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### Sample References

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

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

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

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## Original Articles



## Short Communication



## Case Reports



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## CONGRESS CALENDAR

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

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

ORIGINAL ARTICLE

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J Clin Res Pediatr Endocrinol 2017;9(3):185-193

# Clinicopathological Characteristics of Papillary Thyroid Cancer in Children with Emphasis on Pubertal Status and Association with BRAF$^{V600E}$ Mutation

**Şükran Poyrazoğlu[1], Rüveyde Bundak[1], Firdevs Baş[1], Gülçin Yeğen[2], Yasemin Şanlı[3], Feyza Darendeliler[1]**

*[1]İstanbul University İstanbul Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey*
*[2]İstanbul University İstanbul Faculty of Medicine, Department of Pathology, İstanbul, Turkey*
*[3]İstanbul University İstanbul Faculty of Medicine, Department of Nuclear Medicine, İstanbul, Turkey*

### What is already known on this topic?

Papillary thyroid cancer (PTC) is more disseminated in prepubertal children. Recurrence rate was reported to be higher in the prepubertal group.

### What this study adds?

BRAF$^{V600E}$ mutation is not correlated with a more extensive or aggressive disease in pediatric PTC patients. Frequency of BRAF$^{V600E}$ mutation is similar between prepubertal and pubertal children with PTC.

## Abstract

**Objective:** Papillary thyroid cancer (PTC) may behave differently in prepubertal children as compared to pubertal children and adults. BRAF gene activating mutations may associate with PTC by creating aberrant activation. We aimed to evaluate the clinicopathological characteristics of PTC patients with emphasis on the pubertal status and also to investigate the association of BRAF$^{V600E}$ mutation with disease characteristics.

**Methods:** The medical records of 75 patients with PTC were reviewed retrospectively. BRAF$^{V600E}$ mutation status was available only in the medical records of 56 patients.

**Results:** Mean age at diagnosis was 12.4 ± 3.8 years. There was no difference in sex, initial signs, tumor histopathology, and pathological evidence of tumor aggressiveness between prepubertal and pubertal children. Although not statistically significant, lateral neck nodal metastasis and lung metastasis at diagnosis were more prevalent in prepubertal children. After excluding patients with microcarcinoma, prepubertal children were found to require lateral neck dissection and further doses of radioactive iodine more frequently than pubertal patients. Recurrence was also more frequent in prepubertal children ($p = 0.016$). Frequency of BRAF$^{V600E}$ mutation was similar in prepubertal and pubertal patients. BRAF$^{V600E}$ mutation was found in 14/56 (25%) patients and was high in the classic variant PTC ($p = 0.004$). Multicentricity was high in BRAF$^{V600E}$ mutation ($p = 0.01$). There was no relation between BRAF$^{V600E}$ mutation and lymph node and pulmonary metastasis at diagnosis, or between BRAF$^{V600E}$ mutation and pathological evidence of tumor aggressiveness.

**Conclusion:** PTC is more disseminated in prepubertal children. BRAF$^{V600E}$ mutation does not correlate with a more extensive or aggressive disease. BRAF$^{V600E}$ mutation is not the cause of the differences in the biological behavior of PTC in prepubertal and pubertal children.

**Keywords:** Children, pediatric thyroid cancer, papillary thyroid cancer, puberty, BRAF mutation

**Address for Correspondence:** Şükran Poyrazoğlu MD,
İstanbul University İstanbul Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
**Phone:** +90 212 414 20 00 **E-mail:** sukranpoyrazoglu@yahoo.com **ORCID ID:** orcid.org/0000-0001-6806-9678

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**Conflict of interest:** None declared
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## Introduction

Differentiated thyroid carcinomas (DTCs) are the most common endocrine neoplasia in childhood. Papillary thyroid cancer (PTC) which arises from follicular epithelial cells constitutes more than 90% of thyroid cancer cases (1). Over the last decades, the incidence of thyroid cancer showed an increase worldwide and the incidence of DTC in children has been reported to increase by 1.1% per year (2).

DTC has been considered a distinct disease in both children and adults. Pediatric DTC differs from the condition in adults in terms of clinical manifestations and outcomes. Lymph node metastasis, extrathyroidal involvement, and pulmonary metastasis at diagnosis are more common in children (3,4,5). Despite extensive disease at diagnosis, children have a more favorable outcome and a lower mortality. Also, transformation to less differentiated tumors is less common in children (4,5,6). Similarly, PTC has been found to behave differently in prepubertal children than in pubertal children. Some studies report an increased prevalence in extrathyroidal involvement, regional lymph node metastases, distant metastases, and lymph node recurrence in younger children (7,8,9,10), but these features are not observed in all studies (11,12). It remains uncertain whether prepubertal children are at greater risk for more extensive disease or for higher rates of recurrence. The recent pediatric guidelines of the American Thyroid Association in 2015 recommend that prepubertal and pubertal status should be included in studies to increase uniformity and to evaluate more accurately the potential influence of pubertal development on the incidence and behavior of DTC within the pediatric population (13).

B-type RAF kinase (BRAF) is a cytoplasmic serine/threonine kinase and an essential molecule in the mitogen-activated protein kinase (MAPK) pathway (14,15). *BRAF* gene activating mutations cause PTC in thyroid follicle cells by creating aberrant activation and the most common mutation is $BRAF^{V600E}$ (a valine by glycine substitution at codon 600) (14). $BRAF^{V600E}$ mutation may be associated with aggressive character in PTC, reducing differentiation of the cancer and radioactive iodine (RAI) retention capacity by decreased expression of the sodium-iodide symporter (15,16). Although the impact of $BRAF^{V600E}$ mutations in PTC is controversial, some studies in adults showed that $BRAF^{V600E}$ mutation may associate with more aggressive disease, higher risk for lymph node involvement, distant metastasis, and poor prognosis (17,18). It has been suggested that the different behavior of PTC in children and in adults might result from the differences in the prevalence of the $BRAF^{V600E}$ mutation (19,20).

In this study, we aimed to evaluate the clinicopathological characteristics of PTC patients with emphasis on the pubertal status of the patients. We also investigated the association of $BRAF^{V600E}$ mutation with disease characteristics in our patients with PTC.

## Methods

Between 1983 and 2015, eighty-four patients (56 girls, 28 boys) with TC were followed in our unit. The medical records of these patients were reviewed retrospectively. PTC was the most common type (75 patients, 89.2%), followed by medullary thyroid cancer (MTC) (5 patients, 6%), and follicular thyroid cancer (FTC) (4 patients, 4.8%). MTC and FTC cases were excluded and PTC patients were evaluated in this paper.

The details of the patients' presentations, family history, pathological findings of the tumor, treatment, and outcome data were evaluated from the medical records of the patients. A history of external radiotherapy to the cervical region prior to admission was questioned in all cases. The dose and frequency of radioiodine ($I^{131}$) ablation therapy and the presence of metastases, recurrences, and other complications in the follow-up were noted. $BRAF^{V600E}$ mutation status was found in the medical records of 56 patients. $BRAF^{V600E}$ mutations of some of the cases were reported previously (21). The patients were classified with respect to pubertal status at diagnosis. The onset of puberty was defined according to Tanner standards as attainment of breast budding in girls and testicular volume ≥4 mL in boys (22).

Treatment for PTC was based on surgery and $I^{131}$ ablation and suppressive thyroid hormone therapy. Sixty-five (86.7%) patients had their thyroid surgery performed at our hospital, whereas the other 10 patients (13.3%) underwent thyroid surgery at other hospitals, and then were referred to our hospital for follow-up and $I^{131}$ therapy. Except the early years when subtotal thyroidectomy was performed; all patients underwent total thyroidectomy with or without neck lymph node dissection. Radioiodine treatment was used for ablation of thyroid remnants and/or lung metastases. All patients except those with microcarcinoma with no risk factors were treated with radioiodine to ablate the postsurgical thyroid remnant. The postoperative radioiodine treatment was prescribed as follows: 30-100 mCi for patients with a tumor limited to the thyroid gland; 150 mCi for tumors invading the thyroid capsule surrounding tissues and/or with metastases in the neck or mediastinal lymph nodes; 175-200 mCi for distant metastases. Dose adjustments in younger children were made on a per kg basis using the doses for a standard

70 kg person as a reference point. Repeated $I^{131}$ treatments were given to patients with evidence of recurrence or metastases. One week after $I^{131}$ administration, a whole-body scintigraphy (WBS) was performed. A second WBS was performed using 2-5 mCi at 12 months after $I^{131}$ treatment concurrent with measurement of stimulated thyroglobulin (Tg) levels before WBS. All patients were maintained on L-thyroxin suppressive therapy.

Patients were followed according to a standard protocol. During follow-up, all patients were evaluated clinically at 3 to 6 month intervals and were given L-thyroxin treatment postoperatively aiming to keep thyroid-stimulating hormone (TSH) levels below 0.1 μU/mL and Tg levels as undetectable. The follow-up protocol included assessment of free thyroxine, TSH, and Tg levels every 3-6 months. Ultrasonography was performed every six months to assess for presence of residual thyroid tissue and evaluation of the lymph nodes. $I^{131}$ WBS was performed using 2-5 mCi 12 months after diagnosis. Concurrent measurements of stimulated Tg levels were also done before WBS and repeated 6-12 months after each radioiodine therapy. Beginning in the 1990s, thyroglobulin levels and thyroglobulin antibodies were monitored in all patients.

Patients were classified as remission, persistent disease, or recurrence. Remission is defined as negative diagnostic results on WBS, neck ultrasound, chest computed tomography, and undetectable or low serum stimulated Tg levels. Patients who never entered remission were accepted as having persistent disease by one or more than one of the following criteria: detectable serum Tg levels either suppressed or after TSH stimulation, lymph nodes on the neck ultrasound, confirmed by fine needle aspiration biopsy and positive WBS. Recurrence was accepted as the appearance of cancer with new RAI uptake or biopsy in any patient who had been free of cancer. If tumor diameter was ≤1 cm, PTC was classified as microcarcinoma.

All of the histological examinations were reviewed at our hospital. Indicators of tumor aggressiveness such as multicentricity, vascular invasion, perineural invasion, thyroid capsular invasion, extrathyroidal extension, lymph node metastasis, and lung metastasis were evaluated.

$BRAF^{V600E}$ mutation analysis was performed in formalin fixed, paraffin-embedded papillary thyroid carcinoma specimens. For genomic DNA preparation, the QIAamp DNA tissue kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions. $BRAF^{V600E}$ mutation was determined by pyrosequencing using the Qiagen PyroMark Q24 pyrosequencer (Qiagen, Venlo, Netherlands) following the manufacturer's instructions, as has been described (23).

Statistical analyses were performed using SPSS statistical package version 17 (SPSS Inc., Chicago, IL, USA). The results are presented as mean ± standard deviation or as percentage figures. Chi-square test, Fisher's exact test, t-test, and Kruskal-Wallis test were used in the statistical analyses. A p-value $< 0.05$ was accepted as statistically significant.

This study was approved by the local ethics committee.

## Results

Mean age of the patients at diagnosis was 12.4 ± 3.8 years (range 1.3 to 17.8). The male/female ratio was 24/51 (1:2.1) in the total group. The most common presenting sign was presence of a nodule (70%). Twelve patients had a history of radiotherapy for conditions including Hodgkin's lymphoma (n = 7), medulloblastoma, neuroblastoma, pinealoblastoma, pons glioma, and liposarcoma (each in 1 patient). Hodgkin's lymphoma and PTC were diagnosed simultaneously in one patient. The mean interval between radiotherapy and presentation with PTC was 9.2 ± 2.1 years (range: 6-11 years). PTC was detected in these patients as a result of prospective follow-up based on their history of external radiotherapy. Twenty patients had a family history of thyroid disease. Eighteen patients had an associated disease at diagnosis (13 Hashimoto's thyroiditis, 2 type 1 diabetes, 2 multinodular goiter, 1 Graves' disease). Except one, none of the patients had a family history of thyroid cancer.

Total and subtotal thyroidectomies were performed in 92% and 8% of the patients, respectively. Central compartment neck dissection was performed in 42 (56%) patients, lateral neck dissection in 14 (18.7%). Because of multicentricity and history of radiotherapy, 8 patients with microcarcinoma underwent central neck dissection. Neck dissection was performed bilaterally in 7 (9.3%) patients. Temporary hypoparathyroidism was observed in 3 patients (4%) and permanent hypoparathyroidism in 6 (8%) patients after surgery.

The mean tumor diameter was 2.2 ± 1.6 cm (range: 0.2-7 cm) and 23 (30.7%) tumors were microcarcinomas. In 23 microcarcinoma patients, the thyroid nodule was identified incidentally on ultrasound during follow-up.

Twelve patients were followed for history of thyroid disease [Hashimoto thyroiditis (n = 6), goiter (n = 5), hypothyroidism (n = 1)], 8 patients for history of radiotherapy, and 3 patients had thyroid ultrasound because of family history of thyroid disease.

Forty patients (53.3%) had the classical variant of PTC, 29 patients (38.7%) had been diagnosed with the follicular

variant, 5 patients (6.7%) with the variant with diffuse sclerosis, and 1 patient (1.3%) with the solid variant of PTC. At diagnosis, the incidence of multicentricity, capsule invasion, lymph node metastasis, and lung metastasis were calculated as 49.3%, 40%, 45.3%, and 13.3%, respectively. In Table 1, some clinical and laboratory features of PTC in prepubertal and pubertal children are presented. Male/female ratio was similar in prepubertal and pubertal children (p = 0.56). There was no difference in sex, clinical signs at diagnosis, and tumor histopathology between prepubertal and pubertal children. Presence of $BRAF^{V600E}$ mutation was also similar in prepubertal and pubertal children. Because frequency of microcarcinoma was higher in the pubertal group (36.8%, p = 0.04) as compared to the prepubertal group (11.1%), prepubertal children had greater tumor size than pubertal children (p = 0.03). However, after excluding microcarcinoma, tumor size was similar between the two groups (Table 2, p = 0.24). Similarly, there was no difference in pathological evidence of tumor aggressiveness (multicentricity, vascular invasion, perineural invasion, capsule invasion, extrathyroidal extension) between prepubertal and pubertal children.

Total thyroidectomy and subtotal thyroidectomy ratio was also similar between prepubertal and pubertal children (p = 0.62). Radioiodine therapy was administered to 61 patients with PTC in a total dose ranging from 13 mCi to 605 mCi. A total of 17 patients underwent a second or more doses of RAI treatment due to presence of lymph node and lung metastasis. Extent of metastasis, response to treatment, and outcome in patients with clinically detected PTC in prepubertal and pubertal groups are given in Table 2. To be able to compare the two groups, patients with microcarcinoma detected during follow-up for other reasons were excluded. Although lateral neck nodal metastasis and lung metastasis at diagnosis were more frequent in prepubertal children (43.7% and 33.3%, respectively) than

**Table 1. Comparison of some clinical and laboratory features of papillary thyroid cancer in prepubertal and pubertal patients**

| | Prepubertal n = 18 | Pubertal n = 57 | p |
|---|---|---|---|
| Age (years, mean ± SD) | 7.4 ± 2.2 | 14.8 ± 2.1 | < 0.001* |
| **Sex (n, %)** | | | |
| Female | 11 (61.1%) | 40 (68.3%) | 0.56** |
| Male | 7 (38.9%) | 17 (31.7%) | |
| **Initial signs (n, %)** | | | |
| Nodule | 13 (75%) | 39 (67.3%) | 0.61** |
| Goiter/Lymphadenopathy | 5 (25%) | 15 (32.7%) | |
| **History of radiotherapy (n, %)** | 3 (16.6%) | 9 (15.8%) | 0.59** |
| **Pathological evidence of tumor aggressiveness (n, %)** | | | |
| Multicentricity | 10 (55.5%) | 27 (47.4%) | 0.24** |
| Vascular invasion | 6 (33.3%) | 22 (38.6%) | 0.61** |
| Perineural invasion | 5 (27.8%) | 12 (21.1%) | 0.30** |
| Capsule invasion | 9 (50%) | 21 (36.8%) | 0.14** |
| Extrathyroidal extension | 6 (33.3%) | 17 (29.8%) | 0.76** |
| **Mean tumor diameter (cm, mean ± SD)** | 2.9 ± 1.7 | 1.9 ± 1.5 | 0.03* |
| Microcarcinoma (n, %) | 2 (11.1%) | 21 (36.8%) | 0.04** |
| Tumor histopathology (n, %) | | | |
| Classical variant | 8 (44.4%) | 32 (56.1%) | 0.42** |
| Subtype | 10 (55.6%) | 25 (43.9%) | |
| With diffuse sclerosis | 2 | 3 | |
| Follicular | 8 | 21 | |
| Solid | - | 1 | |
| **$BRAF^{V600E}$ (n, %)** | | | |
| Positive | 2 (15.4%) | 12 (27.9%) | 0.48** |
| Negative | 11 (84.6%) | 31 (72.1%) | |

*t-test, **Chi-square test or Fisher's exact test

SD: standard deviation

in the pubertal group (19.4% and 13.9%, respectively), there was no statistically significant difference in frequency of lymph metastasis and lung metastasis between the two groups after excluding microcarcinoma. Although not statistically significant, a greater proportion of prepubertal children required lateral neck dissection and a second or more doses of RAI treatment for lymph node and lung metastasis compared to pubertal children (Table 2; 43.7% and 19.4% vs. 50% and 25%, respectively). Frequency of persistent disease was similar between prepubertal and pubertal groups. However, recurrence was more frequent in prepubertal children after excluding microcarcinoma patients (25% vs. 2.7%, respectively; p = 0.016). Frequency of surgical complications was similar in the prepubertal and pubertal groups. There was no difference with regard to presence of $BRAF^{V600E}$ mutation between prepubertal and pubertal patients after excluding microcarcinoma patients (Table 2, p = 0.68).

The mean follow-up period was 4.3 ± 3.4 years (range: 1-14.1). During the follow-up, tumor recurrence was detected in 5 PTC patients. Pulmonary recurrence was observed in 3, cervical lymph node recurrence in 1, and cervical and mediastinal lymph node recurrence in 1 patient. Four of these patients were prepubertal at diagnosis. One patient diagnosed prepubertally died with pulmonary and mediastinal involvement 10.3 years after diagnosis. She had undergone two surgical interventions (total thyroidectomy, neck dissection) and had also received RAI treatment (cumulative dose of 560 mCi).

$BRAF^{V600E}$ mutation was found in 14/56 (25%, 2 prepubertal, 12 pubertal) patients. None of the patients with $BRAF^{V600E}$ mutation had a history of external radiotherapy. The relationship between clinicopathological characteristics of PTC and $BRAF^{V600E}$ mutation is shown in Table 3. While the frequency of $BRAF^{V600E}$ mutation was significantly higher in patients with classical PTC histology (p = 0.004), it was similar in girls and boys (p = 0.73), and in tumors larger or smaller than 1 cm in diameter (p = 0.33). Multicentricity was significantly high in $BRAF^{V600E}$ mutation positive patients (p = 0.01), but lymphovascular invasion, perineural invasion, thyroid capsular invasion, and extrathyroidal extension of the tumor were similar between $BRAF^{V600E}$

**Table 2. Comparison of type of treatment, outcome, and presence of $BRAF^{V600E}$ mutation in prepubertal and pubertal patients after excluding microcarcinoma**

| | Prepubertal n = 16 | Pubertal n = 36 | p |
|---|---|---|---|
| **Mean tumor diameter (cm, mean ± SD)** | 3.3 ± 1.6 | 2.8 ± 1.5 | 0.24* |
| **Metastasis** | | | |
| Lymph nodes | 10 (62.5%) | 24 (66.7%) | 0.76** |
| Lateral neck nodal metastasis | 7 (43.7%) | 7 (19.4%) | 0.093** |
| Lung metastasis | 5 (33.3%) | 5 (13.9%) | 0.25** |
| **Total thyroidectomy** | 14 ((87.5%) | 34 (94.4%) | |
| **Subtotal thyroidectomy** | 2 (12.5%) | 2 (5.6%) | 0.57** |
| **Neck dissection** | | | |
| Central dissection | 8 (50%) | 26 (72%) | 0.20** |
| Lateral dissection | 7 (43.7%) | 7 (19.4%) | 0.093** |
| **Radioactive iodine therapy** | | | |
| One dose | 8 (50%) | 27 (75%) | |
| Two or more doses | 8 (50%) | 9 (25%) | 0.11** |
| Remission | 7 (43.7%) | 27 (84.2%) | 0.055** |
| Persistent disease | 5 (31.2%) | 8 (22.2%) | 0.26** |
| Recurrence | 4 (25%) | 1 (2.7%) | 0.016** |
| **Hypoparathyroidism** | | | |
| Temporary | - | 3 (5.2%) | |
| Permanent | 2 (11.1%) | 4 (7.0%) | 0.62** |
| **$BRAF^{V600E}$ (n, %)** | | | |
| Positive | 2 (12.5%) | 9 (25%) | 0.68** |
| Negative | 8 (50%) | 18 (50%) | |

*t-test, **Chi-square test or Fisher's exact test

SD: standard deviation

mutation positive and negative patients. There was no relationship between $BRAF^{V600E}$ mutation and lymph node and pulmonary metastasis at diagnosis. Within the pubertal group, there was no difference in tumor aggressiveness with respect to presence of $BRAF^{V600E}$ mutation.

After excluding patients with a history of radiotherapy, when numbers of patients diagnosed per each 10 years during the study period were evaluated, an increase was noted in numbers of cases in the last decade, especially in microcarcinoma (Table 4). The increase, especially in the last decade, of the frequency of PTC in girls and in pubertal patients was noteworthy.

**Table 3. Comparison of some features of patients with respect to the presence of $BRAF^{V600E}$ mutation**

| | $BRAF^{V600E}$ positive n = 14 | $BRAF^{V600E}$ negative n = 42 | p |
|---|---|---|---|
| **Gender** | | | |
| Female | 11 (76.9%) | 29 (69%) | 0.73* |
| Male | 3 (23.1%) | 13 (31%) | |
| **Age at diagnosis (years)** | 13.5 ± 2.5 | 12.9 ± 4.0 | 0.96** |
| **Tumor size** | | | |
| ≤1 cm | 3 (23.1%) | 16 (38%) | 0.33* |
| > 1 cm | 11 (76.9%) | 26 (62%) | |
| **Histological type** | | | |
| Classical variant | 12 (85.8%) | 16 (38%) | 0.004* |
| Subtype | 2 (14.2%) | 26 (62%) | |
| With diffuse sclerosis | 1 | 4 | |
| Follicular | 1 | 21 | |
| Solid | - | 1 | |
| **Extent of disease** | | | |
| Multicentricity | 12 (85.7%) | 18 (42.8%) | 0.01* |
| Vascular invasion | 7 (50%) | 17 (40.5%) | 0.55* |
| Perineural invasion | 4 (28.6%) | 9 (21.4%) | 0.48* |
| Capsule invasion | 7 (50%) | 17 (40.5%) | 0.75* |
| Extrathyroidal extension | 6 (42.8%) | 12 (28.6%) | 0.34* |
| Lymph node metastasis | 8 (57.1%) | 16 (38%) | 0.20* |
| Lung metastasis | 1 (7.1%) | 4 (9.5%) | 0.66* |
| **Radioactive iodine therapy** | | | |
| One dose | 9 (64.3%) | 28 (66.6%) | |
| Two or more doses | 4 (28.6%) | 5 (11.9%) | 0.24* |
| Remission | 10 | 37 | |
| Persistent disease | 4 | 5 | 0.20* |
| Recurrence | - | - | |
| **Remission** | | | |
| Prepubertal | 1 (10%) | 7 (18.9%) | 0.66* |
| Pubertal | 9 (90%) | 30 (81.1%) | |
| **Persistent disease** | | | |
| Prepubertal | 1 (25%) | 2 (40%) | 0.59* |
| Pubertal | 3 (75%) | 3 (60%) | |

*Chi-square test or Fisher's exact test, **t-test

## Discussion

In our cohort, at diagnosis, although not statistically significant, lateral neck nodal metastasis and lung metastases were more frequent in prepubertal children after excluding patients with microcarcinoma. Frequency of microcarcinoma was high in pubertal patients; PTC was also more disseminated in prepubertal children compared to pubertal children. As a result of this, the prepubertal children in our cohort required more lateral neck dissection and more than one dose of RAI therapy than pubertal children. While PTC was more disseminated in the prepubertal group at diagnosis, pathological evidence of tumor aggressiveness was similar between the prepubertal and pubertal groups.

Similar to our results, it was reported that at diagnosis, DTC is generally more widespread at presentation in prepubertal children than in adolescents and tumor invasion, expressed by extension beyond the thyroid capsule and the presence of metastases in regional lymph nodes and lungs, was more prominent in the prepubertal than in the pubertal patients (8,9). Lazar et al (8) reported that DTC is more aggressive in prepubertal children with an increased incidence of extrathyroidal extension, lymph node involvement, and lung metastases at presentation compared with pubertal children.

In our cohort, in addition to the more disseminated presentation, recurrence rate was higher in the prepubertal group during follow-up. Some pediatric studies have shown that younger age is associated with persistent disease or recurrence. PTC with an onset at ages younger than 10 years appears to have higher recurrence and mortality rates than PTC with onset at older ages (7). In a study from Belarus on 740 children, younger age was related to an increased risk of recurrent lymph node and lung metastases after correcting for other risk factors (10). However, some studies have not confirmed these results (11,12). Lazar et al (8) reported that despite the aggressive presentation of DTC in prepubertal children, its course and outcome were similar to that of the pubertal group.

It has been advanced that PTC is a distinct disease not only in children and adults but also in prepubertal and pubertal children (7,8,9,10). $BRAF^{V600E}$ mutation in pediatric PTC might be important to explain the difference encountered among prepubertal, pubertal children, and adults. In our cohort, although frequency of $BRAF^{V600E}$ mutation is not so high, we did not find any association of $BRAF^{V600E}$ mutation with lymph node or lung metastases or extrathyroidal

**Table 4. Number of patients with papillary thyroid cancer per every 10 years after excluding patients with a history of radiotherapy**

| | 1983-1993 | 1994-2004 | 2005-2015 | p |
|---|---|---|---|---|
| **Patients (n, %)** | 5 (7.9%) | 7 (11.1%) | 51 (81%) | |
| **Age (years)** | 10.28 ± 2.3 | 9.26 ± 3.1 | 13.1 ± 3.4 | 0.006* |
| **Gender (n, %)** | | | | |
| Female | 3 (60%) | 2 (28.6%) | 39 (76.5%) | 0.031** |
| Male | 2 (40%) | 5 (71.4%) | 12 (23.5%) | |
| **Pubertal status (n, %)** | | | | |
| Prepubertal | 2 (40%) | 4 (57.1%) | 9 (17.6%) | 0.048** |
| Pubertal | 3 (60%) | 3 (42.9%) | 42 (82.4%) | |
| **Tumor size (n, %)** | | | | |
| ≤1 cm | 1 (20%) | 0 | 14 (27.5%) | 0.27** |
| >1 cm | 4 (80%) | 7 (100%) | 37 (72.5%) | |

*Kruskal-Wallis test, **Chi-square test

involvement, findings consistent with most pediatric reports (19,20,24,25). While some adult studies have shown more aggressive clinical behavior and worse prognosis for patients with $BRAF^{V600E}$ mutation, others have not (15,17,26,27). Meta-analyses in adults report a significant association of $BRAF^{V600E}$ with lymph node metastases, tumor size, and extrathyroidal extension (17,27). $BRAF^{V600E}$ mutation is a criterion for tumor aggressiveness in papillary microcarcinoma in some adult studies (28,29). However, Gouveia et al (26) demonstrated in a study of 429 adult patients with PTC that while 73.2% of patients had the $BRAF^{V600E}$ mutation, there was no association with $BRAF^{V600E}$ mutation and lymphovascular invasion, or extrathyroidal involvement. The prognostic value of $BRAF^{V600E}$ mutation is not clear in pediatric patients. Henke et al (24) reported a high $BRAF^{V600E}$ mutation rate (63%) in a pediatric population, but they did not find any correlation with $BRAF^{V600E}$ mutation and a more extensive or aggressive disease. Some other pediatric studies also reported no association between $BRAF^{V600E}$ mutation and the presence of an extensive disease (20,25,30). A need for large pediatric cohort studies is obvious.

Recent studies have shown that $BRAF^{V600E}$ mutations are common in adult PTC patients (29%–83%) (31,32). The prevalence of $BRAF^{V600E}$ mutation is variable in children ranging from 0% to 63% (19,24). We found $BRAF^{V600E}$ mutation in 25% of our PTC patients. We were not able to show any correlation of $BRAF^{V600E}$ mutation with age and pubertal status. There are controversial results on the correlation of BRAF mutation and age in the pediatric literature. Although it was reported that BRAF mutation increases with increasing age, our results are in line with other studies which demonstrated no correlation between BRAF and age (24,25,33).

The $BRAF^{V600E}$ mutation is most commonly found in classical PTC in adults (17,26,33,34). As in adult patients, $BRAF^{V600E}$ mutation in pediatric patients occurs more commonly in classical PTC than in non-classical subtypes (24,25). We also observed that $BRAF^{V600E}$ mutation occurred mostly in classical PTC patients. In our group, there was no sex difference with respect to $BRAF^{V600E}$ mutation. Henke et al (24) showed a male predominance in pediatric patients consistent with the findings on an adult population (17,35).

We found increasing numbers of cases of PTC diagnosed in the last 10 years at our unit after excluding patients with a history of radiotherapy. The reason for this increase in rate of PTC in pediatric patients, especially in the pubertal group, could be related to improved scrutiny for early diagnosis in recent years, since in our cohort 27.5% of the patients were diagnosed to have microcarcinoma within the last decade. Similarly, several studies have reported dramatic increases over recent decades in incidence of thyroid cancer, predominantly small papillary carcinomas (36,37,38). It was proposed that sudden changes in thyroid cancer incidence have resulted from large scale thyroid gland surveillance in high risk populations, improved diagnostic technology (ultrasonography, computed tomography, magnetic resonance imaging), and increased access to health care services (37,38).

In conclusion, our findings indicate that PTC is more disseminated in prepubertal children with an increased incidence of lateral neck lymph node involvement and lung metastases at presentation as compared with pubertal children. Extensive surgical treatment and repeated RAI treatment are required in prepubertal children. $BRAF^{V600E}$

mutation frequency was not high in our patients and was comparable to most other pediatric studies. We showed that $BRAF^{V600E}$ mutation was not correlated with a more extensive or aggressive disease process. Genetic factors other than $BRAF^{V600E}$ may also be involved in the expression of biological features and clinical behavior of PTC in prepubertal and pubertal children and adults.

### Ethics

**Ethics Committee Approval:** This study was approved by the local ethics committee.

**Informed Consent:** The medical records of patients were reviewed retrospectively.

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

### Authorship Contributions

Concept: Şükran Poyrazoğlu, Feyza Darendeliler, Firdevs Baş, Rüveyde Bundak, Yasemin Şanlı, Gülçin Yeğen, Design: Şükran Poyrazoğlu, Data Collection and Processing: Şükran Poyrazoğlu, Analysis and Interpretation: Şükran Poyrazoğlu, Feyza Darendeliler, Gülçin Yeğen, Yasemin Şanlı, Literature Research: Şükran Poyrazoğlu, Writing: Şükran Poyrazoğlu, Feyza Darendeliler, Gülçin Yeğen, Yasemin Şanlı.

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# Congenital Hyperinsulinism in China: A Review of Chinese Literature Over the Past 15 Years

**Wei-Yan Wang[1], Yi Sun[1], Wen-Ting Zhao[1], Tai Wu[1], Liang Wang[2], Tian-Ming Yuan[1], Hui-Min Yu[1]**

*[1]Zhejiang University School of Medicine, Children's Hospital, Clinic of Neonates, Hangzhou, China*
*[2]Zhejiang Cancer Hospital, Clinic of Chest Surgery, Hangzhou, China*

### What is already known on this topic?

Several studies have already summarized the clinical and genetic characteristics of congenital hyperinsulinism (CHI) in Beijing and Shanghai cities.

### What this study adds?

Studies throughout China are still scarce. Our article reviewed the clinical presentation, therapeutic outcomes, and genetic mutations of CHI in the Chinese population over the past 15 years and also compared the CHI in China with that in other countries.

## Abstract

**Objective:** Congenital hyperinsulinism (CHI) is a rare but severe cause of hypoglycemia. The present study investigates the clinical presentation, therapeutic outcomes and genetic mutations of CHI in Chinese individuals over the past 15 years.

**Methods:** The authors retrospectively reviewed one case in their department and 206 cases reported from January 2002 to October 2016 in China. PubMed, Ovid Medline, Springer and Wanfang Database, CBMD database, and CKNI database were the sources used to collect the data.

**Results:** In total, 207 cases were recruited. Of these, the ages of 100 (48.3%) were within the 4$^{th}$ week after birth. Seventy-seven cases (37.2%) were born large for gestational age (LGA). Seizures occurred in 140 cases (67.6%). Among 140 cases (67.6%) who were administered diazoxide treatment, 90 (64.3%) were responsive. Seven cases (3.4%) received octreotide treatment and 19 cases (9.2%) underwent surgery. 63/129 cases (48.8%) were detected to have gene mutations, including *ABCC8* (69.8%), *KCNJ11* (12.7%), *GLUD1, GCK, HADH*, and *HNF4A*. Among the diazoxide-unresponsive cases, gene mutations were detected in 20/36 (55.6%) cases with *ABCC8* and in 2 (5.6%) cases with *KCNJ11*. Among the diazoxide-responsive cases, gene mutations were detected in 8 patients with ABCC8, 4 with *KCNJ11*, 5 with *GLUD1*, and 1 with *GCK*.

**Conclusion:** The present study indicates that most CHI cases occurred in neonates and that 1/3 of the cases were born LGA. *ABCC8* and *KCNJ11* are the most common gene mutations. More than half of the diazoxide-unresponsive CHI detected mutations are in *ABCC8* and *KCNJ11* genes. The *GLUD1* gene mutations cause diazoxide-responsive CHI. Identifying the gene mutations can assist in the diagnosis and treatment of CHI.

**Keywords:** Congenital hyperinsulinism, neonate, clinical presentation, gene mutation

## Introduction

Congenital hyperinsulinism (CHI) is due to inappropriate insulin secretion in the course of hypoglycemia (1). It is the most common cause of severe and persistent hypoglycemia in newborns and infants. The incidence of CHI is reported to be 1/50.000 live births in a random mating population; however, it can be as high as 1/2500 in communities with high rates of consanguinity (2,3). The clinical presentation of CHI is heterogeneous and varies by age. The severity of hypoglycemia varies from asymptomatic hypoglycemia revealed by a routine blood glucose test to a state of serious hypoglycemic coma or seizures. Major clinical manifestations of CHI reported by other studies include

**Address for Correspondence:** Hui-Min Yu MD,
Zhejiang University School of Medicine, Children's Hospital, Clinic of Neonates, Hangzhou, China
**Phone:** +86 571 86670752 **E-mail:** 0087436@zju.edu.cn

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macrosomia, large for gestational age (LGA), seizures, cyanosis, food refusal, lethargy, hypoglycemia, and atypical facial appearance including high forehead with a thin upper lip. Although the clinical symptoms of CHI can reflect the severity to some extent, they serve little to aid the clinicians in the selection of regimen, which is usually correlated with the histopathology of CHI.

Histologically, there are focal and diffuse forms of conditions leading to CHI (4). Focal lesions, which account for approximately 40-50% of all cases, require a partial pancreatectomy. This form typically occurs during infancy rather than in older children. On the other hand, a similar number of cases present with a dysfunctional ATP sensitive potassium ($K_{ATP}$) channel involving the entire pancreas which leads to severe hypoglycemia, requiring a near total pancreatectomy (5). The clinical presentations and prognosis of patients with CHI depend primarily on the histopathology of the pancreas. Most patients with focal forms recover well after surgery, while some diffuse forms show persistent hypoglycemia even after surgery. Thus, it is necessary to differentiate the two forms. However, the clinical symptoms of CHI are non-specific, and thus, additional tools are essential in distinguishing the focal lesions from diffuse forms.

In recent years, the understanding of the genetic mechanisms of CHI has made progress. Mutations in 11 genes, including *ABCC8*, *KCNJ11*, *GLUD1*, *GCK*, *HADH*, *UCP2*, *SLC16A1 (MCT1)*, *HNF4A*, *HNF1A* (6), *HK1* (7), and *PGM1* (8) are known to cause CHI. Among them, the most common and most severe forms of CHI are speculated to be associated with the mutations of the $K_{ATP}$ channel genes (*ABCC8* and *KCNJ11*), encoding the sulfonylurea receptor 1 (*SUR1*) and *Kir6.2* subunits, respectively. Fournet and Junien (9) demonstrated that in about 50% of CHI cases, recessive mutations in the $K_{ATP}$ channel genes cause a diffuse pathology that necessitates a near total pancreatectomy, whereas the loss of heterozygosity together with the inheritance of a paternal mutation causes focal lesion that needs a partial pancreatectomy. Thus, the gene mutation type could assist in diagnosing, differentiating, and identifying the histological type of CHI and in treating the disease.

Though the disease is uncommon, the hypoglycemia caused by CHI can sometimes be extremely severe, frequently leading to severe neurological damage or even death in infancy. Therefore, it is important to diagnose and treat these infants with CHI at the earliest to alleviate the degree of brain damage. Several studies (10,11,12,13) have already summarized the clinical and genetic characteristics of CHI in Beijing and Shanghai; however, studies throughout China are yet lacking. In this article, we aimed to review the present knowledge on the clinical presentation, therapeutic outcomes, and genetic mutations of CHI in the Chinese population and to compare this knowledge with that in other countries.

## Methods

The search for case reports and case series on confirmed cases of CHI between January 2002 and October 2016 from PubMed, Ovid Medline, Springer and Wanfang Database, CBMD database, and CKNI database retrieved 19 case reports and case series published in core Chinese journals and 4 in journals publishing in English. Three series that summed up the earlier case reports, 4 case reports in Chinese, and 1 case in English were excluded to avoid overlapping. Thus, 12 articles in Chinese and 3 in English, encompassing 207 patients (including 1 case from our department) were included in the survey. The clinical presentation, therapeutic outcomes and genetic mutations of all these 207 CHI patients were analyzed.

A low level of fasting hypoglycemia (<2.8 mmol/L) requiring a high rate of IV glucose infusion (>8 mg/kg/min) to maintain a normal blood glucose level, presence of a detectable insulin level during hypoglycemia, a glycemic response to glucagon injection, undetectable fatty acid and ketone levels and a normal or increased serum ammonia level constituted the diagnostic criteria for CHI (14).

## Results

A total of 207 cases, 1 case from our own department together with the 206 cases of CHI reported previously from China (11,12,13,15,16,17,18,19,20,21,22,23,24,25,26) were available for analysis. Among them, 154 cases were from Beijing city, 32 from Shanghai city, 14 from Guangdong province, 4 from Zhejiang province, and 1 case each from Hunan, Sichuan, and Shandong provinces.

As shown in Table 1, of the 207 cases, 157 (75.8%) were less than 1-year-old. Among these, 100 cases (48.3%) were only 4 weeks old. The patients were males in 114 cases (55%), females in 93 cases (45%), and the male-to-female ratio was 1.23:1. The birth weight of the series ranged from 1.9 to 5.8 kg. Seizures occurred in 140 cases (67.6%), whereas other symptoms such as cyanosis, food refusal, and lethargy were reported in 67 cases (32.3%). Seventy-seven out of 207 (37.2%) cases were born LGA.

Blood tests showed that the blood glucose level of all cases was <2.8 mmol/L and the lowest glucose level reported was 0.3 mmol/L. High levels of insulin (2.4-220 μlU/mL) were observed in all patients. High levels of ammonia (17-

128 mmol/L) were detected among a subgroup of cases. Only 1 case was found with focal lesions in the head of pancreas using F-dihydroxyphenylalanine positron emission tomography/computed tomography (F-DOPA PET/CT).

In this series, of the 140 cases (67.6%) who were treated with oral diazoxide, 90 cases (64.3%) reached a normal blood glucose level before discharge from the hospital. Only 7 cases (3.4%) received octreotide treatment, and 4 of these 7 cases (57.1%) reached a normal blood glucose level. Nineteen cases (9.2%) underwent surgery, and in 14 of these 19 cases (73.7%), blood glucose reached a normal level.

One hundred-twenty nine cases underwent gene mutation test, and 63 of these cases (48.8%) showed mutations in *ABCC8, KCNJ11, GLUD1, GCK, HADH,* and *HNF4A* genes. Two of these cases were found to be homozygous for ABCC8 mutation (1 case unresponsive to diazoxide, and the other with unknown diazoxide response). Three cases were found

**Table 1. Clinical characteristics of congenital hyperinsulinism in Chinese patients**

| Characteristic | |
|---|---|
| Sex (Male:Female) | 114:93 (1.23:1) |
| **Age of onset** | |
| Neonate (0-4 weeks) | n = 100 (48.3%) |
| Infancy (1-12 months) | n = 57 (27.5%) |
| Childhood (>12 months) | n = 50 (24.2%) |
| Birth weight (range) | 1.9-5.8 kg |
| Macrosomia | n = 77 (37.2%) |
| **Complaints** | |
| Seizures | n = 140 (67.6%) |
| Other (cyanosis, food refusal, lethargy) | n = 67 (32.4%) |
| Blood glucose level (range) | 0.3-2.8 mmol/L |
| Blood ammonia level (range) | 17-128 mmol/L |
| Blood insulin level (range) | (2.4-220) µlU/mL |

**Table 2. Diazoxide-responsive congenital hyperinsulinism cases and gene mutations**

| Case | Sex | Birth weight (kg) | Disease onset | Gene | Mutation | Inherited from | |
|---|---|---|---|---|---|---|---|
| | | | | | | Father | Mother |
| 3 | Male | 4.4 | Neonate | *ABCC8* | c.331G>A;p.G111R* | p.G111R | Negative |
| 5 | Male | 3.2 | Infancy | *ABCC8* | c.1473T>G;p.Y491* | Negative | Negative |
| 12 | Male | 5.12 | Neonate | *ABCC8* | c.4478G>A;p.R1493Q** | p.R1493Q | Negative |
| 13 | Female | 3.15 | Infancy | *ABCC8* | c.4374G>C;p.Q1458H | NA | NA |
| 20 | Female | 4.2 | Neonate | *ABCC8* | c.3650G>A;p.R1217K* | Negative | p.R1217K |
| 21 | Female | 3.6 | Infancy | *ABCC8* | c.4478G>A;p.R1493Q** | R1493Q | Negative |
| 38 | Not mentioned | Not mentioned | Infancy | *ABCC8* | c.3832G>A;p.G1255S | NA | NA |
| 41 | Female | 4.8 | Neonate | *ABCC8* | c.4613G>A;p.R1538Q | NA | NA |
| 42 | Female | 3.2 | Infancy | *KCNJ11* | c.881C>T; p.T294M | p.T294M | Negative |
| 43 | Female | 3.2 | Neonate | *KCNJ11* | c.407G>T; p.R136L | NA | NA |
| 45 | Male | 4.415 | Neonate | *KCNJ11* | c.146T>A;p.I49N | NA | NA |
| 48 | Not mentioned | Not mentioned | Neonate | *KCNJ11* | c.91 G>A;p.R31W | NA | NA |
| 50 | Female | 3.35 | Infancy | *GLUD1* | c.820C>T; R221C | NA | NA |
| 52 | Male | 3.2 | Infancy | *GLUD1* | c.1516G>A; V453M | NA | NA |
| 53 | Male | 3.6 | Infancy | *GLUD1* | c.978G>A;R269H* | Negative | Negative |
| 54 | Male | 3.5 | Infancy | *GLUD1* | c.1506C>T;S445L | Negative | Negative |
| 55 | Male | 3.2 | Infancy | *GLUD1* | c.978G>A;R269H | R269H | Negative |
| 57 | Male | 5 | >1 year | *GCK* | c.295C>T;p.W99R | Negative | Negative |
| 59 | Female | 3.35 | Infancy | *HNF4A* | c.*7G>A; Unkown | NA | NA |
| 60 | Female | 4 | Neonate | *HNF4A* | c.416C>T;p.T139I | NA | NA |

✶ Means the gene mutation appears twice in the study

✦ Means the gene mutation appears three times in the study

Negative means negative result of gene mutation test

NA means gene mutation test is not available

" Means the case was from the authors' department of neonate

* Means missense mutation altering protein function

to be compound heterozygous for ABCC8 mutation (2 cases diazoxide-responsive, 1 case with unknown diazoxide response), 15 cases were found to have heterozygous mutation in *ABCC8* gene (9 cases diazoxide-unresponsive, 4 cases diazoxide-responsive, and 2 cases with unknown diazoxide responsiveness). Two cases were found to possess heterozygous mutation in *KCNJ11* gene (1 case diazoxide-unresponsive, the other one diazoxide-responsive); 1 case was found to have heterozygous mutation in *GLUD1* gene (diazoxide-responsive), 8 cases had de novo mutations (5 *ABCC8*, 2 *GLUD1*, 1 *GCK*), and 32 cases were not analyzed for the parents' gene mutation test (Figure 1).

In this series, 63 cases were detected to possess 60 gene mutations, including 39 mutations in *ABCC8* gene, 8 in *KCNJ11*, 6 in *GLUD1*, 2 in *GCK*, 3 in *HADH,* and 2 mutations in *HNF4A* gene. Mutations in *ABCC8* gene were detected in 8 cases; one mutation in *KCNJ11* gene and one in *GLUD1* gene appeared twice.

We recruited 90 cases who went through gene mutation test and received diazoxide treatment. Among them, 54 cases were responsive to diazoxide and 20 were detected to have positive gene mutations. These mutations were as follows: in *ABCC8* gene-*8* cases, in *KCNJ11-4*, in *GLUD1-5*, in *HNF4A-2*, and in *GCK* gene-1 case (Table 2). The other 36 cases were unresponsive to diazoxide treatment, and 22 cases were detected to have positive gene mutation. These gene mutations were in *ABCC8* gene in 20/36 cases (55.6%) and in *KCNJ11*gene in 2 cases (5.6%) (Table 3).

## Discussion

Most of the patients in the series were the offspring of families who resided in the eastern part of China. The vast majority of CHI cases were from Beijing and Shanghai cities and Guangdong and Zhejiang provinces, though a small number of cases were also reported from other regions such as Hunan, Sichuan, and Shandong provinces. To our knowledge, this is the first report involving the distribution of CHI. We speculated that this geographic disequilibrium was partially due to the disequilibrium of the economy and to missed cases due to ignorance and misdiagnosis of this disease in different areas of China related to social, economic factors. Possibly, the medical personnel in those areas did not realize the significance of identifying the cause of persistent hypoglycemia and did not attempt to investigate the gene mutation status in these patients.

