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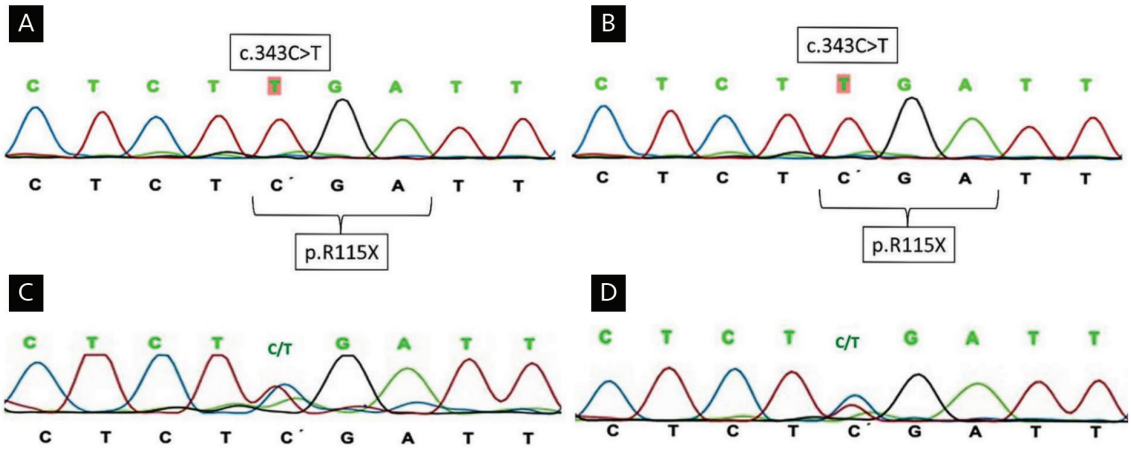


A) (Case 1), B) (Case 2): A novel homozygous nonsense pathogenic variant p.R115X (c.343 C > T) was detected in the CYP19A1 gene sequence analysis. C) (Mother), D) (Father): The parents were heterozygous for the same mutation

Aromatase Deficiency in Two Siblings with 46,XX Karyotype Raised as Different Genders: A Novel Mutation (p.R115X) in the CYP19A1 Gene

Özen S et al.


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REVIEW

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J Clin Res Pediatr Endocrinol 2020;12(1):1-16

# Syndromic Disorders Caused by Disturbed Human Imprinting

**Diana Carli, Evelise Riberi, Giovanni Battista Ferrero, Alessandro Mussa**

*University of Torino, Department of Pediatric and Public Health Sciences, Torino, Italy*

## Abstract

Imprinting disorders are a group of congenital diseases caused by dysregulation of genomic imprinting, affecting prenatal and postnatal growth, neurocognitive development, metabolism and cancer predisposition. Aberrant expression of imprinted genes can be achieved through different mechanisms, classified into epigenetic - if not involving DNA sequence change - or genetic in the case of altered genomic sequence. Despite the underlying mechanism, the phenotype depends on the parental allele affected and opposite phenotypes may result depending on the involvement of the maternal or the paternal chromosome. Imprinting disorders are largely underdiagnosed because of the broad range of clinical signs, the overlap of presentation among different disorders, the presence of mild phenotypes, the mitigation of the phenotype with age and the limited availability of molecular techniques employed for diagnosis. This review briefly illustrates the currently known human imprinting disorders, highlighting endocrinological aspects of pediatric interest.

**Keywords:** Imprinting disorders, epimutation, genotype, phenotype

## Introduction

The imprinting disorders are a group of congenital diseases caused by dysregulation of genomic imprinting that can affect fetal and postnatal growth, neurocognitive development, metabolism and cancer predisposition with relevance to pediatricians, geneticists, endocrinologists and other specialists (1,2,3,4,5,6). Genomic imprinting mediates the expression of specific genes in a parent of origin specific manner. While most genes are expressed biparentally, imprinted genes are expressed only from the paternal or the maternal allele. Imprinted genes are often arranged in clusters and expressed under a coordinated epigenetic regulation (4,7). Human imprinting disorders result from dysregulation of the normal expression of imprinted genes, causing altered dosage or function of such gene transcripts. This can be achieved through different mechanisms, which may involve DNA expression only (epigenetic mechanisms) or may also encompass DNA sequence (genomic mechanisms). While the former are mostly sporadic, the latter result in familial forms with a parent of origin inheritance pattern (5).

The molecular mechanisms responsible for altered imprinted gene expression (Figure 1) are classified into:

1. Uniparental disomy (UPD), which consists of the inheritance of two copies of a chromosome (or part of a chromosome) from one parent and no copy from the other parent. UPD can be heterodisomy, when both homologue chromosomes from the transmitting parent are present, or isodisomy, when two identical chromosomes from the same parental homologue are present (8).

2. Abnormal methylation (also termed epimutation) including excessive methylation (hypermethylation or gain of methylation - GoM) and reduced methylation [hypomethylation or loss of methylation (LoM)]. Abnormal methylation can be primary (i.e. in the absence of an underlying genomic cause) or secondary (i.e. due to an underlying genomic cause). While the former is sporadic, the latter is associated with a recurrence risk, in an autosomal dominant manner with parent of origin effect.

3. Chromosomal abnormalities (deletions, duplications and balanced rearrangements).

4. Intragenic variants in imprinted genes resulting in loss or gain of function.

For all these four mechanisms, the phenotype depends on the affected parental allele; in some cases, aberrations at the same locus involving either the maternal or the

**Address for Correspondence:** Alessandro Mussa MD, University of Torino, Department of Pediatric and Public Health Sciences, Torino, Italy
**Phone:** +39-011-313-1985 **E-mail:** alessandro.mussa@unito.it **ORCID:** orcid.org/0000-0003-2795-6013

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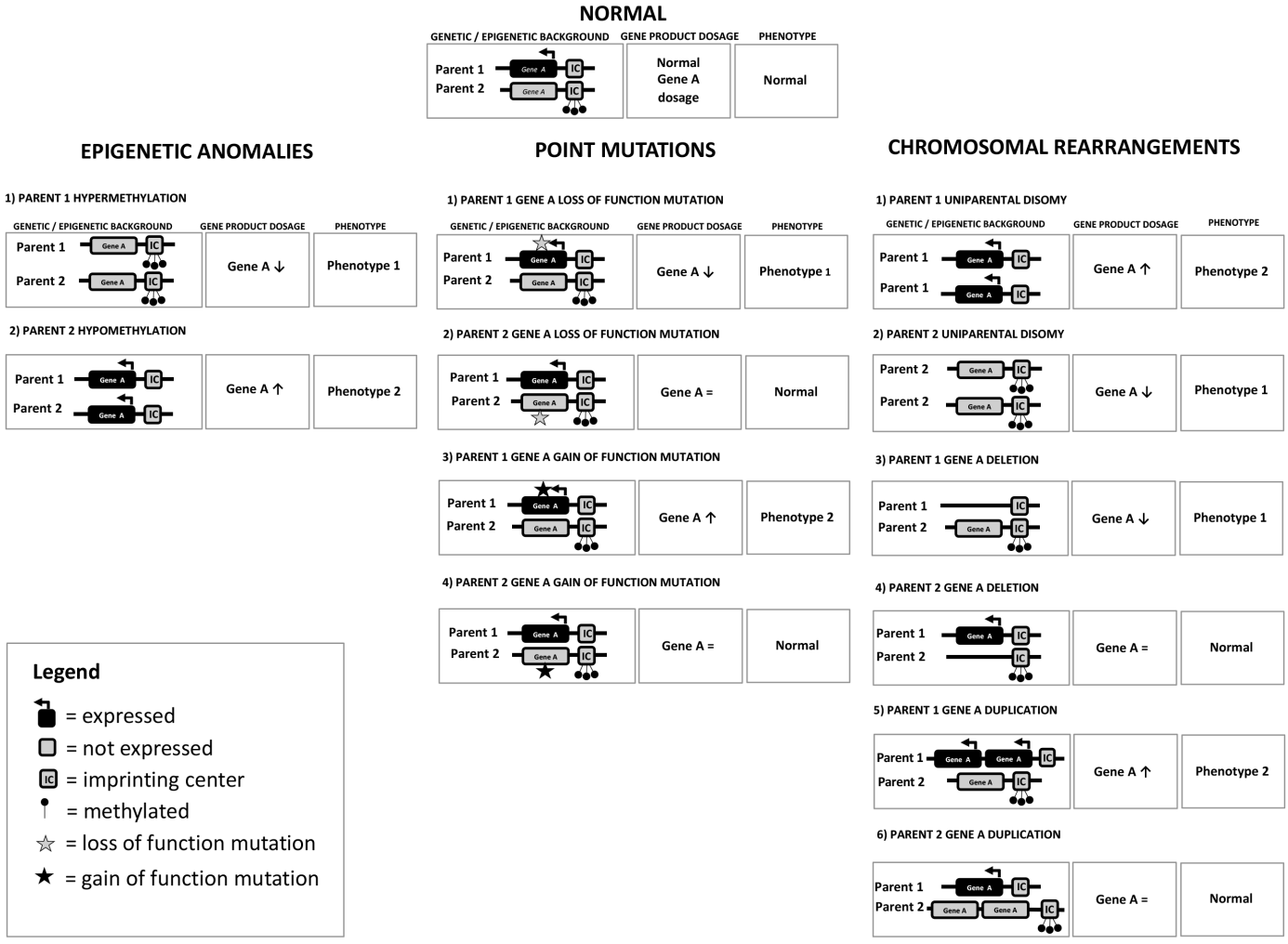


**Figure 1.** Schematic representation of the molecular mechanisms responsible for altered imprinted gene expression. At the top normal functioning of a paradigmatic chromosomal region subjected to imprinting is reported: on the allele inherited from parent 1, the imprinting center (IC) is unmethylated and gene A is expressed, while on the allele inherited from parent 2, gene A is silenced by IC methylation. This leads to a balanced expression of gene A, corresponding to the normal phenotype. Conversely, imbalance between the expression of the imprinted gene leads to a pathological phenotype: a deficiency of gene A leads to phenotype 1, while an excess of gene A leads to phenotype 2. Phenotype 1 and phenotype 2 may have antithetical characteristics (mirror phenotypes). In the left column, epigenetic anomalies leading to disturbed expression of imprinted genes are shown. In the middle column, point mutations and in the right column, uniparental disomy, deletion and duplication affecting the imprinted gene are reported. If the point mutation or the deletion/duplication hits the expressed gene, it will lead to a phenotype while, on the opposite, if they involve a normally silenced gene, they will not result in a phenotype: in both cases, the genetic anomaly could be transmitted to the offspring

paternal chromosome result in opposite phenotypes (Table 1). Although each imprinting disorder is characterized by specific clinical features, shared phenotypic features are common and clinical overlap occurs. Moreover, mild phenotypes, a broad clinical spectrum, mitigation of the presentation with age and limited availability of the molecular techniques employed for diagnosis probably lead to a relevant underdiagnosis (4,5).

Most patients with an imprinting disorder are affected by a single disease-specific locus with a definite phenotype. However, cases with multilocus methylation imprinting disturbances (MLID) and consequent complex phenotypes are increasingly described and further complicate the clinical evaluation. Of interest, the frequency of some of the imprinting disorders is increased in the offspring of subfertile parents and likely connected with artificial reproductive techniques (9,10).

**Table 1. Summary of the clinical features and the molecular mechanisms of the human imprinting disorders**

| | Imprinting syndromes | | |
|---|---|---|---|
| **Chr 6** | | **Transient neonatal diabetes mellitus type 1** | **Maternal uniparental disomy of chromosome 6 (controversial)** |
| | Phenotype | Hyperglycemia without ketoacidosis, IUGR, macroglossia, umbilical hernia, type 2 or gestational diabetes later in life | IUGR, heterogeneous clinical features |
| | Mirror mechanisms | UPD(6)pat | UPD(6)mat |
| | | Paternal duplication 6q24 | |
| | | LoM at *PLAGL1:alt-TSS-DMR* | |
| **Chr 8** | | **Birk-Barel mental retardation syndrome** | |
| | Phenotype | Severe neonatal hypotonia, transient neonatal hypoglycemia, joint contractures, wide alveolar ridges, cleft palate, microretrognathia, developmental delay, intellectual disability | |
| | Mechanism | Maternal c.770G > A, p.Gly236Arg mutation in the *KCNK9/TASK3* gene (chromosomal region 8q24) | |
| **Chr 11** | | **Beckwith-Wiedemann syndrome** | **Silver-Russell syndrome** |
| | Mirror phenotypes | Neonatal macrosomia | IUGR |
| | | Postnatal overgrowth | Postnatal growth failure |
| | | Lateralized overgrowth | Body hemihypoplasia |
| | | Relative microcephaly | Relative macrocephaly |
| | | Macroglossia | Micrognatia and microstomia |
| | | Hyperinsulimenic hypoglycaemia | Non-hyperinsulinemic hypoglycemia |
| | Others | Abdominal wall defects, ear pits and creases, glabellar *naevus flammeus*, organomegaly, nephroureteral malformations, embryonal tumors in infancy | Feeding difficulties, triangular face, low muscle mass, fifth finger clinodactyly, central precocious puberty, insulin resistance in adulthood |
| | Mirror mechanisms | UPD(11)pat | UPD(11)mat |
| | | Paternal duplication 11q15.5 | Maternal duplication 11q15.5 |
| | | GoM at *H19/IGF2:IG-DMR* (IC1) | LoM at H19/IGF2:IG-DMR (IC1) |
| | | LoM at *KCNQ1OT1:TSS-DMR* (IC2) | GoM at *KCNQ1OT1:TSS-DMR* (IC2) (associated with genomic imbalances) |
| | | Chromosomal rearrangements 11q15.5 | Chromosomal rearrangements 11q15.5 |
| | | Maternal *CDKN1C* loss of function mutations | Maternal mutations increasing *CDKN1C* stability |
| | Others | | UPD(7)mat |
| | | | Paternal *IGF2* loss of function mutation chromosomal rearrangements 7q, 7p |
| | | | *HMGA2* and *PLAG1* mutations |
| | | | **IMAGE syndrome** |
| | Phenotype | | IUGR, metaphyseal dysplasia, congenital adrenal hypoplasia, genital anomalies |
| | Mechanism | | Maternal *CDKN1C* gain of function mutations |
| **Chr 14** | | **Kagami-Ogata syndrome** | **Temple syndrome** |
| | Mirror phenotypes | Placentomegaly and neonatal macrosomia | IUGR |
| | | Overgrowth | Failure to thrive, short stature |
| | Others | Polyhydramnios, abdominal wall defects, hypotonia, developmental delay, intellectual disability, hepatoblastoma | Hypotonia, motor delay, joint laxity, precocious puberty, truncal obesity |
| | Mirror mechanisms | UPD(14)pat | UPD(14)mat |
| | | Maternal deletion 14q32 | Paternal deletion 14q32 |
| | | GoM at *MEG3:TSS-DMR* | LoM at *MEG3:TSS-DMR* |

**Table 1. Continued**

| | | Imprinting syndromes | |
|---|---|---|---|
| **Chr 15** | | **Prader-Willi syndrome** | **Angelman syndrome** |
| | Phenotype | Hyperphagia | Anorexia and eating disorders |
| | | Central apnoea and hypoventilation | Seizures, sleep disruption |
| | | Hypotonia, reduced spontaneous motility | Excessive and unmotivated laugh, hyperexcitability, hyperactivity and hyperreflexia, happy demeanor |
| | | Hypothermia and absence of fever response | |
| | | | Sensitivity to heat |
| | Others | Mild to moderate intellectual disability, central obesity, hypogonadotrophic hypogonadism, short stature | Severe intellectual disability, microcephaly, severe speech impairment, ataxia |
| | Mirror mechanisms | UPD(15)mat | UPD(15)pat |
| | | Paternal deletion 15q11q13 | Maternal deletion 15q11q13 |
| | | GoM at *MKRN3:TSS-DMR* | LoM at *MKRN3:TSS-DMR* |
| | Others | | Maternal *UBE3A* loss of function mutations |
| | | **Schaaf-Yang syndrome** | |
| | Phenotype | Neonatal hypotonia, developmental delay, intellectual disability, hypogonadism, autistic behavior, joints contractures | |
| | Mechanism | Paternal *MAGEL2* truncating mutations | |
| | | **Central precocious puberty 2** | |
| | Phenotype | Premature activation of the reproductive axis | |
| | Mechanism | Paternal *MKRN3/ZFP127* loss of function mutations | |
| **Chr 16** | | | Maternal uniparental disomy of chromosome 16 (controversial) |
| | Phenotype | | IUGR, elevated risk of malformation |
| | Mechanism | | UPD(16)mat |
| **Chr 20** | | **PHP1A** | POH |
| | Phenotype | Rickets and poor mineralization due to hypoparathyroidism, Albright hereditary osteodystrophy, generalized hormone resistance, obesity | Heterotopic bone formation with progressive cutaneous and subcutaneous ossification |
| | Mirror mechanism | Maternally-inherited inactivating *GNAS* mutations | Paternally-inherited inactivating *GNAS* mutations |
| | | **PHP1B** | **Maternal uniparental disomy of chromosome 20** |
| | Phenotype | Isolated renal PTH resistance | IUGR, short stature, extreme feeding difficulties, failure to thrive |
| | Mirror mechanism | UPD(20)pat | UPD(20)mat |
| | Other mechanism | LoM at *GNAS A/B:TSS-DMR* | |

IUGR: intrauterine growth restriction, UPD: uniparental disomy, GoM: gain of methylation, LoM: loss of methylation, PTH: parathyroid hormone, POH: progressive osseous heteroplasia, DMR: differentially methylated region

Recent advances in this field suggest that the range of imprinting disorders could be greater than those currently described. In this article we review those described hitherto, ordered by chromosome.

