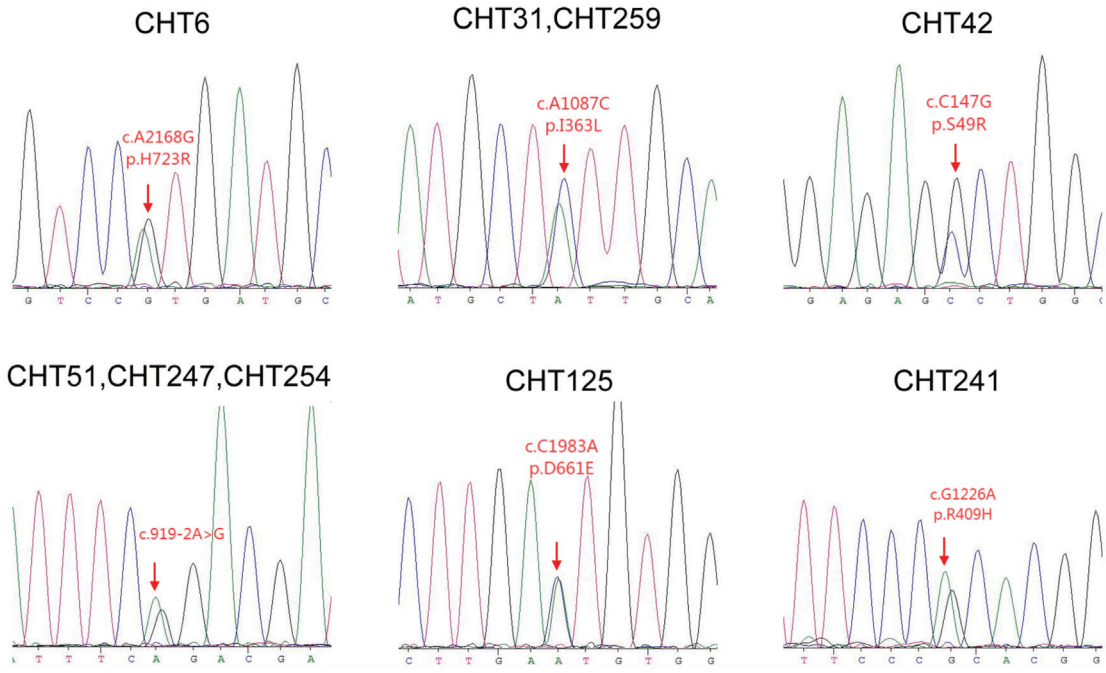


**Figure 1.** Sanger sequencing of *SLC26A4* mutation

sites were scattered in the 12 TM regions of *SLC26A4* (Figure 2).

### Cellular Localization of *SLC26A4* Mutants

*SLC26A4* has been shown, by immunohistochemical analysis, to be located at the apical membrane of thyroid follicular cells (18). To assess the effect of mutations on membrane location of *SLC26A4*, we expressed WT and mutants of the *SLC26A4*-pEGFP-N2 plasmid in 293T cells and observed location using a confocal fluorescence microscope. Although these cells lack the polarization of thyroid follicular cells, WT *SLC26A4* was clearly present at the cell membrane and significant co-localization with marker of cell membrane. Mutant p.S49R showed a cell membrane protein distribution similar to that of the WT *SLC26A4*. Mutants p.R409H and p.H723R did not express on the cell membrane obviously. Novel mutant p.I363L showed partly express in the cytoplasm, but was mostly expressed at the cell membrane. The remaining mutants, p.T485M, p.D661E, and p.S49R had no effect on cell membrane localization (Figure 3).

### Assessment of Iodide Transport in the WT and Identified *SLC26A4* Mutants

It has been suggested that *SLC26A4* mediates iodide efflux at the apical membrane of thyroid follicular cell (10). In

order to assess the effect of mutations on the ability of iodide transport, we co-expressed NIS with WT or mutant *SLC26A4*-pEGFP-N2 plasmids in 293T cells to provide a cell model by which cells could uptake iodide from culture medium. The 293T cells transfected with *NIS* only showed an accumulation of radioiodide ($^{131}$I) in the cells. In contrast, only a small amount of $^{131}$I was retained in the 293T cells after co-transfection with *NIS* and WT *SLC26A4* plasmids. However, compared to the 293T cells co-transfected with *NIS* and WT *SLC26A4* plasmids, with one exception (p.S49R), cells co-transfected with NIS and the identified *SLC26A4* mutants resulted in significantly decreased iodine efflux, indicating that these mutants lead to a decrease in cellular ability of iodide transport in 293T cells (Figure 4).

## Discussion

CH is a relatively common endocrine disease with a prevalence ranging from 1:2000 to 1:4000 in newborns (19). Most cases are dysgenetic although a substantial proportion are due to dyshormonogenesis. There is a close association between genetic abnormalities and dyshormonogenesis but the search for genetic mechanisms in dysgenesis has identified <5% to have a genetic pathogensis. Therefore, it is important to expand the spectrum of the pathogenic genes in patients with CH, given the relatively common occurrence. In this study, mutations in *SLC26A4* were investigated in Chinese patients with CH and seven different heterozygous variants in 10 individuals (10/273, 3.66%) were found. The prevalence of *SLC26A4* mutations in our study was similar to a previous study that screened *SLC26A4* mutation in CH patients from Guangxi Zhuang Autonomous Region, China (20). Fu et al (20) reported that all the mutations detected were heterozygous mutations, and thus cannot be assumed to be pathogenetic. These findings suggested that *SLC26A4* might be an uncommon pathogenic gene for CH in the Chinese population.

*SLC26A4* is a member of the SLC26 anion transporter family that encodes the pendrin protein which was originally predicted to contain 12 TM domains (21). This has since been shown to be incorrect as 14 TM domains were subsequently confirmed by Gorbunov et al (22). These regions contain many anion-binding sites or substrate-binding sites, including TM1, TM3, and TM10, which would affect the function of this protein (5). The protein also has a STAS domain in the cytosol, which is critical for membrane targeting of many SLC26 anion transporters, and STAS domain mutations are associated with at least three human recessive diseases (22). In our study, a total of seven mutation sites in *SLC26A4* were identified, including a novel variant which, to the best of our knowledge, has not previously been reported.

The variant S49R, located in the N-terminal intracellular segment, was shown by functional experiments to have no effect on membrane localization or ion transport. It may be that the site is not an ion binding site, so the mutation has little effect on gene function. The novel variant, I363L was located in TM8, which is an anion-binding domain (5). Our study confirmed that mutation I363L affects the membrane localization of *SLC26A4* slightly and reduced its ability to transport iodine ions by about 53%. We speculate that

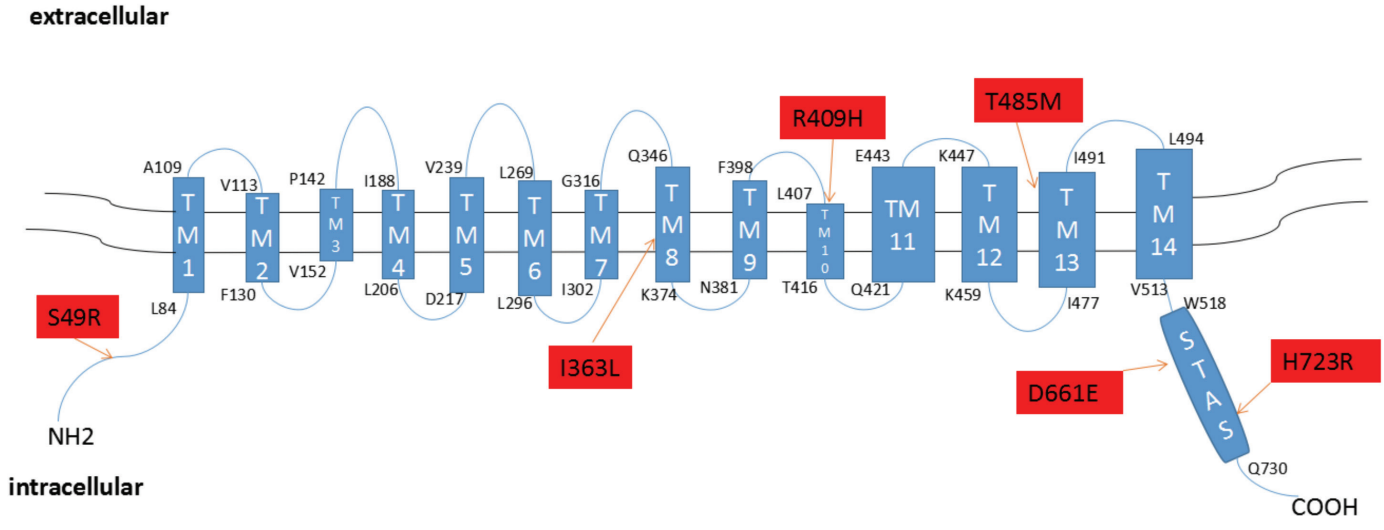


**Figure 2.** The mutations, identified from our patients with congenital hypothyroidism, located in the protein domain of *SLC26A4*. The mutation p.S49R is located in the N-terminal intracellular region, p.D661E and p.H723R are located in the STAS domain of the C-terminal intracellular region of the *SLC26A4*. The remainer 3 mutations were scattered in 12 transmembrane domains of the *SLC26A4*

because TM8 is an ion binding region, mutations in this region may affect the overall ion binding ability, thus affecting gene function. R409H, located in TM10, significantly reduced the membrane localization and iodine transport capacity by 83.7%, a result which is consistent with previous reports. Related studies have shown that this site is an anion-binding site, the mutation directly affect the anion-binding site would have a great impact on gene function. His723 is a conserved site that is located in the STAS domain. The mutation H723R would disrupt the π-cation interaction and polar contact between Tyr530 and His723, again affecting protein function (5). D661E and T485M are located in the STAS domain and TM13, respectively. The two mutation sites have no effect on the localization of the pendrin protein to the cell membrane, but have a significant impact on ion transport, reducing it by about 74% and 67% respectively.

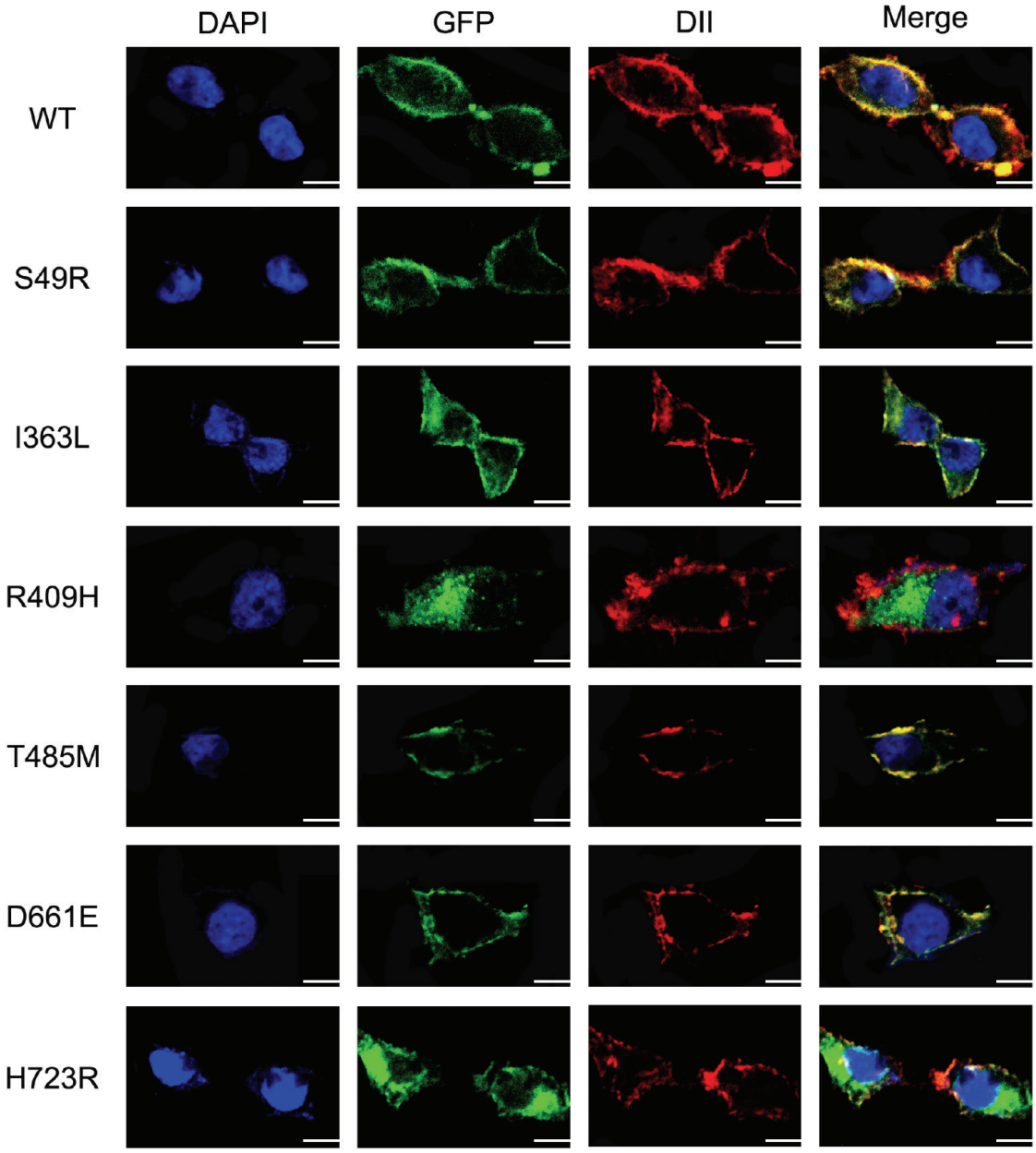


**Figure 3.** Cellular localization of the six mutants of *SLC26A4* in 293T cells detected by confocal microscopy. Mutant p.S49R, p.T485M, p.D661E of *SLC26A4* show strong membrane fluorescence in 293T cells, which is similar to the WT of *SLC26A4* expressed in the 293T cells, and there was no obvious cytoplasmic retention. Mutants p.I363L, p.R409H, and p.H723R reduce the localization on the cell membrane, and p.R409H, p.H723R show obvious cytoplasmic retention, p.I363L is less. All mutant plasmids were homozygous. Scale: 10 μm

*WT: wild-type*

There are mutants that have been characterized as having an effect at the cell surface but with reduced function, such as G209V, F335L, M775T. So, the mechanism of the effect of D661E and T485M on the protein may be similar to these loci, which needs further study (5,17,23).

*SLC26A4* is expressed in the inner ear and thyroid (17). In the inner ear, *SLC26A4* functions as a $Cl^-/HCO_3^-$ exchanger and regulates the balance of endolymphatic ions, thus affecting the function of the inner ear (24). Homozygous mutations in *SLC26A4* lead to EVA, which is the most common inner ear malformation associated with sensorineural deafness in children (25). However, some patients with EVA carried a heterozygous mutation in *SLC26A4*, rather than a biallelic mutation, suggesting that there are other genetic factors involved in the occurrence of EVA. A study conducted by Yang et al (26) confirmed this hypothesis. They identified heterozygous mutations in *SLC26A4* and KCNJ10 from one patients with EVA. In our study, through follow-up, we found that these 10 patients with CH did not have deafness, but they refused to carry out imaging examination of the inner ear, and thus we were unable to determine whether there was EVA.

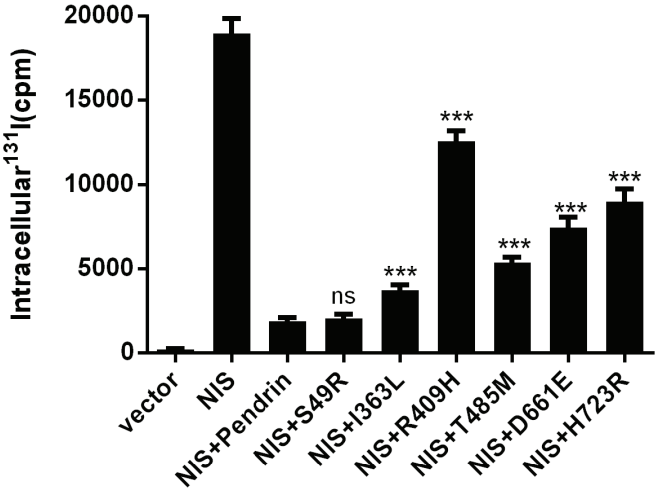


**Figure 4.** The effect of the mutations in *SLC26A4* on iodine transport capacity in 293T cells. Intracellular iodide accumulation in 293T cells after co-transfection with sodium-iodide symporter (*NIS*) and wild-type (WT) or the mutant *SLC26A4*-pEGFP-N2 plasmids were detected by γ-counter. Intracellular iodide accumulation in 293T cells after co-transfection with *NIS* and mutants palsmids from the p.I363L, p.R409H, p.T485M, p.D661E and p.H723R variants were significantly increased, compared to those co-transfected with NIS and WT (pendrin) *SLC26A4*-pEGFP-N2 plasmid, indicating that these mutants reduced iodide efflux mediated by *SLC26A4*. All mutant plasmids were homozygous. The data are shown as mean ± standard error for three independent experiments. Statistical analysis used Welch's t-test

*ns: No statistical difference, ***p < 0.001*

Pathogenic mutations in *SLC26A4* are well recognized as being the pathogenic mutation in Pendred syndrome (PS), which is an autosomal recessive disorder characterized by sensorineural hearing loss, and goiter and some cases may be identified in the neonatal period with CH (27). However, the thyroid phenotype of PS patients is not clearly defined. In 2014, Ladsous et al (28) found that about 30% of PS patients will present with CH and 78.9% patients have goiter in PS patients with biallelic mutations of *SLC26A4*. The researchers speculated that these differences in PS thyroid phenotype in patients with biallelic mutations of *SLC26A4* might be due to different iodine intakes, as most of the patients with PS presented with hypothyroidism in a moderately iodine deficient region in France, but PS patients from Japan and Korea, regions with high iodine intake, were euthyroid (28,29). Although the thyroid phenotype in human patients with PS seemed to be related to iodine intake, lower iodine intake did not lead to goiter and hypothyroidism in the *SLC26A4* knockout mice (30), indicating that other genetic factors or environmental factors might be involved in the pathogentic mechanism resulting in goiter and hypothyroidism in humans with *SLC26A4* mutation. Indeed, among the 10 patients with *SLC26A4* heterozygous mutation, eight patients carried other mutations in genes associated with CH in our cohort (Table 1). Patient 42 and 259 carried compound heterozygous mutations in the *TG* gene, which is the key gene in the thyroid hormone synthesis and was the pathogenic gene in these two patients. Patient 51 and 247 carried biallelic mutation in DUOX2, which is also involved in thyroid hormone synthesis, but the association between DUOX2 variants and CH is less definite and thus the DUOX2 variants in our patients may be pathogenetic. Four of the remaining six patients carried at least one heterozygous mutation in another candidate gene for CH, while the remaining two patients were found to only have heterozygous mutation in *SLC26A4* amongst the 21 CH-associated genes tested. Although we confirmed that the mutations in *SLC26A4* in our patients with CH could decreased the ability of the iodide transport *in vitro*, none of the parents of these patients, some of whom were also carriers, did not have hypothyroidism, suggesting that these heterozygous mutations in *SLC26A4* are probably not the pathogenic gene for our patients who had all been diagnosed with CH and that there are other genetic or environmental factors which might lead to CH. As EVA could be caused by the heterozygous mutation in *SLC26A4* combining with the heterozygous mutation of KCNJ10, and a previous study has reported that heterozygous mutations in DUOXA2 and DUOX2 might lead to CH in a 4-year-old patient (31), it cannot be excluded that those

patients with monoallelic mutation of *SLC26A4* may combine with other, unidentified, gene variants to cause hypothyroidism.

### Study Limitations

The sample size of this study was limited, and no individuals with a homozygous mutation in *SLC26A4* were found. In addition, we could not further elucidate the pathogenesis of *SLC26A4*. Furthermore, the candidate gene panel used in this study did not include SLC26A7, which can also lead to hypothyroidism.

## Conclusion

In this study, we identified seven distinct variants of *SLC26A4* in 10 patients from a cohort of 273 Chinese patients with CH. Functional studies showed that five out of six missense mutations in *SLC26A4*, including one novel mutation, p.I363L, have variable effects on protein function. However, because these mutations were all heterozygous mutations, and 8 out of ten patients also carried variants in other CH candidate genes, the pathogenesis of CH in these patients cannot be explained by these *SLC26A4* variants. The pathogenesis of CH in these patients needs further study.

### Acknowledgments

We would like to thank all patients and their families for the participation in our study and for allowing us to publish their cases.

### Ethics

**Ethics Committee Approval:** The study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine (decision no: 2016-76-T33, date: 2016-08-03).

**Informed Consent:** Informed consent was obtained from all patients or their legal guardians, and all unaffected family members who participated in the study.

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

### Authorship Contributions

Surgical and Medical Practices: Cao-Xu Zhang, Feng Sun, Wen-Jiao Zhu, Rui-Jia Zhang, Ya Fang, Chen-Yan Yan, Concept: Shuang-Xia Zhao, Huai-Dong Song, Design: Chang-Run Zhang, Data Collection or Processing: Chang-Run Zhang, Qian-Yue Zhang, Ying-Xia Ying, Analysis or Interpretation: Chang-Run Zhang, Yuan-Ping Shi, Literature Search: Chang-Run Zhang, Writing: Chang-Run Zhang, Shuang-Xia Zhao.

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# 17-Hydroxyprogesterone Response to Standard Dose Synacthen Stimulation Test in *CYP21A2* Heterozygous Carriers and Non-carriers in Symptomatic and Asymptomatic Groups: Meta-analyses

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### What is already known on this topic?

Standard dose synacthen stimulation test (SDSST) is a gold standard biochemical-screening test for evaluating adrenal gland function. Despite studies investigating the use of SDSST to identify heterozygous *CYP21A2* mutation, the reliability of the test for this is still controversial.

### What this study adds?

This meta-analysis was performed to determine if there were differences in 17-hydroxyprogesterone (17-OHP) response to SDSST (0.25 mg) in the identification of *CYP21A2* heterozygous carriers, with or without clinical sign of androgen excess disorders, to investigate the utility of the SDSST for this purpose and to determine the cut-off levels of 17-OHP for this purpose. The results support the hypothesis that stimulated 17-OHP level after SDSST had the potential to identify *CYP21A2* carriers, although basal 17-OHP level was not sufficiently informative. Additionally, the median level of stimulated 17-OHP was higher in symptomatic mutation-free controls than in asymptomatic mutation-free healthy controls and similarly, the median level of stimulated 17-OHP was higher in symptomatic mutation carriers than in asymptomatic mutation carriers. Clinical phenotype may affect the evaluation of the test.

## Abstract

**Objective:** Standard dose synacthen stimulation test (SDSST) is a gold standard screening test for evaluating adrenal gland function. Despite studies using SDSST to identify heterozygosity in *CYP21A2*, the reliability of the test for this purpose is still controversial. Therefore, the meta-analyses were performed to determine the differences in 17-hydroxyprogesterone (17-OHP) responses to standard dose (0.25 mg) SDSST in the diagnosis of *CYP21A2* heterozygous individuals, with or without clinical signs of androgen excess disorders.
**Methods:** PubMed and MEDLINE databases were searched. A total of 1215 subjects (heterozygous carriers n = 669, mutation-free controls n = 546) were included in the meta-analyses.
**Results:** Basal 17-OHP median/mean levels were 4.156 (3.05-10.5)/5.241 (± 2.59) nmol/L and 3.90 (2.20-9.74)/4.67 (± 2.62) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP median/mean levels were 17.29 (14.22-37.2)/19.51 (± 7.63) nmol/L and 9.27 (7.32-15.9)/10.77 (± 3.48) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP median/mean levels were 3.21 (2.64-4.78)/3.33 (± 0.84) nmol/L and 3.12 (1.82-3.6)/2.83 (± 0.71) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Stimulated 17-OHP median/mean levels were 14.16 (12.73-16.37)/14.16 (± 1.37) nmol/L and 6.26 (4.9-8.23)/6.48 (± 1.2) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. The cut-off levels for stimulated 17-OHP were 10.48 nmol/L and 13.48 nmol/L for asymptomatic heterozygous and symptomatic heterozygous, respectively.
**Conclusion:** The meta-analyses support the idea that stimulated 17-OHP level has potential for use in identifying *CYP21A2* carriers. Besides, considering differences in the basal and stimulated 17-OHP levels in symptomatic heterozygous individuals compared to those who were asymptomatic heterozygous could increase the accuracy of the test.
**Keywords:** Adrenal insufficiency, synacthen stimulation test, 17-OHP level, heterozygous, *CYP21A2*

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## Introduction

Adrenal insufficiency is caused by a failure of the adrenal cortex to produce cortisol. The most common cause of adrenal insufficiency is autosomal recessive inherited congenital adrenal hyperplasia (CAH) (OMIM 201910), characterized by excess adrenal androgen production resulting from impairment of the adrenal 21-hydroxylase (21OH) enzyme. Androgen excess affects approximately 10% of women (1). Disorders that result from hyperandrogenism include polycystic ovary syndrome (PCOS) (OMIM 184700) and 21OH-deficient non-classic adrenal hyperplasia (21OHD-NCAH) (OMIM 201910). The etiology of PCOS is not fully understood but it is a familial disorder that appears to be inherited as a complex genetic trait with a risk to siblings of ~50%. There is no accepted precise mode of inheritance (2,3,4) but it is one of the most common endocrine disorders, affecting 6-10% of reproductive-age women (5). It may be difficult to distinguish PCOS from NCAH clinically (6). Of hyperandrogenic women, 1-10% is reported to be affected by NCAH due to 21OHD and NCAH may even be asymptomatic (7). In childhood, hyperandrogenism may present with premature pubarche (PP) and 5-20% of PP cases were diagnosed with NCAH, mainly due to 21OHD-NCAH (8,9,10,11,12).

Standard dose synacthen stimulation test (SDSST) is the gold standard screening test to evaluate adrenal gland function (13). It is the principal challenge test to estimate the relative activity of adrenocortical enzymes (14) and it has been widely used for the biochemical diagnosis of NCAH, due to various adrenocortical enzyme deficiencies including 21OHD. A consensus is not available about whether heterozygous individuals with *CYP21A2* mutations have a higher risk of developing clinical hyperandrogenism. In some selected populations, being heterozygous for *CYP21A2* seems to be related to irregular menses, hirsutism, PCOS, premature adrenarche (PA), acne, and central precocious puberty (12,15,16,17,18,19,20,21). In contrast, other investigators concluded that heterozygosity for *CYP21* mutations did not increase the risk of clinical androgen excess above that expected in the general population (22,23). The prevalence of asymptomatic carriers for the disease in the general population was estimated to range from 1:50 to as high as 1:16 (24) and even higher among Ashkenazi Jews, according to a single report (8). The frequency of mutation carriers of *CYP21A2* was almost 1 in 4 in PP and hirsute groups (21,25).

The objectives of this meta-analysis were to determine if there was a difference in 17-hydroxyprogesterone (17-OHP) response to standard dose (0.25 mg) SDSST in the diagnosis of *CYP21A2* heterozygous individuals, with or without clinical androgen excess, to investigate the utility of the SDSST to identify heterozygous *CYP21A2* carriers and to determine the cut-off levels of 17-OHP for this purpose.

## Methods

### Search Strategy

PubMed and MEDLINE databases were searched for relevant literature. The search strategy was kept broad, included several synonymous expressions, and performed using the keywords ("21-hydroxylase" OR "*CYP21A2*" OR "21 α-hydroxylase" OR "CYP21") AND "heterozygous" AND ["hirsutism" OR "hyperandrogenemia" OR "polycystic ovary syndrome" OR "PCOS" OR "acne" OR "alopecia" OR "oligomenorrhea" OR "adrenal hyperplasia" OR "adrenocorticotropic hormone (ACTH)]". Only peer-reviewed, original articles were included in the study. Additional publications from the references of the included studies were manually searched by the investigators to identify the articles that may be missed by the electronic search.

### Study Selection

Human studies, published between January 1995 and May 2020, were considered further. Studies without control groups were excluded, as well as those written in languages other than English. Since genetic mutation screening performed before 1995 was based on human leukocyte antigen (HLA) typing in most cases, studies performed before this date were excluded from the search criteria.

In the publications included in the study, mutation analyses were performed using a range of methods, including amplification-refractory mutation system, allele-specific oligonucleotide hybridization, Sanger sequencing, single-strand conformation polymorphism, multiplex ligation-dependent probe amplification, Southern Blot, sequence-specific oligonucleotide probes, real-time quantitative reverse transcription-polymerase chain reaction and multiplex mini-sequencing. 17-OHP measurement was done using radioimmunoassay (RIA), enzyme-linked immunosorbent assay, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods.

The selection criteria were: 1) case-control studies; 2) studies that evaluated the relationship between basal and/or stimulated 17-OHP levels after SDSST in *CYP21A2* carriers and non-carriers with one of the clinical hyperandrogenic symptoms including hirsutism, and/or oligo/amenorrhea

and/or acne and/or elevation of at least one serum androgen; 3) useable data on 17-OHP levels to identify 21OH deficiency in patients with PP, PA and premature thelarche (PT); and 4) studies focused on differential diagnosis between NCAH and PCOS. The exclusion criteria were: 1) case or family reports; 2) studies that evaluated genetically confirmed CAH and NCAH patients; 3) studies focused on diagnosis, treatment, review, method and general information; and 4) studies related to other diseases including Cushing syndrome, acromegaly, adrenal tumor, 11-hydroxylase, cytochrome P450 oxidoreductase (*POR*), 3-beta (β)-hydroxysteroid dehydrogenase (*HSD3B2*), and 17α-hydroxylase/17,20-lyase (*CYP17A1*) deficiencies (Figure 1).

### Data Extraction and Analysis

Two authors independently screened the title, abstract and full text of potentially eligible studies twice at two different time points. Any disagreements were resolved by discussion or by seeking an independent third opinion. The titles and abstracts of the articles were examined and irrelevant ones were excluded. The full texts of the remaining articles were reviewed to find relevant studies that met the inclusion criteria. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline is aimed at improving systematic reviews and formed the basis for the selection protocol used in the current study (Figure 1).

### Statistical Analysis

For all meta-analyses, Review Manager (2014) version, 5.3 was used. A random-effects model and fixed-effect model were used while performing the meta-analyses. Due to the large degree of heterogeneity, a random-effects model was applied, which does not adjust heterogeneity but it is a more conservative approach when the heterogeneity exists. Summary statistics were reported as standardized mean difference (SMD) and mean difference (MD) with 95% confidence intervals (CI). SMD levels of $<0.2$, $>0.2$ and $<0.7$, or $>0.8$ were considered small and moderate, or large effects, respectively (26). Study-level mean (standard deviation; SD) and median (minimum-maximum) levels were reported. Study-level mean levels were used to generate receiver operating characteristic (ROC) curve using Statistical Package for the Social

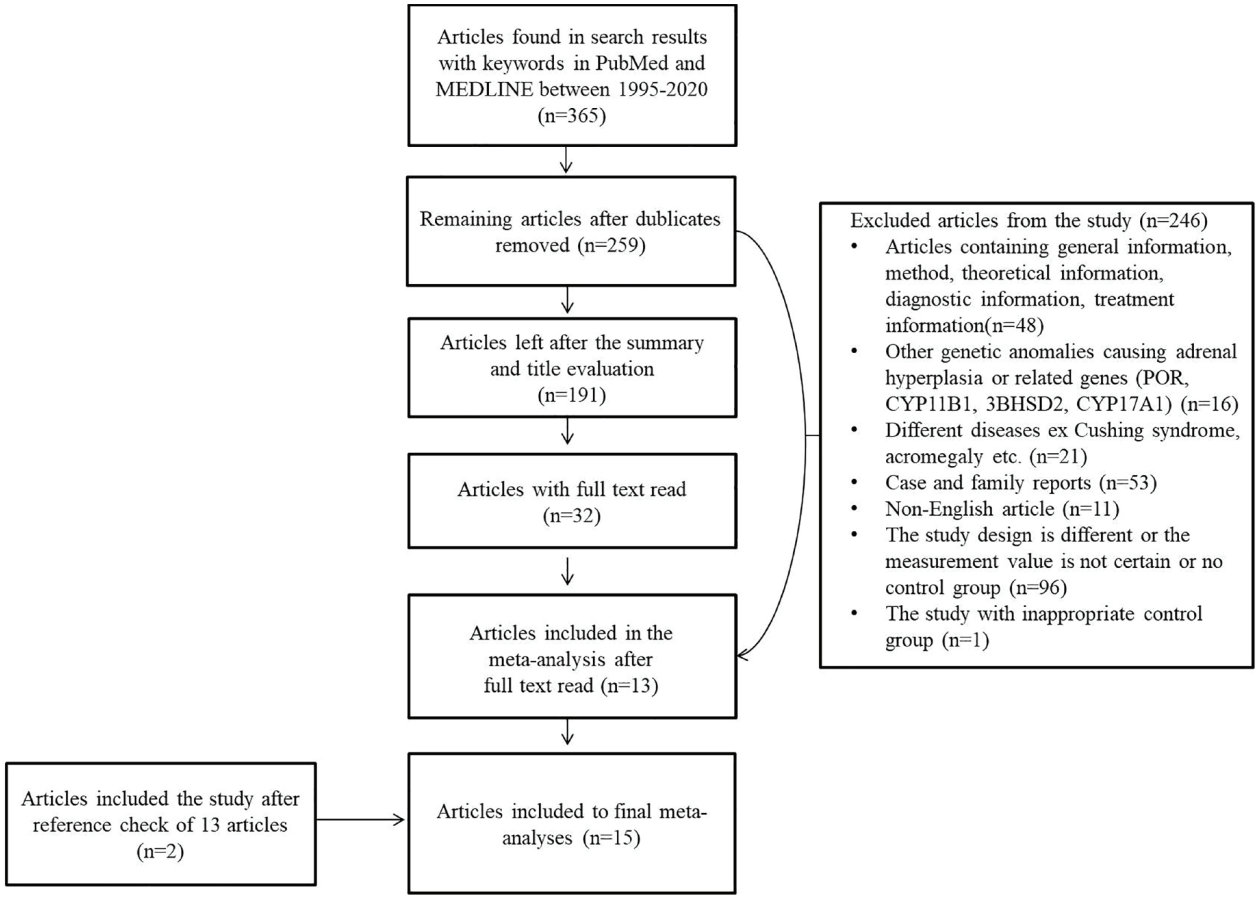


**Figure 1.** Flow chart to illustrate the process by which articles were selected or rejected based on the inclusion and exclusion criteria of the study

Sciences, version 22 (IBM Inc., Armonk, NY, USA) (27). When cut-off values were determined, Youden's index was used and diagnostic accuracy measures are reported.

To convert all reported results to a standard units of measurement (nmol/L) in all the included articles, the 17-OHP values were multiplied by 0.0303 and 3.0261 to convert ng/dL to nmol/L and ng/ml to nmol/L, respectively.

## Patients

Individuals from both "female and male" gender, who were *CYP21A2* heterozygous mutation carriers and non-carriers and aged between 0.7-65 years, were included in the study (Table 1). The study groups consisted of females and/or males with PCOS, PP, PA, PT and clinical hyperandrogenism, relatives of patients with CAH or NCAH, and healthy controls.