In our study, 48.3% of cases were found to present clinical manifestation of CHI within 4 weeks after birth. This finding was similar to the results of a Taiwanese study with 46% of cases with neonatal onset (27). Our study also demonstrates that macrosomia can often be observed among the patients, and LGA accounts for 37.2%. We speculate that the underlying reason for macrosomia should be ascribed to be the severe prenatal hyperinsulinism. A study comprising 114 patients (28) revealed that 27% of patients with neonatal-onset CHI had a birth-weight SDS of >2. Shen et al (22) reported 15 neonatal cases of CHI, of which 10 cases were LGA and blood glucose level returned to normal in only 3 patients in this series. Thus, we propose that an early-onset

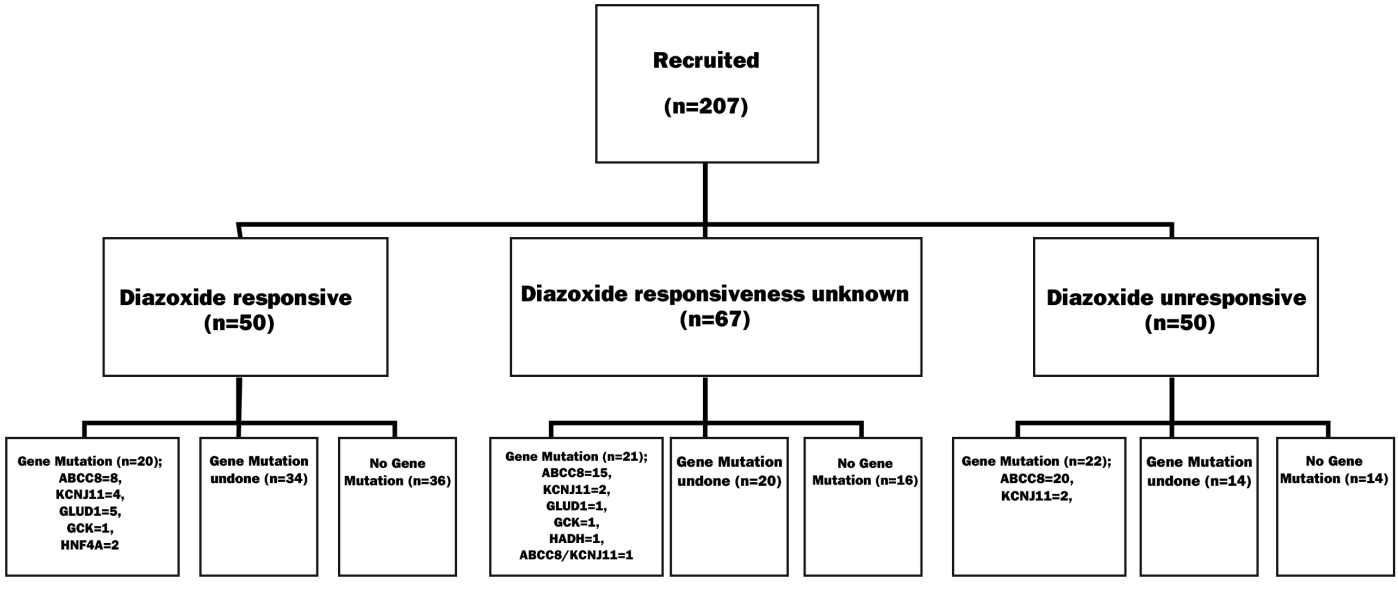


**Figure 1.** Gene mutation distribution of 63 cases detected in 129 cases

**Table 3. Diazoxide-unresponsive congenital hyperinsulinism cases and gene mutations**

| Case | Sex | Birth weight (kg) | Disease onset | Gene | Mutation | Inherited from | |
|---|---|---|---|---|---|---|---|
| | | | | | | Father | Mother |
| 6 | Male | 4.4 | Neonate | *ABCC8* | c.3000C > A;p.C1000*✶ | p.C1000* | Negative |
| 7 | Male | 4.6 | Neonate | *ABCC8* | c.4661G > A;p.G1554D; c.1501G > A;p.E501K | p.G1554D | p.E501K |
| 8 | Male | 3.7 | Neonate | *ABCC8* | c.3888G > A;p.W1296* | p.W1296* | Negative |
| 9 | Male | 3.4 | Infancy | *ABCC8* | c.86A > G;p.D29G | NA | NA |
| 10 | Male | 2.52 | Neonate | *ABCC8* | c.331G > A;p.G111R✶ | NA | NA |
| 11 | Female | – | Infancy | *ABCC8* | c.863G > A;p.W288*✶; c.2506C > T; p.R836*✶ | p.W288* | p.R836* |
| 14 | Female | 3.6 | Neonate | *ABCC8* | c.1792C > T;p.R598*✶ | p.R598* | Negative |
| 15 | Female | 3.6 | Neonate | *ABCC8* | c.276_277insCATC;p.Ile93Hisfs*3 | p.Ile93Hisfs*3 | Negative |
| 16 | Female | 4.6 | Neonate | *ABCC8* | c.4478G > A;p.R1493Q✶ | p.R1493Q | Negative |
| 17 | Female | 4.7 | Neonate | *ABCC8* | c.1887delc; P629PfsX17 | P629PfsX17 | Negative |
| 18 | Male | 4.4 | Neonate | *ABCC8* | c.863G > A;p.W288*✶ | p.W288* | Negative |
| 19 | Female | 4.2 | Infancy | *ABCC8* | c.1919C > T;A640V;c.3586C > T;p.Q1196* | p.Q1196* | Negative |
| 23 | Male | 3.4 | Infancy | *ABCC8* | c.3000C > A;p.C1000*✶ | p.C1000* | Negative |
| 24 | Female | 5.28 | Neonate | *ABCC8* | c.4513G > C; D1505H | Negative | Negative |
| 25 | Female | 4.5 | Neonate | *ABCC8* | c.1421A > G; Q474R | Negative | Negative |
| 26 | Female | 4.05 | Neonate | *ABCC8* | c.1792C > T;p.R598*✶ | Negative | Negative |
| 37 | Male | 3.9 | Neonate | *ABCC8* | c.2990G > A;p.W997* | NA | NA |
| 39 | Not mentioned | Not mentioned | Infancy | *ABCC8* | c.1861C > T;p.R598X | NA | NA |
| 40" | Male | 5.8 | Neonate | *ABCC8* | c.2797C > T;p.R933*✦ | p.R933* | p.R933* |
| 44 | Male | 4.2 | Infancy | *KCNJ11* | c.703C > G; Q235E | Q235E | Negative |
| 49 | Not mentioned | Not mentioned | Neonate | *KCNJ11* | c.101 C > T;p.R34H | NA | NA |
| 61 | Male | 4.25 | Neonate | *ABCC8/ HADH* | c.2506C > T;p. R836*✶;c.2797C > T;p. R933*✦/c.719G > T;p.T240M | NA | NA |

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✦ Means the gene mutation appears three times in the study

Negative means negative result of gene mutation test

NA means gene mutation test is not available

"Means the case was from the authors' department of neonate

*Means missense mutation altering protein function

age of CHI together with a high birth weight are indicators of severity of this condition.

In addition, our findings demonstrate that seizures frequently occurred in CHI, as reported by Ferry et al (29) and that cyanosis, food refusal, lethargy, and hypoglycemia are also features of CHI. The clinical presentation is nonspecific, and the conventional ultrasound scan, computed tomography, and magnetic resonance imaging could not detect any lesion.

The findings of this study show that diazoxide is the first-line treatment in this condition. The positive response rate (64.3 %) to diazoxide in our sample is in accordance with that of 66 % in 175 cases of CHI reported by de Lonlay et al (30). We recruited only 7 cases receiving octreotide treatment, 4 of these cases were responsive. Although octreotide treatment could be used in diazoxide-unresponsive cases, it could cause cholestasis and hepatitis (31,32). Elevated levels of liver enzymes and gallbladder pathology were detected in the patients treated. Octreotide treatment may also impose an enormous economic burden on the family, thereby, limiting its clinical usage.

Surgery is the last option for drug-resistant CHI patients. In our review, 19 such cases underwent the operation and 14 of these cases were restored to normal glucose level after

surgery. However, since F-DOPA PET/CT is not available in Mainland China, most patients are obliged to undergo a subtotal pancreatectomy and endure the risk of becoming a diabetic as a side-effect.

Nevertheless, now that gene mutation test is available, several researchers are focusing on the relationship between diazoxide treatment and gene mutation type. Previously, the reasons for the differences in responsiveness to diazoxide treatment were unknown and were ascribed by some researchers to different histological patterns of CHI (33). Snider et al (6) reported that 91 % of diazoxide-unresponsive cases were correlated with recessive KATP channel gene mutations, while 41 % of diazoxide-responsive cases were correlated with dominant $K_{ATP}$ mutations.

In our study, 129 cases underwent gene mutation test and 48.8 % of these cases were detected to have a gene mutation, which is a much lower frequency than that reported by Park et al (33). These authors reported a frequency of 82 % as mutation findings in a Korean population. However, our results are similar to those of other researchers who reported a frequency of 45.3 % mutations in CHI patients (34). These differences in frequency may be partially due to differences in ethnicity-related genetics. Moreover, Park et al (33) recruited only 17 patients, and thus, a large-scale cohort is essential to illustrate the frequency in a population. Among the 63 cases of gene mutation detected in our series, the $K_{ATP}$ channel gene mutations (*ABCC8* or *KCNJ11*) account for 82.5 % of the gene mutations; this result is similar to the 84.2 % frequency reported by Yorifuji et al (34) and 80.1 % by Kapoor et al (35).

We detected 60 gene mutations out of 63 cases in the Chinese population, with 8 *ABCC8*, 1 *KCNJ11,* and 1 *GLUD1* gene mutations appearing twice, and 2 *ABCC8* gene mutations appearing three times. This is the first report to sum up the occurrence rate of gene mutations in a population and we hope these findings will facilitate the clinicians' work in CHI cases.

Our results indicate that all *GLUD1* gene mutations contribute to diazoxide responsiveness. This finding is in accordance with the study by Snider et al (6). Some cases with a heterozygous *ABCC8*/*KCNJ11* mutation are also diazoxide-responsive, which is similar to the result of Kapoor et al (35). *GCK* mutation may also be attributed to diazoxide-responsive CHI; in most cases it is medically responsive, although in some cases, surgery may be required (36). Two *HNF4A* gene mutation cases were diazoxide-responsive in our study; Kapoor et al (37) also elucidated that CHI patients with *HNF4A* can exhibit mild, transient to severe, persistent hypoglycemia and are diazoxide-responsive.

We also demonstrated that the cases with compound heterozygous recessively acting *ABCC8/KCNJ11* mutations, or homozygous *ABCC8/KCNJ11* recessively acting mutation, or some cases with a heterozygous *ABCC8/KCNJ11* mutation may show diazoxide-unresponsiveness, which is in accordance with the results of Kapoor et al (35).

In our study, one *ABCC8* gene mutation (*c.331G > A; p.G111R*) occurred twice, but one case was responsive to diazoxide treatment, while the other was unresponsive. The reason for this difference in response is unknown, but we see that the responsive case had a paternally inherited heterozygous mutation, while a gene mutation test was not performed in the parents of the unresponsive case. It can be speculated that the unresponsive case may have had a paternally inherited mutation and loss of heterozygosity that caused focal CHI.

Nowadays, the gene mutation test can be conducted easily with rapid results in some developed countries. However, in China, the cost of gene mutation test is high and it takes a long time to obtain the results. One of the aims of this study was to facilitate the diagnosis and treatment of CHI by the clinician in China, where F-DOPA PET/CT is not available. Some gene mutations appearing two or three times may indicate their frequent occurrence in the Chinese population.

In conclusion, our study suggests that nearly half of CHI cases occur in neonates, and the most common symptom is seizures. The first-line treatment of CHI is diazoxide treatment, octreotide is not used often, and surgery is the option due to drug unresponsiveness. F-DOPA PET/CT is not available in Mainland China. In Chinese patients, *ABCC8* and *KCNJ11* are the most common gene mutations, and *GLUD1* ranks second. Half of the gene mutations of diazoxide-unresponsive CHI are *KATP* (*ABCC8* and *KCNJ11*) mutations, homozygous or compound heterozygous mutations, and some are a heterozygous *ABCC8* mutation. *GLUD1* always caused diazoxide-responsive CHI.

CHI is a complex disorder with non-specific presentations. Our study suggests that gene mutation test is now performed more frequently in China, although not as often as in other developed countries. Obtaining rapid results in genetic testing is uniquely valuable for CHI patients in China.

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### Ethics

**Ethics Committee Approval:** No ethics approval was required.

**Informed Consent:** No informed consent was required.

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### Authorship Contributions

Concept: Hui-Min Yu, Design: Tian-Ming Yuan, Data Collection and Processing: Wen-Ting Zhao, Analysis and Interpretation: Tai Wu, Literature Research: Yi Sun, Liang Wang, Writing: Wei-Yan Wang.

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# The Relationship Between Glycemic Variability and Inflammatory Markers in Obese Children with Insulin Resistance and Metabolic Syndrome

**Abdurrahman Kaya[1], Cemil Koçyiğit[2], Gönül Çatlı[2], Elif Büşra Özkan[3], Bumin Nuri Dündar[2]**

*[1]Tepecik Training and Research Hospital, Clinic of Pediatrics, İzmir, Turkey*
*[2]İzmir Katip Çelebi University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey*
*[3]İzmir Katip Çelebi University Faculty of Medicine, İzmir, Turkey*

### What is already known on this topic?

Glycemic variability describes the fluctuations in blood glucose and it is associated with vascular complications and mortality in patients with metabolic syndrome and diabetes mellitus.

### What this study adds?

Glycemic variability is not different among obese adolescents with insulin resistance and metabolic syndrome. Elevated interleukin-6 levels and metabolic syndrome diagnostic criteria such as hypertension and dyslipidemia do not cause further increase in glycemic variability.

## Abstract

**Objective:** Increased glycemic variability (GV) is associated with increased oxidative stress, vascular complications, and mortality in metabolic syndrome (MS) and diabetes mellitus patients. To investigate the relationship between GV and inflammatory parameters in obese children with insulin resistance (IR) and to elucidate their effects on the development of MS.

**Methods:** Fifty obese adolescents with IR were included in the study. All patients underwent anthropometric measurements, body fat analysis, and continuous glucose monitoring system (CGMS) for 24 hours. Serum lipids, adiponectin, and interleukin-6 (IL-6) levels were measured. GV coefficient (GVC) was calculated using the standard deviation and the average glucose value obtained by CGMS. IR was diagnosed according to the results of oral glucose tolerance test (OGTT). MS was diagnosed according to the modified World Health Organization and the International Diabetes Federation criteria.

**Results:** Twenty-seven of the patients had MS and the remaining had only IR. Body fat mass, HbA1c, IL-6 levels, and peak insulin levels in the OGTT were significantly higher in the group with MS, but there was no difference in adiponectin levels. GVC was not different between the groups, but GVC significantly positively correlated with homeostasis model of assessment for IR, as well as with fasting, peak, and total insulin levels when all the patients were analyzed, while no significant relation was detected with adiponectin and IL-6 levels.

**Conclusion:** This study suggests that GV is not different among obese adolescents with IR and MS. There seems to be a significant association between GV and IR parameters. However, other diagnostic criteria of MS (hypertension and/or dyslipidemia) or elevated IL-6 levels does not cause further increase in GV.

**Keywords:** Glycemic variability, metabolic syndrome, interleukin-6, adiponectin

**Address for Correspondence:** Bumin Nuri Dündar MD,
İzmir Katip Çelebi University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
**Phone:** +90 232 469 69 69 **E-mail:** bumindundar@gmail.com **ORCID ID:** orcid.org/0000-0002-7506-061X

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## Introduction

Glycemic variability (GV) describes the fluctuations in blood glucose levels throughout the day. Regardless of the average blood glucose concentration, increased GV has been shown to increase oxidative stress and cause endothelial dysfunction. It is also associated with vascular complications and mortality in patients with metabolic syndrome (MS) and diabetes mellitus (DM) (1). The fluctuations in blood glucose concentration have been reported to adversely affect endothelial function even in non-diabetic individuals (2). Furthermore, people who develop diabetes have an increased cardiovascular risk even before the appearance of diabetes and subjects with MS are at high risk for both cardiovascular events and diabetes (3). MS is associated with increased circulating levels of proinflammatory cytokines such as interleukin-6 (IL-6) and decreased anti-inflammatory factors such as adiponectin, which are both able to influence insulin sensitivity and endothelial dysfunction (4,5). Although there are studies investigating the relationship of GV with endothelial dysfunction and DM complications in adults with MS, studies in children are limited to type 1 diabetes. There are no reports investigating the relationship between GV and inflammatory markers in childhood obesity.

In this study, we aimed to investigate the relationship between GV by continuous glucose monitoring system (CGMS) and inflammatory parameters in obese children with insulin resistance (IR) and to elucidate their effects on the development of MS.

## Methods

This study was conducted at İzmir Tepecik Training and Research Hospital in patients attending the Pediatric Endocrinology outpatient clinic between November 2014 and May 2015. The study protocol was approved by the Ethics Committee of İzmir Katip Çelebi University Faculty of Medicine. Written consent was obtained from all subjects and their parents before the study. The criteria defined by the World Health Organization and International Diabetes Federation were used in the diagnosis of MS (6,7). Patients who met both criteria were accepted as MS.

Fifty patients 10-18 years of age with obesity and IR were enrolled in the study. All subjects underwent a detailed physical examination including evaluation for syndromes and endocrine diseases as well as a laboratory evaluation including thyroid function tests and diurnal cortisol levels. Participants with syndromic obesity, endocrine disorders accompanied by obesity, a history of drug use (glucocorticoid, antipsychotics, etc.), and metabolic, cardiovascular, respiratory or hepatic disease were excluded.

Anthropometric measurements [height, body weight, waist circumference (WC)] were performed by the same person using the same tools and the results were recorded. The height and weight of each participant were measured while participants were wearing a light robe and no shoes. Subjects with a body mass index (BMI) value greater or equal to the 95$^{th}$ percentile according to the age and gender were considered as obese. The BMI percentile and BMI standard deviation (SD) were evaluated using the reference values developed by Bundak et al (8). WC was measured at the narrowest point between the lower border of the rib cage and the iliac crest. WC was evaluated using the percentile curves of healthy Turkish children (9). BMI was calculated by dividing weight by height in meters squared (kg/m$^2$). Blood pressure was measured twice from the right brachial artery in a sitting position following a 10-minute rest. The average of these two measurements was recorded. Children with systolic and/or diastolic blood pressure greater than the 95$^{th}$ percentile (adjusted for height, age, and sex) were considered to have hypertension (10). Body composition of patients, i.e. body fat percentage (%), fat mass (FM), fat-free mass, and muscle mass, was analyzed using the bioelectrical impedance device (TBF-310GS$^{TM}$, Tanita, Tokyo, Japonya).

Following overnight fasting, the blood samples were collected for estimation of biochemical parameters such as glucose level, lipid profile [triglyceride, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol levels] and insulin levels. An oral glucose tolerance test (OGTT) was performed in all patients. Blood glucose and insulin concentrations were measured before and at 30, 60, 90, and 120 minutes after the consumption of a glucose load in a dose of 1.75 g per kilogram of body weight (up to a maximum of 75 g of glucose). The homeostasis model of assessment for IR (HOMA-IR) index was implemented using the following equation: [fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5]. Impaired fasting glucose (fasting glucose 100-125 mg/dL), impaired glucose tolerance (2-h glucose 140-199 mg/dL), and diabetes (fasting glucose ≥126 mg/dL or 2-h glucose ≥200 mg/dL) were defined by glucose levels obtained during the OGTT according to the American Diabetes Association guidelines (11). IR was considered if peak insulin, 2-h insulin, and total insulin values obtained by OGTT were greater than 150 (μU/mL), 75 (μU/mL), and 300 (μU/mL), respectively (12). For serum adiponectin and IL-6 levels, 8-10 mL of venous blood were taken from all patients. Following centrifugation at 3000 rpm for 10 min in sterile conditions, the serum samples were stored in clean and dry Eppendorf tubes in the freezer at -20 °C until

analyzed. The ELISA method was used for measurements of plasma adiponectin (Ebioscience, Vienna, Austria reference no: BMS2032 lot no: 102162008) and IL-6 levels (Ebioscience, Vienna, Austria, reference no: BMS213/2, lot No: 101174060).

For about 24 hours, all patients underwent CGMS by means of a microdialytic system (Guardian® REAL-Time CGMS and Sof-Sensor®, Medtronic MiniMed, Northridge, CA). In this system, a semipermeable microdialytic fiber is placed in the subcutaneous adipose tissue of the abdominal wall as a catheter guide. Hence, the membrane and interstitial space are in contact. Serum concentrations of glucose are roughly similar to those of the interstitial fluids. By using a biosensor based on the glucose-oxidase reaction, the glucose concentration in the device is measured. This device, which can be used in an outpatient basis, allows for the recording of daily routine activities. The registered data at the end of the test are downloaded and analyzed using software and are displayed in a final report showing the glycemic values recorded every 5 minutes. The mean 24-hour glycemia, its SD, and GV coefficient [GVC% = (SD/mean) x 100] were calculated for each CGMS test. The GVC % was assumed to represent GV.

### Statistical Analysis

Statistical analysis was performed using SPPS 21.0 (SPSS Inc., Chicago, IL, USA) software.

All data were given as mean ± SD values. The chi-square test was used to compare the frequency of the data. Homogeneity of the data was assessed using the Kolmogorov-Smirnov test. Differences in the means between the two groups were tested using the student's t-test for data with normal distribution and the Mann-Whitney U test for data without normal distribution. Correlations were expressed by the Pearson's or Spearman correlation coefficient according to data distribution. The results were expressed with a 95% confidence interval, and a p-value of less than 0.05 was considered statistically significant.

## Results

A total of 50 patients (female/male: 31/19) with obesity and IR were included in the study. MS was diagnosed in 27 patients. The remaining patients had only IR without MS. Table 1 shows the clinical and laboratory characteristics of the groups. There was no difference between WC, BMI, total cholesterol, LDL-C, and adiponectin levels in the groups. Body FM, systolic and diastolic blood pressure, triglycerides, peak insulin in the OGTT, glycated hemoglobin (HbA1c), HOMA-IR, and IL-6 levels were significantly higher in the group with MS. WC, BMI, body fat FM, and triglyceride levels were negatively correlated with adiponectin level. Body weight, HbA1c, and triglyceride levels were positively correlated with IL-6. The results of the OGTT and 24-h continuous glucose monitoring in the patients with or without MS are shown in Table 2. GV was not different among the groups. Fasting insulin, 2-h insulin, peak insulin, total insulin, 2-h glucose levels in the OGTT, and HOMA-IR were positively correlated with GV, while no significant

**Table 1. Clinical and laboratory characteristics of patients with or without metabolic syndrome**

| Variables | MS (-) (n = 23) (46%) | MS (+) (n = 27) (54%) | p |
|---|---|---|---|
| Males/females % | 47.8/52.2 | 29.6/70.4 | **0.045** |
| Age (years) | 13.9 ± 2.3 | 13.9 ± 2.3 | 0.947 |
| Systolic blood pressure (mmHg) | 117.0 ± 9.1 | 134.9 ± 11.4 | **0.001** |
| Diastolic blood pressure (mmHg) | 72.7 ± 6.8 | 86.1 ± 10.0 | **0.001** |
| **Anthropometric measurements** | | | |
| Body weight (SDS) | 4.7 ± 1.8 | 5.4 ± 2.1 | 0.131 |
| BMI (percentiles) | 99.7 ± 0.3 | 99.8 ± 0.2 | 0.090 |
| Waist circumference (cm) | 104.3 ± 9.0 | 108.4 ± 11.5 | 0.167 |
| Fat % | 39.7 ± 7.4 | 42.6 ± 6.1 | 0.147 |
| Fat mass (kg) | 34.3 ± 10.2 | 40.8 ± 11.2 | **0.044** |
| **Biochemical parameters** | | | |
| Total cholesterol (mg/dL) | 169.5 ± 36.8 | 167.1 ± 41.8 | 0.835 |
| Triglycerides (mg/dL) | 97.7 ± 27.7 | 148.7 ± 73.7 | **0.002** |
| HDL cholesterol (mg/dL) | 48.2 ± 9.8 | 38.7 ± 6.2 | **0.001** |
| LDL cholesterol (mg/dL) | 101.8 ± 31.9 | 98.7 ± 38.5 | 0.752 |
| Uric acid (mg/dL) | 5.4 ± 1.0 | 5.9 ± 1.2 | 0.221 |
| ALT (mg/dL) | 34.9 ± 34.4 | 24.4 ± 12.6 | 0.176 |
| AST (mg/dL) | 28.3 ± 14.3 | 25.7 ± 8.4 | 0.455 |
| HbA1c (%) | 5.2 ± 0.2 | 5.5 ± 0.3 | **0.020** |
| IL-6 (pg/mL) | 3.0 ± 10.2 | 4.7 ± 6.5 | **0.002** |
| Adiponectin (ng/mL) | 12810 ± 8675 | 8992 ± 3976 | 0.069 |

SDS: standard deviation score, MS: metabolic syndrome, BMI: body mass index, HDL: high-density lipoprotein, LDL: low-density lipoprotein, AST: aspartate transaminase, ALT: alanine transaminase, IL-6: interleukin-6, HbA1c: glycated hemoglobin

**Table 2. Oral glucose tolerance test and 24-h continuous glucose monitoring in patients with or without metabolic syndrome**

| OGTT | MS (-) (n = 23) (46%) | MS ( + ) (n = 27) (54%) | p |
|---|---|---|---|
| **Plasma glucose (mg/dL)** | | | |
| Basal | 88.3 ± 7.4 | 91.3 ± 8.8 | 0.208 |
| 2 h post-load | 106.6 ± 16.8 | 112.4 ± 25.0 | 0.343 |
| **Plasma insulin (mIU/L)** | | | |
| Basal insulin | 26.1 ± 14.9 | 32.6 ± 16.4 | 0.089 |
| 2 h post-load | 82.4 ± 66.5 | 123,2 ± 115.4 | 0.231 |
| Peak insulin | 138.1 ± 58.1 | 204.7 ± 115.2 | **0.030** |
| Total insulin | 418.1 ± 208.6 | 604.7 ± 413.3 | 0.058 |
| HOMA-IR | 5.8 ± 3.9 | 6.9 ± 3.1 | **0.044** |
| **24-h CGM** | | | |
| Mean glycemia | 92.2 ± 15.9 | 91.9 ± 18.8 | 0.952 |
| Mean GV (%) | 18.5 ± 7.3 | 19.1 ± 8.33 | 0.802 |

MS: metabolic syndrome, OGTT: oral glucose tolerance test, HOMA-IR: homeostatic model assessment-insulin resistance, CGM: continuous glucose monitoring, GV: glycemic variability

relation was detected with adiponectin and IL-6 levels (Table 3). Impaired fasting glucose and impaired glucose tolerance were detected in 7 and 6 patients, respectively when OGTT results were evaluated. Serum glucose levels during the day were found to exceed 200 mg/dL in 2/7 of patients with impaired fasting glucose and 1/6 of patients with impaired glucose tolerance according to CGMS data. The HbA1c values of these three patients were not different from the average (4.9%, 5.4%, and 5.7%).

## Discussion

Adipokines secreted by adipose tissue and some cytokines are known to influence insulin sensitivity directly or indirectly by modulating insulin signal, glucose and lipid metabolism (4). In the literature, there are studies investigating the role of cytokines in obesity, diabetes, and MS and analyzing the efficacy of anti-inflammatory and inflammatory markers on endothelial dysfunction. Makni et al (5) reported that IL-6 levels are greater in children with MS than in children with IR only. Another study has found lower adiponectin levels in children diagnosed with MS compared with obese children without MS. In our study, IL-6 levels were significantly higher in the MS group compared to the other group. Although adiponectin levels were lower in the MS group, this difference was not statistically significant. These findings support the hypothesis that inflammation plays a role in the development of MS. Garanty-Bogacka et al (13) reported a significant reduction in IL-6 levels in obese children after an average weight reduction of 5.3 kg with a low-calorie diet for 6 months. IL-6 levels were significantly higher in the high HbA1c group than the low HbA1c group in a study in which type 2 DM diagnosed cases were divided into two groups according to HbA1c levels (14). We found a positive correlation between IL-6 levels and HbA1c in our study. These findings suggest that inflammation plays an important role in the increase in glycosylated hemoglobin levels and in the development of MS complications such as atherosclerosis, hypertension, and type 2 DM. In our study, a negative correlation was found between adiponectin levels and the parameters of obesity (waist circumference, BMI, FM) which were consistent with other studies (15,16). This result supports the studies which has reported that adiponectin strongly associates with obesity.

GV has been reported to contribute to the development of subclinical atherosclerosis, which leads to endothelial dysfunction by inducing oxidative damage (3). It is believed that oxidative damage occurs due to the excessive amounts of reactive oxygen species caused by fluctuations in blood glucose. Recent studies have shown that fluctuations in blood glucose levels have a worse effect on endothelial function compared to chronic hyperglycemia in both diabetic and non-diabetic individuals (2,3). There are studies in the literature investigating the relationship of GV with endothelial dysfunction and diabetes complications, but these studies are usually conducted in adult patients. There are no reports in the literature investigating the relationship of GV with MS and inflammatory markers in childhood obesity. Buscemi et al (3) reported that GV was higher in patients with type 2 DM compared to other obese individuals without diabetes and there was no difference found between adults with or without MS. In the same study, GV was found to positively correlate with WC, BMI, fasting insulin levels, HOMA-IR, and IL-6 levels. We did not find significant differences between the groups in terms of GV, however, GV positively correlated with BMI, IR parameters (HOMA-IR, fasting insulin, total and peak insulin levels in OGTT), and 2-h glucose in our study. Despite this positive association between GV and IR parameters, we did not find any significant relationship between GV and serum lipids and/or blood pressure. These results suggest that IR increases the fluctuations in blood glucose levels during the day. However, there is no evidence that dyslipidemia or hypertension increases GV in MS patients.

There is a limitation of this study that needs to be addressed. We did not have a healthy control group or a group of obese

**Table 3. Correlations of clinical and laboratory variables with adiponectin, interleukin-6, and mean glycemic variability**

| Parameters | Mean GV (%) | | Adiponectin (ng/mL) | | IL-6 (pg/mL | |
|---|---|---|---|---|---|---|
| | r | p | r | p | r | p |
| Basal insulin (μIU/mL) | 0.303 | 0.032 | -0.274 | 0.054 | 0.098 | 0.499 |
| Prandial (2 h post-load) insulin (μIU/mL) | 0.315 | 0.026 | -0.159 | 0,271 | 0.084 | 0.563 |
| Peak insulin (μIU/mL) | 0.280 | 0.049 | -0.226 | 0.115 | 0.195 | 0.174 |
| Total insulin (μIU/mL) | 0.291 | 0.041 | -0.247 | 0.084 | 0.170 | 0.237 |
| HOMA-IR | 0.303 | 0.032 | -0.265 | 0.063 | 0.130 | 0.368 |
| Fasting glucose (mg/dL) | 0.189 | 0.189 | 0.143 | 0.323 | 0.017 | 0.907 |
| Prandial glucose (2 h post-load) (mg/dL) | 0.281 | 0.048 | 0.023 | 0.875 | 0.005 | 0.971 |
| HbA1c (%) | -0.254 | 0.075 | 0.116 | 0.424 | 0.390 | 0.005 |
| Triglycerides (mg/dL) | 0.190 | 0.186 | -0.359 | 0.010 | 0.353 | 0.012 |
| Total cholesterol (mg/dL) | -.0.151 | 0.297 | 0.229 | 0.110 | -0.100 | 0.490 |
| HDL-C | -.0.179 | 0.213 | 0.428 | 0.002 | -0.271 | 0.057 |
| LDL-C (mg/dL) | -0.189 | 0.188 | 0.264 | 0.064 | -0.136 | 0.348 |
| Adiponectin (ng/mL) | -.0128 | 0.375 | - | - | -0.199 | 0.165 |
| IL-6 (pg/mL) | -.0.120 | 0.406 | -0.199 | 0.165 | - | - |
| Mean GV (%) | - | - | -0.099 | 0.495 | 0.086 | 0.556 |
| Fat mass (kg) | 0.079 | 0.585 | -0.425 | 0.002 | 0.260 | 0.068 |
| WC (cm) | 0.029 | 0.843 | -0.432 | 0.002 | 0.215 | 0.134 |
| Body weight (SDS) | 0.141 | 0.329 | -0.207 | 0.150 | 0.415 | 0.003 |
| BMI (percentiles) | 0.114 | 0.432 | -0.316 | 0.025 | 0.125 | 0.387 |
| BMI (kg/m$^2$) | 0.108 | 0.456 | -0.316 | 0.025 | 0.230 | 0.109 |
| Systolic pressure (mm/Hg) | -0.135 | 0.351 | -0.321 | 0.023 | 0.343 | 0.015 |
| Diastolic pressure (mm/Hg) | 0.002 | 0.986 | -0.380 | 0.007 | 0.218 | 0.128 |

MS: metabolic syndrome, WC: waist circumference, BMI: body mass index, GV: glycemic variability, HOMA-IR: homeostatic model assessment-insulin resistance, SDS: standard deviation score, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HbA1c: glycated hemoglobin, IL-6: interleukin

patients without IR in this study. If the MS group could be compared with controls, a significant difference in GV might have been detected.

In conclusion, this study drew attention to three important points. GV is not different among obese adolescents with IR and MS. There is a significant association between GV and IR parameters. However, other diagnostic criteria of MS (hypertension and/or dyslipidemia) or inflammation (elevated IL-6 levels) does not cause further increase in GV.

### Ethics

**Ethics Committee Approval:** The study protocol was approved by the Ethics Committee of İzmir Katip Çelebi University Faculty of Medicine.

**Informed Consent:** Written consent was obtained from all subjects and their parents before the study.

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

### Authorship Contributions

Concept: Bumin Nuri Dündar, Design: Bumin Nuri Dündar, Data Collection or Processing: Abdurrahman Kaya, Cemil Koçyiğit, Analysis or Interpretation: Gönül Çatlı, Literature Search: Abdurrahman Kaya, Cemil Koçyiğit, Writing: Abdurrahman Kaya, Elif Büşra Özkan.

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# Treatment of Pre-pubertal Patients with Growth Hormone Deficiency: Patterns in Growth Hormone Dosage and Insulin-like Growth Factor-I Z-scores

**Megan Oberle, Adda Grimberg, Vaneeta Bamba**

*The Children's Hospital of Philadelphia, Division of Endocrinology and Diabetes, Philadelphia, PA, USA*

### What is already known on this topic?

Insulin-like growth factor-I (IGF-I) can be used to monitor growth hormone (GH) therapy. Individualized IGF-I based dosing may be a more physiologic and objective approach to weight-based dosing. Guidelines by the Pediatric Endocrine Society recommend titrating GH to maintain IGF-I concentrations in the normal range for age and sex.

### What this study adds?

Lack of evidence regarding dosing based on IGF-I z-score values (IGF-1z) contributes to variable clinical practice in GH dosing. This study examined GH prescribing practices and found a prevalence of supraphysiologic IGF-Iz. Our findings demonstrate the need to better understand not just factors that influence IGF-Iz but also the long-term effects of supraphysiologic IGF-Iz.

## Abstract

**Objective:** To describe the range of insulin-like growth factor-I (IGF-I) z-score values (IGF-Iz) and growth hormone (GH) dose adjustments in pre-pubertal patients with GH deficiency (GHD) treated with GH in a single tertiary care center.

**Methods:** This is a retrospective review of GH-treated patients of ages ≤9 years with GHD, seen in an endocrinology clinic in 2013-2014. Patient demographics and pre-treatment anthropometrics, GH treatment duration, IGF-Iz, and GH dosage (mg/kg/week) were extracted. Multipredictor linear regression was used to evaluate the associations between IGF-Iz and GH dosage and subject gender, race, insurance type, age, and clinical characteristics. Logistic regression was used to calculate the odds ratio of direction of GH dose adjustment (decrease/no change versus increase) and IGF-Iz category based on patient clinical characteristics, accounting for provider random effect.

**Results:** Forty-one percent (57/139) of IGF-Iz were outside the "normal" range of between -2 and +2 standard deviation; the majority of IGF-Iz beyond the "normal" range (93%) were supraphysiologic [> +2 standard deviation score (SDS)]. Of the IGF-Iz > +2, 10/53 (18%) were followed by a GH dose increase and 30/53 (57%) had no dose change. Patient clinical characteristics and demographics did not significantly increase the odds of being in the IGF-Iz > +2 SDS category or having a dose increase in multipredictor logistic regression models.

**Conclusion:** GH dosages and IGF-Iz varied, without significant patient clinical predictors. IGF-Iz was frequently supraphysiologic, and these levels often did not prompt a reduction in GH dose, likely influenced by a variety of factors. Our study emphasizes the need for better understanding of long-term safety and efficacy of maintaining supraphysiologic levels of IGF-Iz.

**Keywords:** Growth, insulin-like growth factor-I, growth hormone deficiency, growth hormone therapy, clinical decision making

**Address for Correspondence:** Megan Oberle MD,
The Children's Hospital of Philadelphia, Division of Endocrinology and Diabetes, Philadelphia, PA, USA
**Phone:** +215 590 31 74 **E-mail:** oberlem@email.chop.edu

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## Introduction

Historically, growth hormone (GH) therapy for pediatric patients with GH deficiency (GHD) has been guided by multiple clinical factors, including weight or body surface area, growth velocity, progression of skeletal maturation, side effects, and measurement of serum concentration of insulin-like growth factor-I (IGF-I). Individualized IGF-I-based GH dosing has been suggested as a more physiologic and objective approach to GH dose titration (1). Indeed, the Pediatric Endocrine Society guidelines recommend titrating GH dose "to maintain serum IGF-I concentration in the normal range for age and sex" (2). Reference ranges of IGF-I differ across commercial laboratories, but the recent advent of z-score reporting allows more standardized comparison of IGF-I levels across age, gender, pubertal status, and measuring laboratory. By comparing z-scores, clinicians compare normalized data to one another, just as one might compare body mass index (BMI) z-scores in children at different ages.

Despite these advances, the optimal target IGF-I level has not been established to balance height outcomes, safety, and cost. Short-term studies of IGF-I-based GH dosing have shown increased height outcomes when targeting an IGF-I z-score value (IGF-Iz) of 0 or +2 standard deviation score (SDS) compared to weight-based dosing (3,4). Although a greater increase in height was found to be associated with targeting an IGF-Iz of +2 SDS, higher doses of GH were required leading to supraphysiologic (> +2 SDS) IGF-I levels compared to targeting IGF-Iz of 0 (4). While there have been reports of adverse side effects, such as intracranial hypertension associated with higher doses of GH and supraphysiologic IGF-Iz, there is a lack of clinical data demonstrating a direct dose-response effect (5,6,7,8,9).

In practice, dosing decisions are influenced by subjective factors and therefore vary across clinicians and patients. Thus, we sought to retrospectively describe the range in IGF-Iz and patterns of GH dose adjustments in pre-pubertal patients with GHD treated with GH in a single tertiary care center and secondarily, to determine if GH dosage and IGF-Iz are associated with patient demographic and clinical factors.

## Methods

The Children's Hospital of Philadelphia Institutional Review Board approved this retrospective chart review with waiver of consent prior to data collection.

**Subjects:** The electronic health record (EHR) system was queried to identify all patients under age 9 years (chosen as a surrogate marker for pre-pubertal status) with ICD-9 code 253.3/ ICD-10 code E23.0 (pituitary dwarfism) or 253.2/E.23.6 (panhypopituitarism) who were treated with GH in the outpatient clinic of the Diagnostic and Research Growth Center of the Children's Hospital of Philadelphia between January 1, 2013 and December 31, 2014. A member of the study team (M.O.) reviewed the records for each patient identified by the EHR query to confirm study eligibility. Patients were included if they had GHD defined by peak GH level < 10 ng/mL on both arginine, clonidine, and glucagon stimulation testing or multiple pituitary hormone deficiencies (MPHD) with low GH or IGF-I concentrations based on age, sex, and reference range documented GH treatment during the study period, and measurements of IGF-I and IGF-Iz during the study period. IGF-Iz were reported by two commercial laboratories, based on their reference data, together with the absolute values of the IGF-I measurements. Patients were excluded if IGF-Iz were not reported. Patients also were excluded if they were Tanner stage 2 or greater on physical examination (10), were receiving active treatment for precocious puberty, or were being treated with GH for indications other than GHD.

**Procedures:** The following data were collected from the EHR: gender, age at the start of GH treatment, race/ethnicity, endocrinologist, insurance type, mid-parental height, baseline IGF-Iz, IGF-Iz on treatment, initial GH dose, GH dose at time of IGF-Iz measurement, and both pre-treatment and on-treatment weight, height (Ht), and BMI z-scores (z). Subjects' gender-adjusted mid-parental heights were calculated and transformed into z-scores (mid-parental Htz) (10). Race/ethnicity by parental report was recorded at the time of the clinical visit. The commercial laboratory analyzing each IGF-Iz was also recorded. For each IGF-Iz obtained during the study period, the corresponding clinical notes were reviewed to determine if a GH dose adjustment was made. The majority of patients had IGF-I concentrations evaluated 2-4 times a year, so we also performed subgroup analyses on only the last IGF-Iz that was measured during the specified timeframe (last IGF-Iz).

### Statistical Analysis

Sample size was not calculated as subjects were drawn from a convenience sample of all cases that matched inclusion criteria. Statistical analyses were performed on two separate datasets: all IGF-Iz scores collected and the last IGF-Iz during the study period. Demographic and clinical characteristics were summarized by standard descriptive statistics. Continuous variables are presented as mean ± standard deviation (SD). The continuous variable, IGF-Iz, was categorized into three groups: low (IGF-Iz

< -2 SDS), normal (IGF-Iz between -2 SDS and +2 SDS), and supraphysiologic (IGF-Iz > +2 SDS). Each IGF-Iz was also assigned to a category based on clinical decision: GH dosage increase, decrease, or no dose adjustment. Student's t-test was used to compare IGF-Iz and GH dose by categorical variables (gender, race, and insurance type). Chi-squared test or Fisher's exact test was used to compare categorical variables, including IGF-Iz categories and GH dose adjustment groups. Multipredictor linear regression, accounting for provider random effect, was used with the outcome variables IGF-Iz and GH dosage, and the potential predictors: gender, race, insurance type, age, and clinical characteristics. Logistic regression was used to calculate the odds ratio of direction of GH dose adjustment (decrease/no change versus increase) and IGF-Iz category based on patient clinical characteristics, accounting for provider random effect. All statistical calculations were performed on Stata Data Analysis and Statistical Software 14.0 (StataCorp LP, College Station, TX, U.S.). Statistical significance was defined as p-value ≤0.05.

## Results

A total of 139 IGF-Iz were recorded from 55 subjects who met inclusion criteria (Figure 1). At the time of the last IGF-Iz assessment during the study period, subjects had a mean age of 6.1 ± 1.5 years, and mean duration of GH therapy of 2.9 ± 2.1 years. Sixty-four percent of subjects were male, 67% were white, and 65% had private insurance as their primary coverage (Table 1). Of the 55 subjects, 65% (36) had isolated GHD and 34% (19) had panhypopituitarism. Eighty-two percent (45) underwent stimulation testing and had peak GH levels less than 10 ng/mL. The 18% who did not undergo stimulation testing had MPHD (hypothyroidism, adrenal insufficiency, and/or diabetes insipidus) and low IGF-I and IGF-binding protein 3 concentrations based on age, sex, and reference range. Subjects with MPHD received replacement therapy for their other pituitary deficiencies per clinical routine. Clinical characteristics, including peak GH concentration on stimulation testing, baseline Htz, mid-parental Htz, or initial GH dosage, did not differ significantly between male and female patients.

### Patterns in Insulin-like Growth Factor-I Z-score and Growth Hormone Dosing

The mean of all IGF-Iz obtained during the study period was 1.57 ± 1.8. The mean IGF-Iz was higher in males than females; this difference approached, but did not reach, statistical significance (1.79 ± 1.9 vs. 1.20 ± 1.3, p = 0.06). The mean GH dose (mg/kg/week) prescribed during the study period was 0.28 ± 0.9 and did not differ between males and females. The mean last IGF-Iz was 1.18 ± 1.6, and the mean GH dose at last IGF-Iz assessment was 0.27 ± 0.1 mg/kg/week. These measurements did not differ significantly between genders.

All 3 IGF-Iz below -2 SD were associated with subjects who had septo-optic dysplasia with central hypothyroidism, and 2 had low thyroxine at the time of their low IGF-Iz. After adequate thyroid replacement and normalization of thyroxine level, one subject's IGF-Iz continued to be below -2 SD; the provider of this subject also documented non-adherence with GH at the time of the low IGF-Iz. The other subject did not have a repeat IGF-Iz obtained during the study period.

### Supraphysiologic Insulin-like Growth Factor-I Z-score

Of all 139 IGF-Iz measurements, 57 (41%) were outside of the generally accepted normal range and most of these (53/57, 93%) were supraphysiologic. More males were outside of the generally accepted normal range than females (47% vs. 30%, p = 0.06).

### Predictors of Insulin-like Growth Factor-I Z-score

Using multipredictor linear regression accounting for provider random effect, an increase by 0.27 SDS in IGF-Iz was significantly associated with an increase by 1 SDS in most recent Htz adjusting for patient gender, race, insurance

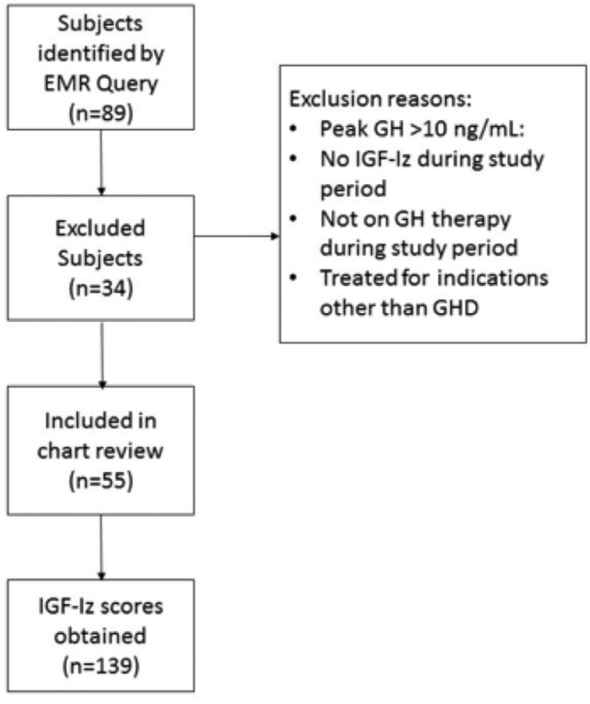


**Figure 1.** Flow diagram of included subjects and insulin-like growth factor-I z-score

GH: growth hormone, GHD: growth hormone deficiency, IGF-Iz: insulin-like growth factor-I z-score

type, and age [p = 0.03, 95% confidence interval (CI) 0.3, 0.5]. This association remained significant when including peak GH level on stimulation testing, pre-treatment Htz, GH dose, and mid-parental Htz in the linear regression model (ß = 0.6, p = 0.01, 95% CI 0.2, 1.0). Peak GH level on stimulation testing, GH dose, pre-treatment Htz, and mid-parental Htz were not significantly associated with IGF-Iz when adjusting for patient age, gender, race, and insurance type. Using logistic regression and controlling for provider random effect, patient clinical characteristics and demographics did not significantly increase odds of being in the supraphysiologic category. Logistic regression did not determine that etiology (isolated GHD vs. panhypopituitarism) was a significant predictor of IGF-Iz or GH dosage.

In the multipredictor models using IGF-Iz as the outcome, 49% of variation ($R^2$) in the model came from patient

**Table 1. Clinical and demographic characteristics of subjects**

| Characteristic | All | Male | Female |
|---|---|---|---|
| **n (%)** | 55 | 35 (64) | 20 (36)* |
| Age at start of treatment (years ± SD) | 3.2 ± 2.4 | 3.2 ± 2.5 | 3.2 ± 2.2 |
| Duration of GH therapy from beginning of therapy to last study IGF-I (years ± SD) | 2.9 ± 2.1 | 3.0 ± 2.2 | 2.6 ± 1.8 |
| **Race/Ethnicity, n (%)** | | | |
| White | 33 (60) | 21 (38) | 12 (22) |
| Black | 8 (15) | 6 (11) | 2 (4) |
| Asian | 3 (5) | 2 (4) | 1 (1) |
| Multiple races | 7 (13) | 4 (7) | 3 (6) |
| Other | 4 (7) | 2 (3.5) | 2 (3.5) |
| **Insurance type, n (%)** | | | |
| Private | 36 (65) | 21 (38) | 15 (27) |
| Medicaid | 17 (31) | 13 (24) | 4 (7) |
| Self-Pay | 2 (4) | 1 (2) | 1 (2) |
| **Etiology, n (%)** | | | |
| Isolated GHD | 36 (65) | 14 (25) | 22 (40) |
| Panhypopituitarism | 19 (35) | 13 (24) | 6 (11) |
| Pre-treatment height z-score ± SD (n = 43) | -2.5 ± 1.6 | -2.3 ± 1.3 | -2.8 ± 2.0 |
| Most recent height z-score ± SD | -1.1 ± 1.6 | -1.4 ± 1.7 | -0.9 ± 1.5 |
| Most recent BMI z-score ± SD | 0.7 ± 1.2 | 0.9 ± 1.0 | 0.3 ± 1.4 |
| Mid-parental height z-score ± SD (n = 47) | -0.32 ± 0.93 | -0.21 ± 0.8 | -0.5 ± 1.1 |
| Peak GH (ng/mL) on stimulation testing ± SD (n = 45) | 4.9 ± 2.6 | 4.9 ± 2.8 | 4.9 ± 2.4 |
| Most recent GH dosage (mg/kg/week) ± SD | 0.27 ± 0.1 | 0.27 ± 0.1 | 0.26 ± 0.1 |
| Most recent IGF-Iz ± SD | 1.18 ± 1.6 | 1.3 ± 1.7 | 0.94 ± 1.4 |

*p-value < 0.05

All other comparisons between genders have a p-value > 0.05

SD: standard deviation, BMI: body mass index, GH: growth hormone, GHD: growth hormone deficiency, IGF-Iz: insulin-like growth factor-I z-score

demographics and clinical characteristics: gender, age at the start of GH treatment, race/ethnicity, provider, insurance type, mid-parental Htz, baseline IGF-Iz, IGF-Iz on treatment, baseline GH dose, GH dose at time of IGF-Iz measurement, pre-treatment weight, height, and BMIz, and on-treatment weight, height, and BMIz. Twenty-seven percent of variation was related to individual clinician. A remaining 24% of the variation in IGF-Iz was unidentified.