## Chromosome 6

### Transient Neonatal Diabetes Mellitus Type 1

Transient neonatal diabetes mellitus type 1 (TNDM1, OMIM #601410) has a prevalence of approximately 1 in 500,000 births (11) and it is characterized by intra-uterine growth

restriction (IUGR) and infantile hyperglycemia in the absence of ketoacidosis. Macroglossia and umbilical hernia are often present. TNMD1 features are evident in infants during the first weeks of life, usually presenting with dehydration, and generally disappearing by the age of 18 months. Insulin treatment is usually required. However, diabetes may relapse later in life in approximately half of the patients, showing characteristics of type 2 diabetes mellitus. Women may relapse during pregnancy presenting with gestational diabetes mellitus (12).

TNDM1 can be caused by three different molecular mechanisms (12):

1. Paternal UPD of chromosome 6 (41%).

2. Duplication of the paternal allele at 6q24 (29%).

3. Hypomethylation of the maternal differentially methylated region (DMR), *PLAGL1: alt-TSS-DMR* (30%).

This latter mechanism can be due to either an isolated imprinting variant or as part of a generalized hypomethylation at imprinted loci (MLID), due to recessive loss of function *ZFP57* mutations in almost half of the cases (13). TNDM1-MLID patients may have further phenotypic manifestations, such as structural brain abnormalities, developmental delay and congenital heart disease (14).

All three molecular mechanisms accounting for TNDM1 lead to over-expression of the *PLAGL1/ZAC* gene which regulates apoptosis and cell cycle arrest (15). The protein encoded by the *PLAGL1/ZAC* gene is a zinc finger protein and regulates *PACAP1* that has a key role in stimulating insulin secretion by pancreatic beta cells. Moreover, *PLAGL1/ZAC1* gene overexpression may reduce the number of beta cells or impair their function, stopping cell cycling and inducing apoptosis (12).

## Maternal Uniparental Disomy of Chromosome 6

Maternal UPD of chromosome 6, abbreviated to UPD(6) mat, has been hypothesized to be associated with IUGR and other heterogeneous clinical features, especially intellectual disability (16). However, homozygosity of a recessive allele and/or placental trisomy 6 mosaicism is likely to be the pathogenic mechanism in some of these patients. These data suggest that a specific imprinting disorder associated with UPD(6)mat does not exist and that the heterogeneous clinical features in UPD(6) mat patients are either caused by placental trisomy 6, undetected trisomy 6 cell lines or by homozygosity for recessive mutations (5,17). However, given the small number of patients described to date and the presence of an imprinted region on chromosome 6q24 further studies are required to clarify this contentious issue.

## Chromosome 7

Maternal UPDs of chromosome 7 are responsible for a small subset (5-10%) of Silver-Russell syndrome (SRS). Since the majority of SRS cases are due to chromosome 11 abnormalities, this topic is extensively described in the chromosome 11 section.

## Chromosome 8

### Birk-Barel Syndrome

Birk-Barel syndrome (OMIM #612292) is characterized by severe neonatal hypotonia, transient neonatal hypoglycemia, joint contractures, wide alveolar ridges, cleft palate, microretrognathia, developmental delay and variable intellectual disability. Distinctive facial features include dolichocephaly, bitemporal narrowing, short philtrum, tented upper lip and medially flared eyebrows (18,19).

This disorder is caused by a specific missense mutation (c.770G>A, p.Gly236Arg) in the maternal copy of the *KCNK9/TASK3* gene, located in chromosomal region 8q24.

The 8q24 chromosomal region includes two imprinted genes: *PEG13*, expressed by the paternal allele and *KCNK9*, expressed by the maternal allele. The reciprocal expression of these genes is regulated by a maternal methylated region located within the *PEG13* transcript, named *PEG13:TSS-DMR* (20).

The *KCNK9/TASK3* gene encodes a member of the two pore-domain potassium channel subfamily (18,19). TASK3 channels are widely expressed, especially in the brain, where they play a role in the migration of cortical pyramidal neurons regulating both neuronal activity and neuronal development. Of note, nonsteroidal anti-inflammatory fenamic acid drugs, especially flufenamic acid, are able to stimulate the two pore-domain potassium channels, partially rescuing the reduced outward current through mutated *KCNK9* channels, suggesting that fenamic acid compounds might be useful in treating this condition (18).

## Chromosome 11

### Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS) (OMIM #130650) is the most common congenital overgrowth condition (1:10,500 live births) (21) and represents the paradigm of genetic imprinting disorders and cancer predisposition

syndromes. Clinical features include neonatal macrosomia, postnatal overgrowth, macroglossia, abdominal wall defects ranging from severe (omphalocele, gastroschisis) to moderate (umbilical hernia) and mild (*diastasis recti*), ear pits and creases, glabellar *naevus flammeus*, lateralized overgrowth (previously termed hemihyperplasia) (22), organomegaly, nephroureteral malformations (23), hyperinsulinism or transient hypoglycaemia (1), placental mesenchymal dysplasia and predisposition to the development of embryonal tumors in infancy (24). These features combine variably accounting for the different degree of severity of presentation and depicting a broad phenotypic spectrum (25,26,27,28,29), including cases with isolated lateralized overgrowth (22). The diagnosis is clinical, based on criteria and a scoring system which has been recently revised (1).

BWS is caused by several epigenetic and genetic defects. In approximately 85% of patients disturbed expression of imprinted genes located into two separate domains on chromosome 11p15.5 is found. In this chromosomal region, two differentially methylated imprinting centers (IC) (*H19/IGF2:IG-DMR* and *KCNQ1OT1:TSS-DMR*, commonly referred to as IC1 and IC2, respectively) control the expression of genes involved in cell cycle progression and somatic growth control. Five mechanisms leading to the disruption of the expression of such genes are currently known:

1. Approximately 50% of cases are caused by LoM at IC2 (IC2-LoM) leading to reduced expression of *CDKN1C*, normally expressed by the maternal chromosome only. IC2-LoM is usually a sporadic primary epigenetic defect, however rare familial cases carrying genetic mutations causing secondary hypomethylation have been described (30). An increasingly growing fraction of patients with IC2-LoM also display methylation abnormalities at other imprinted loci leading to additional phenotypes (MLID) (31,32). Disruption of trans-acting mechanisms regulating the normal imprinting at the 11p15.5 ICs as well as other differentially methylated regions can be responsible for such cases; rare inheritable mutations in the *NLRP* family genes have been described (33,34,35). NLRP proteins are members of the NLR family of proteins and are important components of inflammasomes with a major role in innate immunity (36). Interestingly, a subset of *NLRP* genes is expressed in oocytes and early embryos (37). Females with mutations in *NLRP2* and *NLRP7* gave birth to few or no liveborn children (38). Germline mutations in *NLRP2* are responsible for a familial form of BWS caused by a trans-acting mechanism, consistent with the hypothesis that *NLRP2* has a role in establishing or maintaining genomic imprinting in humans (33). *NLRP5* mutations have also been reported in five mothers of offspring with MLID, linking this gene with a maternal effect on reproductive fitness, epigenetic and developmental reprogramming of zygotes and reproductive outcomes (32,39).

2. Mosaic segmental paternal UPD of chromosome 11, accounting for 20% of the cases, leads to altered expression at both gene clusters (1) with IC2-LoM and IC1-GoM. Genome-wide UPD of chromosome 11 is found in a subset of cases and associated with higher cancer risk (40).

3. IC1-GoM results in biallelic expression of the *IGF2* gene which is normally expressed by the paternal allele only and reduced expression of the *H19* gene, an oncosuppressor gene normally expressed by the maternal allele. IC1-GoM is found in 5-10% of cases and in a subset of patients is caused by microdeletions encompassing the *OCT4/SOX2* binding site localized inside IC1, leading to a maternally transmitted BWS phenotype (41,42).

4. Maternal *CDKN1C* loss-of-function mutations are responsible for maternally inheritable BWS and account for 5-10% of cases.

5. Finally, approximately 1% of BWS cases are caused by chromosomal rearrangements (duplications, translocations, inversions, deletions) involving the 11p15.5 chromosomal region and causing secondary IC1-GoM or IC2-LoM (24).

About 15% of clinically diagnosed BWS cases have no detectable molecular defect when investigated using commonly employed diagnostic molecular techniques. However, low somatic mosaicism of the above mentioned defects is increasingly found by using novel molecular techniques (43) and analysing tissues other than blood (e.g. buccal smear) (44). It cannot be excluded that in a fraction of patients the molecular defect has not yet been discovered.

Besides providing diagnostic confirmation and the possibility of genetic counselling, molecular anomalies detected in BWS have implications for the clinical management of patients and prognostic value. Indeed, specific correlation between epigenotype and phenotypic features are present, especially concerning cancer risk (26,27,28,45). BWS molecular subtypes are characterized by a gradient in cancer development probability and display different histotypes allowing differentiation of tumor surveillance protocols according to the epigenotype. This facilitates the early detection of relevant associated tumors, with special reference to Wilms' tumor and hepatoblastoma (26,45,46,47,48,49,50,51,52).

## Silver-Russel Syndrome

SRS (OMIM #180860) is the phenotypic and genetic opposite disorder of BWS, has an estimate incidence of 1:30,000 to 1:100,000 (2) and represents the paradigm of genetic restricted growth imprinting disorders and poor feeding predisposition.

The phenotypic clinical spectrum of SRS includes severe IUGR, postnatal growth failure with no catch-up, body hemihypoplasia with body asymmetry, relative macrocephaly with triangular face, typical facial appearance (prominent forehead, narrow chin, small jaw and downturned corners of the mouth), low muscle mass, fifth finger clinodactyly, feeding difficulties, recurrent hypoglycemia, premature adrenarche, rapidly progressing and/or central precocious puberty (CPP) and insulin resistance in adulthood (2,53).

The diagnosis of SRS is clinical and molecular testing is used for confirmation and phenotype stratification. Given the broad spectrum of presentation, the diagnosis is based on the Netchine-Harbison scoring system (54), having high sensitivity and predictive value. A molecular cause can be identified in approximately 60% of patients with a clinical diagnosis (2), while the molecular aetiology remains unknown in a substantial proportion of patients:

1. The most common mechanisms is LoM at IC1 on the paternal chromosome 11p15 (IC1-LoM), which is detected in 40-60% of patients. IC1-LoM results in reduced *IGF2* expression and increased *H19* expression (2,55).

2. Besides IC1-LoM, a variety of rearrangements involving the 11p15.5 region resulting in a SRS phenotype have been described (56,57).

3. From 5 to 10% of cases are caused by maternal UPD of chromosome 7 (2).

4. Mirroring BWS molecular alterations in chromosomal region 11p15.5, the SRS phenotype also results from alterations at the centromeric IC2 of 11p15.5. Genomic imbalances involving IC2 resulting in gain of methylation at this center have been rarely described (58).

5. Rare monogenic causes have been described including a mutation increasing CDKN1C stability in a family with maternally transmitted SRS (59), *IGF2* loss-of-function mutation in a family with paternally transmitted SRS (60) and *HMGA2* and *PLAG1* mutations with dominant transmission regardless of maternal or paternal transmission (61,62,63). Coding variants in these genes are overall very rare (2).

Differential diagnosis of SRS includes other genetic syndromes characterized by growth restriction, including single gene disorders such as IMAGE syndrome (discussed immediately below) and Temple syndrome (discussed in the chromosome 14 section) and chromosomal anomalies and copy number variants (2). The differential diagnosis can have extremely important implications for management since SRS treatment may include growth hormone (GH) therapy (53) and response to treatment. For instance, GH treatment is contraindicated in patients with chromosome breakage disorders due to the associated risk of malignancy (2).

## IMAGE Syndrome

IMAGE syndrome (OMIM #614732) results from a gain-of-function mutation in the *CDKN1C* gene, negatively regulating cellular proliferation. Since *CDKN1C* is expressed only from the maternal allele, IMAGE syndrome occurs only when the *CDKN1C* gain-of-function mutation is inherited from the mother (64). This syndrome is characterized by SRS phenotype associated with metaphyseal dysplasia, congenital adrenal hypoplasia with adrenal insufficiency, and almost always includes genital anomalies (65).

## Chromosome 14

### Temple Syndrome

Temple syndrome (OMIM #616222) is characterized by prenatal and postnatal growth failure and early onset of puberty with final short stature, hypotonia, feeding difficulties in early childhood, motor delay, joint laxity, truncal obesity and minor dysmorphic features such as broad forehead and short nose with wide nasal tip and small hands and feet (66). Due to relatively mild and age-dependent characteristics, the prevalence of Temple syndrome in the general population is unknown and the disorder is likely underdiagnosed in clinical practice (66).

Temple syndrome shows several nonspecific clinical features overlapping with Prader-Willi syndrome (PWS) and SRS (67,68,69). The treatment may include GH therapy (70).

The syndrome is caused by alteration of imprinted gene expression at chromosome 14q32.2. This region contains a cluster of imprinted genes including three paternally expressed genes (*DLK1*, *DIO3* and *RTL1*) and multiple maternally expressed non-coding RNAs (*MEG3*, *RTL1as*, *MEG8*, *snoRNAs*, and *microRNAs)* (71). The parental origin-dependent expression patterns are regulated by a germline-derived primary intergenic DMR (*MEG3/DLK1:IG-DMR*) and a postfertilization-derived secondary DMR (*MEG3:TSS-DMR*), both normally methylated only on the paternal allele (72). Mechanisms that result in functional hemizygosity of

14q32 imprinted genes can cause the clinical phenotypes (4), including:

1. Chromosome 14 maternal UPD (78%) (73).

2. Isolated methylation deficiency at *MEG3:TSS-DMR* in the 14q32.2 imprinted region (12%) (74).

3. 14q32 deletions of paternal origin (10%) (71).