**Table 1. Main characteristics of the included studies**

| Author | Year | Nationality | Age range (year) | Gender | Case (21-HTZ) | Control (mutation free) | Mutation analyses method | 17-OHP measurement method |
|---|---|---|---|---|---|---|---|---|
| Oriolo et al (34) | 2020 | Italy | HTZ: 22.2 + 7.2<br>Control: 24.0 + 9.2 | F | 15 (PCOS diagnosis) | 32 (PCOS diagnosis) | Sanger sequencing | RIA |
| Polat et al (21) | 2019 | Turkey | Range: 18-45 | F | 14 (PCOS diagnosis) | 40 (PCOS diagnosis) | Sanger sequencing | RIA |
| Grodnitskaya and Kurtser (40) | 2017+ | Russia | HTZ: 26.4 ± 5.3<br>Control: 26.1 ± 7.2 | F | 7 (one of the clinical androgenic symptoms (oligo/amenorrhea, hirsutism or acne) and/or elevation of at least one serum androgen) | 43 (one of the clinical androgenic symptoms (oligo/amenorrhea, hirsutism or acne) and/or elevation of at least one serum androgen) | ARMS, MLPA | ELISA |
| Neocleous et al (30) | 2017 | Greek Cypriot | Not stated | F | 52 (clinical hyperandrogenism) | 52 (clinical hyperandrogenism) | Sanger sequencing, MLPA | RIA |
| Settas et al (31) | 2013+ | Greece | HTZ: 23.4 ± 5.4<br>Control: 29.5 + 5.5 | F | 15 (PCOS diagnosis) | 68 (PCOS diagnosis) | ARMS + Sanger sequencing | RIA |
| Napolitano et al (35) | 2011* | Italy | Not stated | F + M | 161 (relatives of patients with CAH and NCAH) | 73 (relatives of patients with CAH and NCAH and healty volunteers) | Southern Blot, Multiplex minisequencing, LR-PCR | RIA |
| Costa-Barbosa et al (37) | 2010* | Brazil | HTZ: 23-62<br>Control: 23-65 | F + M | 61 (parents of affected patients with 21OHD) | 27 (healthy volunteers) | ARMS, MLPA | LC-MS/MS |
| Paris et al (38) | 2010 | France | HTZ: 6.8 + 0.7<br>Control: 6.7 + 1.3 | F | 8 (PP diagnosis) | 25 (PP diagnosis) | Sanger sequencing | RIA |
| Bidet et al (39) | 2009* | France | 23.4 + 8.8 (range: 13-52) | F | 211 (relatives of patients with CAH and NCAH) | 36 (relatives of patients with CAH and NCAH) | Sanger sequencing, Southern Blot, RT-qPCR | RIA |
| Admoni et al (41) | 2006 | Israel | Not stated | F + M | 24(PP and/or hirsutism, and/or premature telarche) | 43 (hirsutism, PP, precocious puberty, menstrual irregularity) | SSOP | RIA |
| Bachega et al (36) | 2000 | Brazil | Not stated | F + M | 13 (precocious pubarche, acne, hirsutism and/or menstrual irregularities) | 8 (precocious pubarche, acne, hirsutism and/or menstrual irregularities) | ARMS | RIA |
| Dacou-Voutetakis and Dracopoulou (15) | 1999 | Greece | Not stated | F + M | 18 (premature adrenarche) | 26 (premature adrenarche) | ARMS, Southern Blot | RIA |

**Table 1. Continued**

| Author | Year | Nationality | Age range (year) | Gender | Case (21-HTZ) | Control (mutation free) | Mutation analyses method | 17-OHP measurement method |
|---|---|---|---|---|---|---|---|---|
| Witchel and Lee (33) | 1998* | USA | Not stated | F + M | 28 (relatives of patients with 21-hydroxylase deficiency) | 23 (healthy control and relatives of patients with 21-hydroxylase deficiency) | ASOH, SSCP | RIA |
| Witchel et al (32) | 1997[a] | USA | Not stated | F + M | 28 (relatives of patients with 21OHD) | 22 (healthy control and relatives of patients with 21-hydroxylase deficiency) | ASOH, SSCP | RIA |
| Witchel et al (18) | 1997[b#] | USA | Not stated | F + M | 10 (PP diagnosis) | 18 (PP diagnosis) | ASOH, SSCP | RIA |
| Witchel et al (18) | 1997[b#] | USA | Not stated | F + M | 4 (clinical hyperandrogenism) | 10 (clinical hyperandrogenism) | ASOH, SSCP | RIA |

*Shows the study included in the analysis group formed from asymptomatic heterozygous carriers and asymptomatic mutation-free controls, [+]shows the study that only had basal 17-OHP levels [#]shows the same study used two times in the same analyses with two different data set.
F: female, M: male, RIA: radioimmunoassay, LC-MS/MS: liquid chromatography with tandem mass spectrometry, ELISA: enzyme-linked immunosorbent assay, SSCP: single-strand conformation polymorphism, ASOH: allele-specific oligonucleotide hybridization, SSOP: sequence-specific oligonucleotide probes, ARMS: amplification-refractory mutation system, MLPA: multiplex ligation-dependent probe amplification, RT-qPCR: quantitative polymerase chain reaction, LR-PCR: long range PCR, 21-HTZ; CYP21A2 heterozygous, HTZ: heterozygous, PCOS: polycystic ovary syndrome, CAH: congenital adrenal hyperplasia, NCAH: non-classic CAH, PP: premature pubarche, USA: United States of America

ΩMutation analysis of the *CYP21A2* gene and SDSST were applied to all volunteers participating in the study.

### Heterogeneity and Publication Bias

Between-study variability was compared for within-study variability (i.e., heterogeneity of effect size) using the $I^2$ statistic, which measures the percentage of variation due to heterogeneity (28). An $I^2$ level less than 25% indicated low heterogeneity, whereas levels between 35 to 50% showed moderate heterogeneity and those above 50% showed high heterogeneity (28). Publication bias was assessed using contour-enhanced funnel plots (29).

## Results

### Search Results

Three hundred and sixty-five relevant publications were found after the screening of studies published between 1995 and 2020. After excluding repetitive and irrelevant publications, fifteen high-quality, peer-reviewed publications that met inclusion criteria were included in the meta-analysis. These studies were carried out in Europe, the United States of America, and Russia. Three studies were carried out in Greece (15,30,31), three in the United States of America (18,32,33), two in Italy (34,35), two in Brazil (36,37), two in France (38,39), one in Turkey (21), one in Russia (40) and one in Israel (41). *CYP21A2* mutation analysis and basal and/or stimulated 17-OHP measurements were available in all study subjects. The SDSST was performed in patients by administration of a single, intravenous dose of 0.25 mg synacthen (synthetic ACTH). Measurements of the basal and stimulated serum 17-OHP levels were done after 30 or 60 minutes of synacthen administration.

Among the included studies, two were added to the relevant sub-analysis since they only included basal 17-OHP measurements (31,40). Two different control groups were identified in the included studies; a mutation-free asymptomatic healthy volunteer group (asymptomatic heterozygous vs. asymptomatic mutation-free healthy control), and a mutation-free but clinically symptomatic volunteer group (symptomatic heterozygous vs. symptomatic mutation-free control). Therefore, these two control sub-groups were analysed in two separate analyses to make clear discrimination. In a study, since 17-OHP levels were given separately based on two different clinical findings, the same study was included twice with different data sets (18). Whereas 17-OHP level measured in a publication was remarkably high in both groups compared to given 17-OHP levels in other publications used in the meta-analysis, the study was included in the analysis since it met the inclusion criteria (36). One thousand, two hundred and fifteen subjects including 21OH-heterozygous carriers (n = 669) and mutation-free controls (n = 546) were included in the meta-analysis (Table 1).

## Comparison of Basal and Stimulated 17-OHP Levels

### Symptomatic Heterozygous vs. Symptomatic Mutation-free Control

The fixed-effects model was used with basal 17-OHP levels because the heterogeneity of the studies was low ($I^2 = 0\%$, $p = 0.620$). In the symptomatic heterozygous carriers, the level of the MD was found to be higher than symptomatic mutation-free controls (MD: 0.70 nmol/L, 95% CI: 0.21-1.18, $Z = 2.81$, $p = 0.005$). When the MD was standardized, the difference was determined to have a medium effect size (SMD: 0.33 nmol/L, 95% CI: 0.14-0.51, $Z = 3.42$, $p < 0.001$) (Figure 2).

When comparing the stimulated levels of 17-OHP in symptomatic heterozygous carriers and symptomatic mutation-free controls, the random-effects model was used due to significant heterogeneity ($I^2 = 72\%$, $p < 0.001$). The MD were found to be higher in heterozygous carriers (MD: 7.20 nmol/L, 95% CI: 5.15-9.25, $Z = 6.87$, $p < 0.001$). The SMD (SMD: 0.9 nmol/L, 95% CI: 0.46-1.34, $Z = 4.01$, $p < 0.001$) shows that the difference has a large effect size (Figure 2).

The ROC curves for symptomatic heterozygous and symptomatic mutation-free controls are shown in Figures 3A, 3B, demonstrating that stimulated 17-OHP provides good discrimination (area under ROC curve = 0.80, $p = 0.034$) between symptomatic heterozygous and symptomatic mutation-free controls, with an optimal cut-off of 13.41 nmol/L, yielding a sensitivity of 100% and a specificity of 66.7%. Basal 17-OHP level was not found to be capable of discriminating heterozygous from wild type in the symptomatic group (Table 2).

### Asymptomatic Heterozygous vs. Asymptomatic Mutation-free Healthy Control

The fixed-effects model was used in the analysis of basal 17-OHP levels in asymptomatic heterozygous carriers with the asymptomatic mutation-free healthy controls due to the low heterogeneity of the studies ($I^2 = 0\%$, $p = 0.53$). Basal 17-OHP level was higher in asymptomatic heterozygous carriers than in asymptomatic mutation-free healthy controls (MD: 0.62 nmol/L, 95% CI: 0.20-1.04, $Z = 2.92$, $p < 0.001$). The SMD in the groups was 0.27 nmol/L (SMD: 0.27, 95% CI: 0.10-0.45, $Z = 3.04$, $p = 0.002$), and this difference was determined to have a medium effect size (Figure 4).

When comparing the stimulated level of 17-OHP in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, the random-effects model was used due to significant heterogeneity ($I^2 = 85\%$, $p < 0.001$). The 17-OHP level was higher in asymptomatic

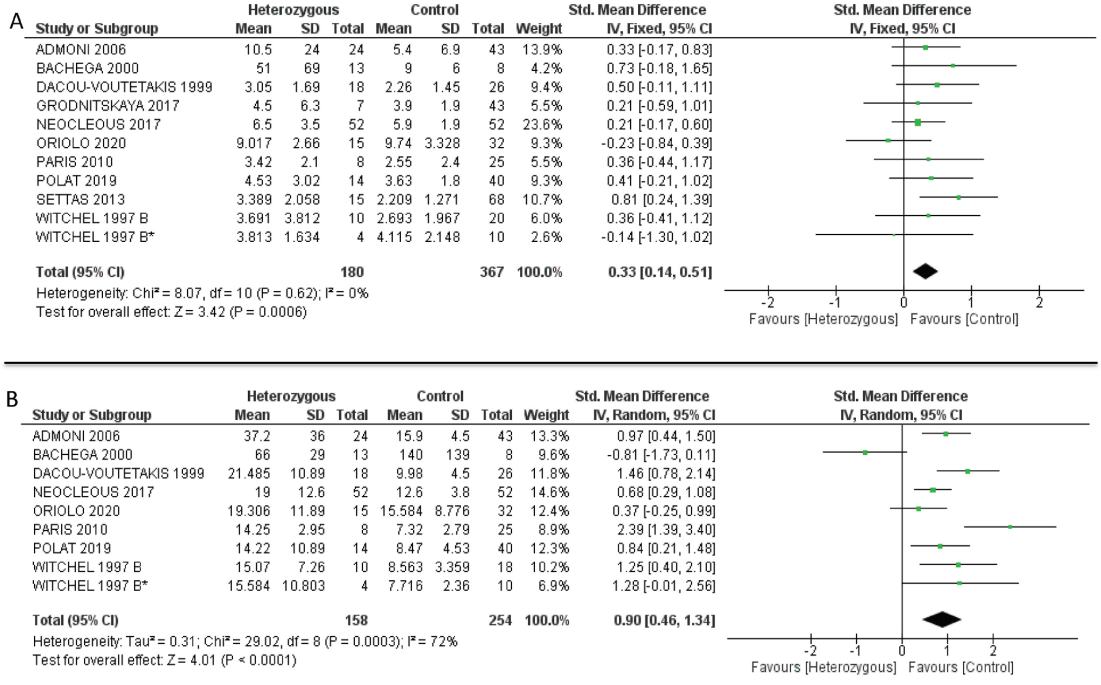


**Figure 2.** Forest plot of 17-hydroxyprogesterone response to standard dose synacthen stimulation test of symptomatic heterozygous vs. symptomatic mutation-free volunteers

**Shows the same study used two times in the same analyses with two different data set*

*CI: Confidence interval, SD: Standard deviation*

heterozygous carriers than in asymptomatic mutation-free healthy controls (MD: 7.57 nmol/L, 95% CI: 6.82-8.32). The SMD in the groups was 1.34 nmol/L (SMD: 1.34, 95% CI: 0.81-1.87, Z = 4.99, p < 0.001), and the effect size for this difference was similar to the symptomatic heterozygous vs. symptomatic mutation-free controls comparison (Figure 4).

The ROC curves for asymptomatic heterozygous and asymptomatic mutation-free healthy controls are shown in Figures 3C, 3D, demonstrating that stimulated 17-OHP provided good discrimination (area under ROC curve = 1.0, p = 0.009) between asymptomatic heterozygous and asymptomatic mutation-free healthy control with an optimal cut-off of 10.48 nmol/L, yielding a sensitivity of 100% and a specificity of 100%. Basal 17-OHP level was not found to be capable of discriminating heterozygous from wild type in the asymptomatic group (Table 2).

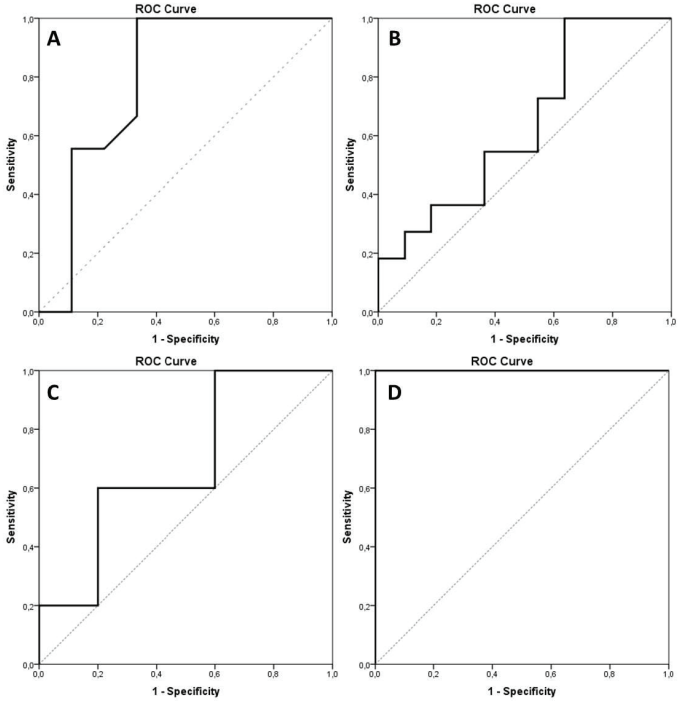


**Figure 3.** Receiver operating characteristic-curve analysis for basal and stimulated 17-hydroxyprogesterone (17-OHP) levels in symptomatic and asymptomatic groups. A: Basal 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers B: Stimulated 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers C: Basal 17-OHP levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers D: Stimulated 17-OHP of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers

### Median and Mean of Basal and Stimulated 17-OHP Levels

Study-level median and mean were calculated, after elimination of the publication with extreme basal and stimulated 17-OHP levels (36). Basal 17-OHP median (range) levels were 4.156 (3.05-10.5) nmol/L and 3.90 (2.20-9.74) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP mean ± SD levels were 5.24 ± 2.59 nmol/L and 4.67 ± 2.62 nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP median levels were 17.29 (14.22-37.2) nmol/L and 9.27 (7.32-15.9) nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Stimulated 17-OHP mean levels were 19.51 ± 7.63 nmol/L and 10.77 ± 3.48 nmol/L in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively. Basal 17-OHP median levels were 3.21 (2.64-4.78) nmol/L and 3.12 (1.82-3.6) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Basal 17-OHP mean levels were 3.33 ± 0.84 nmol/L and 2.84 ± 0.71 nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. Stimulated 17-OHP median levels were 14.16 (12.73-16.37) nmol/L and 6.26 (4.9-8.23) nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively (Figure 5). Stimulated 17-OHP mean levels were 14.16 ± 1.37 nmol/L and 6.48 ± 1.2 nmol/L in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively.

### Heterogeneity and Publication Bias

Funnel plots were drawn in both fixed and random-effect models to determine whether there was a publication

**Table 2. The cut-off value for predicting heterozygous individuals with standard dose synacthene stimulation test**

| SDSST | AUC | 95% CI for AUC | p | Cut-off value* | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| Basal 17-OHP symptomatic | 0.67 | 0.39-0.87 | 0.279 | - | - | - |
| Stimulated 17-OHP symptomatic | 0.80 | 0.57-1.0 | 0.034 | 13.41 | 100 | 66.7 |
| Basal 17-OHP asymptomatic | 0.68 | 0.33-1.0 | 0.347 | - | - | - |
| Stimulated 17-OHP asymptomatic | 1.0 | 1.0-1.0 | 0.009 | 10.48 | 100 | 100 |

*ROC curve analysis from study-level data.
SDSST: standard dose synacthen stimulation test, AUC: area under ROC curve, CI: confidence interval, 17-OHP: 17-hydroxyprogesterone, ROC: receiver operating characteristic

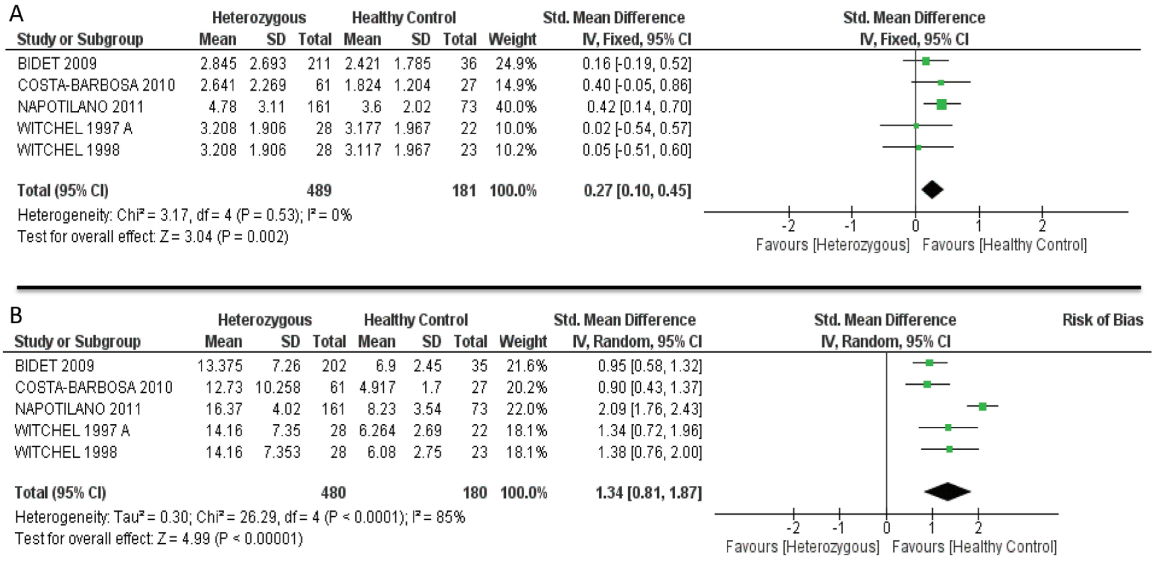


**Figure 4.** Forest plot of 17-hydroxyprogesterone response to standard dose synacthen stimulation test in asymptomatic heterozygous vs. asymptomatic mutation-free healthy controls

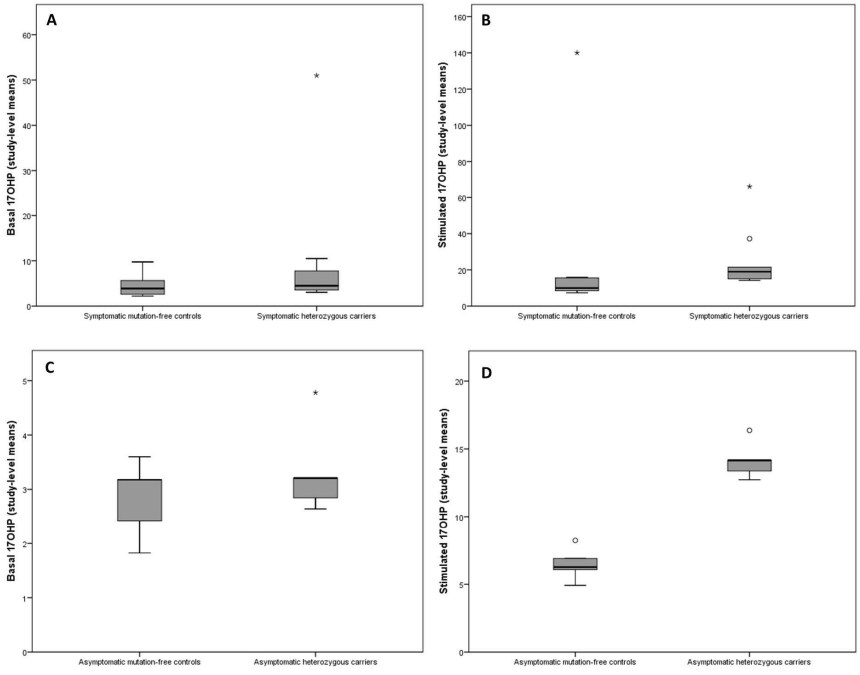


**Figure 5.** Box plot for median of basal and stimulated 17-hydroxyprogesterone levels in symptomatic and asymptomatic groups

bias in the included papers. In both models, CI were also presented in the funnel plot. As funnel plots seemed almost symmetrical in all meta-analyses, it was concluded that publication bias was weak (Figure 6).

## Discussion

Measurement of serum 17-OHP was introduced in 1968 (42), and it is now used most widely for the diagnosis of adrenal enzymatic defects (43) in combination with SDSST, a gold standard and commonly used biochemical test in the evaluation of adrenal gland function. The SDSST for evaluation of adrenal gland function has been investigated in various clinical conditions, such as pre-clinical Addison's disease (44), immediately after pituitary surgery (45), patients with primary hypothyroidism (46), and in patients with primary fibromyalgia syndrome (47), in women with PCOS (48) and hirsutism and/or oligomenorrhea (49),

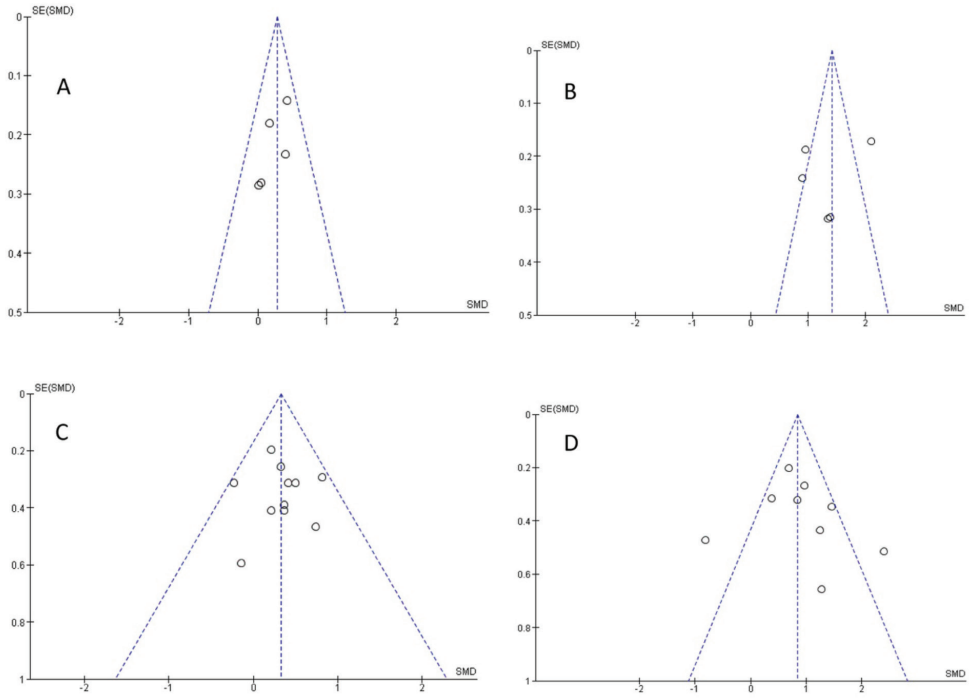


**Figure 6.** Funnel plots to detect publication bias of the meta-analysis. A: Basal 17-hydroxyprogesterone (17-OHP) levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers B: Stimulated 17-OHP levels of asymptomatic heterozygous vs. asymptomatic mutation-free healthy volunteers C: Basal 17-OHP levels of symptomatic heterozygous vs. symptomatic mutation-free volunteers D: Stimulated 17-OHP of symptomatic heterozygous vs. symptomatic mutation-free volunteers. The standardized mean difference (SMD) on the x-axis is plotted against the standard error of the SMD on the y-axis. Symmetrical distribution of studies indicates the absence of publication bias

in adolescents with PP (15), and in patients with CAH or NCAH (50). Compared to normal female individuals, female carriers of 21-OHD frequently demonstrate an exaggerated secretion of the 21-OH precursor 17-OHP after ACTH administration (51). It has been reported that 50-80% of carriers exhibit 17-OHP levels above the 95$^{th}$ percentile of the control level after ACTH stimulation (51,52,53).

To our knowledge, this is the first meta-analysis of differences in 17-OHP responses to SDSST in heterozygous and mutation-free symptomatic and asymptomatic volunteers, considering only high-quality studies with the same SDSST criteria and *CYP21A2* mutation analyses at a molecular level excluding HLA typing. In the literature, SDSST was recommended when the basal 17-OHP ≥6 nmol/L. So 21OHD-NCAH was unlikely in cases with lower basal 17-OHP has become widely accepted. Possible heterozygote carrier status was considered for the patients with baseline 17-OHP >6 nmol/L or those with baseline 17-OHP <6 nmol/L and ACTH stimulated 17-OHP <30 nmol/L (54,55). Stimulated 17-OHP >30 nmol/L was considered as the criterion for 21OH deficiency-related NCAH (56). In another study, patients were considered to be heterozygote carriers of 21OHD with ACTH-stimulated 17-OHP concentrations between 12.1 nmol/L and 30.2 nmol/L (57). In yet another study, stimulated 17-OHP levels >45 nmol/L after ACTH stimulation were suggested to have NCAH while levels between 15 and 45 nmol/L were suggested to be probable mutation carriers and levels below 15 nmol/L were interpreted as normal (41).

In our study, MD and SMD of the basal 17-OHP were 0.7 nmol/L and 0.33 nmol/L in the symptomatic group while MD and SMD in the asymptomatic group were 0.62 nmol/L and 0.27 nmol/L compared to the control group, respectively. SMD levels in both groups were slightly higher than the small effect size limit of <0.2. In our meta-analyses, in the asymptomatic group, both the basal median 17-OHP level and the SMD level were found to be lower than in the symptomatic group. The authors of some of the included studies had attempted to identify a cut-off level for basal 17-OHP that will exclude the diagnosis of NCAH and avoid unnecessary SDSST, especially for countries where synthetic ACTH is not widely available (58). Temeck et al (59) found that 13-14% of patients with NCAH would be missed if a basal 17-OHP level of 6 nmol/L

was used. Escobar-Morreale et al (60) proposed a cut-off level of 5.1 nmol/L with 100% sensitivity and 88.6% specificity in a cohort of women with hyperandrogenism. Leite et al (11) showed that basal level of 17-OHP >3 nmol/L was sufficient for the diagnosis of NCAH. Gönç et al (61) determined that only one of the NCAH cases would be missed when 4.69 nmol/L was used as the basal 17-OHP cut-off level in patients with PA and these authors also suggested that including the patient's clinical phenotype in the evaluation of the basal 17-OHP level can increase the accuracy of the SDSST. On the other hand, there is no consensus for identification of heterozygosity, and so 17-OHP levels between those of NCAH and normal are accepted as indicating heterozygousity (41).

In these meta-analyses, MD and SMD of the stimulated 17-OHP were 7.2 nmol/L and 0.9 nmol/L in the symptomatic group, and 7.57 nmol/L and 1.34 nmol/L in the asymptomatic group, respectively. SMD levels in both groups were higher than the large effect size limit of >0.8. The stimulated 17-OHP median levels were determined to be 17.29 nmol/L (14.22-37.2) and 9.27 nmol/L (7.32-15.9) in symptomatic heterozygous carriers and symptomatic mutation-free controls, respectively, and median stimulated 17-OHP levels were 14.16 nmol/L (12.73-16.37) and 6.26 nmol/L (4.9-8.23) in asymptomatic heterozygous carriers and asymptomatic mutation-free healthy controls, respectively. ROC analysis showed that the basal 17-OHP level was not discriminative in both symptomatic and asymptomatic groups, but the stimulated 17-OHP level was informative. The cut-off level for the asymptomatic heterozygous individuals was 10.48 nmol/L, while the cut-off level for the symptomatic group was 13.41 nmol/L. Both the cut-off levels were lower than 15 nmol/L, which were interpreted as normal. Besides, the stimulated 17-OHP median level of symptomatic mutation-free controls was higher than that of asymptomatic mutation-free healthy controls (9.27 vs. 6.26 nmol/L). Similarly, the stimulated 17-OHP median level of symptomatic heterozygous carriers was higher than that of asymptomatic heterozygous carriers (17.29 vs. 14.16 nmol/L). The stimulated 17-OHP level of the symptomatic group was in the heterozygous range (>15 nmol/L and <45 nmol/L), while it was below the level considered heterozygous (<15 nmol/L) in the asymptomatic group. This result supports the hypothesis that clinical phenotype effects the test. Similarly, Admoni et al (41) made a comparison between symptomatic heterozygous and family member carriers, which revealed that the symptomatic carriers had a significantly higher ACTH-stimulated 17-OHP than family member carriers.

*CYP21A2* gene mutation is not the only factor causing androgen excess symptoms. Other hormones or genes may also play a role, resulting in a similar clinical phenotype. Additionally, both adrenal and gonadal steroid hormone biosynthesis is a complex phenomenon, regulated by the feedback mechanism between different tissues and dozens of genes belonging to different gene families. The biosynthetic pathways have not been fully elucidated yet and there is still much to be understood (62).

The *CYP21A2* heterozygous mutation carrier should be considered in the differential diagnosis of hyperandrogenic symptoms (63). Determination of carrier status is also compulsory for genetic counselling of the parents affected by CAH/NCAH and in families that includes a parent with confirmed heterozygous mutation, since genetic counselling plays an important role in the control of genetic diseases. The heterozygous individuals may be diagnosed with NCAH due to false-positivity of the SDSST, especially in V281L heterozygous mutation (21). Therefore, there is a need for up-to-date studies on the specificity and sensitivity of the SDSST to distinguish *CYP21A2* carriers and 21OHD-NCAH.

The V281L mutation is compatible with the NCAH allele and the resulting protein exhibits 30-50% residual enzymatic activity (64). It has been shown that the ACTH test result gave a level close to the cut-off level used for NCAH in heterozygous V281L mutation (21,41). Escobar-Morreale et al (23) hypothesized one possible explanation for the abnormal 21-hydroxylase function in subjects with one normal allele and a "half functioning" allele was a dominant-negative mutation. This is postulated to happen because the product of a mutation adversely affects the wild-type gene product within the same cell, as seen in different diseases such as familial hypertrophic cardiomyopathy (65) and alfa-mannosidosis (66).

Considering the developing technology, more precise basal and stimulated 17-OHP cut-off levels can be identified because it will be possible to combine genetic analysis, now more widely available and less expensive, with more precise hormone measurements obtained through LC/MS-MS. This kind of work can be valuable under conditions in which hormone determinations are possible but access to genetic testing is limited due to financial restrictions of health care systems or health insurance. Moreover, the SDSST is faster and cheaper than genetic analyses. We suggest that adding the clinical phenotype and the type of mutation to basal and stimulated 17-OHP evaluation may increase the accuracy of the test and yield better results.

### Study Limitations

Our study had a few limitations. Firstly, the included articles did not report data separately by gender. Secondly, the ages of the subjects in the included publications varied widely. Thirdly, copy number variation of *CYP21A2* was not investigated in all studies included. Fourthly, study assays, number of individuals and 17-OHP units differed between included studies. Our study also had some strengths. Firstly, a genetic analysis was performed in all the subjects. Secondly, almost all 17-OHP measurements were performed by using the same hormone measurement method (RIA), with the exception of two studies (37,40).

## Conclusion

In conclusion, this meta-analysis supports the idea that stimulated 17-OHP level has the potential to identify *CYP21A2* carriers by SDSST, although basal 17-OHP level may not be informative. ROC curve analysis from study-level data may produce some bias, and so the cut-off values from our results should be used with caution. In addition, the SDSST needs further investigation to increase specificity and sensitivity to determine heterozygosity. A greater increase in stimulated 17-OHP levels was found in mutation-free symptomatic individuals than those who were asymptomatic. Therefore, we suggest that individuals might be better evaluated by SDSST by considering the clinical phenotype and type of mutation to increase the accuracy of the test.

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### Ethics

**Ethics Committee Approval:** This study is a meta-analysis study.

**Informed Consent:** This study is a meta-analysis study.

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

### Authorship Contributions

Surgical and Medical Practices: Seher Polat, Concept: Seher Polat, Yusuf Kemal Arslan, Design: Seher Polat, Yusuf Kemal Arslan, Data Collection or Processing: Seher Polat, Yusuf Kemal Arslan, Analysis or Interpretation: Seher Polat, Yusuf Kemal Arslan, Literature Search: Seher Polat, Yusuf Kemal Arslan, Writing: Seher Polat.

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# Serum Neudesin Levels in Obese Adolescents

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### What is already known on this topic?

Neudesin is a neurotropic factor that contributes to the complex control of energy homeostasis. In addition, neudesin can influence appetite control in the hypothalamus or the anxiety-like behaviour controlled by the dentate gyrus of the hippocampus.

### What this study adds?

We assessed the relationship between obesity, metabolic complications and neudesin levels in adolescents. Neudesin levels were significantly decreased in obese and morbidly obese adolescents. Neudesin may have a significant potential role in the regulatory mechanisms of obesity and other metabolic disorders.

## Abstract

**Objective:** Advances in knowledge of neurotrophic factors are now revealing the complex control of energy homeostasis and appetite, as well as the crucial role these factors play in nervous system function. The aim of this study was to assess serum levels of neudesin in adolescents with obesity and to examine the relationship between these levels and metabolic outcomes.

**Methods:** Adolescents, aged 10-17 years were enrolled. Subjects were divided into normal weight, obese and morbidly obese subgroups. Serum neudesin concentrations were compared between the groups.

**Results:** In total, 88 adolescents were recruited, of whom 30 (34.1%) were normal weight, 15 (17.0%) were obese and 43 (48.9%) were morbidly obese. Neudesin levels were significantly lower in obese adolescents than in the control group ($p = 0.013$). A correlation analysis applied to the whole study group revealed a negative correlation between serum neudesin concentration and body mass index (BMI) z scores ($r = -0.40$, $p < 0.001$). Serum neudesin levels tended to increase in adolescents with metabolic syndrome, insulin resistance, dyslipidaemia, and hypertension but the differences were not significant ($p = 0.259$, $p = 0.246$, $p = 0.259$, and $p = 0.523$, respectively).

**Conclusion:** Serum neudesin levels were significantly correlated with BMI z score in obese adolescents. Generally, serum neudesin levels were low in obese and morbidly obese adolescents and tended to increase with the appearance of metabolic disorders. Both obesity and associated metabolic disorders have multifactorial causes. Therefore, we suggest that the role of the neudesin molecule in the regulatory mechanisms of obesity and metabolic disorders should be further investigated with well-designed studies enrolling larger groups.