### Growth Hormone Dosage Titration

GH dose adjustments were categorized into three groups: no dose adjustment, dose increase, and dose decrease (Figure 2). For all IGF-Iz, the odds of a dose increase were not significantly associated with IGF-Iz category [p = 0.8, odds ratio (OR) 1.1 95% CI 0.6, 2.0]. Of 82 measures of normal IGF-Iz, there was one instance (1/82, 1%) of subsequent dose decrease, 28 instances (28/82, 34%) of dose increase, and 53 instances (53/82, 65%) that were not associated with a dose change. Males were not more likely to receive a dose increase than females when IGF-Iz was normal (p = 0.9, OR 1.0, 95% CI 0.4, 2.7).

When IGF-Iz were in the supraphysiologic category, 10/53 (19%) instances resulted in a dose decrease, 13/53 (25%) had dose increase, and 30/56 (56%) had no dose change. Patient clinical characteristics and demographics were not significantly associated with the odds of dose increase in the supraphysiologic IGF-Iz category. The odds ratio of an increase in GH dosage was not higher in males than females (p = 0.95, OR 0.96, 95% CI 0.26, 3.53) when adjusting for patient demographics and provider.

Notably, one subject with low IGF-Iz was non-adherent to treatment (Figure 2). This was the only instance of the 4 with low IGF-Iz that did not result in a dose increase.

### Predictors of Growth Hormone Dosage

The mean GH dosage (mg/kg/week) did not differ significantly between IGF-Iz categories. Using multipredictor linear regression accounting for provider random effect, mid-parental Htz was found to be significantly associated with GH dosage when adjusting for patient gender, race, insurance type, and age (ß = -0.024, p = 0.04, 95% CI -0.05, -0.001). Even when adjusting for other clinical characteristics (peak GH value on stimulation testing, pre-treatment Htz, IGF-Iz, and most recent Htz), an increase by 1 SDS in mid-parental Htz was associated with 0.036 mg/kg/week decrease in GH dosage (p = 0.01, 95% CI -0.06, -0.01).

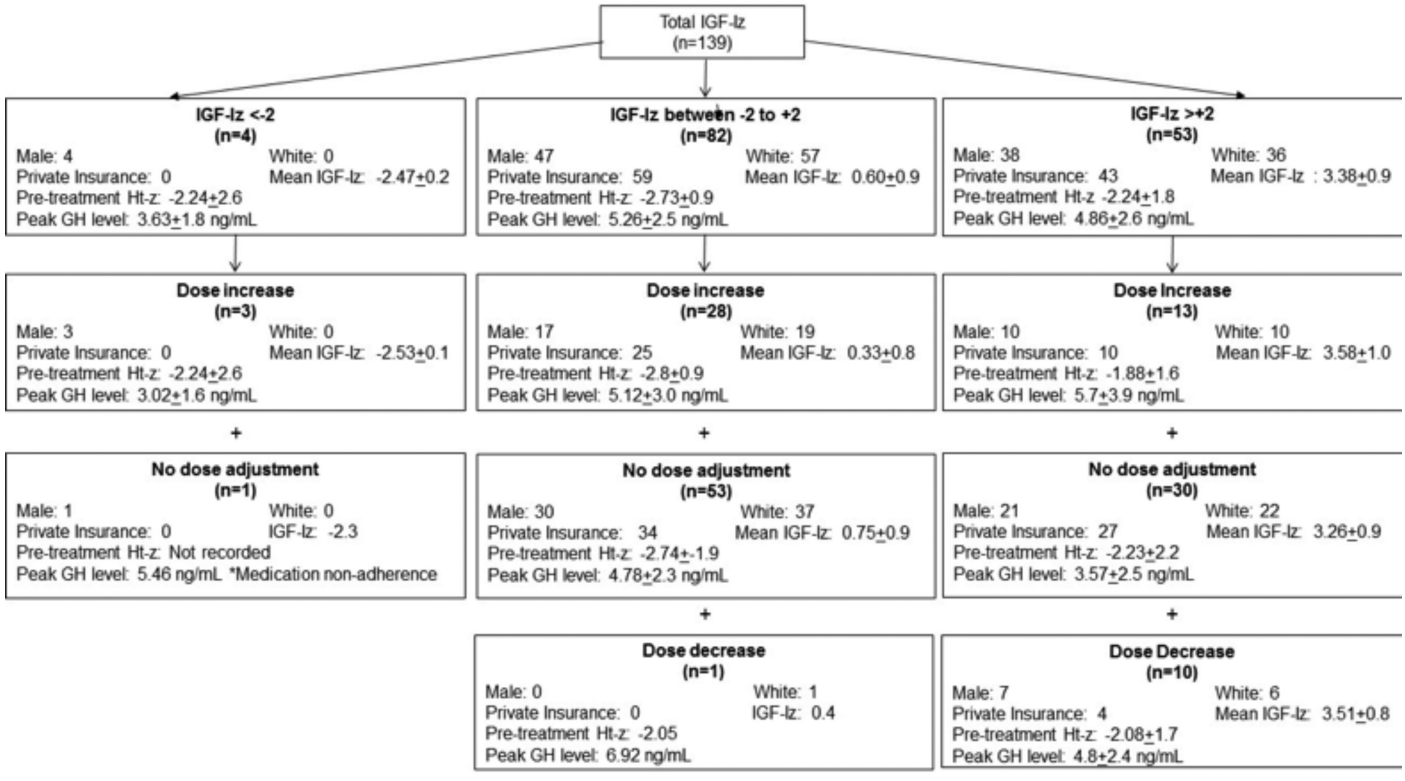


**Figure 2.** Flow diagram of all insulin-like growth factor-I z-score determinations arranged by insulin-like growth factor-I z-score category and dose adjustment category. Number of male subjects, white subjects, and subjects insured under private insured are listed for each category. Mean insulin-like growth factor-I z-score and Htz-scores are z-scores ± 2 standard deviation. Peak growth hormone concentration is provided in ng/mL as mean value ± 2 standard deviation

GH: growth hormone, IGF-Iz: insulin-like growth factor-I z-score

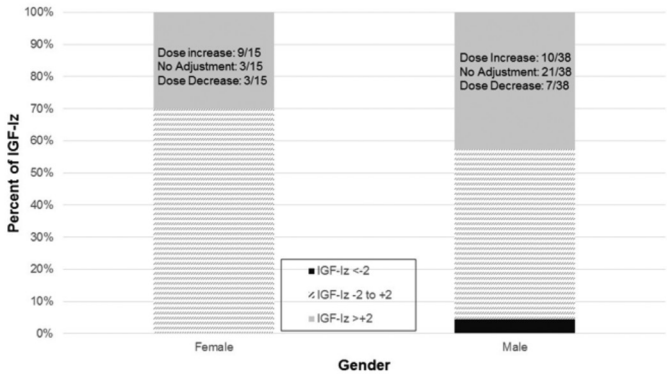


**Figure 3.** Proportions of insulin-like growth factor-I z-score in and outside of target range by patient gender. By Fisher's exact test, insulin-like growth factor-I z-score obtained from male patients had a higher percentage of being outside of -2 to +2 standard deviation score ($p = 0.06$). For those with supraphysiologic insulin-like growth factor-I z-score, decisions about growth hormone dose titration are provided

GH: growth hormone, IGF-Iz: insulin-like growth factor-I z-score

Other clinical characteristics in the model (peak GH value on stimulation testing, pre-treatment Htz, IGF-Iz, and most recent Htz) were not found to be statistically significant ($p > 0.05$). Patient demographics and clinical characteristics accounted for 47% ($R^2$) of the variation in the prediction of GH dosage when accounting for provider random effect.

## Discussion

Although the Pediatric Endocrine Society recommends titrating GH dose "to maintain serum IGF-I concentration in the normal range for age and sex" (2), we found that 41% of IGF-Iz obtained during a 2-year period at a large pediatric endocrinology center were outside of the laboratory-specified normal range of between -2 and +2 SDS, with the majority above +2 SDS (supraphysiologic). In addition, GH dose was increased in 25% (13/53) of instances where IGF-Iz was elevated, compared to 34% (28/82) when IGF-Iz was in the normal range. This observation suggests that IGF-z need not be the primary determinant of GH dose adjustments.

Htz and height velocity are clinical characteristics used to assess response to current GH dosage (2). Other clinical factors, such as mid-parental height, age, and gender may also influence clinical decision making. Mid-parental Htz was the only patient demographic and clinical characteristic tested in our study that significantly predicted GH dosage when adjusting for IGF-Iz. As mid-parental Htz increased, GH dosage decreased, suggesting that our patients with taller parents were more sensitive to lower doses of GH. This finding is consistent with other studies demonstrating that GH-deficient children can achieve comparable increases in growth velocity with smaller doses of GH than children with idiopathic short stature (4,5,11). Short children of tall parents are more likely to have more severe GHD, whereas short children of short parents might have familial short stature and are not truly GH deficient.

Clinician characteristics, such as age and gender, may also influence GH dose adjustment. Our study accounted for the bias of individual clinicians by accounting for healthcare provider in our statistical models. In a short informal survey of endocrinologists in our clinical practice, we found that the majority of providers base the initial GH dose on weight and then subsequently adjust the GH dose using the IGF-Iz in combination with other clinical factors (growth velocity, age, and pubertal status) to titrate GH therapy.

Although previous studies have suggested that physician beliefs and practices as well as consumer preferences play major, yet subjective, roles in referrals to subspecialists for short stature evaluation and even potential access to GH therapy (12,13,14,15), our findings do not demonstrate influence of patient gender on GH dose at the subspecialist level. However, there were more males than females with supraphysiologic IGF-Iz, with the result approaching statistical significance. A larger sample size may show gender bias in medical decision-making and GH dose titration.

The clinical variables included in our analysis, such as mid-parental height, Htz, and GH stimulation test results, were insufficient in the prediction of IGF-Iz associated with GH dosage. In our IGF-Iz prediction models, about half the variation ($R^2$) was explained by patient demographics and clinical characteristics. A quarter of the variation was explained by individual clinician decisions (Figure 3), highlighting the degree of variability in GH titration amongst clinicians. The percentage of variation unexplained by the predictions models could be attributable to GH therapy adherence, height velocity, genetics, and co-morbidities or concomitant medications. Our statistical models also did not determine significant predictors of GH dosage or dose adjustment.

Similarly, prior studies have investigated the use of IGF-Iz and GH response prediction models in patients with GHD, such as the Pfizer International Growth Study (KIGS), the Gothenburg, and the Cologne models (16). Between 50-80% of the variation of growth velocity in the first year of GH treatment was explained by predictors such as age, gender, etiology of short stature, height velocity, change in height SDS, peak GH value on stimulation testing, serum IGF-I and IGF-binding protein 3 levels, and biomarkers of bone

metabolism (16). IGF-I-based dose titration reduced this variation (16). Other factors contributing to the variation in IGF-Iz may include unidentified underlying conditions such as celiac disease, hypothyroidism, or nutritional deficits (16).

GH dose titration based primarily on IGF-Iz may prevent some subjectivity in GH dose adjustments, and because the target is generally normal IGF-I levels, decrease exposure to potential adverse side effects. Prescription of higher doses of GH and tolerance of supraphysiologic IGF-Iz may be done in an attempt to maximize adult height. However, it should be noted that short-term increases in height velocity may not translate to increase in adult height. Supraphysiologic IGF-Iz may accelerate bone age progression and with the subsequent loss of time for growth, result in a shorter adult height; a study using IGF-I therapy demonstrated that high doses of IGF-I may accelerate bone age (17).

At this time, there is limited clinical evidence to determine if long-term exposure to supraphysiologic IGF-Iz increases the risk of adverse events (9). GH therapy is associated with the development of increased insulin resistance, intracranial hypertension, slipped capital femoral epiphysis, and subsequent second neoplasms in patients with prior cancer treatment particularly radiation (5,6,7,18). The French subgroup of the Safety and Appropriateness of Growth hormone treatments in Europe (SAGhE) study found that higher doses of GH (greater than 50 µg/kg/day) were associated with increased all-cause mortality than expected in adults who had been treated with GH in childhood for isolated GHD, small for gestational age, or idiopathic short stature (7). These findings were controversial and not reproducible with other populations (8), which further highlights the need for additional research on the predictors and consequences of supraphysiologic IGF-Iz. Further research is necessary to balance positive outcomes of treatment with health care costs and adverse effects.

The retrospective nature of our study introduced several limitations. Most notably, this study was performed blinded to growth velocity, an important factor in GH treatment. Growth velocity is often used by clinicians in titrating GH dose. In our study, IGF-Iz and height measurements were often asynchronous in the EHR, and height measurements were taken by multiple specialties participating within a single patient's care, leading to discrepant growth velocity calculations; therefore, we determined that growth velocity could not be accurately calculated. Despite this limitation, this study contributes information describing the range of IGF-Iz in the clinical setting. We were also limited in that adherence was not consistently ascertained and may influence variable bias, though likely less contributory to the supraphysiologic group. Selection bias may have been present since we only included data from commercial laboratories that reported IGF-Iz, and insurance preferences dictate the use of designated commercial labs, which may change over time. Our strengths include the use of IGF-Iz, which allows standardized comparisons of data across labs, inclusion of a diverse population, and the use of a prepubertal population, thereby eliminating effects of estrogen on the GH/IGF-I axis.

This report is a novel examination of GH prescribing practices by physicians and sheds light on the prevalence of supraphysiologic IGF-Iz. We took advantage of commercial lab z-score calculations to better understand GH prescribing practices in a large academic center. We did not find gender-specific differences in IGF-Iz and GH dosage when controlling for both provider and patient characteristics, although we had more male subjects who had IGF-Iz outside of the physiologic range. Our results suggest that multiple factors contribute to medical decision making related to GH surveillance and dosing.

### Ethics

**Ethics Committee Approval:** The Children's Hospital of Philadelphia Institutional Review Board approved this retrospective chart review with waiver of consent prior to data collection.

**Informed Consent:** Retrospective chart review.

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

### Authorship Contributions

Concept: Megan Oberle, Adda Grimberg, Vaneeta Bamba, Design: Megan Oberle, Adda Grimberg, Vaneeta Bamba, Data Collection or Processing: Megan Oberle, Analysis or Interpretation: Megan Oberle, Adda Grimberg, Vaneeta Bamba, Literature Search: Megan Oberle, Adda Grimberg, Vaneeta Bamba, Writing: Megan Oberle, Adda Grimberg, Vaneeta Bamba,

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# Melanocortin-4 Receptor Gene Mutations in a Group of Turkish Obese Children and Adolescents

**Selma Tunç[1], Korcan Demir[2], Fatma Ajlan Tükün[3], Cihan Topal[4], Filiz Hazan[5], Burcu Sağlam[6], Özlem Nalbantoğlu[1], Melek Yıldız[1], Behzat Özkan[1]**

*[1]Dr. Behçet Uz Children's Hospital, Clinic of Pediatric Endocrinology, İzmir, Turkey*
*[2]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey*
*[3]Ankara University Faculty of Medicine, Department of Medical Genetics, Ankara, Turkey*
*[4]Dr. Behçet Uz Children's Hospital, Clinic of Pediatrics, İzmir, Turkey*
*[5]Dr. Behçet Uz Children's Hospital, Clinic of Medical Genetics, İzmir, Turkey*
*[6]Düzen Laboratory, Division of Genetic Diagnosis Center, Ankara, Turkey*

### What is already known on this topic?

Melanocortin-4 receptor (MC4R) mutations are the most common known cause of monogenic obesity. Prevalence of MC4R mutations in children with severe obesity varies from 0.3% up to 6.3%, but there is no relevant published data on Turkish subjects.

### What this study adds?

The present study reports a novel mutation and suggests that MC4R mutations are more frequent in Turkish children and adolescents with severe obesity as compared to the existing literature.

## Abstract

**Objective:** Melanocortin-4 receptor (MC4R) mutations are the most common known cause of monogenic obesity. Data regarding MC4R mutations in Turkish subjects are limited. To determine the prevalence of MC4R mutations in a group of Turkish morbid obese children and adolescents.

**Methods:** MC4R was sequenced in 47 consecutive morbidly obese children and adolescents (28 girls and 19 boys, aged 1-18 years) who presented during a one-year period. Inclusion criterion was a body mass index (BMI) ≥120% of the 95$^{th}$ percentile or ≥35 kg/m$^2$. Patients with chronic diseases, Cushing syndrome, hypothyroidism, or suspected syndromes that could cause obesity were excluded. Onset of obesity was before age 10 years in all subjects.

**Results:** Mean age was 13.2 ± 4.1 years, age at onset of obesity 5.1 ± 2.1 years, height standard deviation (SD) score 1.21 ± 0.93, BMI 40.0 ± 8.8 kg/m$^2$, and BMI SD score was 2.72 ± 0.37. One novel (c.870delG) and two previously reported (c.496 G > A, c.346_347delAG) mutations were found in four (8.5%) obese children and adolescents. The novel mutation (c.870delG) was predicted to be a disease-causing frame-shift mutation using *in silico* analyses. Fasting glucose and lipid levels of the patients with MC4R mutation were normal, but insulin resistance was present in two of the subjects. Six more individuals with MC4R mutation (1 child, 5 adults) were detected following analyses of the family members of affected children.

**Conclusion:** MC4R mutations are frequently found in morbid obese Turkish children and adolescents.

**Keywords:** Melanocortin-4 receptor, obesity, mutation

**Address for Correspondence:** Korcan Demir MD,
Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
**E-mail:** korcandemir@gmail.com **ORCID ID:** orcid.org/0000-0002-8334-2422

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## Introduction

Genetic background in obesity is frequently polygenic and rarely monogenic (1). Among the monogenic types of non-syndromic obesity, melanocortin-4 receptor (MC4R) deficiency is presumably the most frequent and the best understood form (2). *MC4R* encodes the 322-amino acid 7-transmembrane G-protein-linked receptor (3). This receptor is expressed in many neurons in several areas of brain including hypothalamus and contributes to appetite regulation. Activation of MC4R by alpha-melano-stimulating hormone, which is produced following interaction of leptin with its receptor, stimulates the anorexigenic pathways and increases energy expenditure (4).

*MC4R* mutations result in hyperphagia, early-onset obesity, increased linear growth in childhood, increased body fat and fat-free mass, increased bone mineral density, and hyperinsulinemia (5). To date, over 150 different mutations have been reported in *MC4R* (4). Prevalence of *MC4R* mutations in children with severe obesity varies from 0.3% up to 6.3% (6,7). However, there is no such data from Turkey. The single study of *MC4R* in obese Turkish children was on evaluation of two polymorphisms (8).

Assessment of *MC4R* mutations would further be of benefit regarding treatment. Recently, setmelanotide, a MC4R agonist, was shown to be effective in treatment of patients with proopiomelanocortin deficiency (9). It might also be effective in treatment of MC4R deficiency.

The aim of this study was to establish the prevalence of *MC4R* mutations in a group of Turkish children and adolescents with morbid obesity.

## Methods

The study was conducted in one of the major tertiary children's hospitals in the region. Consecutive subjects with morbid obesity were recruited from the pediatric endocrinology clinic during a 1-year period. Morbid obesity was defined as body mass index (BMI) ≥120 percent of the 95$^{th}$ percentile values or a BMI ≥35 kg/m$^2$ (whichever is lower). This corresponds to approximately the ≥99$^{th}$ percentile or BMI standard deviation (SD) score ≥2.33 (10). Cases with chronic diseases (cardiovascular, gastrointestinal, and respiratory), a history of drug use (steroids and antipsychotics), endocrine pathology resulting in secondary obesity, or suspected syndromes associated with obesity (including Prader-Willi and Laurence-Moon-Biedl syndromes) were excluded. Following written informed consent from their legal representatives consistent with the Helsinki declaration, 47 unrelated Turkish morbid obese children and adolescents of ages 1-18 years (28 girls and 19 boys) were included in the study. Onset of obesity was before the 10$^{th}$ year of life in all subjects.

Height was measured to the nearest 0.5 cm. Body weight (barefoot, wearing light clothes) was measured using an electronic scale sensitive to the nearest 100 g. Body weight, height, and BMI were recorded, and their SD scores were calculated using Turkish national anthropometric references (11).

All subjects underwent a clinical examination and blood samples were obtained after 12-h fasting for biochemical parameters including glucose, insulin, triglycerides, total cholesterol, high density lipoprotein cholesterol, and low density lipoprotein cholesterol; genetic analyses were performed. The study was approved by the institutional ethics committee (2015/17-01).

### Genetic Analyses

Peripheral blood samples were collected in EDTA tubes. Genomic DNA was extracted from blood lymphocytes by standard procedures. All exons and adjacent intronic regions of *MC4R* were amplified by polymerase chain reaction (PCR) using previously reported primer pairs (12). The products of PCR were purified and directly sequenced using the Big Dye Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were analyzed using the SeqScape Software version 2.5 and Sequencing Analysis Software version 5.1 for the identification of mutations. Genetic analyses were also made in the parents and siblings of the index cases.

### Statistical Analysis

The data were statistically analyzed using SPSS 15.0 (Chicago, IL, USA). Mann-Whitney U-test and chi-square test were used to compare numerical and categorical variables, respectively. A p-value of $<0.05$ was chosen to represent statistical significance. Data were presented as mean ± SD or n (%).

## Results

The study included 47 morbid obese children and adolescents (28 girls and 19 boys, aged 1-18 years). Mean age was 13.2 ± 4.1 years, mean age at onset of obesity 5.1 ± 2.1 years, mean height SD score 1.21 ± 0.93, mean BMI 40.0 ± 8.8 kg/m$^2$, and BMI SD score was 2.72 ± 0.37. Comparison of mutation carriers and non-carriers regarding anthropometric (BMI SD score, height SD score, weight SD score) and biochemical (fasting blood glucose, lipids,

insulin, free thyroxine, thyroid-stimulating hormone, adrenocorticotropic hormone, and cortisol) variables revealed no statistically significant differences except for age at onset of obesity (Table 1).

We detected 3 distinct variants of *MC4R* (c.870delG, c.496 G > A, c.346_347delAG) in four patients (8.5%). The c.870delG mutation was novel; the remaining mutations have been reported previously (4). The families with a *MC4R* mutation are presented below in chronological order of diagnosis and evaluation of cases. Genotypes and phenotypic characteristics of the index cases are summarized in Table 2.

### Family 1

An 8-year-old boy (Patient 1-II-2) who suffered from obesity since age 3 years was the first index case. He was born at term (3800 g) following an eventless pregnancy. Motor and mental developmental stages were normal. Hyperphagia (demanding more food immediately after a meal) was present. His parents were not relatives. His father was obese since childhood. The height of the index case was 148 cm (SD score 3.4), weight 134 kg (SD score 4.27), BMI 61 kg/m$^2$ (SD score 3.05). Physical examination revealed acanthosis nigricans. Fasting insulin and glucose levels were 29.8 mIU/L and 90 mg/dL, respectively. *MC4R* analysis revealed

**Table 1. Comparison of anthropometric and biochemical variables between *MC4R* mutation carriers and non-carriers**

| | Whole group (n = 47) | Mutation-positive group (n = 4) | Mutation-negative group (n = 43) | p* |
|---|---|---|---|---|
| Gender (F/M) | 28/19 | 2/2 | 26/17 | 0.68 |
| Age (years) | 13.2 ± 4.1 | 10 ± 4.4 | 13.5 ± 4.0 | 0.14 |
| Age at onset of obesity (years) | 5.1 ± 2.1 | 2.5 ± 1.25 | 5.3 ± 2 | 0.01 |
| BMI (kg/m²) | 40 ± 8.8 | 45.3 ± 14.2 | 39.5 ± 8.2 | 0.26 |
| BMI SD score | 2.7 ± 0.38 | 2.9 ± 0.28 | 2.7 ± 0.38 | 0.17 |
| Triglycerides (mg/dL) | 119 ± 54.9 | 101 ± 29 | 121 ± 59 | 0.59 |
| Total cholesterol (mg/dL) | 161.7 ± 33.1 | 157 ± 14.5 | 165 ± 30.7 | 0.66 |
| Insulin (mIU/L) | 21.6 ± 12.4 | 22.4 ± 7.5 | 21.7 ± 13.5 | 0.59 |
| Glucose (mg/dL) | 89 ± 7 | 87 ± 3 | 89 ± 7 | 0.60 |
| $fT_4$ (ng/dL) | 1.23 ± 0.15 | 1.17 ± 0.17 | 1.24 ± 0.15 | 0.62 |
| TSH (mIU/L) | 2.75 ± 0.99 | 2.47 ± 0.82 | 2.78 ± 1.01 | 0.62 |
| Cortisol (µg/dL) | 8.4 ± 1.6 | 7.5 ± 1.4 | 8.5 ± 1.6 | 0.69 |
| ACTH (pg/mL) | 17.1 ± 4.7 | 17.6 ± 6.4 | 17.1 ± 4.6 | 0.62 |

*Comparisons were made between cases with mutation carriers and non-carriers.

F: female, M: male, BMI: body mass index, BMI SD: body mass index standard deviation, $fT_4$: free thyroxine, TSH: thyroid-stimulating hormone, ACTH: adrenocorticotropic hormone

**Table 2. Genotypic and phenotypic characteristics of mutation carriers**

| Patient | Genotype | Age (years) | Sex | BMI SD Score | Height SD Score | Age at onset of obesity (years) |
|---|---|---|---|---|---|---|
| 1-II-2 | c.496 G > A | 8 | M | 3.05 | 3.4 | 3 |
| 2-II-1 | c.496 G > A | 16 | F | 2.47 | 1.36 | 4 |
| 3-II-2 | c.870delG | 6 | M | 3.01 | 1.94 | 2 |
| 4-II-2 | c.346_347delAG | 10 | F | 3.07 | 1.06 | 1 |

M: male, F: female, BMI SD: body mass index standard deviation

a previously reported heterozygous c.496G>A (p.V166I) mutation. A family segregation analysis for this mutation showed that his father (Patient 1-I-2, BMI 43 kg/m$^2$) had the same mutation as well (Figure 1a).

### Family 2

The second index case was a 16-year-old female (Patient 2-II-3) who was known to be obese since age 4 years. She was born at term (3600 g) following an eventless pregnancy. She had attained normal motor and mental developmental stages. Hyperphagia was not reported. Her parents were not consanguineous. The father was slightly obese (BMI 30 kg/m$^2$). Her height was 171.3 cm (SD score 1.36), weight 122 kg (SD score 2.47), BMI 42 kg/m$^2$ (SD score 2.47). Physical examination revealed no other findings. *MC4R* analysis revealed the same mutation as in Family 1: heterozygous c.496G>A (p.V166I) (Figure 1b). Family 1 and Family 2 were not related. Among the family members, only the father was carrying the mutation.

### Family 3

A novel mutation (heterozygous c.870delG, Figure 2) was detected in a 6-year-old boy who was reported to be obese since the age of 2 years (Patient 3-II-2). He was born at term (3900 g) following a normal pregnancy. His motor and mental developmental stages were normal. Hyperphagia was reported to be present. His parents were not relatives. His mother was obese since childhood. The height of the index case was 126 cm (SD score 1.94), weight 41 kg (SD score 3.41), BMI 26 kg/m$^2$ (SD score 3.01). Remaining physical examination was normal. The novel *MC4R* mutation was predicted to be a disease-causing frame-shift mutation (p.I291SfsX10) using in silico analyses. Results of bioinformatics analyses of the mutation with PolyPhen2 and Mutation Taster were in agreement: probably damaging (score, 0.999) and disease-causing (probability, 1.000), respectively. His mother (BMI 30 kg/m$^2$) and sister (10.5 years, BMI 32 kg/m$^2$, SD score 2.3) were found to have the same mutation (Figure 1c).

### Family 4

A previously reported c.346_347delAG (p.S116Ffsx6) mutation was found in homozygous state in a 10-year-old female (Patient 4-II-2) with consanguineous parents. She was obese since 1 year of age. She was born by cesarean section at term (4200 g). Her motor and mental developmental stages were normal. Hyperphagia was described. BMI values of her mother and father were 32.4 kg/m$^2$ and 24 kg/m$^2$, respectively. Her height was 147 cm (SD score 1.06), weight 114 kg (SD score 3.87), BMI 53 kg/m$^2$ (SD score 3.01). Physical examination revealed acanthosis nigricans. Fasting insulin and glucose levels were 28 mIU/L and 84 mg/dL, respectively. Both parents were heterozygous for the mutation (Figure 1d).

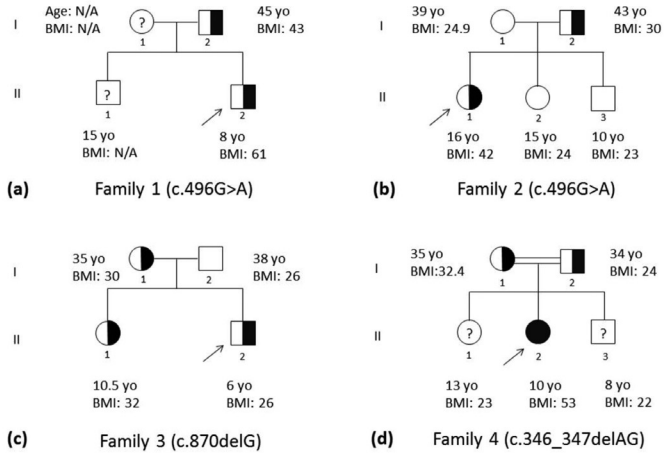


**Figure 1.** Pedigrees of the four families with melanocortin-4 receptor mutations

BMI: body mass index, N/A: non-available, yo: year-old, arrows indicate the index cases, and question marks indicate unknown mutation status

## Discussion

To the best of our knowledge, this is the first published study to assess MC4R mutations in Turkish children and adolescents with morbid obesity. We found three different *MC4R* mutations in four of 47 subjects (8.5%). Screening of family members revealed more affected cases.

Until now, a variable frequency of *MC4R* mutations (0.3-6.3%) was reported in obese children. This wide range

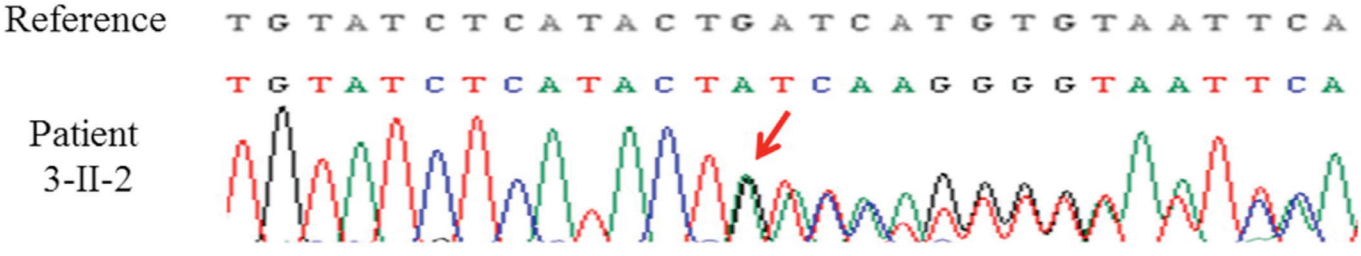


**Figure 2.** Heterozygous deletion of guanine (arrow) at nucleotide 870 results in a frame-shift mutation

apparently seems to be due to different inclusion criteria and ethnic background in relevant studies. Wang et al (13) included non-syndromic Chinese children with a BMI > 97$^{th}$ percentile (nearly 2 SD score) and found that 1.5% of the cases were carrying a *MC4R* mutation. Santoro et al (14) included tall (> 2 SD score) and severely obese (BMI > 3 SD score) Italian children who started to gain weight before 10 years of age and with at least one obese parent. They have found three mutations in five obese children (1.6%) (14). Interestingly, frequency of *MC4R* mutations was only 0.95% among 210 Slovak children whose mean BMI SD score was 4.86 ± 1.7 (7). However, Dubern et al (15) included 63 severely obese (BMI > SD score) French children with non-syndromic and early-onset obesity and found a higher prevalence: 6.3%. In the present study, we found an even higher rate of *MC4R* mutations compared to the existing literature. This might be due to inclusion of cases who had more severe obesity (approximately ≥99$^{th}$ percentile or BMI SD score ≥2.33) which started early in life. Of note, in an unpublished study from another center in our city, frequency of *MC4R* mutations was reported to be 8.6% among 93 obese children and adolescents (mean age 7.3 ± 3.7 years) who started to gain weight before 6 years of age and had a history of early-onset obesity in a first-degree relative (16).

There is only one study assessing *MC4R* mutations in morbidly obese Turkish adults. Mergen et al (17) included 40 subjects with onset of severe obesity before 10 years of age and a history of obesity in at least one family member. There was only one affected case (BMI 41.7 kg/m$^2$) with a p.N247S mutation. They reported a lower mutation rate (2.5%) despite having the same ethnic background. However, we cannot make a comparison since definition of severe obesity and BMI values of the study group were not provided (17). Furthermore, it is known that some of the *MC4R* mutation carriers are obese during childhood but not in adulthood (7,14,18).

We did not detect any differences in the anthropometric and biochemical variables between mutation carriers and non-carriers (Table 1). However, the age of onset of obesity was significantly lower in mutation carriers compared to non-carriers. These findings were similar to those of other studies (7,13,14,19). In addition, hyperphagia, tall stature, and hyperinsulinemia were not present in all affected cases. Farooqi et al (20) reported that only some of MC4R mutation carriers had hyperinsulinemia.

One of the mutations (c.870delG) we detected was not reported previously. This novel mutation was present only in the affected cases in Family 3 and it was predicted to be a disease-causing frame-shift mutation using in silico analyses.

In case 4-II-2, a homozygous *MC4R* mutation was detected. It was reported that age at onset of obesity was earlier and obesity was more severe in homozygous mutation carriers compared to heterozygous mutation carriers (5,20). In our case, obesity began at an earlier age, but BMI SD score was not higher. In addition, while both parents were heterozygous for the mutation, only the mother was obese. Several studies have also reported that mutation carriers would have a normal BMI value (7,14,18). According to Dubern et al (1), the phenotypic difference between the parents may be caused due to incomplete penetrance of mutations. The remaining mutation that was detected in Families 1 and 2 (c.496G > A) was first reported by Wang et al (13). Our cases were more severely affected (BMI values of patients 1-II-2 and 2-II-1: 42 and 61, respectively) than their case who was a seven-year-old patient with a BMI value of 30.7 kg/m$^2$ (13). Other genetic and environmental modifiers would explain differences in the severity of the phenotype of c.496G > A mutation (21).

In summary, the present study provides data regarding *MC4R* mutations in severe obese children and adolescents from Turkey. We found a higher frequency of MC4R mutations compared to the existing literature.

### Ethics

**Ethics Committee Approval:** This study was approved by the Dr. Behçet Uz Children Hospital Ethics Committee, (2015/17-01).

**Informed Consent:** Following written informed consent from their legal representatives consistent with the Helsinki declaration, 47 unrelated Turkish morbid obese children and adolescents of ages 1-18 years (28 girls and 19 boys) were included in the study.

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

### Authorship Contributions

Concept: Filiz Hazan, Korcan Demir, Selma Tunç, Design: Selma Tunç, Korcan Demir, Behzat Özkan, Data Collection and Processing: Selma Tunç, Özlem Nalbantoğlu, Melek Yıldız, Analysis and Interpretation: Fatma Ajlan Tükün, Filiz Hazan, Burcu Sağlam, Literature Research: Selma Tunç, Korcan Demir, Writing: Selma Tunç, Korcan Demir, Cihan Topal.

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# Management of Childhood Thyroid Nodules: Surgical and Endocrinological Findings in a Large Group of Cases

**Emre Divarcı[1], Ülgen Çeltik[1], Zafer Dökümcü[1], Orkan Ergün[1], Geylani Özok[1], Samim Özen[2], Damla Gökşen Şimşek[2], Şükran Darcan[2], Nazan Çetingül[3], Aylin Oral[4], Yeşim Ertan[5], Bengü Demirağ[6], Ahmet Çelik[1]**

*[1]Ege University Faculty of Medicine, Department of Pediatric Surgery, İzmir, Turkey*
*[2]Ege University Faculty of Medicine, Division of Pediatric Endocrinology, İzmir, Turkey*
*[3]Ege University Faculty of Medicine, Division of Pediatric Oncology, İzmir, Turkey*
*[4]Ege University Faculty of Medicine, Department of Nuclear Medicine, İzmir, Turkey*
*[5]Ege University Faculty of Medicine, Department of Pathology, İzmir, Turkey*
*[6]Dr. Behçet Uz Children's Hospital, Division of Pediatric Oncology, İzmir, Turkey*

### What is already known on this topic?

The management of thyroid nodules in children is still a big challenge for clinicians. The rarity of this clinical entity limits the surgical and endocrinological experience in most pediatric centers.

### What this study adds?

In this study, we aimed to present our surgical and endocrinological experience in more than one hundred pediatric cases. We emphasized the importance of a standard management approach including detailed ultrasonography, fine-needle aspiration biopsy, and frozen section examination due to high rates of malignancy and metastasis in children.

## Abstract

**Objective:** The management of childhood thyroid nodules is still a big challenge for clinicians. In this study, we aimed to present our surgical and endocrinological experience in more than one hundred pediatric cases.

**Methods:** A retrospective analysis of patients admitted with a thyroid nodule between 2006 and 2014 was performed. Detailed ultrasonography and fine-needle aspiration biopsy (FNAB) were the cornerstones of the diagnostic approach.

**Results:** One hundred-three children (72 female, 31 male) with a mean age of 13.1 ± 3.6 years (3-18 years) were admitted to our center. Management strategy was surgery in 58 patients and follow-up in 45 patients. Mean nodule size was 17 ± 12.7 mm (2-45 mm). The diagnoses were listed as benign solitary nodule (48 patients), thyroid carcinoma (26 patients), multinodular goiter (23 patients), Hashimoto thyroiditis (4 patients), and Graves' disease (2 patients). Surgical procedures were nodulectomy/lobectomy (32 patients), total thyroidectomy (TT) (13 patients), or TT + neck dissection (13 patients). The rate of malignancy was 25% in the total group and 44% in the surgery group. The malignancy rate was higher in patients younger than 12 years compared to older children (41% vs. 17%, p = 0.040). Metastasis was seen in 38% of the malignant nodules. Postoperative complications were transient hypocalcemia (8%), permanent hypocalcemia (1.7%), and unilateral vocal cord paralysis (1.7%). Recurrence or mortality was not encountered in the 5.4 ± 1.2-year follow-up period.

**Conclusion:** Thyroid nodule in a child requires an aggressive diagnostic approach due to increased risk of malignancy and metastasis. Intraoperative frozen section examination must be done as a useful adjunct to determine the surgical strategy. Incidence of complications is small in thyroid surgery when performed by experienced surgeons.

**Keywords:** Thyroid nodule, thyroidectomy, papillary carcinoma, frozen section, fine-needle aspiration biopsy

**Address for Correspondence:** Emre Divarcı MD,
Ege University Faculty of Medicine, Department of Pediatric Surgery, İzmir, Turkey
**Phone:** +90 535 726 87 63 **E-mail:** emre.divarci@ege.edu.tr **ORCID ID:** orcid.org/0000-0002-8519-8794



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## Introduction

The management of thyroid nodules in children is still a big challenge for clinicians. The treatment strategies were usually derived from adult guidelines until the recent publication of pediatric guidelines for the management of thyroid nodules in children by the American Thyroid Association (ATA) (1,2,3). While most of the main principles in the treatment of pediatric cases are similar to those of adults, there are certain specific and distinctive characteristics in the diagnosis and surgical treatment of children with thyroid nodules.

Thyroid nodules are seen less frequently in childhood than adults (0.2-5% of children, 13% of adolescents) (4). However, the risk of malignancy of a pediatric thyroid nodule is higher than that in an adult patient (7-15 % in adults vs. 22-26% in children) (1,5,6). The rarity of this clinical entity limits the acquisition of surgical and endocrinological experience in most pediatric centers (7,8,9). In this study, we aimed to present our surgical and endocrinological experience in a large group of pediatric cases with thyroid nodules.

## Methods

A retrospective analysis of patients with a thyroid nodule who presented or referred to our center between 2006 and 2014 was performed. The medical records of patients including preoperative, intraoperative, and postoperative data were reviewed. We analyzed the following information: preoperative data which comprised demographics, clinical symptoms, physical examination, family history, radiological features, fine-needle aspiration biopsy (FNAB) results; intraoperative data which included frozen section findings and surgical procedures; and postoperative data which covered histopathological findings, complications, follow-up findings, and final prognosis.

The management of a thyroid nodule initially started with identification of the malignancy risk for differentiated thyroid carcinoma (DTC). Preoperative work-up consisted of clinical and family history, physical examination, detailed thyroid and neck ultrasonography, laboratory tests (thyroid hormones, thyroid antibodies, calcitonin, and electrolyte levels), and FNAB.

Thyroid nodules were identified as low risk or high risk for DTC by the preoperative diagnostic studies listed above. Ultrasonography findings including increased nodular size, hypoechogenicity, invasive and irregular nodule margins, increased nodular blood flow, microcalcifications, and abnormal cervical lymph nodes were accepted as potential signs of high risk malignancy. Patients with such findings were investigated by ultrasonography-guided FNAB and/or surgical sampling. Patients with thyroid nodules underwent surgery or follow-up in accordance with this management protocol (Figure 1).

Patients in the follow-up group with low risk for DTC were followed by ultrasonography and laboratory tests for 6-12-month periods. Patients with high-risk thyroid nodules underwent surgical excision by local nodule excision/lobectomy. In the earlier period of the study, we preferred local nodule excision, but subsequently we began to perform lobectomy as a standard procedure. We performed intraoperative frozen section examination in all of the nodules to identify the histopathological diagnosis during surgery. Patients with malignant results underwent total thyroidectomy and those with metastatic cervical lymph nodes underwent central or lateral neck dissection. In benign results on frozen section examination, lobectomy was accepted to be sufficient to terminate the surgery. In suspicious results on frozen biopsy, surgery was terminated and a second surgical procedure was planned for 2-3 weeks

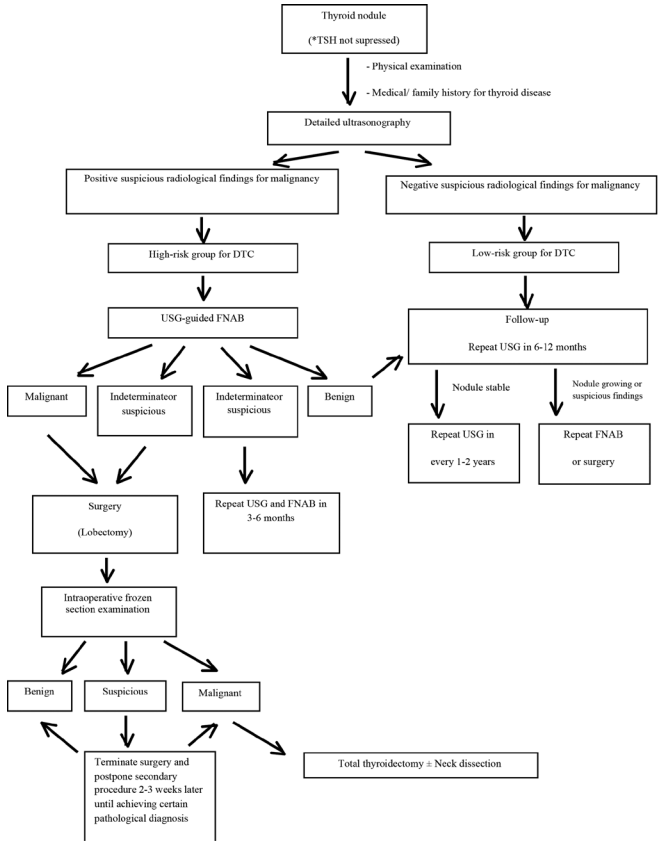


**Figure 1.** Management protocol in a pediatric patient with a thyroid nodule

DTC: differentiated thyroid carcinoma, USG: ultrasonography, FNAB: fine-needle aspiration biopsy, TSH: thyroid-stimulating hormone

later to ascertain the pathological diagnosis. Radioactive iodine therapy was applied in the postoperative period to all patients with thyroid malignancy. The dose of the radioiodine therapy was determined by body weight (37-74 MBq/kg).

All parents and adolescent patients gave their informed consent prior to their inclusion in the study. SPSS for Windows 20.0 was used for the statistical analysis. Pearson's chi-square test was also used in the data analysis. A p-value lower than 0.05 was considered to be statistically significant.

## Results

During the time period between 2006 and 2014, a total of 103 children (72 female, 31 male) were admitted to our center with a thyroid nodule. The diagnostic management protocol presented above was used to investigate the patients and to identify the potential risk for DTC. Fifty-eight patients with high risk for DTC underwent surgery and were labelled as the surgery group. Forty-five patients with low risk for DTC were followed without surgical intervention and named as the follow-up group. The data pertaining to the preoperative, intraoperative, and postoperative periods are presented below.

### Preoperative Data

Mean age of all patients was 13.1 ± 3.6 years (3-18 years) (Table 1). Mean ages for the surgery and follow-up groups were 12.6 ± 3.8 years (3-18 years) and 13.8 ± 3.2 years (4-18 years), respectively (p = 0.086). There was a female dominancy (2-3/1 ratio) in the total group. Visible or palpable swelling in the neck was the presenting admission symptom in 70 patients (68%). Twenty-three patients were diagnosed during examination for an increased risk for thyroid pathologies due to a positive medical or family history (21%). Ten patients had atypical symptoms like growth retardation or obesity and were identified incidentally (11%).

**Table 1. Preoperative data**

| | Number of patients (n) | Mean value |
|---|---|---|
| Age | | |
| Surgery group | 58 | 12.6 ± 3.8 years |
| Follow-up group | 45 | 13.8 ± 3.2 years |
| Total | 103 | 13.1 ± 3.6 years |
| Gender | | |
| Female | 72 (70%) | |
| Male | 31 (30%) | |
| Admission symptoms | | |
| Palpable swelling | 70 (69%) | |
| Medical/family history | 23 (21%) | |
| Incidentally | 10/10%) | |
| Mean nodule size | | |
| Surgery group | 58 | 22.1 ± 12.2 mm |
| Follow-up group | 45 | 10.2 ± 10.1 mm |
| Total | 103 | 17 ± 12.7 mm |

Twenty-four patients had a family history for thyroid diseases (23%). Twenty of these patients had multinodular goiter and four patients had a positive family history for DTC (2 with medullary carcinoma and 2 with papillary carcinoma). The patient's medical history or his/her family history were not found to be an independent risk factor for malignancy of a thyroid nodule (p = 0.931). Seven patients had a suspicious individual medical history for thyroid disorders. Four of them had been treated for a previous Hodgkin lymphoma or neuroblastoma and had a history of radiotherapy to the neck 3 to 12 years ago. The size of the nodules in these patients who had a history of radiotherapy was smaller than 1 cm (6-8 mm). However, all of the nodules were malignant. The other three patients had underlying thyroid disorders as Hashimoto thyroiditis (2 patients) or Graves' disease (1 patient).

Thyroid ultrasonography was performed in all patients in the preoperative work-up. Mean nodule size was 17 ± 12.7 mm (2-45 mm) for the total group. In the group who underwent surgery, mean nodule size was 22.1 ± 12.2 mm (5-45 mm) and larger than that of the follow-up group, which was 10.2 ± 10.1 mm (2-30 mm). Mean nodule size was significantly larger in the surgery group (p = 0.000). Chest CT scan was applied to five patients due to the high risk for distant metastasis. Two of them had pulmonary metastasis.

Thyroid scintigraphy was performed in 40 patients. Eighteen patients had hypoactive cold nodules. DTC was detected in only four of these 18 patients (22%). Fourteen patients had hyperactive hot nodules on scintigraphy. Seven of 14 patients had DTC (50%). The results of our patients were not in accordance with classical knowledge about thyroid scintigraphy.

In this group of patients, FNAB was performed more frequently in recent years. Thirty-one patients underwent FNAB. The results of FNAB revealed that the histology was benign (16 patients), non-diagnostic (10 patients), suspicious (4 patients), and malignant (1 patient). Patients with malignant results or suspicious results in biopsy but who showed characteristic ultrasonographic features for malignancy underwent surgical excision.

Thyroid hormone levels were normal in most of the patients -98 of 103 patients were euthyroid (95%). Two patients with

benign nodules had a mild increase in thyroid-stimulating hormone (TSH) levels. One patient with Hashimoto thyroiditis had a significantly increased TSH level (>100 μIU/L). TSH levels were low in two patients with multinodular goiter.