Maternal UPD of chromosome 14 represents the major molecular cause of Temple syndrome. However, some evidence indicate that UPD over-representation among the molecular causes of Temple's syndrome could be due to an ascertainment bias and it is possible that frequencies of the molecular findings in Temple syndrome will be updated in the coming years (75).

## Kagami-Ogata Syndrome

Kagami-Ogata syndrome (OMIM #608149) includes overgrowth (typically with birth weight disproportionately greater than length), polyhydramnios, placentomegaly, poor sucking and hypoventilation in the neonatal period, abdominal wall defects ranging from omphalocele to *diastasis recti*, a distinctive facial appearance (full cheeks, depressed nasal bridge, micrognathia, short webbed neck and protruding philtrum), small bell-shaped thorax with coat-hanger ribs, and variable developmental delay and/or intellectual disability. Some features are rather nonspecific and can be also observed in BWS. Kagami-Ogata syndrome is associated with increased risk of developing hepatoblastoma (9%) and a neonatal mortality rate as high as 20-25% (76).

Kagami-Ogata syndrome can be caused by three different molecular mechanisms (4):

1. Paternal UPD of chromosome 14 (65%).

2. Microdeletion affecting the maternal 14q32.2 imprinted region (20%).

3. Hypermethylation (15%) affecting the *MEG3:TSS-DMR* in the maternal 14q32.2 imprinted region (77).

While UPD(14)pat and hypermethylation are sporadic, microdeletions can lead to a maternally transmitted Kagami-Ogata syndrome. Recently it has been shown that causal deletions do not necessarily include the DMRs; therefore, a normal methylation pattern does not exclude the syndrome (78).

As discussed for Temple's syndrome, it has been proposed that over-representation of UPD(14)pat among the molecular causes of the Kagami-Ogata syndrome could be secondary to an ascertainment bias and the frequencies of the molecular causes could change as availability of specific molecular tests increases (75).

## Chromosome 15

### Angelman Syndrome

Angelman syndrome (AS) (OMIM #105830) is characterized by developmental delay, intellectual disability with severe speech impairment, microcephaly and seizures. The symptoms usually appear in the first year of life (79). Seizures typically occur between one and three years of age and can be associated with generalized, specific electroencephalographic changes (80). Patients also present with sleep disruption, excessive laughter, happy demeanor, gait ataxia, tremulousness of the limbs and protruding tongue. AS prevalence is approximately one in 12,000-24,000 live births (80).

AS can be caused by four different mechanisms:

1. Maternally derived *de novo* deletion of 15q11-q13 (70-75%).

2. Paternal UPD of chromosome 15 (3-7%).

3. Imprinting defect at *MKRN3:TSS-DMR* in the maternal chromosome 15q11.2-q13 locus (2-3%).

4. Maternally inherited mutations in *UBE3A* gene (10-15%) (5).

The phenotype is usually more severe in patients with large deletions. All genetic mechanisms result in lack of expression of the maternally expressed 15q11-q13 *UBE3A* gene. *UBE3A* is normally expressed exclusively from the maternal allele in human fetal brain and in adult frontal cortex. Duplications of this gene have been linked to autism spectrum disorder, developmental delay and neuropsychiatric phenotypes (81), further supporting the hypothesis that *UBE3A* plays a pivotal role in neurodevelopment. AS patients have a paternal copy of *UBE3A*, but it is silenced by a nuclear localized long non-coding RNA, known as *UBE3A* antisense transcript (*UBE3A-ATS*) (82). Antisense oligonucleotides treatment aimed at reducing the *UBE3A-ATS* in order to unsilence the paternal *UBE3A* gene is under study (82).

## Prader-Willi Syndrome

PWS (OMIM #176270) includes variable characteristics according to the age of the patient. Decreased fetal movement, abnormal fetal position at delivery, and increased incidence of assisted delivery or cesarean section are common. Hypotonia of central origin with poor sucking and feeding difficulties resulting in failure to thrive are prevalent in the neonatal period and in the first year of life.

Subsequently, progressive hyperphagia with central obesity occurs. Hyperphagia is linked to a hypothalamic dysfunction resulting in lack of satiety and food-seeking behavior with central obesity being the result of both hyperphagia and a reduced total energy expenditure connected with decreased physical activity and decreased lean body mass. Extreme obesity and related complications represent the major causes of morbidity and mortality in PWS (83). Hypothalamic hypogonadism with cryptorchidism, incomplete genital development, delayed and incomplete puberty and infertility are typical (84). Short stature is very common and is usually treated with GH replacement therapy, with the additional benefit of acquisition of lean mass. Abnormalities of GH function in PWS have been reported and other hypothalamic hormones can also be deficient causing tertiary hypothyroidism, and central adrenal insufficiency (85). PWS patients may exhibit developmental delay of variable severity. Behavior problems are common and manifest with a typical pattern including temper tantrums, controlling and manipulative behavior and compulsivity. Current trials are underway to evaluate oxytocin as a potential therapeutic agent for controlling behavior issues in PWS patients (86,87).

Characteristic facial features may develop over time and include narrow bifrontal diameter and nasal bridge, almond-shaped palpebral fissures, thin vermilion of the upper lip with down-turned corners of the mouth.

Diagnosis and molecular testing is based on clinical criteria (88).

PWS is caused by lack of expression of imprinted genes on chromosome *15q11.2-q13* gene cluster, defined as the "PWS critical region". Alterations not involving this critical region are not associated with PWS. The PWS critical region encompasses imprinted genes normally expressed only on the paternal allele: *MKRN3*, *MAGEL2*, *NDN*, *PWRN1*, *C15orf2*, *SNURF-SNRPN* and several snoRNA genes. The deficiency of one of these snoRNA (*SNORD116*) is believed to elicit the key features of PWS phenotype (89,90).

Altered expression can be caused by four mechanisms:

1. Deletion of the 15q11-q13 imprinted loci on the paternal allele (up to 70-75% of cases).

2. Maternal UPD of chromosome 15 (up to 20-25%).

3. Imprinting defects due to primary epimutations at *MKRN3:TSS-DMR* (2%) (84,91).

4. Small deletions within the IC critical region which may or may not lead to an imprinting deficiency detectable by methylation analysis (<0.5%) (84,91,92).

Most PWS cases are sporadic. Inheritable PWS is rare and can be due to deletions caused by unbalanced chromosome rearrangement or paternally inherited IC deletion. The diagnosis is confirmed through DNA methylation analysis, with subsequent cytogenetic testing, fluorescence *in situ* hybridization and microsatellite marker analysis, which define the genotype classifications (93).

## Schaaf-Yang Syndrome

Schaaf-Yang syndrome (OMIM #615547) is a PWS-like disease, due to truncating mutations in the *MAGEL2* gene, which is located in the PWS critical region (chromosome 15q11-q13) and is normally maternally imprinted and paternally expressed. Schaaf-Yang syndrome is characterized by neonatal hypotonia, developmental delay and intellectual disability, hypogonadism, autistic behavior and joints contractures. The typical PWS features of hyperphagia and obesity are usually absent. Consequently, the phenotypic overlap with PWS is preeminent in the neonatal period. The phenotypic spectrum ranges from severe fetal akinesia to mild expression including intellectual disability and finger contractures (94).

Paradoxically, while truncating mutations in the *MAGEL2* gene cause Schaaf-Yang syndrome, *MAGEL2* whole gene deletions cause on slight or even absent expression of the clinical phenotype (94). It is likely, as *MAGEL2* is a one-exon gene, that truncating mutations may result in a shortened protein with a dominant-negative effect. As an alternative explanation to this phenomenon, the deletion of the entire paternal copy of the gene, including its promoter, could lead to leaky expression of the maternal copy of the gene (94).

## Central Precocious Puberty 2

CPP (OMIM #176400) also known as gonadotropin dependent precocious puberty, is characterized by a premature activation of the reproductive axis, before the age of eight years in girls and nine years in boys (95). Prevalence of CPP has been estimated at approximately 1.1:100,000 with an overall male to female ratio of at least 1:10 (96). Subjects affected by CPP present with pubertal signs such as breast development or testicular enlargement and acceleration of growth and bone age, consistent with elevated basal and GnRH-stimulated LH levels (97).

CPP 2 (CPPB2, OMIM #615346) is caused by heterozygous loss of function mutations in the *MKRN3/ZFP127* gene, located in the PWS critical region (chromosome 15q11-q13). An antisense RNA of unknown function overlaps this gene, probably regulating *MKRN3/ZFP127* expression. *MKRN3/*

*ZFP127* is maternally imprinted and paternally expressed. Therefore only mutations inherited from fathers are disease-causing (97). It is noteworthy that a high frequency of *MKRN3/ZFP127* mutations was reported in a cohort of CCP males with anticipated puberty (98).

Puberty in humans normally starts when pulsatile GnRH is released from hypothalamic neurons. Indeed, the onset of puberty requires both a decrease in factors that inhibit the release of GnRH and an increase in stimulatory factors. MKRN3/ZFP127 protein levels declined prior to clinical onset of puberty and thereafter through puberty, which correlated negatively with gonadotropin concentrations in prepubertal girls (99) and its circulating levels declined during puberty in healthy boys (100). The expression pattern of *MKRN3/ZFP127* suggests the hypothesis of an inhibitory effect on GnRH secretion (101) but the precise mechanism by which its deficiency leads to an early reactivation of pulsatile GnRH secretion remains to be elucidated (95).

GnRH agonists have been the standard of care for the management of CPP in order to decrease bone maturation, growth velocity and progression of clinical signs of puberty (102).

## Chromosome 16

### Maternal Uniparental Disomy of Chromosome 16

UPD(16)mat has a high frequency since it is caused by trisomy 16 rescue (103). UPD(16)mat is associated with IUGR with an elevated risk of malformation but without a unique and specific phenotype. The heterogeneity of the phenotype suggests that placental insufficiency or mosaicism for trisomy 16 may be responsible for symptoms in such patients (36,104,105). Taken together, these data seem to indicate, as for UPD(6)mat, that a specific chromosome 16 associated imprinting disorder does not exist (105). On the other hand, some imprinted genes with unknown function have been identified on chromosome 16 and further studies are required to clarify the issue (106).

## Chromosome 20

### Pseudohypoparathyroidism

Pseudohypoparathyroidism (PHP) is a heterogeneous group of endocrine disorders characterized by renal resistance to parathyroid hormone (PTH), causing hypocalcaemia, hyperphosphatemia and elevated circulating PTH levels (3,107). Depending on the molecular defect, PHP includes other endocrine deficiencies related to hormone action resistance and other non-endocrine features. Overall, prevalence of PHP has been estimated to be 1.1 in 100,000 (108,109,110).

*GNAS* is a complex imprinting locus resulting in maternally, paternally, or biallelically expressed transcripts in differentially imprinted tissues: *Gsα*, the alpha-stimulatory subunit of the G protein; *XLαs*; *A/B*; *NESP*; and the antisense transcript *GNAS-AS1*. The antisense transcript *GNAS-AS1, A/B* and *XLαs* are transcribed from the paternal allele only; *NESP* is transcribed from the maternal allele only, and *Gsα* has a biallelical expression in most tissues, while its expression is restricted to the maternal allele in some others, including renal proximal tubule, thyroid, pituitary gland and gonads (111), even if the promoter of *Gsα* is not differentially methylated. The *GNAS* locus has two different IC regions (112); the first one is located within the *STX16* gene and controls the establishment of imprinting at the *GNAS A/B:TSS-DMR* only, while the second one, encompassing the antisense transcript *GNAS-AS1* on exons 3-4, controls the establishment of imprinting over the entire *GNAS* locus (111). Isolated imprinting defects at *GNAS A/B:TSS-DMR* are associated with deletions in the maternal allele affecting *STX16* and/or *NESP*, while overall imprinting alteration at the four DMRs of the *GNAS* locus is caused by maternal deletions at exons 3 and 4, or 40 and 33bp microdeletions at introns 4 and 3 of *GNAS-AS1* (3,111).

PHP type 1a (PHP1A, OMIM #103580) is caused by loss of function mutations in the maternal allele of *GNAS* gene. PHP1A patients present with generalized hormone resistance of variable degree, intellectual disability, obesity connected with decreased resting energy expenditure (113), and Albright hereditary osteodystrophy (AHO). AHO includes short stature, round facies, subcutaneous ossifications, brachydactyly and other skeletal anomalies (107).

Loss of function of *Gsα* on the paternal allele can cause pseudopseudohypoparathyroidism (PPHP) (OMIM #612463). Since renal tubular cells predominantly express the maternal allele of *GNAS*, a paternally inherited mutation results in a normal renal response to PTH, causing AHO without concurrent endocrine abnormalities (114). Paternal loss of function mutations can also cause progressive osseous heteroplasia (OMIM#166350), a condition characterized by subcutaneous ossifications presenting during childhood and progressing to involve subcutaneous and deep connective tissues, in the absence of AHO or hormone resistance (115).

Both PHP1A and PPHP individuals have halved *Gsα* expression in erythrocytes, which normally have a biallelic expression of *GNAS*. AHO may be caused by *Gsα* haploinsufficiency in tissues with *GNAS* biallelic expression (116).

In contrast, PHP type 1b (PHP1B, OMIM #603233) is clinically characterized by isolated renal PTH resistance and

in some cases by thyroid stimulating hormone resistance. Rarely, these patients show an AHO phenotype (117). Interestingly, *Gsα* expression in erythrocytes is mildly reduced in patients with AHO (116). All patients with PHP1B have, at least, LoM at *GNAS A/B:TSS-DMR*, likely leading to the downregulated expression of the *GNAS-Gsa* transcript in imprinted tissues (111). Hormonal resistance is caused by LoM on the maternally inherited allele (118). Overall, 20% of PHP1B cases are inherited and caused by the previously mentioned deletions at the ICs, while the remaining 80% are sporadic and associated with methylation defects encompassing the whole *GNAS* locus. A small subset of the sporadic PHP1B cases is due to paternal UPD of chromosome 20q (6). Duplications and deletions in the *GNAS* locus have been identified in a few patients (119) but the majority of cases are still of unknown aetiology.

PHP patients should be screened for GH deficiency with the aim of eventually starting GH replacement therapy. Hypocalcaemia should be treated with an active form of vitamin D and calcium supplementation. Associated endocrinopathies, such as hypothyroidism and hypogonadism, should be treated. Surgical excision of AHO subcutaneous ossifications should only be considered in the presence of delimited, superficial lesions associated with pain and/or movement impairment (3).

## Maternal Uniparental Disomy of Chromosome 20

UPD(20)mat, generally caused by trisomy rescue after meiosis 2 nondisjunction, is characterized by IUGR, short stature and extreme feeding difficulties with failure to thrive from birth, often requiring gastric tube feeding in the first years of life. GH supplementation has been suggested as probably safe and effective for this condition (120). UPD(20)mat presents with phenotypic overlap with SRS, and must be considered in the SRS differential diagnosis (2).

## Conclusion

The imprinting disorders represent a rapidly evolving field in medicine and genetics. Their paradigm challenges traditional molecular diagnostic techniques and genetic counselling. A precise molecular diagnosis is essential and further clinical phenotyping is needed to provide the appropriate means for accurate management of these disorders.