**Keywords:** Obesity, neudesin, insulin resistance, metabolic syndrome

## Introduction

Obesity in children and adolescents has increased worldwide in the last 30 years. The most important reasons for this increase are the decrease in physical activity in children and the negative effects of wider use of developing technology and poorer eating and drinking habits (1). The epidemic of obesity is closely associated with an increase in metabolic disorders, including diabetes, dyslipidemia and cardiovascular diseases, and this has prompted major interest in the regulation of adipose functions.

Adipose tissue can be divided into two distinct types: white and brown. White adipose tissue (WAT) is specialised for the storage of excess energy as triglycerides, whereas brown adipose tissue (BAT) dissipates energy as heat, thereby counteracting obesity. The regulation of adipose tissue function depends on the sympathetic nervous system

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(SNS), which plays a fundamental role in maintaining energy homeostasis in living organisms (2). The SNS also modulates the development of obesity because it stimulates lipolysis in WAT and enhances heat production in BAT by activating adrenergic signalling (3).

The functioning of the SNS depends on neurotrophic factors which promote the survival, differentiation and maintenance of neurons in the vertebrate nervous system (4,5,6). One newly identified neurotropic factor is neudesin, a compound that contributes to the complex control of energy homeostasis. Human neudesin is a 172 amino acid protein that shares a high sequence similarity to neudesin found in other vertebrates. Neudesin activates the mitogen-activated protein kinase and phosphoinositide 3-kinase signalling pathways and has a role in neural cell differentiation, cell proliferation and tumorigenesis (7). Neudesin is preferentially expressed in the central nervous system and the spinal cord, where it promotes neural cell differentiation. Neudesin mRNA expression has also been documented in other tissues, including adipose tissue, heart, lungs, and kidney. In addition to its neurotropic effects, neudesin can influence appetite control in the hypothalamus or the anxiety-like behaviour controlled by the dentate gyrus of the hippocampus (3).

Neudesin has been examined in only a limited number of studies to date, but the current consensus is that neudesin may have potential for the treatment of obesity and obesity-related disorders (3,7). The aim of the present study was to investigate the levels of circulating serum neudesin in a group of adolescents with obesity and to examine the relationship between these levels and metabolic outcomes that develop due to obesity.

## Methods

### Participants

This prospective study included adolescents aged 10 to 17 years. Of these adolescents, around two thirds were referred to the paediatric endocrinology outpatient clinic because of weight gain and were diagnosed with obesity. The other third were healthy age- and sex-matched children who served as the control group. Exclusion criteria were: chronic or hereditary diseases; endocrinological disorders including syndromes associated with obesity; and a history of drug use. Pubertal stage was assessed according to the criteria described by Marshall and Tanner (8).

A challenge in determining the prevalence of metabolic syndrome (MS) is the existence of multiple definitions and criteria used to identify this condition. In response, the International Diabetes Federation (IDF) released the IDF Consensus Worldwide Definition of MS as a single, universally accepted tool. The IDF defines MS in children and adolescents as the presence of abdominal obesity (waist circumference ≥90$^{th}$ percentile by age and sex) and the presence of two or more of the following clinical features: an elevated triglyceride level (≥1.7 mmol/L), a low high-density lipoprotein cholesterol (HDL-C) level (< 1.03 mmol/L), high blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg and/or a diagnosis of hypertension) and an elevated glucose level (≥5.6 mmol/L and/or a diagnosis of type 2 diabetes) (9). Written informed consent was obtained from the children's parents after they were informed of the aim and procedures of the study. The study was approved by the Tekirdağ Namık Kemal University University of Non-Invasive Clinical Research Ethics Committee (protocol number: 2019.137.08.09, date: 01.08.2019).

### Study Design

The height of each participant was measured using a wall-mounted stadiometer sensitive to the nearest 0.1 cm (Harpenden, Holtain, Crymych, UK) and with the adolescent in a standing position, without shoes. Weight was measured using a portable calibrated scale sensitive to the nearest 0.1 kg (SECA762; Voge & Hakle, Hamburg, Germany), with the participants wearing light clothing. The body mass index (BMI) was calculated as weight (kg) divided by height ($m^2$). The height, weight and BMI were expressed as standard deviation scores (SDS) using the 2007 growth reference percentiles for Turkish children and adolescents (10). Children were divided into three groups - normal weight, obese, or morbidly obese (11). Blood pressure was measured using an automated sphygmomanometer. Elevated blood pressure (≥95$^{th}$ percentile for height) was determined using tables provided by the Task Force Report (12).

### Statistical Analysis

The Kolmogorov-Smirnov test was used to assess normality of data distribution from the control and patient groups. Parameters were evaluated according to their nonparametric and parametric distributions. Differences between different groups were assessed using the Student's t-test for parametric data, while the Mann-Whitney U test was used for data with nonparametric distribution. Relationships between parameters were investigated using Pearson correlation analysis. The correlations were modelled by linear regression analysis of different sizes and were found to be similarly significant. All statistical analyses were performed with the Statistical Package for the Social

Sciences, version 22.0 (IBM Inc., Armonk, NY, USA). A p value of $< 0.05$ was considered statistically significant.

### Laboratory Measurements

In both the patient and control groups, peripheral venous blood samples were collected after 12-hour overnight fasting and used for enzymatic measurements of the concentrations of glucose, insulin, free thyroxine, thyroid-stimulating hormone, low-density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides, total cholesterol (TC) and alanine aminotransferase (Roche Modular DP Automatic Biochemical Analyser; Roche Diagnostics, Indianapolis, IN, USA). We used the homeostasis model assessment of insulin resistance (HOMA-IR) to determine the presence of IR by employing the following formula: fasting glucose (mmol/L) × fasting insulin (IU/L)/22.5. The HOMA-IR cut-off values for IR were taken as 5.22 in boys and 3.82 in girls (13).

## Results

In total, 58 adolescents with obesity/morbid obesity (mean age, 13.68 ± 2.01 years) and 30 healthy controls (mean age, 12.96 ± 2.5 years) were enrolled in this study. On pubertal assessment all participants were stage ≥2, based on clinical examination and palpation. No significant difference in sex distribution was observed between the two groups ($p > 0.05$). The characteristics and baseline laboratory values of the patients and control subjects are shown in Table 1. The BMI, BMI-SDS, waist circumference, glucose, insulin, HOMA-IR, TC, triglycerides, systolic blood pressure and diastolic blood pressure were significantly higher and the neudesin level significantly lower in the patients with obesity than in the controls (Table 1). Significant differences were noted in the presence of acanthosis and IR between the patient and control groups ($p < 0.01$).

The characteristics and baseline laboratory values of the patients with obesity and morbid obesity are shown in Table 2. The BMI, BMI-SDS, insulin and HOMA-IR values were significantly higher and the neudesin level significantly lower in the patients with morbid obesity than in the patients with obesity (Table 2). No statistically significant relationships were found between these groups in terms of blood pressure, glucose or lipid levels ($p > 0.05$).

There was a significant correlation between neudesin and BMI-SDS (Figure 1) and the systolic and diastolic blood pressures, which were all negatively correlated ($r = -0.401^{**}$, $r = -0.246^{*}$ and $r = -0.250^{*}$, respectively) (Table 3). No significant correlation was found for the other parameters assessed.

**Table 1. Baseline characteristics and metabolic parameters of the obese adolescents and controls**

| | Obese patients (n = 58) Median (min-max) | Controls (n = 30) Median (min-max) | p value |
|---|---|---|---|
| Age (years) | 14.25 (10.10-17.00) | 12.78 (9.00-17.00) | 0.150 |
| Gender (female/male) | 37/21 | 16/14 | 0.348 |
| Height (cm) | 161.93(138.00-180.00) | 165.25 (127.0-178.20) | 0.670 |
| Height-SDS | 0.66 (-2.15-2.97) | -0.07 (-1.80-2.24) | **0.001**** |
| Weight (kg) | 82.20 (55.50-130.00) | 53.75 (25.00-76.00) | **< 0.001***** |
| Weight-SDS | 3.14 (1.58-6.36) | 0.09 (-1.41-1.80) | **< 0.001***** |
| BMI (kg/m²) | 31.83 (24.96-44.70) | 20.47 (15.50-24.70) | **< 0.001***** |
| BMI-SDS | 2.73 (1.35-4.27) | 0.18 (-0.91-1.40) | **< 0.001***** |
| SBP (mm/Hg) | 120.06 (90-160) | 110.20 (100-120) | **< 0.001***** |
| DBP (mm/Hg) | 80.94 (52-105) | 74.14 (70-80) | **< 0.001***** |
| Waist/hip ratio | 0.87 (0.69-1.02) | 0.83 (0.78-0.89) | **0.026*** |
| Glucose (mg/dL) | 96.33 (78-118) | 90.33 (79-97) | **0.001**** |
| Insulin (µU/mL) | 27.18 (7.46-93.21) | 7.53 (3.40-12.00) | **< 0.001***** |
| HOMA-IR | 6.38 (1.46-26.01) | 1.70 (0.76-2.81) | **< 0.001***** |
| TC (mg/dL) | 146.75 (100-205) | 126.40 (96-169) | **< 0.001***** |
| TG (mg/dL) | 95.50 (33-342) | 72.67 (44-122) | **0.019*** |
| HDL (mg/dL) | 44.25 (33-90) | 42.40 (34-69) | 0.245 |
| LDL (mg/dL) | 81.17 (37-132) | 73.80 (41-101) | 0.091 |
| Neudesin (ng/mL) | 2.64 (1.14-6.17) | 3.12 (1.76-10.91) | **0.013*** |

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and significant values are indicated in bold.
The data are expressed as medians and (min-max).
BMI-SDS: body mass index-standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, TG: triglycerides, HDL: high-density lipoprotein, LDL: low-density lipoprotein, min-max: minimum-maximum

Of the 58 patients with obesity, 31% (n = 18) were diagnosed with MS by the IDF Consensus Worldwide definition. Interestingly, neudesin levels were higher in patients with MS compared to those without MS but the differences were not significant (3.27 ± 1.54 vs 2.86 ± 1.04 ng/L, respectively). We also investigated neudesin levels in further subgroups stratified by the presence or absence of dyslipidemia, hypertension and IR but no significant relationships were observed (Table 4).

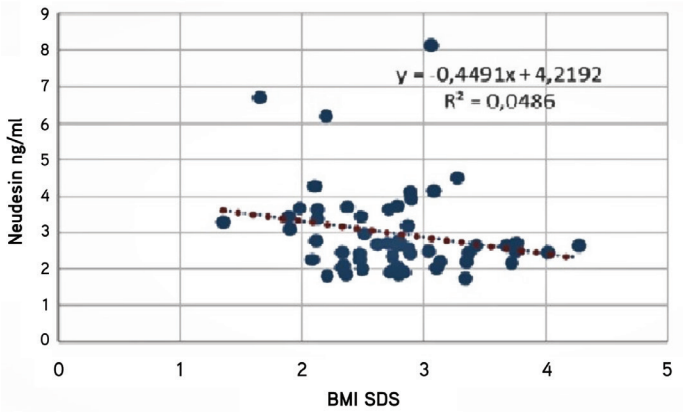


**Figure 1.** Correlation between neudesin and BMI-SDS

*BMI-SDS: body mass index-standard deviation score*

## Discussion

Experimental studies suggest that neudesin may be a novel regulator of energy homeostasis and food intake, with a potential role in the development of obesity and its complications. Previously published studies reported a preferential expression of neudesin in the paraventricular nuclei and arcuate nucleus, which are important areas of the hypothalamus for regulating appetite (14,15,16). It has also been proposed that neudesin may affect melanocortin signalling, as administration of recombinant neudesin in a mouse model via an intracerebrovascular cannula increased the hypothalamic expression of proopiomelanocortin (POMC) and melanocortin 4 receptor (MC4R) mRNA and decreased food intake and body weight (14). Su et al (17) showed that neudesin (NENF) acts as a negative regulator of myogenesis in bovines and that knockdown of NENF inhibited pre-adipocyte differentiation and promoted myoblast myogenesis.

In the present study, we found significantly lower neudesin levels in adolescents with obesity, as well as a negative correlation between neudesin levels and BMI, with neudesin levels decreasing as BMI-SDS increased. The specificity of serum neudesin was 83.33%, with a likelihood ratio of 4.14 and diagnostic accuracy of 73.86% between healthy and obese adolescents. To the best of our knowledge, this

**Table 2. Characteristics and metabolic parameters of the obese and morbid obese adolescents**

| | Obese patients (n = 15) Median (min-max) | Morbid obese (n = 43) Median (min-max) | p |
|---|---|---|---|
| Age (years) | 14.70 (10.11-17.00) | 14.11 (10.10-16.70) | 0.192 |
| Gender (female/male) | 10/5 | 27/16 | 0.793 |
| Height (cm) | 166.10 (145.80-180.00) | 161.00 (138.00-179.30) | **0.046** |
| Height-SDS | 0.64 (-2.15-2.97) | 0.72 (-0.43-2.96) | 0.198 |
| Weight (kg) | 76.00 (56.80-94.50) | 83.20 (55.50-130.00) | 0.069 |
| Weight-SDS | 2.46 (1.58-3.84) | 3.19 (1.77-6.36) | **0.005** |
| BMI (kg/m²) | 27.87 (24.96-32.00) | 33.90 (27.10-44.70) | **< 0.001** |
| BMI-SDS | 2.10 (1.35-2.51) | 2.83 (2.08-4.27) | **< 0.001** |
| SBP (mm/Hg) | 120.00 (100-133) | 120.00 (90-160) | 0.201 |
| DBP (mm/Hg) | 80.00 (65-90) | 81.00 (52-105) | 0.631 |
| Waist/hip ratio | 0.82 (0.75-0.95) | 0.88 (0.69-1.02) | 0.066 |
| Glucose (mg/dL) | 92.00 (81-109) | 97.00 (78-118) | 0.239 |
| Insulin (µU/mL) | 21.60 (10.69-31.88) | 28.00 (7.46-93.21) | **0.036** |
| HOMA-IR | 4.58 (2.81-7.36) | 6.94 (1.46-26.01) | **0.029** |
| TC (mg/dL) | 162.00 (107-205) | 144.00 (100-204) | 0.482 |
| TG (mg/dL) | 81.00 (45-157) | 96.00 (33-342) | 0.597 |
| HDL-C (mg/dL) | 45.00 (35-90) | 44.00 (33-74) | 0.289 |
| LDL-C (mg/dL) | 81.00 (48-106) | 81.00 (37-132) | 0.791 |
| Neudesin (ng/mL) | 3.19 (1.714-6.177) | 2.47 (1.144-4.502) | **0.043** |

Significant values are indicated in bold.
The data are expressed as medians and (min-max).
BMI-SDS: body mass index-standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, TG: triglycerides, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol, min-max: minimum-maximum

is the first description of a relationship between obesity, metabolic complications and neudesin levels in children. Very few studies have reported a similar correlation between neudesin and BMI in adults and animal models (3,18).

Bozkaya et al (19) found that circulating neudesin levels were decreased in subjects with polycystic ovary syndrome (PCOS) compared to controls. They proposed, based on their BMI data, that neudesin levels were notably lower in overweight and obese subjects than in lean subjects in both the PCOS and control groups. The work of Byerly et al (14), in their experimental study of recombinant neudesin administration showing increased hypothalamic POMC and MC4R mRNA expression and decreased food intake, suggests that neudesin acts as an anorexigenic neurotrophic factor.

Kratochvilova et al (20) also investigated serum neudesin levels and its mRNA expression in subcutaneous and visceral adipose tissue in obese subjects with or without type 2 diabetes. They found that serum neudesin concentrations in obese subjects, both with and without type 2 diabetes, did not differ from healthy lean control subjects. Similarly, Polkowska et al (21) assessed the serum levels of neudesin in children with type 1 diabetes and showed a statistically significant correlation between BMI and the levels of neudesin in the diabetic children with the longest disease duration. They found statistically higher mean neudesin concentrations in diabetic patients and proposed that IR was greater in these children with diabetes than in their healthy peers and that this difference may explain why neudesin levels were higher in patients with type 1 diabetes than non-diabetic children.

Ohta et al (3) examined the physiological role of neudesin in various tissues of wildtype mice fed a high-fat diet or normal chow food. They showed decreased food intake by

**Table 3. Correlation analysis of anthropometric and metabolic parameters**

| | | BMI-SDS | Neudesin (ng/mL) | Waist/hip ratio | Glucose (mg/dL) | Insulin (µU/mL) | TC (mg/dL) | TG (mg/dL) | HDL-C (mg/dL) | LDL-C (mg/dL) | SBP (mm/Hg) | DBP (mm/Hg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BMI-SDS | | 1 | | | | | | | | | | |
| Neudesin (ng/mL) | r | -0.401** | 1 | | | | | | | | | |
| | p | **< 0.001** | | | | | | | | | | |
| Waist/hip ratio | r | 0.293** | -0.042 | 1 | | | | | | | | |
| | p | **0.006** | 0.705 | | | | | | | | | |
| Glucose (mg/dL) | r | 0.284** | -0.073 | 0.092 | 1 | | | | | | | |
| | p | **0.007** | 0.511 | 0.395 | | | | | | | | |
| Insulin (µU/mL) | r | 0.634** | -0.228* | 0.258* | 0.497** | 1 | | | | | | |
| | p | **< 0.001** | **0.037** | **0.015** | **< 0.001** | | | | | | | |
| TC (mg/dL) | r | 0.317** | -0.090 | 0.120 | 0.195 | 0.185 | 1 | | | | | |
| | p | **0.003** | 0.415 | 0.265 | 0.069 | 0.085 | | | | | | |
| TG (mg/dL) | r | 0.283** | 0.040 | 0.198 | -0.041 | 0.298** | 0.230* | 1 | | | | |
| | p | **0.008** | 0.720 | 0.064 | 0.703 | **0.005** | **0.031** | | | | | |
| HDL-C (mg/dL) | r | 0.032 | -0.034 | -0.033 | -0.012 | -0.168 | 0.333** | -0.296** | 1 | | | |
| | p | 0.766 | 0.760 | 0.760 | 0.915 | 0.118 | **0.002** | **0.005** | | | | |
| LDL-C (mg/dL) | r | 0.173 | -0.071 | -0.033 | 0.196 | 0.085 | 0.743** | 0.185 | -0.029 | 1 | | |
| | p | 0.108 | 0.520 | 0.757 | 0.067 | 0.431 | **< 0.001** | 0.084 | 0.786 | | | |
| SBP (mm/Hg) | r | 0.474** | -0.246* | 0.089 | 0.357** | 0.430** | 0.058 | 0.214* | -0.078 | 0.042 | 1 | |
| | p | **< 0.001** | **0.024** | 0.409 | **0.001** | **< 0.001** | 0.592 | **0.045** | 0.472 | 0.695 | | |
| DBP (mm/Hg) | r | 0.461** | -0.250* | 0.187 | 0.241* | 0.442** | 0.996** | 0.297** | **-0.043** | **-0.028** | 0.509** | 1 |
| | p | **< 0.001** | **0.022** | 0.082 | **0.024** | **< 0.001** | **< 0.001** | **0.005** | 0.690 | 0.797 | **< 0.001** | |

Significant values are indicated in bold.
*Correlation is significant at the 0.05 level (two-tailed).
**Correlation is significant at the 0.01 level (two-tailed).
BMI-SDS: body mass index-standard deviation score, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol, TG: triglycerides, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol

**Table 4. Comparison of median neudesin levels in subgroups**

| | Waist/hip ratio > 90 percentile (n = 29) | Waist/hip ratio < 90 percentile (n = 26) | p value |
|---|---|---|---|
| Neudesin (ng/mL) | 2.63 (1.14-4.50) | 2.62 (1.71-6.17) | 0.745 |
| | Hypertensive (n = 25) | Non-hypertensive (n = 33) | |
| Neudesin (ng/mL) | 2.64 (1.14-4.50) | 2.46 (1.35-6.17) | 0.523 |
| | Insulin resistance (n = 43) | Non-insulin resistance (n = 15) | |
| Neudesin (ng/mL) | 2.64 (1.14-6.17) | 2.40 (1.35-4.15) | 0.246 |
| | Impaired fasting glucose (n = 24) | Non-impaired fasting glucose (n = 34) | |
| Neudesin (ng/mL) | 2.47 (1.14-4.50) | 2.64 (1.35-6.17) | 0.730 |
| | Dyslipidemia (n = 18) | Non-dyslipidemia (n = 40) | |
| Neudesin (ng/mL) | 2.64 (1.35-6.17) | 2.43 (1.14-4.10) | 0.379 |
| | MS (n = 18) | Non-MS (n = 40) | |
| Neudesin (ng/mL) | 2.64 (1.14-4.50) | 2.52 (1.35-6.17) | 0.259 |

The data are expressed as medians and (minimum-maximum).
MS: metabolic syndrome, min-max: minimum-maximum

neudesin knockout mice fed normal chow, suggesting that neudesin increased food intake. By contrast, the food intake was similar between wildtype and neudesin knockout mice when fed a high-fat diet, showing that resistance to diet-induced obesity in neudesin knockout mice was independent of food intake. Neudesin administration might therefore only be efficacious in lean mice, while its effects on food intake are blunted in obese mice fed a high-fat diet. Ohta et al (3) also suggested that high fat diet-induced adipocyte hypertrophy was significantly suppressed in neudesin knockout mice. The weights of the liver and BAT were significantly smaller in neudesin knockout mice fed a high-fat diet, and they proposed that these changes might be attributed to increased sympathetic nerve activity (14).

In our study group, 18 obese children were diagnosed with MS, and this prevalence of MS was similar to previous reports from our country (22,23). Neudesin levels tended to be higher in patients with MS; however, the levels were statistically comparable between obese children with or without MS. Triglyceride levels, TC levels, the HOMA-IR index and systolic and diastolic blood pressures were significantly higher in our obese group, but no statistically significant relationships were found between the neudesin levels and IR, dyslipidemia, or hypertension subgroups. In addition, neudesin levels were not different when stratified by waist/hip ratio. Waist/hip ratio was significantly greater in our obese adolescent group compared to the healthy control group, but not strikingly so. In fact, since waist/hip ratio did not show any difference between obese and morbidly obese groups, no significant difference could be found also in neudesin levels. We found no previous studies on neudesin levels in obese children with which to compare our findings.

The study performed by Ohta (18) showed that neudesin knockout mice were protected from obesityinduced metabolic dysfunction and exhibited increased energy expenditure due to increased sympathetic activity. This, in turn, resulted in increased heat production and fatty acid oxidation in BAT and enhanced lipolysis in WAT. They proposed that glucose tolerance was impaired and insulin sensitivity was aggravated by a high-fat diet, whereas neudesin knockout mice were protected from IR induced by a high-fat diet (3).

Kratochvilova et al (20) also reported a possible role of neudesin in the development of obesity-related metabolic disturbances, based on the observed effects of acute fasting and selected weight-reducing interventions, like endoscopic duodenojejunal bypass liner implantation or gastric plication. An obese diabetic group undergoing duodenojejunal bypass liner implantation showed significantly increased concentrations of circulating neudesin. Their serum neudesin levels were also positively correlated with insulin levels and inversely with BMI, while BMI and insulin levels were correlated in subjects undergoing acute fasting.

## Study Limitations

There were some limitations in our study. IR was derived by calculation rather than by insulin clamp technique, the gold standard but an invasive method. In addition, we examined the relationship between neudesin levels and metabolic outcomes. However, we could not assess its possible significance in body weight and metabolic control after weight reducing interventions.

## Conclusion

In conclusion, the serum neudesin levels were low in obese and morbidly obese adolescents and tended to increase with the appearance of metabolic disorders. These findings suggest that neudesin may play a role in the regulation of adipose tissue or energy metabolism. However, the potential role of neudesin in MS-related disorders appears to be limited, based on the findings of this study. More research is needed for a better and more robust understanding of the role of the neudesin molecule in the regulatory mechanisms in obesity and related metabolic disorders.

### Ethics

**Ethics Committee Approval:** The study was approved by the Tekirdağ Namık Kemal University University of Non-Invasive Clinical Research Ethics Committee (protocol number: 2019.137.08.09, date: 01.08.2019).

**Informed Consent:** Written informed consent was obtained from the children's parents after they were informed of the aim and procedures of the study.

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

### Authorship Contributions

Surgical and Medical Practices: Çiğdem Binay, Concept: Aliye Çelikkol, Çiğdem Binay, Design: Aliye Çelikkol, Çiğdem Binay, Savaş Güzel, Data Collection or Processing: Aliye Çelikkol, Çiğdem Binay, Özge Ayçiçek, Savaş Güzel, Analysis or Interpretation: Aliye Çelikkol, Çiğdem Binay, Özge Ayçiçek, Savaş Güzel, Literature Search: Aliye Çelikkol, Çiğdem Binay, Özge Ayçiçek, Savaş Güzel, Writing: Aliye Çelikkol, Çiğdem Binay, Savaş Güzel.

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# Genetic Indices Relationship to Hyperglycemia-associated Biomarkers: Consistency with miRNA Expression in Egyptian Children with T1DM

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

MicroRNAs (miRNAs) are oligonucleotide sequences, some of which are known to exert a specific biological function.

**What this study adds?**

New evidence about miRNA expression patterns in type 1 diabetes mellitus (T1DM). Gives insights into the criteria for biomarkers to be used in T1DM. Some miRNA molecules appear to be highly predictive for disease-associated glycemic derangement, depending on the reliability of the used methodology.

## Abstract

**Objective:** Micro RNAs (miRNAs) are gaining acceptance as novel biomarkers for the autoimmune disorders. However, miRNA profiles have not been investigated in individuals at risk of or diagnosed with type 1 diabetes mellitus (T1DM). To study the expression pattern of miRNAs in plasma obtained from patients with T1DM and compare with matched healthy controls

**Methods:** Equal numbers of patients with T1DM (90) and healthy-matched control children (90) were assessed for the expression profile of plasma miRNAs including miRNA-101-5p, miRNA-146-5p, miRNA-21-5p, miRNA-375, miRNA-126, and Let7a-5p using reverse transcriptase polymerase chain reaction methodology and quantitative real-time testing.

**Results:** Analysis showed that miRNA-101, miRNA-21 and miRNA-375 were highly expressed, whereas, miRNA-146-5p, miRNA-126, and miRNA-Let7a-5p showed significantly low levels of expression in T1DM patients compared to controls ($p < 0.05$). In addition, miRNA-101 and miRNA-146 correlated with age at diagnosis of T1DM and disease duration, respectively. Furthermore, multivariate analysis showed that miRNA-126 and Let7a-5p had a significant negative correlation with mean hemoglobin A1c (HbA1c) values.

**Conclusion:** Dysregulation of the six miRNAs analyzed suggested a possible role as biomarkers in T1DM. miRNA-101 was correlated with age at diagnosis while miRNA-146 correlated with disease duration. Two further miRNAs correlated with the existing biomarker, HbA1c.

**Keywords:** Type 1 diabetes, miRNAs, plasma, q RT-PCR, gene expression

## Introduction

Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic beta-cells by autoimmune mechanisms (1). When beta-cell destruction exceeds 80-90% by the infiltrating immune system, the disease can be diagnosed. The development of T1DM is slow, with a long latent phase during which it is possible to discover and treat individuals at risk (2,3).

It is estimated that about 80,000 children may develop T1DM annually.

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Complications related to vascular pathology associated with T1DM have a major impact on quality of life, morbidity, and mortality rates, posing an enormous burden on healthcare systems worldwide. Diabetic nephropathy is a major cause of end-stage renal disease (ESRD) and T1DM also increases the risk of cardiovascular diseases. In addition, severe diabetic retinopathy may result in blindness in adult patients. Thus, the identification of novel targets for improved treatment options and innovative, non-invasive biomarkers are urgently needed to enhance risk prediction, early diagnosis, and prognostic assessment (4).

Short (22 nucleotides), non-coding microRNA (miRNA) molecules have been shown to be important agents that regulate the pattern of organic phenomenon underlying disease pathogenic mechanisms in a post-transcriptional manner (5). Generally, miRNAs exert their respective function through binding with the 3' untranslated regions of their specific genes, leading to translational inhibition or direct degradation of the targeted mRNA with a resultant decrease in protein expression (5,6). Observed alterations in miRNA expression have been closely associated with many human inflammatory and autoimmune disorders (7,8). The estimated regulatory control of miRNAs of more than 60% of the protein-coding genes had consequently been linked to many diseases, including cancer, endocrine disorders, and autoimmune diseases, including T1DM (9). MiRNA-specific profiles were observed in peripheral blood mononuclear cells or serum from T1DM patients and these important molecules seem to modulate mRNA expressions of the major T1DM auto-antigens (10). Previously, it was reported that miRNAs, especially those expressed in human pancreatic islets, including miRNA-375 and miRNA-376, may be involved in the regulation of beta-cell pancreatic function (11). Later, a number of miRNAs were discovered to be regulatory factors for beta-cell pancreatic function (12).

We selected these beta-cell associated miRNAs, miR-101, miR-21 and-375, miR-146-5p, miR-126, and miR-Let7a-5p, as they are considered good indicators of beta-cell pancreatic function and diabetic state. The aim of the present study was to investigate the variable pattern of miRNA expression profiles in plasma obtained from patients with T1DM and matched control subjects through quantitative real-time (RT) polymerase chain reaction (PCR).

## Methods

This case-control study was prospectively conducted in children with T1DM having variable disease duration and variable degrees of glycemic control, who were diagnosed according to ADA criteria (group 1) (1). A group of apparently healthy, age- and sex-matched children served as controls (group 2). All subjects were enrolled from the Pediatric Department in collaboration with the Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Menoufia University Hospitals, Egypt. Demographic data, anthropometric measurements, treatment regimens, and other clinically important parameters were extracted from medical sheet records.

Cases that were suspected of being diagnosed as maturity onset diabetes of youth, type 2 diabetes, or secondary diabetes mellitus, or with evidence of chronic systemic/rheumatic diseases, inflammatory disorders, and recent febrile illness, or on long-term steroid therapy, were excluded from the study.

Upon approval of the study protocol from the Ethical Committee of Menoufia University, according to the Helsinki II Declaration criteria, written informed consent was obtained from all participants.

Following complete history taking and thorough clinical examination, all studied subjects underwent sampling of 7-10 mL whole blood after an overnight fast of at least 12 hours via sterile technique. One mL of blood was transferred into a sodium fluoride tube and another sample of blood was obtained after 2 hours for an enzymatic colorimetric determination of blood glucose, using a commercially available kit, (Spinreact, Spain) (13).

Another 4 mL of blood was transferred into two EDTA tubes: one 2 mL sample was used for quantitative colorimetric determination of glycated hemoglobin expressed as a percentage of the total hemoglobin by the use of (Teco Diagnostics, USA) (14) and hemoglobin A1c (HbA1c) values of ≥6.5% was accepted as the limit for diagnosing T1DM (1).

For molecular analysis, 2 mL of blood was transferred into the other EDTA tube and centrifuged for ten minutes at 4000 rpm. The clear supernatant was separated and kept frozen at -80 °C until further processing. Determination of miRNA levels was performed through a process to obtain cDNA via reverse transcription of previously isolated RNA, together with the measurement of miRNA levels using specific primer sets after being referenced against endogenous control U6B. These steps were as follows.

### RNA Isolation

A total RNA, including miRNA molecules was extracted from plasma using Qiagen™ RNA Blood Mini Kit (Qiagen, Applied Biosystems, USA) according to the manufacturer's instructions.

### Real-time PCR

The Qiagen® miScript II RT Kit (Qiagen, Applied Biosystems, USA) was used to reverse transcribe RNA. Then, complementary DNA (cDNA) was assayed with the universal SYBR Green Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen).

The preparation of the RT Master Mix was made as follows. 4 µL 5 × miScript HiSpec Buffer, 2 µL 10 × miScript Nuclease Mix, 2 µL RNase-free water, 2 µL miScript reverse transcriptase Mix, then a 10 µL template RNA to achieve a total reaction volume of 20 µL. Reverse transcription was carried out at 37 °C for 60 minutes and 95 °C for 5 minutes on an Applied Biosystems 2720 thermal cycler (Bioline, USA). Diluted cDNA was the template for the second step, RT-PCR using Qiagen-produced SYBR Green miScript kit. The addition of universal primers was based on mRNA sequences delivered from the miR-database for miRNA-101-5p, miRNA-146a-5p, miRNA-375, miRNA-21-5p, miRNA-126, and miRNA Let 7 a-5p, as shown in Table 1. Each reaction for RT-PCR was finalized to 25 µL volume, as follows: 12.5 µL 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µL 10x miScript specific Primer, 2.5 µL 10x miScript primer assay, 4 µL Template c DNA and 3.5 µL RNase-free water. The mixture was incubated at 95 °C for 15 min (as initial denaturation), then denaturation at 94 °C for 15 seconds duration, annealing for 30 seconds at a temperature of 55 °C and final extension for 30 seconds adjusted to 70 °C, for 60 cycles. Amplification of small RNA RNU6B was carried out with each sample as an endogenous control. Data analysis was performed by the RT cycler in-built software (Applied Biosystems® 7500 Software version 2.0.1 thermal cycler, Applied Biosystems, Foster City, CA, USA).

### Validation of the Quantified miRNAs

Subsequently, relative quantification of gene expression was performed using the comparative ΔΔCt method, in which the amount of targeted miRNAs were normalized to RNU6B as an endogenous reference for both patients and controls. It should be noted that the target miRNAs were selected because of evidence from available databases and literature that showed the association of these molecules to pathways involved in T1DM development in humans (2,9,15).

### Statistical Analysis

Statistical Package for the Social Sciences (SPSS), version 20 (SPSS Inc., Chicago, USA) was used for data analysis. For non-parametric data, median and range values were used. A chi-square test was used to examine the relationship between qualitative variables. For quantitative data, a comparison between two groups was done using either Student's t-test or Mann-Whitney test (non-parametric t-test), as appropriate. The correlation between numerical variables was assessed using Spearman's correlation method. For the determination of T1DM risk, multivariate logistic regression analysis was additionally used, aided by the calculation of Odds ratio (OR) and 95% confidence Interval (CI). A p-value less than 0.05 was considered significant.

## Results

In this study, a total of 180 children were enrolled, including 90 (46 males and 44 females) children with T1DM (group 1), and 90 (55 males and 35 females) control subjects (group 2) who were age and sex matched. The mean age of patients and controls was $10.93 \pm 4.51$ and $10.15 \pm 2.56$ years, respectively. Demographic and clinical data of the studied groups are shown in Table 2.

Unsurprisingly, results of laboratory investigations for the T1DM children including fasting blood sugar, 2-hour post-prandial blood sugar (2-hPP) and mean HbA1c% were significantly different to the healthy controls ($p < 0.001$ for all). All of the newly diagnosed cases with T1DM disease duration of 6-12 months were diagnosed because of being positive for anti-insulin autoantibodies (IAAs), where the levels ranged from 10-130 mIU/L with a mean value of $10.42 \pm 19.59$ compared to healthy controls with IAA range of 0-7.0 mIU/L and mean value of $2.08 \pm 2.36$ ($p = 0.002$).