### Intraoperative Data

The initial surgical procedure in a group of patients was local excision of the suspected lesion by nodulectomy or lobectomy. This surgical sampling by local nodule excision was performed in the early period of the study (15 patients, 25%), but we preferred lobectomy primarily in recent years (43 patients, 75%) (Table 2). Frozen section examination was performed in all patients who underwent surgery. The results of the frozen examination during surgery showed that the nodule was benign in 28 patients, malignant in 23 patients, and suspicious in seven patients. Surgery was terminated in the patients with benign and/or suspicious results. Seven patients with intraoperative suspicious results underwent further histopathological exploration in the postoperative period. Four of these 7 patients had benign results with a diagnosis of cystic nodular goiter (3 patients) or follicular adenoma (1 patient) and did not require any additional surgical intervention. However, three patients with suspicious results on frozen examination had subsequent malignant results as papillary carcinoma (2 patients) and follicular carcinoma (1 patient) which necessitated total thyroidectomy 2-3 weeks after the initial surgery.

Thus, 26 of the patients had malignancy and required total thyroidectomy. Mean age in these patients with malignant nodules was statistically lower than that of the patients with benign nodules ($11.9 \pm 3.6$ vs. $13.6 \pm 3.5$) ($p = 0.047$). Most of these patients were female (20 patients, 77%). Median nodule size was similar in patients with benign and malign nodules as 14 mm (2-45 mm and 5-50 mm).

The overall rate of malignancy in this group of patients with thyroid nodules was 25% (26/103). The rate of malignancy in the surgery group was 44.8% (26/58). The standard surgical procedure was total thyroidectomy in patients without cervical lymph node metastasis. In patients with preoperative suspicion of metastatic lymph nodes on ultrasonography, frozen section examination was performed to lymph nodes. Also, patients with suspected nodules during surgery were investigated for metastasis. 13 patients underwent neck dissection for suspicion of lymph node metastasis (22%). The surgical approach was central dissection in 10 patients and additional lateral neck dissection in 3 patients.

**Table 2. Intraoperative data**

| | Number of patients (n) |
|---|---|
| Frozen section examination | |
| Benign | 28 |
| Malignant | 23 |
| Suspicious | 7 |
| Surgical procedure | |
| Nodulectomy/Lobectomy | 32 (56%) |
| Total thyroidectomy | 13 (22%) |
| Total thyroidectomy + neck dissection | 13 (22%) |

### Postoperative Data

The spectrum of diagnosis ranged from solitary benign nodules, multinodular goiter, medical disorders like Graves' disease and Hashimoto thyroiditis to malignancies such as papillary, medullary, and follicular carcinoma (Table 3). Most patients had benign etiologies (75%, 77/103). The most common disorder was a solitary benign thyroid nodule in 48 patients (46%). Twenty-eight of these patients were followed with ultrasonography and FNAB. The other 20 patients with benign solitary nodules underwent surgical excision. The diagnosis was multinodular goiter in 23 of these patients. Follow-up (15 patients) or surgery (8 patients) were the preferred management approaches in multinodular goiter. Surgery was preferred in cases with a large goiter located bilaterally. Total thyroidectomy was preferred in these 8 patients for cosmetic concerns. A decision for surgery (2 patients) or follow-up (2 patients) was made in the 4 patients with Hashimoto thyroiditis according to the medical condition of the patients. Two children with Graves'

**Table 3. Postoperative data**

| | Number of patients (n) |
|---|---|
| **Diagnosis** | |
| Benign etiologies | 77 (75%) |
| Solitary benign nodule | 48 (47%) |
| Multinodular goiter | 23 (22%) |
| Hashimoto thyroiditis | 4 (4%) |
| Graves' disease | 2 (2%) |
| Malignancy | 26 (25%) |
| Papillary carcinoma | 22 (21%) |
| Medullary carcinoma | 2 (2%) |
| Follicular carcinoma | 2 (2%) |
| Malignancy rate | 25% (26/103) |
| Metastasis rate | 38% (10/26) |
| **Complications** | |
| Transient hypocalcemia | 5 (8%) |
| Permanent hypocalcemia | 1 (1.7%) |
| Unilateral vocal cord paralysis | 1 (1.7%) |

disease underwent subtotal thyroidectomy. Twenty-six patients had malignant etiologies as papillary carcinoma (22 patients), medullary carcinoma (2 patients), and follicular carcinoma (2 patients). Metastasis was detected in 10 of these 26 patients during initial diagnosis (38%). Cervical lymph nodes were involved in all of these 10 patients. In addition, 2 of these patients had diffuse lung metastasis and underwent radioactive iodine therapy. Patients with medullary carcinoma had a positive family history for medullary carcinoma. These patients did not have any clinical symptoms or physical examination findings, but 5 and 10 mm solitary thyroid nodules were detected on ultrasonography. Prophylactic thyroidectomy was preferred in these patients, and medullary carcinoma was identified on pathological examination. Follicular carcinoma was detected in 2 patients. The size of the nodules were larger as 37 and 45 mm in these 2 patients. FNAB was performed in one of them revealing suspicious cytology. No surgical complications occurred in patients with follicular and medullary carcinomas. All of the complications were seen in patients with papillary carcinomas.

The age of the children with a thyroid nodule varied from 3 years to 18 years in this in this group of patients. The malignancy rate was 41 % (14/34) in patients of ages ≤12 years and higher than the rate of 17% (12/69) in patients ≥12 years. This difference was statistically significant ($p = 0.040$).

In the early postoperative period, all patients were monitored for possible complications such as hypocalcemia and vocal cord paralysis. Permanent unilateral vocal cord paralysis was detected in one patient (1.7%). The hoarseness did not require tracheostomy. Transient hypocalcemia was determined in five patients (8%) and resolved spontaneously in the postoperative period. Permanent hypocalcemia occurred in one patient and required permanent oral calcium and calcitriol replacement (1.7%).

Mean postoperative follow-up was $5.4 \pm 1.2$ years (2-10 years). No complications were noted in the late postoperative period. Two patients who had undergone nodule excision with the diagnosis of multinodular goiter required total thyroidectomy in the late postoperative period due to cosmetic concerns. Recurrence or mortality was not encountered in any of the patients (0%).

## Discussion

The management protocol for a thyroid nodule in children starts with a detailed preoperative examination which usually necessitates an operative procedure and continues with long-term follow-up in the postoperative period. The malignancy rate and metastasis risk for a thyroid nodule in a child is higher than those in an adult (1,5,6). In our study, the rates of malignancy and metastasis were identified as 25% and 44 %, respectively. Lower age is a significant risk factor for malignancy in a thyroid nodule. In this study, we found that there was a two times increased risk in children younger than 12 years old as compared to those who were older (41% vs. 17%). Therefore, a more aggressive approach is necessary in younger children. The metastasis rate was also higher. For these reasons, the nature of a thyroid nodule needs to be detected immediately to achieve effective treatment. A history of neck radiotherapy is a significantly important risk factor for malignancy in a thyroid nodule. In our study, papillary carcinoma was identified in all of the nodules which were smaller than 1 cm in patients with a history of a previous neck radiotherapy.

A management protocol should be initiated with identification of the risk of malignancy in a nodule. Detailed thyroid ultrasonography is essential to reveal the risk by demonstration of specific radiological findings like microcalcifications, invasive nodule borders, hypoechogenicity, and increased vascularity (10). Also, the nodule size must be monitored regularly to detect any enlargement as a critical parameter for additional diagnostic intervention. FNAB should be performed under ultrasonography guidance (11,12,13,14). After identification of high risk for DTC, surgical sampling by lobectomy must be preferred as an initial surgical intervention. Nodulectomy is given up in solitary nodules to avoid the surgical risks of subsequent surgical procedures with a new nodule formation in residual thyroid lobe. Routine intraoperative frozen section examination must be applied in all patients with an increased risk of malignancy in a thyroid nodule (15,16,17). In our study, we achieved a high rate of useful results by frozen examination which were helpful in deciding on the surgical strategy during surgery. Surgery is terminated in cases with benign results. Suspicious results which cannot identify the risk of malignancy necessitate further pathological examination and possibly indicate a need for complete total thyroidectomy.

Frozen section examination during surgery is a useful adjunct in the decision of surgical strategy but has some limitations (18). Lobectomy must be performed to investigate the nodule with extranodular margins. Frozen section biopsy could diagnose classical papillary thyroid carcinoma (PTC) successfully. However, it has limitations for the diagnosis of follicular variant PTC and may not identify follicular thyroid carcinoma which requires the evaluation of the entire lesion and vascular/capsular invasion (19,20).

In the early period of our study, we performed routine thyroid scintigraphy as a diagnostic tool. We found conflicting results about the activity of nodules as cold or hot. Routine scintigraphy is not essential in the management of thyroid nodules. In the ATA pediatric guideline, routine scintigraphy is not recommended in patients with normal TSH levels (3).

The complication rate in thyroid surgery is very low in experienced hands. The most frequent complication we encountered was transient hypocalcemia in five patients in the early postoperative period (8%) which resolved spontaneously. Unilateral vocal cord paralysis occurred in one patient with an incidence of 1.7%. This patient's diagnosis was follicular variant papillary carcinoma and she had bilateral fixed large nodules. Permanent hypocalcemia was seen in one patient with the diagnosis of solid variant papillary carcinoma. In conformity with previous reports from experienced teams, no major complications such as bleeding, signs indicating a need for tracheostomy or wound infection during or after surgery developed in any of our patients (21).

Thus, we conclude that children admitted with a thyroid nodule must be evaluated by an experienced multidisciplinary team including experts in pediatric endocrinology, radiology, pathology, oncology, nuclear medicine, and surgery. A thyroid nodule in a child requires an aggressive diagnostic approach due to increased risk of malignancy and metastasis. The cornerstone of the diagnostic approach is a detailed thyroid and neck ultrasonography. Enlarged nodules larger than 1 cm and nodules which have specific ultrasonographic findings need to undergo ultrasonography guided FNAB. Also, risk of malignancy is higher in patients with a previous history of radiotherapy to the neck and in those with nodules smaller than 1 cm. In patients with a high-risk thyroid nodule, surgical intervention must be performed under frozen section examination guidance during surgery. Malignant results on frozen examination necessitate total thyroidectomy. Also, metastatic cervical lymph nodes require central or lateral neck dissection.

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### Ethics

**Ethics Committee Approval:** Retrospective study.

**Informed Consent:** All parents and adolescent patients gave their informed consent prior to their inclusion in the study.

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

### Authorship Contributions

Concept: Emre Divarcı, Ülgen Çeltik, Design: Emre Divarcı, Zafer Dökümcü, Ahmet Çelik, Orkan Ergün, Geylani Özok, Data Collection and Processing: Emre Divarcı, Ülgen Çeltik, Samim Özen, Bengü Demirağ, Yeşim Ertan, Aylin Oral, Analysis and Interpretation: Emre Divarcı, Zafer Dökümcü, Ahmet Çelik, Samim Özen, Damla Gökşen Şimşek, Şükran Darcan, Nazan Çetingül, Literature Research: Emre Divarcı, Ülgen Çeltik, Zafer Dökümcü, Writing: Emre Divarcı, Zafer Dökümcü.

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# Clinical and Genetic Findings of Turkish Hypophosphatasia Cases

**Halil Sağlam[1], Şahin Erdöl[2], Sevil Dorum[2]**

*[1]Uludağ University Faculty of Medicine, Department of Pediatrics, Division of Metabolism and Endocrinology, Bursa, Turkey*
*[2]Uludağ University Faculty of Medicine, Department of Pediatrics, Division of Metabolism, Bursa, Turkey*

### What is already known on this topic?

Hypophosphatasia (HPP) is a bone mineralization disorder, commonly presenting with findings such as early loss of deciduous teeth and growth retardation. All HPP cases reported from Japan have an inherited autosomal recessive disorder.

### What this study adds?

Some minor abnormalities such as mild short stature and osteopenia can be observed in asymptomatic heterozygote carriers whose laboratory findings are normal. Turkish HPP cases can exhibit an autosomal recessive or dominant inheritance similar to cases of European origin.

## Abstract

**Objective:** Hypophosphatasia (HPP) is a rare, commonly unrecognized hereditary mineralization defect with a dramatically poor prognosis in severe cases. This study is the first to examine the detailed clinical and laboratory characteristics of patients with HPP and healthy carriers in Turkey.

**Methods:** The study data were obtained retrospectively from the files of 10 healthy carriers and of 16 cases with HPP (12 children and 4 adults) who were followed in our center from 2012 to 2016.

**Results:** The annual incidence of perinatal lethal hypophosphatasia (PLH) was estimated to be approximately 1 case per 435,517 live births,, which is the first report from Turkey. The clinical courses of the cases differed depending on the type of HPP. All of the seven cases (58.3% of all cases) with perinatal lethal form of HPP died. A need for respiratory support ($p = 0.001$), a history of pyridoxine-dependent seizures ($p = 0.001$), a low chest circumference measurement ($p = 0.017$), younger age at diagnosis ($p = 0.029$), a small head circumference at the time of presentation ($p = 0.042$), a low arm span to height ratio ($p = 0.048$), and a low serum alkaline phosphatase (ALP) level ($p = 0.042$) seemed to be predicting factors for mortality. The mean height standard deviation score of the patients and those of the healthy carriers did not differ significantly ($p = 0.173$). Different mutations were detected in nine of 14 cases (64.2%) in whom an *ALPL* gene mutation analysis could be performed, and five of these cases (35.7%) had novel mutations. The most common mutations were c746G > T (five alleles), c346G > A (three alleles), and c.140C > T (three alleles). In addition, the most frequently observed genotype in Turkish HPP cases was autosomal-dominant c.346G > A (p.A116T) mutations which were detected in three cases in two different families.

**Conclusion:** Because of the respiratory problems, especially the lung hypoplasia, the clinical course is poor in cases with the perinatal lethal form of HPP. Some minor abnormalities such as mild short stature and osteopenia could be observed in asymptomatic heterozygote carriers. Laboratory findings were normal in these cases.

**Keywords:** Turkish children, hypophosphatasia

## Introduction

Hypophosphatasia (HPP) (OMIM# 241500, 241510, 146300) is a rare hereditary metabolic disease characterized by deficiency of alkaline phosphatase (ALP) due to mutations in the gene coding tissue non-specific ALP (TNSALP) enzyme (1p36.1-34) (1). ALP is a dephosphorylation enzyme that removes the phosphate group from many molecules, including nucleotides, proteins, and alkaloids. It is found in all tissues in the human body, most intensively in the liver, bile duct, kidneys, bones, small intestine mucosa, and placenta. TNSALP is the ALP originating from the liver, bones, and

**Address for Correspondence:** Şahin Erdöl MD,
Uludağ University Faculty of Medicine, Department of Pediatrics, Division of Metabolism, Bursa, Turkey
**E-mail:** dr_sahinerdol@hotmail.com **ORCID ID:** orcid.org/0000-0003-4402-9609



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kidneys (2). TNSALP activity is found in bones, cartilage, and tooth tissue at almost all levels, and it is known to remove phosphorus from molecules containing phosphate, such as pyridoxal phosphate (PLP), phosphoethanolamine (PEA), and pyrophosphate (PPi). Bone mineralization is realized upon the placement of hydroxyapatite crystals composed of free calcium (Ca) and inorganic phosphate (Pi) in the collagen matrix. On the other hand, PPi is known as one of the most significant suppressors of hydroxyapatite formation. When TNSALP activity is low, PPi cannot be transformed into Pi, and as a result, serum PPi levels increase; the increasing PPi levels prevent bone and tooth development (2,3). Low TNSALP activity leads to low circulating pyridoxal levels within the central nervous system, negatively affecting γ–carboxyglutamic acid neurotransmitter synthesis and leading to pyridoxine-dependent seizures (4).

The ages at which the disease is initially observed cover a broad spectrum, from the fetal period to adulthood depending on enzyme activity, as is the case for almost all hereditary diseases. For this reason, the disease has been divided into the following clinical sub-groups according to age at presentation: perinatal lethal hypophosphatasia (PLH), prenatal benign hypophosphatasia (PBH), infantile hypophosphatasia, childhood hypophosphatasia, adulthood hypophosphatasia, and odontohypophosphatasia (odonto HPP). The ages at which the symptoms/findings appear and the severity of the disease are generally inversely proportional (2).

Cases may be identified through findings including insufficient mineralization in bones and teeth, early shedding of deciduous teeth, rickets, fractures, bone deformities, respiratory failure, growth and developmental retardation, hypotonia, pyridoxine-dependent seizures, hypercalcemia, hyperphosphatemia, nephrocalcinosis, craniosynostosis, and lower back pain, again depending on enzyme activity. When osteochondral formations called Bowdler projections (observed in the fibula and ulna) are detected, it can be deemed pathognomonic for the disease. Those formations can be observed in both fetal and benign perinatal forms (5).

It is estimated that the incidence of severe forms of the disease that affect both sexes equally is approximately 1 in 300.000 in Europe. Severe cases of the disease are generally inherited in an autosomal recessive manner, having a broad clinical spectrum, whereas inheritance varies in less severe cases (6).

The most significant key to the diagnosis is awareness of the disease. When HPP is brought into mind in the differential diagnosis of patients who present with bone, teeth, and other systemic symptoms and findings, it is very easy to diagnose the disease by establishing the low ALP levels (5).

An ALP replacement at bone level is required for treatment of cases with HPP. HPP has been recently added into the group of rare diseases that cannot be treated. Fortunately bone-directed recombinant human TNSALP (asfotase alfa) was developed and recently proved to be useful in a relatively broad group of patients with HPP (7).

In this study, we aimed to examine the clinical and laboratory characteristics of HPP cases and heterozygote carriers with normal laboratory findings, including TNSALP levels.

## Methods

### Patients

The study data were obtained retrospectively from the files of 16 cases with a diagnosis of HPP and 10 healthy carrier cases that were followed in our center during the four years from 31 October 2012 to 1 November 2016. HPP cases whose age- and sex-matched serum ALP levels were low, who had no vitamin D insufficiency, and had not received bisphosphonate treatment, and healthy carrier family members were included in the study. The diagnosis was confirmed through an *ALPL* gene mutation analysis. Genetic analysis of the parents and siblings of the index cases was also performed. Healthy carriers, all of whom were parents or siblings of the HPP cases, were defined as those whose serum ALP, PEA, PLP levels were normal, did not have any complaints, and had a heterozygous mutation in the *ALPL* gene.

Detailed history and physical examination findings were obtained from the case files. The early loss of deciduous tooth and the existence of easy bone fractures were particularly questioned. Weight, height, head circumference, breast circumference from nipple and arm span were measured in all cases by the same specialist doctor using calibrated, standardized measurement tools. Arm span to length/height ratios were calculated and compared to the age- and sex-matched reference values (8). All of the comparisons of the anthropometric measurements were evaluated according to the age- and sex-matched centiles and/or standard deviation score (SDS). The data on number of teeth were obtained from the file. All of the cases were assessed at baseline and at each visit in terms of the tooth status which is defined as the number of missing teeth compared to the number they should actually have according to his/her age. A neurological examination was performed by the same child development specialist by using the Bayley developmental motor scale test in cases up to 42 months old and by using

the Peabody developmental motor scale test in cases from 42 months to 18 years old. The motor development of adults was evaluated by an adult neurologist. Gross motor development, which was expected to be mostly affected, was evaluated in the comparisons.

Serum Ca, P, ALP, PTH and vitamin D levels, urine Ca/Cr ratios, bone radiographs, lumber region dual-energy X-ray absorptiometry (DEXA) measurements, *ALPL* gene mutation analysis, urine PEA levels, and plasma PLP values were obtained from the medical records. In DEXA assessments, T-scores in adults and Z-scores in children were considered. A detailed eye examination was performed to identify any papilledema or calcifications, and a renal ultrasound was performed to identify the existence of nephrocalcinosis in all cases by the same pediatric ophthalmologist and radiologist.

### Classification of the Patients

Cases whose findings were evident in the intrauterine and newborn periods and whose clinical course was poor were classified as those with the perinatal lethal form of the disease, and those whose clinical course was good were classified as being afflicted with the prenatal benign form. Those who revealed symptoms between 1 and 6 months were classified as infantile HPP. Cases whose symptoms became apparent between 6 months and 18 years were classified as childhood, and after the age of 18 years were classified as adulthood HPP. Cases whose course included only a tooth finding in both childhood and adulthood were classified as odonto HPP (9).

### Radiology

All of the radiologic studies were performed in our center. Bone mineralization data of the cases were determined using both conventional X-ray radiographies and DEXA. Conventional X-ray radiographs were studied with the Xgeo GC80 (Samsung Electronics Co. Ltd., Suwon, South Korea) device and DEXA was studied with a horizon (Hologic, Inc., Bedford, MA, USA) device. Anterior-posterior skull and chest x-rays, and bilateral upper and lower extremity radiographs were taken using conventional X-ray radiography. Lumbar L1–L4 vertebral bone mineral densities were measured using DEXA. All the assessments were performed by the same specialist radiologist and by the authors.

### *TNSALP* Gene Mutation Analysis

PCR products were purified and sequenced using the direct sequencing method and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer Corp., Foster City, CA, USA), and they were moved to an ABI PRISM 310 electrophoresis system (Applied Biosystems, USA). The method of Mornet et al (10) was used in the PCR amplification of exons and single strand conformation polymorphism analysis.

### Statistical Analysis

The compliance of the data included in the study with a normal distribution was assessed using the Shapiro-Wilk test. Data not in compliance with a normal distribution were compared using the Mann-Whitney U test, and their descriptive statistics were expressed as the median (minimum-maximum). A Pearson chi-square test was used to compare the categorical variables between groups. The IBM SPSS Statistics 23 software package was used for statistical analysis. In comparisons, p-value $<0.05$ was considered statistically significant.

## Results

During the study period of 4 years, due to our involvement with an international HPP study, all cases diagnosed as having HPP throughout Turkey were being directed or referred to our center. The number of PLH cases referred to us over the four years is seven, and in addition, five other PLH cases in other cities died before reaching our center. Thus, the number of PLH cases diagnosed in Turkey within this period of four years is 12, as far as we could detect. The total number of newborn babies in Turkey from 2012 to 2016 is 5,226,213 according to the data of the Turkish Statistical Institute (11). Consequently, the incidence of PLH in our country is estimated to be approximately 1 case per 435,517 live births. The estimated annual number of PLH cases is three. It was not possible to estimate an annual incidence for cases with other HPP forms which could easily go unrecognized because of the relatively subtle findings.

All 16 HPP and 10 healthy carrier cases participating in the study were of Turkish ethnic origin and were directed to our center from various provinces of Turkey from 2012 to 2016 with a suspicion of HPP.

Of the HPP cases, seven (43.8%) were PLH, three (18.7%) PBH, three (18.7%) odonto HPP, two were (12.5%) adult HPP, and one (6.3%) was childhood HPP. The clinical and laboratory characteristics of those cases are summarized in Table 1. The median follow-up duration of all the patients was 1 year (min = 0.04 years; max = 3.66 years).

Median age at HPP diagnosis was 0.58 years (range, 0-35 years), whereas median age of heterozygote cases whose laboratory characteristics were normal was 8.15 (range, 6-55) years.

In total, 43.8% (n = 7) of HPP cases were female and 56.2% (n = 9) were male. This proportion was similar in carrier cases as well (40% vs. 60%). The male gender predominated in PLH (71.4%), but no statistically significant difference was detected in terms of gender between HPP cases and their heterozygous family members with normal laboratory findings (p = 0.85).

Clinical findings differed depending on the form of HPP. The most frequently observed findings were short stature, short extremities, epilepsy, large fontanel, motor retardation, and respiratory failure (Table 2).

At presentation, no statistically significant difference was detected between the height SDS of HPP cases and those of carrier cases with normal laboratory values (-2.06 vs. -1.62; p = 0.173). Regarding height and arm span/height ratios, mean arm span/height ratio in HPP cases at presentation was 0.94, and this ratio was found to be low compared to normal reference data in all cases whose age at diagnosis was below three years (n = 10). As well, this proportion was found to be normal in all patients diagnosed after age three years with a mild clinical course (n = 6). The average arm span/height proportion of carrier cases not having HPP (n = 10) was 0.98, and all were within the normal limits (Table 3) (8).

During the follow-up interval of about 4 years, no change was observed in terms of rough motor functions in 43.8% (n = 7) of cases, whereas 37.5% (n = 6) had deterioration and 18.8% (n = 3) had improvement. Gross motor functions of all healthy carrier cases were normal.

None of the heterozygote carriers not having HPP experienced early loss of deciduous teeth; while six of nine HPP cases (66.6%) whose teeth had erupted, experienced early loss of deciduous teeth.

The tooth status of four (44.4%) of nine cases with HPP whose teeth had erupted worsened when the basal and final assessments were considered, while the tooth status of the 10 heterozygote carriers not having HPP did not change.

Conventional radiography revealed osteopenia in 10 (62.5%) and rickets in 7 (43.7%) of the HPP cases. All of the cases having rickets also had osteopenia. Rickets or osteopenia were found in none of the carriers not having HPP (Table 2).

DEXA T-/Z-scores of cases having HPP were found to be lower than those of healthy carriers, but the difference was not statistically significant [-1.7 (range, -3.1-0.1) vs. -0.9 (range, -1.1- -0.9)], (p = 0.381).

**Table 1. Clinical and laboratory characteristics of cases with hypophosphatasia**

| Case | Age at diagnosis | HPP type | ALP level at diagnosis | Follow-up period (years) | Mutation analysis | Height SDS at diagnosis | Last height SDS | Outcome |
|---|---|---|---|---|---|---|---|---|
| A | 0.06 | PLH | 11 | 1.16 | c.659G > C (Gly220Ala) hom. | -4.56 | -5.11 | Exitus |
| B | 0.06 | PBH | 24 | 3.66 | c.815G > A (Arg272His) hom.[a] | -4.41 | -4.28 | Alive |
| C | 0.17 | PLH | 10 | 0.2 | c.799-804delCACTTC (p.267-268 del HF) hom.* | -1.97 | -2.51 | Exitus |
| D1 | 0.66 | PBH | 45 | 1.75 | c.746G > T (p.G249V) hom.[b] | -2.23 | -2.75 | Alive |
| D2 | 8 | PBH | 31 | 1.08 | c.746G > T (p.G249V) hom.[b] | -0.54 | 0.07 | Alive |
| E | 0.25 | PLH | 10 | 0.5 | c.194C > A mut tas:chr1:21887602 C > A hom.* | -2.54 | -3.95 | Exitus |
| F | 0.06 | PLH | 6 | 0.04 | c.140C > T (p.N47I) hom.* | -4.64 | -5.12 | Exitus |
| G | 0.08 | PLH | 6 | 0.16 | c.1243T > G (p.Y415D) hom.* | ND | ND | Exitus |
| H | 0.5 | CH | 49 | 1.5 | c.346G > A (p.A116T) het.c | 0.4 | -2.14 | Alive |
| I1 | 0.0 | PLH | 10 | 0.0 | Could not be performed | ND | ND | Exitus |
| I2 | 0.0 | PLH | 38 | 0.0 | Could not be performed | ND | ND | Exitus |
| J | 34 | Adult | 2.39 | 3.5 | c.1444C > A (p.H482N) het. + cDNA 1557A > G (Y436C) het.* | -2.06 | -2.06 | Alive |
| K | 3.75 | OH | 70 | 2 | c.346G > A (p.A116T) OD het.[c] | -1.64 | -1.42 | Alive |
| L | 30 | OH | 22 | 2 | c.346G > A (p.A116T) OD het.[c] | -0.32 | -0.32 | Alive |
| M | 35 | OH | 24 | 1.66 | c.746G > T (p.G249V) het. | -0.7 | -0.7 | Alive |
| N | 23 | Adult | 26 | 1 | c.140C > T (p.N47I) het.* | -3.47 | -3.47 | Alive |

[a]Reported to be related to infantile form of hypophosphatasia in the literature, [b]reported to be related to perinatal lethal hypophosphatasia in the literature, [c]reported to be related to adult form of hypophosphatasia in the literature

HPP: hypophosphatasia, D1, D2 and I1, I2 cases are siblings, *: novel mutation, hom: homozygote, het: heterozygote, ND: no data, PLH: perinatal lethal hypophosphatasia, PBH: prenatal benign hypophosphatasia, CH: childhood hypophosphatasia, OH: odonto hypophosphatasia

Survival significantly differed among HPP forms (p = 0.001). All cases who succumbed were of PLH form and all PLH cases died. Moreover, a statistically significant difference was found between cases who survived and cases who died with respect to age at diagnosis (p = 0.029), head circumference at the time of presentation (p = 0.042), arm span to height ratio (p = 0.048), chest circumference (p = 0.017), and serum ALP level (p = 0.042). The chest circumferences of all of the seven cases who died were < 3 percentile, and the chest circumference of only one (11.1%) of the nine living HPP cases was < 3 percentile. Serum ALP levels of PLH cases, who all died, and of PBH cases, who all survived, were 10.0 IU/L (range, 6.0-38.0) and 31.0 IU/L (range, 24.0-45.0), respectively. Major clinical and laboratory findings of the HPP forms are given in Table 4.

One of the most significant factors related to survival was the need for respiratory support (p < 0.001). Respiratory support was needed in all of the seven cases who died, while none of the nine living cases needed respiratory support. While all PLH cases had respiratory failure, respiratory assessment showed normal function in all PBH cases.

Moreover, there was a significant difference between patients who survived and those who died with respect to existence of pyridoxine-dependent seizures (p = 0.001). While all seven cases who died had pyridoxine-dependent seizures, there was no history of pyridoxine-dependent seizures in any of the nine surviving cases.

### TNSALP Mutation Analysis

Genetic analysis was available for 14 of 16 HPP cases. Of these, 9 (64.2%) had different mutations and five (35.7%) were novel disease-causing mutations according to the Mutation Taster pathogenicity prediction program. The c746G > T (five alleles), c346G > A (three alleles), and c.140C > T (three alleles) were the most frequently observed mutations. The most frequently observed genotype in HPP cases was the autosomal dominant c.346G > A (p.A116T) heterozygote mutation, detected in three cases in two different families. Five of eight cases (62.5%) having a homozygote mutation had PLH, whereas none of the heterozygote cases had PLH (Table 1).

## Discussion

The clinical and genetic characteristics of Turkish HPP cases were specified with this retrospective study. No data on HPP incidence in Turkey are available, and it differs across the world. The data on incidence are more often related to PLH as mild forms could be missed. The incidence of PLH in Japan is 2-3 in 1 million live births annually (12), whereas this value was estimated to be 1 in 100,000 in Canada (13). The annual PLH incidence in our country was found to be approximately 3.06 cases per 1 million live births in this study. PLH is the form most frequently observed (43.7%) in the patients referred to our center, similar to the data in Japan (12), and this form is observed more frequently in males (71.4%).

**Table 2. Clinical and radiological characteristics of hypophosphatasia cases**

| | Perinatal lethal (n = 7) | Prenatal benign (n = 3) | Odonto HPP (n = 3) | Adult (n = 2) | Childhood (n = 1) | Total (%) (n = 16) |
|---|---|---|---|---|---|---|
| **Clinical features** | | | | | | |
| Short stature | 7 | 2 | 0 | 2 | 1 | 12 (75.0) |
| Short extremities | 7 | 2 | 0 | 0 | 0 | 9 (56.2) |
| Seizures | 7 | 1 | 0 | 0 | 0 | 8 (50.0) |
| Large fontanelle | 7 | 0 | 0 | 0 | 1 | 8 (50.0) |
| Motor retardation | 7 | 1 | 0 | 0 | 0 | 8 (50.0) |
| Respiratory failure | 7 | 0 | 0 | 0 | 0 | 7 (43.7) |
| Early loss of deciduous teeth | N/A | 1 | 3 | 1 | 1 | 6 (37.5) |
| Dimple in extremities | 0 | 3 | 0 | 0 | 0 | 3 (18.7) |
| **Radiological findings** | | | | | | |
| Osteopenia | 7 | 1 | 0 | 1 | 1 | 10 (62.5) |
| Long bone deformity | 7 | 2 | 0 | 1 | 0 | 10 (62.5) |
| Narrow thorax | 7 | 1 | 0 | 0 | 0 | 8 (50.0) |
| Rickets | 7 | 0 | 0 | 0 | 0 | 7 (43.7) |
| Osteochondral spurs | 0 | 3 | 0 | 0 | 0 | 7 (18.7) |
| Bone loss | 1 | 0 | 0 | 0 | 0 | 1 (6.25) |

HPP: hypophosphatasia, N/A: not applicable

**Table 3. Comparison of clinical and laboratory characteristics of cases with hypophosphatasia and of carriers not having hypophosphatasia**

| Case | Male Gender (%) | Height SDS at diagnosis Median (min;max) | Average arm span/ height ratio | Ca Median (min;max) (mg/dL) | P Median (min;max) (mg/dL) | ALP Median (min;max) (U/L) | PTH Median (min;max) (pg/mL) | D vit Median (min;max) (µg/L) | PEA Median (min;max) (µmol/L) | PLP Median (min;max) (µg/L) | DEXA T/Z Score Median (min;max) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PLH cases | 71.4 | -3.55 (-6.64;-1.97) | 0.88 (0.86;0.91) | 11.04 (9.10;15.2) | 5.2 (2.4;5.50) | 10.0 (6.0;38.0) | 5.25 (3.0;42.0) | 24.1 (4.3;42.2) | NA | NA | NA |
| All HPP cases | 56.3 | -2.06 (-6.64;0.40) | 0.94 (0.86;1.13) | 9.95 (9.10;15,2) | 6.0 (2.60;7.70) | 23.0 (2.39;70) | 21.3 (3.0;70.8) | 22.5 (4.3;60.0) | 340.8 (74.9;1594) | 40.9 (10.6;910.0) | -1.70 (-3.10;0.10) |
| Carriers not having HPP | 60 | -1.62 (-1.95;0.49) | 0.98 (0.98;0.98) | 9.60 (9.30;10) | 4.30 (3.70;5.10) | 46.0 (31;171) | 53.8 (36.9;120.1) | 19.0 (15.2;131.4) | 45.1 (16.8;123.7) | 36.3 (18.0;39.4) | -0.9 (-1.1;-0.6) |
| *p value | 0.85 | 0.173 | 0.727 | **0.027** | **0.049** | **0.001** | **0.001** | 1 | 0.067 | 0.51 | 0.38 |

HPP: hypophosphatasia, PEA: Phosphoethanolamine, PLP: Pyridoxal phosphate, DEXA: dual-energy X-ray absorptiometry, SDS: standard deviation score, NA: not available; *: between all hypophosphatasia cases and carriers not having hypophosphatasia

Bone deformities were detected in fetal ultrasonography (USG) of two PLH and two PBH cases included in the study, and fetal USG findings in both forms were similar. In line with previous literature data, these results support the argument that the findings obtained through fetal USG, do not predict the clinical course.

Since the ratio of severe clinical forms was high among our HPP cases, clinical findings were more pronounced and more common in most of the cases. For example, the incidence of pyridoxine-dependent seizures was observed at a rate of 42.8% in Japanese PLH cases, whereas pyridoxine-dependent seizures were detected in all of the Turkish PLH cases followed in our center. Similarly, in a study conducted by Taketani et al (12), the ratio of short stature in cases with HPP was 42.8%, a ratio higher than that reported from western countries. This figure was even higher (75.0%) in our patients. Also, three of four cases without short stature were odonto HPP cases and one was an 8-year-old PBH case.

The most significant prognostic factors detected in our HPP cases were respiratory failure and pyridoxine-dependent seizures, both indicating a worse prognosis and this finding conformed with previous studies (2,5). In addition, chest circumference, age at diagnosis, head circumference, arm span to height ratio, and serum ALP levels were found to be predictors of prognosis in our HPP cases. In other words, as the measurements of chest circumference, age at diagnosis, head circumference, arm span to height ratio, and serum ALP levels decrease, the prognosis worsens.

To our knowledge, heterozygote family members of HPP cases with normal laboratory values have not been studied so far. Our attention has been drawn to the fact that the serum ALP levels of heterozygote family members of HPP cases were generally normal but within the lower third of the sex-matched adult reference values, and this could have led to some mild abnormalities. For example, the mean height SDS of the carrier family members without HPP was -1.62 which was similar to that of HPP cases (-2.06). Similarly, the median T-/Z-score of carrier heterozygote cases was -0.9 which is not statistically different from that of the HPP cases (-1.7) (Table 3). These findings may indicate that the carriers have slightly low height and low bone mineral density compared to the mean of population, although they are healthy.

Certain mutations can lead to severe HPP forms in some societies, such as the existence of a homozygote 1559delT mutation in the *ALPL* gene leading to PLH in a Japanese society (12). However, this was not the case in the present study as the mutations of all Turkish PLH cases were different.

**Table 4. Principal clinical and laboratory characteristics by hypophosphatasia forms**

| HPP Form | n | Age at diagnosis median year (min:max) | Baseline serum ALP median IU/L (min;max) | Age adjusted reference values for ALP | Baseline height SDS median (min;max) | Baseline arm span/ height ratio median (min;max) |
|---|---|---|---|---|---|---|
| Perinatal lethal | 7 | 0.17 (0.00;0.75) | 10.0 (6.0;38.0) | 70-400 | -3.55 (-6.64;-1.97) | 0.88 (0.86;0.91) |
| Perinatal benign | 3 | 0.66 (0.06;8.0) | 31.0 (24.0;45.0) | 100-360 | -2.23 (-4.41;-0.54) | 0.90 (0.87;0.97) |
| Odonto HPP | 3 | 30.0 (3.75;35.0) | 24.0 (22.0;70.0) | 31-129 | -0.70 (-1.64;-0.32) | 0.98 (0.98;0.98) |
| Adult | 2 | 28.5 (23.0;34.0) | 14.1 (2.32;26) | 31-129 | -2.76 (-3.47;-2.06) | 1.01 (1.0;1.02) |
| Childhood | 1 | 0.5 (0.5;0.5) | 49.0 (49.0;49.0) | 100-360 | 0.4 (0.4;0.4) | 1.13 (1.13;1.13) |

HPP: hypophosphatasia, ALP: alkaline phosphatase, SDS: standard deviation score, min;max: minimum-maximum, p-value was not given since the number of cases was insufficient for comparisons in certain hypophosphatasia forms

Mutations observed in the *ALPL* gene could vary between societies; for example, the mutations among all the reported Japanese HPP cases were inherited in an autosomal recessive manner (12). The mutations among the Turkish HPP cases could exhibit an autosomal recessive or dominant heritage, similar to cases of European origin. An autosomal recessive heritage leads to HPP forms that are more serious, whereas an autosomal dominant heritage leads to more minor forms, such as odonto HPP and childhood HPP. Because the family members of the HPP cases were assessed through detailed physical, laboratory, and radiologic examinations in this study, some mild HPP forms were detected in the mothers of cases with severe forms of HPP who have a homozygote mutation. For example, the case having the novel c.140C > T (p.N47I) homozygote genotype had PLH, whereas her heterozygote mother had adult-type HPP. Moreover, the case having the c.746G > T (p.G249V) homozygote genotype had clinical PBH, while his heterozygote mother had clinical odonto HPP. Interestingly, fathers of both patients having the same heterozygous mutations were completely normal. Though most of the heterozygote cases in many other inherited metabolic diseases do not reveal clinical phenotype, this may not be the case in HPP.

A genotype-phenotype correlation for HPPs except PLH has not been suggested so far in previous studies. For example, HPP of different clinical severity was observed in two siblings having the same mutation (12). In our study, we also observed similar findings. Two siblings having PBH with the c.746G > T (p.G249V) homozygote mutation exhibited a different clinical course. Although both had highly elevated serum PLP and urinary PEA levels, the boy exhibited a mild phenotype and all his clinical findings spontaneously improved at age 9, while the girl showed more pronounced clinical findings and was still having abnormal clinical findings at the age of 2.5 years. On the other hand, it has been suggested that the 1559delT mutation, which is only observed in Japanese cases, could lead to PLH (14).

The three mutations detected in our study led to a different clinical course compared to the cases with the same mutations in the literature. For instance, while the c.815G > A (p.A272H) homozygote mutation in the literature led to infantile HPP (15), it led to PBH in our case. Similarly, while the c.746G > T (p.G249V) homozygote mutation in the literature was suggested to lead to PLH (16), it led to PBH in our two sibling cases, and again, though the c.346G > A (p.A116T) heterozygote mutation in the literature was related to odonto HPP (17), it led to both childhood and odonto HPP in our study (Table 1). This suggests that the same mutation may lead to different clinical outcomes in different cases.

As expected, clinical HPP forms having a homozygote mutation in the *ALPL* gene generally have a more serious clinical outcome and the clinical findings appear early compared to heterozygote cases. Interestingly, in this peculiar disease, some cases with a homozygote mutation in the *ALPL* gene exhibiting very striking clinical findings at an early age, even in the intrauterine period, may have a very favorable clinical outcome, as is the case in most of the PBH cases.

In conclusion, in this first HPP study from Turkey, annual PLH incidence was estimated to be approximately 1 per 435,517 live births. PLH was the most frequently observed form. The most frequently observed findings were short stature, short extremities, epilepsy, large fontanel, motor retardation, and respiratory failure. The most significant predictors of poor prognosis in HPP cases are pyridoxine-dependent seizures and respiratory failure mainly caused by a hypoplastic thorax. Although intrauterine bone anomalies may indicate HPP, they fail to predict the course of the disease. Mild short

stature and osteopenia can be observed in asymptomatic heterozygote cases whose laboratory findings are normal. Both autosomal recessive and dominant mutations can cause HPP phenotype in the Turkish population.

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### Ethics

**Ethics Committee Approval:** The study was approved by the Ethics Committee of Uludağ University, Bursa (Approval #2016-19/13).

**Informed Consent:** Informed consent was obtained from the parents of all patients.

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

### Authorship Contributions

Concept: Şahin Erdöl, Halil Sağlam, Design: Şahin Erdöl, Halil Sağlam, Data Collection or Processing: Şahin Erdöl, Sevil Dorum, Analysis or Interpretation: Şahin Erdöl, Halil Sağlam, Literature Search: Şahin Erdöl, Sevil Dorum, Writing: Şahin Erdöl, Halil Sağlam.

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# The Relationship of Disordered Eating Attitudes with Stress Level, Bone Turnover Markers, and Bone Mineral Density in Obese Adolescents

**Aslı Okbay Güneş[1], Müjgan Alikaşifoğlu[2], Ezgi Şen Demirdöğen[3], Ethem Erginöz[4], Türkay Demir[3], Mine Kucur[5], Oya Ercan[6]**

*[1]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatrics, İstanbul, Turkey*
*[2]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, İstanbul, Turkey*
*[3]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Child and Adolescent Psychiatry, İstanbul, Turkey*
*[4]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Public Health, İstanbul, Turkey*
*[5]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Biochemistry, İstanbul, Turkey*
*[6]İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine and Endocrinology, İstanbul, Turkey*

### What is already known on this topic?

In the literature, there is only one study investigating the relationship between disordered eating attitudes and bone health in obese adolescents. In this study, bone mineral density was found to be lower in obese individuals who had a high level of body shape concern than other obese individuals.

### What this study adds?

In this study, no significant correlation was found between disordered eating attitudes (except binge eating disorder) and bone mineral density. The femoral neck bone mineral density was significantly higher in subjects who had binge eating disorder compared to the ones who did not have the disorder.

## Abstract

**Objective:** To investigate the effect of stress caused by disordered eating attitudes on bone health in obese adolescents.

**Methods:** A cross-sectional study comprising 80 obese adolescents was performed from November 2013 to September 2014. Twenty-four-hour urinary free cortisol levels were measured as a biological marker of stress. Bone turnover was evaluated using bone-specific alkaline phosphatase, serum osteocalcin, and urinary N-telopeptide concentrations. Bone mineral density was measured using dual-energy X-ray absorptiometry. The Eating Disorder Examination Questionnaire, Dutch Eating Behavior Questionnaire, Children's Depression Inventory, and the State-Trait Anxiety Inventory for Children were used to assess eating disorders, depression, and anxiety. Psychiatric examinations were performed for binge eating disorders.

**Results:** In the Pearson's correlation test, a positive correlation was found between the 24-hour urinary cortisol level and Dutch Eating Behavior Questionnaire total and restrained eating subscale scores ($p < 0.05$ for both). In linear regression analyses, the Dutch Eating Behavior Questionnaire total and restrained eating subscale scores were found to be significant contributors for urinary cortisol level ($\beta = 1.008$, $p = 0.035$; $\beta = 2.296$, $p = 0.014$, respectively). The femoral neck areal bone mineral density was found to be significantly higher in subjects who had binge eating disorder compared with those without binge eating disorder ($p = 0.049$).

**Conclusion:** Despite the lack of apparent effects on bone turnover and bone mineral density in our obese adolescents at the time of the study, our results suggest that disordered eating attitudes, and especially restrained eating attitudes, might be a source of stress. Therefore, studies in this area should continue.

**Keywords:** Adolescence, obesity, cortisol, disordered eating attitude, stress, bone turnover, bone mineral density

**Address for Correspondence:** Müjgan Alikaşifoğlu MD, İstanbul University Cerrahpaşa Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, İstanbul, Turkey **E-mail:** kasif@istanbul.edu.tr **ORCID ID:** orcid.org/0000-0002-0463-6597



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## Introduction

The frequency of overweight and obesity is gradually increasing worldwide (1). According to the World Health Organization (WHO), the frequency of obesity increased more than two-fold between 1980 and 2014 (1). It is known that intake of food in obese individuals increases in the presence of negative feelings including anger, fear, boredom, anxiety, stress, and sadness (2). In addition, it has also been shown that obese individuals work hard to restrict food intake (3). The efforts of obese individuals to restrict food intake may cause excessive weight gain by leading to binge eating which has the completely opposite effect (4).

Some studies showed that restriction of food intake increased endogenous cortisol secretion by leading to stress (5,6). It has been reported that increased cortisol levels inhibit bone formation by decreasing the number of osteoblasts and their function, by stimulating osteoclastogenesis, and thus affecting bone health negatively by disrupting bone turnover (7). A limited number of studies have investigated the effect of stress caused by disordered eating attitudes on bone health and the results of these studies showed variations (8,9,10,11). In studies involving premenopausal women and adolescent girls, it was found that cognitive eating restraint did not affect cortisol levels, but negatively affected bone health (8,9). In other studies conducted on premenopausal women, it was shown that cognitive eating restraint increased cortisol levels by leading to stress, and the increased cortisol levels affected bone health negatively (10).

Adolescence is an important period in terms of skeletal development (12). The results of studies directed at understanding the effect of obesity in adolescence on bone health are variable (13,14,15). In some studies, it was found that the bone mineral content (BMC) and bone mineral density (BMD) were higher in obese children and adolescents compared with those who were not obese (13,14). In contrast, in another study, it was reported that obesity in adolescents decreased BMC and BMD (15). As far as we know, there is only one study which investigated the relationship between disordered eating attitudes and bone health in obese adolescents (11). In that study, urinary free cortisol level was found to be increased in individuals who had a high level of concern with weight compared with those who had no weight concerns and BMD was found to be lower in individuals who had a high level of body shape concern compared with those who had no body shape concern (11). Based on these findings, it can be speculated that stress caused by disordered eating attitudes in obese adolescents might negatively affect bone health by increasing endogenous cortisol secretion. In this study, we aimed to investigate the effects of disordered eating attitudes and stress on bone health in adolescent obese individuals.

## Methods

This is a prospective cross-sectional study. Eighty adolescents aged between 11 and 18 years who were referred to Cerrahpaşa Faculty of Medicine, Department of Pediatrics, Adolescent Outpatient Clinic, between November 2013 and September 2014, considered obese according to their body mass index (BMI) values as specified by Cole et al (16) were included in the study. Subjects with a chronic disease, substance addiction, or other psychiatric disorders which could prevent compliance with the study, and those who did not use any method to lose weight in the last six months were excluded.

Ethics Committee approval was obtained for the study from the Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee (date: 12.07.2013, number: 18857). A second approval was obtained from the same Committee (date: 11.03.2014, number: 6281) for the addition of the State-Trait Anxiety Inventory for Children and psychiatric face-to-face evaluation for binge eating disorder (BED). Detailed information about the study was given to the subjects who accepted to participate in the study as well as to their parents and they were asked to sign an informed consent form.

A detailed history about the subject's physical and psychosocial health status was taken from the adolescents and their parents.

Height and body weight measurements and physical examination of the subjects were performed by the same physician. BMI was calculated using the following formula: BMI = [weight/height$^2$ (kg/m$^2$)] (1). Pubertal staging was performed in accordance with the Tanner staging system (17). Testicular volume was evaluated using the Prader orchidometer in boys and recorded. Telarche in girls and a testicular volume of 4 mL in boys was considered as puberty (17).