Besides those described, it is likely that more imprinting disorders remain to be identified. This review briefly illustrated the rapidly evolving advances in the understanding of human genomic imprinting and related disorders. Novel discoveries in this field will likely occur in the next decade and will offer the potential for more precise molecular diagnosis and clinical definition, as well as the model for novel diagnostic and therapeutic techniques directed towards personalized medicine in the fields of growth, metabolism and cancer.

### Ethics

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

### Authorship Contributions

Concept: Alessandro Mussa, Design: Giovanni Battista Ferrero, Alessandro Mussa, Data Collection or Processing: Diana Carli, Evelise Riberi, Analysis or Interpretation: Giovanni Battista Ferrero, Alessandro Mussa, Literature Search: Diana Carli, Evelise Riberi, Writing: Diana Carli, Evelise Riberi.

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REVIEW

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# Current Diagnosis, Treatment and Clinical Challenges in the Management of Lipodystrophy Syndromes in Children and Young People

**Samim Özen[1], Barış Akıncı[2,3], Elif A. Oral[3]**

*[1]Ege University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey*
*[2]Dokuz Eylül University Faculty of Medicine, Department of Internal Medicine, Division of Endocrinology and Metabolism, İzmir, Turkey*
*[3]University of Michigan Medical School, Department of Medicine, and Brehm Center for Diabetes, Division of Metabolism, Endocrinology, and Diabetes, Michigan, USA*

## Abstract

Lipodystrophy is a heterogeneous group of disorders characterized by lack of body fat in characteristic patterns, which can be genetic or acquired. Lipodystrophy is associated with insulin resistance that can develop in childhood and adolescence, and usually leads to severe metabolic complications. Diabetes mellitus, hypertriglyceridemia, and hepatic steatosis ordinarily develop in these patients, and most girls suffer from menstrual abnormalities. Severe complications develop at a relatively young age, which include episodes of acute pancreatitis, renal failure, cirrhosis, and complex cardiovascular diseases, and all of these are associated with serious morbidity. Treatment of lipodystrophy consists of medical nutritional therapy, exercise, and the use of anti-hyperglycemic and lipid-lowering agents. New treatment modalities, such as metreleptin replacement, promise much in the treatment of metabolic abnormalities secondary to lipodystrophy. Current challenges in the management of lipodystrophy in children and adolescents include, but are not limited to: (1) establishing specialized centers with experience in providing care for lipodystrophy presenting in childhood and adolescence; (2) optimizing algorithms that can provide some guidance for the use of standard and novel therapies to ensure adequate metabolic control and to prevent complications; (3) educating patients and their parents about lipodystrophy management; (4) improving patient adherence to chronic therapies; (5) reducing barriers to access to novel treatments; and (5) improving the quality of life of these patients and their families.

**Keywords:** Lipodystrophy, childhood, adolescence, progeria, metreleptin

## Introduction

Lipodystrophy is the general term for a heterogeneous group of disorders characterized by near total [generalized lipodystrophy (GL)] or partial [partial lipodystrophy (PL)] lack of body fat (1). Lipodystrophy can be genetic or acquired. Congenital GL (CGL), familial PL (FPLD), acquired GL (AGL), and acquired PL (APL) make up the four major categories of lipodystrophy in clinical practice, although there are several others such as progeria associated lipodystrophy, auto-inflammatory syndromes, and complex syndromes associated with lipodystrophy (1,2,3,4,5). The current classification schema, which is based on clinical presentation, may change as our understanding of the disease processes improve. In this paper, we will focus on presentation of various forms of lipodystrophy during childhood and adolescence and then review the general clinical approach for these patients.

## Types of Lipodystrophy in Children and Young People

### Genetic Lipodystrophy Syndromes

### Congenital Generalized Lipodystrophy

CGL (Berardinelli-Seip syndrome) is the most common lipodystrophy subtype in infancy and early childhood, while the incidence of FPLD increases close to puberty (3,4,5,6). CGL cases made up almost half (519 of 1141) of pediatric patients with non-human immunodeficiency

**Address for Correspondence:** Samim Özen MD, Ege University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
**Phone:** +90 232 390 12 30 **E-mail:** samimozen@gmail.com **ORCID:** orcid.org/0000-0001-7037-2713

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virus lipodystrophy identified by a recent systematic review of a total of 351 studies (6). CGL is a rare disorder with autosomal recessive inheritance, in which patients are born with near total lack of body fat. These patients have a remarkable phenotype characterized by near total absence of adipose tissue, muscular overdevelopment and prominent subcutaneous veins, which can be noticed either at birth or in the first year of life (7). The prevalence of CGL is uncertain, but it has been estimated at approximately 1:10 million (8). A recent study reported an estimated prevalence of 0.23 cases/million for diagnosed GL (9). However, CGL has a higher prevalence in certain parts of the world as a result of consanguineous marriages. We reported an estimated CGL prevalence of 1:2 million in Turkey, considerably higher than in other reports but still quite rare (10).

There are four major subtypes of CGL based on mechanism:

CGL1 [Online Mendelian Inheritance in Man (OMIM) #608594] is caused by pathogenic variants of the 1 acylglycerol 3-phosphate acyltransferase β2 (*AGPAT2*) gene (11). The AGPAT2 enzyme converts lysophosphatidic acid into phosphatidic acid, a critical step in triglyceride synthesis (12). Homozygous pathogenic variants that eliminate enzyme activity have been demonstrated in the majority of CGL1 cases. Compound heterozygous or homozygous pathogenic variants with low levels of *in vitro* enzyme activity have also been reported (7,13). Although patients with CGL1 lack metabolically active adipose tissue, the preservation of residual mechanical adipose tissue in the palms, soles, scalp, orbital and periarticular regions and the perineum is clinically apparent (14,15).

CGL2 (OMIM #269700) is caused by pathogenic variants of the Berardinelli-Seip congenital lipodystrophy 2 (*BSCL2*) gene (16), which encodes the transmembrane protein seipin. This protein is involved in the fusion of small lipid droplets in adipocytes, as well as in the differentiation of adipocytes (17). The majority of identified variants have been classified as null pathogenic variants, based on functional investigations, which lead to severe disruption of the protein. Missense pathogenic variants have also been reported (7,13,18).

Pathogenic variants of the caveolin 1 (*CAV1*) gene, which encodes caveolin 1, a principal component of the caveolae, causes CGL3 (OMIM #612526). A homozygous nonsense pathogenic variant in *CAV1* was reported in a patient with CGL from Brazil (19). Magnetic resonance imaging of the proband confirmed the absence of metabolically active adipose tissue, while bone marrow adipose tissue was preserved (19). Heterozygous *CAV1* pathogenic variants have also been associated with PL (20).

CGL4 (OMIM #613327) is caused by homozygous or compound heterozygous pathogenic variants in the polymerase 1 and transcript release factor (*PTRF*, or cavin-1) gene (21). PTRF regulates the expression of caveolins 1 and 3. Cavin-1 plays a major role in the formation of caveolae and caveolin stabilization through interaction with the cellular cytoskeleton. Cavin-1 regulates adipocyte differentiation and it is a determining factor in the capacity of adipose tissue to expand (22). CGL3 and CGL4 have distinct clinical characteristics (Table 1) (23,24,25).

In addition to these four major groups of CGL, patients with GL associated with Lamin A/C (*LMNA*) p.T10I (26), and biallelic peroxisome proliferator activated receptor gamma (*PPARG*) (27) pathogenic variants have been reported. Patients with biallelic loss-of-function pathogenic variants in phosphate cytidylyltransferase 1 alpha (*PCYT1A*) gene were reported to have a severe PL phenotype (28), and potentially can be classified in the CGL category.

Table 1 summarizes subtypes of CGL.

## Familial Partial Lipodystrophy

FPLD is a subtype of lipodystrophy with a genetic background, which in adults is more common than any other subtype of lipodystrophy (4,8,9,29). FPLD exhibits a typical fat tissue distribution. Phenotypic features are more prominent in females. Loss of adipose tissue is predominantly observed in the upper and lower extremities. Patients may exhibit accumulation of fat in certain areas such as the face and neck, and perineal and intra-abdominal depots (2,4,30,31,32). A Cushingoid-appearance can be observed, due to thin limbs, facial fat accumulation and increased fat in the dorsocervical region resembling a 'buffalo hump' (5). The partial loss of fat may be apparent in early life, but typically becomes more pronounced over time in FPLD, and most patients start to lose adipose tissue after puberty (30). For this reason, it is difficult to recognize these patients in childhood. However, a few patients with FPLD have been reported from pediatric endocrinology practices (6).

Most FPLD subtypes are inherited in an autosomal dominant (AD) manner. However, recent evidence suggests that a large group of patients with FPLD1, also known as Koberling-type FPLD (OMIM #608600), follow a polygenic inheritance pattern (33,34,35). FPLD2, the Dunnigan variety, (OMIM #151660), is caused by AD pathogenic variants in the *LMNA* gene (36), which encodes nuclear lamins A and C (36,37). *LMNA* R482W and R482Q are the most common pathogenic variants (38,39). FPLD3 (OMIM #604367) is caused by AD pathogenic variants in the peroxisome proliferator-

activated receptor gamma gene (*PPARG*) on chromosome 3p25 (40,41). PPARG plays a major role in the regulation of adiposity differentiation (40). Other subtypes of FPLD are rare. A recent review of these subtypes is available (1). The subtypes of FPLD are presented in Table 2.

## Acquired Lipodystrophy Syndromes

### Acquired Generalized Lipodystrophy, Lawrence Syndrome

AGL affects the whole body and causes generalized fat loss. Patients develop typical metabolic complications of lipodystrophy including severe insulin resistance, diabetes, hypertriglyceridemia and non-alcoholic steatohepatitis (NASH) (42). The clinical phenotype is very similar to that of CGL. However, patients with AGL are born with normal fat tissue. Loss of adipose tissue typically begins in childhood or adolescence. Marked phenotypic features occur over differing lengths of time, from a few weeks to a year. The cause of the disease is still unknown. The disease coincides with other autoimmune diseases such as juvenile dermatomyositis, type 1 diabetes and autoimmune hepatitis. AGL is associated with panniculitis in some patients. Complement abnormalities may also be present (8,42,43).

### Acquired Partial Lipodystrophy, Barraquer-Simons Syndrome

APL (OMIM #608709) is characterized by the loss of fat from the face, neck, arms, chest and abdomen with preservation of lower extremity fat. Clinical onset typically occurs in childhood or adolescence and females predominate at a ratio of 4:1. Loss of fat first manifests in the face, and gradually progresses to the upper extremities, thorax and upper abdomen, symmetrically and in a cephalocaudal fashion. Excessive deposition of fat may be observed in the lower limbs (44). The etiology of APL remains unknown, however, there is a link to autoimmune abnormalities and coincidental autoimmune conditions can be observed. APL is associated with low complement factor 3 (C3) levels and membranoproliferative glomerulonephritis in some patients, which may cause end stage renal disease in some

**Table 1. Subtypes of congenital generalized lipodystrophy, and their genetic and clinical characteristics**

| Subtype | Gene | Molecular basis | Phenotype |
|---|---|---|---|
| CGL1 | *AGPAT2* | AGPAT2 plays a major role in the metabolism of lysophosphatidic acid, a potent signaling molecule responsible for activating a G-protein-linked receptor | Near total fat loss, preserved mechanical fat, severe insulin resistance, metabolic abnormalities, cystic lesions in long bones |
| CGL2 | *BSCL2* | Seipin, encoded by *BSCL2*, plays a key role in the fusion of small lipid droplets in the adipocytes and in adipocyte differentiation | Severe near total fat loss, almost no mechanical fat, severe insulin resistance, metabolic abnormalities and occasionally cystic lesions in long bones. May be associated with delayed mental development and cardiomyopathy |
| CGL3 | *CAV1* | Caveolin 1 is an integral component of caveolae, which are present on adipocyte membranes. Caveolae translocate fatty acids and other lipids to lipid droplets | Near total fat loss, preserved bone marrow fat, severe insulin resistance, metabolic abnormalities, delayed growth, short stature, hypocalcemia, vitamin D resistance, and osteopenia |
| CGL4 | *PTRF* | *PTRF* is involved in the biogenesis of caveolae and regulates expression of caveolins 1 and 3 | Near total fat loss, partially preserved bone marrow fat, myopathy with elevated CK levels, percussion-induced muscle wounding, scoliosis, atlantoaxial instability, pyloric stenosis, gastrointestinal dysmotility, cardiac arrhythmias (catecholaminergic polymorphic ventricular tachycardia, atrial fibrillation, prolonged QT), sudden death |
| | *LMNA* p.T10I | *LMNA* codes nuclear envelope proteins, lamin A and C. Mutant lamins disrupt the interaction between the nuclear lamina and chromatin and may result in apoptosis, which may be followed by premature adipocyte death | Near total loss of fat developing in early childhood, severe insulin resistance, metabolic abnormalities, and progeroid features |
| | *LMNA* (Lamin C-specific) | Mutant lamin C may disrupt its interaction with other cellular proteins resulting in defective development and maintenance of adipose tissue | Juvenile-onset generalized fat loss, severe insulin resistance, metabolic abnormalities, phenotype very similar to CGL1 |
| | *PPARG* (Biallelic) | PPARG is a key regulator of adipocyte differentiation | Generalized fat loss similar to CGL1 or CGL2, severe insulin resistance, metabolic abnormalities |

*AGPAT2*: 1-acylglycerol-3-phophate O-acyltransferase 2, *BSCL2*: Berardinelli-Seip congenital lipodystrophy type 2, *CAV1*: caveolin 1, CGL: congenital generalized lipodystrophy, CK: creatine kinase, *LMNA*: lamin A/C, *PPARG*: peroxisome proliferator-activated receptor gamma, *PTRF*: polymerase 1 and transcript release factor

patients (44,45,46). Drusen deposition in the macula has also been reported.

### Progeroid Disorders and Other Rare Genetic Lipodystrophy Syndromes

Lipodystrophy is a part of the clinical picture in many progeroid syndromes (1,47,48). There are also several other complex syndromes associated with lipodystrophy (1). The characteristics of these syndromes are presented in Table 3.