**Table 1. Primers used for the quantitative reverse transcriptase-polymerase chain reaction assay for micro-RNA determination in all samples**

| Mature miRNA symbol | Accession No. | Sequence | Catalogue No. |
|---|---|---|---|
| miR 101-5p | MIMAT0004513 | 5-CAGUUAUCACAGUGCUGAUGCU-3 | MS00008379 |
| miR 146 a-5p | MIMAT0000449 | 5-UGAGAACUGAAUUCCAUGGGUU-3 | MS00003535 |
| miR 21 a-5p | MIMAT0004494 | 5-CAACACCAGUCGAUGGGCUGU-3 | MS000009086 |
| miR-375 | MIMAT00000728 | 5-UUUGUUCGUUCGGCUCCGUGA-3 | MS000031829 |
| miR 126 a-3p | MIMAT0000445 | 5-UCGUACCGUGAGUAAUAAUGCG-3 | MS00003430 |
| Let 7 a-5p | MIMAT0000062 | 5-UGAGGUAGUAGGUUGUACAGUU-3 | MS00031220 |
| RNU6B | Internal control | 5-CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT-3 | MS00033740 |

Comparative results regarding the levels of miRNAs studied in both groups revealed that miRNA-101-5p, miRNA-21-5p, miRNA-375 were present in significantly higher concentration in patients with T1DM ($p < 0.05$), whereas miRNA146-5p, miRNA-126, and miRNA Let 7a-5p had significantly lower plasma levels in T1DM ($p < 0.05$) (Table 3). One of the remarkable findings in this study were significant negative correlations between miRNA-101-

**Table 2. Comparison between the two studied group as regarding to demographic, clinical and laboratory data**

| | T1DM (n = 90) | | Control (n = 90) | | Test of sig. | p |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | | |
| **Sex** | | | | | | |
| Male | 46 | 51.1 | 55 | 61.1 | $\chi^2 = 1.827$ | 0.176 |
| Female | 44 | 48.9 | 35 | 38.9 | | |
| **Consanguinity** | | | | | | |
| Positive | 48 | 48 | 65 | 65 | 2.9 | 0.006 |
| Negative | 52 | 52 | 35 | 35 | | |
| **Family history** | | | | | | |
| Positive | 21 | 21 | 0 | 0 | | < 0.001* |
| Negative | 79 | 79 | 100 | 100 | 20.1* | |
| **Age (years)** | | | | | | |
| Mean ± SD | 10.93 ± 4.51 | | 10.15 ± 2.56 | | t = 1.434 | 0.154 |
| **Systolic blood pressure (mm/Hg)** | | | | | | |
| Mean ± SD | 110.4 ± 6.10 | | 107.0 ± 4.50 | | t = 3.199* | 0.002* |
| **Diastolic blood pressure (mm/Hg)** | | | | | | |
| Mean ± SD | 67.78 ± 5.95 | | 67.0 ± 6.44 | | t = 0.841 | 0.401 |
| **BMI (kg/m²)** | | | | | | |
| Mean (SD) | 19.6 ± 3.98 | | 20.22 ± 5.51 | | t = 0.91 | 0.32 |
| Range | 13-29 | | 13.3-32.5 | | | |
| **Age of onset** | | | | | | |
| Mean ± SD | 6.93 ± 3.29 | | | | | |
| Median (IQR) | 7.0 (5.0-10.0) | | | | | |
| **Duration of illness** | | | | | | |
| Mean ± SD | 4.41 ± 3.35 | | | | | |
| Median (IQR) | 4.42 (1.0-7.0) | | | | | |
| **Presentation** | | | | | | |
| DKA | 30 | 33.3 | | | | |
| Classic symptoms | 60 | 66.7 | | | | |
| **HbA1c%** | | | | | | |
| Mean ± SD | 9.03 ± 2.04 | | 5.94 ± 0.34 | | t = 14.165* | < 0.001* |
| Median (IQR) | 9.0 (7.2-10.0) | | 5.95 (5.7-6.2) | | | |
| **FBS (mg/dL)** | | | | | | |
| Min-max | 105.0-300.0 | | 75.0-105.0 | | | |
| Mean ± SD | 210.52 ± 46.46 | | 91.0 ± 8.76 | | t = 23.985* | < 0.001* |
| Median (IQR) | 200.0 (180.0-250.0) | | 91.0(85.0-98.0) | | | |
| **2 hr PP (mg/dL)** | | | | | | |
| Min-max | 130.0-310.0 | | 140.0-172.0 | | | |
| Mean ± SD | 257.11 ± 40.31 | | 154.80 ± 11.47 | | t = 23.156* | < 0.001* |
| Median (IQR) | 260.0 (220.0-300.0) | | 155.5 (145.0-165.0) | | | |
| **Micro albuminuria** | | | | | | |
| No | 82 | 91.1 | 91 | 100.0 | $\chi^2 = 8.372$ | FEp = 0.007* |
| Yes | 8 | 8.9 | 0 | 0.0 | | |
| Min-max | 206.20-394.0 | | - | | | |
| Mean ± SD | 265.55 ± 80.19 | | - | | - | - |
| Median (IQR) | 231.0 (215.1-316.0) | | - | | | |

$\chi^2$: Chi-square test, FE: Fisher Exact, MC: Monte Carlo, IQR: interquartile range, t: Student's t-test U: Mann Whitney test, p: p value for comparing between the studied groups, *: Statistically significant at p≤0.05.
SD: Standard deviation, Min-max: minimum-maximum, 2 hr PP: 2 hour post-prandial, DKA: diabetic ketoacidosis, BMI: body mass index

5p and the age of T1DM onset (r = -0.264, p = 0.015) and with the duration of T1DM (r = -0.162, p = 0.02) while MiR-146 correlated positively with disease duration (r = 0.239, p = 0.023). Furthermore, miRNA-126 (r = -0.214, p = 0.042) and miRNA-Let7a-5p (r = -0.216, p = 0.043) correlated negatively with mean HbA1c levels (Table 4).

Results of multivariate logistic regression analysis for T1DM risk are shown in Table 5. miRNA-126 and miRNA-Let7a-5p markers remained highly significant after adjustment for age, sex and mean HbA1c levels with OR (95% CI) of 0.016 (0.0-0.544), p = 0.021 for miRNA-126 and 1.808 (1.006-3.249) p = 0.048 for miRNA-Let7a-5p.

**Table 3. Comparison between the two studied groups according to different miRNAs expression**

| miRNA expressions | T1DM (n = 90) | Control (n = 90) | U | p |
|---|---|---|---|---|
| **miRNA-101-5p** | | | | |
| **Min-max** | 0.0-1640.55 | 0.09-1.66 | 3652.0 | 0.028* |
| **Mean ± SD** | 65.33 ± 270.39 | 0.39 ± 0.14 | | |
| **Median (IQR)** | 0.60 (0.08-1.95) | 0.26 (0.10-0.35) | | |
| **miRNA-146a-5b** | | | | |
| **Min-max** | 0.0-328.49 | 0.0-1.21 | 2646.0* | < 0.001* |
| **Mean ± SD** | 7.89 ± 48.63 | 0.63 ± 0.44 | | |
| **Median (IQR)** | 0.16 (0.02-0.43) | 0.77 (0.0-0.88) | | |
| **miRNA-375a-3p** | | | | |
| **Min-max** | 0.0-2127.09 | 0.07-2.93 | 1908.0* | < 0.001* |
| **Mean ± SD** | 70.36 ± 332.53 | 0.95 ± 1.02 | | |
| **Median (IQR)** | 2.10 (1.08-3.11) | 0.53 (0.09-1.05) | | |
| **miRNA-21-3p** | | | | |
| **Min-max** | 0.0-89.39 | 0.0-0.06 | 2682.0* | < 0.001* |
| **Mean ± SD** | 2.27 ± 13.21 | 0.03 ± 0.02 | | |
| **Median (IQR)** | 0.08 (0.0-0.48) | 0.02 (0.0-0.05) | | |
| **miRNA-126** | | | | |
| **Min-max** | 0.00-0.62 | 0.00-152.32 | 1569.50* | < 0.001* |
| **Mean ± SD** | 0.19 ± 0.15 | 4.50 ± 22.39 | | |
| **Median (IQR)** | 0.15 (0.07-0.31) | 0.69 (0.26-1.61) | | |
| **miRNA Let 7a-5p** | | | | |
| **Min-max** | 0.0-35.80 | 0.0-1.92 | 2356.50* | < 0.001* |
| **Mean ± SD** | 0.94 ± 5.21 | 0.50 ± 0.51 | | |
| **Median (IQR)** | 0.09 (0.02-0.23) | 0.12 (0.10-1.02) | | |

U: Mann-Whitney test, p: p value for comparing between the studied groups, *: Statistically significant at p≤0.05.
SD: Standard deviation, IQR: interquartile range, Min-max: minimum-maximum, T1DM: type 1 diabetes mellitus

**Table 4. Correlation between different parameters and all miRNAs in patients with T1DM**

| | | miRNA-101-5p | miRNA-146a-5b | miRNA-375a-5p | miRNA-21-3p | miRNA-126 | miRNA Let 7a-5p |
|---|---|---|---|---|---|---|---|
| **Duration of illness** | $r_s$ | -0.162 | 0.239 | 0.095 | -0.151 | -0.167 | -0.034 |
| | **P** | 0.02* | 0.023* | 0.386 | 0.167 | 0.128 | 0.756 |
| **Age of onset** | $r_s$ | -0.264 | 0.149 | -0.008 | 0.211* | 0.089 | -0.062 |
| | **P** | 0.015* | 0.160 | 0.943 | 0.046* | 0.402 | 0.563 |
| **HbA1c** | $r_s$ | 0.090 | 0.183 | 0.173 | 0.054 | -0.214 | -0.216 |
| | **P** | 0.413 | 0.162 | 0.201 | 0.610 | 0.042* | 0.043* |
| **FBS** | $r_s$ | -0.194 | 0.176 | -0.147 | 0.166 | -0.032 | 0.241 |
| | **P** | 0.067 | 0.097 | 0.166 | 0.119 | 0.764 | 0.022 |
| **2 hr PP** | $r_s$ | -0.215 | 0.134 | -0.008 | 0.096 | 0.006 | 0.123 |
| | **P** | 0.042 | 0.209 | 0.939 | 0.368 | 0.954 | 0.247 |

$r_s$: Spearman coefficient, *: Statistically significant at p≤0.05.
T1DM: type 1 diabetes mellitus, FBS: fasting blood sugar, HbA1c: hemoglobin A1c, 2 hr PP: 2 hour post-prandial

Correlations between some of the studied mi-RNAs and some clinical parameters of T1DM are shown in Figures 1, 2. The amplification for miRNAs expression patterns [normalized fluorescent signal (ΔRn) that was plotted against the number of cycles] is shown in Figures 3,4,5.

## Discussion

The understanding of the role of miRNAs in modulating gene expression has greatly improved and these molecules are being implicated in the disease mechanisms of various genetic disorders (16,17,18). In light of this, miRNAs have been proposed as biomarkers of disease pathogenesis and prognosis (19,20). This suggestion was supported by the relationship of these molecules to 60% or more of the coding genes that were thought to be associated with a number of endocrine and autoimmune diseases (21,22,23).

Based on the underlying autoimmune background of T1DM, it seemed reasonable to include children with recently diagnosed T1DM who still had circulating IAA. Some studies have suggested that miRNAs may be helpful as biomarkers in the early phases of T1DM (22). Other miRNAs are closely associated with glucose homeostasis that determines the progressive pattern in at-risk individuals (22). There is recent evidence of changes in proinflammatory cytokines and autoimmune markers which is asociated with particular patterns of circulating miRNAs in children with T1DM (24,25,26).

Consistent with this, we found that miRNA-146a-5p, miRNA Let-7a-5p, and miRNA-126 were down-regulated, whereas miRNA-101, miRNA-21-5p, and miRNA-375a-5p were consistently up-regulated in patients compared to controls.

As for miR-375, its abundance in pancreatic tissue has been suggested to make it a good biomarker for beta-cell mass

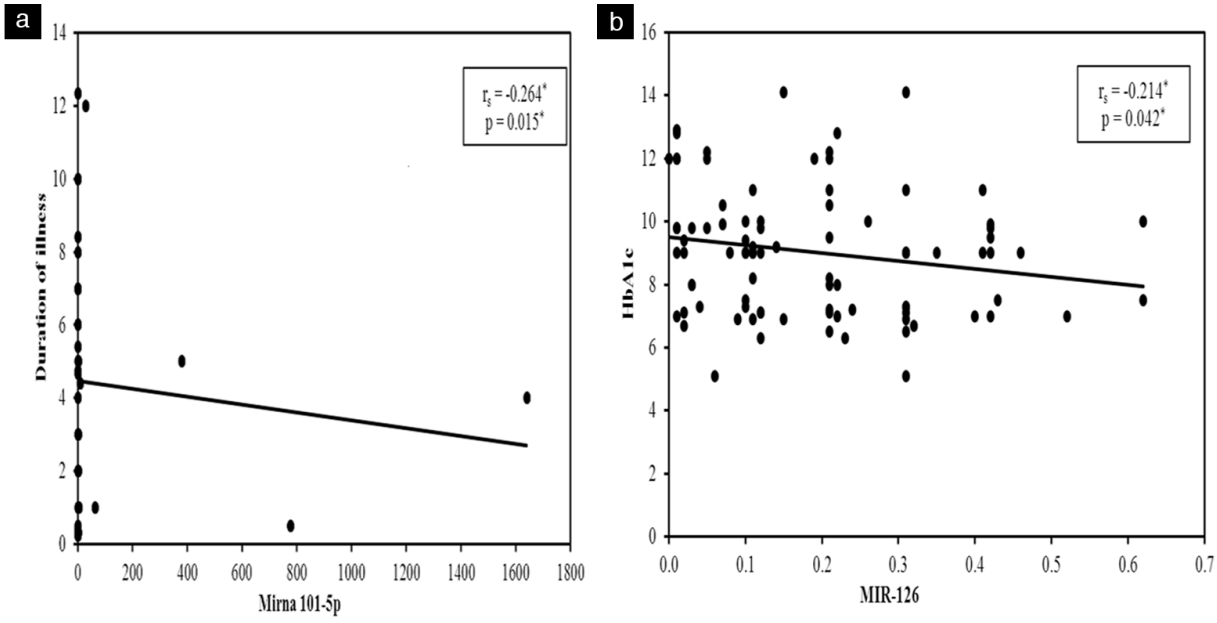


**Figure 1.** a) Correlation results between miRNA-101-5p with age of type 1 diabetes mellitus onset. b) Correlation results between miRNA-126 and hemoglobin A1c

**Table 5. Univariate and multivariate analysis for the parameters affecting T1DM risk (n = 90)**

| | Univariate | | Adjust OR | |
|---|---|---|---|---|
| | p | COR (95% CI) | p | AOR# (95% CI) |
| **miRNA-101-5p** | **0.009*** | 1.565 (1.121-2.185) | 0.307 | 1.783 (0.587-5.414) |
| **miRNA-146a-5p** | **0.616** | 1.017 (0.951-1.088) | 0.844 | 1.009 (0.924-1.101) |
| **miRNA Let 7 a-5p** | **< 0.001*** | 1.845 (1.370-2.483) | 0.048* | 1.808 (1.006-3.249) |
| **miRNA-21-5p** | **< 0.001*** | 11.62 (3.63.69-71.85) | 0.026* | 7.180 (2.554-20.187) |
| **miRNA-126** | **< 0.001*** | 0.013 (0.002-0.064) | 0.021* | 0.016 (0.0-0.544) |
| **miRNA-375** | **0.453** | 1.037 (0.943-1.140) | 0.921 | 1.014 (0.776-1.324) |

*: Statistically significant at p≤0.05.
OR: Odds ratio, CI: confidence interval, AOR#: adjust OR by family history and HbA1c, HbA1c: hemoglobin A1c, miRNA: micro RNA

and changes in beta-cell function (27). We found that the level of miRNA-375 in the plasma of patients with T1DM was significantly increased. However, in our cohort there was no correlation between miRNA-375 and HbA1c. In contrast, Marchand et al (28) reported dysregulated miRNA-375 levels in the blood of newly diagnosed children with T1DM when quantified to high levels in human islet tissue and conferred as a hallmark in the etiology of T1DM. These same authors suggested miRNA-375 may be a marker of the early phases of the disease.

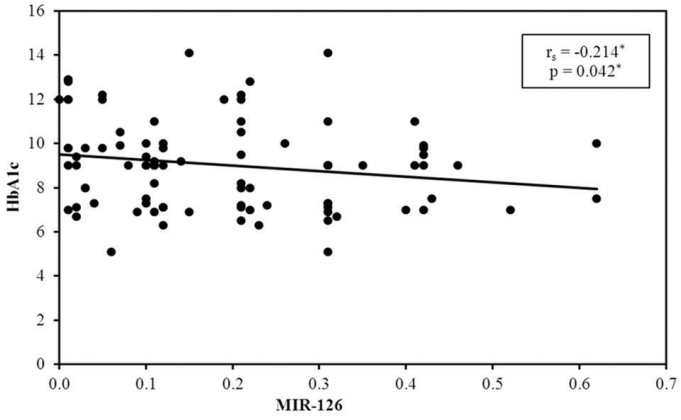


**Figure 2.** Amplification plot for miRNA-21-5p expression pattern [normalized fluorescent signal (ΔRn) plotted against the number of the cycle]

We found that miRNA-21-5p was present at significantly higher concentrations in T1DM patients than in the controls. Pan et al (29) investigated this same miRNA in terms of the effect of enteroviral infection on miRNA-21-5p expression and subsequent contribution to T1DM. Earlier studies have shown that has-miRNA-21-5p was highly expressed in the plasma of T1DM patients in comparison to controls (30). Ongoing research has linked miRNA-21-5p to inflammatory cytokines (31). Furthermore, it was suggested that miRNA-21 overexpression was believed to influence the Bax group/apoptotic signaling pathway, and hence may be involved in pancreatic beta-cell death (32,33). This could suggest a new target for T1DM therapy.

Another up-regulated miRNA in our study was miRNA-101-5p, which has been associated with reduction of insulin secretion and beta-cell mass although it is known to play a role in cytokine release regulation and altered signaling of STAT3, HGF/C-Met, and Ephrin receptor/pathway mechanisms. We found a significant association of miRNA-101-5p and IAA positive cases in recent onset T1DM. Interestingly, Santos et al (34) reported that the expression

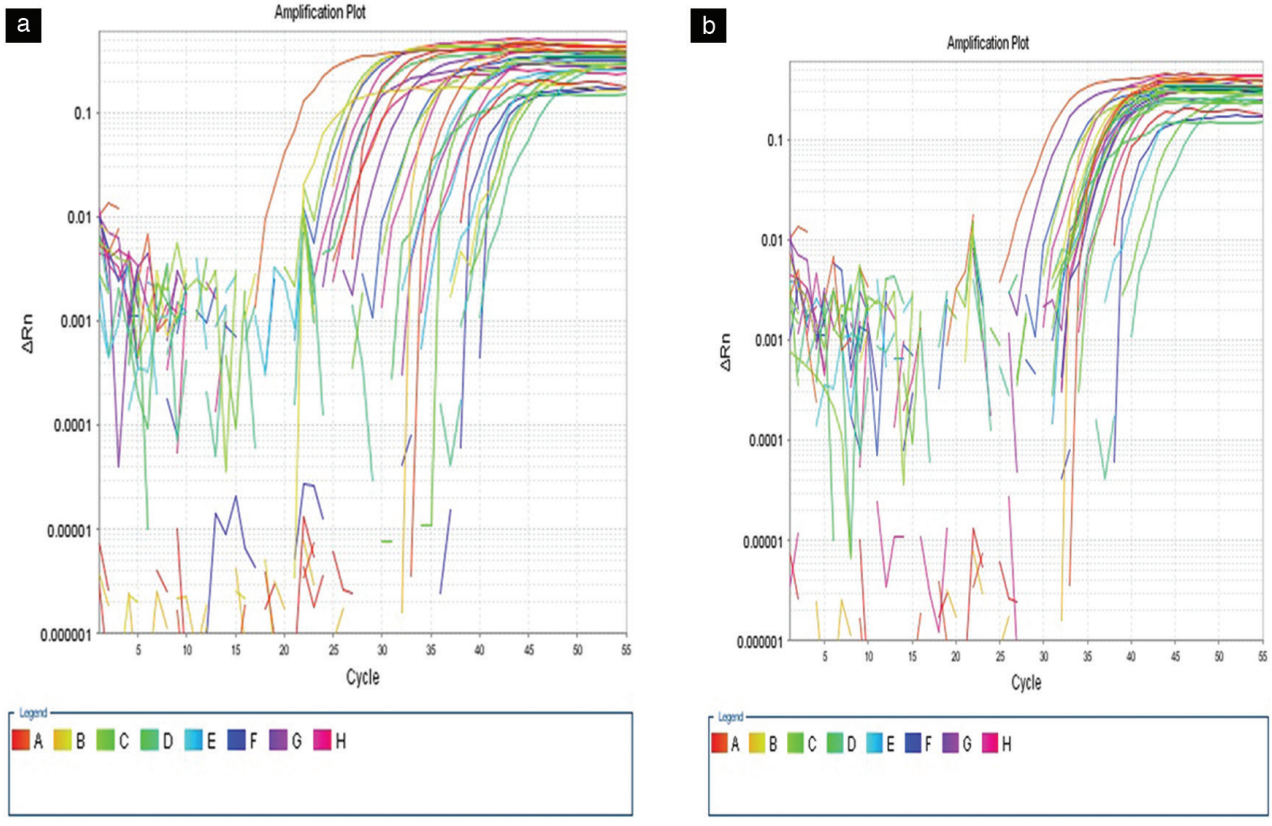


**Figure 3.** a) Amplification for miRNA-101-5p expression [normalized fluorescent signal (ΔRn) plotted against the number of the cycle]. b) Amplification for miRNA-146a-5p expression [normalized fluorescent signal (ΔRn) plotted against the number of the cycle]

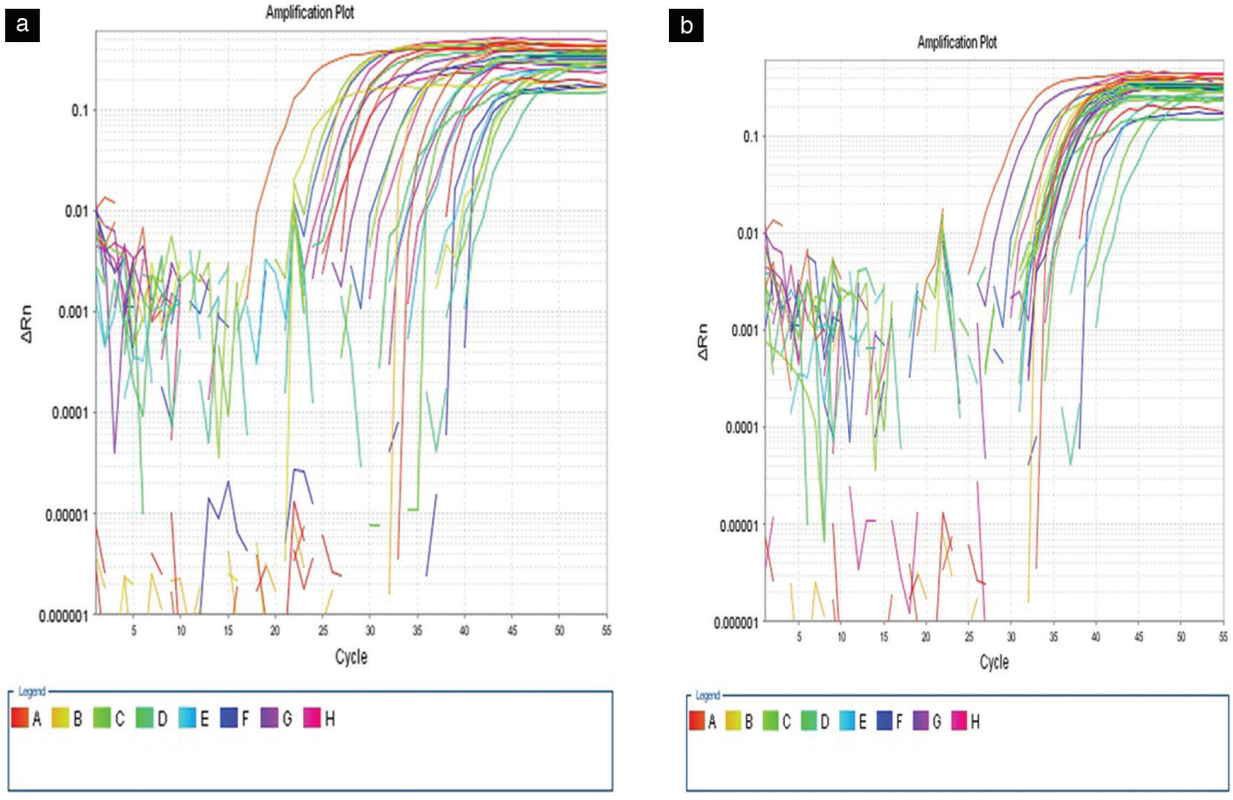


**Figure 4.** a) Amplification for miRNA-375 expression [normalized fluorescent signal (ΔRn) plotted against the number of the cycle]. b) Amplification plot of miRNA Let 7 a-5p expression [normalized fluorescent signal (ΔRn) against the number of the cycle]

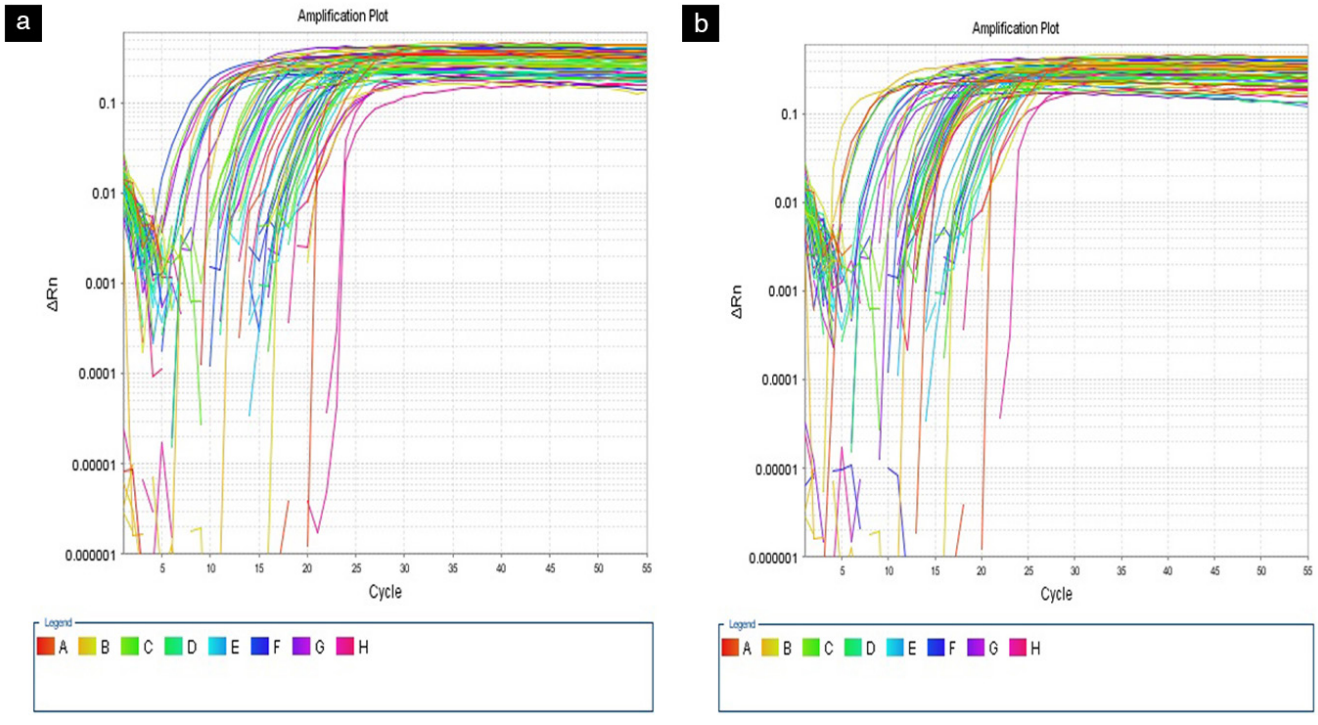


**Figure 5.** a) Amplification of miRNA-126 gene expression [normalized fluorescent signal (ΔRn) against the number of the cycle]. b) Amplification of miRNA-126 gene expression [normalized fluorescent signal (ΔRn) against the number of the cycle]

of miRNA-101 was about threefold higher in patients with multiple autoantibodies levels.

miRNA Let7 a-5p was expressed at lower levels our patient group and demonstrated a statistically significant difference in comparison to that of controls. Similar results were reported by Tian et al (35) where miRNA Let7a was downregulated in both human and mouse tissue derivatives. The miRNA Let7a is known to be involved in the regulation of glucose metabolism. This molecule was found to be negatively correlated to HbA1c by Erener et al (36), which is consistent with our findings.

Assessing the level of miRNA-126 expression revealed contradictory findings. Osipova et al (30) reported lower urinary levels in patients with T1DM, with no significant difference in plasma concentration comparing patients and controls. However, Wang et al (37) found decreased plasma levels of miRNA-126 in those with chronic ESRD. We also found significantly lower plasma level of the miRNA-126 in T1DM patients in agreement with Wang et al (37) but in contrast to Osipova et al (30). It has been shown that decreased levels of miRNA-126 are associated with deranged response to vascular endothelial growth factor and endothelial dysfunction (38,39). In addition, previous reports considered miRNA-126 to be a controlling factor for various biological processes (40,41,42), through linkage of decreased circulatory miRNA-126 levels to micro-vascular change, which in turn is known to influence the development of later chronic T1DM complications (42,43).

The significant negative correlation between miRNA-126, mi-RNA Let7a-5p and higher mean HbA1c values suggested a significant association of the altered levels of the circulating miRNAs to hyperglycemic state (36). These findings are in agreement with the hypothesis of Akerman et al (22) who suggested that the expression of miRNAs may be of value as complementary markers in at-risk individuals with abnormal OGTT results. Satake et al (44) reported the association of dysregulated miRNAs with hyperglycemia in children with T1DM that may contribute to further development of diabetic complications.

It has been proposed that, in T1DM, miRNAs may be a cornerstone in pathogenesis and are more than merely markers of active beta-cell dysfunction (45). Previous studies reported the effect of miRNAs on pancreatic cellular biology, especially for beta-cell differentiation, insulin production, mediation of inflammation, and apoptosis (46).

There was a negative correlation between miRNA-101 with the age of onset of T1DM in our cohort. Earlier studies have suggested a greater rate of beta-cell turnover and pancreatic injury in young children with T1DM and lower levels of miRNA-101 as it functions to mediate Treg function in controlling autoimmune cellular destruction (47).

Another important miRNAs molecule that showed significant down-regulation besides being indicated in patients with recent-onset T1DM was miRNA-146 a-5p. This was evident through a lowered expression level in T1DM cases. This is of interest because miRNA-146-5p has been associated with genes linked to apoptotic and innate immune regulatory pathway mechanisms and, as such, warrants further investigation in T1DM (47).

### Study Limitations

Small sample size was a limitation in our study so that it was not possible to reach any definite causative conclusions but merely report associations. Other limitations of our study include no calculation of sample size or power of the study. This was because published data on the link between T1DM and miRNAs are very scarce and so were not sufficient for an exact calculation of statistical sample size. Lastly, the relationship between miRNAs and the presence and/or levels of T1DM-associated autoantibodies was not investigated. Further research is necessary to overcome all of these limitations.

## Conclusion

In this study there was a significant difference between plasma concentrations of some miRNAs in children with T1DM and healthy controls. In the patients with T1DM there was evident down-regulation of miRNAs-146-5p, 126-5p, and Let 7a-5p molecules and up-regulation of miRNAs-101-5p, 21-5p, and 375. As miRNA-126-5p and Let7a-5p miRNAs have a significant negative correlation with mean HbA1c they could be used as alternative biomarkers for hyperglycemia-associated pathophysiologic changes in T1DM and further work may reveal additional benefits of using these miRNAs compared to measuring HbA1c.

Given the known gene regulatory action of miRNAs and that we found significant changes in circulating levels in children with T1DM compared to healthy age- and sex-matched controls, there is a potential for T1DM-associated miRNAs to become useful biomarkers and even possible therapeutic targets in the future. Further larger-scale functional studies are required to investigate genetic interactions which may lead to improvement in the quality and life expectancy of children with T1DM.

### Ethics

**Ethics Committee Approval:** The study were approved by the Menoufia University Faculty of Medicine of Ethical Committee (approval number: 59601070, date: 09.02.2020).

**Informed Consent:** All subjects gave written, informed consent through their parent or guardian before enrollment in the study.

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

### Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: All authors.

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ORIGINAL ARTICLE

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# Investigating Genetic Mutations in a Large Cohort of Iranian Patients with Congenital Hyperinsulinism

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### What is already known on this topic?

It is well known that congenital hyperinsulinism (CHI) is the most frequent cause of severe and persistent hypoglycaemia in the neonatal period, infancy, and childhood. To date, mutations in at least nine different genes have been reported to cause CHI*: ABCC8, KCNJ11, GLUD1, GCK, HADH, HNF4A, SLC16A1, HNF1A* and *UCP2*. Data are mainly limited to European populations while the occurrence of the pathogenic mutations underlying CHI are higher in consanguineous families which are more prevalent in Asian societies.

### What this study adds?

We report the frequency of causal gene mutations in a cohort of Iranian children with a diagnosis of CHI and add five novel mutations. Based on our findings we recommend screening of *HADH* gene variants in all patients with diazoxide-responsive CHI if there is no access to targeted next generation sequencing.

## Abstract

**Objective:** Congenital hyperinsulinism (CHI) is the most frequent cause of severe and persistent hypoglycaemia from birth. Understanding the pathophysiology and genetic defects behind hyperinsulinism and its complications provides clues to timely diagnosis and management. The aim of this study was to evaluate the underlying genetic aetiology of a specific Iranian pediatric cohort with CHI.

**Methods:** A total of 44 unrelated children, 20 girls and 24 boys, with an initial diagnosis or history of CHI from all regions of Iran were recruited between 2016 and 2019. Targeted next generation sequencing (tNGS) was performed for the genes found in about half of CHI patients.

**Results:** Mutations were identified in 24 cases (55%). Patients with a confirmed genetic cause were mainly diagnosed below age of one year old ($p = 0.01$), had fewer other syndromic features, excluding seizure, ($p = 0.03$), were less diazoxide responsive ($p = 0.04$) and were more diazoxide unresponsive leading to pancreatectomy ($p = 0.007$) compared to those with no identified mutations. Among 24 patients with identified genetic mutations, 17 (71%) had a mutation in *ABCC8*, 3 (12%) in *KCNJ11*, 3 (12%) in *HADH*, and 1 patient had a mutation in *KMT2D*. These included five novel mutations in *ABCC8*, *KCNJ11*, and *KMT2D*.

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**Conclusion:** This is the biggest genetic study of CHI in Iran. A high frequency of recessive forms of CHI, especially HADH mutations, in our study could be due to a high rate of consanguineous marriage. We recommend tNGS to screen for all the CHI genes.
**Keywords:** Congenital hyperinsulinism, genetic mutations, diazoxide, targeted next generation sequencing

## Introduction

Congenital hyperinsulinism (CHI) is the most frequent cause of severe and persistent hypoglycaemia and the most common metabolic abnormality in the neonatal period, infancy, and childhood (1,2). There is an unregulated secretion of insulin from pancreatic β-cells in the course of low blood glucose in CHI patients, leading to severe and persistent hypoglycaemia due to genetic defects (3,4). CHI is rare but a very high incidence has been reported in isolated European populations and also communities with a high rate of consanguinity (5,6,7). About 60 % of infants with CHI develop hypoglycaemia during the first months of life. This condition typically goes through remission and flare-up cycles due to hypoglycaemia. Age of onset is variable and symptoms can range from asymptomatic and mild to severe symptoms, including medically unresponsive hypoglycaemia (5,6).

The clinical manifestations, histological subtypes and underlying molecular mechanisms of CHI are heterogeneous (4). Based on histological assessments, there are two major subtypes of CHI including diffuse and focal forms (3,8). The differentiation between these two subtypes is important for clinical management. The diffuse form, where abnormality is in all pancreatic β-cells, is inherited in an autosomal recessive or dominant mode, most commonly due to mutations in *ABCC8* and *KCNJ11*. Recessive mutations in *ABCC8/KCNJ11* are usually severe and require high concentrations of intravenous glucose to maintain normoglycemia while dominant mutations usually cause milder disease. The focal form is confined to a small region of the pancreas with sporadic inheritance. This subtype results from a heterozygous paternal mutation in *ABCC8/KCNJ11* and somatic loss of maternal chromosome 11p15 in the focal pancreatic lesion (9).

Understanding the pathophysiology and genetic defects behind hyperinsulinism and its complications has provided clues to diagnosis and management of the disease. Since CHI causes a set of complex and heterogenous metabolic complications, cases will have different clinical presentation with different age of onset and prognosis. The conservative treatment includes diazoxide as first choice. However, some children, mainly those with the recessive mutations in *ABCC8/KCNJ11*, develop a form of disease unresponsive to medical therapy and so pancreatectomy is the only option (3,10).