The subjects were evaluated through a face-to-face interview by a child psychiatrist in the outpatient clinic of child psychiatry regarding the presence of BED according to the Diagnostic and Statistical Manual of Mental Disorders 5$^{th}$ Edition diagnostic criteria. The parents were not present during the interview. Other eating attitudes of the patients were evaluated using the Eating Disorder Examination Questionnaire (EDE-Q), which was adapted to Turkish by Yucel et al (18), and the Dutch Eating Behavior Questionnaire (DEBQ), which was adapted to Turkish by Bozan et al (19).

The EDE-Q: The internal consistency of the Turkish version of this scale was found high (Cronbach $\alpha = 0.93$) and the Cronbach $\alpha$ was found as 0.70 or above for each subscale (18). The scale consists of four subscales and 28 items. The subscales measure restraint, eating concerns, shape concerns, and weight concerns, and the items evaluate eating attitudes of the individual over the last four weeks. The subjects are asked to mark one of the options ranging from 0 (never) to 6 (every day) for items 1-12 and 19-21, and from 0 (none) to 6 (significantly) for items 22-28. In order to obtain a certain subscale score, the scores of the relevant items in that subscale are added up divided by the total number of items in that subscale. The scores obtained from the four subscales are also added up and divided into the number of subscales (four) in order to calculate the total score. The scale has no cut-off point. The score obtained increases with the severity of the disordered eating attitude.

The DEBQ: The internal consistency coefficients of the whole and subscales of the Turkish version are considerably high [Cronbach $\alpha$ (whole scale) = 0.94, emotional eating = 0.97, external eating = 0.90, restrained eating = 0.91] (19). The scale comprises thirty-three items and three subscales which measure restrained eating, emotional eating, and external eating behaviors (19). Each item has options ranging between 0 (not at all) and 5 (frequently). In order to obtain a certain subscale score, the scores of the items related with that subscale are summed. The scores obtained from the three subscales are summed in order to calculate the total score. The scale has no cut-off point. As the score obtained increases, the severity of disordered eating attitudes increases.

Children's Depression Inventory [(CDI), adapted to Turkish]: This method was used to assess depression as a covariate, because relevant studies in the literature that were conducted on adults showed that depression negatively affected bone health by increasing cortisol levels (20,21,22). This scale involves 27 items that can be applied to children aged between 6 and 17 years. The internal consistency of the Turkish version is high (Cronbach $\alpha = 0.77$) (20). The child or adolescent is asked to mark the most appropriate option for the last two weeks. Each item is given a score of 0, 1, or 2 according to the severity of the symptom. The highest score is 54. The cut-off point has been recommended as 19.

State-Trait Anxiety Inventory for Children (STAI-C): Based on studies which indicate that cortisol levels increase as the level of anxiety increases and that increased levels of anxiety affect bone health (23,24), anxiety was assessed as a covariate using STAI-C. Adaptation studies of this scale showed that it could be applied to children aged 9-16 years. The internal consistency of the Turkish version is high (Cronbach $\alpha = 0.82$ for state anxiety and $= 0.81$ for trait anxiety) (25). The STAI-C can be applied to groups or individuals. The scale is composed of two subscales as state anxiety and trait anxiety, each involving 20 items. The trait anxiety subscale was used in this study. In this scale, the severity of anxiety is graded using one of the options including "almost never", "sometimes", and "frequently". These options are given one, two, and three points, respectively. The possible scores range between 20 and 60, and an increase in the score expresses an increase in anxiety.

Venous blood samples were obtained from the adolescents after a 12-hour fasting period; serum bone-specific alkaline phosphatase (B-ALP) was measured using a human B-ALP ELISA kit (Hangzhou Eastbiopharm, Hangzhou, China, Cat. No: CK-E10874), serum osteocalcin (OC) was measured using an EDI OC (1-43/49)-specific ELISA kit (Epitope Diagnostics Inc., San Diego, CA, USA, KT809), 24-hour urine N-terminal telopeptide (NTx) was measured using an Osteomark NTx Urine kit (Alere Scarborough, Inc., Scarborough, ME, USA, Ref 9006), and 24-hour urine free cortisol was measured using a DRG Urinary Cortisol kit (DRG International Inc., Springfield Township, NJ, USA, EIA-2989) with an ELISA assay. The adolescents and their parents were informed about how to collect a 24-hour urine sample, discarding the first urine in the morning and collecting urine in the next 24 hours including the first urine in the next morning.

The 25-hydroxy vitamin D (25-OH vit D) level was taken as a covariate in the linear regression analyses to investigate the relationship between disordered eating attitudes and bone health. Vitamin D deficiency is reported to be more frequent in obese individuals compared with individuals of normal body weight and vitamin D deficiency is known to negatively affect bone health (26,27). The measurement was performed using electrochemiluminescence with a Cobas vitamin D total kit (Roche Diagnostics GmbH, Mannheim, Germany, Ref 05894913).

Areal BMD (aBMD) was measured in the femoral neck and lumbar 1-4 vertebral (L1-4 vertebra) area. The measurements were performed by two experienced technicians using a Hologic QDR 4500W measurement device with dual-energy X-ray absorptiometry (DEXA). The femoral neck was used in measurements because it is the most appropriate BMD measurement region to evaluate the risk of hip fracture. Moreover, the fracture prediction model of the WHO involves femoral neck BMD measurements (28). Lumbar vertebrae were used for measurements because they are one of the most common body regions where osteoporotic fractures occur (29). The z-scores of the subjects were calculated using the reference values of femoral neck and L1-4

vertebral aBMD by age and sex for healthy Turkish children and histories of fracture were interrogated (30). After taking age and sex into account, z-scores of ≤-2 indicate clinically low bone mass, and z-scores between -1 and -2 indicate that an individual is at risk for low bone mass.

The Statistical Package for Social Sciences version 21.0 was used for statistical analyses. The data were assessed for normality using visual and analytic methods. Continuous variables were defined as mean ± standard deviation and categorical variables were defined as percentages. In the comparison of continuous variables by groups, Student's t-test was used for the variables that showed normal distribution and the Mann-Whitney U test was used in the absence of normal distribution. In the assessment of the correlations between variables, Pearson's correlation test was used for variables with parametric distribution and Spearman's correlation test was used for those that had non-parametric distribution.

A series of linear regressions were conducted to determine whether disordered eating attitudes, as measured by the EDE-Q and DEBQ, and BED, as assessed by clinical interview, significantly contributed to the 24-hour urinary cortisol level, bone turnover markers, and BMD. Sex, age, height, weight, pubertal stage, CDI, and STAI-C scores were considered as covariates in the aforementioned models. In addition to the variables above, 25-OH vit D level was taken as a covariate in the linear regression models examining the contribution of disordered eating attitudes to bone turnover markers and BMD. Height and weight were used, rather than BMI, because height serves as an adjustment measure for bone size. A p-value of $< 0.05$ was considered statistically significant.

## Results

Mean age of the subjects was $14.01 \pm 1.59$ years. Forty-six (57.5%) of the subjects were girls. Mean BMI was found as $31.29 \pm 3.06$ kg/m². The relationship of disordered eating attitudes with 24-hour urine cortisol levels, bone turnover markers, femoral neck aBMD, and L1-4 vertebral aBMD values is shown in Table 1. A positive correlation was found between the 24-hour urinary cortisol level and DEBQ total score and DEBQ restrained eating subscale score ($p < 0.05$ for both). No significant difference was found between subjects with and without BED in terms of 24-hour urinary cortisol levels.

No significant difference was found between subjects with and without depression in terms of 24-hour urinary cortisol levels, bone marker levels, and BMDs. In Spearman's correlation test, no significant correlation was found between the level of anxiety and 24-hour urinary cortisol levels, bone marker levels, and BMD.

In the linear regression model running for determination of the contribution of the DEBQ total score to the urine free cortisol level, the DEBQ total score ($\beta = 1.008$, $p = 0.035$) was the only significant contributor to the model (adjusted $R^2 = 0.124$), which means that an increase of one unit in the DEBQ total score led to an increase of 1.008 units in 24-hour urinary cortisol level (Table 2).

In the linear regression model running for determination of the contribution of the DEBQ restrained eating score to the urine free cortisol level, the DEBQ eating restrained score ($\beta = 2.296$, $p = 0.014$) and age ($\beta = 12.067$, $p = 0.018$) were the significant contributors to the model (change in model $R^2 = 0.143$), which means that an increase of one unit in the DEBQ restrained eating subscale score led to an increase of 2.29 units in 24-hour urinary cortisol level (Table 3).

The femoral neck aBMDs of the subjects who had BED were found significantly higher compared with the femoral neck aBMDs of subjects without BED ($p = 0.049$). Although this p-value was lower than the value which was considered as a statistical significance limit, it should be approached with suspicion, because the confidence interval values of both groups were overlapped (Table 4). In the linear regression model in which gender, age, height, weight, pubertal stage, vitamin D, CDI, and STAI-C scores were considered as covariates, the contribution of BED to femoral neck aBMD was investigated. When the beta coefficients were examined, no association was found between BED and femoral neck aBMD ($p = 0.896$).

## Discussion

In this study, we investigated the associations of disordered eating attitudes with cortisol, bone markers, and BMD. Significant associations were found between the DEBQ total score and DEBQ restrained eating subscale score. As a biological stress marker, 24-hour urinary cortisol was also found to be associated with these scores. In the literature, the results of studies that investigated the relationship between disordered eating attitudes and cortisol level were variable (5,6,8,9,10,11). In some studies conducted with pre- and postmenopausal women, cortisol levels were found to be higher in subjects who showed restrained eating attitudes compared with those without restrained eating attitudes (5,6). This finding was related with increased activation of the hypothalamo-pituitary-adrenal (HPA) axis caused by restrained eating attitudes by way of stress (5). On the

**Table 1. Correlations of disordered eating attitudes (measured by Dutch Eating Behavior Questionnaire and Eating Disorder Evaluation Questionnaire) with cortisol, bone turnover, and bone mineral density (Pearson's correlation test)**

| | Range, mean | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Urine free cortisol | 6.72-333.84 µg/24 h, 109.58 | - | | | | | | | | | | | | | |
| 2. Osteocalcin | 10.09 - 329.21 ng/mL, 50.78 | -.004 | - | - | | | | | | | | | | | |
| 3. Bone specific alkaline phosphatase | 2.64 - 1481.84U/L, 340.07 | -.042 | **.919**** | - | | | | | | | | | | | |
| 4. N-Telopeptide 24 hours | 0 - 8891.96 nMBCE/mM creatinine, 761.02 | **.054** | **.046** | **.075** | - | | | | | | | | | | |
| 5. L1-4 vertebral aBMD | .628 - 1.196 g/cm$^2$, 0.903 | -.010 | .171 | .204 | -.028 | - | | | | | | | | | |
| 6. Femoral neck aBMD | 0.695 - 1.241g/cm2, 0.885 | -.039 | **.192** | .241* | **.077** | .668** | - | | | | | | | | |
| 7. DEBQ restrained eating | 4-40, 27.53 | **.227*** | -.140 | -.127 | .113 | -.104 | -.119 | - | | | | | | | |
| 8. DEBQ emotional eating | 0-61, 23.39 | .118 | .180 | .149 | .053 | .181 | .039 | -.154 | - | | | | | | |
| 9. DEBQ external eating | 11-47, 24.44 | .080 | 0.068 | 0.009 | -.168 | .177 | .088 | **-.273**** | **.363**** | - | | | | | |
| 10. DEBQ total score | 31-116, 75.36 | .225* | .104 | .058 | .011 | .171 | .017 | .198 | **.843**** | **.616**** | - | | | | |
| 11. EDE-Q restraint | 0-6, 1.82 | **.128** | **.069** | **.046** | **.143** | **.066** | .121 | **.438**** | .029 | .005 | .216 | - | | | |
| 12. EDE-Q eating concern | 0-5.6, 1.61 | .159 | .126 | .055 | .005 | .010 | .123 | .137 | **.268**** | .036 | **.276**** | **.436**** | - | | |
| 13. EDE-Q shape concern | 0-5.88, 3.42 | -.130 | .054 | -.048 | .026 | .001 | .003 | .212 | .312** | -.021 | **.314**** | **.383**** | **.660**** | - | |
| 14. EDE-Q weight concern | 0.4-5.8, 3 | **-.149** | **.068** | **.006** | **.013** | **-.047** | -.004 | .161 | .200 | -.096 | .174 | **.357**** | **.617**** | **.847**** | - |
| 15. EDE-Q total score | 0.25-5.02, 2.47 | .010 | .098 | .018 | .055 | .001 | .067 | **.289**** | **.250*** | -.013 | **.306**** | **.667**** | **.842**** | **.884**** | **.858**** |

aBMD: areal bone mineral density, DEBQ: Dutch Eating Behavior Questionnaire, EDE-Q: Eating Disorder Evaluation Questionnaire, *p < 0.05, **p < 0.01

other hand, some studies conducted on adult women and adolescent girls showed no such correlation (8,9). In the study of Schvey et al (11) conducted on obese adolescents, no relationship was found between cognitive eating restraint as assessed by the results of the Eating Disorder Examination interview and the 24-hour urinary cortisol levels. However, the 24-hour urinary cortisol level was found to show an increase as the level of weight concern increased. This finding was interpreted to mean that cognitive eating restraint did not cause stress in the study participants, but that it actually helped treatment because the participants were obese adolescents who were in search of treatment. It also suggested that the main source of stress in these individuals was their body weight (11). In contrast to the results reported by the above authors, the fact that we found significant associations between the increase in 24-hour urinary cortisol level and DEBQ total score and DEBQ restrained eating subscale score suggests that disordered eating attitudes (restrained, emotional, external eating), and especially restrained eating, causes an increase in endogeneous cortisol production as a the main source of stress.

**Table 2. Multiple linear regression analysis: Dutch Eating Behavior Questionnaire total score and other contributors of 24-hour urine free cortisol level**

| Variable | Beta | Significance |
|---|---|---|
| Sex | -29.073 | 0.058 |
| Age | 10.117 | 0.050 |
| Height | -0.944 | 0.528 |
| Weight | -0.061 | 0.946 |
| Pubertal stage | 11.295 | 0.169 |
| Depression | -0.924 | 0.449 |
| Anxiety | 0.219 | 0.861 |
| DEBQ total score | 1.008 | 0.035 |

DEBQ: Dutch Eating Behavior Questionnaire

**Table 3. Multiple linear regression analysis: Dutch Eating Behavior Questionnaire restrained eating score and other contributors of 24-hour urine free cortisol level**

| Variable | Beta | Significance |
|---|---|---|
| Sex | -22.232 | 0.132 |
| Age | 12.067 | 0.018 |
| Height | -1.299 | 0.376 |
| Weight | 0.463 | 0.596 |
| Pubertal stage | 10.626 | 0.190 |
| Depression | -0.693 | 0.565 |
| Anxiety | 0.742 | 0.539 |
| DEBQ restrained eating score | 2.296 | 0.014 |

DEBQ: Dutch Eating Behavior Questionnaire

In our study, no significant difference was found between the subjects with and without depression in terms of 24-hour urinary cortisol levels, bone markers, and BMD values. Depression was found to increase cortisol levels and/or negatively affect bone health in some studies conducted with adult women and adolescents (21,22,23,24). Another study in adult women failed to demonstrate any effect of depression on cortisol level and bone health (31). The investigators who found the cortisol level to be increased in individuals with depression explained this finding by increased activation of the HPA axis (21,23). In a study which reported that depression had no effect on the cortisol level and bone health, the authors related this finding to the fact that their patient group had mild or moderate depression and thus the HPA axis was not activated (31). When Mathew et al (32) reevaluated subjects ten years after they were diagnosed as having major depression in adolescence, in whom no relation was found between depression and serum cortisol level, the authors showed that the HPA was abnormal and cortisol levels were increased in those who attempted suicide. This finding suggested that factors including severity of depression, duration of depression, and an accompanying diagnosis of psychosis also had an impact on cortisol secretion (32). Schvey et al (11) conducted a study with obese adolescents

**Table 4. Comparison of areal bone mineral density values according to binge eating disorder status**

| Variables | | Lumbar 1-4 vertebral aBMD (g/cm²) | | p value* | Femoral neck aBMD (g/cm²) | | | p value* |
|---|---|---|---|---|---|---|---|---|
| | | Mean | ± SD | | Mean | ± SD | CI** (lower -upper) | |
| Binge eating disorder | Yes | 0.920 | 0.147 | 0.490 | 0.927 | 0.136 | 0.868-0.986 | 0.049 |
| | No | 0.895 | 0.138 | | 0.868 | 0.112 | 0.837-0.898 | |

*Student's t-test, **CI: Confidence interval

aBMD: areal bone mineral density, SD: standard deviation, CI: confidence interval

and showed that depression had no effect on cortisol levels and bone health, and related this finding to the fact that the prevalence of depression was low (5.8%) in the subjects included in their study. In our study group, the prevalence of depression was considerably high (25%), but it could not be shown that depression, as a cause of stress, affected the 24-hour urinary cortisol level. This finding may be related with the fact that none of our patients were diagnosed with severe depression (need for hospitalization) or that the depression periods were short, because the subjects were in adolescence and the HPA axis was not yet activated.

In our study, no correlation was found between the level of anxiety and 24-hour urinary cortisol levels, bone markers, and BMD. In a study conducted on adults, it was found that the 24-hour urinary cortisol level increased as the level of anxiety increased, a finding interpreted as an effect of anxiety on the activity of the sympathetic nervous system (23). In a study conducted with adolescent girls, anxiety was found to negatively affect bone health (24). In contrast, another study conducted with adolescents found no correlation between the level of anxiety and cortisol, findings similar to our results. This lack of correlation was related with the fact that the effect of anxiety on the HPA axis did not develop until adulthood, similar to the relationship between depression and cortisol levels (33). Similarly, the anxiety level in our subjects probably did not affect the HPA axis and did not disrupt bone health, because they were still in their adolescent years.

In this study, no significant associations were found between disordered eating attitudes, as evaluated with EDE-Q and DEBQ, and L1-4 vertebral and femoral neck aBMD, whereas the femoral neck aBMDs of subjects who had BED were found to be significantly higher compared with those who had no BED ($p = 0.049$). The number of studies investigating the relationship between disordered eating attitudes and bone health are limited and the results are variable (8,9,10,11,34,35). Some studies reported that restrained eating in adult women and adolescent girls disrupted bone turnover (8,9) and decreased BMD or BMC (8,9,10,34,35). In the study by Barrack et al (34) with adolescent female athletes, it was shown that restrained eating was the disordered eating attitude that negatively affected bone health to the greatest extent. In this same study, whole-body and lumbar vertebral BMD z-scores were also found to be higher in subjects who had BED compared with those without BED, a finding which is in line with our results. This finding may be attributed to the fact that these individuals consumed large quantities of food during a given period of time and this behavior, if practiced frequently, might have prevented development of chronic energy deficiency (34). No studies in the literature have investigated bone health in obese adolescents with BED. Additional studies are needed in this area to confirm the accuracy of our results. Two studies have investigated the relationship between disordered eating attitudes other than BED and bone health in obese adolescents and adults (11,35). Schvey et al (11) found no association between restrained eating attitude and bone health in obese adolescents, whereas it was reported that BMD decreased as body shape concern increased. Based on this finding, the investigators thought that psychological distress caused by body shape concern affected bone density (11). There are also studies showing that obesity increases bone mass (13,14). In one study, the BMD z-scores of obese adolescents were found to be higher compared with adolescents with a normal body weight, despite their low levels of 25-OH vit D and of physical activity (13). It was thought that leptin levels were high in obese individuals due to their high amount of biologically active adipose mass, and leptin was thought to be the cause of the increased BMD (13). In addition, higher insulin levels were reported in obese individuals compared with individuals with normal body weight; it was thought that insulin could lead to an increase in BMD through a direct anabolic effect on bone by way of bone receptors (13). In another study, it was found that bones were wider and stronger in obese adolescent boys compared with controls, and it was proposed that this was related with increased mechanical load on the bones in obese individuals and with the peripheral impact of estradiol (14). The results of our study suggest that the negative effects of disordered eating attitudes on bone health could have been masked with the bone mass increasing effect of obesity, as reported in the literature (13,14). We think that follow-up studies with larger sample sizes investigating the relationship between disordered eating attitudes and bone health in obese adolescents are needed. To date, only a limited number of studies have examined these parameters in obese adolescents and adults, and the study groups were small (11,35). The first strength of our study is that BMD was measured using DEXA which is the most commonly used measurement technique for BMD and has the largest normal database (36). The assessment of stress with 24-hour urine cortisol levels and the fact that our study group comprised both girls and boys were other strengths of our study. Our study was a cross-sectional study, the number of participants was limited, and the study group was composed of obese adolescents who presented to our outpatient clinic in order to lose weight; these may be considered among the limitations of our study. Despite the lack of apparent effects on bone turnover and BMD in our obese adolescents at the time of the study, our results suggest that disordered eating attitudes, and

restrained eating attitude especially, might be a source of stress. Thus, studies investigating long-term bone health in obese individuals with disordered eating attitudes may be useful in this respect. In addition, screening of obese adolescents in terms of disordered eating attitudes might be recommended.

### Ethics

**Ethics Committee Approval:** Ethics Committee approval was obtained for the study from the Cerrahpaşa Faculty Clinical of Medicine Research Ethics Committee (date: 12.07.2013, number: 18857). A second approval was obtained from the same Committee (date: 11.03.2014, number: 6281) for the addition of the State-Trait Anxiety Inventory for Children and psychiatric face-to-face evaluation for binge eating disorder.

**Informed Consent:** Detailed information about the study was given to the subjects who accepted to participate in the study as well as to their parents and they were asked to sign an informed consent form.

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

### Authorship Contributions

Concept: Aslı Okbay Güneş, Müjgan Alikaşifoğlu, Design: Aslı Okbay Güneş, Müjgan Alikaşifoğlu, Oya Ercan, Türkay Demir, Data Collection and Processing: Aslı Okbay Güneş, Ezgi Şen Demirdöğen, Ethem Erginöz, Mine Kucur, Analysis and Interpretation: Ethem Erginöz, Aslı Okbay Güneş, Müjgan Alikaşifoğlu, Literature Research: Aslı Okbay Güneş, Müjgan Alikaşifoğlu, Ezgi Şen Demirdöğen, Writing: Aslı Okbay Güneş, Müjgan Alikaşifoğlu, Oya Ercan.

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# Phenotype Heterogeneity in Glucokinase–Maturity-Onset Diabetes of the Young (GCK-MODY) Patients

**Anna Wędrychowicz[1]*, Ewa Tobór[2]*, Magdalena Wilk[2]*, Ewa Ziółkowska-Ledwith[2], Anna Rams[2], Katarzyna Wzorek[2], Barbara Sabal[2], Małgorzata Stelmach[1], Jerzy B. Starzyk[1]**

*[1]Polish-American Pediatric Institute, Jagiellonian University Collegium Medicum, Department of Pediatric and Adolescent Endocrinology, Cracow, Poland*

*[2]Polish-American Pediatric Institute, Jagiellonian University Collegium Medicum, Students' Scientific Group at the Department of Pediatric and Adolescent Endocrinology, Cracow, Poland*

**Equal main/first authors*

### What is already known on this topic?

Monogenic glucokinase-maturity-onset diabetes of the young (GCK-MODY) is the second most common type of diabetes mellitus (DM) after type 1 DM in a population of children and adolescents in Central Europe. Since it has been possible to genetically test patients with DM, the number of CGK-MODY patients in Poland has been increasing.

### What this study adds?

This paper presents the detailed clinical presentation of GCK-MODY patients. Only 32% of all analyzed GCK-MODY carriers fulfilled DM diagnostic criteria, the rest presented with impaired fasting glucose or glucose intolerance. Our clinical data could help to identify GCK-MODY patients among patients with DM. The proper diagnosis could avoid insulin therapy in young patients which had previously been misdiagnosed as type 1 DM.

## Abstract

**Objective:** The aim of the study was to evaluate the clinical phenotypes of glucokinase-maturity-onset diabetes of the young (GCK-MODY) pediatric patients from Southwest Poland and to search for phenotype-genotype correlations.

**Methods:** We conducted a retrospective analysis of data on 37 CGK-MODY patients consisting of 21 girls and 16 boys of ages 1.9-20.1 (mean 12.5 ± 5.2) years, treated in our centre in the time period between 2002 and 2013.

**Results:** GCK-MODY carriers were found in a frequency of 3% among 1043 diabetes mellitus (DM) patients and constituted the second most numerous group of DM patients, following type 1 DM, in our centre. The mean age of GCK-MODY diagnosis was 10.4 ± 4.5 years. The findings leading to the diagnosis were impaired fasting glucose (IFG) (15/37), symptoms of hyperglycemia (4/37), and a GCK-MODY family history (18/37). Mean fasting blood glucose level was 6.67 ± 1.64 mmol/L. In the sample, there were patients with normal values (4/37), those with DM (10/37), and IFG (23/37). In OGTT, 120 min glucose level was normal in 8, diabetic in 2, and characteristic for glucose intolerance in 27 of the 37 cases. Twelve of the 37 cases (32%) were identified as GCK-MODY carriers. In the total group, mean C-peptide level was 2.13 ± 0.65 ng/mL and HbA1c was 6.26 ± 0.45% (44.9 ± -18 mmol/mol). Thirty-two patients had a family history of DM. DM autoantibodies were detected in two patients. The most common mutations were p.Gly318Arg (11/37) and p.Val302Leu (8/37). There was no correlation between type of mutations and plasma glucose levels.

**Conclusion:** The phenotype of GCK-MODY patients may vary from those characteristic for other DM types to an asymptomatic state with normal FG with no correlation with genotype.

**Keywords:** Glucokinase-Maturity-Onset Diabetes of the Young, GCK-MODY, children, adolescents, genotype, phenotype

**Address for Correspondence:** Anna Wedrychowicz MD, Polish-American Pediatric Institute, Jagiellonian University Collegium Medicum, Department of Pediatric and Adolescent Endocrinology, Cracow, Poland
**Phone:** +48 12 658 12 77 **E-mail:** anna.wedrychowicz@uj.edu.pl **ORCID ID:** orcid.org/0000-0003-0864-6810

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## Introduction

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes inherited in an autosomal dominant way (1,2). There are over 800 known mutations associated with MODY and new ones are being discovered all the time (3). Glucokinase-maturity-onset diabetes of the young (GCK-MODY), also known as MODY type 2, is the most common type of the monogenic diabetes in Poland (4), and, along with a HNF1A- MODY, is one of the most common in the world. It is caused by a heterozygous mutation in the glucokinase gene on chromosome 7 (5). Glucokinase in the pancreatic beta cells senses increased blood glucose levels and controls the release of insulin. The heterozygous mutation in the glucokinase-coding gene results in a changed insulin threshold and therefore persistent hyperglycemia (6). As the hyperglycemia is mild and does not progress or cause any long-term complications, it may remain unnoticed (7).

GCK-MODY patients are usually non-obese, do not require treatment, and do not have vascular complications. This is the reason why it is important to differentiate this type from diabetes mellitus type 1 and type 2 (DM1 and DM2) in order to avoid unnecessary treatment (3,8,9,10). It should also be kept in mind that at the beginning, MODY was considered as a rare form of diabetes, however, it is probably much more common than assumed but often remains undiagnosed. By spreading knowledge of the existence of groups of diabetes such as MODY, and through the possibility of molecular testing, we should be able to change this situation.

The aim of this present study was to evaluate the clinical phenotype of GCK-MODY patients from Southwestern Poland treated in our department and also to search for phenotype-genotype correlations.

## Methods

For this retrospective analysis, of all 1043 patients with DM treated in the Department of Pediatric and Adolescent Endocrinology in Cracow, we selected 37 (21 girls and 16 boys) aged between 1.92 and 20.1 years, with a mean age of $12.5 \pm 5.2$ years, and with genetically confirmed GCK-MODY, which were included in the study. All participants and/or their parents gave their written informed consent to use their clinical data in scientific publications. All patients had been treated in our department in the years 2002-2013.

The following data were analyzed in details: age at GCK-MODY diagnosis, anthropometric data at diagnosis and during treatment, signs and symptoms at the time of diagnosis, medical history including course of pregnancy, birth parameters, and family history. Results of oral glucose tolerance test (OGTT), C-peptide, HbA1c, lipid profile, autoantibodies, and presence of other co-morbidities were also analyzed.

The clinical molecular testing was performed in an approved laboratory, with results interpreted by a board-certified clinical molecular geneticist or molecular genetic pathologist or the equivalent, in accordance with the American College of Medical Genetics (ACMG) Standards and Guidelines 2015 (11). The gene mutations were assessed in the Laboratory of Immunopathology and Genetics, Medical University of Lodz, Poland, which has achieved the International Quality Certificate ISO 9001:2008, a certificate of the Polish Society of Human Genetics. Molecular testing was performed by DNA sequencing performed using fluorescent-labeled terminating deoxynucleoside triphosphates with gene-specific oligonucleotide primers and multiplex ligation-dependent probe amplification to detect ezon deletions (MRC-Holland, Amsterdam, The Netherlands). Details of the molecular methods used in our patients were reported previously (4,12). The molecular testing was also performed in family members of patients including parents, siblings, and grandparents with impaired fasting glucose (IFG), glucose intolerance (GI), or DM.

Anthropometric measurements were taken in all patients. Height was measured to the nearest millimeter using a rigid stadiometer. Weight was measured to the nearest 0.1 kg using a calibrated balance scale. Reference data for Polish children were used for assessment (13). Body mass index (BMI) was calculated as weight in kilograms (kg) divided by the square of the height in meters ($m^2$).

The statistical analysis was performed using Statistica/MS Exel programs. Student's t-test was used to compare the analyzed groups. A p-value $< 0.05$ was considered as statistically significant.

## Results

GCK-MODY carriers amounted for 3% of all DM patients in our center (37/1043). The mean age at diagnosis was $10.4 \pm 4.5$ years. The suspicion of GCK-MODY was based on different heterogeneous signs, symptoms, and results of laboratory tests. 14/37 patients presented with an IFG, 4/37 patients were admitted with symptoms of hyperglycemia (polydipsia, polyuria, fatigue), 1 patient was obese and presented with IFG, and 18/37 patients had had monogenic DM previously diagnosed in their family. Before the confirmation of GCK mutation, 9/37 patients were treated as DM1 and in 6/37 cases insulin had been administered.

The mean serum fasting glucose level was $6.67 \pm 1.64$ mmol/L and the level ranged between 5.2 and 9.2 mmol/L.

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According to their blood glucose levels, the patients could be divided into three groups: those (4/34 patients) with normal values with a glucose level below 5.5 mmol/L (100 mg/dL), those (10/34 patients) with a glucose level above 6.9 mmol/L (125 mg/dL) characteristic for DM, and the remaining group (20/34) with an IFG 5.5-6.9 mmol/L (100-125 mg/dL). The profiles for OGTT results were also variable. The 120 min glucose level was normal in 8/26 patients and lower than 7.8 mmol/L (140 mg/dL). Two of the 26 patients had a result characteristic for DM, namely, higher than 11.1 mmol/L (200 mg/dL), and the remaining patients (16/26) had GI with values between 6.9 and 11.1 mmol/L (140-200 mg/dL). Thus, 12 of the 37 patients (32%) who were CGK-MODY carriers fulfilled the criteria of DM according to the Polish Diabetes Association recommendations (14). This figure is equivalent to 12/1043 (1.15%) of all patients with DM in our center.

The mean fasting C-peptide level was $2.13 \pm 0.65$ ng/mL (normal ranges 0.2-4.2 ng/mL) and in 18/19 cases the result was above 0.75 ng/mL. Mean HbA1c level at diagnosis was $6.26 \pm 0.45$ % (44.9 mmol/mol). This changed during the diet alone treatment into $6.13 \pm 0.39$ % (43.5 mmol/mol) and the difference between the results was statistically significant ($p < 0.013$). Six patients (6/37) had an elevated LDL cholesterol and five patients (5/37) had elevated total cholesterol levels. The mean levels of total cholesterol ($4.58 \pm 1.02$ mmol/L), LDL cholesterol ($2.52 \pm 1.02$ mmol/L), high-density lipoprotein (HDL) cholesterol ($1.4 \pm 0.23$ mmol/L), and triglycerides ($0.99 \pm 0.35$ mmol/L) were within normal ranges.

One patient was obese and one was underweight, the rest had BMI results normal for age. Two children (siblings) were diagnosed because of short stature and in both cases, pathological causes of short stature were excluded. Height of the other children were within normal ranges for age and compatible with their mid-parental height values.

Eighteen of the mothers of these children (18/37) had a confirmed GCK mutation, usually during family genetic tests. Two mothers (2/37) are being treated for DM2. Six mothers (6/37) were diagnosed with diabetes during pregnancy, found through routine screening, and all of them are GCK mutation carriers. In two of their children (2/37), birth weights were greater or equal to 4000 g. In both of these cases, children had the same GCK mutations as their mothers. Three patients (3/37) were small for gestational age babies (15). All these patients got their mutations from their fathers. Two of them were twins.

The patients presented highly incriminating family histories. In the case of 32 patients (32/37) one of the parents (18 mothers, 12 fathers) was diagnosed with diabetes GCK-MODY and had the same mutation as her/his child. A clear autosomal dominant mode of inheritance was presented. In five cases (5/37), the histories are not known. In an interview with 21 patients (21/37), diabetes also appears in one of the grandparents. The great-grandparents of 4 patients (4/37) had a confirmed GCK mutation.

Autoantibodies typical for DM1 such as islet cell autoantibodies (ICA), insulin autoantibodies, glutamic acid decarboxylase (GAD) autoantibodies, tyrosine kinase autoantibodies, were detected in 2 patients (2/37). One of these two patients had all four above-mentioned antibodies positive, while only ICA autoantibodies were positive in the second patient. Autoimmune diseases were not observed in this group of patients. Three patients (3/37) had Gilbert disease.

Thirteen mutations of the GCK gene were identified. Eleven (11) of these were missense mutations, one nonsense mutation, and one deletion (Table 1). The most common were p.Gly318Arg (11/36) and p.Val302Leu (8/36). Seven subjects with the second mutation (7/8) are members of the same family.

The variability of glucose levels was also observed within the carriers of the same mutation (p.Gly318Arg, p.Val302Leu).

Regarding the OGTT profile, we observed two different patterns in p.Gly318Arg mutation carriers. In 3 of these 11 patients, the mean increase in plasma glucose level was 5.56 mmol/L and the 120 min result was typical for DM, whereas

**Table 1. Types of glucokinase mutations in our patients**

| Type of mutation/Protein effect | Number of patients |
|---|---|
| G > A p.(Gly318Arg) | 11 |
| G > C p.(Val302Leu) | 8 |
| C > T p.(Ser383Leu) | 4 |
| G > A p.(Gly44Asp) | 3 |
| delCTT p.(Ser212del) | 2 |
| C > A p.(Arg377Gln) | 1 |
| A > T p.(Asp198Val) | 1 |
| G > A p.(Gly176Arg) | 1 |
| G > T p.(Glu157Ter) | 1 |
| G > A p.(Val226Met) | 1 |
| G > A p.(Glu221Lys) | 1 |
| C > A p.(Arg37Gln) | 1 |
| T > C p.(Ser212Pro) | 1 |

in two other subjects (2/11), the mean increase was 1.45 mmol/L and at 120 min, the glucose level was within the normal range. At baseline, the glucose level was increased but similar in both groups (5.2-6.7 mmol/L) (Table 2).

A similar situation was observed in the carriers of p.Val302Leu mutation. Baseline glucose levels were elevated in all patients in this group, ranging from 5.5 mmol/L to 6.23 mmol/L (Table 3). Three patients (3/8) had a mean increase of 0.5 mmol/L in OGTT at 120 min and normal glucose levels, while two others (2/8) showed an increase of 2.95 mmol/L and the glucose levels suggest GI. Presented differences had no relationship with the age of the patients.

## Discussion

GCK-MODY is mostly described as an asymptomatic condition, with mild fasting hyperglycemia (5.5-8 mmol/L), minor postprandial glucose extrusion, and a family history of diabetes. Usually, only a proper diet is sufficient to maintain an appropriate glucose level and prevent diabetic complications (1,8,9,10). However, new studies have revealed that GCK-MODY patients are not such a homogeneous group and that their phenotypes may vary considerably depending on the type of mutation (16,17,18). Results of these studies indicate that about half of GCK-MODY patients fulfill the criteria of DM, while the rest present with IFG or GI (16). Missense mutations have variable effects on glucokinase activity ranging from a small change in affinity for glucose to complete inactivity (19). Analysis of the clinical data of our patients led to similar conclusions. The mean features of the whole group were similar to those described in the literature, although findings pertaining to characteristics such as autoantibodies, obesity, fasting glucose levels were radically different in some of the patients from those characteristics for other types of diabetes. Compared to previous reports, we observed a lower percentage (32% vs. 50%) of GCK-MODY carriers fulfilling the criteria of DM. OGTT results of patients with two of the most frequent missense mutations (p.Gly318Arg, p.Val302Leu) show that their affinity for glucose does not correlate to the type of the mutation and even within the same mutation, postprandial glucose levels may vary significantly. There is no strong correlation between certain types of mutations and plasma glucose levels, although the threshold of insulin secretion is hypothetically the same. Differences in insulin sensitivity (20,21), diet, and physical activity might be probable reasons for those findings. Potential roles of other genes that modulate GCK function are also possible, considering that the GCKR regulatory protein gene has already been shown to interact with polymorphisms with GCK in a clinically significant way (22,23). GCK mutations affect not only the pancreatic function of this enzyme but the liver function too, where the decrease in the glycogen synthesis and storage, as well as increase in glucogenesis after standard meals, is reported. This defect in hepatic glucose metabolism contributes to postprandial hyperglycemia of GCK-MODY patients (24,25,26).

Our study reports the clinical presentation of 37 patients with confirmed GCK-MODY from a single, pediatric centre in Central Europe. The analysis of the clinical data includes a detailed process of diagnostics and also the assessment of the results of GCK-MODY treatment. The diagnosis of GCK-MODY was suspected on the basis of atypical signs and symptoms of diabetes, or atypical results of treatment, or previous family history of GCK-MODY. The limitation of the study is a lack of genetic tests in the whole group of 1043 patients with DM treated in our department due to financial limitations.

Recent studies report that specific gene mutations can present clinically as a neonatal form as well as 'type 2-

**Table 2. Oral glucose tolerance test profile in p.Gly318Arg mutation carriers**

| Patients with p.Gly318Arg mutation | Blood glucose (mmol/L) 0 min | Blood glucose (mmol/L) 120 min | Increase of blood glucose in OGTT (mmol/L) |
|---|---|---|---|
| 1 | 6.7 | 12.2 | 5.5 |
| 2 | 5.2 | 10.9 | 5.7 |
| 3 | 5.8 | 11.3 | 5.5 |
| 4 | 6.4 | 7.5 | 1.1 |
| 5 | 6 | 7.8 | 1.8 |

OGTT: oral glucose tolerance test

**Table 3. Oral glucose tolerance test profile in p.Val302Leu mutation carriers (blood glucose)**

| Patients with p.Val302Leu mutation | Blood glucose (mmol/L) 0 min | Blood glucose (mmol/L) 120 min | Increase of blood glucose in OGTT (mmol/L) |
|---|---|---|---|
| 1 | 5.5 | 9.3 | 3.8 |
| 2 | 6.2 | 8.3 | 2.1 |
| 3 | 6.2 | 6.7 | 0.5 |
| 4 | 5.6 | 6.1 | 0.5 |
| 5 | 6.1 | 6.6 | 0.5 |

OGTT: oral glucose tolerance test

like' or 'type 1-like' forms during adulthood (27) which makes diagnosis very difficult especially when symptoms, laboratory tests, and phenotype correspond to 'double' diabetes or even 'triple' diabetes with features of DM1, DM2, and presence of monogenic mutation. It is not an exception that patients with GCK-MODY are diagnosed as DM1 and treated with insulin. According to the literature, those subjects require higher than replacement doses to improve metabolic control of DM (28) and it may be a reason for their physicians to extend their diagnosis. In our group of patients, two boys presented with such inexplicably high requirement for insulin, whereas in another four children, the requirement for insulin was lower than that of other patients with DM1. These facts together with a strong positive family history of DM should also lead to a suspicion of monogenic diabetes.

Despite wide ranging glucose levels, HbA1c values were similar in all patients and mean levels were below the 10$^{th}$ percentile for diabetic children (22). Furthermore, we observed a significant reduction of HbA1c levels after treatment with diet alone. Reported fasting C-peptide levels above 0.75, persisting for 3-5 years from diagnosis, are suggestive of DM2 or monogenic diabetes. Similar values are consistent with short-term insulin independence in an individual who has not previously 'failed' non-insulin therapy but may occur in the DM1 diabetes honeymoon period (29).

The prevalence of dyslipidemia encountered in some patients may occur in GCK-MODY but is characteristic rather for DM2 (19,30). The carriers of GCK mutations usually show lower levels of fatty acids and triglycerides in circulation than the healthy population (31). Reduced GCK activity is likely to reduce glycolytic flux and production of both glycogen and malonyl-CoA. The latter is an important regulator of lipid metabolism; reduced levels alleviate inhibition of carnitine palmityl transferase 1, thereby increasing fatty acid oxidation. In addition, malonyl-CoA is the precursor of fatty acid synthesis; this will potentially also be reduced when GCK activity drops. Moreover, esterification of fatty acids into TAGs would be insufficient owing to reduced production of glycerol-3-phosphate via glycolysis. Thus, overall, hepatic fatty acid and TAG production and glucose metabolism would be decreased in the face of reduced GCK activity (31). Moreover, HDL cholesterol values measured in individuals with likely monogenic diabetes may be useful in screening for GCK-MODY and its differentiation from DM1 and HNF1A-MODY, regardless of treatment or metabolic control (12). The studies performed by Fendler et al (32) showed that individuals with GCK-MODY exhibit a strongly protective profile HDL cholesterol (high concentration of large HDL and low levels of intermediate and small HDL subpopulation). Next to constitutively moderately elevated glycemia observed in these patients, this lipid profile may be also a factor contributing to the low frequency of cardiovascular complications (32).

A twofold increase in incidence of Gilbert disease in our patients compared to the general population appears to be due to inter-family relationships in some of these individuals.

The positive GAD antibodies observed in two patients are not characteristic for GCK-MODY. The prevalence of GAD antibodies and a confirmed genetic diagnosis of MODY may represent the 1-2% of the population with detectable islet antibodies with no associated pathogenesis. In general, the finding of islet autoantibodies makes the diagnosis of MODY very unlikely, and genetic testing should only be performed if other clinical characteristics strongly suggest this form of diabetes rather than DM1 (33). Furthermore, positive diabetes autoantibodies can be transient in GCK-MODY patients and are not markers of prediabetes. It is possible that autoantibody titers are aggravated by obesity or by other factors, such as drugs (34).

Individuals with GCK-MODY are usually not obese and achieve normal growth. The imposition of obesity cannot be excluded, in view of the overall increase in obesity in all populations.

Large population cohort studies of pregnant women estimate the population prevalence of GCK-MODY as 1.1 in 1000 (35,36). The percentage of mutations in females diagnosed with diabetes in pregnancy could be significantly greater. Most importantly, maternal hyperglycemia in pregnancy is the primary risk factor for newborn macrosomia caused by fetal hiperinsulinism. GCK-MODY patients present with good, sometimes even high, insulin production and function but have increased set point stimulated insulin secretion. Usually babies with maternally inherited GCK-MODY and which have an increased set point stimulated insulin secretion, secrete a normal amount of insulin with maternal hyperglycemia, and have normal birth weights. Very high maternal hyperglycemia could result in fetal insulin hypersecretion, and ultimately, an overweight newborn, as was the case in two of our patients. The low birth weight observed in our three patients with paternally inherited GCK-MODY and non-diabetic mothers may be an effect of fetal hyperglycemia due to GCK mutation. Hyperglycemia in these cases is a result of low insulin secretion in the milieu of maternal normoglycemia due to increased set point stimulated insulin secretion.

According to the guidelines for GCK-MODY diagnosis, mild fasting hyperglycemia (5.5-8.0 mmol/L), small increase in OGTT (< 4.6 mmol/L) at 120 min, negative autoantibodies,

and MODY diagnosed in one of the parents are key indications for the genetic screening for GCK mutations. Our study shows that the diagnosis of this type of diabetes is more challenging in some cases. The phenotype of GCK-MODY patients may vary from one that is characteristic of other types of diabetes to an asymptomatic state with normal fasting glucose levels. Differences in insulin sensitivity, diet, and physical activity may be the probable causes of these findings. Potential roles of other genes modulating GCK function are also possible. The most important observation is that the proper diagnosis of GCK-MODY could lead to cessation of insulin treatment with improvement in the patients' quality of life.

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### Ethics

**Ethics Committee Approval:** Retrospective analysis study.

**Informed Consent:** All participants and/or their parents gave their written informed consent to use their clinical data in scientific publications.

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

### Authorship Contributions

Concept: Anna Wedrychowicz, Design: Anna Wedrychowicz, Ewa Tobór, Magdalena Wilk, Ewa Ziółkowska-Ledwith, Anna Rams, Katarzyna Wzorek, Barbara Sabal, Data Collection and Processing: Anna Wedrychowicz, Ewa Tobór, Magdalena Wilk, Ewa Ziółkowska-Ledwith, Anna Rams, Katarzyna Wzorek, Barbara Sabal, Małgorzata Stelmach, Analysis and Interpretation: Anna Wedrychowicz, Ewa Tobór, Magdalena Wilk, Ewa Ziółkowska-Ledwith, Anna Rams, Katarzyna Wzorek, Barbara Sabal, Małgorzata Stelmach, Literature Research: Anna Wedrychowicz, Ewa Tobór, Magdalena Wilk, Ewa Ziółkowska-Ledwith, Anna Rams, Katarzyna Wzorek, Barbara Sabal, Małgorzata Stelmach, Writing: Anna Wedrychowicz, Ewa Tobór, Magdalena Wilk, Ewa Ziółkowska-Ledwith, Anna Rams, Jerzy B. Starzyk.

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# The Relationship Between Perceived Family Climate and Glycemic Control in Type 1 Diabetes Mellitus Adolescent Patients

**Şafak Eray[1], Halit Necmi Uçar[1], Fatma Çetinkaya[2], Erdal Eren[3], Pınar Vural[4]**

*[1]Van Training and Research Hospital, Clinic of Child and Adolescent Psychiatry, Van, Turkey*
*[2]Taksim Training and Research Hospital, Clinic of Child Health and Diseases, İstanbul, Turkey*
*[3]Uludağ University Faculty of Medicine, Department of Pediatric Endocrinology, Bursa, Turkey*
*[4]Uludağ University Faculty of Medicine, Department of Child and Adolescent Psychiatry, Bursa, Turkey*

### What is already known on this topic?

Expressed emotion has been linked to poor glycemic control in the literature. Although some studies have reported a positive or negative correlation between emotional over-involvement and glycemic control, there are also studies in which no correlation was found.

### What this study adds?

In this study, the relationship between perceived expressed emotion and glycemic control in adolescents diagnosed with type 1 diabetes mellitus not accompanied by psychopathology will be investigated. To the best of our knowledge, no study concerning expressed emotion and glycemic control has been carried out in Turkey to date. We aimed to emphasize the role of family climate in the treatment of diabetic patients.

## Abstract

**Objective:** Type 1 diabetes mellitus (T1DM) is a chronic disease which ranks third in children under age 16 years. Expressed emotion (EE) is a term that indicates a specific family climate including lack of emotional support (LES), irritability, and emotional over-involvement. It is known that the family environment is highly important for glycemic control in diabetic adolescents. In this study, the relationship between perceived EE and glycemic control in adolescents diagnosed with T1DM not accompanied by psychopathology were investigated.

**Methods:** The study included 49 adolescents with T1DM and 50 adolescents as a control group. Adolescents with psychopathology and intellectual disability were excluded from the study. Perceived EE was measured by the Shortened Level of Expressed Emotion Scale (SLEES) and blood sugar regulation was assessed by HbA1c levels.

**Results:** The adolescents with T1DM showed a significant difference in perceived EE ($p = 0.020$) and LES ($p = 0.014$) when compared with the control group. When diabetic adolescents were compared among themselves, the diabetic adolescents with poor glycemic control perceived greater EE ($p = 0.033$) and less emotional support ($p = 0.049$). In regression analyses, the predictive power of mother's educational level, the employment status of mothers and the subscale "LES" of SLEES combined to explain HbA1c level was determined to be 37.8%.

**Conclusion:** The strong relationship between perceived EE and glycemic control showed us that perceived EE can hinder treatment compliance without causing psychopathology. For this reason, it is recommended that not only patients with psychopathology, but all diabetic adolescents receive psychosocial support and family interventions.