### Other Causes of Acquired Lipodystrophy

The development of lipodystrophy after whole body irradiation in preparation for bone marrow transplant (49,50,51,52,53,54), following cranial irradiation and as a result of hypothalamic tumors (55) has been described

**Table 2. Subtypes of familial partial lipodystrophy and their genetic and clinical characteristics**

| Subtype | Gene | Molecular basis | Phenotype |
|---|---|---|---|
| FPLD1 (Koberling type) | Unknown | Polygenic etiology? | Fat loss mainly limited to extremities, truncal obesity, palpable "ledge" between lipodystrophic and non-lipodystrophic areas, severe insulin resistance, and metabolic abnormalities |
| FPLD2 (Dunnigan type) | *LMNA* | *LMNA* codes nuclear lamina proteins, lamin A and C. Mutant lamins disrupt the interaction between the nuclear lamina and chromatin and may result in apoptosis, which may be followed by premature adipocyte death | Fat loss dominantly affects the limbs, increased muscularity, excess fat in the face and neck, labial pseudohypertrophy, increased mon pubis fat, severe insulin resistance and metabolic abnormalities |
| FPLD3 | *PPARG* | *PPARG* plays a major role in the regulation of adiposity differentiation | Milder fat loss mostly affects the distal limbs, no accumulation of adipose tissue in the face and neck, severe insulin resistance and metabolic abnormalities |
| FPLD4 | *PLIN1* | Perilipin coats lipid droplet and its required for optimal lipid incorporation and release from droplet | Fat loss in the lower limbs and femorogluteal depot, severe insulin resistance, and metabolic abnormalities. |
| FPLD5 | *CIDEC* | *CIDEC* is expressed in lipid droplets. Pathogenic variants of the *CIDEC* gene may result in the loss of ability of lipid droplets to store fat and defects in adipocyte differentiation | Partial lipodystrophy, acanthosis nigricans, severe insulin resistance, metabolic abnormalities, and diabetic ketoacidosis |
| **Lipodystrophy syndromes associated with lipomatosis** | | | |
| FPLD6 | *LIPE* | *LIPE* encodes hormone-sensitive lipase, involved in lipolysis regulation | Late-onset partial fat loss affecting the lower limbs, multiple lipomatosis, and progressive distal symmetric myopathy |
| | *MFN2* | *MFN2* gene encodes mitofusin 2, a membrane-bound mediator of mitochondrial membrane fusion and inter-organelle communication | Partial lipodystrophy with upper body adipose hyperplasia (lipomatosis without encapsulation), and low leptin levels |
| **Other FPLD syndromes** | | | |
| | *ADRA2A* | ADRA2A activation inhibits cAMP production and reduces lipolysis | FPLD phenotype, increased muscularity, needs to be confirmed in further pedigrees |
| | *AKT2* | AKT is a serine/threonine protein kinase involved in insulin-stimulated glucose transport | Partial fat loss affecting the limbs, severe insulin resistance, and metabolic abnormalities |
| | *CAV1* (heterozygous) | *CAV1* is an integral component of caveolae, which are present on adipocyte membranes. Caveolae translocate fatty acids and other lipids to lipid droplets | Partial fat loss from the upper body with preservation of fat in the lower body, neurodegeneration, cerebellar ataxia, congenital cataracts and neurosensory deafness |
| | *PCYT1A* (biallelic) | Rate-limiting enzyme in the Kennedy pathway of *de novo* phosphatidylcholine synthesis | Severe lipodystrophy with near total lack of subcutaneous fat in the arms, legs, and buttocks. Preservation of fat in the trunk, in the dorsocervical and submandibular regions, and over the mons pubis. Severe insulin resistance, and metabolic abnormalities, potentially can be classified in the CGL category |

*ADRA2A*: adrenoceptor α-2A, *AKT2*: AKT serine/threonine kinase 2, *CAV1*: caveolin 1, *CIDEC*: cell death inducing DFFA like effector C, FPLD: familial partial lipodystrophy, *LIPE*: hormone sensitive type lipase E, *LMNA*: lamin A/C, *MFN2*: mitofusin 2, *PCYT1A*: phosphate cytidylyltransferase 1 alpha, *PLIN1*: perilipin 1, *PPARG*: peroxisome proliferator-activated receptor gamma

and is a clinically neglected cause of lipodystrophy. When children have aggressive treatments for childhood cancers, they should be assessed for signs of fat loss and ensuing metabolic abnormalities. In a young child presenting with lipodystrophy, it is very important to consider the possibility of central nervous system tumors, especially when the clinical presentation does not fit with AGL. Other forms of cancer therapy, such as checkpoint inhibitors, may lead to

**Table 3. Progeroid disorders and other rare complex genetic disorders associated with lipodystrophy syndromes**

| Disorder/Syndrome | Gene | Lipodystrophy pattern | Clinical features |
|---|---|---|---|
| Hutchinson-Gilford progeria syndrome | *LMNA* | Severe partial lipodystrophy which may progress to an almost complete absence of subcutaneous fat | Short stature, low body weight, and progeroid features |
| Atypical progeroid syndrome | *LMNA* | Fat loss more extensive than the typical pattern in FPLD2 | Muscular symptoms, skin defects, cardiomyopathy and rhythm abnormalities, and progeroid features |
| MADA | *LMNA* | Partial loss of subcutaneous fat from the extremities along with normal or excessive fat in the face and the neck | Craniofacial, skeletal and cutaneous abnormalities |
| MADB | *ZMPSTE24* | More generalized loss of subcutaneous fat than MADA | Mandibular and clavicular hypoplasia, acroosteolysis, premature renal failure, and progeroid features |
| MDP syndrome | *POLD1* | Progressive loss of fat | Mandibular hypoplasia, deafness, and progeroid features |
| JMP syndrome | *PSMB8* | Panniculitis-induced lipodystrophy | Autoinflammatory syndrome, joint contractures, muscle atrophy, and microcytic anemia |
| CANDLE syndrome | *PSMB8* | Partial loss of adipose tissue from the upper limbs and face | Autoinflammatory syndrome, chronic atypical neutrophilic dermatitis, and recurrent fever |
| SHORT syndrome | *PIK3R1* | Variable loss of subcutaneous fat | Short stature, hyperextensibility, ocular depression, teething delay |
| Néstor-Guillermo progeria syndrome | *BANF1* | Decreased subcutaneous fat | Progeroid features, growth retardation, thin limbs, and stiff joints |
| Neonatal progeroid syndrome | *CAV1* | Generalized loss of body fat | Progeroid appearance at birth |
| Neonatal Marfan progeroid syndrome | *FBN1* | Generalized loss of fat | Progeroid appearance, Marfanoid habitus, skeletal features, dilated aortic bulb, bilateral subluxation of the lens, myopia, no significant metabolic abnormality associated with insulin resistance |
| Keppen-Lubinsky syndrome | *KCNJ6* | Generalized loss of fat | Severe developmental delay and intellectual disability, microcephaly, large prominent eyes, open mouth, progeroid appearance |
| Werner syndrome | *WRN* | Partial lipodystrophy affecting the limbs | Progeroid features |
| Bloom syndrome | *BLM* | Paucity of adipose tissue and low BMI | Short stature, sun-sensitive, telangiectasia, and risk of cancers |
| Cockayne syndrome | *ERCC6, ERCC8* | Generalized loss of fat | Short stature, mental retardation, chorioretinitis, and progeroid features |
| AREDYLD syndrome | Unknown | Generalized loss of fat | Acrorenal field defect, ectodermal dysplasia, and multiple abnormalities |
| | *SPRTN* | Lipodystrophy | Progeroid features, hepatocellular carcinoma |
| | *OPA3* | Lipodystrophy | Optic atrophy, cataracts, and peripheral neuropathy |

*BANF1*: barrier to autointegration factor 1, *BLM*: bloom syndrome RecQ helicase-like, *CAV1*: caveolin 1, *ERCC6*: excision repair cross-complementing group 6, *ERCC8*: excision repair cross-complementing group 8, *FBN1*: fibrillin-1, *KCNJ6*: potassium inwardly-rectifying channel subfamily J member 6, *LMNA*: lamin A/C, *OPA3*: optic atrophy 3, *PIK3R1*: phosphatidylinositol 3-kinase, regulatory subunit 1, *POLD1*: DNA polymerase delta 1, catalytic subunit, *PSMB8*: proteasome subunit beta-type 8, *SPRTN*: spartan, *WRN*: Werner syndrome RecQ like helicase, *ZMPSTE24*: Zinc metallopeptidase STE24, BMI: body mass index, MADA: mandibulo-acral dysplasia type A, MADB: mandibulo-acral dysplasia type B, FPLD2: familial partial lipodystrophy type 2

fat loss to varying extents (56,57). These unusual forms of acquired lipodystrophy will be reviewed in a specific review in the near future.

## Clinical Features of Lipodystrophy

### Leptin Levels

GL is characterized by very low or undetectable levels of leptin. However, in PL leptin concentrations may range from low to normal (58). Several studies have suggested that baseline serum leptin measurement may assist physicians to identify PL patients who are more likely to benefit from leptin replacement (59,60). Leptin concentrations correlate with fat mass, and females have higher leptin concentrations than men in adulthood (61). However, it is challenging to interpret leptin levels in infancy, childhood and adolescence. Girls generally have higher leptin levels after the completion of puberty, as might be expected given the difference in concentrations seen in adults of different genders (62,63). Prepubertal levels of leptin are similar in both sexes, although concentrations fluctuate during infancy (62,64). Younger infants have higher leptin levels than older infants presumably secondary to an initial increase in breast milk leptin from colostrum to mature milk, which is followed by a decreasing trend during the first months of lactation and a subsequent increase during the late period of lactation (65,66,67). Leptin concentrations correlate with age in prepubertal girls and boys and increase in both boys and girls during early puberty. In boys, this early pubertal leptin peak is followed by a decrease in leptin concentrations several years after the rise in serum testosterone levels. However, in girls, leptin concentrations continue to increase during puberty in parallel with increasing levels of estrogen. The concentration of leptin peaks in mid-puberty and maintains this plateau level thereafter (62). Thus, clinicians should pay attention to many factors when interpreting leptin concentrations. Serum leptin measurement may help clinicians with the management of lipodystrophy, but it should not be considered as a reliable tool to diagnose or rule out lipodystrophy. In addition to leptin, adiponectin concentrations are helpful in certain patients. A relatively high concentration of adiponectin is a distinguished characteristic of CGL2, although serum leptin is extremely low in all subtypes of CGL (10,68).

### Metabolic Abnormalities

Patients with lipodystrophy may develop metabolic abnormalities associated with insulin resistance before adulthood, which are more severe and have an earlier onset in GL (3,31,43,69). These metabolic abnormalities include, but are not limited to, diabetes, hypertriglyceridemia, hepatic steatosis, and NASH (3). Most females with lipodystrophy suffer from polycystic ovary syndrome (PCOS). Some of the factors determining the severity of metabolic abnormalities are the degree of adipose tissue loss, type of lipodystrophy, age, and sex. However, the severity of metabolic abnormalities may vary even among subjects with the same genetic pathogenic variant (30,32,70,71).

Hyperphagia usually develops due to severe leptin deficiency in early childhood in subjects with CGL (7). Accelerated linear growth, advanced bone age and signs indicative of acromegaly, such as enlargement of the hands, feet and jaw, may be observed (3). Cystic bone lesions are frequently noted in CGL1 patients (10,72,73). Hyperinsulinemia and severe insulin resistance develop in the majority of patients with CGL due to near total adipose tissue loss and leptin deficiency (10,74). Acanthosis nigricans (AN), a clinical marker of insulin resistance, can be observed in body folds such as the axilla and neck during childhood, with a strong likelihood of a further increase after puberty (3,8). Approximately 45% of patients with CGL develop diabetes mellitus before puberty (7,10). The treatment of diabetes is challenging, and high-doses of insulin (> 100 units/day) may be required. Although most patients are poorly controlled, diabetic ketoacidosis rarely develops, but has been reported, probably as a result of severe hyperinsulinemia and lack of fat tissue (4). Hypertriglyceridemia usually develops in childhood. Eruptive xanthomas and recurrent episodes of pancreatitis caused by severe hypertriglyceridemia may emerge after puberty (3). Hepatic disease is more aggressive in patients with CGL2 (10). Hepatomegaly is remarkable at very young ages (3,10). Patients may develop cirrhosis during childhood (10). PCOS, hyperandrogenism, and menstrual irregularity are common in adolescent girls (75).

AN, metabolic abnormalities associated with insulin resistance and hepatic steatosis may be observed in young people with FPLD (31), albeit milder in presentation than in CGL. Hypertriglyceridemia is common and can be severe. Episodes of acute pancreatitis may be observed. Cardiomyopathy and myopathy, as well as features resembling muscular dystrophy, may be detected in patients with FPLD2 (8,31,59). In contrast to other forms of lipodystrophy, in APL metabolic complications have rarely been reported (44). However, our previous observations suggested that a subgroup of patients with APL developed metabolic abnormalities associated with insulin resistance, which can also be progressive in some cases. Although the majority of these patients were adults, a few of them developed metabolic abnormalities in their younger years (46).

## Treatment of Lipodystrophy in Children and Young People

### General Guidelines

Although there is no curative treatment for lipodystrophy syndromes, early diagnosis of these patients may prevent mortality or serious morbidity. The aim of medical treatment is to correct metabolic abnormalities associated with lipodystrophy and prevent end-organ complications. Medical nutrition therapy (MNT) and exercise are important tools in the management of patients with lipodystrophy, although it is not easy for patients and families to comply with these therapies. Hyperphagia due to leptin deficiency is a serious problem in these children. However, weight increase is only part of the clinical problem. Since overeating exacerbates hepatic steatosis, diabetes and hyperlipidemia, particularly in babies and children, dietary fat intake should be reduced, and fat should be predominantly taken in the form of cis-monounsaturated fats and long chain omega-3 fatty acids. Medium chain triglycerides in infant formulas may be helpful to reduce triglyceride levels. In patients developing acute pancreatitis secondary to hypertriglyceridemia the amount of dietary fat should be severely restricted. Patients with diabetes also have to balance their intake of carbohydrates (4). Exercise may improve metabolic parameters in patients with lipodystrophy. Sedentary time should be reduced as much as possible, with a focus on minimizing time spent on computer and television. Physical activity should be advised, unless contraindicated, and a detailed cardiac examination should be performed before advising an exercise plan. Special attention should be given to patients with CGL4, FPLD2 and progeroid syndromes. CGL patients with lytic bone lesions and also patients with hepatosplenomegaly should avoid contact sports (1,4).

The use of lipid lowering drugs should be considered in children and adolescents with lipodystrophy when diet and exercise fail to control triglyceride levels. Fibrates are commonly used in children with very high triglyceride levels who are at risk for pancreatitis (76). Omega-3 fish oil therapy may also help to reduce triglyceride levels (76,77). Although fibrates, alone and in combination with statins, have been shown to effectively reduce triglyceride levels in adults, data are very sparse in children (6,77). Physicians should pay attention to safety measures while using fibrates for hypertriglyceridemia in children. Liver enzymes should be monitored. The risk of rhabdomyolysis should be kept in mind.

Metformin is the first choice to treat insulin resistant diabetes in children, in addition to MNT and lifestyle modification. The American Food and Drug Administration (FDA) approved the use of metformin for pediatric patients 10 years of age or older, noting that the safety and effectiveness of metformin have been shown in pediatric patients ages 10 to 16 years (78). Lipodystrophy patients with diabetes usually require insulin injections in combination with metformin (6). Insulin doses required to cause an effect may need to be very high, and patients may need to use concentrated forms of insulin (4).

Most patients with lipodystrophy desire a better cosmetic appearance. Patients with lipodystrophy may consider having cosmetic surgery, which may help them feel better about their physical appearance and may offer an improved quality of life. Excess unwanted localized fat can be removed from several locations that include the chin, buffalo hump, and vulvar region by liposuction or surgical excision. Lipoatrophic areas may also benefit from autologous adipose tissue transplantation, facial reconstruction and implants (1).

## Metreleptin Therapy

Recombinant human leptin (metreleptin) therapy can be used to minimize and prevent complications of lipodystrophy (79). Severe hypoleptinemia causes hyperphagia and exacerbates metabolic complications associated with insulin resistance in patients with lipodystrophy (80). Several long-term studies have shown beneficial effects of metreleptin in GL with severely low serum leptin levels (81,82,83,84). Metreleptin therapy has been associated with a significant reduction in triglyceride and hemoglobin A1c (HbA1c) levels, and improvements in appetite control, insulin sensitivity and hepatic steatosis (82,85,86,87,88,89).