Since severe hypoglycaemia has a negative effect on neural system function, especially during the early years of life, early diagnosis and treatment are important. In recent decades there has been a substantial expansion in information of genetic defects leading to CHI. To date, mutations in at least nine different genes have been reported to cause CHI: *ABCC8, KCNJ11, GLUD1, GCK, HADH, HNF4A, SLC16A1, HNF1A* and *UCP2* (4,9). These genes are involved in regulating insulin secretion from β-cells (4). Data about CHI are mainly limited to European populations. However, the occurrence of the pathogenic mutations underlying CHI is increased in consanguineous families compared with non-consanguineous families (4). Therefore, investigating the aetiologies of this disorder is even more momentous in Asian countries with highly consanguineous populations, including Iran.

The aim of this study was to assess genetic mutations underlying CHI by recruiting individuals diagnosed with CHI. All the genetic and clinical data was combined to provide an Iranian CHI database for patients and specialists. We also report the frequency of causal gene mutations and describe a number of novel mutations.

## Methods

### Study Participants

Unrelated participants diagnosed with CHI were recruited to the study. Patients were from all regions of Iran and who had been referred to two centres in Iran, Imam Reza Hospital, Mashhad, Iran and the Division of Endocrinology and Metabolism in the Department of Paediatrics at the Children's Medical Centre in Tehran, Iran (Table 1). Clinical information was supplied by the referring clinicians. Informed consent was obtained from parents on behalf of their children. Peripheral blood samples were collected from affected participants and their parents at the time of referral and used to perform genetic testing.

### Clinical Data

CHI was defined as fasting hypoglycaemia [glucose < 50 mg/dL (2.8 mmol/l)] occurring simultaneously with an inappropriately detectable plasma insulin ( > 2.0 µU/mL) (11). Paediatric subjects whose hyperinsulinemic hypoglycemia (HH) did not remit after at least three months follow-up were eligible to be enrolled in this study. A detailed clinical

and demographical history, along with EDTA blood sample from the affected individual, both parents and affected siblings, were obtained. In the clinical history, diazoxide-responsiveness was defined as the ability to achieve elevated intravenous glucose and maintain normoglycemia (12). Low birth weight was determined based on birth weight adjusted by the gestational age (13).

### Genetic Analysis

The genetic testing and variant interpretation were performed by the Exeter Molecular Genetics Laboratory (Exeter, UK). Briefly, DNA was extracted using standard methods and the samples were analysed for coding and flanking intronic regions of the *KCNJ11* (NM_000525.3) and *ABCC8* (NM_001287174.1) genes by Sanger sequencing. If no mutation was found, targeted next generation sequencing (tNGS) (Agilent custom capture v5.3/Illumina NextSeq500) for the coding regions and exon/intron boundaries of the genes found in about half of CHI patients (*ABCC8, KCNJ11, AKT2, GLUD1, GCK, GPC3, HADH, HNF4A, KDM6A, KMT2D, SLC16A1, CACNA1D, PMM2, TRMT10A and HNF1A*) (14) was performed. This assay can also detect partial/whole gene deletions and duplications (15). For a patient with mosaicism in *KMT2D*, a confirmatory dosage analysis of exons 51-54 of the *KMT2D* genes (NM_003482.3) by Droplet Digital PCR using EvaGreen was performed.

Variants were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology standards and guidelines for the interpretation of sequence variants (16). The frequencies of the identified variants were checked in GnomAD [>120 000 individuals (http://gnomad.broadinstitute.org)] and in human variant and mutation databases, such as ClinVar and Human Gene Mutation Database, as well as in the literature via PubMed and Google searches. The *in silico* tools SIFT, PolyPhen-2 and Align-GVGD were used to assess the pathogenicity of missense variant effects, and the prediction of variant effect on mRNA splicing was made using SpliceSiteFinder-like, MaxEntScan, GeneSplice, NNSPLICE and Human Splicing Finder. All *in silico* programs were accessed through the ALAMUT Visual software version 2.7.1 (Interactive Biosoftware, Rouen, France). Conservation of amino acids and nucleotides across multiple species was performed using the University of California Santa Cruz genome browser (http://genome.ucsc.edu).

### Ethical Considerations

The study was approved by the Ethical Committee of the Endocrinology and Metabolism Research Institute (ethical code: IR.TUMS.EMRI.REC.1397.009, date: 18.07.2018). The consent form was signed by all participants, or in the case of minors, the consent form was signed by their parents or legal guardian. A signed, written consent form was separately obtained for genetic testing. All procedures performed in this study were in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

### Statistical Analysis

We used the chi-square test to assess the differences between patients with confirmed genetic cause and those with no identified mutations groups.

**Table 1. The clinical features of congenital hyperinsulinism patients in our study**

| | n (%) | Gender n | | Clinical features n | | | | | | | Novel mutation n |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Girls | Boys | Low birth weight | Hypoglycaemia | Diagnosed < 1 year | Positive consanguinity | Other clinical features | Diazoxide responsive | Pancreatectomy | |
| **Total** | 44 (100%) | 20 | 24 | 8 | 30 | 34 | 21 | 7 | 35 | 8 | - |
| **No mutation** | 20 (45%) | 8 | 12 | 7 | 14 | 12 | 7 | 7 | 20 | 0 | - |
| **With mutation** | 24 (55%) | 12 | 12 | 1 | 16 | 22 | 14 | 0 | 15 | 8 | 5 |
| ***ABCC8*** | 17 (71%) | 9 | 8 | 1 | 11 | 16 | 10 | 0 | 9 | 7 | 2 |
| ***KCNJ11*** | 3 (12%) | 3 | 0 | 0 | 3 | 3 | 2 | 0 | 2 | 1 | 2 |
| ***HADH*** | 3 (12%) | 0 | 3 | 0 | 1 | 3 | 2 | 0 | 3 | 0 | 0 |
| ***KMT2D*** | 1 (4%) | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |

## Result

### Patient Characteristics

Forty-four unrelated children, 20 (45.45%) girls, with an initial diagnosis or history of CHI from all regions of Iran, diagnosed between 2016 and 2019 were recruited. Of the 44, 21 children (48%) came from consanguineous families (Table 1). Age of diagnosis varied but 36 (81.8%) were diagnosed before their first birthday. Only two (4.5%) patients had a positive family history of hypoglycaemia and 13 (29.5%) had a family history of diabetes. Low birth weight was reported for 10 (22.7%) patients. Insulin level ranged from 7.2 to 147 mU/L within our population and pancreatectomy had already been performed in eight (18.2%). Other symptoms, including hypokalaemia, autism, renal failure, seizure, oesophageal atresia, and failure to thrive were observed in 14 (31.8%). Nine out of 44 patients (20.45%) were Diazoxide-unresponsive including eight patients who had a history of pancreatectomy.

Disease causing mutations were identified in 24 cases (55%). Patients with a confirmed genetic cause in any of the known genes, were mainly diagnosed below the age of one year old ($p = 0.01$), had fewer other syndromic features, excluding seizure ($p = 0.03$), were less diazoxide responsive ($p = 0.04$) and were more diazoxide unresponsive leading to pancreatectomy ($p = 0.007$) compared to those with no identified mutations. Birth weight, hypoglycaemia and consanguinity rate was similar between the two groups (Tables 1, 2, 3).

### Genetic Findings

Among 24 patients with identified genetic mutations, 17 (71%) had a mutation in *ABCC8*, 3 (12%) in *KCNJ11*, 3 (12%) in *HADH*, and one patient had a mutation in *KMT2D* (Table 1). These included five novel mutations in *ABCC8, KCNJ11,* and *KMT2D*. Details of the mutations and the clinical features of the patients are described below and in Table 2.

Patients with *ABCC8* mutations. A total of 17 probands, including eight males and nine females were found with mutations in *ABCC8* gene, of whom two patients were carrying novel mutations; a homozygous in-frame deletion (c4724-4732del) and a heterozygous missense *de novo* mutation (c.1109G > C; p.Arg370Thr). Both of these patients were diazoxide responsive. In total, five patients had heterozygous mutations, one compound heterozygous and 11 patients had homozygous mutations. Ten patients (59%) were from consanguineous families. The median (interquartile range) birth weight was 3550 (3200-4200) g. Eight patients (47%) were diazoxide unresponsive and had undergone pancreatectomy. Other clinical features included seizure in two (11.8%) patients.

Patients with *KCNJ11* mutations. All three patients were homozygous, including two with novel missense mutations (c.362T > G; p.Phe121Cys and c.370T > A; p.Ser124Thr) who were both diazoxide responsive. Birth weight was between 3800 g and 4880 g. The patient with c.287_288delinsTG mutation (p.Ala96Val) had a history of seizure, was diazoxide unresponsive and underwent pancreatectomy.

Patients with *HADH* mutations. Three cases were detected with *HADH* gene mutations, of whom one case was homozygous for a frameshift variant (c.617del; p.Lys206fs) and the other two were homozygous for a nonsense variant (c.706C > T; p.Arg236Ter). Two patients were from consanguineous families and had seizures. Birth weight ranged from 3250g to 4800g. The response to diazoxide was good in all the patients.

Patient with *KMT2D* mutation. One patient was mosaic for a *KMT2D* partial gene deletion of exons 51-54. The level of mosaicism within his leukocyte DNA was estimated to be at least 20%, consistent with a post-zygotic origin. Genetic testing in the parents indicated a *de novo* change. The patient was from a non-consanguineous family and was diagnosed with hyperinsulinism at the age of two years. Birth weight was 4500g and the response to diazoxide was good. The patient had a history of seizure and did not have any facial dysmorphism.

### Diazoxide Responsiveness and Pancreatectomy

Diazoxide unresponsiveness was seen in nine (38%) of the patients with genetic mutations. These included eight patients (89%) with mutations in *ABCC8* and one with *KCNJ11* mutation. This figure means 45% of those with $K_{ATP}$-channel mutations were diazoxide-unresponsive. Seven out of eight (88%) patients who had had pancreatectomy had *ABCC8* gene mutations and one had *KCNJ11* mutation. Pathogenic mutation was invariably found in diazoxide-unresponsive patients (9/9; 100%), although more than half of cases in the diazoxide-responsive group (20/35; 57%) had no genetic variant identified in the genes investigated (Figure 1).

## Discussion

We described the spectrum of genetic mutations in CHI in an Iranian population, as well as the frequency of each mutation and their related clinical features. Genetic mutations were found in 55% of our patients, consistent with previous studies which identified mutations in 12 out of 19 (63%) patients in Turkey (10) or in only 47% (56 of 118)

**Table 2. Mutation description and clinical features of patients with identified genetic cause**

| ID | Gene variant | Location | Effect | Inheritance | DNA description | Protein description | Novel | Consanguineous | Clinical features | Pancreatectomy | Age of diagnosis | BW | Response to diazoxide |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HI 1 | *ABCC8* | Exon 21 | Nonsense | Homozygous | c.2524C > T | p.Arg842Ter | No | Yes | Seizure | No | < 1 year | 4500 | Good |
| HI 12 | *ABCC8* | Exon 1 | Missense | Heterozygous | c.96C > G | p.Asn32Lys | No | No | Seizure | No | < 1 year | 3590 | Good |
| HI 2 | *ABCC8* | Exon 1 | Missense | Homozygous | c.96C > G | p.Asn32Lys | No | Yes | No | Yes | < 1 year | 3000 | Poor |
| HI 4 | *ABCC8* | Exon 16 | Missense | Heterozygous | c.2159C > T | p.Ser720Phe | No | No | No | Yes | < 1 year | 4100 | Poor |
| M-10 | *ABCC8* | Exon 3 | Missense | Homozygous | c.331G > A | p.Gly111Arg | No | Yes | No | No | < 1 year | 4200 | Poor |
| M-12 | *ABCC8* | Intron 14 | Aberrant splicing | Homozygous | c.2041-21G > A | p.? | No | Yes | No | No | < 1 year | 3400 | Good |
| M-15 | *ABCC8* | Exon 28 | Frameshift | Heterozygous | c.3438dup | p.Thr1147fs | No | No | No | Yes | < 1 year | 3300 | Poor |
| M-17 | *ABCC8* | Intron 11 | Aberrant Splicing | Homozygous | c.1672-5C > G | p.? | No | Yes | No | Yes | < 1 year | 5500 | Poor |
| M-23 | *ABCC8* | Exon 25 | Frameshift | Homozygous | c.3151dup | p.Cys1051fs | No | Yes | No | No | < 1 year | 5530 | Good |
| M-24 | *ABCC8* | Exon 28 | Frameshift | Heterozygous | c.3438dup | p.Thr1147fs | No | No | No | Yes | < 1 year | 3550 | Poor |
| M-28 | *ABCC8* | Intron 14 | Aberrant splicing | Homozygous | c.2041-21G > A | p.? | No | No | No | No | < 1 year | 2000 | Good |
| M-3 | *ABCC8* | Intron 11 | Aberrant splicing | Homozygous | c.1671 + 1G > A | p.? | No | Yes | No | Yes | < 1 year | 4350 | Poor |
| M-30 | *ABCC8* | Intron 14 | Aberrant splicing | Homozygous | c.2041-21G > A | p.? | No | Yes | No | No | < 1 year | 3600 | Good |
| M-5 | *ABCC8* | Exon 23 | Nonsense | Homozygous | c.2809C > T | p.Cln937Ter | No | No | No | Yes | < 1 year | 2600 | Poor |
| M-6 | *ABCC8* | Intron 14 | Aberrant splicing and missense | Compound heterozygous | c.2041-21G > A / c.96C > G | p.? / p.Asn32Lys, c.96C > G | No | Yes | No | No | < 1 year | 3150 | Good |
| HI 3 | *ABCC8* | Exon39 | In-Frame Deletion | Homozygous | c4724-4732del | p.A1575-F1577 | Yes | Yes | No | No | < 1 year | 3500 | Good |
| M-21 | *ABCC8* | Exon 7 | Missense | Heterozygous | c.1109G > C | p.Arg370Thr | Yes | No | No | No | < 1 year | 3200 | Good |
| HI 17 | *HADH* | Exon 6 | Nonsense | Homozygous | c.706C > T | p.Arg236Ter | No | Yes | Seizure | No | < 1 year | 4800 | Good |
| HI 5 | *HADH* | Exon 5 | Frameshift | Homozygous | c.617del | p.Lys206fs | No | Yes | Seizure | No | < 1 year | 3600 | Good |
| M-19 | *HADH* | Exon 6 | Nonsense | Homozygous | c.706C > T | p.Arg236Ter | No | No | No | No | < 1 year | 3250 | Good |
| M-14 | *KCNJ11* | Exon 1 | Missense | Homozygous | c.287_288delinsTG | p.Ala96Val | No | Yes | Seizure | Yes | < 1 year | 3800 | Poor |
| M-20 | *KCNJ11* | Exon 1 | Missense | Homozygous | c.362T > G | p.Phe121Cys | Yes | No | No | No | < 1 year | 3950 | Good |
| M-32 | *KCNJ11* | Exon 1 | Missense | Homozygous | c.370T > A | p.Ser124Thr | Yes | Yes | No | No | < 1 year | 4880 | Good |
| HI 13 | *KMT2D* | Exons 51-54 | Partial Deletion | Post-zygotic | Chr12 (GRCh37): g.(49415449) (49416715)del | | Yes | No | Seizure | No | 2 years | 4500 | Good |

of the diazoxide-responsive cases in the US (17). This figure is lower than some studies, which could reflect differences in referral patterns and inclusion or exclusion criteria (18).

Consistent with previous studies (17,19,20), mutations in $K_{ATP}$-channel including SUR1 (*ABCC8*) and Kir6.2 (*KCNJ11*), accounted for 83 % of all CHI-causing mutations in our study. Mutations in the *ABCC8* gene with 71 % occurrence was the most frequent cause of CHI in our population. In addition, eleven participants were homozygous for a mutation in *ABCC8* which confirmed a diagnosis of autosomal recessive CHI. One patient was homozygous for a novel, in-frame deletion in *ABCC8*, which requires further investigation to determine its clinical significance.

The majority of recessive mutations in the $K_{ATP}$-channel has been shown to lead to medically unresponsive CHI (21,22), but in our study, six out of 11 patients with homozygous *ABCC8* mutations and two out of three patients with homozygous *KCNJ11* mutations were diazoxide-responsive. Although Salomon-Estebanez et al (23) had suggested that this may be due to the reduction of severity of disease over time, it is not reasonably likely and may be due to variable criteria used to determine diazoxide responsiveness. In contrast, *ABCC8* and *KCNJ11* heterozygous mutations were characterized by various presentations and treatment responses; three out of six (50 %) patients with heterozygous mutations were diazoxide-unresponsive which is in agreement with other studies (21). In our study, all heterozygous mutations were in the *ABCC8* gene, which may indicate a dominant pattern of inheritance for certain mutations in the *ABCC8* gene.

**Table 3. Clinical features of patients with no identified genetic cause**

| ID | Genetic test | Consanguineous | Clinical features | Pancreatectomy | Age of diagnosis | BW | Response to diazoxide |
|---|---|---|---|---|---|---|---|
| HI 11 | tNGS | No | No | No | 4 years | 3800 | Good |
| HI 14 | tNGS | Yes | Renal cyst, abnormal internal genitalia | No | 13 months | NA | Good |
| HI 6 | tNGS | Yes | Hypocalcemia, autism, renal failure, nephrectomy | No | 10 years | 3700 | Good |
| M-1 | tNGS | No | No | No | 2 years | 1930 | NA |
| M-11 | tNGS | Yes | No | No | Diagnosed < 1 year | 3200 | Good |
| M-18 | tNGS | Yes | No | No | After birth | 3250 | Good |
| M-2 | tNGS | No | No | No | 4.5 months | 3300 | Good |
| M-25 | tNGS | No | No | No | 2 years | 3500 | Good |
| M-27 | tNGS | Yes | Precocious puberty, hypothyroidism | No | 4 years | 3100 | Good |
| M-9 | tNGS | No | GI obstruction | No | 7.5 years | 1890 | Good |
| HI 10 | *ABCC8* & *KCNJ11* | Yes | No | No | Diagnosed < 1 year | 3500 | Good |
| HI 9 | *ABCC8* & *KCNJ11* | No | Esophageal atresia | No | Diagnosed < 1 year | 1069 | Good |
| M-13 | *ABCC8* & *KCNJ11* | No | No | No | Diagnosed < 1 year | 3050 | Good |
| M-16 | *ABCC8* & *KCNJ11* | Yes | Macroglossia | | Diagnosed < 1 year | 4200 | Good |
| M-22 | *ABCC8* & *KCNJ11* | No | No | No | 2 years | 1700 | Good |
| M-26 | *ABCC8* & *KCNJ11* | No | No | No | Diagnosed < 1 year | 3970 | NA |
| M-29 | *ABCC8* & *KCNJ11* | No | No | No | Diagnosed < 1 year | 1600 | Good |
| M-31 | *ABCC8* & *KCNJ11* | No | No | No | Diagnosed < 1 year | 2200 | Good |
| M-4 | *ABCC8* & *KCNJ11* | No | No | No | 14 month | 4070 | Good |
| M-7 | *ABCC8* & *KCNJ11* | No | IUGR | No | Diagnosed < 1 year | 1900 | Good |

tNGS: targeted next generation sequencing

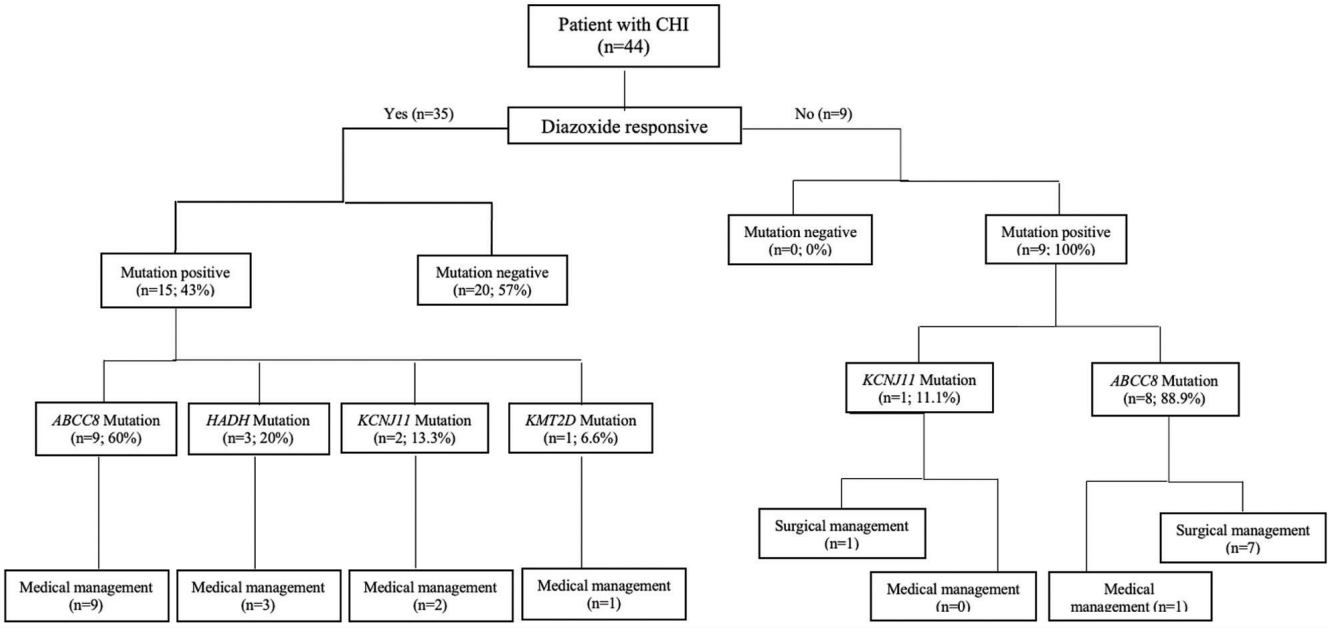


**Figure 1.** Mutation analysis results and treatment choices for patients with diazoxide-responsive congenital hyperinsulinism (CHI) vs. diazoxide-unresponsive CHI

Although, a subtotal pancreatectomy is necessary in most cases of CHI (24), the vast majority of our participants were diazoxide-responsive (79%). Previous studies have reported different rates of diazoxide responsiveness in their cohorts. In studies of de Lonlay et al (8) and Snider et al (17) the ratio of diazoxide-responsive participants were 28% and 34%, respectively. In a study from Turkey 59% (13/22) of patients were diazoxide-responsive (10). In 27 infants who were born small-for-gestational age in the UK and developed HH, diazoxide-response was 100% (25). Diazoxide-responsiveness in two studies of Iranian patients has been reported to be 18/23 and 3/6 (18,26). This discrepancy is probably due to the number of cases in some studies, variable criteria used to define diazoxide responsiveness, differences in sensitivity of the methods used for detecting the mutations and/or occurrence of mutations in combination with a second mutant allele that complicated the prediction of consequences. Our results, however, are consistent with previous studies (8,27), and indicate that *ABCC8* gene defects are the most important cause of diazoxide-unresponsive CHI (8/9 of diazoxide-unresponsive patients) in Iranian children.

The three patients with *HADH* homozygous mutations were all diazoxide-responsive. This is consistent with other studies showing hyperinsulinism due to *HADH* gene mutations responds relatively well to diazoxide (12,18,24). Mutations in the *HADH* gene result in a diffuse form of CHI (28). Interestingly, the rate of observed *HADH* mutations in our study was the same as for *KCNJ11* mutations. This result is in concordance with some other studies (18,26). A high frequency of *HADH* mutations in our study could be due to a high rate of consanguineous marriage. Based on our results, screening for *HADH* gene variants is recommended in all patients with diazoxide-responsive CHI.

One patient in our cohort was mosaic for a *KMT2D* partial gene deletion. Pathogenic variants in the *KMT2D* gene cause Kabuki syndrome (29). People with Kabuki syndrome also have facial and some other specific congenital anomalies, including growth delays, mental retardation and skeletal abnormalities, which were not observed in our participant.

In nearly 20% of patients, no mutation was detected using tNGS, which may miss large deletions and chromosomal rearrangements. Furthermore, negative results do not exclude a monogenic aetiology and further unidentified mutations in other unidentified genes should be considered as new aetiologies.

## Study Limitations

The strength of our study is that we have collected genetic data on the largest cohort of CHI in an Iranian population to date and identified novel variants causing the disease. Our samples were referred from different parts of Iran. Study limitations include the retrospective collection of clinical data and the lack of follow-up data to assess detailed clinical significance of novel findings.

## Conclusion

Most novel mutations identified in this study were inherited in a homozygous fashion. Variation in reported rates of diazoxide responsiveness suggest a need for revision of the criteria used to define diazoxide responsiveness. All novel mutations that we report for the first time were medically responsive, and yet should now be considered in CHI analysis. More studies on the molecular basis of CHI are necessary for societies with highly consanguineous families. We recommend tNGS for all CHI patients but screening of *HADH* gene variants in patients with diazoxide-responsive CHI seems to be required if there is no access to tNGS.

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### Ethics

**Ethics Committee Approval:** The study was approved by the Ethical Committee of the Endocrinology and Metabolism Research Institute (ethical code: IR.TUMS. EMRI.REC.1397.009, date: 18.07.2018).

**Informed Consent:** Informed consent was obtained from parents on behalf of their children.

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

### Authorship Contributions

Surgical and Medical Practices: Maryam Razzaghy-Azar, Farzaneh Abbasi, Somayyeh Hashemian, Peyman Eshraghi, Siroos Karimdadi, Parisa Tajdini, Rahim Vakili, Concept: Mahsa M. Amoli, Hanieh Yaghootkar, Design: Mahsa M. Amoli, Data Collection or Processing: Sepideh Borhan Dayani, Samaneh Enayati, Analysis or Interpretation: Saeedeh Saeedi, Mahsa M. Amoli, Hanieh Yaghootkar, Literature Search: Saeedeh Saeedi, Mahsa M. Amoli, Writing: Saeedeh Saeedi, Mahsa M. Amoli, Hanieh Yaghootkar.

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BRIEF REPORT

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# Comparison of Indonesian Growth Reference Chart and World Health Organization Child Growth Standard in Detecting Stunting: A Systematic Review and Meta-analysis of 15,874 Children

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

The World Health Organization Child Growth Standards (WHOCGS) overestimates the stunting population in several countries, including Indonesia. An accurate growth standard is needed to avoid overdiagnosis and overtreatment of stunting.

**What this study adds?**

This systematic review and meta-analysis combine the previous findings that compare WHOCGS and the 2018 Indonesian Growth Reference Chart (IGRC). Pooled analysis showed that the IGRC resulted in a lower prevalence of stunted and severely stunted but not normal and tall children.

## Abstract

Recognition of an overestimation of stunted children in Indonesia when using the World Health Organization Child Growth Standards (WHOCGS) led to the creation of the Indonesian Growth Reference Chart (IGRC) in 2005, with further improvement in 2018. This systematic review aimed to determine whether there is a difference in the diagnosis of stunting when using these two charts. This systematic review is registered in the PROSPERO database (CRD42021259934). Literature research was performed on PubMed, Science Direct, Medline, Scielo, Medrxiv, Research Square, SSRN, and Biorxiv to identify studies published from 2018 onwards that examined the comparison of IGRC and WHOCGS in detecting stunting. Three studies were included in this review. Pooled analysis showed that IGRC resulted in a lower prevalence of stunted and severely stunted children [risk ratio (RR): 0.28 (95% confidence intervals (CI): 0.15-0.51), $p < 0.0001$, $I^2 = 97\%$]. Comparison between IGRC and WHOGCS for prevalence of normal height children showed that there was no difference, and this finding was not significant [RR: 1.56 (95% CI: 0.92-2.66), $p = 0.1$, $I^2 = 100\%$], and the comparison for prevalence of tall children also showed that there was no difference when using IGRC or WHOGCS, and this finding was also insignificant [RR: 2.02 (95% CI: 0.78-5.20), $p = 0.14$, $I^2 = 98\%$]. This meta-analysis showed that stunted and severely stunted Indonesian children are over-represented using WHOCGS. The difference between IGRC and WHOCGS has occurred because of the sample population, as IGRC includes children from all 33 provinces in Indonesia, better reflecting the growth of all children in Indonesia.

**Keywords:** Indonesian Growth Reference Chart, WHO Growth Chart, stunting

## Introduction

Despite improvements in accessibility to basic needs, such as food and water and primary medical care, stunting is still prevalent amongst Indonesian children (1). Although the percentage of children with stunting decreased to 11.6% in 2020, some provinces still have more than 20% of children who suffer from stunting (2).

It was previously believed that undernutrition was the leading cause of stunting (3), so aggressive nutrition has been provided for stunted children, which resulted in increased prevalence of obesity (4). Starvation inhibits growth, but intervention at a nutritional level does not show any beneficial effect (3). Similarly to other countries that have adopted local reference growth charts, an

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overestimation of stunted children in Indonesia was noted when using the World Health Organization Child Growth Standards (WHOCGS) (5). Some other factors may influence the potential growth of South East Asian children, such as variation in genetic growth potential and intergenerational epigenetic growth limitations (6). When investigating stunted and stunting children, other anthropometric measurements, such as body mass index (BMI), skinfold thickness, or even height standard deviation scores, need to be considered. This is because one or more obvious clinical symptoms of malnutrition are present in stunted children (3).

Therefore, an Indonesian Growth Reference Chart (IGRC) was created in 2005 (7) and was further improved in 2018 (8). However, the adoption of IGRC is still slow, even though there are apparent disparities between findings using WHOCGS and IGRC (7,8). Therefore, the primary aim of this systematic review was to determine whether there is a difference in diagnosis of stunting when using these two charts (2006 WHOCGS vs 2018 IGRC). The secondary aim was to assess whether IGRC charts also detect normal height children and tall children better than WHOGCS.

## Methods

### Eligibility Criteria

The Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) 2020 statement was followed in this systematic review (9,10). The protocol for this systematic review has been uploaded to the International Prospective Register of Systematic Reviews (PROSPERO) database (CRD42021259934).

The literature search was limited to the period from 2018 onwards, with no restrictions on language. The reason for the timeframe restriction was because the version of IGRC used in this study was published in 2018 (8). All cross-sectional studies and cohort studies were eligible for inclusion in this review. The inclusion criteria were children aged 0-60 months with their height measured and plotted against both IGRC and WHOCGS. Exclusion criteria comprised studies making comparison of stunting using charts other than the specific two in question - IGRC and WHOCGS. Abstracts, letters to the editor, and reviews were screened for references to ensure literature saturation before they were excluded.

Stunting was defined as length/height below -2 standard deviation (SD) for children under the age of two, while severe stunting was defined as length/height below -3 SD for children under the age of three for both WHOCGS and IGRC, taking into account their sexes.

### Search Strategy and Study Selection

The literature search started on July 10, 2021, and ended on the same day. The authors utilized four public databases, PubMed, Science Direct, Medline, and Scielo, and four different preprint databases, Medrxiv, Research Square, SSRN, and Biorxiv. Table 1 contains a list of keywords used to search each database.

**Table 1. Keywords used in each database platform**

| Database | Keyword or medical subject headings |
|---|---|
| Medline | (((Indonesian[All Fields] AND ("federal government" [MeSH Terms] OR ("federal" [All Fields] AND "government" [All Fields]) OR "federal government" [All Fields] OR "national" [All Fields]) AND ("growth and development" [Subheading] OR ("growth" [All Fields] AND "development" [All Fields]) OR "growth and development" [All Fields] OR "growth" [All Fields] OR "growth" [MeSH Terms]) AND reference[All Fields] AND "chart" [All Fields]) AND (WHO[All Fields] AND ("growth charts" [MeSH Terms] OR ("growth" [All Fields] AND "charts" [All Fields]) OR "growth charts" [All Fields] OR ("growth" [All Fields] AND "chart" [All Fields]) OR "growth chart" [All Fields]))) AND ("thinness" [MeSH Terms] OR "thinness" [All Fields] OR "underweight" [All Fields])) OR ("growth disorders" [MeSH Terms] OR ("growth" [All Fields] AND "disorders" [All Fields]) OR "growth disorders" [All Fields] OR "stunting" [All Fields]) |
| Research Square | (Indonesian growth chart) AND (WHO growth chart) AND (underweight) OR (stunting) |
| Google Scholar | Indonesian growth chart AND WHO growth chart AND underweight OR stunting |
| PubMed | (("indonesian" [All Fields] OR "indonesians" [All Fields]) AND ("growth charts" [MeSH Terms] OR ("growth" [All Fields] AND "charts" [All Fields]) OR "growth charts" [All Fields] OR ("growth" [All Fields] AND "chart" [All Fields]) OR "growth chart" [All Fields]) AND ("WHO" [All Fields] AND ("growth charts" [MeSH Terms] OR ("growth" [All Fields] AND "charts" [All Fields]) OR "growth charts" [All Fields] OR ("growth" [All Fields] AND "chart" [All Fields]) OR "growth chart" [All Fields]))) AND (2018:2021[pdat]) |
| Science Direct | (Indonesian growth chart) AND (WHO growth chart) AND (underweight) OR (stunting) |
| Scielo | (Indonesian growth chart) AND (WHO growth chart) AND (underweight) OR (stunting) |
| Medrxiv | (Indonesian growth chart) AND (WHO growth chart) |
| Biorxiv | (Indonesian growth chart) AND (WHO growth chart) AND (underweight) OR (stunting) |
| SSRN | (Indonesian growth chart) AND (WHO growth chart) |

### Data Extraction and Quality Assessment

Three independent reviewers (CP, CT, and CF) compiled the data in a standardized format, including demographic characteristics of the included participants (age, sex, and height) and prevalence of stunting according to IGRC and WHOCGS. It was planned that if there was any missing data needed for this systematic review in any identified study, the corresponding author of the research would be contacted directly.

The same independent reviewers conducted the quality assessment of each study. The Newcastle Ottawa Quality Assessment Scale (NOS) was used to assess the quality of cross-sectional and longitudinal studies (11). Any differences between NOS results were discussed until a consensus was reached. If there were still any unresolved disagreements, two expert reviewers (GSO and AJ) were consulted, and the final decision was made based on their expertise and consensus. A score of ≥7 was the cut-off used for a study to be considered of good quality (11).

### Statistical Analysis

The meta-analysis was carried out using the Review Manager 5.4 (Cochrane Collaboration) software. The risk ratios (RR) and their 95 percent confidence intervals (CI) were calculated using Mantel-Haenszel's formula. In contrast, the mean difference and its SD were calculated using the Inverse Variance technique. Low, moderate, and high degrees of heterogeneity was determined using the $I^2$ statistic, with values of 25 percent, 26 percent -50 percent, and > 50 percent, respectively. When the two-tailed p-value was 0.05 or less, the results were considered significant. Begg's funnel plot analysis was used to estimate the qualitative risk of publication bias.

## Results

The study selection process is listed in Figure 1, where ultimately, three studies were selected for inclusion in this review (12,13,14). Two studies (12,13) had good quality with a NOS of eight each, while Hilmy and Fatharani's (14) (2021) study only scored five using NOS (Table 2). All of the studies were cross-sectional studies. There were 15,874 children included in total in this review, with 7372 children being male (46.4%). Using WHOCGS, there were 7627 stunted children (48.04%), while there were only 1884 stunted children (11.87%) when plotted against the IGRC.

Three studies (n = 15,874) reported on the prevalence of stunted and severely stunted children. Pooled analysis showed that IGRC resulted in a lower prevalence of stunted and severely stunted children [RR: 0.28 (95% CI: 0.15-0.51), $p < 0.0001$, $I^2 = 97\%$, random-effect modelling; Figure 2A]. When comparing IGRC and WHOGCS in terms of normal height children, pooled analysis of the three studies (n = 15,874) showed that there was no difference, and this finding was not significant 'RR 1.56 (95% CI: 0.92-2.66), $p = 0.1$, $I^2 = 100\%$, random-effect modelling; Figure 2B]. Lastly, pooled analysis of tall children from two studies (n = 15,656) showed that there was also no difference between IGRC and WHOGCS, and this finding was also insignificant [RR: 2.02 (95% CI: 0.78-5.20), $p = 0.14$, $I^2 = 98\%$, random-effect modelling; Figure 2C]. The funnel plot was not used to visualize publication bias as there were less than ten studies (15).