**Keywords:** Type 1diabetes mellitus, adolescents, perceived expressed emotion, glycemic control

**Address for Correspondence:** Şafak Eray MD,
Van Training and Research Hospital, Clinic of Child and Adolescent Psychiatry, Van, Turkey
**E-mail:** drsafakeray@gmail.com **ORCID ID:** orcid.org/0000-0002-4847-7751



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## Introduction

Adolescence is a transitional period from childhood to adulthood during which the individual has unique physical and psychological needs. Chronic illnesses such as diabetes mellitus hinder the physical and mental development of adolescents (1). Type 1 diabetes mellitus (T1DM) is a chronic disease which ranks third in frequency after asthma and cerebral palsy in children under 16 years of age (2). Chronic illnesses such as T1DM, which necessitate lifestyle changes, increase the likelihood of psychiatric disorders, including depression, in adolescents (3). This situation affects the compliance of patients with T1DM as well as the regulation of their blood sugar. It has been reported that family support is one of the most important indicators of compliance and treatment success (4,5,6,7,8).

Expressed emotion (EE) is an empirical concept that is accepted as a barometer of the emotional climate at home. This concept was formulated because of the strong relationship between environmental changes in the family system and the mental health of family members (9). EE is a measure of environmental stress at home, which is estimated by communication styles including the amount of criticism made by family members about the patients, the presence or absence of hostile attitudes, the level of intrusiveness, and emotional over-involvement (EOI) (10). EE is not only a predictor of psychological diseases but also may predict the state of physical disease (10). When the effects of family attitudes on glycemic control are taken into account, the relationship between T1DM and EE has captured the attention of the researchers (11,12,13,14,15). However, when the literature is examined, contradictory results are seen (12,13,14,15). In general, EE has been linked to poor glycemic control (11). Although some studies have reported a positive or negative correlation between EOI and glycemic control, there are also studies in which no correlation was found (12,13,14,15).

When these studies are examined, it is observed that the assessments were oriented toward the parents rather than the adolescents. Non-reliance on self-reporting by the adolescents and the failure to use assessments developed especially for adolescents can be viewed as a flaw of these studies. When the family climate is considered a reciprocal concept of emotional tone, the question of whether it is an individual characteristic of reciprocal interaction arises. How EE is perceived becomes important (16).

To the best of our knowledge, no studies concerning this subject have been conducted in Turkey to date. In this study, the relationship between perceived EE and glycemic control in adolescents diagnosed with T1DM not accompanied by psychopathology will be investigated. We aimed to emphasize the role of family climate in the treatment of diabetes.

## Methods

The study was carried out with 99 adolescents-49 T1DM patients and 50 healthy control subjects. The patient group consisted of T1DM cases who had been diagnosed in the Uludağ University Pediatric Endocrinology Department at least one year prior to the study. These adolescents were living with both their father and mother and had no apparent disease or condition, such as the use of exogenous steroids, to disrupt their blood sugar regulation. Psychopathology and mental retardation were accepted as exclusion criteria. All participants in the study and their parents gave informed consent after being informed of the methods and objectives of this study. The necessary legal permission and approval were obtained from the Uludağ University Faculty of Medicine Ethics Committee before proceeding to the data collection stage.

The participants were initially requested to fill out the sociodemographic form prepared by the researchers and then were evaluated using the Affective Disorders and Schizophrenia Schedule for School Age Children Present and Lifetime Version (K-SADS-PL), a form for detection of psychopathology used by child and adolescent psychiatrists. Cases diagnosed to have a mental disorder such as depression, an anxiety disorder, or an attention deficit hyperactivity disorder were excluded. The participants were evaluated using the WISC-R test, and those whose scores were less than 85 were also excluded. The Shortened Level of Expressed Emotion Scale (SLEES) was filled out by the adolescents under supervision of the researchers. One additional participant was also excluded from the study because of unreliable answers. Blood sugar regulation over the previous three months was assessed by glycosylated hemoglobin (HbA1c). All patients were on multiple daily injections of insulin.

The control group of children was matched with the patient group with respect to age, gender, parental employment status, education, and family structure. The control group consisted of 50 adolescents with no psychopathology or mental retardation and was selected from among adolescents who had presented with physical complaints to the pediatric department and who volunteered to participate to the study.

Glycemic control in DM was assessed in accordance with standards suggested by the International Society for

Pediatric and Adolescent Diabetes (ISPAD) in 2007, with optimal control defined as HbA1c < 7.5%, suboptimal control as 7.5-9%, and poor control as > 9% (17). The diabetic adolescents were divided into two groups based on their HbA1c levels, either above or below 9%, and each group was compared with the other group and the control group.

### Information Collection Form

The Information Collection Form was created by the researchers to collect information on the socio-demographic characteristics of the participants including age, gender, education level of the parents, employment status of the parents, number of siblings, birth order, economic status, as well as physical and mental health of family members. Socioeconomic status (SES) was determined based on the official starvation and poverty limits of 2015.

### Kiddie Schedule for Affective Disorders and Schizophrenia-Present and Lifetime Version (K-SADS-PL)

K-SADS-PL is a semi-structured diagnostic interview that was created to assess psychopathology in children and adolescents according to DSM-III and DSM-IV diagnostic criteria. It was developed by Kaufman et al (18) in 1997 and subsequently translated into Turkish. A validity and reliability study of the schedule for Turkish children was conducted by Gökler et al (19) in 2004. This tool serves to assess psychopathology in children and adolescents based on the data obtained by interviewing the child and his/her parents.

### Shortened Level of Expressed Emotion Scale in Adolescents

The scale developed by Nelis et al (20) was translated into Turkish by Vural et al (21) in 2013. SLEES consists of 33 items measuring the EE of the person perceived to be most important individual in the participant's life over the previous 3 months. The three subscales of the SLEES include lack of emotional support (LES), irritability, and intrusiveness. Higher scores indicate higher levels of EE.

### Data Analysis

Data were evaluated using IBM Statistical Package for the Social Sciences Statistics 22 statistical software package program. The mean and the standard deviation values with minimum and maximum levels were used for the statistical expression of the groups. While the comparison of the continuous variables between the groups was performed using the student's t-test for normally distributed variables, comparison of the abnormally distributed variables and non-parametric parameters was performed using the Mann-Whitney U test. For comparison of the categorical variables, a chi-square test was utilized. We used multiple linear regressions to examine HBA1C levels as predictors of the relationship between sociodemographic variables and EE scores. A p-value of $< 0.05$ was considered statistically significant.

## Results

The diabetic group and the control group were similar in age ($14.46 \pm 1.24$ years and $14.00 \pm 1.39$ years, respectively) ($t = 1.763$, $p = 0.081$). Fifty-one percent ($n = 25$) of the diabetic group and 60.0% ($n = 30$) of the control group were girls.

To compare the income level, the poverty line defined as a monthly income under 3300 Turkish lira per month by the Turkish Statistical Institute was used, and the groups were further divided into high and low socio-economic status groups. In terms of family income, the difference between the patient and control groups was not statistically significant ($\chi^2 = 2.541$, $p = 0.111$). Regarding the parents' cohabitation, all participants were living with both parents. To compare educational status, the parents were divided into two groups, as those who completed high school and those who did not. No significant difference was found between the two groups ($\chi^2 = 0.990$, $p = 0.320$) ($\chi^2 = 0.093$, $p = 0.761$). Similarly, the employment status of the parents did not show a statistically significant difference ($\chi^2 = 0.542$, $p = 0.461$) ($\chi^2 = 2.926$, $p = 0.087$) (Table 1).

The SLEES scores of the patient and control groups are shown in Table 2. There was a significant difference in the total perceived EE scores between the groups and in the subscale of LES scores. There were no differences between the two groups in the subscales of irritability and intrusiveness.

Mean HbA1c of diabetic adolescents was $10.36 \pm 2.62$%. The median value was 10.00, the minimum was 6.50, and the maximum value was 17.10. There was no statistically significant differences in HbA1c values of girls [standard deviation (SD) = $10.57 \pm 2.39$] and boys (SD = $10.15 \pm 2.89$) ($t = 0.547$, $p = 0.587$). In addition, there was no relationship between age and HbA1c values ($p = 0.368$).

The diabetic patients were divided into two groups, those with HbA1c levels at or below 9% (group 1) and those with levels above 9% (group 2), to assess the relationship between perceived EE and glycemic control. The mean age of the two groups was similar ($14.21 \pm 0.97$ and $14.63 \pm 1.37$ years, respectively). The female to male ratio in the two groups was 63.2% and 40.0%, respectively ($p = 0.114$).

Means of the SLEES by groups were: $X = 54.8$, $SD = 10.4$ for group 1 and $X = 64.4$, $SD = 19.6$ for group 2. An

**Table 1. Sociodemographic variables of the participants**

| n (%) | | Patient group | Control group | p-value |
|---|---|---|---|---|
| | | n (%) | | |
| Gender | Male | 24 (51.0) | 20 (40.0) | 0.369 |
| | Female | 25 (49.0) | 30 (60.0) | |
| Age (years) | | 14.46 ± 1.24 | 14.00 ± 1.39 | 0.081 |
| Family income | High SES | 5 (10.2) | 11 (22.0) | 0.111 |
| | Low SES | 44 (89.8) | 39 (78.0) | |
| Cohabitation of parents | Coexistence | 49 (100.0) | 50 (100.0) | |
| Mother's educational level | High school graduate | 7 (14.3) | 4 (8.0) | 0.320 |
| | Did not finish high school | 42 (85.7) | 46 (92.0) | |
| Mother's employment status | Unemployed | 31 (63.3) | 28 (56.0) | 0.461 |
| | Employed | 18 (36.7) | 22 (44.0) | |
| Father's educational level | High school graduate | 10 (20.4) | 9 (18.0) | 0.761 |
| | Did not finish high school | 39 (79.6) | 41 (82.0) | |
| Father's employment status | Unemployed | 5 (10.2) | 1 (2.0) | 0.087 |
| | Employed | 44 (89.8) | 49 (98.0) | |

SES: socioeconomic status

independent sample t-test was conducted to evaluate the differences between the two groups. There were significant differences in the perceived EE scores of the groups (t = -2.199) (p = 0.033). There was also a significant difference in the subscale of LES scores between the groups (t = -2.018) (p = 0.049). There were no differences in the subscales of irritability and intrusiveness between the groups (Table 3).

To evaluate the variables that influence HbA1c levels of the diabetic adolescents, regression analysis was performed. Starting with 10 different variables, we proceeded by eliminating the least significant variables (Table 4). All models were found to be significant, but some variables were not. The significant variables were educational level of mothers, employment status of mothers, and subscale LES of SLEES. It was observed that maternal educational level was negatively associated with HbA1c. The employment status of mothers was positively associated with HbA1c. An increase in HbA1c was found when the LES subscale scores of SLEES increased. In conclusion, the combined predictive power of these three variables to explain HbA1c levels was determined to be 37.8%.

**Table 2. Expressed emotion scores in the diabetic group and control group**

| | Diabetic group | Control group | p-value |
|---|---|---|---|
| | X ± SD | X ± SD | |
| Total Score | 60.7 ± 17.2 | 53.8 ± 10.5 | **p = 0.020** |
| LES | 28.9 ± 9.8 | 24.7 ± 6.3 | **p = 0.014** |
| Intrusiveness | 18.3 ± 6.5 | 16.3 ± 4.6 | p = 0.118 |
| Irritability | 13.4 ± 3.9 | 12.8 ± 2.8 | p = 0.340 |

LES: lack of emotional support, SD: standard deviation

**Table 3. Relationship between perceived expressed emotion and glycemic control in the diabetic group**

| | Group 1 | Group 2 | |
|---|---|---|---|
| | X ± SD | X ± SD | p-value |
| Total score | 54.8 ± 10.4 | 64.4 ± 19.6 | **0.033** |
| LES | 25.7 ± 7.1 | 30.9 ± 10.8 | **0.049** |
| Intrusiveness | 16.7 ± 4.2 | 19.3 ± 7.5 | 0.549 |
| Irritability | 12.4 ± 3.1 | 14.1 ± 4.3 | 0.135 |

LES: lack of emotional support, SD: standard deviation

**Table 4. Regression analysis of the variables influencing HbA1c**

| Model | Predictors | R2 | F | Model p | B | Predictor p |
|---|---|---|---|---|---|---|
| 1. | Gender | 0.397 | 2.507 | 0.020 | -0.431 | 0.548 |
| | Age | | | | -0.080 | 0.786 |
| | Mother education | | | | -1.286 | 0.008 |
| | Father education | | | | -0.107 | 0.818 |
| | Mother employment | | | | 1.831 | 0.053 |
| | Father employment | | | | 0.977 | 0.425 |
| | Monthly income | | | | -0.050 | 0.941 |
| | EE-LES | | | | 0.083 | 0.090 |
| | EE-Intrusiveness | | | | -0.002 | 0.981 |
| | EE-Irritability | | | | -0.046 | 0.692 |
| 2. | Gender | 0.397 | 2.859 | 0.011 | -0.433 | 0.536 |
| | Age | | | | -0.078 | 0.782 |
| | Mother education | | | | -1.284 | 0.007 |
| | Father education | | | | -0.108 | 0.811 |
| | Mother employment | | | | 1.832 | 0.050 |
| | Father employment | | | | 0.976 | 0.420 |
| | Monthly income | | | | -0.049 | 0.941 |
| | EE-LES | | | | 0.082 | 0.031 |
| | EE- Irritability | | | | -0.048 | 0.609 |
| 3. | Gender | 0.397 | 3.297 | 0.006 | -0.437 | 0.526 |
| | Age | | | | -0.077 | 0.781 |
| | Mother education | | | | -1.282 | 0.007 |
| | Father education | | | | -0.123 | 0.758 |
| | Mother employment | | | | 1.810 | 0.038 |
| | Father employment | | | | 0.978 | 0.413 |
| | EE-LES | | | | 0.083 | 0.027 |
| | EE-Intrusiveness | | | | -0.047 | 0.608 |
| 4. | Gender | 0.396 | 3.844 | 0.003 | -0.402 | 0.548 |
| | Mother education | | | | -1.267 | 0.006 |
| | Father education | | | | -0.125 | 0.753 |
| | Mother employment | | | | 1.805 | 0.036 |
| | Father employment | | | | 0.975 | 0.409 |
| | EE-LES | | | | 0.081 | 0.025 |
| | EE-Irritability | | | | -0.048 | 0.600 |
| 5. | Gender | 0.395 | 4.565 | 0.001 | -0.384 | 0.560 |
| | Mother education | | | | -1.362 | < 0.001 |
| | Mother employment | | | | 1.895 | 0.019 |
| | Father employment | | | | 0.835 | 0.439 |
| | EE-LES | | | | 0.079 | 0.025 |
| | EE-Intrusiveness | | | | -0.056 | 0.513 |
| 6. | Mother education | 0.390 | 5.493 | 0.001 | -1.381 | < 0.001 |
| | Mother employment | | | | 1.981 | 0.012 |
| | Father employment | | | | 0.706 | 0.500 |
| | EE-LES | | | | 0.077 | 0.027 |
| | EE-Intrusiveness | | | | -0.051 | 0.546 |
| 7. | Mother education | 0.385 | 6.872 | < 0.001 | -1.355 | < 0.001 |
| | Mother employment | | | | 1.938 | 0.013 |
| | Father employment | | | | 0.717 | 0.490 |
| | EE-LES | | | | 0.071 | 0.031 |
| 8. | Mother education | 0.378 | 9.106 | < 0.001 | -1.387 | < 0.001 |
| | Mother employment | | | | 1.963 | 0.011 |
| | EE-LES | | | | 0.073 | 0.026 |

EE: expressed emotion, LES: lack of emotional support

## Discussion

In our study, adolescents with diabetes mellitus showed significantly higher perceived EE and perceived LES when compared with the control group. When diabetic adolescents were compared among themselves, the diabetic adolescents with poor glycemic control perceived greater EE and LES from their families.

Factors that may affect family climate, such as integrity of the family structure, a history of psychiatric disease, or newly diagnosed diabetes which may be associated with poor glycemic control, were accepted as exclusion criteria. During psychiatric evaluations, psychopathology, as expected, was observed to be more frequent in the diabetic group than in the control group. Adolescents with psychopathology were excluded from the study.

Mean HbA1c levels in our subjects were similar to those found in other studies and there were no significant differences with respect to gender and age, again consistent with the literature (22). When we examined the factors that influence glycemic control, it was found that low socio-economic level affected HbA1c levels negatively, as expected (23,24). Our study also showed that high maternal educational level affected glycemic control positively. However, paternal educational level did not have the same effect. Mothers with higher educational levels can deal better with diabetes and its treatment requirements, which include physical activity, proper diet, and insulin dosage. The role of mothers in family structure has been emphasized in previous studies (22,25). Father's employment status was not determined to be an influencing factor. The results of our study were similar to those made in other countries. However, although the educational level of mothers was negatively associated with the Hba1c levels**,** the same was not true for maternal employment status. This situation can be explained by the lack of time that working mothers have to devote to regulating the lifestyle of their diabetic adolescents. However, this result, which may have been affected by sample size and socio-cultural characteristics, needs to be confirmed by further studies.

When diabetic adolescents and control groups were examined in terms of perceived EE, it was observed that diabetic adolescents perceived a higher EE than the control group. This result was mainly due to higher scores on the LES subscale, indicating that diabetic adolescents feel less emotional support. There are conflicting results in the literature concerning blood sugar regulation and EE. While Koenigsberg et al (12) stated that EE can predict glycemic control, Worrall-Davies et al (14) observed no relationship between the two. When the subscales of EE, EOI, and criticism were evaluated separately, there were also conflicting results regarding the relationship between glycemic control and EOI. While Stevenson et al (13) reported a positive association between glycemic control and EOI, Liakopoulou et al (15) found a negative correlation. Worrall-Davies et al (14) found no association between glycemic control and EOI. These conflicting results with regard to EOI in adolescents can be associated with the perception of EOI by adolescents. Excessive protection may be perceived negatively by adults, although children may perceive it in a positive way. No association between poor glycemic control and intrusiveness was found in our study. We also have observed no relationship between poor glycemic control and perceived irritability in our study.

In order to resolve the conflicting results regarding EOI, we used an assessment tool specially designed for adolescents, which had subscales including LES, irritability, and intrusiveness. We observed that LES and high EE were associated with poor glycemic control. There was no difference between the two groups in terms of irritability and intrusiveness.

The relationship between psychopathology and EE has been clearly demonstrated in several studies (26,27,28). It is also known that psychopathology rates are higher in patients with chronic diseases. Psychopathology affects glycemic control negatively, both because it is a source of chronic stress and because it tends to lower compliance rates. The aim of our study was to evaluate the effect of family climate on glycemic control by comparing diabetic adolescents without psychopathology to a control group.

The strengths of our study were the exclusion of psychopathology in both the control and diabetic groups based on clinical interviews and evaluating EE by using a self-report scale specially designed for adolescents. Limitations of our study were not evaluating for psychopathology in other family members by psychiatrists and the low number of the diabetic patients.

The strong relationship between perceived EE and glycemic control showed that it can hinder treatment compliance without causing psychopathology. For this reason, not only patients with psychopathology, but all diabetic adolescents require psychosocial support and family interventions. As psychiatric consultation may play a critical and positive role in the treatment of chronic diseases, we recommend that psychosocial interventions be part of any diabetes treatment. Although most diabetics do not have psychiatric disorders, psychiatric intervention is recommended to improve compliance by the diabetics themselves and their families. We suggest that our study be repeated with a larger sample size and longer follow-up with family intervention, as such studies are lacking in the literature.

### Ethics

**Ethics Committee Approval:** The necessary legal permission and approval were obtained from the Uludağ University Faculty of Medicine Ethics Committee before proceeding to the data collection stage (2015).

**Informed Consent:** All participants in the study and their parents gave informed consent after being informed of the methods and objectives of this study.

**Peer-review:** External and internal peer-reviewed.

### Authorship Contributions

Concept: Şafak Eray, Design: Halit Necmi Uçar, Data Collection or Processing: Fatma Çetinkaya, Analysis or Interpretation: Halit Necmi Uçar, Literature Search: Şafak Eray, Writing: Şafak Eray, Erdal Eren, Pınar Vural.

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SHORT COMMUNICATION

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# An *ABCC8* Nonsense Mutation Causing Neonatal Diabetes Through Altered Transcript Expression

**Sarah E. Flanagan[1], Vũ Chí Dũng[2], Jayne A. L. Houghton[1], Elisa De Franco[1], Can Thi Bich Ngoc[2], Annet Damhuis[1], Frances M. Ashcroft[3], Lorna W. Harries[1], Sian Ellard[1]**

*[1]University of Exeter Medical School, Institute of Biomedical and Clinical Science, Department of Molecular Genetics, Exeter, United Kingdom*
*[2]National Children's Hospital, Department of Endocrinology, Metabolism and Genetics, Hanoi, Vietnam*
*[3]University of Oxford, Henry Wellcome Centre for Gene Function, Department of Physiology, Anatomy and Genetics, Oxford, United Kingdom*

### What is already known on this topic?

The pancreatic ATP-sensitive $K^+$ (K-ATP) channel regulates insulin secretion. Gain-of-function mutations in the genes encoding the two subunits of the channel, *KCNJ11* and *ABCC8*, cause neonatal diabetes, whilst loss-of-function mutations in these genes result in congenital hyperinsulinism.

### What this study adds?

This is the first report of a loss-of-function mutation in *ABCC8* resulting in gain-of-channel function and neonatal diabetes.

## Abstract

The pancreatic ATP-sensitive $K^+$ (K-ATP) channel is a key regulator of insulin secretion. Gain-of-function mutations in the genes encoding the Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*) subunits of the channel cause neonatal diabetes, whilst loss-of-function mutations in these genes result in congenital hyperinsulinism. We report two patients with neonatal diabetes in whom we unexpectedly identified recessively inherited loss-of-function mutations. The aim of this study was to investigate how a homozygous nonsense mutation in *ABCC8* could result in neonatal diabetes. The *ABCC8* p.Glu747* was identified in two unrelated Vietnamese patients. This mutation is located within the in-frame exon 17 and RNA studies confirmed (a) the absence of full length SUR1 mRNA and (b) the presence of the alternatively spliced transcript lacking exon 17. Successful transfer of both patients to sulphonylurea treatment suggests that the altered transcript expression enhances the sensitivity of the K-ATP channel to Mg-ADP/ATP. This is the first report of an *ABCC8* nonsense mutation causing a gain-of-channel function and these findings extend the spectrum of K-ATP channel mutations observed in patients with neonatal diabetes.

**Keywords:** Neonatal diabetes, nonsense mutation, splicing

## Introduction

The pancreatic beta cell K-ATP channel is crucial for the controlled release of insulin as evidenced by the identification of *KCNJ11* and *ABCC8* mutations in patients with neonatal diabetes and congenital hyperinsulinism (1). Loss-of-function mutations in these genes cause hyperinsulinism by leading to a loss of K-ATP channels at the plasma membrane via effects on gene expression, protein synthesis, protein maturation, or membrane trafficking or by impairing the ability of SUR1 to regulate channel activity (2,3). This latter effect is brought about by reducing or abolishing channel activation by MgADP and/or MgATP. In contrast, gain-of-function mutations cause neonatal diabetes by reducing the ability of ATP to inhibit channel activity (at Kir6.2) or enhancing the ability of Mg-nucleotides to stimulate channel activity (at SUR1) (4).

**Address for Correspondence:** Sarah E. Flanagan PhD,
University of Exeter Medical School, Institute of Biomedical and Clinical Science,
Department of Molecular Genetics, Exeter, United Kingdom
**E-mail:** S.Flanagan@exeter.ac.uk **ORCID ID:** orcid.org/ 0000-0002-8670-6340

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ATP-sensitive potassium (K-ATP) channels are hetero-octomeric complexes consisting of four pore-forming $K^+$ inward rectifying ($K_{IR}6.x$) subunits and four regulatory sulphonylurea receptor (SURx) subunits. The $K_{IR}6.x$ protein has a pore loop flanked by two transmembrane and cytoplasmic domains, whilst SURx consists of 17 transmembrane helices with 2 regulatory nucleotide binding domains (NBD). Binding of ATP to Kir6.x inhibits channel activity, whilst interaction of cytosolic Mg-nucleotides with SURx causes channel activation (5).

Different isoforms and splice variants of K-ATP channel subunits exist with different combinations of $K_{IR}6.x$ and SURx acting to increase channel diversity. The K-ATP channel within the pancreatic beta cell is composed of four Kir6.2 and four SUR1 subunits. Kir6.2 is encoded by the single exon of the *KCNJ11* gene whilst *ABCC8*, which encodes SUR1, has 39 exons. At least five different SUR1 alternatively spliced transcripts have been identified in rodent cell lines which result from exon skipping (SUR1Δ17, Δ19, Δ17/19, Δ33) or truncation of the C-terminus (SUR1C) (6,7).

Recessively inherited K-ATP channel mutations are most common in patients with congenital hyperinsulinism, whilst dominant mutations are the commonest cause of neonatal diabetes. In a few patients with neonatal diabetes, recessive inheritance of two *ABCC8* or *KCNJ11* gain-of-function mutations or compound heterozygosity for an activating and an inactivating *ABCC8* mutation have been described (8,9). We now report two patients with diabetes diagnosed before 6 months of age: one with homozygosity for a novel *ABCC8* nonsense variant and one with compound heterozygosity for the same nonsense variant and a previously reported loss-of-function missense mutation.

## Methods

For the genetic analysis of *ABCC8* and *KCNJ11* genes, genomic DNA was extracted from peripheral leukocytes using standard procedures and the coding regions and intron/exon boundaries of the *ABCC8* and *KCNJ11* genes were amplified by polymerase chain reaction (PCR) (primers available on request). Amplicons were sequenced using the Big Dye Terminator Cycler Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions and reactions were analysed on an ABI3730 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the reference sequences (NM_000525.3 and NM_000352.3) using Mutation Surveyor v3.24 software (SoftGenetics, State College, PA).

Clinical information was provided by the referring clinicians via a neonatal diabetes request form (available at www.diabetesgenes.org) or by provision of clinical notes. The study was conducted in accordance with the Declaration of Helsinki principles with informed parental consent given on behalf of the children.

### Real-time Quantification of *ABCC8* Transcripts with and without Exon 17

Total pancreatic RNA was purchased from Clontech (Oxford, UK), islets were sourced from the National Disease Resource Interchange (NDRI; Philadelphia, USA). These samples were taken from donors who were otherwise free of disease. Control samples were free of cancer, pancreatic fibrosis or systematic sepsis. Samples must have had a maximum of 5 mins 'downtime' before sample processing, and donors must have had no abdominal injuries or pancreatic trauma and no transmissible diseases. RNA was extracted using the Eppendorf Perfect RNA mini Kit (Eppendorf, Hamburg, Germany). 500 ng total RNA from each sample were reverse transcribed using the Thermoscript real-time-PCR (RT-PCR) system (Invitrogen, Paisley, UK) using 50 °C as the incubation temperature as per manufacturer's instructions. TaqMan qRTPCR probes specific to the exon16:exon17 boundary or the exon16:exon18 were purchased from Life Technologies (Warrington, UK) and validated by standard curve analysis. Assay details are available on request. RT-PCR reactions were carried out using the ABI Prism 7000 platform (Applied Biosystems). Each sample was amplified in triplicate to ensure accuracy of quantification. Where multiple samples per tissue were tested, each sample was from a separate mRNA extraction and reverse transcription. PCRs contained 10 µL TaqMan Universal Mastermix (no AMPerase) (Applied Biosystems), 0.9 µmol/L each primer, 0.25 µmol/L probe, and 2 µL cDNA reverse transcribed as above in a total volume of 20 µL. PCR conditions were a single cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Transcript abundance for the full length and exon 17 deleted mRNAs were calculated using the Comparative Ct approach, relative to the endogenous control beta 2 microglobulin (*B2M*) and normalised to the level of full length *ABCC8* transcript in each sample.

## Results

The first patient is a male who presented with hyperglycaemia (blood glucose: 30.9 mmol/L [normal range 4-6 mmol/L (556 mg/dL)] on the 36$^{th}$ day of life. At the age of 4 years, he was referred for genetic testing and at this time was treated with 0.7 U/kg/day of insulin and had a HbA1c of 8% (normal range < 6.5%). Sequence analysis identified a novel homozygous nonsense variant, p.Glu747* (c.2239G > T), in

exon 17 of *ABCC8*. No *KCNJ11* mutation was identified and testing of the unaffected parents confirmed that both were heterozygous for the p.Glu747* variant.

Patient 2 is a male who presented with a blood glucose level of 26.5 mmol/L [normal range 4-6 mmol/L (477 mg/dL)] during the 6th week of life. At the time of genetic testing, he was being treated with 1.2 U/kg/day of insulin and had a HbA1c of 10.3%. Sequence analysis identified two heterozygous *ABCC8* variants: p.Glu747* (c.2239G>T) in exon 17 and p.Glu128Lys (c.382G>A) in exon 3. The p.Glu128Lys variant has been reported previously as a recessively acting loss-of-function mutation in multiple unrelated patients with congenital hyperinsulinism (10,11,12). No *KCNJ11* mutations were identified and testing of the unaffected parents confirmed that the two variants were *in trans* with p.Glu128Lys inherited from the mother and p.Glu747* inherited from the father. A summary of the clinical characteristics and genetic results for these two unrelated patients are provided in Table 1.

The identification of biallelic *ABCC8* loss-of-function variants in patients with neonatal diabetes was unexpected as recessive loss-of-function K-ATP channel mutations usually cause congenital hyperinsulinism. The *ABCC8* p.Glu747* nonsense variant is located within the small (36 bp) in-frame exon 17 that encodes part of the nucleotide binding domain 1 and there is a rodent SUR1 isoform which lacks exon 17 (6). We therefore undertook mRNA studies to investigate (a) the relative proportion of messenger RNA transcripts with and without this exon in control samples and (b) the effect of the p.Glu747* variant. We found that the isoform lacking exon 17 was expressed at only 44% of the level of the reference *ABCC8* transcript in human islets and at 53% of levels of the reference *ABCC8* transcript in whole pancreas (Figure 1). We next isolated total RNA from patient-derived leukocyte cell lines and amplified cDNA using PCR primers targeted to exons 16 and 18 (primer sequences available on request). Sequence analysis showed complete absence of transcripts containing exon 17 in the patient who was homozygous for the p.Glu747* variant. His father, who is heterozygous for the variant, was shown to have a mixture of transcripts with or without exon 17 (Figure 2).

Sulphonylureas are an effective treatment for individuals with neonatal diabetes caused by mutations in *ABCC8* or *KCNJ11* and result in improved glycaemic control (13,14). These drugs exert their effects by binding to the SUR1 subunit of the K-ATP channel and closing the channel

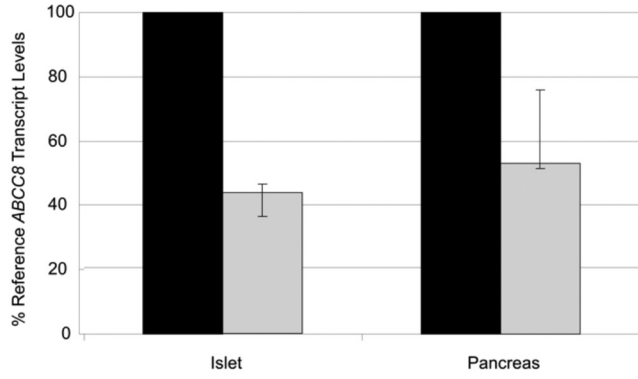
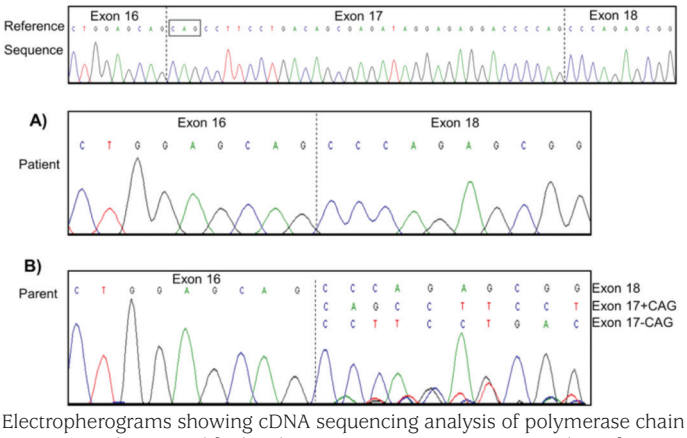



**Figure 1.** Expression pattern of *ABCC8* transcripts with and without exon 17 in human pancreas and islets. The graphs illustrate the levels of messenger RNA transcripts either containing exon 17 (black bars) or excluding exon 17 (grey bars) expressed relative to levels of the full length *ABCC8* reference transcript in human islets (3 technical replicates from RNA pooled from 2 independent donors) and from human total pancreas (3 technical replicates from RNA pooled from 3 donors). Transcript levels were determined by isoform-specific qRTPCR, calculated relative to the endogenous control beta 2 microglobulin (*B2M*) and normalised to the amount of full length transcript present in each sample. Error bars represent the range of quantification for each sample

**Table 1. Clinical characteristics and results of genetic analysis in the two patients**

| | **Patient 1** | **Patient 2** |
|---|---|---|
| **Gender** | **Male** | **Male** |
| Current age | 11 years | 6 years |
| Country | Vietnam | Vietnam |
| Age at diagnosis of diabetes | 5 weeks | 6 weeks |
| Blood glucose at presentation | 30.9 mmol/L | 26.5 mmol/L |
| Insulin dose (pre-transfer) | 0.7 U/kg/day | 1.2 U/kg/day |
| HbA1c on insulin | 8.3% | 13.6% |
| Glibenclamide dose (post-transfer) | 0.43 mg/kg/day | 0.24 mg/kg/day |
| HbA1c on SU | 5.2% | 5.2% |
| ABCC8 mutation identified | p.Glu747*/p.Glu747* | p.Glu747*/p.Glu128Lys |

SU: sulphonylureas




Electropherograms showing cDNA sequencing analysis of polymerase chain reaction products amplified with primers targeting exons 16 and 18 of *ABCC8*

**Figure 2.** The reference genomic DNA sequence is provided. A dashed line denotes an exon-exon boundary. (A) Sequence analysis identified homozygosity for the SUR1Δ17 in patient 1 with the homozygous p.Glu747* *ABCC8* variant in exon 17. (B) Sequence analysis of cDNA from the unaffected father who is heterozygous for the p.Glu747* variant identified a transcript which lacked exon 17 and two transcripts which contained exon 17. An alternate splice recognition site at the 3' intron 16/exon 17 boundary results in two transcripts containing either 36 or 39 basepairs (+/- CAG boxed) (GenBank L78208 and L78224)

independently of ATP. Studies undertaken by Hambrock et al (6) demonstrated high-affinity binding of sulphonylureas to the rat SUR1Δ17 isoform suggesting that sulphonylurea treatment may be effective in our two patients. We therefore undertook a controlled trial of glibenclamide in each patient which resulted in an improvement in glycaemic control (8.3% on insulin vs. 5.2% on glibenclamide in the first patient and 13.6% vs. 5.2% in the second). The patients are currently 11 years and 6 years and are well-controlled on 0.43 mg/kg/day and 0.24 mg/kg/day of glibenclamide, respectively. As sulphonlyureas bind directly to the SUR1 subunit, this observation confirms that the p.Glu747* variant is not preventing expression or trafficking of K-ATP channels to the cell surface. Patient 2 was compound heterozygous for the p.Glu747* variant and a p.Glu128Lys mutation. Functional studies have shown that p.Glu128Lys results in reduced expression of mutant channels (11). It is therefore likely that in both of our patients, all K-ATP channels at the cell surface are homomeric for the SUR1Δ17 isoform.

## Discussion

To date, all reported gain-of-function *KCNJ11* and *ABCC8* mutations cause neonatal diabetes by altering the sensitivity of the K-ATP channel to intracellular MgADP or ATP. As SUR1 exon 17 encodes part of NBD1 which is required for nucelotide handling, it seems likely that a protein lacking this crucial regulatory domain would have an altered sensitivity to ATP. However, whilst an SUR2 splice variant lacking exon 17 has been identified in mice which had a 2-fold reduction in sensitivity to ATP compared to the full-length isoform (15), a study using rat SUR1Δ17 found no differences in sensitivity to MgATP between the mutant and wild-type channels (6). The reason for this discrepancy is not clear but may reflect differences between rodent and human K-ATP channel physiology. Therefore, whilst further studies are required to assess the effect of the p.Glu747* variant upon channel activity in humans, the identification of the same variant in two patients with sulphonylurea-responsive neonatal diabetes, and its location within an alternatively spliced exon encoding a functionally important domain, provides strong evidence that p.Glu747* is an etiological mutation.

In conclusion, we have identified a novel recessively-inherited *ABCC8* nonsense variant in two unrelated patients with neonatal diabetes. We hypothesize that this variant causes a gain-of-channel function by changing the relative abundance of alternatively spliced SUR1 transcripts which enhances the sensitivity of the channel to intracellular MgADP/ATP. These results expand knowledge on genotype/phenotype relationships in neonatal diabetes due to K-ATP channel mutations and highlight the importance of considering the impact on different transcripts when unexpected mutations are identified in genes that are alternatively spliced.

### Ethics

**Informed Consent:** The study was conducted in accordance with the Declaration of Helsinki principles with informed parental consent given on behalf of the children.

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

### Authorship Contributions

Concept: Sarah E. Flanagan, Sian Ellard, Design: Sarah E. Flanagan, Sian Ellard, Lorna W. Harries, Frances M. Ashcroft, Data Collection and Processing: Sarah E. Flanagan, Vu Chí Dung, Jayne A. L. Houghton, Elisa De Franco, Can Thi Bich Ngoc, Annet Damhuis, Lorna W. Harries, Analysis and Interpretation: Sarah E. Flanagan, Vu Chí Dung, Jayne A. L. Houghton, Elisa De Franco, Can Thi Bich Ngoc, Annet Damhuis, Lorna W. Harries, Sian Ellard, Frances M. Ashcroft, Literature Research: Sarah E. Flanagan, Sian Ellard, Lorna W. Harries, Frances M. Ashcroft, Writing: Sarah E. Flanagan, Sian Ellard.

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CASE REPORT

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# Pituitary Adenoma Apoplexy in an Adolescent: A Case Report and Review of the Literature

**Hero Zijlker[1], Sebastian Schagen[1], Jan Maarten Wit[1], Nienke Biermasz[2], Wouter van Furth[3], Wilma Oostdijk[1]**

*[1]Leiden University Medical Center, Department of Pediatrics, Leiden, The Netherlands*
*[2]Leiden University Medical Center, Department of Medicine, Division of Endocrinology, Leiden, The Netherlands*
*[3]Leiden University Medical Center, Department of Neurosurgery, Leiden, The Netherlands*

### What is already known on this topic?

Pituitary apoplexy (PA) is a rare clinical syndrome in adolescents that can cause a life-threatening situation. PA is frequently seen in non-functioning adenomas and often results in headache and visual impairments.

### What this study adds?

Our study is to help physicians in differentiating between a PA and a pituitary abscess, to create an overview of the possible clinical symptoms seen in PA, and to create awareness for a possible adrenocorticotropic hormone-deficiency.

## Abstract

We present a 13-year-old boy who was admitted with complaints of a state of progressive sleepiness and a sudden headache with vomiting and fever. Laboratory testing showed hypoglycemia, multiple pituitary hormonal deficiencies, and an elevated C-reactive protein level. A cranial magnetic resonance imaging (MRI) showed an opaque sphenoid sinus and an intrasellar mass suggesting hemorrhage, so that we suspected pituitary apoplexy (PA) originating from a non-functioning adenoma, although a pituitary abscess could not completely be excluded. The boy was treated with antibiotics, hydrocortisone, and levothyroxine. Due to his rapid clinical improvement, no surgery was performed and we considered the diagnosis of PA as confirmed. At follow-up, the MRI scan showed a small residual lesion. Pituitary deficiencies of growth hormone, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone, and vasopressin persisted. A literature search of all well-documented cases of PA in children or adolescents (n = 30, 13 boys and 17 girls) indicated that this condition is rare below 20 years of age but must be considered when a patient experiences headache with or without visual disturbances, even in the presence of clinical and laboratory signals suggestive of pituitary abscess. MRI neuroimaging is helpful in the differential diagnosis. In both conditions, the possibility of ACTH deficiency should always be considered, investigated, and treated. In cases without severe neuro-ophthalmological deficits and/or with a rapid and positive response to acute medical management, one can abstain from surgical treatment.

**Keywords:** Pituitary adenoma, apoplexy, panhypopituitarism, adolescents, pituitary abscess, headache, magnetic resonance imaging

## Introduction

Neoplasms of the pituitary gland are extremely rare in childhood and adolescence (1:1.000,000) (1). Of all pituitary neoplasms, less than 10% are diagnosed in children and adolescents. Most of these are craniopharyngiomas (80-90%) and relatively few (3% of all intracranial neoplasms) are adenomas. Of all adenomas in patients younger than 20 years, approximately 97% secrete hormones and 16% develop pituitary apoplexy (PA) (2).

PA is a clinical syndrome caused by hemorrhage or infarction of the pituitary gland and is predominantly seen in patients with pituitary adenomas, probably due to their relatively high metabolism, limited blood flow, and high

**Address for Correspondence:** Wilma Oostdijk MD, PhD,
Leiden University Medical Center, Department of Pediatrics, Leiden, The Netherlands
**Phone:** +31 71 526 28 24 **E-mail:** w.oostdijk@lumc.nl **ORCID ID:** orcid.org/0000-0003-0180-2470



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intratumoral pressure when compared to other primary central nervous system tumors (3). PA occurs relatively often in large macroadenomas (4). Since non-functioning adenomas (NFAs) are on average larger than endocrine active adenomas, PA is relatively more frequently observed in NFAs (2,4). The presenting symptoms include sudden and severe headache, visual disturbances, and various neurological signs (4). In adults, PA is more common in males between 50-69 years and precipitating factors include angiography, cardiac surgery, anticoagulant therapy, and dynamic hormonal testing or gonadotropin-releasing hormone (GnRH) agonist treatment. However, little is known about this condition in patients younger than 20 years.

In 1972, Dawson and Kothandaram (5) were the first to describe an adolescent with PA. Since then, only a few case reports and individual cases extracted from larger case series have been reported in the literature. In 2015, Jankowski et al (6) presented a case series of nine adolescents with PA, comparing symptomatology, neuroimaging, pathology, and outcomes to those in adults.

In this paper, we report a case of a 13-year-old boy presenting with sudden and severe headache. Additional investigations suggested PA originating from an non-functioning adenoma (NFA), although initially a pituitary abscess [accounting for less than 1% of all pituitary lesions (7)] could not be completely excluded. We reviewed the literature and summarize the clinical, biochemical, and imaging characteristics of all reported cases of PA in patients younger than 20 years. Pediatricians should be aware that this condition which is frequently accompanied by adrenocorticotropic hormone (ACTH) deficiency, although extremely rare, can occur in children and adolescents and that the differential diagnosis with pituitary abscess can be difficult.

## Case Report

A 13-year-old boy, with an uninformative previous medical history, presented at the pediatric clinic of a general hospital with complaints of severe fatigue which had lasted for several months. Four days prior to admission, he had become progressively sleepy and experienced a sudden and severe stabbing frontal headache with vomiting and phonophobia. At physical examination, he had a normal level of consciousness, fever up to 38 degrees Celsius, and no neurological abnormalities. He had no visual disturbances and normal extra-ocular movements. His linear growth and pubertal development had been unremarkable; at admission, his height standard deviation score (SDS) was 0.0 (8). His body mass index was 20 kg/m$^2$ (+1.0 SDS) (9) and Tanner stage was G3P3A2 with testes of 12 mL (assessed with the Prader orchidometer). Initial laboratory results demonstrated a normal white blood cell count (9.2x10$^9$ /L; 54% neutrophils), an elevated serum C-reactive protein (CRP) (201 mg/L) and hypoglycemia (2.8 mmol/L). Despite normal neurological examination, increased intracranial pressure due to a brain tumor or abscess was considered because of the severe headache and vomiting. For this reason, broad-spectrum antibiotics were immediately administered intravenously and he was referred to our academic hospital.

Magnetic resonance imaging (MRI) of the cerebrum showed a sellar mass with suprasellar extension (2.5x2.0 cm) and slight optic chiasm compression (Figure 1). The mass appeared heterogeneously hyperintense on T1-weighted imaging (T1WI) and hypointense to isointense on T2-weighted imaging (T2WI). Also sphenoid sinus mucosal thickening and rim enhancement of the mass after gadolinium contrast were noted. These MRI findings were highly suggestive of hemorrhage that most likely originated from a pre-existing pituitary adenoma.

The endocrine investigations (Table 1) demonstrated central hypothyroidism, hypocortisolism, and hypogonadism, as well as low serum insulin-like growth factor (IGF)-I and IGF

**Table 1. Hormonal values of our case at presentation and 3 months later**

| | At presentation | After 3 months | Normal values |
|---|---|---|---|
| LH (U/L) | 1.3 | 2.1 | 2.0-9.0 |
| FSH (U/L) | 1.8 | 3.8 | 1.5-12.5 |
| ACTH (8 a.m.) (ng/L) | 10 | | 0-75 |
| Cortisol (8 a.m.) (µmol/L) | 0.213 | 0.386 | 0.1-0.6 |
| Cortisol in ACTH-test (0.58 ug/m$^2$) | 0.036* → 0.321** | | >0.50 ** |
| TSH (mU/L) | 0.134 | 0.318 | 0.3-4.8 |
| $fT_4$ (pmol/L) | 6.6 | 15.9 | 12-22 |
| hGH (µg/L) | 0.73 | 0.21 | 0.00-2.42 |
| IGF-I (nmol/L) | 5.2 | 15.9 | 18.5-74.1 |
| IGF-I (SDS) | -4.1 | -2.4 | |
| IGFBP-3 (mg/L) | 2.1 | 5.4 | 2.6-6.3 |
| Prolactin (µg/L) | 2.4 | 3.2 | 4.0-15.0 |
| Testosterone (nmol/L) | <0.1 | 8.9 | 8-31 |
| Osmolarity (mOsmol/kg) | 354 | 573 | 50-1200 |
| Sodium (mmol/L) | 137 | 143 | 136-144 |
| Potassium (mmol/L) | 3.9 | 4.2 | 3.6-4.8 |

*At baseline, **after 30 minutes

LH: luteinizing hormone, FSH: follicle-stimulating hormone, ACTH: adrenocorticotropic hormone, hGH: human growth hormone, IGF-I: insulin-like growth factor, IGFBP-3: insulin-like growth factor binding protein-3, SDS: standard deviation scores, $fT_4$: free thyroxine

binding proteins-3 levels suggestive of growth hormone (GH) deficiency. A stress dose of hydrocortisone was immediately administered followed by substitution with hydrocortisone and levothyroxine. We considered PA originating from an NFA most likely, based on the specific MRI findings.

Because we could not completely exclude pituitary abscess, immediate surgical intervention was considered. However, rapid clinical improvement was noted after administration of broad-spectrum antibiotics, and hydrocortisone was started. One day after initiation of treatment, the fever disappeared and CRP levels gradually declined to 48 mg/L on the fourth post-treatment day. CRP levels were completely normalized after 2 weeks. Surgery was eventually not performed due to the boy's rapid clinical improvement, the sella enlargement, which is unusual for pituitary abscess, and the sphenoid sinus mucosal thickening seen on the MRI, which suggested a possible infectious process on the surgical route. The combination of MRI findings and clinical course has made the diagnosis of PA virtually certain, although not histologically confirmed. Remarkably, diabetes insipidus (DI) developed three days after admission despite conservative treatment.

The patient was discharged after five days with oral broad-spectrum antibiotics, hydrocortisone, levothyroxine, and vasopressin. A small residual lesion was seen on the MRI three months later (Figure 1), which resolved after 6 months. The thyroid-stimulating hormone (TSH) and ACTH deficiencies persisted. GH deficiency was diagnosed by a very low (GH peak 0.9 ug/L) response to GH stimulation tests, so that GH substitution was started. The pituitary-gonadal axis was not affected (normal pubertal GnRH test) and puberty progressed normally. Genetic evaluation showed no abnormalities in the *MEN1* gene.