The metabolic effects of metreleptin are remarkable in pediatric patients with lipodystrophy. In the largest report of the efficacy of metreleptin in pediatric patients with lipodystrophy to date, Brown et al (85) showed a reduction of HbA1c from a mean level of 8.3% to 6.5%. Triglyceride levels significantly decreased from 374 mg/dL to 189 mg/dL. The benefit was more prominent in adolescents (from 9.6% to 7.1% for HbA1c, and from 556 mg/dL to 226 mg/dL for triglycerides), presumably because of the presence of a more severe disease at baseline. Insulin sensitivity improved, and half of the patients who were on insulin at baseline were able to discontinue insulin treatment after metreleptin. The levels of liver enzymes decreased, and liver histology improved in a subset of patients who underwent before and after treatment liver biopsies. Metreleptin therapy was also associated with partial normalization of rapid growth in CGL, and improvements in pubertal development. The average dose of metreleptin was 0.082

mg/kg/day (range: 0.04 to 0.19 mg/kg/day; absolute dose: 4.1 mg/day). The dose per kg was nonsignificantly higher in adolescents. Patients with FPLD were treated with higher doses of metreleptin compared to those with GL.

Metreleptin was approved by the FDA in 2014 for treatment of adult and pediatric patients with GL to treat metabolic complications of lipodystrophy, as an adjunct to diet and lifestyle modifications (90,91). Although metreleptin therapy resulted in heterogeneous improvements in patients with PL, in a subset probably consisting of PL patients with low leptin levels, metreleptin is likely to be beneficial (59,60). Although the FDA has not yet approved metreleptin for treatment of PL, the European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) has approved metreleptin in patients with PL in whom standard treatments have failed to achieve adequate metabolic control (92). There is no age limit for treatment in the US, and it has been used in infants as young as six months of age (85). However, the EMA CHMP's recent positive opinion includes authorization for metreleptin only in children two years of age and above with GL, and in children 12 years of age and above with PL (93). In Turkey, metreleptin therapy is currently available only for GL patients for whom standard treatments have failed to control HbA1c and triglyceride levels (94).

The generally recommended starting dose of metreleptin is 5 mg/day in females greater than 40 kg, 2.5 mg/day in males greater than 40 kg, and 0.06 mg/kg/day (0.012 mL/kg) in males and females less than or equal to 40 kg (91). The dose should be kg based in children, and the physicians should keep in mind that most children will require increasing per kg doses, especially as they reach puberty. However, the dose should be adjusted based on clinical response, and tolerability issues should also be borne in mind including excessive weight loss in pediatric patients. Metreleptin can be given once daily at any time of day regardless of meals (4,5). The most common side-effects are hypoglycemia and injection site reactions, such as erythema and/or urticarial (91). Metreleptin therapy has been shown to have beneficial effects on kidney function (95). However, a few patients have been described with worsening of proteinuria on metreleptin (91). This observation needs to be confirmed empirically as the disease progression itself can cause worsening proteinuria.

Two important issues which led to a "black box" warning in the US need to be specifically discussed. These are the development of neutralizing antibody and T-cell lymphoma. Anti-metreleptin antibodies were reported in a considerable number of patients with lipodystrophy on metreleptin. However, antibodies with *in vivo* neutralizing activity have only been detected in a small number of patients (96). T-cell lymphoma has been reported in a few patients with AGL treated with metreleptin (97,98). Immune dysfunction is a feature of the natural history in patients with AGL (42,44). To date no lymphoma development has been reported in patients with CGL or FPLD treated with metreleptin. Current evidence suggests that lymphoma development in patients with AGL may be associated with the natural history of the disease rather than being a treatment effect associated with metreleptin.

## Challenges in the Management of Lipodystrophy in Childhood and Adolescence

The needs of pediatric patients are different to those of adults and there are several challenges specific to children and young people in the management of lipodystrophy. Crying gives the baby a way to call for help when he/she is hungry or uncomfortable. Babies with lipodystrophy feel hungry all the time because of leptin deficiency. Appetite control is almost impossible in GL especially during active growth without getting help from metreleptin therapy. Even on metreleptin, patients and parents struggle to decide on the right amount of food to consume. Metreleptin causes weight loss in most patients. Parents become stressed when they witness their children losing weight on metreleptin, especially as they already look very thin because of the lipodystrophy.

Small children may have difficulties with verbalizing symptoms, such as abdominal pain caused by acute pancreatitis, symptoms of hyper- or hypoglycemia, muscle symptoms and infections. It may also be difficult to explain to children and adolescents why metabolic control is critical in lipodystrophy. The need for different types of therapies, and tasks such as glucose monitoring, routine blood sampling and regular hospital visits, being careful with what type of food is eaten and how much food is eaten given the associated hyperphagia is overwhelming for many of them. Children with lipodystrophy may need multiple injectable treatments including both insulin and metreleptin. Even though metreleptin therapy may allows the reduction in frequency or even the discontinuation of insulin injections metreleptin is still an injectable agent. In addition the lack of subcutaneous tissue makes the injection technique more challenging in lipodystrophy. It should be also noted that the relatively large injection volume would be another issue with metreleptin injections. It may prove difficult to get an active child to accept injections and self-monitoring of blood glucose at home.

Physical appearance is a big problem for adolescents with lipodystrophy. They may be worried about being with their

friends and in new social environments. Anger and temper outbursts are common. They may also have specific fears associated with the subtype of lipodystrophy.

To provide timely diagnosis and improve the delivery and quality of care, specialized centers for lipodystrophy for affected children and adolescents should be established. Optimizing management algorithms for children and young people can provide guidance for the use of standard and novel therapies to ensure adequate metabolic control and to prevent complications of lipodystrophy. These centers would develop more experience and thus be able to provide better and more efficient education for patients and their parents about lipodystrophy and its management. This should result in improved adherence to therapies and quality of life for these patients and their families. Cost of treatment remains the biggest barrier to access to novel treatments, such as metreleptin. However, the recent EMA approval of metreleptin therapy is a promising development for lipodystrophy patients in Europe and elsewhere in the world where the recommendations of EMA are adopted by the local authorities. It is important to state that regulatory authority approval does not guarantee access, if local health care coverage programs do not include therapies for rare diseases in their formularies. If metreleptin therapy is not an option in a specific country, or for a patient's plan, access to metreleptin can potentially be established through compassionate use programs and other regulatory mechanisms.

## Conclusion

Lipodystrophy syndromes are a heterogeneous group of disorders, characterized by the lack of adipose tissue, and is associated with severe insulin resistance that usually results in metabolic abnormalities leading to serious morbidity and increased mortality. Although there is no definitive cure for lipodystrophy, patients may benefit from an early diagnosis made in childhood, which in turn may improve lipodystrophy outcomes by providing care at the earliest stage possible. Effective strategies should be developed to overcome challenges in the management of lipodystrophy in children and young people.

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# Can Nesfatin-1 Predict Hypertension in Obese Children?

**Hatice Güneş[1], Filiz Alkan Baylan[2], Hakan Güneş[3], Fatih Temiz[4]**

*[1]Sütçü İmam University Faculty of Medicine, Department of Pediatrics, Kahramanmaraş, Turkey*
*[2]Sütçü İmam University Faculty of Medicine, Department of Biochemistry, Kahramanmaraş, Turkey*
*[3]Sütçü İmam University Faculty of Medicine, Department of Cardiology, Kahramanmaraş, Turkey*
*[4]Sütçü İmam University Faculty of Medicine, Department of Pediatric Endocrinology and Metabolism, Kahramanmaraş, Turkey*

### What is already known on this topic?

Childhood obesity is increasing over the years and leads to morbidities such as hypertension.

### What this study adds?

Obesity causes hypertension but the reason/s why all obese individuals are not hypertensive is controversial. This study aimed to clarify part of this issue by comparing obese peers stratified by blood pressure and found that Nesfatin-1 independently predicts hypertension in obese children.

## Abstract

**Objective:** The prevalence of childhood obesity is increasing and leads to co-morbidities such as hypertension. However, it is still not clear why some obese individuals are hypertensive and others not. Nesfatin-1 is a recently discovered anorexigenic peptide which also has effects on blood pressure (BP). Our aim was to evaluate the relationship between obesity-related hypertension and Nesfatin-1.

**Methods:** This cross-sectional study comprised 87 obese children. The patients were divided into two groups; hypertensive (n = 30) and normotensive (n = 57) obese. The American Academy of Pediatrics guidelines were used to diagnose hypertension. Blood samples were collected after 12 hours of fasting to investigate Nesfatin-1 concentrations. We also evaluated serum trace elements in addition to the routine blood tests.

**Results:** Body mass index (BMI), weight and serum Nesfatin-1 concentrations were higher in the hypertensive group (p = 0.002, p = 0.001, and p = 0.007, respectively). There was no difference between serum zinc levels, but Copper (Cu) levels were significantly lower in the hypertensive group (p = 0.248, p = 0.007, respectively). There were positive correlations between BP and BMI and weight Z-scores and a negative correlation with Cu. The optimal cut-off value of Nesfatin-1 to predict hypertension was found to be > 1.8 ng/mL, with a specificity of 71.9% and a sensitivity of 96.7% [area under the curve = 0.703, 95% confidence interval (CI): 0.577-0.809; p = 0.002]. In multiple logistic regression analysis Nesfatin-1 [Odds ratio (OR) = 1.103, 95% CI: 1.039-1.171; p = 0.001], Cu (OR = 0.947, 95% CI: 0.915-0.979; p = 0.001) and BMI for age Z-score (OR = 56.277, 95% CI: 5.791-546.907; p = 0.001) still remained significant predictors of hypertension.

**Conclusion:** Nesfatin-1 levels are higher and are an independent predictor of hypertension in obese subjects.

**Keywords:** Obesity, hypertension, Nesfatin-1, children

## Introduction

The prevalence of childhood obesity has been increasing over the years. The estimated prevalence among the world's children is 6.7% and is expected to be 9% by 2020 (1). In a meta-analysis study, the prevalence of obesity has increased from 0.7% to 7.1% in Turkey, between 1990 and 2015 (2), and is over 10% in some studies (3). This increased prevalence also poses a more serious problem by increasing the incidence of obesity-related co-morbid conditions. In addition to metabolic diseases such as diabetes and insulin resistance, obese patients are prone to various cardiovascular diseases such as hypertension and dyslipidemia. This increased disease burden, starting from childhood, deserves

**Address for Correspondence:** Hatice Güneş MD, Sütçü İmam University Faculty of Medicine, Department of Pediatrics, Kahramanmaraş, Turkey
**Phone:** +90 344 300 37 85 **E-mail:** drhaticegunes82@gmail.com **ORCID:** orcid.org/0000-0002-6940-0964

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detailed research which will also illuminate likely effects on adult health (4).

Obesity-related hypertension is a serious problem in childhood. The underlying etiology is complex and multiple factors such as activation of the renin-angiotensin-aldosterone system, stimulation of the sympathetic nervous system, hyperinsulinemia, peripheral fat tissue compression in the renal parenchyma, a number of cytokines affecting the vascular endothelium, and the abnormalities of some adipokines, such as leptin, are associated with this (1). Obesity is also related to some multi-nutrient and trace element deficiencies. For example, zinc (Zn) deficiency contributes to leptin reduction in rats and humans (5). Since many trace elements are components of antioxidant enzymes, such as cytoplasmic Cu-Zn superoxide dismutase, trace elements have major antioxidant roles affecting the vascular endothelium and contribute to the prevention of hypertension (6,7,8).

Nesfatin-1 has recently been shown to be an anorexigenic peptide which originates from its precursor protein, nucleobindin-2 (NUCB2) (9,10). It has been associated with appetite, food intake and weight loss (11). In addition to regulating food intake, Nesfatin-1 has been shown to regulate energy homeostasis, to contribute to water balance, affect gastrointestinal motility and also to have cardiovascular effects (12,13,14). NUCB2/Nesfatin-1 has been shown to be distributed in the hypothalamus, nucleus tractus solitarius and dorsal vagal complex which exert an influence of cardiovascular function (9,15). Central administration of Nesfatin-1 increases blood pressure (BP) and heart rate by the pressor effects of increased vasopressin, renin and catecholamine levels (16,17,18). Peripheral injection of Nesfatin-1 increases BP (19,20). The expression of NUCB2 mRNA was shown to be increased in the media of the aorta of hypertensive rats, so it may have a role in development of hypertension (21). In one study, serum Nesfatin-1 concentrations were reported to be higher in subjects with essential hypertension than the control group, and were found to correlate with systolic BP (22). Some studies demonstrated elevated Nesfatin-1 levels in hypertensive patients and especially in those who were obese (23). Based on these studies, Nesfatin-1 is considered to be a risk factor for obesity-related hypertension.

Although it has been shown that weight gain contributes to hypertension, it is unclear why some obese individuals are not hypertensive. This issue was our starting point. Thus, the aim of our study was to demonstrate and compare the levels of Nesfatin-1 in obese hypertensive and non-hypertensive or normotensive obese children and to identify the role of this peptide in obesity related-hypertension. In addition, we also aimed to assess the serum levels of Zn and Copper (Cu) in these subjects.

## Methods

Eighty seven obese children (41 male, 46 female) aged between 8 to 18 years, who were referred to the pediatric endocrinology and metabolism outpatient clinic of our hospital were included in this cross-sectional study. We divided the patients into two groups, matched for by age and sex, based on BP into a hypertensive obese group (n = 30) and a normotensive obese group (n = 57) which would serve as controls. Patients who had primary hypertension, hormonal abnormalities such as Cushing syndrome, hyperthyroidism, diabetes mellitus, medication-related hypertension, renal disease, heart disease, and other chronic diseases were excluded from the study.

Weight and height measurements were taken by a pediatric endocrinology nurse. Weight was measured with patients only in their underwear. Height was measured using a Harpenden stadiometer. Body mass index (BMI) was calculated by dividing weight (kg) by height squared ($m^2$). Obesity was defined as a BMI index above the 95$^{th}$ percentile. An individual was considered to be morbidly obese if his/her weight was ≥99$^{th}$ percentile (24).

BP was measured by an experienced nurse using an appropriately sized cuff, by the auscultatory method after at least 10 minutes of rest. The measurements were repeated three times at different clinical visits and mean values were recorded. Hypertension was defined as a systolic BP (SBP) and/or diastolic BP (DBP) ≥95$^{th}$ percentile on the basis of age, sex, and height percentiles (25). Ambulatory BP measurements were performed in patients with normal out-of-hospital blood BP values to investigate a diagnosis of "white coat" hypertension. Patients with this form of hypertension were excluded from the study (Figure 1). Both SBP, DBP, BMI, weight and height Z-scores for age of the patients were calculated using an on-line calculator (www.quesgen.com/BMIPedsCalc.php).

Venous blood samples were collected from all study groups between 8 a.m. and 10 a.m. following a fasting period of 12 hours and centrifuged at 4,000 rpm for 10 minutes. The serum samples obtained were frozen at -80 °C until time of analysis. While samples were taken for Nesfatin-1 routine blood analyses (serum glucose, insulin, high density lipoprotein, low density lipoprotein, total cholesterol, triglyceride, other biochemical parameters and complete blood count) were also performed and the results recorded. Serum concentrations of Nesfatin-1 were analyzed by enzyme-linked immunosorbent assay (ELISA) method using

a commercial kit (Bioassay Technology Laboratory, China and Wuhan Fine Biotech Co. Ltd., China), an automated ELISA reader (Thermo Scientific, Finland) and a computer program (Scanlt for Multiscan FC 2.5.1) according to the manufacturing company's direction. For Nesfatin: Sensitivity was 0.15 ng/mL and assay range was 0.30 ng/mL - 90 ng/mL, intra-assay CV% was <8%, while inter-assay CV% was <10%. The results were expressed in ng/mL units. Serum Zn and Cu concentrations were determined by flame a spectrophotometry method in Perkin Elmer Analyst 800 model atomic absorption spectrometer device after a 1/4 dilution with 5% glycerol for serum Zn and a 1/2 dilution with 10% glycerol for serum Cu. Results were expressed as µg/dL.