## Discussion

Several studies of the Indonesian population have attempted to identify factors associated with stunting in children (16,17,18). However, there is little consensus on determinants that might be associated with stunting, prompting doctors, researchers, and government officials to discuss the potential of developing an IGRC. The need for a local growth reference chart stems from reports that

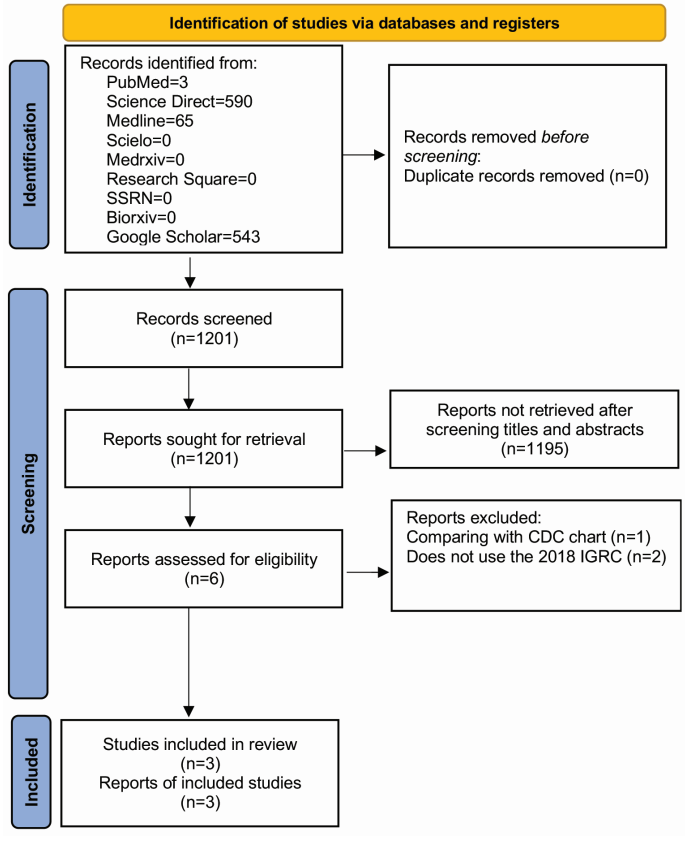


**Figure 1.** PRISMA flow chart of this study

*IGRC: Indonesian Growth Reference Chart*

WHOCGS is more likely to overdiagnose underweight and stunting in otherwise normal children (19). The result of this meta-analysis supports this finding, as stunted and severely stunted were over-represented when using the WHOCGS in the three studies examined. The main difference between IGRC and WHOCGS is in the sample population. While WHOCGS includes children that have followed the feeding recommendations of the WHO, IGRC includes children from all 33 provinces in Indonesia which better reflects the growth of children in the whole population of Indonesia

**Table 2. Summary of studies included in this review**

| Author (year) | Study location | Total sample (% male) | Stunted children according to WHOCGS (%) | Stunted children according to IGRC (%) | Conclusion | NOS | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Selection | Comparability | Outcome |
| Novina et al (12) | Bandung | 12772 (54.6) | 7193 (56.31) | 1698 (13.3) | The WHOCGS grossly underestimates the true prevalence of malnourishment among Indonesian children. | 4 | 1 | 3 |
| Flynn et al (13) | Musi sub-district | 218 (49.5) | 112 (51.4) | 18 (8.3) | In Musi sub-district, WHOCGS is not appropriate for reflecting child growth. | 3 | 2 | 3 |
| Hilmy and Fatharani (14) | Blega sub-district | 2884 (50.8) | 322 (11.2) | 168 (5.8) | When IGRC was used instead of WHOCGS, the frequency of stunting was twice lower. More research is required. | 2 | 1 | 2 |

WHOCGS: World Health Organization Child Growth Standard, IGRC: Indonesian Growth Reference Chart, NOS: Newcastle-Ottawa Scale

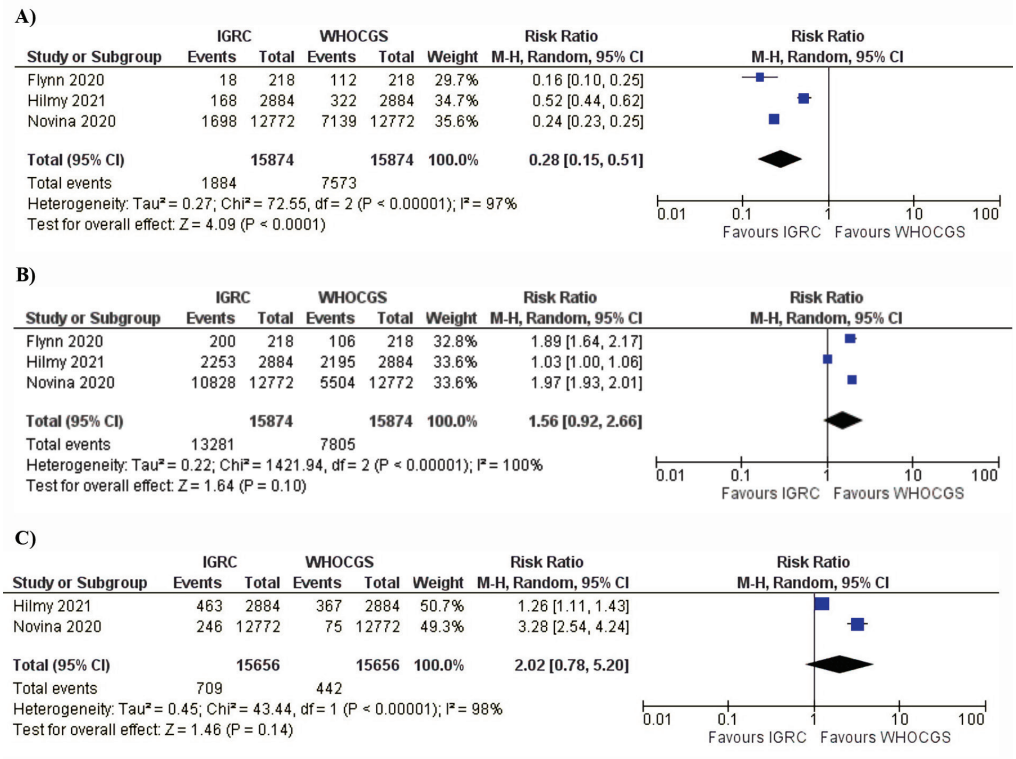


**Figure 2.** Forest plot that demonstrates the association of stunting and severe stunting (A), normal height (B), and tall (C) children when comparing IGRC and WHOCGS

*WHOCGS: World Health Organization Child Growth Standard, IGRC: Indonesian Growth Reference Chart, CI: confidence interval*

(13). Genetic and unknown environmental factors are still debated as the cause of differences between local growth reference charts and WHOCGS population analyses (20).

### Study Limitations

However, a local growth reference chart also has limitations, including both statistical and practical limitations (20). This might explain why there are no differences in IGRC and WHOCGS in detecting normal height children and tall children. As the local guideline recommends using 2006 WHOCGS in children under five years old, we suggest that the IGRC can be incorporated when a child is stunted according to WHOCGS. If the child is clinically normal and the IGRC detects that the child is not stunted, these findings can be discussed and explained to the parents.

Several caveats should be noted when comparing WHOCGS and IGRC. While IGRC plots the development of 0-18 years old children, WHOCGS only plots for the 0-5 years old. Comparing IGRC and WHOCGS is thus like comparing apples with pears. The optimal growth chart, according to Karlberg's (21) infancy-childhood-puberty development model, should include the whole growth spectrum, from infancy to puberty, due to the significant variations in growth rates in each phase of childhood and WHOCGS does not do this.

There are several limitations of this review. Firstly, one study has a low NOS, which indicates that the study is not well-conducted and may have introduced a bias in our synthesis. Secondly, there are only three studies that could be included due to the limited number of studies available. There was one study that was excluded because the study used the 2005 IGRC instead of the 2018 IGRC (22). Lastly, there was significant heterogeneity amongst the studies that study design or publication bias might explain. However, despite the limitations, our meta-analysis might provide a stimulus for clinicians, researchers, or government bodies to conduct more large, well-designed, prospective studies to investigate the continuous growth of children in terms of weight and height, in both Indonesia and other countries that have noted discrepant findings between WHOCGS and local growth reference charts.

## Conclusion

The result of this meta-analysis showed that Indonesian stunted and severely stunted children are over-represented using WHOCGS compared to IGRC. This could be due to the difference in sample population between the two growth charts, because IGRC includes children from all 33 provinces in Indonesia. Despite this, IGRC also has its limitations which might explain why there are no differences in IGRC and WHOCGS in detecting the prevalence of normal height and tall children. Therefore, more well-designed, large, prospective studies are still needed to investigate this matter further.

### Ethics

**Ethics Committee Approval:** Ethics is not required since this is a systematic review.

**Informed Consent:** Informed consent is not required since this is a systematic review.

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

### Authorship Contributions

Concept: Gilbert Sterling Octavius, Andry Juliansen, Design: Gilbert Sterling Octavius, Chelsea Serena br. Pardede, Cindy Clarissa Thandy, Clauvinna Adhityana Lie Fisca, Andry Juliansen, Data Collection or Processing: Gilbert Sterling Octavius, Chelsea Serena br. Pardede, Cindy Clarissa Thandy, Clauvinna Adhityana Lie Fisca, Analysis or Interpretation: Gilbert Sterling Octavius, Andry Juliansen, Literature Search: Chelsea Serena br. Pardede, Cindy Clarissa Thandy, Clauvinna Adhityana Lie Fisca, Writing: Gilbert Sterling Octavius, Chelsea Serena br. Pardede, Cindy Clarissa Thandy, Clauvinna Adhityana Lie Fisca, Andry Juliansen.

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CASE REPORT

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# Using Etomidate in a Two-month-old Infant with Cushing Syndrome due to Adrenocortical Carcinoma

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

Medical treatment of Cushing syndrome (CS) is important, particularly when a surgical cure is impossible. Although etomidate is an adrenal-blocking drug used to treat CS, its use in infants is unreported.

**What this study adds?**

Here we describe the case of a 2-month-old girl treated with etomidate for CS caused by adrenocortical carcinoma. We identified a safe dosage with an infusion rate of 0.03 mg/kg/hour, and observed good control of hypercortisolemia after treatment.

## Abstract

Cushing syndrome (CS) is a rare disease caused by hypercortisolemia. Although surgical treatment is the first-line treatment in CS, the appropriate medication for the patient's condition should be selected when medical treatment is needed. Etomidate is an adrenal-blocking drug used to treat CS and the most suitable for severe hypercortisolemia and adrenocortical carcinoma (ACC), due to cardiovascular stability and an anti-tumorigenic effect. However, its use and safe recommended dosage in infants with CS is unreported. Here we describe the case of a 2-month-old girl treated with etomidate for CS caused by ACC. Even though radical mass excision was performed, severe hypercortisolemia persisted, resulting from metastatic lesions in the liver, and medical treatment was considered. The etomidate doses, no bolus dose and infusion rate of 0.03 mg/kg/hour, may be an appropriate dose for severe hypercortisolemia in infants. This case will help determine future treatment strategies for similar cases in infants.

**Keywords:** Etomidate, infant, Cushing syndrome

## Introduction

Cushing syndrome (CS) is a rare disease, particularly in children (1). First-line treatment of CS is surgical resection of the source of excess glucocorticoid. However, non-surgical medical treatment of hypercortisolemia is important when the mass is unresectable, or in pre-and postoperative emergency situations. Reports of non-surgical medical treatment of CS in infants are very rare.

Medical treatment for CS can be divided into three groups: adrenal-blocking drugs; neuromodulatory drugs; and glucocorticoid receptor-blocking agents (2). Among these, adrenal-blocking drugs are based on inhibition of adrenal steroidogenesis at enzymatic sites, with the most frequently used drugs being oral ketoconazole and metyrapone. Ketoconazole inhibits multiple enzymes involved in adrenal steroidogenesis, but can cause hepatotoxicity (2). Metyrapone blocks the last step of cortisol synthesis, increasing the concentration of several precursor molecules with mineralocorticoid activity. Therefore, metyrapone may cause hypertension, edema, or hypokalemia (2).

Etomidate is an adrenal-blocking drug that functions by inhibiting enzymes in adrenal steroidogenesis. It exhibits a rapid onset of action, does not dramatically change blood

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pressure or heart rate, and does not stimulate histamine release (3). Therefore, it is suitable for patients with severe cardiovascular complications and life-threatening hypercortisolemia. However, to our knowledge, there is only one reported case of etomidate administration in infants with CS. We report the case of a 2-month-old girl presenting with hypertrophic cardiomyopathy (HCMP) and CS, resulting from severe hypercortisolemia due to adrenocortical carcinoma (ACC), that was not resolved through surgery. Our report focuses on the use of etomidate to treat this patient.

## Case Report

A 2-month-old girl was referred to the Pediatric Endocrinology Department in Severance Children's Hospital, South Korea for evaluation of cushingoid features. She was a full-term baby of normal weight, with unremarkable pregnancy and maternal history. During a hospital visit for regular checkup and vaccinations, her vital signs showed hypertension and tachycardia. Two-dimensional echocardiography revealed HCMP (Figures 1, 2). She was admitted to the cardiology department.

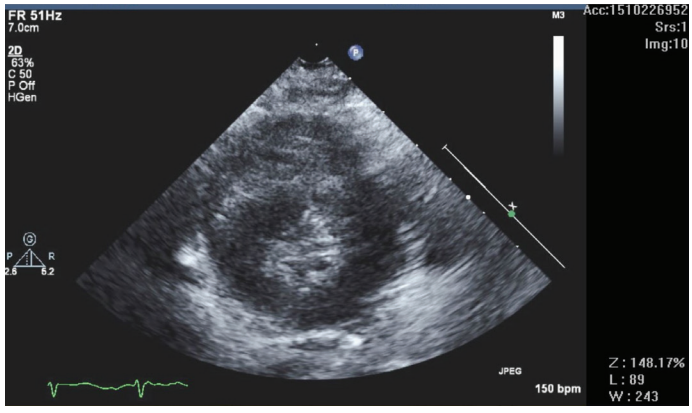


**Figure 1.** Parasternal short axis view of echocardiogram in infant patient with hypertrophic cardiomyopathy

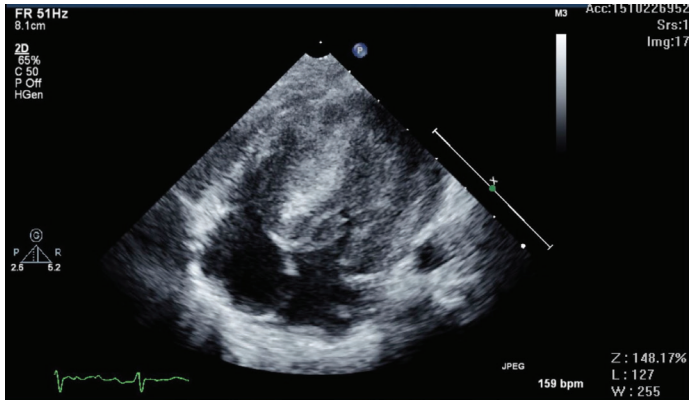


**Figure 2.** Four chamber view of echocardiogram in infant patient with hypertrophic cardiomyopathy

During her stay, she was referred to the endocrinology department due to cushingoid appearance, including a full moon face, purple striae, central obesity, and accumulation of fat in the abdomen, chest, and face. Her body weight, body length, and body mass index were 7.6 kg (> 97$^{th}$ percentile), 52 cm (< 3$^{rd}$ percentile), and 28.1 kg/m$^2$ (> 97$^{th}$ percentile), respectively.

Basal cortisol and adrenocorticotropic hormone (ACTH) were assessed at 8 am, and the cortisol level was 112.7 µg/dL (reference range 6.0-18.4 µg/dL at 7-10 am) with a concomitant suppressed plasma ACTH level of < 1 pg/mL (reference range 7.2-63.3 pg/mL). Further diagnostic studies showed high 24-hour urinary free cortisol levels (1,777.1 µg/day and 2,782.7 µg/day, respectively, reference range 58.0-403.0 µg/day), and low ACTH level, consistent with ACTH-independent CS. Abdominal sonography revealed an approximately 4.5 × 3.5 × 4.7 cm heterogeneously hypoechoic lesion in the right suprarenal space, and several small enhancing lesions in the liver, suspected to be metastatic lesions. Resection of the adrenal gland tumor was performed and histopathologic examination confirmed ACC with poor prognosis according to the Wieneke-index criteria (4). The metastatic liver lesions were also removed, and metastasized ACC was histopathologically confirmed.

The patient's postoperative cortisol level immediately decreased to 22.6 µg/dL, and we closely monitored her cortisol levels to prevent an adrenal crisis (data shown in Table 1). After 24 hours, cortisol levels stabilized at 15.9 µg/dL, but gradually increased 48 hours after surgery without steroid replacement. On postoperative day 18, her cortisol level was 68.0 µg/dL, and imaging studies revealed severe progressive cardiomegaly with compressed lungs and multiple liver metastases. Her general condition and vital signs then deteriorated, with hypertension (blood pressure, 170/100 mmHg) and tachycardia (heart rate, 140 beats/minute). Therefore, medical treatment was considered instead of reoperation. First, the oncology department started chemotherapy for the liver metastases. The planned chemotherapy was cisplatin-etoposide-doxorubicin. In addition, etomidate was selected to treat severe hypercortisolemia because of the patient's severe HCMP, severe hypertension, and liver metastases. We initially administered a 0.3 mg/kg loading dose of etomidate for severe hypercortisolemia, and subsequently maintained a dose of 0.03 mg/kg/hour. Her cortisol level decreased to 32.3 µg/dL after four hours, and to 21.7 µg/dL after eight hours of etomidate infusion. However, her blood pressure decreased, possibly due to relative adrenal insufficiency. Therefore, we stopped the infusion of etomidate. Furthermore, after khree days of cisplatin and etoposide treatment, the patient's

**Table 1. Post operation and after etomidate treatment**

| | Postoperative | | First attempt etomidate (loading 0.3 mg/kg, infusion rate 0.03 mg/kg/hour) | | Second attempt etomidate (infusion rate 0.03 mg/kg/hour) | |
|---|---|---|---|---|---|---|
| | ACTH (pg/mL) | Cortisol (μg/dL) | ACTH (pg/mL) | Cortisol (μg/dL) | ACTH (pg/mL) | Cortisol (μg/dL) |
| Baseline | < 1.0 | 107.8 | < 1.0 | 63.3 | < 1.0 | 80.2 |
| 1 hour | 8.84 | 22.6 | | | | |
| 3 hours | 4.46 | 19.8 | | | | |
| 4 hours | | | | 32.3 | | 46.3 |
| 6 hours | | 17.6 | | | | |
| 8 hours | | | | 21.7 | | 44.6 |
| 12 hours | | 19.6 | | | | 30.6 |
| 24 hours | < 1.0 | 15.9 | | | | 38.5 |
| 36 hours | | 19.5 | | | | 19.4 |
| 48 hours | 1.95 | 23.3 | | | | 17.2 |
| 72 hours | 2.12 | 26.3 | | | 4.01 | 13.2 |
| 96 hours | < 1.0 | 37.6 | | | | 20.9 |
| 7 days | < 1.0 | 34.7 | | | | |
| 8 days | | 33.3 | | | | |
| 18 days | < 1.0 | 68.0 | | | | |

ACTH: adrenocorticotropic hormone

chest radiography revealed severe pneumonia and her oxygen saturation declined, probably due to suppression of the immune system. Thus, the patient's chemotherapy also had to be stopped.

Four days after stopping etomidate, her cortisol level increased to 80.2 μg/dL again, and we restarted etomidate infusion at a dose of 0.03 mg/kg/hour without a loading dose. Subsequently, her cortisol level decreased to 46.3 μg/dL after four hours of etomidate infusion, and continued to slowly decrease, to reach 19.4 μg/dL at 36 hours. As the target cortisol level was 10-20 μg/dL and her vital signs were stable, we maintained the etomidate dose at 0.03 mg/kg/hour without glucocorticoid replacement (Table 1). However, blood tests showed pancytopenia (white blood cell 900/μL, hemoglobin 8.3 g/dL, platelets 35,000/μL) at six days after stopping chemotherapy, and her pneumonia was exacerbated, with an acute respiratory distress syndrome pattern. Even her severe cardiomegaly did not improve, compressing against her lungs on chest radiography. Her oxygen saturation and vital signs rapidly deteriorated and she finally expired at seven days after chemotherapy had been stopped and five days after the second treatment with etomidate.

## Discussion

Although surgery is the first choice in CS, medication should be considered when surgical treatment is not possible. In our case, even after extended surgical resection, cortisol level increased again due to metastatic liver lesions. We therefore chose medical treatment considering her poorer condition. However, there are no specific medication guidelines for infants with CS. Of the available drugs, we chose etomidate for several reasons. First, oral medications were not suitable, due to the patient's feeding and breathing difficulties resulting from severe cardiomegaly that compressed both lungs. Second, ketoconazole was ill-advised, due to increasing serum cortisol levels caused by metastasized ACC liver mass. Last, a drug for cardiovascular stability was indicated due to her general unstable condition as a result of severe HCMP and hypertension.

Etomidate is a member of the imidazole family, initially developed as an anti-fungal agent, but popularly used as an anesthetic due to its potent hypnotic activity and promotion of cardiovascular stability (3). However, it was also found to increase mortality by lowering serum cortisol levels (5). Etomidate inhibits 11β-hydroxylase, catalyzes cortisol and corticosterone production from deoxycortisol and deoxycorticosterone, respectively, and can quickly reduce serum cortisol levels within 12 hours (6). Since then, etomidate has been used as a medical treatment for CS.

Optimal etomidate doses and treatment intervals are uncertain because of inconsistent evidence. In adult patients with CS, cortisol levels were dramatically reduced within hours of receiving a 0.03 mg/kg bolus followed by a 0.03-0.1 mg/kg/hour dose (6). Preda et al (7) reviewed 43 case reports, including pediatric patients,

and recommended low-dose etomidate intravenous infusion at 0.04-0.05 mg/kg/hour for partial and slow blocking, to prevent adrenal insufficiency. However, this review contained only three pediatric patients, without a consistent dose (6 year-old male, 0.03 mg/kg/hour, titrated to 0.08 mg/kg/hour; 14 year-old female, 3-3.5 mg/hour; 17 year-old female, 10 mg bolus then 2.5 mg/hour) (8,9,10), and no infant patients. There is only one report about the use of etomidate in a 14-month old infant who received etomidate for five days at an infusion rate of 0.03 mg/kg/hour (11). In our case, etomidate was administered as a 3-mg (0.3 mg/kg) bolus for induction and then as a continuous infusion at 0.03 mg/kg/hour. Serum cortisol levels began to drop after etomidate induction, and although the infusion rate was lower than previously described dosages (6,7), the abrupt lowering of serum cortisol levels led to adrenal insufficiency, possibly due to a high loading dose. Lower etomidate dose may be needed in adrenal-origin CS because ACTH-independent CS is more sensitive than ACTH-dependent CS to adrenal enzyme blockade (7). Therefore, our second etomidate infusion was performed without a loading dose. Serum cortisol levels began to drop after four hours and slowly decreased to within normal range within 36 hours, accompanied by stable vital signs. In addition, her cortisol levels were stable for four days with the same dose of etomidate. Therefore, the doses used in this study (no bolus dose and infusion rate of 0.03 mg/kg/hour) may be appropriate for severe hypercortisolemia in infants.

The reported side effects of etomidate at anesthetic dose include myoclonic seizure, gastrointestinal issues, and dystonic reactions. Notably, our patient did not receive the anesthetic dose and experienced no adverse side effects. Therefore, we suggest that the described dose is safe and effective for infants. Although etomidate is relatively safe, an intensive care setting is recommended due to its sedative effect and the risk of inducing adrenal insufficiency (7). We also recommend monitoring serum cortisol (maintain between 10 and 20 μg/dL) and potassium levels.

As etomidate has an anti-proliferative effects on adrenal cortical cells, it could be an anti-tumorigenic agent for metastatic ACCs (12). In addition, because cardiovascular complications are a major cause of morbidity and mortality for CS patients, etomidate is a good choice for patients with hypertension and HCMP from severe hypercortisolemia (3). Therefore, although this was, to our knowledge, the second attempt to treat an infant patient with CS using etomidate, we found that it was the best possible option after unsuccessful surgical treatment.

## Conclusion

In conclusion, this is the youngest case, to our knowledge, of etomidate treatment in an infant patient with CS. After etomidate was administered to a 2-month-old infant with CS at a 0.03 mg/kg/hour infusion rate, her serum cortisol levels fell into normal range within 36 hours without any side effects.

### Ethics

**Informed Consent:** A written informed consent was obtained from the patient's parents.

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

### Authorship Contributions

Surgical and Medical Practices: Ahreum Kwon, Yongha Choi, Jo Won Jung, Concept: Ahreum Kwon, Design: Ho-Seong Kim, Ahreum Kwon, Data Collection or Processing: Yongha Choi, Analysis or Interpretation: Ahreum Kwon, Yongha Choi, Literature Search: Junghwan Suh, Yongha Choi, Ahreum Kwon, Writing: Ahreum Kwon, Yongha Choi. Ho-Seong Kim

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CASE REPORT

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# Precocious Pseudo-puberty in a Two-year-old Girl, Presenting with Bilateral Ovarian Enlargement and Progressing to Unilateral Juvenile Granulosa Cell Tumour

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### What is already known on this topic?

Causes of feminising precocious pseudo-puberty of ovarian origin include follicular cysts, McCune-Albright syndrome (MAS) and juvenile granulosa cell tumour (JGCT). MAS and JGCT are associated with mutations in the *GNAS* and *AKT1* genes respectively, but *GNAS* mutations have also been found in JGCT. Ovarian involvement is usually unilateral in JGCT, and unilateral or bilateral in MAS.

### What this study adds?

We present a case in which feminizing precocious pseudo-puberty was initially associated with bilaterally enlarged, cystic ovaries. Shortly after presentation, the signs of feminisation escalated and repeat imaging showed a histologically proven JGCT in one ovary, with return of the other ovary to normal. Although a molecular genetic cause has not yet been identified, with normal *GNAS*, *AKT1* exon 3 and *FOXL2* sequencing, this unique observation shows how a unilateral JGCT was preceded by enlargement and cystic change in both ovaries.

## Abstract

Ovarian causes of precocious pseudo-puberty (PPP) include McCune-Albright syndrome (MAS) and juvenile granulosa cell tumour (JGCT). We describe a case of PPP in which bilateral ovarian enlargement with multiple cysts progressed to unilateral JGCT. A girl aged 2.17 years presented with three months of breast development, and rapid growth. Examination showed tall stature, height +2.6 standard deviations, Tanner stage B3P2A1. A single café au lait patch was noted. Bone age was advanced at 5 years. Pelvic ultrasound showed bilaterally enlarged ovaries (estimated volumes 76 mL on the left, 139 mL on the right), each containing multiple cysts. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) values before/after gonadotrophin administration were 0.43/0.18 and <0.1/<0.1 mUI/mL, serum estradiol 130 pg/mL, (prepubertal range <20 pg/mL). PPP of ovarian origin was diagnosed, and tamoxifen 20 mg daily started. However, after only seven weeks height velocity escalated and breast development increased to B3-4 with menorrhagia. Basal/stimulated LH and FSH were still suppressed at 0.13/0.25 and <0.1/<0.1 mUI/mL and, serum estradiol 184 pg/mL. Repeat imaging now showed normal right ovary (volume 1.8 mL) and a large left-sided vascular solid/cystic ovarian tumour which was excised (weight 850 g). Histology showed JGCT, International Federation of Gynecology and Obstetrics stage IA. DNA from tumour tissue showed no mutation in *GNAS*, exon 3 of *AKT1* (which contains a mutational hotspot) or *FOXL2*. The observation that bilateral ovarian activity progressed to unilateral development of JGCT in this patient is novel. This case highlights current uncertainties in the ontology of JGCT, and its possible relationship with MAS.

**Keywords:** Feminizing precocious pseudo-puberty, ovary, juvenile granulosa cell tumour, McCune-Albright syndrome

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## Introduction

Premature sexual development can be broadly divided into true, or central, precocious and early puberty arising from activation of the hypothalamo-pituitary axis; and precocious pseudo-puberty in which there is either autonomous secretion of sex steroids by the gonads or adrenal glands, or from exogenous sources (1).

Causes of ovarian precocious pseudo-puberty include autonomous follicular cysts, which are usually self-limiting (2), the McCune Albright syndrome (MAS), sex cord tumours arising from granulosa and thecal cells, and from germ cell tumours. MAS usually results from a somatic gain-of-function mutation in the *GNAS1* gene which encodes the G-protein alpha subunit-GNAS, causing a mosaic pattern involvement of various tissues, particularly skin, bone and ovary (3). Principal features are café au lait patches, precocious pseudo-puberty due to ovarian involvement, and polyostotic fibrous dysplasia (4). Renal phosphate wasting may accompany the latter (5). Rarely, MAS occurs in association with Cushing syndrome (6), gigantism/acromegaly (7) and hyperthyroidism (8). The diagnosis can be made clinically when there is multisystem involvement, but isolated monostotic bone lesions may require confirmation by histology and molecular analysis is indicated in atypical cases with single organ involvement (9).

Granulosa cell tumours belong to the group of sex cord-gonadal stromal tumours and account for 5 % of all malignant ovarian tumours (10). Adult granulosa cell tumours are caused by mutations in *FOXL2*, the gene encoding Forkhead box protein L2 (11), usually present after 30 years of age and account for < 1 % of granulosa cell tumours in prepubertal children (12). Juvenile granulosa cell tumours (JGCT), which represent 5-12 % of ovarian tumours in childhood, usually present before 30 years of age with feminizing precocious pseudo-puberty in children, disturbance of menstrual cycle with or without signs of hyperandrogenism in women, and abdominal mass at any age (13). In some cases, torsion of the annex or tumour rupture with haemo-peritonitis result in presentation with an acute abdomen. Biological markers, such as Inhibin B and Anti-Müllerian hormone levels are raised and are of value in tumour monitoring. JGCT has been associated in 60 % of cases with mutations of the *AKT1* gene, which encodes the RAC-alpha serine/threonine-protein kinase enzyme (14). Of note, *GNAS* mutations have been described in some cases of JGCT (15).

We present a case in which feminizing precocious pseudo-puberty was associated with bilateral ovarian enlargement initially, but which then progressed to JGCT with normal appearances of the contralateral ovary.

### Clinical Assessment, Biochemical Investigations and Diagnostic Imaging

These were carried out in the Military Hospital of Tunis. Height and weight were measured using standardized equipment, and values converted to standard deviations (SD) for corresponding ages using the normative French data of Sempe et al (16). Skeletal maturity ("bone age") was assessed by the method of Greulich et al (17). Luteinising hormone (LH) and follicle stimulating hormone (FSH) were sampled before and 15, 30 and 60 minutes after stimulation with 100 mcg of luteinizing hormone (LH) releasing hormone (LHRH) and measured using chemiluminescence immunoassay (UniCel DxI 600 Access Immunoassay System, Beckmann Coulter International S.A., Nyon, Switzerland). Estradiol was measured using enzyme-linked fluorescent immunoassay (VIDAS, Biomérieux F-69280 Marcy l'Etoile, France). Laboratory reference ranges for LH during the follicular phase, mid-cycle, and luteal phase were 1-7, 6-73 and 0.5-10 mIU/mL. For FSH the equivalent ranges were 3-8, 4-18 and 2-8 mIU/mL and for estradiol < 266, 118-255, and 26-165 pg/mL. For prepubertal girls, mean ± SD for LH was 0.03 ± 0.03 mIU/mL and for FSH 2.16 ± 1.14 mIU/mL. Prepubertal range for estradiol was < 20 pg/mL. Limit of detection was < 0.1 mIU/mL for LH and FSH, and < 10 ng/mL for estradiol.

Ultrasound imaging was carried out in the Radiology Department of the Military Hospital of Tunis using a General Electric Logiq S7 model (General Electric, Boston, MA 02210) featuring a 3.6-15 MHz transducer.

Magnetic resonance imaging (MRI) was carried out using a MRI MAGNETOM Verio-Siemens 3 Tesla scanner (Siemens, 80333 Munich, Germany). Since only two of three dimensions - length, width, and depth - were available for the initial ultrasound, approximate volumes were calculated using the prolate ellipsoid formula and assuming that width and depth were the same on the sagittal view and that length was at least the same as width and depth on the transverse view.

Bone imaging was carried out according to a standardised protocol using a gamma camera (Siemens, 80333 Munich, Germany), and scanning the whole skeleton two hours after injecting $^{99}$technetium-labelled hydroxylmethyl diphosphonate

### Tumour Histopathology, Immune Staining and Genetic Studies

Paraffin blocks of tumour were sent first to the Royal National Orthopaedic Hospital in London where histopathology findings were corroborated and mutational analysis for *GNAS1* were performed. DNA was successfully extracted,

and the sample was tested for the hotspots R201H, R201C and Q227L, as previously described (18).

The paraffin blocks were then sent on to the François Jacob Institute of Biology in Paris, where mutational analysis of exon 3, a mutational 'hot spot' of the *AKT1* gene, and *FOXL2*-C134W status were carried out. DNA was extracted from paraffin-embedded tumor material using xylene. Tissue was then rehydrated with successive baths of ethanol, then vortexed and centrifuged, and the supernatant removed. After futher ethanol rehydration steps, tissue was digested with proteinase K and DNA extraction performed with the Nucleospin DNA rapid Lyse kit (Macherey-Nagel, Allentown, PA 18109, United States). Given the poor quality of the DNA extracted from paraffin-embedded tissue, amplification of exon 3 of *AKT1* required a semi-nested polymerase chain reaction (PCR), using three primers. The central part of *FOXL2* was amplified by conventional PCR. PCR was performed with the Herculase II Fusion DNA Polymerase (Agilent, Santa Clara, CA 95051 United States) according to the manufacturer protocol. Sanger sequencing was performed by Eurofins according to their in-house procedures.

Finally, since the original histology report from Tunisia had suggested the presence of Call-Exner bodies, which are associated with adult rather than JGCT, tissue was sent from Paris to the pathology department at Glan Clwyd Hospital in Wales, United Kingdom for further review.

## Case Report

A girl was admitted, aged 2.17 years, for assessment of premature sexual development which took the form of rapid growth and breast development over a 3-month period.

Delivery was by caesarean section at 38 weeks of gestation, birth weight 3650 grams, birth length 50 cm, head circumference 34.5 cm. There was no relevant family history, and parents were unrelated. The mother's menarche was at 12 years.

On examination, the child was well, height 94 cm (+2.6 SD), weight 13.2 kg (+1 SD) compared with a mid-parental height of -0.4 SD (mother's height 160 cm, father's height 174 cm). Bone age was 5.0 years (chronological age 2.2 years). Pubertal stage according to Tanner was B3P2A1. The abdomen was supple and non-tender with no palpable masses. Skin examination showed a single café au lait patch situated on the antero-lateral border of the left thigh, 3 cm in its longest axis, with irregular outline. There were no lentigines, haemangiomas or subcutaneous tumours, no hepato-splenomegaly, and no bony tenderness or deformity.

Biochemical investigations before and after LHRH stimulation showed basal/peak LH values of 0.43/0.18 mUI/mL and FSH values $<0.1/<0.1$ mUI/mL. Serum estradiol was 130 pg/mL, (prepubertal range $<20$ pg/mL).