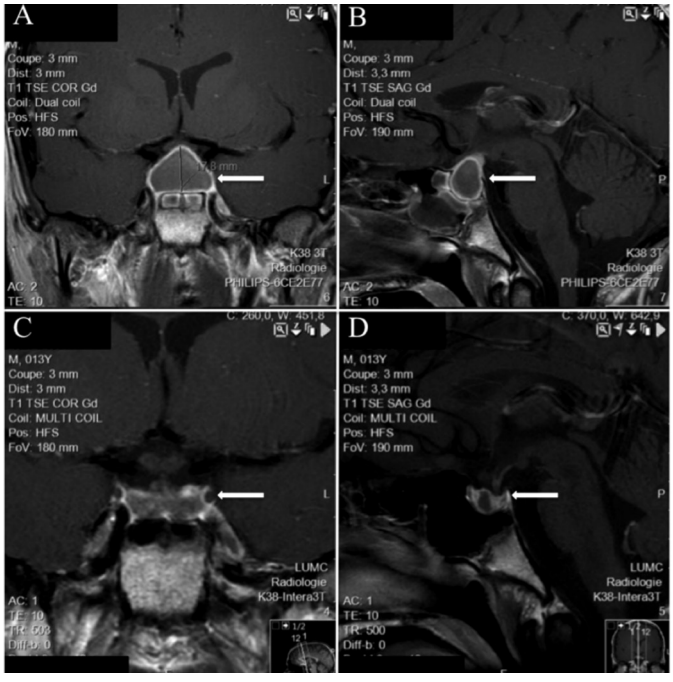


**Figure 1.** Magnetic resonance imaging: T1-weighted imaging. At presentation: coronal (A) and sagittal (B) view showing rim enhancement and sphenoid sinus mucosal thickening. Three months later: coronal (C) and sagittal (D) view showing substantial mass reduction

### Literature Search

A literature search was performed in databases Pubmed, Embase, Web of Science, Medline, and Cochrane to identify all cases of PA originating from an adenoma in patients younger than 20 years. Only publications written in English were included.

Cases found in larger case series with no or scarce individual descriptions were excluded (n = 36) (2,10,11). This resulted in 30 cases published between 1972 and 2016: 16 case reports and 14 cases extracted from 6 case series (Table 2) (5,6,11,12, 13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30). The total group consisted of 13 boys and 17 girls with a mean age of 15.3 years (range 6-19 years, median 16 years).

## Discussion

PA in children and adolescents is a rare entity that requires rapid and adequate treatment to prevent a life-threatening situation. Based upon our literature search, various aspects are discussed.

### Non-Functioning Pituitary Adenomas and Pituitary Apoplexy

Only 3% of all pituitary adenomas in patients younger than 20 years are NFAs (2). This low percentage can be explained by the slow growth of pituitary adenomas and data suggesting that 85-90% of normal pituitary gland and optic chiasm have to be compromised to develop endocrine insufficiencies and visual deficits, respectively (31).

So far, only four extensively described cases of patients younger than 20 years with PA originating from a NFA have been published (13,18,22,23). Based upon these data, we conclude that PA originating from a NFA is an extremely rare entity in this age group, despite the fact that PA is likely to occur in NFAs.

### Adrenocorticotropic Hormone Deficiency

A corticotropic deficiency can lead to serious hemodynamic instabilities causing a life-threatening situation and this was seen in 50-80% of the adult patients with PA (4). No accurate data are available for determining the prevalence of corticotropic deficiencies in children and adolescents with PA. However, 43% (5,12,13,14,16,17,18,19,22,23,25, 27,29) of the reported cases received steroid replacement

**Table 2. Summary of clinical and pathological characteristics of cases younger than 20 years with pituitary apoplexy**

| Case no. | 1st Author (reference) Year of publication | Age Sex | Clinical signs and symptoms | Visual disturbances | Endocrine function and symptoms |
|---|---|---|---|---|---|
| 1 | Dawson and Kothandaram (5) 1972 | 17 F | Headache, fever, URI, confusion (1 week), neck stiffness, hemiparesis, unconscious | Decreased visual acuity, nerve III and VI palsy, acute eyelid edema, papilledema | Amenorrhea |
| 2 | Rovit and Fein (20) 1972 | 18 F | Headache, lethargy, moribund (6 hours, after pneumoencephalography), neck stiffness | Decreased visual acuity (6 hours), nerve III-V palsy | Cushing's syndrome, total adrenalectomy, galactorrhea |
| 3 | Sakalas et al (21) 1973 | 6 M | Headaches, fever (18 months), lethargic and sleepy (3 weeks), neck stiffness | Photophobia (18 months), progressive visual loss (3 weeks), blindness, dilated pupils and unresponsive to light | Gigantism |
| 4 | Arisaka et al (12) 1983 | 11 M | Headache, vomiting, and fever (12 days) | Decreased visual acuity (12 days), bitemporal hemianopsy and bilateral optic atrophy | Gigantism, precocious puberty, galactorrhea |
| 5 | Kaplan et al (15) 1983 | 17 F | Headache (chronic) | None | Amenorrhea, galactorrhea |
| 6 | Kaplan et al (15) 1983 | 15 F | Headache (chronic) | None | Growth faltering, pubertal delay |
| 7 | Lever et al (27) 1986 | 19 F | Headache, nausea, vomiting fever (following TRH stimulation), neck stiffness | Photophobia, diplopia (following TRH stimulation) | Gigantism, acromegaly, irregular menstruation, galactorrhea |
| 8 | Pozzati et al (26) 1987 | 15 M | Headache, nausea, vomiting, lethargy, neck stiffness | Diplopia, nerve III and VI palsy, central facial weakness | None |
| 9 | Vidal et al (23) 1992 | 16 F | Headache, stupor, neck stiffness (1 day) | Photophobia (1 day) | Amenorrhea (5 months), panhypopituitarism |
| 10 | Mizutani et al (18) 1993 | 11 M | 'Visual symptoms' | Gradual decrease of visual acuity for 2 years, bitemporal hemianopsy | None |
| 11 | Kulah et al (17) 1995 | 17 M | Headaches, mild degree of mental dullness (4 months) | None | Growth faltering, pubertal delay, hypopituitarism |
| 12 | Sugita et al (11) 1995 | 14 F | Headache (3 weeks) | Progressive bilateral visual loss (2 weeks), decreased bilateral visual activities, bitemporal hemianopsy | None |
| 13 | Pinto et al (19) 1998 | 14 F | Headache, fever, vomiting, asthenia (after 6 months of bromocriptine) | Diplopia | Amenorrhea, hypopituitarism |
| 14 | Dourakis et al (13) 2002 | 15 M | Headache, vertigo, fever (4 days), neck stiffness | Photophobia (4 days) | Growth faltering, pubertal delay, hypopituitarism |
| 15 | Rotman-Pikielny et al (28) 2003 | 19 F | Headache, fever (2 days after CRH stimulation), nausea, vomiting, neck stiffness | Ptosis, nerve III palsy, diplopia, photophobia | Cushing's syndrome, amenorrhea, galactorrhea, fatigue |
| 16 | Knoepfelmacher et al (16) 2004 | 17 M | Headache, nausea, vomiting, asthenia (after 1 year of cabergoline) | None | Pubertal delay, hypopituitarism |
| 17 | Satyarthee and Mahapatra (22) 2005 | 13 M | Headache (6 hours), meningeal irritability | Decreased visual acuity (6 hours), bitemporal hemianopsy | Normal hormonal status |

**Table 2. Continued**

| | | | | | |
|---|---|---|---|---|---|
| 18 | Kamboj et al (14) 2005 | 18 M | Headache, nausea, lethargy, disoriented, fever (3 days) | Unequal but reactive pupils, acutely deterioration of vision and pupillary responses | Hypopituitarism |
| 19 | Balarini Lima et al (30) 2008 | 15 F | Headache (after 32 weeks of cabergoline) | Left-sided blindness, loss of left visual field | Puberty arrest, amenorrhea, hypogonadotrophic hypogonadism |
| 20 | Wang et al (24) 2011 | 15 F | 'Endocrine symptoms' | None | Amenorrhea (3 years), acromegaly, hypogonadism |
| 21 | Jankowski et al (6) 2015 | 14 M | Headache, nausea, vomiting (6 days) | Photophobia (6 days) | Weight gain, rapid height increase, sleep disturbance, behavioral problems, dysphoric mood |
| 22 | Jankowski et al (6) 2015 | 18 F | Headache, nausea, vomiting, dizziness (15 days) | None | Galactorrhea |
| 23 | Jankowski et al (6) 2015 | 16 F | Headache, dizziness (4 days) | Photophobia (4 days) | Galactorrhea, amenorrhea, fatigue |
| 24 | Jankowski et al (6) 2015 | 14 M | Headache, dizziness (5 months) | None | Fatigue |
| 25 | Jankowski et al (6) 2015 | 16 F | Headache, nausea (2 months) | Decreased visual acuity | Galactorrhea |
| 26 | Jankowski et al (6) 2015 | 16 F | Headache (3 days) | None | Amenorrhea, galactorrhea |
| 27 | Jankowski et al (6) 2015 | 18 F | Headache, dizziness (5 months) | Peripheral field deficit L > R (5 months) | Amenorrhea, galactorrhea |
| 28 | Jankowski et al (6) 2015 | 17 F | Headache (1 year) | Peripheral field deficit L > R (5 months) | Amenorrhea, galactorrhea |
| 29 | Kumar and Sharma (25) 2016 | 18 M | Headache, vomiting (5 days) | Decreased visual acuity (5 days), bilateral papilledema, bitemporal hemianopsy | Acromegaly, hypopituitarism |
| 30 | Özçetin et al (29) 2016 | 9 M | Fever (3 days), vomiting, somnolence | Progressive loss of vision, blindness, nerve VI palsy | SIADH |
| | Our case | 13 M | Headache, vomiting, phonophobia, fever (4 days) | None | Fatigue, hypopituitarism |

F: female, M: male, URI: upper respiratory infection, TRH: thyroid-releasing hormone, SIADH: syndrome of inappropriate antidiuretic hormone secretion, CRH: corticotropin-releasing hormone

therapy, indicating that corticotropic deficiency is commonly seen in young patients with PA. Even if serum cortisol and its response to an ACTH injection appear normal in the acute situation, an ACTH deficiency can become apparent in subsequent days.

Due to the severity of a possible hemodynamic instability seen by an Addisonian crisis, every patient with signs or symptoms of PA should immediately be treated with steroids (4). Our patient immediately received hydrocortisone after hypocortisolism was noticed. In the following days, his clinical condition improved rapidly. However, since antibiotics were also administered, it was hard to say which component of the treatment led to his improvement.

### Differentiation Between Pituitary Apoplexy Originating from Adenoma and Pituitary Abscess

The differentiation between PA and pituitary abscess is of vital importance because of the contrasting therapeutic consequences: pituitary abscess is an indication for immediate operation, while an expectative policy would usually be the best option in patients with PA. Based on the literature, four

elements are discussed for the differential diagnosis between PA and pituitary abscess: 1) Clinical presentation, 2) Infection parameters, 3) Endocrine function, 4) Neuroimaging.

### Clinical Presentation

Liu et al (7) described the largest series (33 patients; mean age 42 years; range 12 to 63 years) of patients with pituitary abscess so far. In their cohort, the most common clinical symptoms were headache (70%), with no common pattern, and visual disturbances (27%). Headache with a sudden onset can also occur in patients with pituitary abscess (32). In the reports on young individuals we reviewed, headache was also the most common described symptom (90%), with a sudden onset in one third of them. Despite the frequently described sudden onset, no common pattern of headache was observed. Visual disturbances were described in 73% of cases.

Regarding the strong clinical similarities between PA and pituitary abscess, no differentiation between these two conditions could be made based on clinical presentation.

Furthermore a low 'PA Score' (0 out of 10 points), based on the absence of visual symptoms and his normal level of consciousness (33), was found in our patient. This clinical tool did not help us differentiate the two conditions.

### Infection Parameters

Initial laboratory results demonstrated a normal white blood cell count, an elevated serum CRP, and hypoglycemia in our patient, which together with the elevated body temperature was initially considered highly suggestive of pituitary abscess. However, in the literature, a 57-year-old man was described with a presentation similar to that of our patient (34). In that patient, there was a strong suspicion of bacterial meningoencephalitis due to the combination of fever, meningeal irritation, elevated CRP (109 mg/L), and neutrophilic leukocytosis (13.600/$mm^3$, 66% neutrophils). MRI imaging showed a sellar mass which initially was defined as a secondary pituitary abscess. However, cerebrospinal fluid contained no microbes, and during surgery, biopsy was obtained that demonstrated PA originating from an adenoma. Also, the presence of fever appears to have a low discriminative power, since this was reported in one third of the cases in young individuals with PA in 18% of patients with pituitary abscess (7). Despite the difference in age in comparison with our patient, we conclude that a very elevated CRP, leukocytosis, and fever can also occur in PA.

### Endocrine Function

Our patient's endocrine analysis demonstrated hypopituitarism. This endocrine condition was reported in 26% of the reviewed cases and in 85% of cases with pituitary abscess (7). In patients with pituitary abscess, 70% presented with DI (7). In contrast, less than 5% of the cases with PA described by Briet et al (4) presented with DI and none of the reviewed were young cases. DI is a condition that usually develops after pituitary surgery (35). It is noteworthy that DI may be masked by secondary adrenal failure and develop after steroid or thyroid replacement. From the reviewed cases, seven (23%) developed DI - five of them were operated and 2 were treated conservatively (Table 3). Persistent DI was described in 3 cases and transient - in 1. Description of DI in the other 3 cases is inconclusive. Eight of nine cases originally described by Jankowski et al (6) are included in our study. Four of them developed transient DI and 1 developed persistent DI. DI after conservative treatment of PA has sporadically been documented in the literature. Only two non-surgically treated cases included in our group developed DI (13,18).

We conclude that endocrine function tests are necessary but can hardly assist in differentiating PA from pituitary abscess.

### Neuroimaging

The MRI showed a space-occupying lesion at the site of the sella turcica in our patient.

The typical cystic features of pituitary abscess are hypointense on T1WI but hyperintense on T2WI. Rim enhancement can be seen after gadolinium in 64% of pituitary abscesses (7). The remaining 36% had hypointense to isointense signaling on T1WI and isointense to hyperintense signaling on T2WI. The MRI performed in our patient showed a heterogeneous hyperintense signal on T1WI, hypointense to isointense signal on T2WI with rim enhancement after gadolinium, as well as sphenoid sinus mucosal thickening. These T1WI and T2WI features were consistent with a hemorrhage (36), although rim enhancement after gadolinium is more often seen in pituitary abscess (64%) than in PA (36%).

In our reviewed young patients, all MRI findings were suggestive of hemorrhage within an adenoma. Also, compression of the surrounding structures was stated in 15 cases, involving the optic chiasm in 14, the infundibulum in 4, and the hypothalamus in one. Furthermore, sphenoid sinus mucosal thickening was observed in our patient, a finding that could suggest an inflammation of the sinus and may constitute a potential cause of a secondary pituitary abscess (37,38). However, mucosal thickening was also seen in 2 of the 9 adolescent patients with PA investigated by Jankowski et al (6) and is probably due to venous engorgement secondary to PA. Thus, when a patient has

**Table 3. Summary of pathological characteristics, treatment and follow-up data of cases younger than 20 years with pituitary apoplexy**

| Case no. | Adenoma type | Surgery | Residual symptoms and endocrine sequelae | Endocrine replacement |
|---|---|---|---|---|
| 1 | Unknown | - | Normal vision, ophthalmoplegia and hemiparesis resolved | Unknown |
| 2 | ACTH | + | Patient died just after surgery | - |
| 3 | GH | + | DI, improved vision and pupil response of left eye, blindness of right eye | Unknown |
| 4 | GH + PRL | + | DI, vision recovered, growth faltering, hypopituitarism | + |
| 5 | PRL | + | Normal menses | - |
| 6 | PRL | + | Impaired menses | + |
| 7 | GH | - | DI, acromegaly facial symptoms and galactorrhea resolved, hypopituitarism | + |
| 8 | Unknown | - | Gradual improvement of condition, DI, ophthalmoplegia and facial paresis resolved, hypopituitarism | + |
| 9 | NFA | + | Photophobia resolved, normal pituitary function | - |
| 10 | NFA | + | Rapid recovery, DI, visual fields almost full range | + |
| 11 | PRL | + | Endocrine and subjective symptoms improved, hypopituitarism | Unknown |
| 12 | GH + PRL | + | Vision improved, normal pituitary function | - |
| 13 | PRL | - | Resolved functional and ophthalmic signs, hypopituitarism | + |
| 14 | NFA | + | Symptoms improved pre-operatively, DI, hypopituitarism | + |
| 15 | ACTH | - | Cushing's and subjective symptoms resolved, hypopituitarism | + |
| 16 | PRL | - | Normal pubertal development, panhypopituitarism | + |
| 17 | NFA | + | Improvement of vision and field defects, headache resolved | + |
| 18 | PRL | + | Vision and mental status improved, panhypopituitarism | + |
| 19 | PRL | + | Absent mammary development | + |
| 20 | GH | + | Impaired menses | + |
| 21 | ACTH | + | No residual symptoms, normal pituitary function, photophobia resolved | - |
| 22 | PRL | + | No residual symptoms, normal pituitary function | - |
| 23 | PRL | + | Normal pituitary function, headaches, mood disturbance and anxiety, photophobia resolved | - |
| 24 | PRL | + | No residual symptoms, normal pituitary function | - |
| 25 | PRL | + | No residual symptoms, normal pituitary function, normal visual acuity | - |
| 26 | PRL | + | No residual symptoms, normal pituitary function | - |
| 27 | PRL | + | No residual symptoms, normal pituitary function, slight improvement of visual fields | - |
| 28 | PRL | + | Mild intermittent headaches, normal pituitary function, slight improvement of visual fields | - |
| 29 | GH | - | No residual symptoms, normal visual fields | + |
| 30 | GH | + | DI, hypopituitarism | + |
| Our case | NFA | - | No residual symptoms, hypopituitarism | + |

ACTH: adrenocorticotropic hormone, NFA: non-functioning adenoma, GH: growth hormone, PRL: prolactin, DI: diabetes insipidus

either PA or pituitary abscess, the differentiation should be made by MRI neuroimaging. Clinical presentation, PA Score, infection parameters, and endocrine function are not helpful in the differentiation of these two conditions. In our patient, based on the MRI neuroimaging findings, PA was more likely than pituitary abscess.

### Treatment

We did not find any publication comparing the outcome of different therapeutic strategies in patients with PA younger than 20 years. In adults, Singh et al (39) analyzed the outcomes of case series of different treatment options of PA (57 males; 30 females; mean age 51 years; range 15 to 91 years). They concluded that the outcome of most patients was excellent and that no statistically significantly differences existed between the surgically and conservatively treated patients. All patients with endocrine deficiencies or electrolyte disturbances were acutely managed with hormonal and electrolytes substitution. Most of the patients who received early surgery (surgery within a median time of 5 days, range 3 to 10 days) had severe neuro-ophthalmological deficits at presentation. On the other hand, patients who lacked severe neuro-ophthalmological deficits, including patients with reduced consciousness, or patients with a rapid response to acute management, were adequately managed conservatively. In line with this advice, our patient was not subjected to a surgical intervention because of his rapid clinical improvement with antibiotics, hydrocortisone, and levothyroxine substitution.

Out of the 30 reported young cases, 23 underwent surgery, mostly via the transsphenoidal route (78%). Nine of these patients received endocrine replacement therapy. Of these 9 patients, 1 died shortly after surgery and there was no mention of symptom relief in 5. Seven cases were managed conservatively, of whom 6 received endocrine replacement therapy.

Our patient received prolonged endocrine replacement therapy due to persisting hormonal deficiencies and this was also seen in 15 reported cases (50%), illustrating the high risk of permanent damage of the pituitary gland caused by PA.

## Conclusions

PA is a rare condition seen in patients younger than 20 years, but must be considered when a patient experiences headache with or without visual disturbances, even in the presence of clinical or laboratory findings suggestive of an infection. There should be a high index of suspicion for ACTH deficiency which must be promptly treated with stress doses of hydrocortisone. Differentiation between pituitary abscess and PA is difficult. Type of headache, elevated CRP, endocrinological status, and fever do not differentiate between the two conditions. MRI neuroimaging is helpful in making the diagnosis since differences exist in T1W1 and T2W1 images of patients with hemorrhage and abscess. We agree with Singh et al (39) that without severe neuro-ophthalmological deficits or with a quick response to the acute management, patients can be treated conservatively. Furthermore, multiple persistent pituitary deficiencies, including DI, are a common outcome.

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### Ethics

**Informed Consent:** Written informed consent for publication of the data was given by the patient and his parents.

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

### Authorship Contributions

Concept: Hero Zijlker, Design: Wilma Oostdijk, Jan Maarten Wit, Nienke Biermasz, Wouter van Furth, Sebastian Schagen, Data Collection and Processing: Hero Zijlker, Analysis and Interpretation: Hero Zijlker, Wilma Oostdijk, Literature Research: Hero Zijlker, Writing: Hero Zijlker.

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CASE REPORT

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# Pancreatic Agenesis due to Compound Heterozygosity for a Novel Enhancer and Truncating Mutation in the *PTF1A* Gene

**Monica Gabbay[1], Sian Ellard[2], Elisa De Franco[2], Regina S. Moisés[1]**

*[1]Federal University of São Paulo, Paulista School of Medicine, Division of Endocrinology, São Paulo, Brazil*
*[2]University of Exeter Medical School, Institute of Biomedical and Clinical Science, Exeter, United Kingdom*

### What is already known on this topic?

Homozygous truncating mutations in PTF1A have been reported in patients with pancreatic and cerebellar agenesis, while recessive mutations located in a distal PTF1A enhancer cause isolated pancreatic agenesis.

### What this study adds?

This is the first report of a patient with isolated pancreatic agenesis resulting from compound heterozygosity for truncating and enhancer mutations in the *PTF1A* gene. This study broadens the spectrum of mutations causing pancreatic agenesis and the phenotypic variability of this condition.

## Abstract

Neonatal diabetes, defined as the onset of diabetes within the first six months of life, is very rarely caused by pancreatic agenesis. Homozygous truncating mutations in the *PTF1A* gene, which encodes a transcriptional factor, have been reported in patients with pancreatic and cerebellar agenesis, whilst mutations located in a distal pancreatic-specific enhancer cause isolated pancreatic agenesis. We report an infant, born to healthy non-consanguineous parents, with neonatal diabetes due to pancreatic agenesis. Initial genetic investigation included sequencing of *KCNJ11*, *ABCC8* and *INS* genes, but no mutations were found. Following this, 22 neonatal diabetes associated genes were analyzed by a next generation sequencing assay. We found compound heterozygous mutations in the *PTF1A* gene: A frameshift mutation in exon 1 (c.437_462 del, p.Ala146Glyfs*116) and a mutation affecting a highly conserved nucleotide within the distal pancreatic enhancer (g.23508442A > G). Both mutations were confirmed by Sanger sequencing. Isolated pancreatic agenesis resulting from compound heterozygosity for truncating and enhancer mutations in the *PTF1A* gene has not been previously reported. This report broadens the spectrum of mutations causing pancreatic agenesis.

**Keywords:** Pancreatic agenesis, neonatal diabetes, *PTF1A* gene

## Introduction

Neonatal diabetes, defined as onset of diabetes within the first 6 months of life, is a genetically heterogeneous condition with 22 known genetic causes (1,2,3). Its causal genes are involved in the development of the pancreas or islets; beta cell apoptosis or destruction; or beta-cell function (1). The *PTF1A* gene on chromosome 10 encodes a transcription factor with a key role in early pancreas development and cerebellar neurogenesis (4,5). Homozygous truncating mutations in *PTF1A* have been reported in patients with pancreatic and cerebellar agenesis, whilst mutations located in a distal pancreatic-specific enhancer cause isolated pancreatic agenesis (5,6,7,8).

Here, we report a patient with isolated pancreatic agenesis due to compound heterozygous mutations in *PTF1A*: A coding frameshift mutation **(**p.Ala146Glyfs*116**)** and a novel regulatory mutation located in the distal enhancer, 25 kb downstream of this gene.

**Address for Correspondence:** Regina S. Moisés MD,
Federal University of São Paulo, Paulista School of Medicine, Division of Endocrinology, São Paulo, Brazil
**Phone:** +55 11 5576 4744 **E-mail:** rmoises@unifesp.br **ORCID ID:** orcid.org/0000-0002-9048-068X



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## Case Report

The index patient is a 1-year and 9-month-old boy, the second child of healthy, Caucasian, non-consanguineous parents. He was born with a birth weight of 1,935 g (below the third percentile), length of 43 cm (below the third percentile), and head circumference of 32 cm (third percentile). Intrauterine growth retardation was noted at 34 weeks of gestation, and the delivery was by cesarean section at 37 weeks of gestation. Physical examination revealed no dysmorphic features. During the first week of life, hyperglycemia (blood glucose = 250 mg/dL) without ketoacidosis was observed, and treatment with subcutaneous NPH insulin was initiated on the 7$^{th}$ day of life, followed by continuous subcutaneous insulin infusion. Despite intensive insulin therapy, the infant did not show satisfactory weight gain and at 4 months of age, his weight was 3,670 g (still below the third percentile). Abdominal distension and fatty stools were noted. Abdominal ultrasound was performed at that time and the pancreas could not be identified. No other abnormality was found. Pancreatic enzyme replacement was begun at the age of 4 months and a catch-up growth occurred (Figure 1). Over the subsequent months, his growth was satisfactory and his developmental milestones were all reached at an appropriate age.

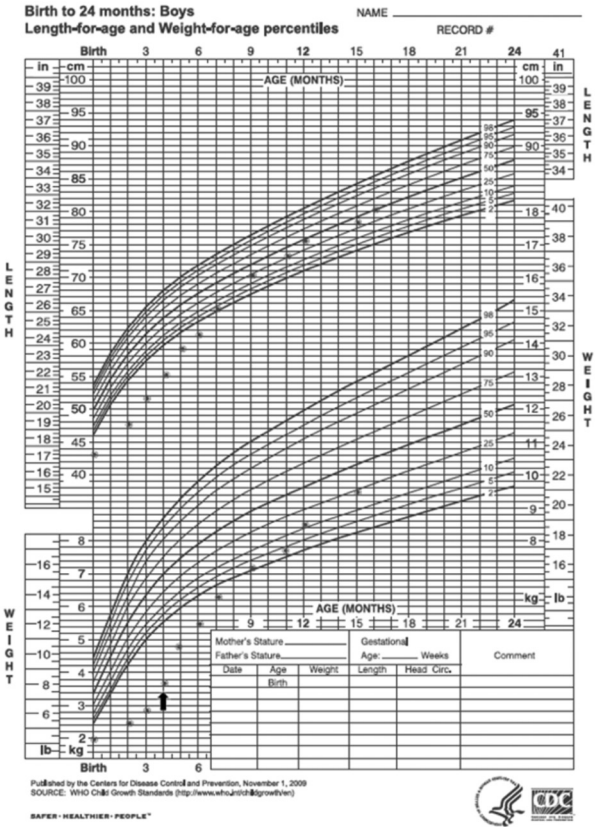


**Figure 1.** Growth of the patient plotted on the World Health Organization growth chart. Arrow denotes the start of pancreatic enzyme replacement

### Genetic Analysis

Initial Sanger sequencing of the coding and flaking intronic regions of the *KCNJ11* (NM_000525), *ABCC8* (NM_000352), and *INS* (NM_000207) genes was undertaken as previously described (3), but no mutations were found. Following this, a next-generation sequencing assay was performed to analyze the coding regions and conserved splice sites of the 22 neonatal diabetes genes: *KCNJ11, ABCC8, INS, EIF2AK3, FOXP3, GATA4, GATA6, GCK, GLIS3, HNF1B, IER3IP1, PDX1, PTF1A, NEUROD1, NEUROG3, NKX2-2, RFX6, SLC2A2, SLC19A2, STAT3, WFS1,* and *ZFP57* (Agilent custom capture v5.1/Illumina HiSeq) at the Molecular Genetics Laboratory, University of Exeter Medical School, UK (9). A compound heterozygous mutations in the *PTF1A* gene was found: a frameshift mutation in exon 1 (c.437_462 del, p.Ala146Glyfs*116) and a mutation affecting a highly conserved nucleotide within the distal pancreatic enhancer (g.23508442A > G) (Figure 2). The two mutations were predicted to be pathogenic and likely to be pathogenic, respectively, according to the American College of Medical Genetics and Genomics variant interpretation guidelines (10). Both mutations were confirmed by Sanger sequencing. Family member testing showed that the proband's mother is a heterozygous carrier of the distal enhancer mutation and the father is a heterozygous carrier of the frameshift mutation.

## Discussion

Pancreatic agenesis characterized by exocrine pancreatic insufficiency and permanent neonatal diabetes (11) is a rare condition. An international cohort study of patients with diabetes diagnosed before 6 months of age found that only 4.9% of them had pancreatic agenesis (12). Mutations in genes that encode transcription factors with a key role in pancreatic development, such as *PDX1*, *GATA6, GATA4,* and *PTF1A*, have been reported as genetic causes of congenital absence of the pancreas (5,6,7,8,12,13,14). However, the origin of this disorder remains unknown in ~15% of patients (De Franco E, unpublished data). Here, we report a patient with neonatal diabetes and exocrine pancreas insufficiency resulting from compound heterozygous mutations in the *PTF1A* gene. PTF1A, a 48kDa binding subunit of the pancreatic transcription factor 1 (PTF1), is required for normal pancreas development (4). This transcription factor is also expressed in embryonic neural tissues and plays a role in cerebellar neurogenesis. *Ptf1a*$^{-/-}$ mice show a size reduction in the cerebellar primordium in embryos, resulting in cerebellar agenesis at birth (5). Consistently with this,

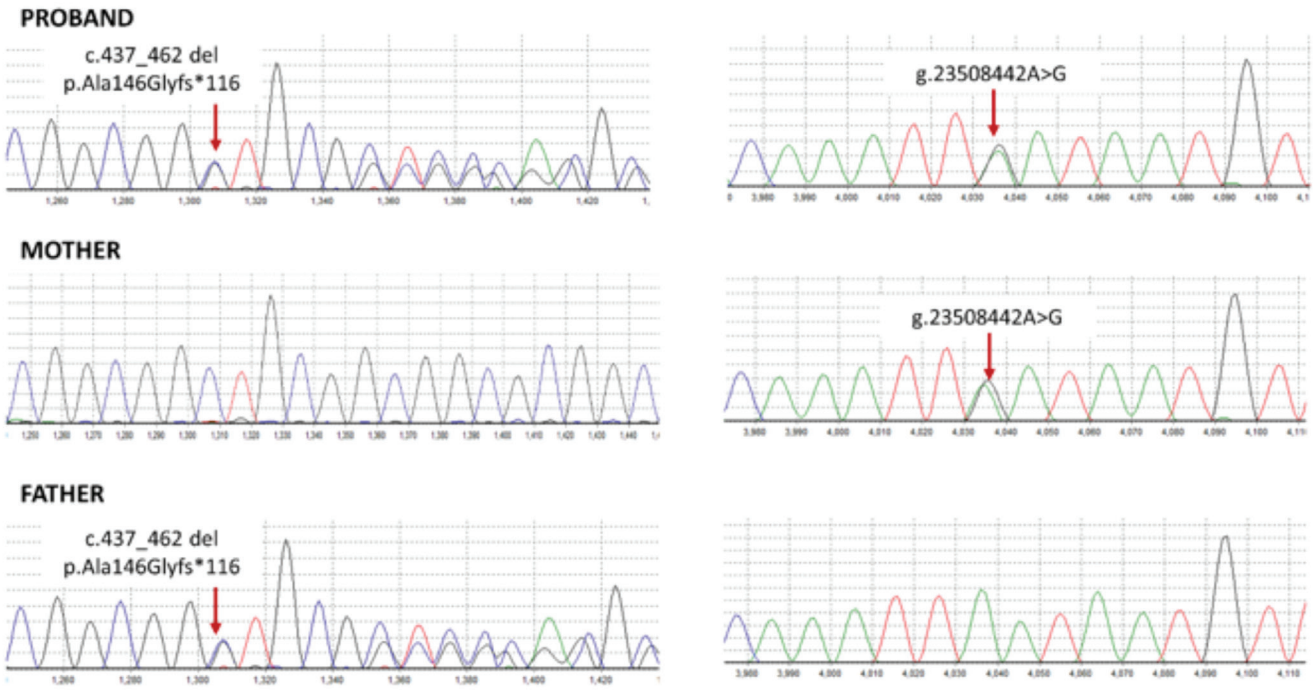


**Figure 2.** Sequence chromatograms showing the *PTF1A* mutations identified in the proband and his parents

recessive loss of function mutations in the *PTF1A* gene have been previously reported to cause agenesis of the pancreas and the cerebellum with additional dysmorphic features (5,6,15). A homozygous missense mutation, p.Pro191Thr, resulting in a protein with a 75% reduced transactivation activity, has been recently reported in patients with isolated pancreatic aplasia/hypoplasia, indicating a correlation between coding mutation severity and phenotype (16). Furthermore, mutations in the enhancer region located 25 kb downstream from the coding region of the *PTF1A* gene, which acts as a developmental enhancer of this gene, have been found to cause isolated pancreatic hypoplasia/agenesis, sparing the cerebellum (7,8).

To the best of our knowledge, this is the first report of a patient with isolated pancreatic agenesis resulting from compound heterozygosity for truncating and enhancer mutations in the *PTF1A* gene. Regarding the age of onset of diabetes, previous reports of patients with *PTF1A* truncating mutations showed that they had diabetes in the first month of life (5,6,15). However, patients with *PTF1A* enhancer mutations had phenotypic variability: The majority of cases are diagnosed in the first month of life, but diabetes at later ages was also observed (8). The patient we report had diabetes diagnosed in the first week of life and his neurological development has been normal, indicating no associated anomaly in the cerebellum. Interestingly, the lack of a severe neurological phenotype in patients with homozygous/compound heterozygous regulatory mutations has been recently reported for another congenital disease, polycystic kidney disease with hyperinsulinemic hypoglycemia (HIPKD) (17). A specific promoter mutation in the *PMM2* gene, either homozygous or in trans with a coding PMM2 mutation, was reported to cause HIPKD in 11 families. Homozygous coding mutations in *PMM2* have been previously reported to cause a congenital disorder of glycosylation type 1a, a severe multisystem disease with prominent neurologic features which were not observed in patients with the promoter mutation. The phenotype-genotype relationship observed in patients with coding versus non-coding mutations in *PTF1A* and *PMM2* highlights the fundamental role of non-coding sequences in development of specific organs.

In summary, we report the case of a patient with isolated pancreatic agenesis due to compound heterozygosity for a truncating and novel enhancer mutation in *PTF1A*, broadening the spectrum of mutations causing pancreatic agenesis and phenotypic variability of this condition.

## Acknowledgements

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## Ethics

**Informed Consent:** Written consent was obtained from the patient's parents.

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

### Authorship Contributions

Concept: Monica Gabbay, Sian Ellard, Elisa De Franco, Regina S. Moisés, Data Collection or Processing: Monica Gabbay, Sian Ellard, Elisa De Franco, Regina S. Moisés, Analysis or Interpretation: Monica Gabbay, Sian Ellard, Elisa De Franco, Regina S. Moisés, Literature Search: Monica Gabbay, Sian Ellard, Elisa De Franco, Regina S. Moisés, Writing: Monica Gabbay, Sian Ellard, Elisa De Franco, Regina S. Moisés.

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CASE REPORT

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# Congenital Central Hypothyroidism Caused by a Novel Thyroid-Stimulating Hormone-Beta Subunit Gene Mutation in Two Siblings

**Bayram Özhan[1], Özlem Boz Anlaş[2], Bilge Sarıkepe[2], Burcu Albuz[2], Nur Semerci Gündüz[2]**

*[1]Pamukkale University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Denizli, Turkey*
*[2]Pamukkale University Faculty of Medicine, Department of Medical Genetics, Denizli, Turkey*

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

Congenital central hypothyroidism (CCH) is a very rare disease. Genes included in pituitary development, as well as mutations of the immunoglobulin superfamily member 1 and transducin β-like protein 1 can cause syndromic CCH. However, isolated CCH is caused by mutations of the thyrotropin-releasing hormone receptor or thyroid-stimulating hormone-beta subunit genes.

**What this study adds?**

This article presents a novel mutation of *TSHB* genes in 2 patients with CCH.

## Abstract

Congenital central hypothyroidism (CCH) is a very rare disease. Alterations in pituitary development genes as well as mutations of immunoglobulin superfamily member 1 and transducin β-like protein 1 can result in CCH and multiple pituitary hormone deficiencies. However, mutations of the thyrotropin-releasing hormone receptor or thyroid-stimulating hormone-beta (*TSHB*) gene are responsible for isolated CCH. In this paper, we present the cases of two siblings with a novel mutation of TSHB. Direct sequencing of the coding regions and exon/intron boundaries of the *TSHB* gene revealed two homozygous nucleotides changes. One of them was c.40A > G (rs10776792) which is a very common variation that is also seen in healthy individuals, the other was c.94G > A at codon 32 of exon 2 which resulted in a change from glutamic acid to lysine (p.E32K). Both patients were homozygous and the parents were heterozygous.
**Keywords:** Hypothyroidism, congenital, thyrotropin deficiency

## Introduction

Congenital central hypothyroidism (CCH) is a very rare disease associated with insufficient thyroid-stimulating hormone (TSH; also called thyrotropin) stimulation of a normally located thyroid gland. Alterations in pituitary development genes, such as *PIT1, PROP1, HESX1, LHX3, LHX4,* and *SOX3* can result in CCH and multiple pituitary hormone deficiencies (1). Recently, mutations of the immunoglobulin super family member 1 (IGSF1) and transducin β-like protein 1 (*TBL1X*) genes have also been described as causes (2,3). IGSF1 is located on Xq 26.2 and its product may act as a signal transduction molecule in the hypophysis. Its precise physiological function is not known, but a deficiency of IGSF1 protein results in deficiencies of TSH and prolactin, pubertal delay and macroorchidism after adolescence (2). In cases with *TBL1X* gene mutations, hearing loss often accompanies central hypothyroidism (3).

Apart from these syndromic CCH causes, isolated CCH can be caused by mutations of the thyrotropin-releasing hormone (TRH) receptor, the least frequent one, or of TSH-beta (*TSHB*) subunit genes. The *TSHB* gene is located on chromosome 1p13 and contains 3 exons. The second and third exons encode a mature protein of 112 amino acids (4).

In this paper, we report two siblings with congenital secondary hypothyroidism who were diagnosed at the ages of 16 and 20 with a new mutation in the *TSHB*-subunit gene.

**Address for Correspondence:** Bayram Özhan MD, Pamukkale University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Denizli, Turkey **Phone:** +90 505 265 62 83 **E-mail:** bayramozhan@yahoo.com **ORCID ID:** orcid.org/0000-0003-4842-9976

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## Case Reports

### Case 1

A 16-year-old girl was admitted to the hospital with a 20-day history of persistent vaginal bleeding. The patient was born to non-consanguineous Turkish parents following an uneventful, full-term second pregnancy. Her past medical history revealed that she was developmentally delayed and that she had received L-thyroxine ($T_4$) treatment for a short period at the age of 2; however, her parents did not continue the treatment. She had 3 siblings. Two of them are dizygotic twins whose ages are 5.26 years. The sister's height is 100.2 cm (Z-score; -1.22), weight 16.2 kg (Z-score; -1.22) and her brother's height is 106.3 cm (Z-score; -1.22), weight 23 kg (Z-score; 1.26). Her oldest brother has short stature.

The physical examination revealed a myxedematous face, thin hair, dry skin, a distended abdomen with an umbilical hernia, and psychomotor retardation. Her weight was 26 kg [standard deviation (SD) score -7.46], and she had a height of 96 cm (SD score -10.45), a body mass index of 28.26 kg/$m^2$, a blood pressure of 88/43 mmHg, and a pulse rate of 117 bpm. Her thyroid gland was not palpable. Her breast development was consistent with Tanner stage 3, she had no pubic or axillary hair.

The laboratory test results (Table 1A) showed that her creatinine phosphokinase, serum aspartate transaminase, alanine transaminase, triglyceride, and cholesterol levels were elevated. Her kidney function was within normal limits. The complete blood count revealed that she had severe anemia with a hemoglobin level of 5.6 g/dL associated with a deficiency of both iron and vitamin B12.

Her free $T_4$ level was 0.05 ng/dL (0.8-2.2), and TSH level was 0.454 uIU/mL (0.51-4.30); prolactin (PRL) level was 15 ng/mL (4.79-23.3). Her follicle-stimulating hormone level (FSH) was 6.10 mIU/mL (0.5-2.41), luteinizing hormone (LH) level was 12.64 mIU/mL (0.18-0.3), and estradiol level 46.08 ng/mL (12.5-166). Insulin-like growth factor 1 level was 38.7 ng/mL (226-903). Radiological investigations revealed delayed bone age, epiphyseal dysgenesis, and kyphoscoliosis (Figure 1). Ultrasonography of her pelvis showed follicles in both ovaries, a uterine length of 57 mm, and an endometrial thickness of 7 mm.

Serum samples were obtained at 0, 20, 40, and 60 minutes after intravenous TRH (200 μg/$m^2$) administration to evaluate her PRL and TSH responses (Table 1B). Her PRL response was normal, but there was no increase in TSH. Based on these physical findings and on the laboratory results which included low levels of free $T_4$ and TSH, a diagnosis of secondary hypothyroidism was made. The pituitary gland was found to be normal in a hypothalamus-pituitary magnetic resonance imaging. To check the integrity of the hypothalamic-pituitary-adrenal axis, a low-dose adrenocorticotropic hormone stimulation test was performed; her peak cortisol level was 21.38 μg/dL. An echocardiogram was performed and it was normal. Usually, bradycardia accompanies hypothyroidism, but in our patient, tachycardia and hypotension were present due to severe anemia. L-$T_4$ treatment was started. Her L-$T_4$ dose was gradually increased from a quarter of the calculated dose to 100 μg/$m^2$/day. Treatment with vitamin B12 and iron was also initiated for anemia.

### Case 2

The 20-year-old brother of Case 1 was also affected (Figure 2). He had coarse facial features, dry skin, cold extremities, and moderate intellectual disability. His weight was 46.4 kg (SD score -3), and he had a height of 133.4 cm (SD score -6), a blood pressure of 100/80 mmHg, and a pulse rate of 70

**Table 1A. Anthropometric measurements and laboratory investigations in the two patients**

| | Case 1 | Case 2 |
|---|---|---|
| Age at diagnosis (years) | 16 | 20 |
| Bone age (years) | 3 | 15 |
| Weight (kg)/Z-score | 26/-7.46 | 47/-3 |
| Height (cm)/ Z-score | 96/-10.45 | 133.4/-6 |
| ALT (IU/L) (< 33) | 44 | 25 |
| AST (IU/L) (< 32) | 55 | 56 |
| Triglyceride (mg/dL) (< 200) | 726 | 126 |
| Cholesterol (mg/dL) (< 200) | 266 | 315 |
| CPK (U/L) (20-180) | 545 | 478 |
| Ferritin (ng/mL) (20-200) | 4.32 | 89 |
| Vitamin B12 (pg/mL) (191-663) | 181.7 | 275 |
| Hemoglobin (g/dL) (12-18) | 5.6 | 12.9 |
| Mean corpuscular volume (fL) (80-100) | 94.4 | 87.3 |

CPK: creatinine phosphokinase, AST: aspartate transaminase, ALT: alanine transaminase

**Table 1B. Thyrotropin-releasing hormone stimulation test results of the index case**

| Time (min) | 0' | 20' | 40' | 60' |
|---|---|---|---|---|
| TSH (uIU/mL) (0.6-4.84) | 0.387 | 0.764 | 0.771 | 0.713 |
| Prolactin (ng/mL) (4.79-23.3) | 15 | 19 | 26 | 14 |

TSH: thyroid-stimulating hormone

bpm. His thyroid gland was not palpable. Testicular volume was 20 mL bilaterally and his pubic hair was consistent with Tanner stage 4. His baseline free $T_4$ level was 0.114 ng/dL (0.8-2.2), TSH level was 1.93 IU/mL (0.51-4.30), and PRL level 30 ng/mL (4.79-23.3). His FSH level was 2.35 mIU/mL (1.5-12.4), LH level 3.98 mIU/mL (1.7-8.6), and total testosterone level was 5.81 ng/mL (2.8-8). His hemoglobin level was normal, and his cortisol level was 10.76 µg/dL. Insulin-like growth factor 1 level was 216 ng/mL (116-358). His bone age was consistent with age 15 years. Based on these physical findings and on the laboratory results (Table 1A), a diagnosis of isolated secondary hypothyroidism was made, and L-T4 therapy was initiated with a low dose and titrated with free $T_4$ values to maintain the serum free $T_4$ concentration in the upper 50 percent of the normal range.

In both patients, euthyroid status was achieved after the first month of therapy. Lipid profile and liver function test abnormalities of the first proband normalized and the anemia improved.

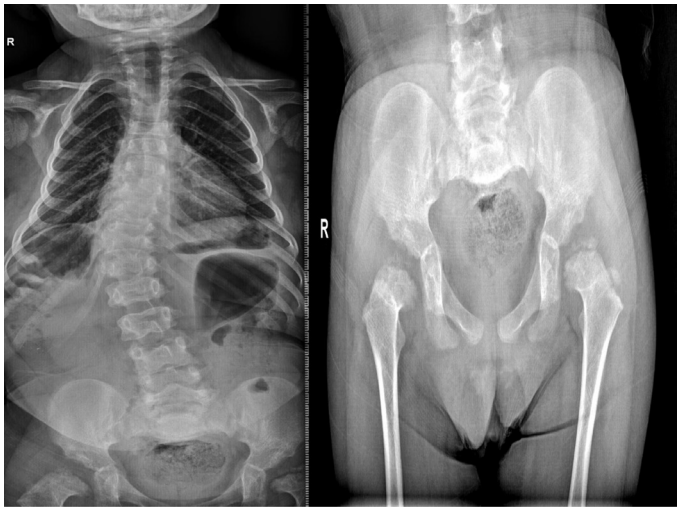

**Figure 1.** Epiphyseal dysgenesis and kyphoscoliosis in case 1

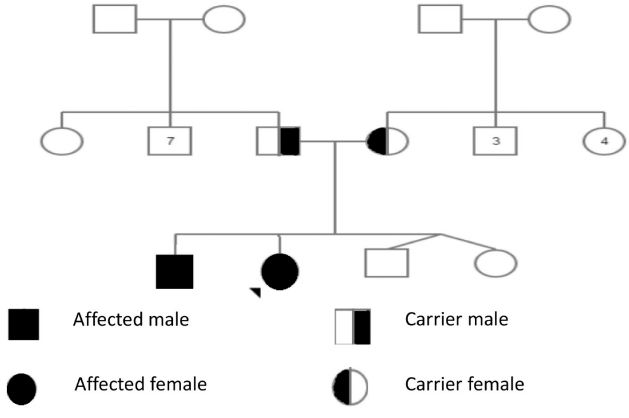


**Figure 2.** Pedigree of the family

## Genetic Analyses

Genomic DNA was extracted from peripheral blood leucocytes of the patients and their family members using a commercially available DNA extraction kit (QuickGene DNA whole blood kit S, Japan). DNA quality control was checked using a spectrophotometer (Thermo Scientific NanoDrop 2000, USA). Specific primers were designed to amplify all the coding regions and the exon/intron boundaries of the *TSHB* gene using a polimerase chain reaction (PCR). For exon II, the forward primer was 5'-GGGATGGTACTGAAGTTTGGT-3', and the reverse primer was 5'-AGATTTGGGAAATGAGGTTGTG- 3'; for exon III the forward primer was 5'-GGCTAAGCAATTCTTTCCCAGT-3' and the reverse primer was 5'-GCTCTCTAACGCCTGTGTAGG-3'. ExPrime Taq Premix was used as the PCR master mix. The quality of the PCR reaction was analyzed using 2 % agarose gel electrophoresis. An OMEGA bio-tek E.Z.N.A. Cycle Pure kit was used to purify the PCR products. The purified specific PCR products were then directly sequenced using a commercial kit according to the manufacturer's instructions (GenomeLab DTCS-Quick Start Kit, USA; BECKMAN Coultur CEQ 8000 Genetic Analysis System, USA).