The study was conducted in accordance with the Declaration of Helsinki. Sütçü İmam University Ethics Committee approval under protocol number 333 (date: 29.08.2018), was granted and informed consent was obtained from the patients and their parents.

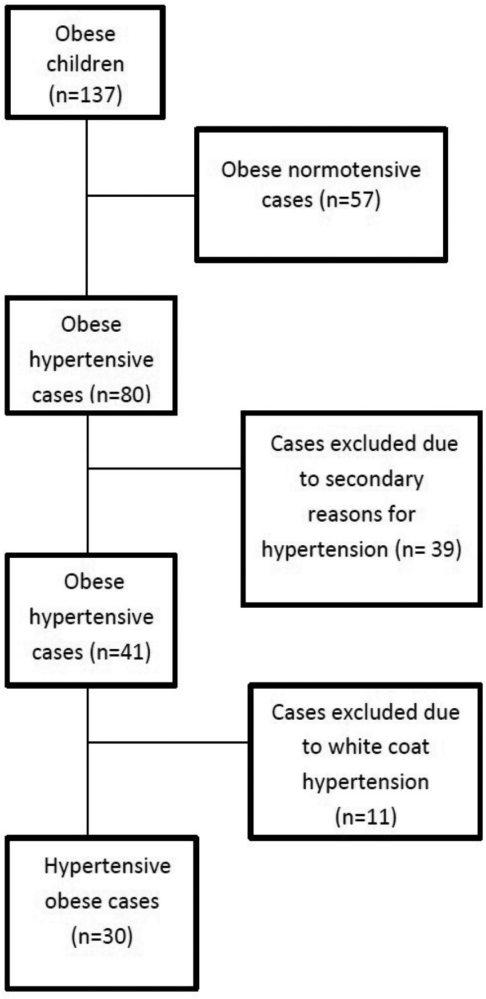


**Figure 1.** Study flow-chart

### Statistical Analysis

Data management and analysis were performed by using SPSS program v.14 (SPSS Inc., Chicago, IL., USA) and a two-sided p value $<0.05$ was considered as statistically significant. Continuous data were expressed as mean ± standard deviation or median and range. Categorical data were expressed as percentages. Mean values were compared by using an independent sample t-test, and in the case of an abnormal distribution, Mann-Whitney U test with median was used. Chi-square test was used for the categorical data. A stepwise multiple regression analysis was used for the definition of the significant determinants of hypertension, and incorporating variables that correlated with a p value of less than 0.1 in the correlation analysis. A value of $p<0.05$ was considered statistically significant.

## Results

The demographic and laboratory characteristics of the study groups are shown in Table 1. Age and gender were similar in the two groups ($p=0.135$, $p=0.607$, respectively), while BMI and weight Z-score values were higher in the obese hypertension group ($p<0.001$, $p=0.002$, respectively). There was a significant difference between each group according to SBP and DBP Z-scores (both $p<0.001$). When laboratory data were compared between the groups, no statistical difference was found, with the exception of creatinine. Creatinine concentrations tended to be higher in the hypertensive group, but within the normal range in both groups (Table 1). Serum Nesfatin-1 concentrations were higher in the hypertensive obese group with than the normotensive obese group ($p=0.007$) (Figure 2).

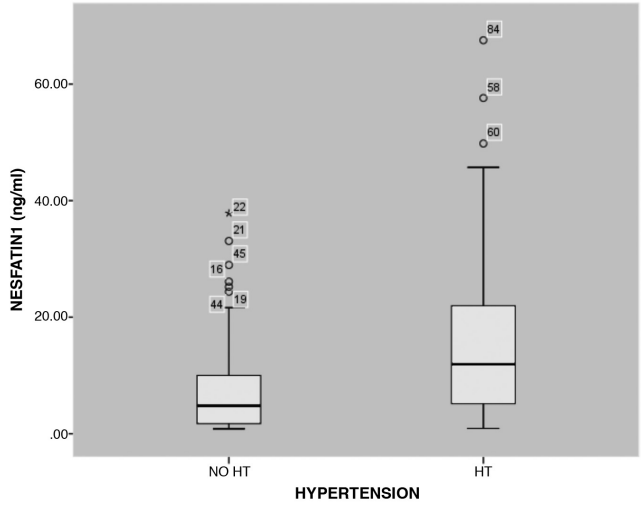


**Figure 2.** Distribution of Nesfatin-1 levels in the obese subjects with and without hypertension

*HT: hypertension*

When both groups were compared in terms of trace element levels, there was no difference in mean serum Zn concentrations (p = 0.248), whereas median serum Cu concentrations were significantly lower in the hypertensive group, (p = 0.007).

Correlation analysis revealed positive correlations between BMI and weight Z-scores and negative correlations between Cu and both SBP and DBP (Table 2).

Receiver operating characteristics curve analysis showed that the optimal cut-off value of Nesfatin-1 to predict hypertension was > 1.8 ng/mL, with a specificity of 71.9% and a sensitivity of 96.7% [area under the curve = 0.703; 95% confidence interval (CI): 0.577-0.809; p = 0.002] (Figure 3).

In the multiple logistic regression model using a backward stepwise method, Nesfatin-1 [Odds ratio (OR) = 1.103, 95%

**Table 1. Demographic and laboratory data of the study groups**

| | Obese hypertensive group (n = 30) | Obese normotensive group (n = 57) | p |
|---|---|---|---|
| Age, years[a] | 13.5 (9-15) | 11 (9-14) | 0.135 |
| Gender, male/female, n | 13/17 | 28/29 | 0.607* |
| Height, m[b] | 1.53 ± 0.18 | 1.51 ± 0.15 | 0.502 |
| HAZ[a] | 1.25 (-0.001-1.69) | 1.17 (0.13-1.72) | 0.724 |
| Weight, kg[b] | 83.7 ± 26.3 | 65.3 ± 18.9 | 0.001 |
| WAZ[a] | 2.56 (2.30-2.95) | 2.19 (1.82-2.54) | 0.002 |
| BMI, kg/m$^2$[a] | 32.8 (27.4-40.3) | 27.2 (24.5-31.8) | 0.002 |
| BAZ[a] | 2.49 (2.18-2.61) | 2.02 (1.87-2.25) | < 0.001 |
| SBP, mmHg[a] | 130 (123.7-140) | 100 (100-110) | < 0.001 |
| SAZ[a] | 1.96 (1.53-2.58) | -0.16 (-0.97-0.48) | < 0.001 |
| DBP, mmHg[a] | 90 (77.5-90) | 70 (60-70) | < 0.001 |
| DAZ[a] | 2.1 (0.80-2.30) | 0.28 (-0.20-0.66) | < 0.001 |
| Fasting blood glucose, mg/dL[a] | 89 (83.2-98) | 90 (85-94) | 0.849 |
| Insulin, µIU/mL[a] | 18.8 (14.2-39.4) | 16.1(10.6-23) | 0.057 |
| HbA1c, %[b] | 5.4 ± 0.33 | 5.4 ± 0.32 | 0.962 |
| ALT, U/L[a] | 22 (16.5-32.5) | 20 (16.7-29) | 0.429 |
| AST, U/L[a] | 22 (20.1-28) | 23.9 (20-28.5) | 0.209 |
| Total protein, g/dL[a] | 7.6 (7.4-7.9) | 7.6 (7.2-8) | 0.859 |
| Albumin, g/dL[b] | 4.7 ± 0.2 | 4.7 ± 0.4 | 0.733 |
| Urea, mg/dL[b] | 9.5 ± 2.4 | 8.8 ± 2.8 | 0.375 |
| Creatinin, mg/dL[b] | 0.6 ± 0.2 | 0.4 ± 0.2 | 0.002 |
| Triglyceride, mg/dL[a] | 122 (90-161) | 114 (85-157) | 0.151 |
| Total cholesterol, mg/dL[b] | 171.2 ± 35.9 | 161.6 ± 29.7 | 0.199 |
| HDL cholesterol, mg/dL[b] | 40.9 ± 7.9 | 43.8 ± 10.4 | 0.191 |
| LDL cholesterol, mg/dL[b] | 100.4 ± 27.2 | 99.5 ± 30.5 | 0.895 |
| WBC, x10$^3$ mm$^3$[a] | 8.8 (7.1-10.3) | 8 (6.8-9.8) | 0.273 |
| Hemoglobin, g/dL[b] | 13.56 ± 1.18 | 13.54 ± 1.11 | 0.925 |
| Platelet count, x10$^3$ mm$^3$[b] | 358 ± 63.6 | 354 ± 58.3 | 0.789 |
| Cu, µ/dL[a] | 85.2 (75.7-103.0) | 110.5 (89.9-125.4) | 0.002 |
| Zn, µ/dL[b] | 102.7 ± 46.7 | 112.9 ± 34.2 | 0.248 |
| Nesfatin, ng/mL[a] | 11.9 (5-22.6) | 4.8 (1.7-10.4) | 0.007 |

[a]median (interquartile range), [b]mean ± standard deviation, *chi-square (p≤0.05 was considered statistically significant).

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, BAZ: body mass index for age Z-scores, Cu: Copper, DBP: diastolic blood pressure, DAZ: diastolic blood pressure for age Z-scores, HDL: high density lipoprotein, HAZ: height for age Z-scores, LDL: low density lipoprotein, SBP: systolic blood pressure, SAZ: systolic blood pressure for age Z-scores, WAZ: weight for age Z-scores, WBC: white blood cell count, Zn: Zinc, HbA1c: hemoglobin a1c

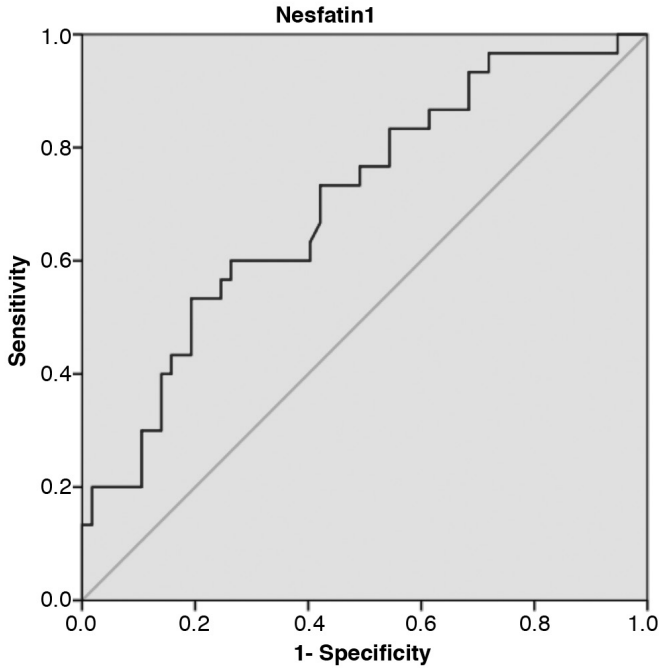


**Figure 3.** Receiver operator characteristic curve of Nesfatin-1 to predict hypertension

**Table 2. Correlation coefficients of systolic and diastolic blood pressures**

| | Systolic blood pressure | | Diastolic blood pressure | |
|---|---|---|---|---|
| | r | p | r | p |
| **BAZ** | 0.404 | < 0.001 | 0.271 | 0.012 |
| **WAZ** | 0.350 | 0.001 | 0.324 | 0.002 |
| **Copper level** | -0.303 | 0.005 | -0.249 | 0.023 |
| **Creatinine level** | 0.248 | 0.021 | 0.094 | 0.389 |

BAZ: body mass index for age Z-scores, WAZ: weight for age Z-scores

CI: 1.039-1.171; p = 0.001], Cu (OR = 0.947, 95% CI: 0.915-0.979; p = 0.001) and BMI Z-score values (OR = 56.277, 95% CI: 5.791-546.907; p = 0.001) still remained significant predictors of hypertension after adjusting for the confounding variables, which were found to be statistically significant in the univariate analysis and for the variables which were also statistically significant in the t-test (Table 3).

## Discussion

In this study, it was shown that Nesfatin-1 levels were independently related to hypertension and higher in obese hypertensive children than their obese normotensive counterparts. BMI Z-scores were higher in the hypertensive group and were positively correlated with BP values. We also found that median serum Cu concentration was low in the hypertensive group.

Weight gain causes hypertension in some individuals but not in others. This may be related to how long the individual is obese and the long-term effects of over-adiposity (26). In our study, although there was no statistical difference in age between the groups, the mean age of the hypertensive group was higher. Our results revealed that serum Nesfatin-1 levels were higher in the obese hypertensive group. This is consistent with the study of Zhao et al (23) who investigated Nesfatin-1 in 40 hypertensive adults and 40 healthy controls, and reported significantly higher levels of Nesfatin-1 in the hypertensive group, especially in obese individuals. In the study of Anwar et al (27) the Nesfatin-1 levels were higher in obese adolescents than their healthy peers and correlated with BMI values. Their results were similar to those reported by Tan et al (28) who compared the levels of Nesfatin-1 in 38 adult subjects. Sahin et al (29) found higher levels of Nesfatin-1 in polycystic ovary syndrome than healthy controls and also reported that these values positively

**Table 3. Univariate and multivariate analyses of study group**

| Univariate analysis | | | | | | | Multivariate analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **B** | **S.E.** | **Wald** | **p** | **OR** | **95% CI** | **B** | **S.E.** | **Wald** | **p** | **OR** | **95% CI** |
| **Statistically significant variables** | | | | | | | | | | | | |
| **Nesfatin-1** | 0.058 | 0.022 | 7.355 | 0.007 | 1.060 | 1.016-1.106 | -5.463 | 2.769 | 3.892 | 0.001 | 1.103 | 1.039-1.171 |
| **Cu** | -0.033 | 0.011 | 8.371 | 0.004 | 0.968 | 0.946-0.989 | -0.055 | 0.017 | 10.228 | 0.001 | 0.947 | 0.915-0.979 |
| **BAZ** | 3.416 | 0.913 | 14.007 | < 0.001 | 30.450 | 5.089-82.199 | 4.030 | 1.160 | 12.067 | 0.001 | 56.277 | 5.791-546.907 |
| **WAZ*** | 1.389 | 0.507 | 7.516 | 0.006 | 4.011 | 1.486-10.829 | | | | | | |
| **Creatinine*** | 2.778 | 0.978 | 8.068 | 0.005 | 16.085 | 2.366-09.361 | | | | | | |

All the variables from Table 1 were examined and only those significant at p < 0.05 level are shown in univariate analysis. Multivariate logistic regression analyses including all the variables in univariate analysis with enter method. A p < 0.05 was considered statistically significant.

*Non-significant variables in multivariate logistic regression analysis are not shown in the Table 1.

B: beta coefficients, BAZ: body mass index for age Z-scores, WAZ: weight for age Z-scores, CI: confidence interval, Cu: Copper, OR: Odds ratio, S.E.: standard error, Wald: wald test

correlated with SBP and DBP. In our study, the Nesfatin-1 levels in both groups were between the commercial kit normal values. This may be due to the gradual increase in serum Nesfatin-1 level with age. In the study of Anwar et al (27) they demonstrated that as the pubertal stage progresses, serum Nesfatin-1 levels increase. In our study the study population is younger.

It was demonstrated that Nesfatin can cross the blood-brain barrier in both directions and this may explain the effect of this peptide on the central control of cardiovascular effects (30). In their study, Yilmaz et al (18) found that BP increased in both groups as a result of intracerebroventricular (i.c.v.) Nesfatin-1 given to hemorrhagic hypotensive rats and control group. The i.c.v. administration of Nesfatin-1 in animal studies increased plasma renin, catecholamine, and vasopressin which resulted in hypertension, too (17,18,31). The central melanocortin system has been implicated in the hypertensive effects of Nesfatin-1 in normotensive animals and also in obesity-related hypertension (17,26,31,32,33). In our study Nesfatin-1 was independently associated with hypertension and was predictive of hypertension in obese subjects. Zhao et al (23) and Sahin et al (29) found a positive correlation between Nesfatin-1 level and BP. However we did not find significant correlation between Nesfatin-1 and BP. This may have been due to the low number of cases in the hypertensive group.