Pelvic ultrasound examination showed uterine length of 3.6 cm with fundo-cervical ratio 1.3 and pubertal configuration, and 4 mm of endometrial thickness. The images of each ovary, recorded at the time of examination, are of relatively inferior quality but show enlargement with multiple cysts and echogenic stroma (Figures 1a, 1b). The left ovary measured 63x48 mm in the sagittal plane, estimated volume 76 mL, right ovary 66x61 mm in the transverse plane, estimated volume 139 mL. Each ovary contained both discrete and coalescent cysts (Figures 1a, 1b).

MAS was considered a possibility in the light of the pelvic ultrasound and skin findings. Further investigations to assess parathyroid, growth hormone (GH), adrenal, thyroid

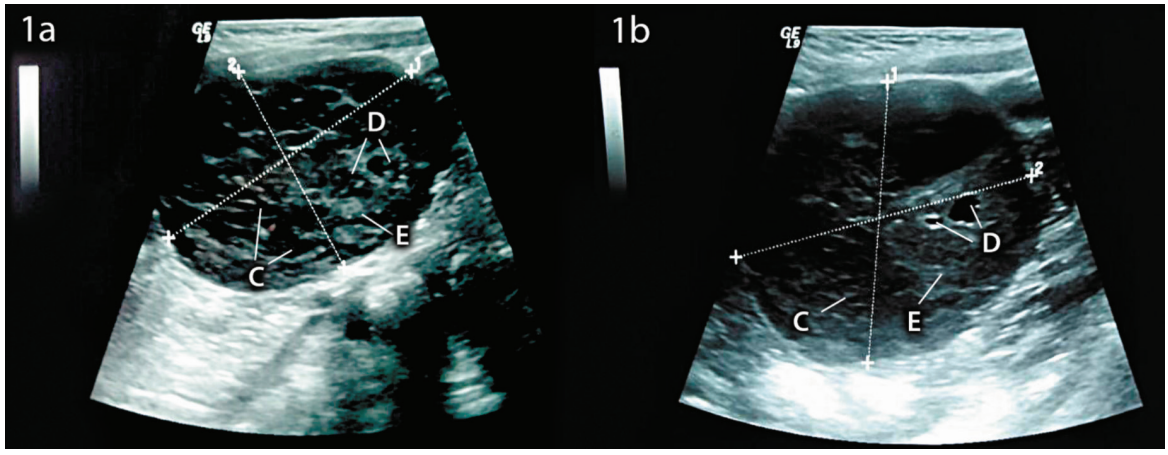


**Figure 1. a, b)** Ultrasound images of left (1a) and right (1b) ovaries in a 2-year-old girl with feminizing precocious pseudo-puberty and suppressed gonadotrophins. Both ovaries are very enlarged (see text) with echo-dense stroma. Both ovaries contain multiple cysts, some discrete and others coalescent as shown by the arrows

and skeletal status were therefore carried out, with normal results: serum calcium 2.36 mmol/L, phosphate 1.59 mmol/L (reference range 1.2-2.0 mmol/L), insulin-like growth factor 1 (IGF-1) 103 µg/L (age-related reference range 82-166 µg/L), urine free cortisol 160 nmol/L (reference range 100-300 nmoL/24 hours), free thyroxine 13.5 pmol/L, TSH 2.37 µUI/mL. Bone scintigraphy of the whole skeleton was also carried out to exclude fibrous dysplasia and no bony lesion was identified.

In view of the precocious pseudo-puberty, tamoxifen 20 mg oral daily was started at 2.3 years but after only seven weeks, aged 2.45 years the features of premature sexual development escalated with an increase in height of 4 cm, progression of breast development, Tanner stage now B3-4P2A1, and development of menorrhagia. Bone age had advanced further to 6.5 years. A second LHRH test, carried out to exclude secondary activation of the hypothalamo-pituitary axis, still showed gonadotrophin suppression with basal/peak LH 0.13/0.25 and FSH <0.1/<0.1 mUI/mL. Serum estradiol was 184 pg/mL.

Repeat pelvic ultrasound now demonstrated a left-sided vascular solid/cystic ovarian tumour measuring 10x8x6 cm lying postero-lateral to the bladder. The right ovary was normal in appearance, volume 1.83 mL.

The left ovarian mass was confirmed by MRI scan (Figure 2) which showed a well-defined solid-cystic abdominal-pelvic mass of left ovarian origin, measuring 12x10x5 cm, extending to the aorta and kidney with no evidence of local invasion. The right ovary was normal. Tumour markers were normal: α feto-protein 4.2 ng/mL (reference range <10 ng/mL), human chorionic gonadotrophin <0 (reference range <2 ng/mL), and ACE 2.3 ng/mL (reference range <5 ng/mL). CA-125 was slightly raised at 43 IU/mL (reference range <35 IU/mL). Inhibin B assay was not available.

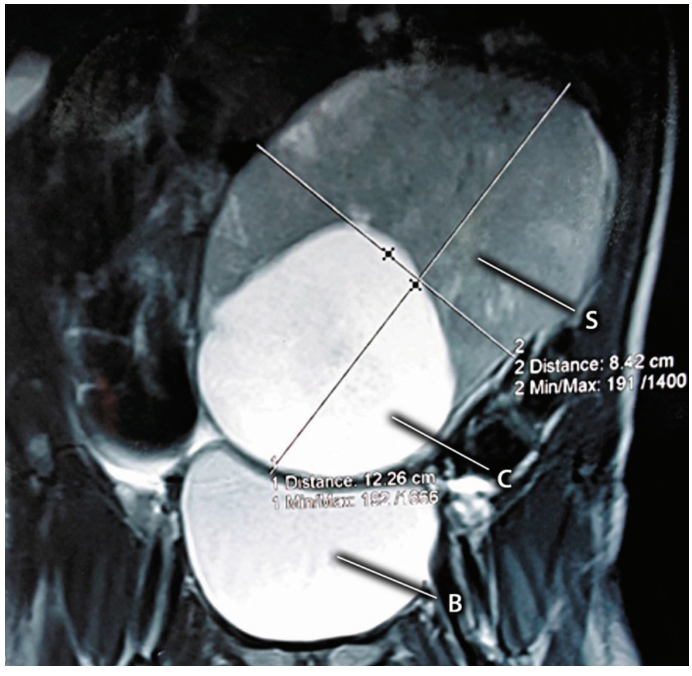


**Figure 2.** Magnetic resonance imaging of right ovary showing solid/cystic tumour, histology of which showed juvenile cell granulosa tumour

*B: bladder, C: cystic tumour, S: solid tumour*

After a week, the child developed symptoms related to torsion of the ovarian annex, requiring urgent laparotomy with removal of left ovary and annexectomy. At surgery, the tumour capsule was intact, tumour weight 850 g. No malignant cells were found on peritoneal lavage and there was no macroscopic evidence of infiltration of the capsule, and no spread to the fallopian tubes.

Histopathology review showed a tumour with nodular architecture, the nodules being encircled by fibrous tissue forming septae (Figure 3a). There were necrotic foci in some nodules (Figure 3b). High power imaging showed that the tumour cells had abundant pale eosinophilic cytoplasm, with oval, irregular vesicular nuclei, rarely showing nuclear grooves, and with rare mitoses (Figure 3c). Reticulin staining showed fibres surrounding groups of granulosa cells (Figure 3d). No follicles or pseudopapillary architecture was noted and no Call-Exner bodies were seen. Features of germ cell tumour or gonadoblastoma were not identified. There was no capsular infiltration.

Immunochemistry showed strongly positive staining for inhibin B but α-fetoprotein and anti-CD30 were not detected. The findings were consistent with a JGCT, graded as International Federation of Gynecology and Obstetrics (FIGO) stage IA in view of its confinement within the tumour capsule. Post-operative progress showed immediate regression of the pubertal signs and metrorrhagia.

At review, aged 5.7 years, height was 113 cm (+0.9 SD), weight 17.2 kg (0 SD), pubertal stage B1P1A1, bone age 7.0 years. Basal gonadotrophins showed LH 2.49 mIU/mL, FSH 2.52 mIU/mL and oestradiol 13 pg/mL. Abdominal and pelvic ultrasound were normal.

Thereafter, the girl has remained prepubertal. At last review, aged 8.1 years, height was 125 cm (-0.53 SD), Tanner stage B1P1A1, bone age now only slightly advanced at 8.7 years. However, basal LH has continued to show mild elevation despite breast stage and estradiol levels remaining prepubertal. Thus LH/FSH and estradiol values at 6.5, 7.0 and 8.1 years were: 0.87/3.78 mIU/mlL and 11 pg/mL, 1.24/1.62 mIU/mlL and 12 pg/mL and 1.46/5.66 mIU/mL and 13 pg/mL, respectively.

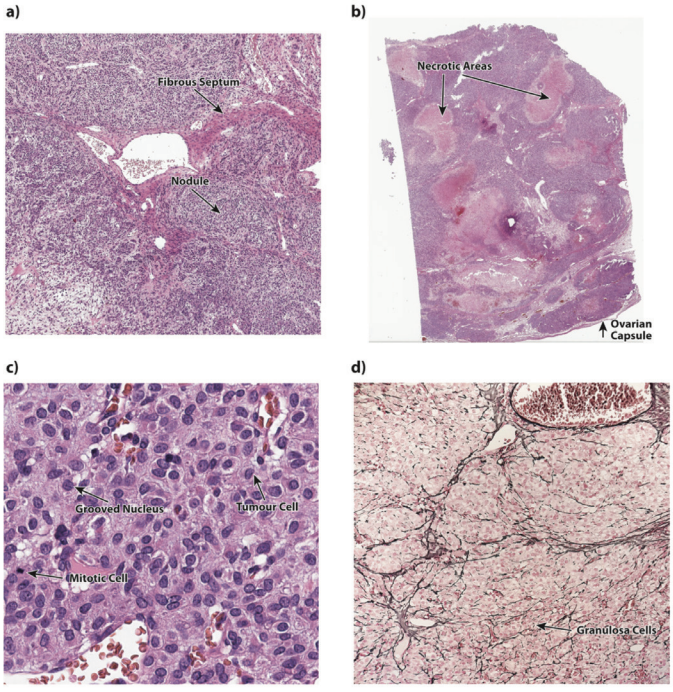


**Figure 3. a, b, c, d)** Histology of juvenile granulosa cell tumour with labelled features showing: a) low power haematoxylin and eosin (H&E) image showing cellular neoplasm with nodules divided by fibrous septae; b) low power H&E showing necrotic areas within some nodules and intact ovarian capsule; c) high power H&E showing abundant eosinophilic cytoplasm with round to oval tumour cells with vesicular chromatin, only a few nuclei showing groove formation, and rare mitoses; and d) medium power reticulin stain showing fibres surrounding groups of granulosa cells

*GNAS1* gene mutation analysis by PCR and restriction enzyme digestion of DNA extracted from paraffin blocks of the tumour was negative for the common hot-spot mutations (R201C, R201H and Q227L). Sequencing of exon 3 in the *AKT1* gene in the tumour tissue did not show a somatic mutation. Finally, sequencing of *FOXL2* was carried out to exclude an adult granulosa cell tumour, and the C134W *FOXL2* pathogenic variant was not found.

## Discussion

Although the feminising precocious pseudo-puberty in this case was clearly of ovarian origin, its precise aetiology remains unclear. At presentation with premature sexual development, the morphology of the ovaries reflected bilateral activity with cysts, gonadotrophin suppression and a single café au lait patch on the thigh. Although this pattern is evocative of MAS there were no other features of MAS, such as bony fibrous dysplasia, with no bony lesion being found on scintigraphy, no abnormality of renal phosphate transport, no disturbance of the GH/IGF-1 axis, Cushing syndrome, or hyperthyroidism. Moreover, the ovarian volumes-estimated at 76 mL on the left and 139 mL on the right-were extraordinarily large, far greater than in the study of eight patients with MAS described by Foster et al (19) in whom mean ovarian volumes overlapped with those seen in girls with central precocious puberty. Also, *GNAS1* analysis in the tumour was negative. However, *GNAS1* mutations are not found in the ovarian tissue of every patient with this MAS. It therefore remains possible that our patient has MAS and may show additional signs of this syndrome as she grows older although she remains well with no additional signs six years after initial presentation.

While it was not possible to diagnose MAS with certainty, it was clear that our patient had precocious pseudo-puberty of ovarian origin, as opposed to true precocious puberty, at presentation and she was therefore treated with tamoxifen, a medication which exerts an anti-estrogen effect through competitive inhibition of binding of estradiol with its receptors.

The clinical situation then changed dramatically in less than two months, with development of a large tumour in a single ovary. The tumour was limited to one side with intact and tumour-free capsule on the surface of the ovary. According to the FIGO classification this corresponds to stage IA, where the tumour is localized to the organ of origin. Histological review showed the tumour to be a granulosa cell tumour of juvenile type. In keeping with this, reticulin staining showed fibres surrounding groups of granulosa cells, contrasting with thecoma and fibroma tumours in which fibres surround individual granulosa cells. Moreover, Call-Exner bodies, which are a feature of adult granulosa cell tumour, were not present while mutational analysis of *FOXL2* was negative. The diagnosis of JGCT is therefore secure.

Just as a diagnosis of MAS in our patient cannot be supported, neither can her case be fully explained by the diagnosis of unilateral JGCT since there was documented evidence of bilateral ovarian activity and enlargement on ultrasound at initial presentation. The present diagnosis therefore is purely descriptive - precocious pseudo-puberty of ovarian origin, with progression to JGCT-in which the underlying mechanism remains unclear. We speculate that some disorder of cell-signalling resulted initially in the bilateral ovarian enlargement with cyst formation, and that the cysts were estrogen-secreting. Following this, the process seems to have spontaneously resolved in one ovary, with transformation, perhaps involving one of the cysts, into JGCT.

We believe that ours is a unique observation. Although bilateral JGCT has been reported (12), presentation with bilateral ovarian enlargement evolving towards unilateral regression and contralateral tumour formation has not. The clinical and pathological features of our case are consistent with previous reports, including the recent paper from Ye et al (20).

There is an intriguing overlap between MAS and JGCT. Mutational analysis using nested PCR is reported to have a 54 % sensitivity for detecting a *GNAS* mutation in suspected MAS (21). The same group also found an activating *GNAS* mutation in 9 of 30 patients with JGCT (15). Bessière et al (14) found, in 10 of 16 JGCT patients, in-frame duplications in exon 3 of the oncogene *AKT1* which lead to its activation. Given that analysis of exon 3 in *AKT1* was negative in our case, whole gene sequencing would be the next diagnostic step in seeking a molecular genetic cause for JGCT. However, given the time and expense involved, this measure would be difficult to justify in an individual patient.

To investigate a putative link between MAS and JGCT, we have examined gene expression in adrenal tissue bearing activating *GNAS1* mutations (Geo Dataset GSE33694 ref PMID: 22259056), and in ovarian tissue from patients with JGCT (22). Interestingly, both the tissues studied showed significant enrichment in genes which are significantly dysregulated by overexpression of the proto-oncogene *B-Raf* (adjusted p value 0.002 for the directly expressed McCune-Albright genes and $<0.001$ for the directly expressed JGCT genes). *B-Raf* is known to be phosphorylated by the *AKT1* gene and also to be differentially regulated by cAMP-dependent protein kinase A activation, which is itself dependant on *GNAS1* activity. Further work examining the interplay between *B-Raf, GNAS1* and *AKT* genes might clarify a possible link between MAS and JGCT.

In practical terms, our patient requires continuing follow up in case other features of MAS develop, paying special attention to the morphology of her remaining ovary. Critical attention is also needed to growth rate and pubertal status, given the risk of developing true central precocious puberty through previous exposure to raised sex steroid levels-the phenomenon of "priming". Fortunately, more than five years since the tumour was successfully removed, our patient remains prepubertal with height now in the lower half of the normal centile range and only marginal bone age advance.

Finally, a puzzling aspect of our case for which we have no explanation is the mild, but consistent, elevation of basal LH observed throughout, with a paradoxical drop from 0.43 to 0.18 mIU/mL after LHRH administration at initial presentation. Since successful surgery, mild basal LH elevation has persisted at a level suggestive of true central puberty. Despite this, the patient has remained clinically prepubertal since surgery, with normal oestradiol values for age.

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### Ethics

**Informed Consent:** Consent for genetic analysis and publication of the case was obtained from the parents.

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

### Author Contributions

Surgical and Medical Practices: Hager Barakizou, Souha Gannouni, Thouraya Kamoun, Fernanda Amary, Reiner Veitia, Concept: Hager Barakizou, Design: Hager Barakizou, Reiner Veitia, Data Collection or Processing: Hager Barakizou, Souha Gannouni, Thouraya Kamoun, Analysis or Interpretation: Hager Barakizou, Souha Gannouni, Thouraya Kamoun, Fernanda Amary, Zilla Huma, Anne-Laure Todeschini, Reiner Veitia, Malcolm Donaldson, Literature Search: Hager Barakizou, Zilla Huma, Anne-Laure Todeschini, Reiner Veitia, Malcolm Donaldson, Muhammed Mehdi, Writing: Hager Barakizou, Zilla Huma, Anne-Laure Todeschini, Reiner Veitia, Malcolm Donaldson.

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CASE REPORT

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# TSHRV656F Activating Variant of the Thyroid Stimulating Hormone Receptor Gene in Neonatal Onset Hyperthyroidism: A Case Review

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

Neonatal hyperthyroidism is a rare condition and is most frequently caused by the transplacental transmission of thyroid receptor stimulating antibodies from a mother with Graves' disease. Activating variants of the *TSHR* gene are rare causes of neonatal hyperthyroidism, which play a significant role in the etiology of familial (autosomal dominant) and sporadic non-autoimmune hyperthyroidism (NAH).

**What this study adds?**

We present a case of neonatal onset congenital NAH with a sporadic germline activating V656F variant in the *TSHR* gene. This variant was described in the literature as a somatic variant in children and adults with toxic thyroid nodule(s) that resulted in the structural activation of the TSH receptor. This study is the first case to highlight the relationship between this variant and neonatal onset NAH.

## Abstract

An activating variant of the thyroid stimulating hormone receptor (*TSHR*) gene is one of the rare causes of neonatal hyperthyroidism. This disorder may occur as a result of an autosomal dominant inheritance or sporadically through *de novo* variation. Here we present a case of neonatal onset congenital non-autoimmune hyperthyroidism (NAH) with a sporadic germline activating *TSHRV656F* variant. A female infant with tachycardia, who was transferred due to hyperthyroidism in the first week of life, displayed no other symptoms or signs. The patient's mother did not have Graves' disease, and TSHR stimulating antibodies were not present in the mother or baby. Imaging showed thyroid gland hyperplasia and left ventricular hypertrophy, the patient was subsequently put on methimazole treatment. After six months undergoing treatment, a heterozygous p.Val656Phe (V656F) (c.1966G > T) variant was detected on exon 10 of the *TSHR* gene. The variant was not identified in the mother and father, so the case was assumed to be sporadic. In conclusion, although the literature describes V656F variant as a somatic variant in children and adults with toxic thyroid nodule(s) that results in the structural activation of the TSH receptor, no previous cases of neonatal hyperthyroidism due to TSHRV656F variant have been reported. This study is the first case review that highlights the relationship between *TSHRV656F* variant and neonatal onset NAH.

**Keywords:** Neonatal hyperthyroidism, activating variant of *TSHR* gene, non-autoimmune hyperthyroidism

## Introduction

Hyperthyroidism in children is a rare, heterogeneous condition characterized by an excessive production of thyroid hormone (1). Approximately 1% of childhood thyrotoxicosis cases emerge in the neonatal period. Neonatal hyperthyroidism, which has an estimated prevalence of 1/50,000, is primarily caused by temporary hyperthyroidism due to maternal Graves' disease and is characterized by the presence of thyroid receptor stimulating antibodies (TRsAB) that have been passed on to the newborn by the mother (2,3). Autoimmune congenital hyperthyroidism continues for nearly four months following birth and is alleviated when the maternal TRsAB is gradually eliminated from the infant's blood (3). Persistent neonatal cases of hyperthyroidism in which no antibodies are detected may be related to

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less common, non-autoimmune genetic etiologies, such as activating variants in the thyroid stimulating hormone receptor (*TSHR*) gene, somatic activating variants of the *GNAS* gene which encodes the stimulant alpha sub-unit of the guanine nucleotide binding protein as is the case in McCune-Albright syndrome (MAS), and less frequently through variants in the thyroid receptor β gene (3,4). MAS is an uncommon etiology of neonatal hyperthyroidism, and is characterized by additional findings, including cafe au lait macules on the skin, skeletal deformities caused by fibrous dysplasia, and signs associated with hormonal hyperfunction such as Cushing syndrome and/or peripheral precocious puberty (5). In the literature, there have been case reports of individuals diagnosed with thyroid hormone resistance due to variants in the thyroid receptor β gene, leading to symptoms of neonatal hyperthyroidism. However, unlike the others, suppression of serum TSH was not observed in these patients (6,7).

In the literature, activating *TSHR* gene variants have been frequently reported in the genetic analysis of children and adults with toxic thyroid nodule(s) (8,9,10,11), and less commonly in cases of neonatal hyperthyroidism (12,13).

In this study, we present a case of neonatal onset, congenital, non-autoimmune hyperthyroidism (NAH) with a sporadic germline activating *TSHR*V656F variant, and whose family history is negative for NAH.

## Case Report

A female patient, the third live born from the fourth pregnancy of a 27-years-old mother, was born at term through normal vaginal birth, weighing 3110 g. The patient exhibited tachycardia on the third day. The patient's family history was negative for thyroid disease. However, the mother was observed to have goiter during evaluation. Physical examination revealed body weight to be 3600 g (50-75p), height 48 cm (10-25p), head circumference 35 cm (25-50p), rhythmic heart rate 160-170/min. Other than mild tachycardia, no signs of pathology were observed and cafe au lait macules were not present on the skin. Laboratory tests revealed a free thyroxine (fT4) level of 3.41 ng/dL (0.93-1.7), and thyroid stimulating hormone (TSH) level of 0.005 mIU/mL (0.35-4.94). Laboratory analysis at seven days of age showed the serum level of free triiodothyronine (fT3) as 12.54 pg/mL (1.8-4.6), fT4 level 3.22 ng/dL (0.83-1.76), and TSH level < 0.01 mIU/L (1.78-12.6) consistent with hyperthyroidism. While serum anti-thyroid peroxidase (anti-TPO) antibody was positive, anti-thyroglobulin (anti-TG) and TSH receptor antibodies (TRAB) were not detected. Thyroid ultrasonography (USG) revealed diffuse hyperplasia of the thyroid gland. Echocardiogram showed mild hypertrophy of the left ventricle. The thyroid function tests of the mother were reported as euthyroid with anti-TPO [60 IU/mL (< 35)] and anti-TG [79.3 IU/mL (< 40)] antibodies being detected in the serum, TRAB [0.3 IU/L (0-1.75)] was not.

At seven days of age, the patient was put on treatment with 0.5 mg/kg/day methimazole (in two doses) and 2 mg/kg/day propranolol (in two doses). On the eighth day of treatment, methimazole treatment was reduced and eventually discontinued due to low fT4 levels. However, on the fifth day following medical discontinuation, thyroid function tests revealed hyperthyroidism and the patient was put back on methimazole. Based on the results of thyroid function tests, the dose of treatment was adjusted between 0.15-0.75 mg/kg/day, and the patient maintained a euthyroid state. Propranolol treatment was discontinued as the patient's tachycardia had resolved.

The patient had tested negative for TRAB from the onset of disease, anti-TPO antibodies had receded, and the patient required more than six months of anti-thyroid treatment. For these reasons, a prediagnosis of NAH was considered. The patient did not demonstrate any additional signs of MAS, and a p.Val656Phe (c.1966G > T) heterozygous variant was detected on exon 10 of the *TSHR* gene. The case was confirmed to be sporadic as the same variant was not detected in the mother and father (Figure 1).

During follow-up of methimazole treatment, the patient's physical examination showed normal sized thyroid glands, normal growth and development, and two periodic thyroid USG evaluations were reported as normal. During the last assessment at 25 months of chronological age, the patient's height was 85 cm (10-25p), which was consistent with her genetic target height, and body weight was 11.1 kg (10-25p). Neuromotor development was appropriate for chronological age. At the time of writing, the patient continues with methimazole treatment (0.45 mg/kg/day) and maintains a euthyroid state.

## Discussion

Here, we present a case of neonatal onset congenital NAH with a sporadic germline activating *TSHR*V656F variant. When hyperthyroidism is detected in a newborn, autoimmune causes must initially be considered and the baby as well as the mother should be assessed for TRsAB. If no autoimmune reasons can be identified in persistent cases of neonatal hyperthyroidism, genetic etiologies should be considered (3).

Since our patient had a persistent NAH and there were no additional findings suggestive of MAS, genetic analysis was conducted initially for *TSHR* activating variants, and a heterozygous sporadic activating V656F (Val656Phe) variant was detected on the *TSHR* gene. Activating germline variants of the *TSHR* gene that display an autosomal dominant inheritance pattern lead to familial or hereditary NAH (FNAH). In contrast, de novo variants lead to sporadic NAH (SNAH), as is the case in our patient. Although uncommon, somatic variants of the *TSHR* gene can also lead to autonomic thyroid adenomas in children (14).

Data in the literature regarding activating *TSHR* variants and their clinical characteristics are accessible on a periodically updated database, started in 1999 (15). In the literature, there are a few case reports of SNAH in which symptoms were present during the neonatal period (12,13). The variant detected in our case was first reported in 1997 by Führer et al (8) in a patient with toxic thyroid nodule. Wonerow et al (9) confirmed this variant to be an activating point variant of the *TSHR* gene. In subsequent years, this variant was observed in some child and adult patients with toxic thyroid nodule(s) (8,9,10,11,16,17). However, after a thorough review of the literature, we did not find any reports of the variant in neonatal cases of hyperthyroidism. In comparison to FNAH, cases of SNAH tend to show symptoms at an earlier age and exhibit more severe clinical manifestations. However, the signs may be affected by genetic, epigenetic, and environmental factors as well as the in vitro activity of *TSHR* gene variants. Akcurin et al (18) reported three cases of the same variant from the same family in which FNAH emerged at different ages. One of the siblings had symptoms during infancy and was diagnosed with hyperthyroidism at age 3.5 years, the other was diagnosed at 12 days old, and their father was diagnosed with toxic multinoduler goiter at 36 years old. Thus, the phenotype-genotype relationship of the disease appears not to be clear.

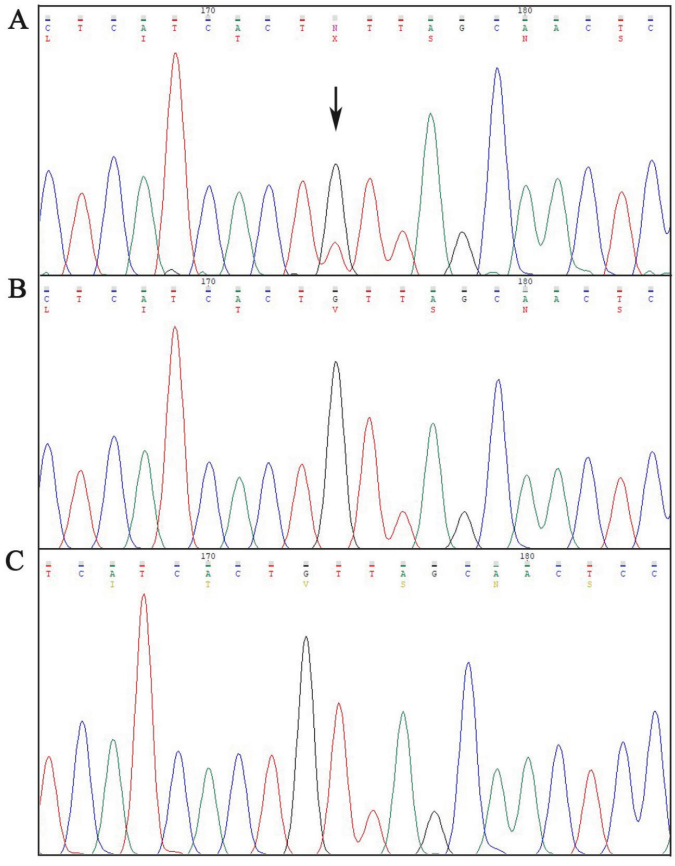


**Figure 1.** A) The index patient's DNA sample showing a Val656Phe (gtt → ttt) missense mutation on exon 10 of the *TSHR* gene. B) The corresponding *TSHR* gene region sequence of the mother (wild type). C) The corresponding *TSHR* gene region sequence of the father (wild type)

Congenital NAH can show clinical signs even in the fetus, which may include tachycardia, arrhythmia, intrauterine growth restriction, and premature birth (12). Our patient was born at term with a normal birth weight. In the first week of the neonatal period, tachycardia was observed in the patient's physical examination. Imaging displayed signs of potential intrauterine involvement, including diffuse hyperplasia of the thyroid gland and mild left ventricular hypertrophy. After the neonatal period, NAH can manifest in babies as tachycardia, growth retardation, accelerated linear growth, advanced bone age, craniosynostosis, restlessness and/or delay in development (14). In our case, these manifestations were prevented in infancy by ensuring a euthyroid state through appropriate management with anti-thyroid medication (ATM). NAH typically causes diffuse enlarged goiter in childhood, and progresses to multinodular goiter at older ages. An important diagnostic criterion for NAH is absence of ophthalmopathy. Unlike Graves' disease, serological testing shows no anti-thyrotropin receptor antibodies, and histopathological examination of thyroid tissue does not show the characteristic mononuclear cell infiltration of Graves' disease; autoimmune markers are not observed during immunohistological analysis (19).

The treatment for hereditary and persistent SNAH differs from that of autoimmune hyperthyroidism in which ATM is temporarily (3-4 months) used (3). Congenital NAH that is caused by activating variants of the *TSHR* gene presents with persistent and severe manifestations of hyperthyroidism. For this reason, ATM may be initially used, but curative approaches, such as surgery or radioactive iodine (RAI) ablation are required (20). Otherwise, the patient

may experience relapses of hyperthyroidism following discontinuation of ATM. Relapses may even be observed in cases of incomplete ablation (subtotal thyroidectomy or a RAI dose that is non-ablative). Remission is observed in nearly 50% of patients one year after ATM. In SNAH, the recovery time of the pituitary-thyroid feedback axis cannot be predicted, and TSH may remain suppressed for more than one year following birth. Due to the rarity of FNAH and SNAH, well-characterized case series have presented valuable information regarding the best treatment methods and various therapeutic modalities. In our case, euthyroidism was quickly attained through ATM, and curative treatment was planned for the future.

## Conclusion

We have reported the first case of neonatal hyperthyroidism associated with a sporadic activating V656F variant of the *TSHR* gene. Sporadic, de novo, activating variants in the *TSHR* gene can cause severe hyperthyroidism in the neonatal period. As in our case, in patients who present with persistent NAH with early onset diffuse goiter, and who have no family history or additional systemic involvement, sporadic activating *TSHR* gene variants should be considered and genetic analysis should be planned. Genetic diagnosis is also a guide for treatment because curative treatment is ultimately required during follow-up in these patients.

### Ethics

**Informed Consent:** Consent form was filled out by the parents of the patient.

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

### Authorship Contributions

Surgical and Medical Practices: Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Concept: Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Design: Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Data Collection or Processing: Leman Kayaş, Emine Çamtosun, Analysis or Interpretation: Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Rıfat Bircan, Literature Search: Leman Kayaş, Emine Çamtosun, Writing: Leman Kayaş, Emine Çamtosun, Ayşehan Akıncı, Rıfat Bircan.

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CASE REPORT

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# Long-term Follow-up of a Toddler with Papillary Thyroid Carcinoma: A Case Report with a Literature Review of Patients Under 5 Years of Age

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

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. However, the frequency of PTC is extremely low in children.

**What this study adds?**

This is the first case report of long-term follow-up and successful outcome of PTC in a patient under the age of two years.

## Abstract

Papillary thyroid cancer (PTC) is extremely rare in children. Herein, we present a case diagnosed with PTC at 15 months of age. We conducted a literature review of the published cases with PTC under five years of age. A 1.25-year-old male patient had initially presented with a complaint of progressively enlarging cervical mass that appeared four months earlier. On physical examination, a mass located in the anterior cervical region with the largest measurements of around 3x3 cm was detected. Cervical and thyroid ultrasonography showed a 50x27 mm solid mass in the right lateral neck. Excisional biopsy revealed a follicular variant of PTC with capsular invasion. Subsequently, he underwent a complementary total thyroidectomy. He was diagnosed with intermediate-risk (T3N0M0) PTC. He developed permanent hypoparathyroidism. In the first year of the operation, he was treated with radioiodine ablation (RAI) since basal and stimulated thyroglobulin (Tg) levels tended to increase. Whole-body scintigraphy was normal in the first year of RAI ablation. On levothyroxine sodium (LT4) treatment, levels of thyroid stimulating hormone (TSH) and Tg were adequately suppressed. He is now 8.5-years-old and disease-free on LT4 replacement therapy for seven years and three months. Pediatric PTC has different biological behavior and an excellent prognosis compared to adults. The optimal treatment strategy for pediatric TC is total thyroidectomy, followed by RAI ablation. Post-operative management should include regular follow-up, TSH suppression by adequate LT4 therapy, serial Tg evaluation, and radioiodine scanning when indicated.

**Keywords:** Papillary carcinoma, thyroid, children

## Introduction

Differentiated thyroid carcinomas (DTC) are the most common endocrine malignancies in childhood. Papillary thyroid cancer (PTC) constitutes 1.4% of new childhood malignancies and 90% of DTCs (1). Notwithstanding that thyroid carcinoma (TC) is rare in childhood, the incidence rate is increasing by 1.1% annually in Europe. Increased frequency of TC may be related to environmental factors or improvement in diagnostic scrutiny (2). Additionally, TC is the most frequently observed secondary malignancy in pediatric cancer survivors. Pediatric TC usually presents

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with neck masses or nodules without any accompanying symptoms. Furthermore, 60% of pediatric patients with PTC have regional lymph node metastases at diagnosis (3). Despite the aggressive course of TC in children compared to adults, it has an excellent prognosis in the pediatric population with 10-year and 30-year survival rates >99% and >96%, respectively (4). DTC is most frequently diagnosed in children between the ages of 11 and 17 (5,6). Although PTC is the most common type of TC, the frequency of PTC is extremely low in children under five years of age. Herein, we report a one-year and three-month-old boy presenting with cervical mass, which was finally diagnosed as PTC, and compared his clinical findings with previously reports.