Direct sequencing of the coding regions and the exon/intron boundaries for the *TSHB* gene revealed 2 homozygous nucleotide changes. The first C.40A > G (rs10776792) is a

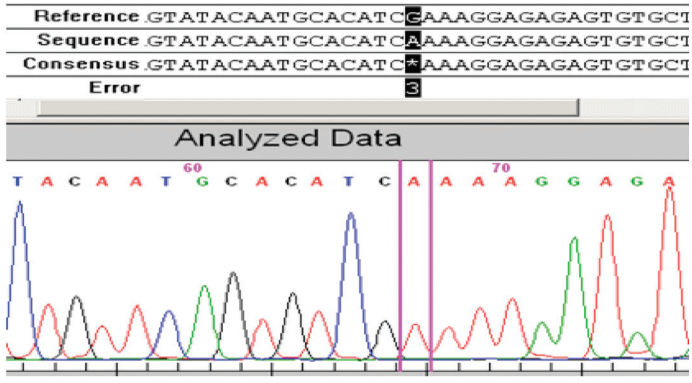


**Figure 3.** Homozygous c.94G > A

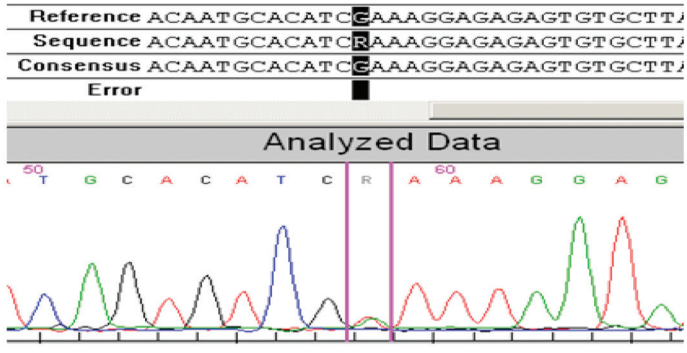


**Figure 4.** Heterozygous c.94G > A

very common variation that can also be seen in healthy individuals, including the healthy members of the family of the two patients (the parents and twin siblings). The other nucleotide change was c.94G>A at codon 32 of exon 2, which results in a change of glutamic acid to lysine (p.E32K). For this novel mutation, both patients were homozygous and the parents were heterozygous (Figures 2, 3, 4). The PolyPhen 2 (score: 0.987), SIFT (score: 0), and Mutation Taster prediction tools (disease causing) all predicted that the mutation was pathogenic.

## Discussion

We herein report two siblings with a new mutation of the *TSHB* gene in a non-consanguineous Turkish family.

The first mutation in TSHB was a single-base substitution in the 29$^{th}$ codon resulting in the replacement of glycine by arginine (G29R) (5). Since the first description of *TSHB* gene mutation, additional mutations have been reported including missense (C108Y, C105R, and G49R), nonsense, frameshift (p.E32*, p.Q69*, p.C125Vfs*10, and p.F77Sfs*6), and splice-site (c.162G>A, c.1625G>A) mutations (1,5,6,7,8,9,10,11,12). The most commonly reported mutation is the C105Vfs114X mutation. This mutation is located on exon 3 of the *TSHB* gene, and it was first described in 1996 (10).

The first reported Turkish cases were two siblings who had a C-to-T transition at nucleotide 654 which led to the conversion of a glutamine into a premature stop codon in codon 49 (Q49X) (12). In 2004, the IVS2+5 G>A mutation resulting in isolated TSH deficiency was reported in 4 children from two consanguineous Turkish families (13). More recently, a homozygous TSHB deletion was found in a 51-day-old male of Turkish descent who was diagnosed during a prolonged jaundice investigation (14).

The mutation in our patients was c.94G>A at codon 32 of exon 2; it resulted in a change from glutamic acid to lysine (p.E32K). Another homozygous mutation at the same position (c.94G>T, p.E32*) was previously reported in 3 Greek children from two families (9). *In silico* analysis of c.94G>A revealed that this alteration was pathogenic. To the best of our knowledge, the mutation detected in our patients has never been reported in central corneal thickness. Unfortunately, we could not perform functional analyses, but the *in silico* analyses were in agreement that the variant has a disease-causing effect.

TSH, LH, and FSH are pituitary glycoprotein hormones. All three are composed of an alpha-subunit and a beta-subunit coupled with non-covalent bonds. Their alpha-subunits are identical, but their beta-subunits are hormone-specific and confer biological specificity. *TSHB* gene mutations cause alterations in the size or shape of the TSHB-subunit by affecting the beta-subunit's seatbelt region or by changing the protein building blocks that are used to make the beta-subunit (1). The production or release of functional TSH from the pituitary gland is thus decreased. This TSH deficiency results in low hormone levels and leads to hypothyroidism. Consequently, TRH levels increase to stimulate both the production of TSH and PRL. There is no increase in TSH levels, because both alpha- and beta-subunits are needed to form intact TSH. On the other hand, serum alpha-subunit concentrations increase, a biochemical hallmark in *TSHB* gene mutations, and the PRL levels increase. For diagnostic purposes, in these patients, high serum alpha-subunit concentrations or a TRH stimulation test can be used to show isolated TSH deficiency and to indicate that the TRH receptor is intact based on an elevated PRL level (1). We performed a TRH stimulation test only for the index case, and the results were compatible with those for an isolated TSH deficiency. The elevated basal PRL levels and the very low TSH levels despite the severe hypothyroidism in the second case were also considered findings indicative of isolated TSH deficiency.

The clinical consequences of CCH are related to the severity and duration of thyroid hormone deprivation. The mutations probably have little impact on phenotype, and no genotype-phenotype correlation has been reported. All of the reported cases showed clinical signs of hypothyroidism, such as prolonged jaundice, coarse facies, large fontanelles, dry skin, umbilical hernia, enlarged tongue, mental and motor retardation. Both patients were in their late teens and both showed all of the clinical and metabolic signs of severe hypothyroidism. They also both had severe motor and mental retardation. The index case had kyphoscoliosis in addition to a very short stature. The radiological appearance was compatible with epiphyseal dysgenesis which is a hallmark of long-standing untreated hypothyroidism (15). Hypothyroidism is treated with L-thyroxine, but measurement of serum TSH cannot be used as a guide to the adequacy of $T_4$ replacement therapy in CCH. Serum TSH is always suppressed to $<0.1$ mU/L, so dosage should be titrated with free $T_4$ value to maintain the serum free $T_4$ concentration in the upper 50 percent of the normal range (16).

Congenital hypothyroidism is the most common preventable cause of mental retardation. Screening programs for newborns were developed to detect this preventable condition as early as possible. Screening by $T_4$ and TSH is highly sensitive. However, TSH-based screening is more frequently used around the world even though it often leads to central hypothyroidism being overlooked (17).

Identification of a TSHB mutation would help in the genetic counseling and early diagnosis of siblings in affected families.

### Ethics

**Informed Consent:** Informed consent forms were obtained from the parents of the patients for publication of the cases, including images.

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

### Authorship Contributions

Concept: Bayram Özhan, Özlem Boz Anlaş, Nur Semerci Gündüz, Design: Bayram Özhan, Özlem Boz Anlaş, Nur Semerci Gündüz, Data Collection or Processing: Bayram Özhan, Özlem Boz Anlaş, Nur Semerci Gündüz, Analysis or Interpretation: Özlem Boz Anlaş, Bilge Sarıkepe, Burcu Albuz, Nur Semerci Gündüz, Literature Search: Bayram Özhan, Özlem Boz Anlaş, Writing: Bayram Özhan, Özlem Boz Anlaş, Bilge Sarıkepe, Burcu Albuz, Nur Semerci Gündüz.

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CASE REPORT

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# Long-Term Follow-up of a Case with Proprotein Convertase 1/3 Deficiency: Transient Diabetes Mellitus with Intervening Diabetic Ketoacidosis During Growth Hormone Therapy

**E. Nazlı Gönç, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir**

*Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey*

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

Diabetes mellitus may develop over time during the course of the disease, which may be due to insufficient conversion of proinsulin into insulin.

**What this study adds?**

Proprotein convertase 1/3 is an enzyme that converts prohormones into active hormones. Thus, proprotein convertase 1/3 deficiency has been reported to be characterized by several hormonal deficiencies. Elevation of proinsulin levels is used in the diagnosis; however, diabetes mellitus has not been reported before.

## Abstract

Proprotein convertase 1/3 (PC1/3) deficiency is a very rare disease characterized by severe intractable diarrhea in the first years of life, followed by obesity and several hormonal deficiencies later. Diabetes mellitus requiring insulin treatment and diabetic ketoacidosis have not been reported in this disorder. We herein present a girl with PC1/3 deficiency who has been followed from birth to 17 years of age. She developed deficiencies of all pituitary hormones over time as well as diabetes mellitus while receiving growth hormone (GH) therapy. She was complicated with diabetic ketoacidosis during dietary management of diabetes mellitus, thus insulin treatment was initiated. Insulin requirement to regulate hyperglycemia was short-lived. Repeat oral glucose tolerance test five years later was normal. The findings of this patient show that diabetes mellitus can develop at any time during follow-up of cases with proportein convertase 1/3 deficiency especially under GH therapy.

**Keywords:** Proprotein convertase 1/3 deficiency, diabetes mellitus, diabetic ketoacidosis, treatment, diabetes insipidus

## Introduction

Proprotein convertase 1/3 (PC1/3) is an enzyme that is responsible for conversion of inactive peptides into active form. It is particularly expressed in neuroendocrine tissues. Thus, its deficiency leads to insufficient activation of several hormones including proinsulin, proopiomelanocortin, pro-thyrotropin-releasing hormone, pro-glucagon, and pro-gonadotropin-releasing hormone (1,2). To date, fewer than 20 patients with PC1/3 deficiency have been reported (3,4,5,6,7,8,9). Clinical presentation of these patients is variable. However, intestinal malabsorption in the first years of life and obesity thereafter are relatively constant findings. Other manifestations such as hypocortisolism, hypothyroidism, diabetes insipidus, hypogonadism, growth deficiency, and disorders of glucose metabolism are not seen in every patient. The time of onset for development of these hormone deficiencies is also variable (3,4,5,6,7,8,9).

Insulin deficiency due to inefficient conversion of proinsulin to insulin is one of the hallmarks of the disease. High proinsulin level is a diagnostic marker for PC1/3 deficiency. However, the patients reported so far did not have a significant disorder related to glucose metabolism.

Herein, we report a long-term follow-up of a 19-year-old girl with PC1/3 deficiency who developed multiple pituitary

**Address for Correspondence:** E. Nazlı Gönç, MD,
Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey
**Phone:** +90 312 305 11 24 **E-mail:** ngonc@hacettepe.edu.tr **ORCID ID:** orcid.org/0000-0003-1385-2563



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hormone deficiencies. She had a transient period of insulin-dependent diabetes mellitus with an intervening diabetic ketoacidosis during growth hormone (GH) therapy.

## Case Report

A female proband was born by cesarean section with a birth weight of 3.5 kg. She was the only child of second-degree cousins of Turkish origin. Chronic diarrhea started in the first week of life. She was hospitalized several times for severe dehydration and metabolic acidosis. Total parenteral nutrition was started at 9 months of age and she was followed at a medical center for six months. Subsequently, the parents managed to offer parenteral nutrition to the patient in the household setting till she reached age 2 years. Since glucose, galactose, lactose, and long-chain fatty acids in the diet increased the amount and frequency of loose stools, they were eliminated from the oral feedings. The intestinal biopsy showed villous atrophy with nonspecific changes. Her appetite was so good that although the diarrheic attacks continued, the patient gained weight. During infections, attacks of metabolic acidosis reappeared, suggesting renal tubular acidosis, and bicarbonate therapy was started. In the following 2 years, diarrhea has nearly resolved, but the restricted diet was continued. At 4.3 years of age, the patient was referred to pediatric endocrinology for polyuria and polydipsia. The parents have been aware of her increased water intake since infancy, but they did not consider it a problem till the cessation of diarrhea. She used to drink 3-4 liters a day.

When the patient was 4.3-year-old, her height was 96 cm (3-10p), weight 22 kg (97p), and body mass index (BMI) was 23.9 kg/m$^2$ (> 95p). Her physical examination was normal, and she did not have any dysmorphic features. The laboratory findings were as follows: hemoglobin (Hb): 12.2, hematocrit: 36, white blood cell: 7600, platelet: 270.000, glucose: 77 mg/dL, Na: 137 mEq/L, K: 4.8 mEq/L, Cl: 116 mEq/L, blood urea nitrogen: 4.1 mg/dL, creatinine 0.53 mg/dL, calcium: 9.9 mg/dL, P: 4.2 mg/dL, alkaline phosphatase: 373 U/L, alanine aminotransferase: 28 U/L, aspartate aminotransferase: 42 U/L. Blood gas analysis revealed: pH: 7.43 and bicarbonate ($HCO_3$): 24.3 mmol/L. Urine density was 1003 and no proteinuria or glucosuria was noted. Water deprivation test yielded an increase in Na level to 151 mEq/L and a urine osmolality to 238 mOsmol/kg, while plasma osmolality was 320 mOsmol/kg. Simultaneous plasma arginine vasopressin (AVP) after water deprivation test was 1.3 pg/mL (0-8). Administration of intranasal DDAVP at a test dose of 5 µg increased the urine osmolality and alleviated the symptoms of polyuria and polydipsia. Diagnosis of central diabetes insipidus was established and intranasal DDAVP at a dose of 1.25 µg per day was started. At that time, morning cortisol, free thyroxine ($fT_4$), and prolactin levels (cortisol: 19.8 µg/dL, $fT_4$: 15. 2 pmol/L, prolactin: 8.2 ng/mL) were normal, insulin-like growth factor 1 (IGF-1) and IGF binding protein-3 (IGFBP-3) levels were low [IGF-1: 15 ng/mL (<-3 standard deviation [SD]) and IGFBP3: 1529 ng/mL (-2 SD–[-3 SD])]. Her weight and height gains are shown in Figures 1a and 1b.

At age 9 years, when her height was at 10$^{th}$ percentile, $fT_4$ was found to be lower than the normal range ($fT_4$: 11.9 pmol/L, normal: 12-22; thyroid-stimulating hormone: 3.6 mIU/L, normal: 0.27-4.2). Fasting morning cortisol level was 4 µg/dL and adrenocorticotropic hormone (ACTH) was 21 pg/mL. Low-dose ACTH test was performed and cortisol peak was subnormal at 15 µg/dL [N: 19.8 µg/dL] (10). The diagnosis of central hypothyroidism and adrenal insufficiency were established, and Na L-thyroxin (100 µg per day) and hydrocortisone (10 mg/m$^2$ per day in three doses) replacements were started accordingly.

At the age of 10.5 years, she was 129.7 cm in height [-1.49 standard deviation score (SDS)], and growth velocity decreased to 1.8 cm/year (Figures 1a, 1b). Her bone age was 8 years. The midparental height was 156.25 cm (-0.99 SDS). The levels of IGF-1 and IGFBP3 were 56 ng/mL (<-3 SD) and 1848 ng/mL (-3 SD), respectively. GH stimulation tests with levodopa and clonidine were carried out and peak GH responses were

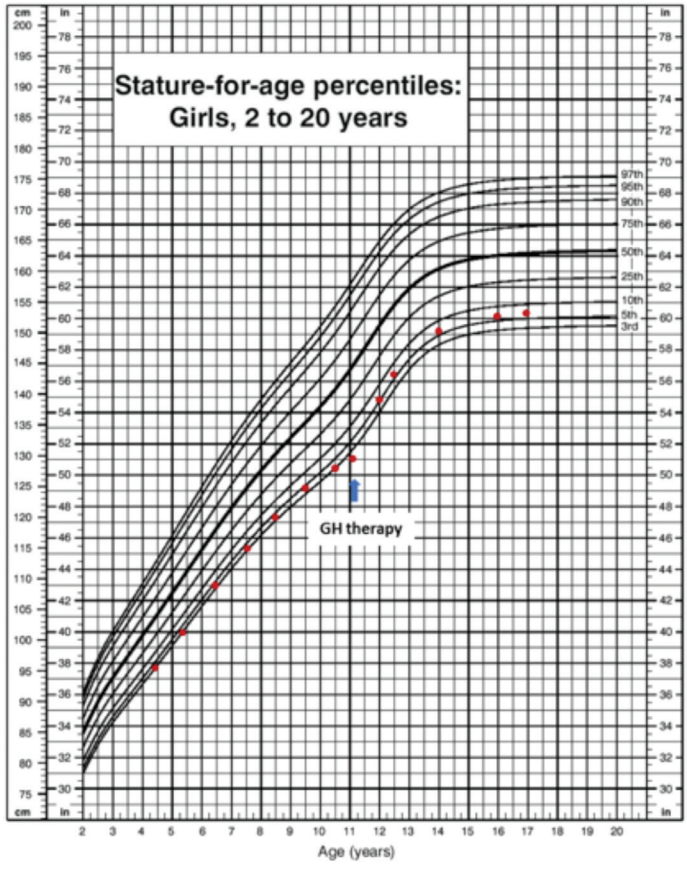


**Figure 1a.** Growth chart of the patient before and after growth hormone therapy

GH: growth hormone

4.03 and 4.6 ng/mL, respectively (normal GH response: > 7 ng/mL). Recombinant human GH (rhGH) was started at a dose of 0.03 mg/kg per day subcutaneously. The repeat magnetic resonance imaging of the pituitary gland was normal with a 6-mm height in the anterior lobe and a normal bright spot on the posterior lobe.

At the age of 11.5 years, after receiving GH therapy for one year, the patient had gained 7 cm. Her height was 134.5 cm and weight 50.3 kg, with a BMI 27.9 kg/m$^2$ (> 97p). The diagnosis of PC1/3 deficiency was established by the mutation analysis of *PCSK1* gene. A novel essential splice site mutation (IVS8 + 1G > T) was identified (7).

Fasting blood glucose level was 85 mg/dL, and there were neither signs and symptoms nor any family history for diabetes mellitus. However, a derangement in glucose metabolism was likely in PC1/3 deficiency, so oral glucose tolerance test was performed. The results revealed diabetes mellitus (Figure 2). HbA1c was 5.8% (4.5-6.2). Anti-insulin, anti-GAD, and anti-IA2 antibodies were negative. Weight loss, physical activity, and diabetic diet were recommended. GH therapy was not discontinued.

Three months later, HbA1c increased to 6% and continued to increase to 6.5% in the next 6 months on GH treatment. At the age of 12.5 years, she was brought to emergency clinic by her parents for lethargy. No signs or symptoms of infection were noted. She was dehydrated. Blood glucose was 725 mg/dL (simultaneous insulin level was 15 mIU/L) with ketosis (urine ketones were 4 + ), and acidosis (blood pH: 7.15 and $HCO_3$: 9.2 mmol/L). HbA1c was 10.5%. Diabetic ketoacidosis was treated with intravenous fluid-electrolyte and insulin therapy. Basal-bolus insulin regimen using rapid-acting insulin three times a day and long-acting insulin, glargine, once a day was started thereafter. Initially, total daily dose of insulin was nearly 1.5 U/kg. However, the daily requirement of insulin progressively decreased to 0.15 U/kg per day within 10 days and eventually it was discontinued within one month. HbA1c levels were between 5.3 and 6.2% and fasting and postprandial glucose levels remained within normal levels thereafter. She received GH treatment till 15.1 years of age under a diabetic diet without any further deterioration in glucose metabolism.

The patient remained prepubertal during her follow-up and at 13 years of age, gonadotropin and estradiol levels were very low (follicle-stimulating hormone < 0.07 mIU/mL, luteinizing hormone < 0.07 mIU/mL, E2: 2.65 pg/mL), so estradiol replacement was started at the age of 13 years and switched to cyclic treatment at 15.5 years.

At 16.5 years, her final height is 153 cm (-1.5 SDS) and weight 69 kg (body mass index: 29.5 kg/m$^2$). She is receiving Na l-thyroxine (2 mcg/kg/day) for hypothyroidism, sublingual lyophilized DDAVP tablet (30 µg two times a day) for central

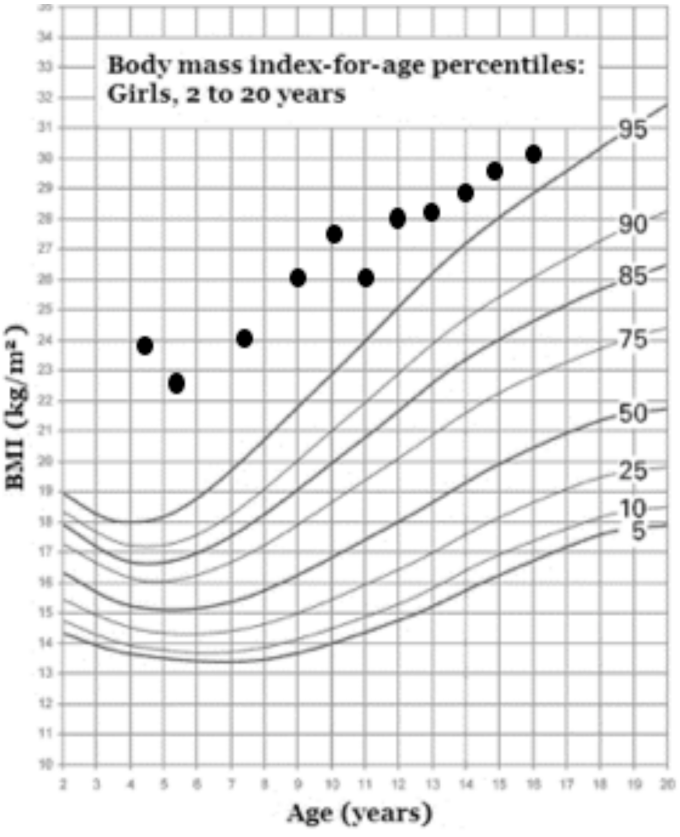


**Figure 1b.** Body mass index chart of the patient

BMI: body mass index

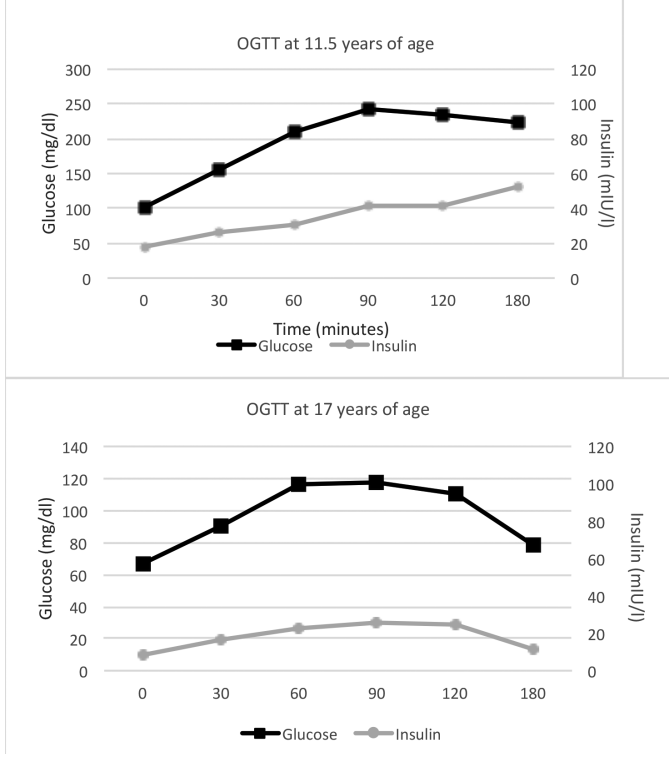


**Figure 2.** Oral glucose tolerance test at two different time points (11.5 and 17 years of age)

OGTT: oral glucose tolerance test

diabetes insipidus, hydrocortisone (10 mg/m$^2$/day) for adrenal insufficiency, combined estrogen-progesterone pills for hypogonadism, and a diabetic diet for diabetes mellitus.

At the age of 17 years, HbA1c was 5.4% and a repeat oral glucose tolerance test showed normal glucose homeostasis (Figure 2).

## Discussion

Multiple hormonal insufficiencies have been reported in patients with PC1/3 deficiency. However, every patient with PC1/3 deficiency varies in the nature of hormonal insufficiency as well as its severity. The first reported case with PC1/3 deficiency was a 43-year-old woman who had obesity, hypogonadotropic hypogonadism and hypoadrenalism (3,11). Although GH response to insulin-induced hypoglycemia was low, she had a normal height of 161 cm. Oral glucose tolerance test showed an elevated two-hour blood glucose level (206 mg/dL) indicating diabetes mellitus. She also had postprandial hypoglycemia after a standardized meal (3).

The second patient was a female infant with intractable diarrhea who subsequently developed obesity (4). She had several episodes of hypoglycemia which were attributed to low cortisol response to hypoglycemia. Hydrocortisone replacement was started. She died of uncertain cause at the age of 18 months (4).

The third case was a boy who was followed till six years of age (5). He developed severe obesity after a period of intractable diarrhea which required 5-week parenteral nutrition in addition to oral feedings with specialized infant formula. At the age of 4 years, he developed polyuria and polydipsia. However, the water deprivation test was not diagnostic for diabetes insipidus. Consequently, following the diagnosis of PC1/3 deficiency, he was further evaluated for hormone insufficiencies; hypocortisolism and hypothyroidism were detected. In the case report, there was no detailed information about glucose metabolism of the boy except a normal fasting glucose and elevated proinsulin levels (5).

The fourth case was the first report in the literature with PC1/3 deficiency who had a documented central diabetes insipidus (6). He had hypocortisolism, hypothyroidism, and low testosterone level with micropenis suggesting hypogonadism as well. He had a normal GH response at the time of hypoglycemia. No further evaluation of glucose metabolism was mentioned in the report (6).

Then, Martín et al (7) reported the clinical, laboratory, and genetic features of 13 children with PC1/3 deficiency from 11 families. Our patient was in that cohort (represented as family 3). Eleven of thirteen cases reported by Martín et al (7) were alive and 8 were younger than 10 years old. Hypothyroidism, hypocortisolism, and diabetes insipidus were relatively more common than GH deficiency in that cohort. It was reported that the patients who received GH had had a good response. Data about glucose metabolism of the patients was scarce except a note of postprandial hypoglycemia in 8 of the cases. Oral glucose tolerance test or HbA1c levels were not determined.

We had the opportunity to follow our patient from birth to 17 years of age and to observe nearly all consequences of PC1/3 deficiency reported so far. PC1/3 activity is essential for the activating cleavage of many peptide hormone precursors including hypothalamic hormones (1,2). So, lack of activation of hypothalamic hormones may mimic multiple pituitary hormone deficiency due to a defect in hypothalamus-pituitary axis. Diabetes insipidus was the earliest hormonal deficiency detected in the current patient. It probably started even before, possibly early in infancy but was disregarded till 4 years of age as the parents assigned the symptoms of polyuria and polydipsia to ongoing diarrhea.

Thyroid hormones and cortisol level were in normal ranges till 9 years of age in the current patient. Although IGF-1 and IGFBP-3 levels were low at 4 years of age, height was at the 10$^{th}$ percentile and growth velocity was normal. At 10.5 years, growth velocity decreased, GH response was low in GH stimulation tests and rhGH was initiated at a conventional dose. One year after the GH therapy, the diagnosis of PC1/3 deficiency was established definitively by genetic analysis. Oral glucose tolerance test was performed since a potential disorder in glucose metabolism was considered and diabetes mellitus was diagnosed. Insulin response to elevated glucose levels indicated neither absolute insulinopenia nor insulin resistance, however suggested a relative insulin deficiency. There was no symptom suggestive of hyperglycemia at the time of testing. HbA1c increased to 6.5% while the patient was on a diabetic diet, and two years after the onset of rhGH therapy (at age 12.5 years), diabetic ketoacidosis developed without any identifiable precipitating cause. Insulin requirement continued for one month only. Although rhGH therapy was continued, a similar picture of insulin insufficiency did not recur till 15.1 years of age. Two years after cessation of rhGH therapy, a repeat oral glucose tolerance test was completely normal.

Diabetes mellitus was not defined as a part of PC1/3 deficiency although it can be speculated that there must be a relative insulin deficiency due to the defect in conversion of proinsulin to insulin. The patients reported so far did not have history of low birth weight suggesting insulinopenia during intrauterine life. Diabetes mellitus was identified only in the first reported patient with PC1/3 deficiency (3). She developed gestational diabetes mellitus requiring insulin treatment (3). The same patient was tested again at age 43 years and at that time, her 2-hour post-load blood glucose level was 206 mg/dL (3).

Our patient is the first patient with PC1/3 deficiency who developed diabetic ketoacidosis. Diabetic ketoacidosis and one month of insulin requirement coincided with rhGH therapy which may be a contributing factor for relative insulin deficiency due to the anti-insulin effect of GH (12). However, since the insulin requirement was transient even in the course of rhGH therapy in our patient, it is difficult to consider GH as the sole factor responsible for deterioration of glucose metabolism. Diabetic ketoacidosis can complicate cases with excess GH secretion such as gigantism or acromegaly (13,14,15). However, we found only one report of a patient developing diabetic ketoacidosis during GH therapy (16). The case was a 13-year-old boy with Prader-Willi syndrome who presented with diabetic ketoacidosis four weeks after initiation of GH treatment (16). The status of glucose metabolism before GH was unknown in this patient and hyperglycemia resolved just 2 months after cessation of GH treatment. Later, this boy was diagnosed as type 2 diabetes (16). Thus, impaired glucose metabolism can associate with GH treatment, but diabetic ketoacidosis is very unlikely to develop and in such a case, presence of a predisposing condition needs to be investigated. Previous reports of patients with PC1/3 deficiency do not include details of routine investigations of glucose homeostasis, especially glucose tolerance test. The most commonly reported disturbance in glucose metabolism was postprandial hypoglycemia. Therefore, the true prevalence of diabetes mellitus in cases with PC1/3 deficiency is yet unknown.

Disorders of glucose homeostasis should be assessed in patients with PC1/3 deficiency. Diabetes mellitus with asymptomatic hyperglycemia may be one of the disorders of hormone metabolism in PC1/3 deficiency. There may be periods with relative or sometimes even severe deficiency of insulin (i.e. leading to ketoacidosis) requiring insulin treatment especially under GH treatment.

### Ethics

**Informed Consent:** Written consent was obtained from the patient's parents.

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

### Authorship Contributions

Concept: E. Nazlı Gönç, Design: E. Nazlı Gönç, Alev Özön, Data Collection and Processing: E. Nazlı Gönç, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir, Analysis and Interpretation: E. Nazlı Gönç, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir, Literature Research: E. Nazlı Gönç, Writing: E. Nazlı Gönç, Alev Özön, Ayfer Alikaşifoğlu, Nurgün Kandemir.

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CASE REPORT

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# Tolvaptan Treatment in Children with Chronic Hyponatremia due to Inappropriate Antidiuretic Hormone Secretion: A Report of Three Cases

**Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo**

*University of Turin, Regina Margherita Children's Hospital, Department of Public Health and Pediatrics, Division of Pediatric Endocrinology, Turin, Italy*

### What is already known on this topic?

Actually, the European Medicines Agency has only approved tolvaptan, a selective V2-receptor antagonist, for the treatment of hyponatremia due to syndrome of inappropriate antidiuretic hormone secretion (SIADH) in adults, whereas the United State Food and Drug Administration recommend tolvaptan and conivaptan for the treatment of both euvolemic and hypervolemic hyponatremia in adults. Many researchers have reported their results with tolvaptan treatment in hypervolemic hyponatremia due to heart failure or polycystic kidney disease. Tolvaptan has been used successfully in two infants to treat hyponatremia due to SIADH. However, treatment with vaptan in paediatric age groups has not been licensed yet neither in Europe nor in the USA.

### What this study adds?

In this paper, we report the use of tolvaptan in 3 children affected by chronic euvolemic hyponatremia due to SIADH. Tolvaptan has been used respectively for 4 and 3 years in the first two patients and for 3 months in the last patient. To date, this is the longest period of drug utilization in children with euvolemic hyponatremia.

## Abstract

Hyponatremia is the most common electrolyte disorder among hospitalized patients and it is sometimes considered as a poor outcome predictor. Its correction is thus indicated, even in asymptomatic patients. The conventional treatment consists of fluid restriction in presence of euvolemia or hypervolemia; loop diuretics are used in some hypervolemic conditions such as cardiac heart failure, liver cirrhosis and nephrotic syndrome, while intravenous isotonic or hypertonic solutions are administered in hypovolemic conditions. The utilization of demeclocycline and urea is not indicated in pediatric ages due to lack of data on their toxicity and poor tolerance. Recently, a new therapeutic option has been developed, a class of non-peptide arginine vasopressin receptor antagonists called vaptans. Tolvaptan is the only such agent approved in Europe for the treatment of hyponatremia caused by syndrome of inappropriate antidiuretic hormone secretion (SIADH) in adults. In USA, tolvaptan and conivaptan have been approved for treatment of euvolemic and hypervolemic hyponatremia. Few data are so far available in paediatric patients, since only one trial has been registered in Europe which includes children and adolescents, but this trial is still ongoing. Here, we report three children with chronic hyponatremia due to SIADH in which tolvaptan has been used successfully.

**Keywords:** Tolvaptan, children, hyponatremia, syndrome of inappropriate antidiuretic hormone secretion

## Introduction

Hyponatremia is defined as a serum sodium level below 135 mmol/L and represents the most frequent electrolyte disorder among hospitalized patients (1,2). Hyponatremia can be classified on the basis of volemic state, i.e. hypovolemic, euvolemic, and hypervolemic, or on the basis of degree of severity of the salt and/or water wasting resulting from different pathogenetic disorders. The symptoms related to this extremely heterogeneous condition depend on many factors and may vary from none to cerebral oedema, seizures, and coma. Particular caution is required in its treatment to avoid rapid correction and consequent osmotic demyelination.

**Address for Correspondence:** Gerdi Tuli MD,
University of Turin, Regina Margherita Children's Hospital, Department of Public Health and Pediatrics, Division of Pediatric Endocrinology, Turin, Italy
**Phone:** + 39 349 3232854 **E-mail:** gtuli@unito.it **ORCID ID:** orcid.org/0000-0001-5862-8958

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Hypovolemic hyponatremia is a frequent condition in paediatric patients and isotonic or hypertonic saline solution is still the mainstay of treatment (1,2,3,4,5,6,7). In older children, hypervolemic hyponatremia usually follows cardiac heart failure, liver cirrhosis, and nephrotic syndrome. Treatment usually consists of fluid restriction together with treatment of the underlying disease. Loop diuretics are also available for treatment in children, while demeclocycline and urea are not allowed due to lack of adequate data on their toxicity and tolerance.

Euvolemic hyponatremia is a typical feature of inappropriate secretion of antidiuretic hormone (SIADH). In children, it is usually associated to hypothalamic-chiasmatic tumours, meningitis-encephalitis, and sepsis. An autonomous arginine-vasopressin (AVP) secretion, independent from plasma osmolality or from the volemic state, is present. AVP is the physiological hypothalamic hormone that regulates osmolality by controlling urinary volume and composition. Normally, it is secreted in response to increases in plasma tonicity or to decreases in plasma volume and activates three types of receptors. In euvolemic hyponatremia, the gold standard treatment is fluid restriction only (2,3), but this approach is often ineffective or difficult to achieve as these patients also have a lowered osmotic threshold for thirst. Compliance is thus really poor and frequently use of isotonic or hypertonic saline solution is needed.

An alternative treatment is actually represented by non-peptide arginine-vasopressin-receptor antagonists, named vaptans (8,9,10). The blocked pathway of AVP signaling inhibits water resorption and results in excretion of diluted urine or "aquaresis" (10). The agents can be used in euvolemic and hypervolemic hyponatremias but are contraindicated in hypovolemic states. Several trials have proven the effectiveness of vaptans in increasing serum sodium levels. As the vaptans-dependent urine generation is electrolyte free, the utilization of these agents makes repletion of electrolytes unnecessary (6).

Actually, the European Medicines Agency (EMA) has only approved tolvaptan, a selective V2-receptor antagonist, for the treatment of hyponatremia due to SIADH, whereas the US Food and Drug Administration (FDA) has approved both tolvaptan and conivaptan as non-selective V1/V2 receptor antagonists for the treatment of both euvolemic and hypervolemic hyponatremia. Their dosage varies from 15 to 60 mg daily or 0.1 to 0.8 mg/kg (9,10). To date, vaptans are not in use in the treatment of acute hyponatremia.

Few data are available on use of vaptans in the pediatric age group (11,12,13,14,15,16,17,18). To date, there is only one ongoing trial registered in Europe which includes children and adolescents. Thus, the use of vaptans in children is not yet included in standard treatment schedules.

We report three pediatric cases of chronic severe hyponatremia due to SIADH who received tolvaptan treatment once the hyponatremia became symptomatic.

## Case Reports

### Case 1

This male patient was a case of ROHHAD syndrome (rapid-onset obesity with hypothalamic dysfunction, hypoventilation, and autonomic dysregulation) referred for endocrinological follow-up. The initial hormonal evaluations at age 5 years had revealed central hypothyroidism requiring treatment with L-thyroxine and then central adrenal insufficiency at age 6 years, at which time, treatment with cortone acetate was started. Over the years of endocrinological follow-up, he presented with usually asymptomatic hypernatremia alternating with hyponatremia due to hypothalamic antidiuretic hormone (ADH) release dysregulation. At age of 7 years, a grand mal seizure episode occurred; serum sodium level was decreased at 125 mmol/L. The acute episode was treated with intravenous phenobarbital and isotonic saline solution. Brain magnetic resonance imaging (MRI) and computed tomography (CT) scan did not show any relevant abnormality, while electroencephalogram showed nonspecific alterations which were attributed to hyponatremia. Evaluation of the clinical (body weight stable at 56 kg, arterial blood pressure 110/70 mmHg, urinary output 1500 mL/24 h) and laboratory findings (plasma sodium 134 mmol/L (reference range 135-145), plasma osmolality 268 mOsm/kg, urea 33 mg/dL (reference range 20-80), creatinine 0.34 mg/dL (reference range 0.25-0.85), copeptin 14 pmol/L (reference range 3-8), urine sodium 96 mmol/L, urine osmolality 484 mOsm/kg) led to a diagnosis of SIADH. Upon cessation of intravenous administration of the isotonic and hypertonic saline solution, the serum sodium level remained unstable ranging from 127 to 133 mmol/L. For this reason, we decided to start an oral low-dose treatment with tolvaptan at 3.75 mg (0.06 mg/kg/day), which was increased to 7.5 mg and then to 11.25 mg after few days (Figure 1).

At present, after 4 years of tolvaptan treatment (present dose 11.25 mg/day, i.e. 0.2 mg/kg/day), the serum sodium levels are stable (ranging from 137 to 144 mmol/L) and no acute nor severe symptoms due to hyponatremia have been observed.

### Case 2

A 4-year-old girl with a large sellar and suprasellar tumour developed chronic euvolemic hyponatremia due to SIADH.

The diagnosis was established after brain MRI and CT scan were performed for neuro-psychomotor development delay, visual loss and hyponatremia. The neurosurgical biopsy revealed a low grade ganglioglioma, so neither chemotherapy nor radiotherapy were proposed. Once the diagnosis was made, regular endocrinological and oncological follow-up was established.

At diagnosis, the patient had asymptomatic hyponatremia (serum sodium ranging from 127 to 131 mmol/L), central precocious puberty, for which a treatment with LHRH-analogue was begun, and severe hypothalamic obesity.

At age 8 years, she had a grand mal seizure episode. Serum sodium level at admission to the Emergency Department was 122 mmol/L. Brain MRI and CT scan did not show any increase of the tumour mass size. Electroencephalogram showed non-specific metabolic wave alterations, thus the pathogenesis of the grand mal seizure was attributed to hyponatremia. Pituitary-thyroid and pituitary-adrenal axis functionality was normal. SIADH was confirmed by clinical and laboratory findings (body weight stable at 35 kg, arterial blood pressure 115/80 mmHg, urinary output 1300 mL/24 h, plasma sodium 133 mmol/L (reference range 135-145), plasma osmolality 265 mOsm/kg, urea 30 mg/dL (reference range 20-80), creatinine 0.30 mg/dL (reference range 0.25-0.85), copeptin 16.6 pmol/L (reference range 3-8), urine sodium 144 mmol/L, urine osmolality 502 mOsm/kg).

The acute episode was treated with intravenous phenobarbital and hypertonic saline solution and with orally administered levetiracetam at discharge. As chronic hyponatremia became symptomatic over time, tolvaptan was started in a dose of 3.75 mg/day (0.1 mg/kg/day, weight 35 kg), then increased to 7.5 mg (Figure 2).

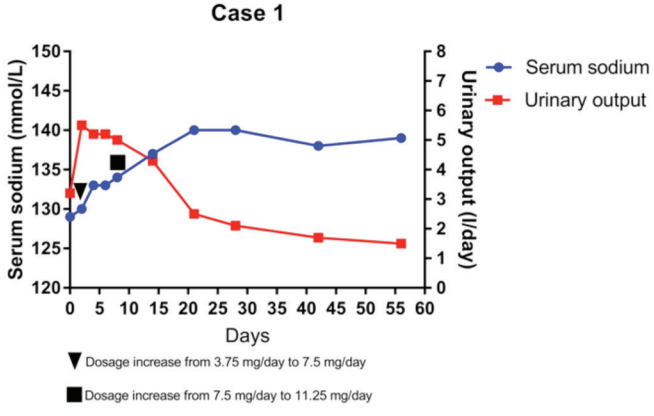


**Figure 1.** Serum sodium and daily urinary output at initiation of tolvaptan treatment and trend in the first two months of treatment

No other seizure episode was observed and levetiracetam withdrawal was decided after 8 months. Actually, after 3 years of treatment, serum sodium levels are nearly normal ranging from 133 to 137 mmol/L and the tolvaptan dosage is 11.25 mg/day (0.32 mg/kg/day). No severe hyponatremia has been registered despite the increase in the size of the tumour mass and central adrenal insufficiency onset.

## Case 3

This 5-year-old boy developed chronic euvolemic hyponatremia due to SIADH after neurosurgical partial removal of a hypothalamic-chiasmatic astrocytoma. Post-surgical chemotherapy regimen treatment was started and an endocrinological follow-up was established to evaluate hypothalamic-pituitary axis functionality. At this time, he had asymptomatic hyponatremia with serum sodium levels ranging from 123 mmol/L to 130 mmol/L. As he developed central hypothyroidism and secondary adrenal insufficiency, a substitutive treatment for these conditions was started. He also developed central precocious puberty for which a LHRH-analogue treatment was started. At age 8 years, after the beginning of a second chemotherapy regimen treatment due to tumour size increase, hyponatremia became symptomatic with headache, nausea, asthenia, and seizures which persisted after chemotherapy withdrawal. Corticosteroid dosage increase (oral cortone acetate 40-50 mg/m$^2$/day), mineralocorticoid treatment (oral fludrocortisone, 0.1 mg/day), and salt supplement (oral NaCl 3 g/day) were not sufficient to maintain serum sodium at acceptable levels, thus we started low-dose tolvaptan treatment (3.75 mg/day, 0.05 mg/kg/day, weight 83 kg) with prompt normalization of serum sodium (136-141 mmol/L) as shown in Figure 3.

After 3 months, the tolvaptan dosage has been increased to 7.5 mg (0.09 mg/kg/day), the corticosteroid dosage was reduced to a substitutive range (10-15 mg/m$^2$/day), the

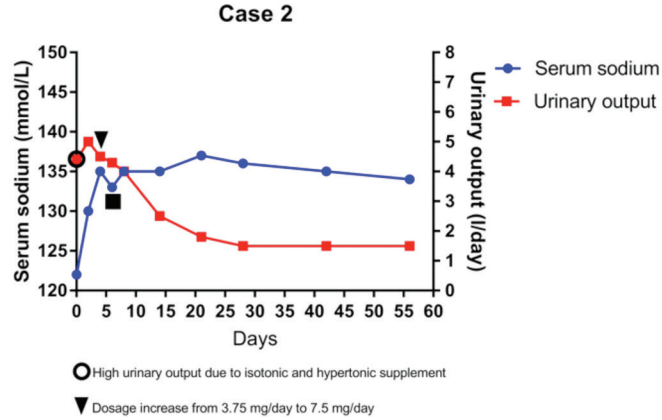


**Figure 2.** Trend of serum sodium and daily urinary output in the first two months after tolvaptan treatment initiation

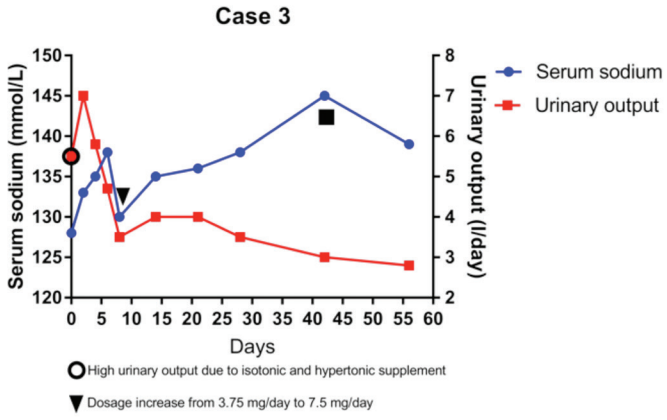


**Figure 3.** Trend of serum sodium and daily urinary output in the first months after tolvaptan treatment initiation and withdrawal timing of associated hyponatremia treatments

mineralocorticoid treatment and the supplemented salt were discontinued, with serum sodium levels remaining stable at 135-144 mmol/L.

## Discussion

A hypothalamic-pituitary dysregulation of the ADH secretion is the most frequent cause of SIADH in pediatric ages, whereas in adults, SIADH is mostly associated with paraneoplastic conditions.

SIADH can be clinically indistinguishable from the nephrogenic syndrome of inappropriate antidiuresis (NSIAD) which is an inherited form of renal water retention. Family history and suppressed ADH levels which were absent in our patients can help orientation towards this diagnosis. Moreover, patients with NSIAD do not respond to AVPR2 antagonists. Gold standard treatment of SIADH is fluid restriction which is difficult to achieve in pediatric ages. This approach is often ineffective as patients affected by SIADH have also a lower osmotic threshold for thirst.

Here, we report the results of tolvaptan treatment in three pediatric patients who developed symptomatic chronic hyponatremia due to SIADH. Other causes of euvolemic hyponatremia such as hypothyroidism or hypocortisolism were excluded in case 2. The patients described in cases 1 and 3 were already taking substitutive treatment for both conditions when they developed severe neurologic symptoms due to hyponatremia. The decision for using tolvaptan was taken due to the severity of symptoms related to the hyponatremic condition and presence of a chronic disease underlying the etiology of hyponatremia. We chose a low-dose treatment at the beginning which was adjusted according to the fluid-electrolyte balance. It would be advisable to start the treatment within the recovery regimen and under hospital conditions, since frequent monitoring of serum sodium levels is needed in the first hours after tolvaptan administration. In the first days of treatment, serum sodium levels, urinary output, and 24-h fluid balance should be closely evaluated. Based on previous experience indicating failure of fluid restriction, we permitted free access to liquids in all our three patients. Since too rapid sodium correction or fluctuations need to be avoided to prevent osmotic demyelination syndrome, withdrawal of other hyponatremia treatment options should be gradual. Tolvaptan should be started at a low dose of usually 3.75 mg and then slowly increased according to sodium values and urinary output. Serum sodium levels get prompt normalization or remain in low-normality range 24-48 hours after tolvaptan administration. In the first days of treatment, an increase in urinary output was observed as expected, but the volume progressively decreased in the following days and stabilized within 15-20 days although the patients remained polyuric.

To date, there are few data regarding the use of vaptans in paediatrics (11,12,13,14,15,16,17,18). The youngest patient treated was 2 years and 4 months old and the treatment dose ranged from 0.22 to 0.8 mg/kg (11). Tolvaptan has been effectively used in 28 patients with hyponatremia and heart failure, but none of the patients were affected by SIADH (12,13). Its use in the pediatric age group in patients with hypervolemic hyponatremia due to congestive cardiac failure with restrictive cardiomyopathy and in patients with massive edema due to nephrotic syndrome have been reported (14,15). There are reports of tolvaptan and conivaptan use in pediatric patients with acute hyponatremia who are resistant to isotonic or hypertonic fluids administration (16,17,18). Tolvaptan treatment was well-tolerated and considered to be a safe treatment in all the reported cases. Actually, EMA and FDA have approved tolvaptan only for the treatment of hyponatremia due to SIADH in adults (19,20,21). Conversely, conivaptan has previously been used in the management of hyperhydration during SIADH, but it is not yet licensed in Europe (16).

In conclusion, while further data are needed to strengthen its effectiveness and safety, we believe that tolvaptan can be a useful treatment option for euvolemic chronic hyponatremia due to SIADH in the pediatric age group. It is indeed noteworthy that through its oral administration, it can improve quality of life compared with intravenous administration of saline solution or fluid restriction. Finally, since increase in urine flow due to vaptans does not cause loss of electrolytes, no repletion is needed. For these reasons, vaptans utilization for the treatment of euvolemic and hypervolemic hyponatremias should actually be

considered even in pediatric ages, especially when chronic diseases or syndromic conditions are responsible for disorders in the SIADH mechanism and in patients with severe hyponatremia symptoms.

### Ethics

**Informed Consent:** Written consent was obtained from the patient's parents.

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

### Authorship Contributions

Concept: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo, Design: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo, Data Collection and Processing: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo, Analysis and Interpretation: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo, Literature Research: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo, Writing: Gerdi Tuli, Daniele Tessaris, Silvia Einaudi, Luisa De Sanctis, Patrizia Matarazzo.

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