## Case Report

A 1.25-year-old male patient initially presented with a complaint of progressively enlarging cervical mass, first noted four months earlier. He was the second child of a healthy 30-year-old mother and a healthy 33-year-old father. There was no consanguinity, and he had a healthy sibling. Except for reactive airway disease, his past medical history was unremarkable, notably without any radiation exposure, family history of TC, or other thyroid diseases. On physical examination, a mass located in the anterior cervical region, measuring 3x3 cm, was detected. His cardiovascular, respiratory, and abdominal physical examination findings were normal. Baseline laboratory analyses were within normal limits. Thyroid hormone levels were normal, and thyroid antibodies were negative. Cervical and thyroid ultrasonography showed a well-circumscribed, solid mass with lobular contours in the right lateral neck, 5x2.7 cm in size. Chest X-ray and abdominal ultrasonography were normal. Subsequently, neck magnetic resonance imaging (MRI) was performed, which revealed a 5x3.5x3 cm mass lesion with well-circumscribed margins in the right thyroid lobe, extending into the upper mediastinum. T2-weighted MRI showed the T2 hyperintense lesion to have diffuse and intense enhancement after contrast material administration (Figure 1). Since imaging did not precisely identify the primary origin and allow a specific diagnosis to be made, with suspicion of a neck tumor with thyroid invasion, total excision of the cervical mass was performed. Macroscopic examination of the excision showed a well-circumscribed, solid, nodular lesion that was gray-white in color and measuring 4.5x3.5x3 cm in size. Hematoxylin-eosin stained sections of the lesion revealed follicular variant PTC (FVPTC) with capsular invasion. There was no vascular invasion or microscopic extra-thyroidal extension. On immunohistochemical evaluation, Hector Battifora mesothelial epitope-1 was found to be diffusely positive. Analysis of $BRAF^{V600E}$ (The B-type Raf kinase) mutation was negative. After total excision of the cervical mass, technetium-$^{99}$m thyroid scintigraphy showed a focus of activity in the middle of the neck. Subsequently, he underwent complementary total thyroidectomy without prophylactic lymph node dissection, since no metastatic lymph node had been observed intraoperatively or on preoperative imaging. In the postoperative period, serum calcium was 9.4 mg/dL [normal range (NR): 8.4-10.4], phosphorus was 5.3 mg/dL (NR: 4-6.5), magnesium was 1.9 mg/dL (NR: 1.5-2.5), alkaline phosphatase: 136 U/L (NR: <281), and parathyroid hormone (PTH) 30 (15-65) pg/mL. Vocal cord movements were normal. Levothyroxine sodium (LT4) replacement (3.5 µg/kg/day) was initiated after surgery. Although serum calcium and PTH levels were normal postoperatively, during the follow-up, hypocalcemia developed due to delayed hypoparathyroidism (calcium 5.1 mg/dL, phosphorus 8.5 mg/dL, magnesium 1.9 mg/dL, alkaline phosphatase 158 U/L, 25-OH vitamin D 20.5 ng/mL and PTH 7 pg/mL) and calcium carbonate and calcitriol replacement therapy were started three month after surgery. Basal thyroglobulin (Tg) level was 1.3 ng/mL. The patient was categorized as stage 3 (T3N0M0) and intermediate-risk with respect to tumor size and other clinical features (7). One year after the operation, stimulated Tg levels tended to increase up to 3.8 ng/mL, and he was treated with 1 mCi/kg radioiodine ablation (RAI), following thyroxine hormone withdrawal and iodine-free diet. Before RAI therapy, TSH was 86.8 mIU/L and stimulated Tg was 6.7 ng/mL. The patient did not show any adverse effects of RAI. Whole-body scintigraphy (WBS), taken one week after radiation therapy, yielded minimal thyroid remnant. Suppressive therapy with LT4 was restarted. Basal Tg levels were 2.8 ng/mL and 0.2 ng/mL, one month and two months after RAI ablation, respectively, and remained at low levels.

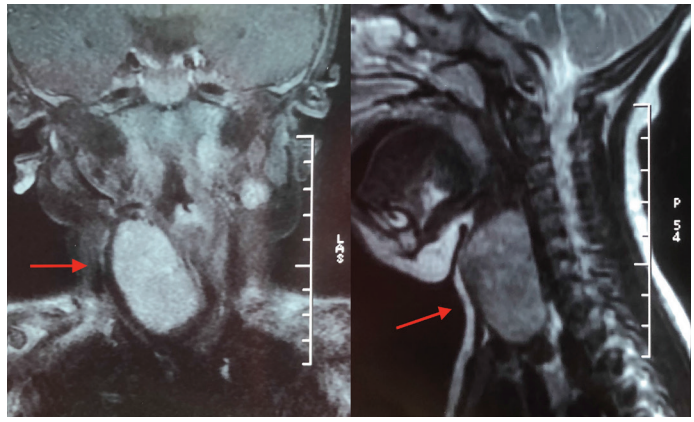

**Figure 1.** Coronal and sagittal planes, showing the mass lesion on magnetic resonance imaging

WBS with a 5 mCi dose of $I^{131}$ was unremarkable with no remnant thyroid tissue, and the serum level of Tg was 0.7 ng/mL in the first year after RAI ablation. The patient was examined and tested periodically every 3-6 months. A level of TSH between 0.1-0.5 mIU/L and a level of fT4 close to the upper limit were maintained. Serum Tg levels remained below 0.04 ng/mL after the first year of RAI therapy. Neck ultrasound was evaluated at 6-month intervals. Given these results, the patient was accepted to be in remission, and he had no evidence of other metastatic foci. Since the patient had developed permanent hypoparathyroidism, calcium and calcitriol supplements were continued.

At the most recent evaluation, he was 8.5-years-old with a height of 136.5 cm [0.8 standard deviation score (SDS)] and a weight of 30 kg (1.3 SDS), and his Tanner stage was 1 (testes volumes 2/2 mL). He is on LT4 (150 µg/day), calcium (50 mg/kg/day), and calcitriol (1 µg/day) supplementation and has been disease-free for seven years and three months.

### Research Strategy and Systematic Review of Literature

A systematic review of the published literature on PTC in patients under five years of age was conducted. The literature was searched from inception to May 2020, using the following keywords: "papillary thyroid carcinoma" and "differentiated thyroid carcinoma" filtered by age, including infant, toddler, and preschool child. Both searches were limited to the English language. Up to date, only ten patients with PTC younger than five years old have been reported (5,8,9,10,11,12,13,14,15,16). Clinical findings, histopathological features, and outcomes of the previous patients and the presented case are summarized in Table 1.

## Discussion

The frequency of DTC has increased in both children and adults over the decades (3). PTC is the most common type of DTC, which usually presents in the adolescent period with a female preponderance. Genetic predisposition, previous thyroid disease, history of malignancy, and radiation exposure are usually the underlying risk factors (17). The data on PTC in early childhood was limited to a few articles and case reports. DTC displays female dominance in adolescence, whereas the female/male ratio is equal or slightly reversed under ten years of age (3). Correspondingly, 6 out of 10 previously reported patients were male (5,8,9,10,13,14), and herein, we present a 1.25-year-old male patient with FVPTC who was successfully treated with total thyroidectomy and RAI ablation.

Up to 70% of the initial manifestation of thyroid cancer is usually asymptomatic solitary neck mass with characteristically normal thyroid hormone levels. Based on pathological examination, thyroid nodules in children are reported to have a higher incidence of malignancy than in adults (22-26% and 5-15%, respectively) (18). Large, hard, fixed, irregular nodules, male sex, being younger than ten years old, and cervical lymphadenopathy should be considered worrisome (19). Cervical mass was the initial finding in our case, as was the case in the previously reported patients (5,8,9,10,11,12,13,14,15,16).

Patients with PTC should be questioned about concomitant thyroid diseases, including autoimmune thyroid disease, and congenital hypothyroidism including thyroid dysgenesis and dyshormonogenesis, environmental factors (iodine deficiency region), medical history of cancer, or neck radiation therapy, and family history of TC. There was no indication of radiation exposure, family history of TC, or any other thyroid disease in our patient. In contrast, 3 out of 10 previously reported patients had a predisposition factor for PTC, including family history (n = 1), congenital hypothyroidism (n = 1), solitary hyperfunctioning nodule with thyrotoxicosis (n = 1) (5,14,16).

The classical diagnostic approach to thyroid nodules comprises evaluating TSH and T4 levels and thyroid ultrasonography. A fine-needle aspiration biopsy (FNA) should be performed for the nodules having highly suspicious features. Considering the probability of diagnostic delay due to inconclusive FNA that may occur owing to the very young age of the child, we preferred excisional biopsy for our patient in the first place. TC in prepubertal children is differentiated from TC in adolescents and adults by exhibiting a more aggressive behavior pattern. Although prepubertal children appear to have more advanced disease with lymph node involvement and distant metastases or recurrent disease, they have a more favorable prognosis than adults (20). Correspondingly, our patient presented with an extensive cervical mass. He was diagnosed with stage 3 PTC and classified as an intermediate-risk group. However, he reached remission rapidly and has had no recurrence during the seven-year follow-up period. Nonetheless, the data on long-term outcome results in children under five years of age is scarce (5,8,9,10,11,12,13,14,15,16).

FVPTC accounts for 22.5% of all PTC. Based on findings from adult studies, tumor size larger than 4 cm and the presence of local invasion are closely associated with poor prognosis, whereas the behavior of well-encapsulated FVPTC is almost always indolent, except for a few rare adult cases in which there was metastasis (21). Nonetheless, the data on prognosis regarding histologic subtypes of DTC are scarce in childhood. Similar to our patient, two girls under five years of age with FVPTC were reported previously (12,15).

**Table 1. Clinical and genetic characteristics of patients under 5 years of age with papillary thyroid carcinoma (5,8,9,10,11,12,13,14,15,16)**

| First author and year of publication | Age at diagnosis | Gender | Presenting symptom | Predisposing factor | Histopathological features | Capsule invasion/ LN involvement/ metastasis | Genetic analysis | Treatment | Follow-up/ Outcome |
|---|---|---|---|---|---|---|---|---|---|
| Srikumar et al (10) 2006 | 2.67 years | Male | Neck mass, 4.5 cm** | Negative | PTC | Negative | N/A | Near TT and RAI ablation | 1 year/R |
| Alkan et al (9) 2008 | 3 years | Male | Neck mass, 2x2 cm* | Negative | PTC | LN involvement | N/A | TT and BND, RAI ablation | N/A |
| Poddar et al (11), 2008 | 11 months | Female | Neck mass, 1-1.5 cm** | Negative | PTC | Negative | N/A | Subtotal thyroidectomy and RAI ablation | 2 months/ NA |
| Khan et al (15) 2008 | 5 years | Female | Neck mass, 3x2.5x2.4 cm** | Negative | FVPTC | LN involvement | N/A | TT and selective right ND, RAI ablation | 6 months/R |
| Drut and Moreno (16) 2009 | 5 years | Female | Thyroid nodule, 0.7 cm* | Congenital hypothyroidism | PTC | Capsule invasion/ LN involvement | N/A | TT and regional LND | N/A |
| Khara et al (5) 2010 | 3.42 years | Male | Neck mass, 4x4 cm** | Family history of TC | PTC | LN involvement | N/A | TT and BND, RAI ablation | N/A |
| Damle et al (14) 2011 | 5 years | Male | Neck mass, 3.4×2.2×2 cm** | Solitary hyperfunctioning nodule, thyrotoxicosis | PTC | N/A | N/A | TT | 6 months/R |
| Gayathri et al (8) 2014 | 5 years | Male | Neck mass, 0.7x0.8x0.9 cm** | Negative | PTC | LN involvement positive | RET positivity | TT and BND, RAI ablation | 1.5 years/R |
| Uhliarova and Hajtman (12) 2016 | 2 years | Female | Neck mass, 5x3 cm* | Negative | FVPTC | Incomplete capsule invasion | N/A | TT and selective LND, RAI ablation | 2 years/R |
| Mahajan et al (13) 2018 | 5 years | Male | Neck mass, (size N/A) | Negative | PTC | Extensive LN and pulmonary involvement | SQSTM1-NTRK3 fusion positive, BRAF negative | Near TT, LND and resection of the bulky mediastinal component, RAI ablation and targeted therapy*** | 5 months/ clinically stable |
| Presented case | 1.25-years | Male | Neck mass, 5x2.7 cm | Negative | FVPTC | Negative | BRAF negative | TT, RAI ablation | 7.25 years/R |

*On physical examination, **On radiology, ***Lenvatinib and larotrectinib.
BND: bilateral neck dissection, FVPTC: follicular variant papillary thyroid carcinoma, LN: lymph node, LND: lymph node dissection, NA: not available, ND: neck dissection, PTC: papillary thyroid carcinoma, R: remission, RAI: radioactive iodine, TC: thyroid carcinoma, TT: total thyroidectomy

However, long term follow-up outcomes were not available in these patients.

Recently, genetic alterations were found to be associated with cancer predisposition and prognosis of TC. It is speculated that the distinct course of disease in childhood is associated with different genetic profiles. Point mutations in B-Raf proto-oncogene (BRAF), telomerase reverse transcriptase (TERT), and rat sarcoma (RAS) genes are more frequent in adults compared to children, whereas neurotrophic tyrosine kinase receptors (NTRK) fusion oncogenes are seen at a high frequency in both children and adults (3,22). Additionally, RET/PTC rearrangements are the most common genetic alteration in childhood DTC, which mainly occurs as a result of radiation exposure and is correlated with an aggressive course. Adult studies showed that BRAF mutations are related to poor prognosis and high risk of recurrence in PTC patients (23), while the impact of $BRAF^{V600E}$ mutation on the prognosis of childhood TC is not yet clear. Furthermore, a variety of genetic syndromes may increase the risk of PTC. The associated hereditary syndromes include familial adenomatous polyposis (APC), Li-Fraumeni syndrome (TP53), Cowden syndrome (PTEN), Werner syndrome (WRN), Carney complex (PRKAR1$\alpha$), and DICER1 syndrome (DICER1) (24,25). Genetic alternations were investigated in only two out of 10 previously reported cases (8,13). One was positive for RET/PTC rearrangement, who presented with extensive lymph node involvement that extended into the mediastinum. Nevertheless, there was no history of radiation exposure in this case, and he was in remission for a 1.5-year follow-up period (8). The other patient was positive for SQSTM1-NTRK3 fusion, which required targeted kinase inhibitors following surgery, and subsequently had RAI ablation (13). BRAF mutation status was tested in our patient and found to be wild type.

The optimal treatment strategy for pediatric TC is total thyroidectomy, followed by RAI ablation when indicated. Neck dissection is recommended for cases with metastatic neck nodes, whereas prophylactic neck dissection is not advised for cases without clinical and radiological lymph node involvement (7). During operation, a rapid frozen section is considered to be beneficial in guiding management and cost-saving via reducing the need for a secondary operation (26). Our patient was treated with mass excision, followed by complementary total thyroidectomy. Owing to the patient's age, not having a locally invasive disease or distant metastasis, low Tg levels, and severe side effects of therapy, RAI ablation was not performed in the immediate postoperative period. Observation with adequate TSH suppression was initially preferred. However, basal and stimulated Tg levels elevated in the first year following surgery, albeit with negative radiological progression, and 1 mCi/kg RAI was performed. He has been on a TSH-suppressive dose of LT4 treatment for over seven years with no recurrence. Both total thyroidectomy and $I^{131}$ RAI ablation have more complications in children than in adults (3). Transient/permanent hypoparathyroidism, recurrent laryngeal nerve damage, and postoperative bleeding/hematoma may occur. Hypoparathyroidism after total thyroidectomy is seen more frequently in young children, due to the fine and delicate structure, leading to damage to parathyroid glands. Other than younger age, central and bilateral lymph node dissection, Graves' disease, thyroid cancer, total thyroidectomy, and reoperation are also predictors of postoperative hypoparathyroidism (27). Recent studies suggest that, along with assessing preoperative and postoperative calcium levels, measuring intraoperative PTH levels may be beneficial for anticipating the risk of postoperative hypocalcemia and the timing of parathyroid gland recovery (28). Although no postoperative complications were seen in our patient, as in the other reviewed patients, interestingly, he developed hypoparathyroidism in the third month after surgery. In the literature, this entity is defined as delayed hypoparathyroidism that can occur months and even years following thyroidectomy due to progressive atrophy of the parathyroid glands resulting in late-onset hypoparathyroidism (29). Secondary to RAI ablation, complications such as transient neck pain and edema, gastrointestinal symptoms, sialadenitis/xerostomia, bone marrow suppression, gonadal damage, dry eyes, nasolacrimal duct obstruction, secondary malignancies, and pulmonary fibrosis may develop. However, RAI is accepted to be safe in children since the side effects are dose-dependent (3,5). Furthermore, molecular targeted therapy has been demonstrated to be beneficial in children with PTC who have an advanced or refractory disease that is not amenable to RAI or further surgery (30). Mahajan et al (13) started targeted therapy (lenvatinib, subsequently switched to larotrectinib) for a five-year-old patient with NTRK3 fusion-positive metastatic PTC. They observed clinical stabilization and no side effects during five months of therapy. Patients with PTC require a regular follow-up by testing serum Tg level and performing neck ultrasonography. Target level of suppressed TSH should be obtained. In addition, if the Tg level increases and thyroid ultrasound is normal, a chest CT scan or a WBS should be performed. Our patient was followed up by a multidisciplinary team consisting of pediatric endocrinologists, surgeons, pathologists, radiologists, and nuclear medicine specialists. A

collaborative approach is essential to maximize long-term survival. Our patient has been examined and tested periodically every 3-6 months for over seven years. This is in contrast to previously published cases, in which follow-up strategy and long-term outcome results were not reported.

## Conclusion

In conclusion, we present a one-year and three-month-old boy with FVPTC, who was successfully treated with total thyroidectomy, followed by RAI ablation. Previously, ten PTC patients under five years of age have been reported, and in most of these earlier cases long-term outcome was unavailable. The presented patient has had more than seven years disease-free. We suggest that this management strategy may be a road map for clinicians dealing with this rare cancer in very young children.

### Ethics

**Informed Consent:** A written informed consent was obtained from the patient's family.

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

### Authorship Contributions

Surgical and Medical Practices: Ayşe Pınar Öztürk, Esin Karakılıç Özturan, Feryal Gün Soysal, Seher Ünal, Göknur Işık, Gülçin Yegen, Semen Önder, Melek Yıldız, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler, Concept: Ayşe Pınar Öztürk, Design: Ayşe Pınar Öztürk, Data Collection or Processing: Ayşe Pınar Öztürk, Analysis or Interpretation: Ayşe Pınar Öztürk, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler, Literature Search: Ayşe Pınar Öztürk, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler, Writing: Ayşe Pınar Öztürk, Şükran Poyrazoğlu, Firdevs Baş, Feyza Darendeliler.

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CASE REPORT

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# Silent Corticotroph Tumor with Adrenocortical Choristoma in an Eleven-year-old Boy

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### What is already known on this topic?

Silent corticotroph adenomas (SCAs) do not manifest biochemical or clinical evidence of hypercortisolism, but are histologically immunopositive for adrenocorticotrophic hormone (ACTH) and Tpit, the transcription factor for functioning and SCAs, which is useful in diagnosis of corticotroph and null cell adenoma. The existence of adrenocortical cells within the pituitary gland, which can be explained as a choristoma, is a very rare entity, and the co-occurrence of these two entities have only been reported in few cases.

### What this study adds?

Adrenocortical choristoma in SCA is a very rare entity, and herein, to the best of our knowledge, we describe the fourth and the youngest patient reported to date.

## Abstract

Silent corticotroph tumors are composed of corticotroph cells, but do not manifest any biochemical or clinical evidence of hypercortisolism. A choristoma is a benign, congenital proliferation of histologically mature tissue elements normally not present at the site of occurrence. The existence of adrenocortical cells within the pituitary gland, which can be explained as a choristoma, is a very rare entity, and the co-occurrence of these two entities have only been reported in few cases. We report an 11-year-old boy with central hypothyroidism. On cranial magnetic resonance imaging a pituitary tumor was detected, and histopathological studies led to a diagnosis of an adrenal choristoma and a silent corticotroph tumor in the pituitary gland. The presence of adrenocortical cells were confirmed by positive calretinin, inhibin and Melan A staining, and the corticotroph cells by immunohistochemistry demonstrating adrenocorticotropic hormone positivity. Herein, we report the fourth and the youngest case of silent corticotroph tumor with adrenocortical choristoma in the literature. Even though the underlying mechanism is not fully understood, suggested mechanisms are discussed.

**Keywords:** Adrenocortical choristoma, corticotroph adenoma, steroidogenic factor 1

## Introduction

Corticotroph adenomas (CAs) comprise approximately 10% of all pituitary tumors (1). Functional CAs are associated with elevated circulating adrenocorticotropic hormone (ACTH) and cortisol levels leading to Cushing disease, with features of hypercortisolism or Nelson's syndrome (2). Up to 20% of CAs are described as silent corticotroph adenomas (SCAs), and they do not manifest biochemical or clinical evidence of hypercortisolism, whereas both silent and functional CAs are immunopositive for ACTH and Tpit, the transcription factor for functioning and SCAs, which is useful in diagnosis of CAs and null cell adenoma (3,4,5). SCAs comprise a very small proportion of the total population of nonfunctional pituitary adenomas. Despite being silent, they show aggressive behavior (6).

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A choristoma is a benign, congenital proliferation of histologically mature tissue elements normally not present at the site of occurrence. This heterotopic congenital mass results from normal tissue elements migrating to or remaining in an abnormal location during embryogenesis. Adrenocortical choristomas have been identified in a wide variety of other non-steroidogenic tissues, including kidney, lung, spinal canal and the leptomeningeal surface in the cranium (7,8). However only four previous reports have described the occurrence of interspersed adrenal cortical cells in three SCAs and in one clinically functioning CA (7,9,10,11).

SCAs are diagnosed incidentally or when they reach a size which leads to clinical symptoms because of mass effects, such as headache or compression findings (1). Adrenocortical choristoma in SCA is a very rare entity, and herein, to our knowledge, we describe only the fourth and the youngest patient reported to date, who was diagnosed during evaluation of central hypothyroidism.

## Case Report

The patient had been on regular follow-up in our clinic with a diagnosis of compensated hypothyroidism due to an exaggerated thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone and had been on L-thyroxine treatment since four months of age. The patient did not have any relevant past medical and familial history, except for the presence of double urethral meatus. At 11 years of age, despite L-thyroxine treatment, findings compatible with central hypothyroidism (free thyroxine: 0.78 ng/dL, normal range: 0.98-1.63 and TSH: 0.47 mIU/mL, normal range: 0.51-4.3) were noted. On physical examination, he was in the 90-97$^{th}$ percentile for weight and 90$^{th}$ percentile for height. His blood pressure was normal (90/50 mmHg, 50-75$^{th}$ percentile). All other pituitary hormones were found to be within normal ranges. Cortisol was found to be low (4.82 ug/dL, normal: >15 ug/dL), and ACTH level was 17.3 pg/mL (relatively low). An ACTH deficiency was confirmed with a peak cortisol of 15.27 ug/dL (normal >18 ug/dL) to low dose ACTH stimulation test (12). Thus, cortisol replacement was added to L-thyroxine replacement.

Magnetic resonance imaging (MRI) identified a tumor measuring 11x11x10 mm in the pituitary region with enhancement characteristics suggestive of a pituitary adenoma (also known as pituitary neuroendocrine tumor) (Figure 1). Transsphenoidal resection of the pituitary tumor was performed due to the tumor mass effect which resulted in central hypothyroidism and central adrenal insufficiency. Pathological examination identified a CA with adrenocortical choristoma. Growth failure was evident after surgery (Figure 2). Based on growth hormone (GH) stimulation tests, complete GH deficiency was confirmed, and GH therapy was initiated. The patient benefited from the treatment with a height velocity of 8.4 cm/year in the first year, and his pubertal development progressed in accordance with his age.

Six years after surgery, tumoral recurrence was observed on MRI in the pituitary gland, with a microadenoma of 5 mm in diameter. However, since the tumor did not cause any clinical findings, the patient was followed up with MRI repeated at 6-month intervals.

Beforehand, informed consent was obtained from the parents of the patient for all steps of treatment and added to the patient's file.

### Histopathological Evaluation

Histopathological evaluation of the tumor revealed the presence of two groups of cells. These were small round cells with amphophilic to basophilic cytoplasm and large spherical, oval cells with abundant, granular, partly vacuolated acidophilic cytoplasm (Figure 3A, 3B).

By immunohistochemistry, the small cells were immunopositive for ACTH and synaptophysin. In addition, these cells were diffusely positive for Periodic Acid-Schiff (PAS), indicating the presence of corticotroph cells with a predominant dense granulation pattern. The larger cells were immunonegative for synaptophysin (Figure 4A, 4B) but positive for mitochondrial antigen, inhibin (Figure 5A, 5B), calretinin and Melan A (clone A103). The cells were rich in mitochondria, and did not stain with PAS, compatible with adrenocortical tissue.

The ratio of the two cell types varied considerably from area to area. Major cellular or nuclear pleomorphism was not noted, and no mitotic figures were seen in either component. The Ki67 labeling index was 3-4%. However,

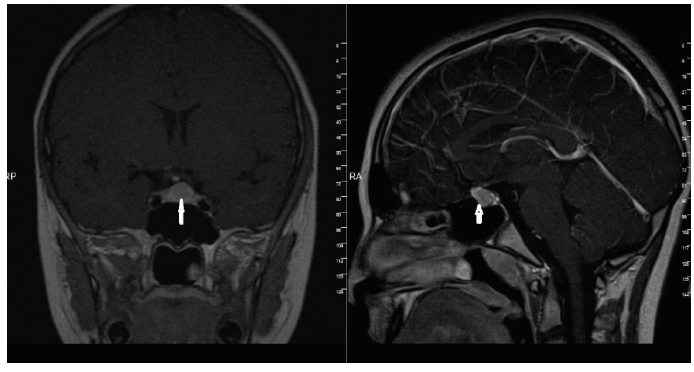

**Figure 1.** Magnetic resonance imaging of the pituitary gland at eleven years of age. The 1 cm adenoma is marked with an arrow

the tumor margins could not be confirmed due to the nature of the specimen excision.

## Discussion

SCAs comprise 3-19% of nonfunctional pituitary adenomas (1). The most frequently reported presenting features of SCAs are tumor mass effects, including headaches, visual disturbance, and hypopituitarism (13). Central hypothyroidism and central adrenal insufficiency developed during the follow-up of our patient. GH deficiency emerged after surgery, as an expected complication of pituitary surgeries (14).

It has been demonstrated that SCAs can be more aggressive than any other clinically nonfunctioning adenomas with a higher prevalence of cavernous sinus invasion and a higher rate of recurrence (5,13). In the other three case reports (7,10,11), data with regard to postoperative follow-up period were lacking, but the tumor in our patient recurred after six years.

The SCA in our patient was associated with adrenocortical cells. Co-existence of CA with adrenocortical choristoma is a very rare entity. In 1996, Oka et al (7) were the first to report such a tumor in a 16-year-old boy who presented with growth retardation (7). Three of the previously described tumors, including the patient reported by Oka et al (7) were all biochemically silent, but the fourth one showed evidence of function (9). Similar to our case, all four previously reported tumors were macroadenomas (tumors exceeding 1.0 cm on MRI studies). Three of the four cases were diagnosed in the teenage period (16-18 years of age), while only one was adult (11). Our 11-year-old patient was the youngest case reported to date.

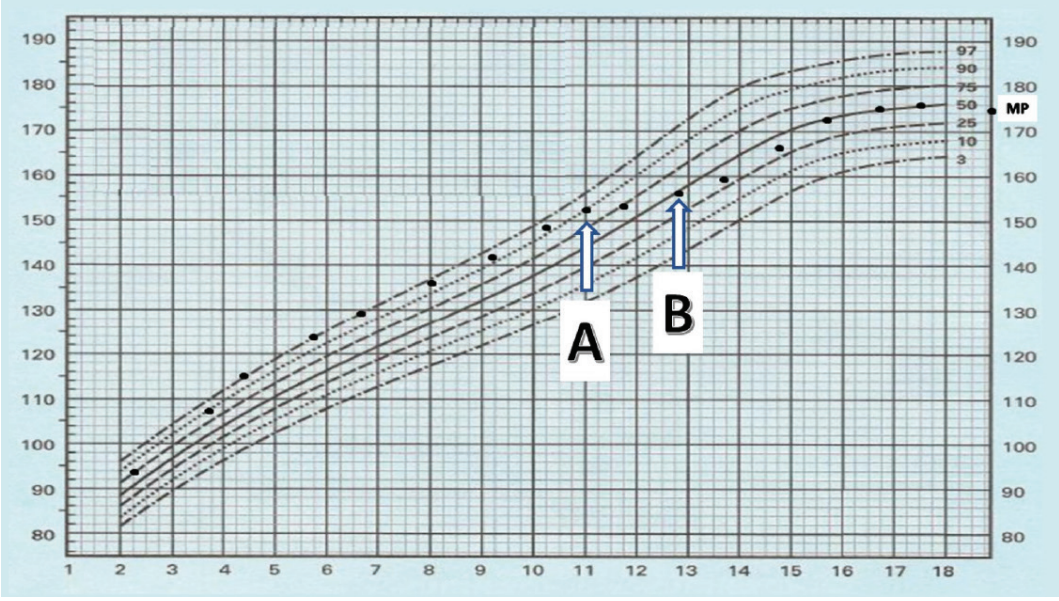


**Figure 2.** Percentile curve of the case. A) Pituitary surgery; B) Initiation of the growth hormone therapy

*MP: midparental height*

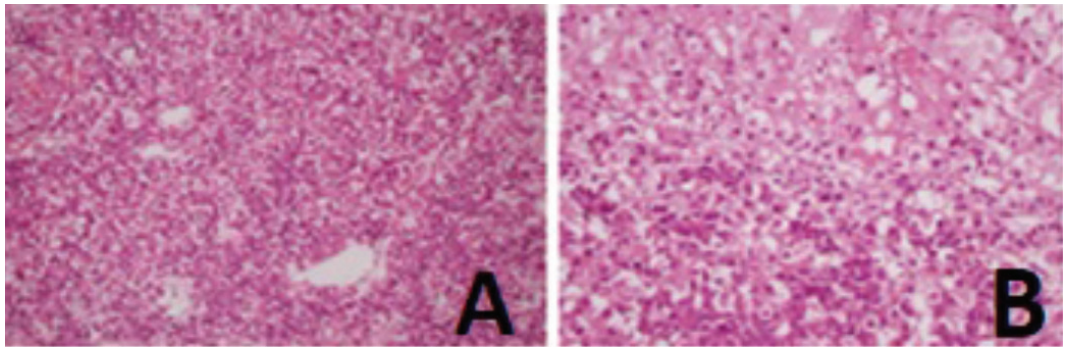


**Figure 3.** A, B) Mixture of the small, round, well-granulated cells with amphophilic or basophilic cytoplasm (corticotroph cells) and the large spherical or oval cells with abundant, granular, partly vacuolated cytoplasm (adrenocortical cells) form groups (H&E; magnification x100-400)

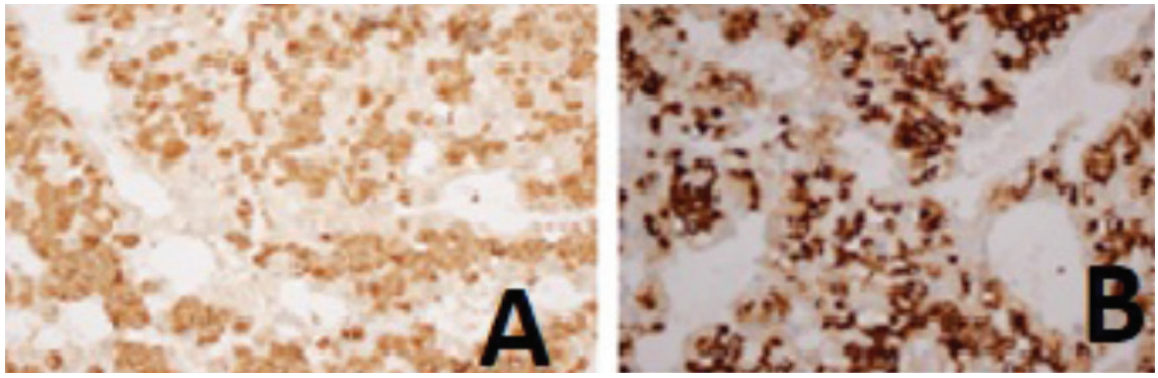


**Figure 4.** A) Small cells immunopositive for adrenocorticotrophic hormone (adrenocorticotrophic hormone; magnification x400). B) Adrenocortical cells are immunonegative and corticotroph cells immunpositive for synaptophysin (synaptophysin; magnification x400)

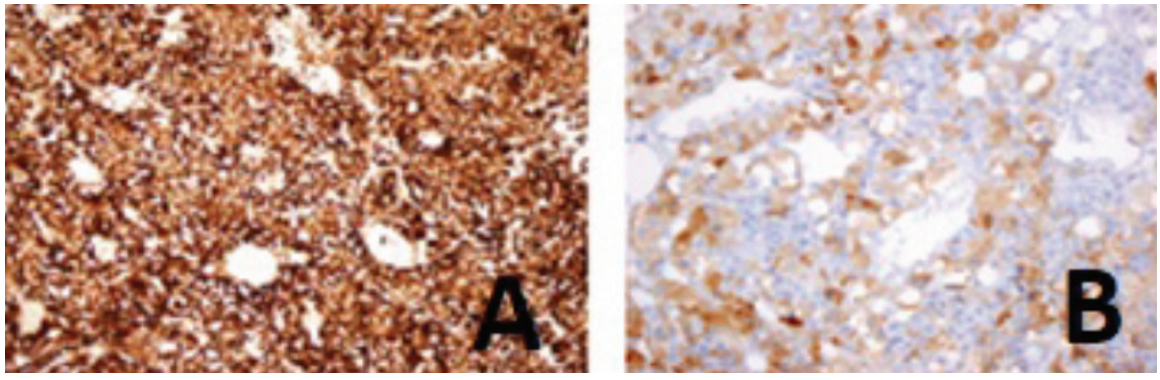


**Figure 5.** A) Large adrenocortical cells with vacuolated cytoplasm are densely immunpositive for mitochondrial antigen (mitochondrial ag; magnification x100). B) Adrenocortical cells are immunpositive for inhibin (inhibin; magnification x400)

The adult patient was a 35-year-old male patient, and he had secondary hypothyroidism, hypogonadotropic hypogonadism and "low insulin-like growth factor-1 with growth hormone" (11). The other three teen-aged patients had either growth retardation (basal GH and insulin-like growth factor-1 levels were reported to be low) or delayed pubertal development. However, our patient had a diagnosis of central hypothyroidism and ACTH insufficiency, which may be associated with the compression due to the tumor that resulted in selective adenohypophyseal dysfunction. Coversely, in a silent adenoma, endocrine hypoactivity may be due to defective production, packaging or release of hormones by pituitary cells. In our case growth retardation developed later during follow-up, and GH deficiency was confirmed. Pubertal development progressed in accordance with age and thus he did not have hypogonadotropic hypogonadism.

The origin of adrenocortical cells in CAs and the reason for co-existence with corticotroph cells is not clearly understood. It is suggested that they might be a random mixture of the two types of cells proliferating in the sella (15). As discussed in previous reports, there might be two explanations for the existence of the two cell types together. The first one is that adrenocortical cells might have differentiated from stem cells, such as undifferentiated mesenchymal cells, under prolonged ACTH stimulation. Studies in humans and in experimental animals support the hypothesis that ACTH stimulation in CAs converts mesenchymal cells to adrenocortical-like cells (16). Groat (16) observed differentiation of the adrenocortical-like cells from ovarian and other mesenchymal tissues in adrenalectomized ground-squirrels. It was also suggested that these two cell types might interact in a paracrine manner due to the close relationship between ACTH and adrenocortical cells. However, our patient had ACTH deficiency which makes this mechanism unlikely. Mete et al (11) suggested that steroidogenic factor-1, which is present in both pituitary and adrenal cortex, may have an important role in the proliferation and differentiation of uncommitted mesenchymal stem cells within the sella. The second explanation is that the adrenocortical cells may have migrated to the wrong place in early embryonic development (7,8,10). In the present case, the latter explanation appears to be more likely.

## Conclusion

The lack of biochemical and clinical evidence of Cushing syndrome, despite corticotroph tumor, indicated the presence of a SCA. In our patient, the presence of the second group of cells, similar to adrenocortical cells in this heterotopic location is compatible with choristoma. The younger age of our patient than previously reported cases and clinical significance of SCA, make this case remarkable, and the recurrence of the tumor in the present case after surgery also makes this case report unique. Another point of note is that endocrinologists should not ignore unexpectedly suppressed TSH values while evaluating thyroid function tests during the follow-up of the patients with primary hypothyroidism and should be careful in terms of newly developing central hypothyroidism. In such a situation, we suggest that other pituitary hormone levels should be measured and pituitary imaging should be undertaken.

### Ethics

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

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

### Authorship Contributions

Surgical and Medical Practices: Özgür Mete, Büge Öz, Concept: Hande Turan, Ada Bulut Sinoplu, Oya Ercan, Design: Hande Turan, Oya Ercan, Data Collection or Processing: Hande Turan, Gürkan Tarçın, Özgür Mete, Ada Bulut Sinoplu, Oya Ercan, Analysis or Interpretation: Hande Turan, Oya Ercan, Literature Search: Hande Turan, Gürkan Tarçın, Oya Ercan, Writing: Hande Turan, Gürkan Tarçın, Özgür Mete, Saadet Olcay Evliyaoğlu, Büge Öz, Oya Ercan.

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