We also evaluated the serum trace elements between the groups and found a significant difference in Cu levels, with lower levels in the hypertensive group and a negative correlation with BP. Cu levels were independently associated with hypertension. However, there was no correlation between BP and Zn levels and there was no significant difference between the groups in terms of Zn. There are several reports of the association of trace elements with hypertension in the literature. It has been suggested that Zn and Cu may play a role in the pathogenesis of hypertension due to the role of these electrolytes in the regulatory enzymes of the vascular system (34). Low serum Cu levels were detected in association with hypertension in both human and animal studies and negative correlations were found (35,36,37,38). This can be related to the inhibitory effect of Cu on angiotensin converting enzyme activity (39,40). In addition Cu deficiency causes hypercholesterolemia and increased oxidative stress, which can lead to hypertension (41).

### Study Limitations and Strengths

There were some limitations of our study. Firstly, the number of cases is low, especially in the hypertensive group. It may have been possible to include a healthy control group for comparison but the primary aim of the study was to investigate the effect of Nesfatin-1 in obesity. In our study, the Nesfatin-1 levels in both groups were between the commercial kit normal values and may be related to the young age groups. Another limitation is the heterogeneity in BMI and weight. This may be related to the cross-sectional study design. However, there are also some strengths of our study. It is the first study in the literature to show the effect of Nesfatin-1 on obese children. We compare only obese peers to understand the effect of Nesfatin-1 on BP is another strength of our study.

## Conclusion

To the best of our knowledge, this is the first study evaluating serum Nesfatin-1 in obese hypertensive children and adolescants. Nesfatin-1 level independently predicts hypertension in obese subjects. This study may begin to illuminate why some obese patients have hypertension while others do not although this is a complex, multifactorial problem which may require a better understanding of the biology of Nesfatin-1. Needless to say, further, larger, randomized controlled trials are needed to provide conclusive evidence concerning this.

### Ethics

**Ethics Committee Approval:** The study was approved by the Ethics Committee of Sütçü İmam University (decision no: 333, date: 29.08.2018).

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

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

### Authorship Contributions

Surgical and Medical Practices: Hatice Güneş, Hakan Güneş, Filiz Alkan Baylan, Fatih Temiz, Concept: Hatice Güneş, Hakan Güneş, Filiz Alkan Baylan, Fatih Temiz, Design: Hatice Güneş, Hakan Güneş, Fatih Temiz, Data Collection or Processing: Hatice Güneş, Hakan Güneş, Fatih Temiz, Analysis or Interpretation: Hatice Güneş, Hakan Güneş, Filiz Alkan Baylan, Fatih Temiz, Literature Search: Hatice Güneş, Hakan Güneş, Filiz Alkan Baylan, Fatih Temiz, Writing: Hatice Güneş, Hakan Güneş, Filiz Alkan Baylan, Fatih Temiz.

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# Treatment with Depot Leuprolide Acetate in Girls with Idiopathic Precocious Puberty: What Parameter should be Used in Deciding on the Initial Dose?

**Doğuş Vurallı[1], Ayfer Alikaşifoğlu[1], İrem İyigün[2], Dicle Canoruç[2], Alev Ozon[1], Nazlı Gönç[1], Nurgün Kandemir[1]**

*[1]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Ankara, Turkey*
*[2]Hacettepe University Faculty of Medicine, Department of Pediatrics, Ankara, Turkey*

### What is already known on this topic?

Insufficient suppression due to inadequate dose of gonadotropin releasing hormone analogues (GnRHa) in central precocious puberty (CPP) may result in continued advancement of bone limiting final height whereas unnecessarily high doses may increase the risk of side effects, as well as total treatment costs. Monthly GnRHa injections are administered at different doses in different countries. For leuprolide acetate (LA), lower doses (3.75 mg/28 days, 80-120 µg/kg/28 days) are preferred in Europe and Asia, while higher doses (7.5-15 mg/28 days, 200-300 µ/kg/28 days) are used in the United States of America.

### What this study adds?

LA treatment at doses of 3.75 mg/28 days is effective in suppressing the hypothalamo-pituitary-gonadal (HPG) axis in the majority of girls with idiopathic CPP. Higher initial doses may be preferred in patients with a body weight ≥36 kg or body mass index-standard deviation scores ≥1.6 for effective suppression of HPG axis.

## Abstract

**Objective:** Doses of gonadotropin releasing hormone (GnRH) analogues used to treat idiopathic central precocious puberty (iCPP) vary among clinicians. Study aims were to evaluate the efficacy of a monthly 3.75 mg dose of leuprolide acetate (LA) to suppress the hypothalamo-pituitary-gonadal (HPG) axis in girls with iCPP and to determine factors that may have an impact on the supressing dose.

**Methods:** Study subjects were 220 girls receiving LA for iCPP. LA was started at a dose of 3.75 mg/28 days. Suppression was assessed using the GnRH test at the third month. To assess clinical suppression signs and symptoms of puberty were also evaluated. The dose of LA was increased to 7.5 mg/28 days in those who had a peak luteinising hormone (LH) ≥2 IU/L and in whom adequate clinical suppression of puberty was absent. Receiver operating characteristic curves were used to determine thresholds for clinical and hormonal factors affecting the suppressing dose of LA. Logistic regression analyses were used to investigate thresholds which might differentiate between those requiring high dose for suppression and those in whom lower dose LA was adequate.

**Results:** Peak stimulated LH $< 2$ IU/L was achieved in 88.6% with a dose of LA of 3.75 mg ($0.11 \pm 0.03$ mg/kg). Significant variables for differentiating the two doses were body weight (Wt) of 36.2 kg and/or body mass index (BMI)-standard deviation scores (SDS) of 1.64 ($p < 0.001$). Multiple logistic regressions showed that Wt and BMI-SDS values above thresholds indicated requirement of LA at a dose of 7.5 mg/28 days ($p < 0.001$).

**Conclusion:** Monthly injections of 3.75 mg LA is an effective treatment in the majority of girls with iCPP. However, a higher initial dose may be preferred in patients with a Wt ≥36 kg or BMI-SDS ≥1.6 for effective suppression of the HPG axis.

**Keywords:** Central precocious puberty, leuprolide, GnRH, GnRH analogue, gonadotropin releasing hormone agonist, precocious puberty, puberty

**Address for Correspondence:** Doğuş Vurallı MD, Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Ankara, Turkey
**Phone:** +90 312 305 11 24 **E-mail:** dvuralli@hotmail.com **ORCID:** orcid.org/0000-0002-4011-2299

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## Introduction

The aim of gonadotropin releasing hormone (GnRH) analogue (GnRHa) treatment in central precocious puberty (CPP) is to allow normal growth, enabling a normal adult height and relieve psychosocial stress associated with early puberty (1,2,3). The intended long-term goals in such treatment include suppression of bone advancement and attainment of an age appropriate growth rate, in order to achieve a normal adult height parallel to target height (1,4). While short-acting nasal and daily injectable forms of GnRHa have been used previously, currently long-acting (monthly) or very-long-acting (three monthly) depot formulations or yearly implants that facilitate adherence to treatment are more commonly preferred (1,3,5). Insufficient suppression due to an inadequate dose of GnRHa may result in continued advancement of bone age (BA) thus limiting final height whereas unnecessarily high doses may increase the risk of side effects, as well as total treatment costs. Higher doses have been shown to suppress both growth and bone mineral accrual rates (6,7). The doses of GnRH analogues used in CPP may vary with clinican preference, as well as local regulatory approvals. Monthly GnRHa injections are administered in different doses in different countries. For leuprolide acetate (LA), lower doses (3.75 mg/28 days, 80-120 µ/kg/28 days) are preferred in Europe and Asia (8,9,10), while higher doses (7.5-15 mg/28 days, 200-300 µ/kg/28 days) are used in the United States of America (11). In the face of such dosage variation, the best dose for optimal pituitary desensitization during monthly leuprolide treatment is still a matter of discussion. The aim of this study was to evaluate the efficacy of a monthly 3.75 mg dose of LA to suppress the hypothalamo-pituitary-gonadal (HPG) axis in girls with idiopathic CPP (iCPP) and to determine factors that may have an impact on the supressing dose. We also aimed to define the best predictor among these factors for the optimal initial dose of LA.

## Methods

A total of 220 girls with a diagnosis of iCPP who were followed between January 2012 and January 2018 who had received 3.75 mg LA (Lucrin depot, subcutaneous or intramuscular) once every 28 days were evaluated. Age at diagnosis, BA, body weight (Wt), height, pubertal stage, basal estradiol levels, basal and stimulated gonadotropin levels, pelvic ultrasonography and magnetic resonance imaging (MRI) findings of the pituitary gland were recorded. CPP was diagnosed based on breast development being at Tanner stage 2 or higher before eight years of age, and peak luteinizing hormone values ≥5 IU/L during the GnRH test (12). A GnRH test was performed in all patients at the time of diagnosis. Blood samples were collected at baseline (zero minutes) for follicle stimulating hormone (FSH) and LH measurements. Then the patients were intravenously administered 100 µg/m$^2$ of GnRH (gonadorelin acetate, Ferring®). Following drug administration, blood samples were collected at 20, 40, 60, and 120 minutes for FSH and LH measurement (13). With the exception of four patients presenting with menarche, all patients were followed for 3-6 months before the treatment decision. GnRHa treatment was given to patients with progressive CPP, determined according to the following criteria: a) Growth velocity above 6 cm/year; b) Advanced BA defined as BA ≥2 years compared with chronological age; c) Rapid progression in pubertal stages defined as progression of puberty from one stage to the next in less than six months; and d) Decrease in predicted adult height compared to target height (14). Pituitary MRI was performed in all cases and the underlying organic pathology was investigated. Cases with no pathological MRI findings were considered to be idiopathic and were included in the study. Subjects were excluded from the analysis if they had any additional conditions that might affect puberty onset such as hypothyroidism, growth hormone deficiency or congenital adrenal hyperplasia. LA was started at an initial dose of 3.75 mg/28 days for all patients with iCPP. For all patients who were started on GnRHa treatment, the GnRH test was repeated in the third month of treatment and the HPG axis was considered to be suppressed if peak LH levels were < 2 IU/L (15,16,17). Clinical signs and symptoms of puberty were also evaluated every 3-6 months to determine whether pubertal suppression was achieved clinically. Parameters of good clinical control included stabilization or regression of pubertal findings, decrease in height velocity to prepubertal levels, cessation of BA progression, and improvement in final height prediction. The dose of LA was increased to 7.5 mg/28 days in those who have a peak LH ≥2 IU/L and in whom clinical suppression of puberty was not achieved. All patients who had a peak LH ≥2 IU/L in the third month GnRH test did not have adequate clinical suppression of puberty and dose LA dose was increased in all of these cases. The higher dose was similarly tested with GnRH test for appropriate suppression of HPG three months later. We compared clinical and hormonal characteristics of the two populations whose HPG axis was suppressed either with 3.75 mg/28 days or 7.5 mg/28 days of LA. Follow up included clinical and hormonal evaluation of all patients every six months after the initial treatment and, during long-term follow-up continuous clinical and hormonal suppression was observed.

### Auxological Parameters

Body Wt were measured with a digital body weighing scale and heights were measured in the standing position with a Harpenden stadiometer by a nurse trained in height measurements and auxology. The percentile curves of the Centers for Disease Control and Prevention (CDC) were used to interpret the growth data (18). Height standard deviation scores (SDS) for chronological age and BA were calculated using CDC charts. Body mass index (BMI) was calculated using the standard equation (Wt in kg/height in meters squared). BMI-SDS was calculated according to the LMS method using CDC charts (19). Puberty staging was evaluated using Marshall and Tanner staging (20). The BA was evaluated according to the Greulich and Pyle atlas (21).

### Hormone Assays

The immunochemiluminometric assay method using commercial kits (ARCHITECT System, Abbott Laboratory Diagnostics, USA) were used to measure FSH, LH and estradiol levels. The sensitivity of the FSH, LH, and estradiol assays was 0.3 IU/L, 0.07 IU/L, and 10 pg/mL respectively.

### Ethics Statements

The study protocol was approved by the Ethics Commitee of Hacettepe University (approval number: GO 19/453-41). The requirement for informed consent was waived due to the retrospective nature of the study.

### Statistical Analyses

Statistical analyses were performed using the Statistical Package for Social Sciences software package for Windows (version 19.0; SPSS Inc., Chicago, IL, USA). Testing for normality was performed by Shapiro-Wilk test and the data was found to be normally distributed. Data are shown as mean ± standard deviation values. Student's t-test was used in comparisons of independent samples. Receiver operating characteristic (ROC) curves were used to determine threshold levels for factors with an impact on the dose of LA that suppressed HPG axis (age, body Wt, BMI, BMI-SDS, basal LH, basal estradiol, peak stimulated LH). Threshold values were analyzed to investigate if they differentiated the two populations of patients whose HPG axis was suppressed either with 3.75 mg/28 days or 7.5 mg/28 days of LA using univariate logistic regression. Pubertal stages were grouped into early (Tanner 2 and 3) vs advanced (Tanner 4 and 5), and impact of pubertal stages on suppressing doses of LA were also analyzed. Statistically significant factors in univariate analysis were re-evaluated using multiple logistic regression analysis. A p value of less than 0.05 was considered statistically significant.

## Results

Peak stimulated LH was < 2 IU/L after three months of treatment in 88.6% (195/220) of the patients with the initial LA dose of 3.75 mg/28 days. In the remaining 11.4% (25/220), the LA dose was increased to 7.5 mg/28 days, as puberty suppression was not achieved clinically and hormonally. The GnRH test was repeated in patients who received 7.5 mg/28 days at the third month of dose escalation. The peak LH levels were found to be < 2 IU/L in all patients and hormonal puberty suppression was achieved in all of them. Regression in the clinical signs and symptoms of puberty and cessation in BA progression were observed. Growth rates decreased to prepubertal levels in all patients with successful hormonal suppression. Consequently, suppression of the HPG axis was achieved in all patients by the sixth month of treatment (Table 1).

Among cases that achieved HPG suppression at the dose of 3.75 mg LA/28 days, the pubertal stage at the time of diagnosis was Tanner stage 2 in 35.9% (70/195), Tanner stage 3 in 54.4% of cases (106/195), and Tanner stage 4 in 9.7% (19/195). Among the cases with successful suppression with a dose of 7.5 mg LA/28 days, 60% (15/25) were at Tanner stage 3, 24% (6/25) were at Tanner stage 4 and 16% (4/25) were at Tanner stage 5 at the time of diagnosis. These latter four patients presented with menarche. There were no cases presenting with menarche among the patients whose puberty were suppressed with 3.75 mg LA. The stage of puberty at the time of diagnosis was significantly advanced among patients for whom the effective dose was 7.5 mg ($p < 0.001$). Suppression was achieved with LA 3.75 mg/28 days in all patients (70/70) who were at Tanner stage 2, in 87.6% of patients (106/121) at Tanner stage 3 and 76% of patients (19/25) at Tanner stage 4 at the time of diagnosis, while all patients (4/4) at Tanner stage 5 required 7.5 mg LA for the suppression of the HPG axis.

A comparison of the clinical and laboratory findings at the time of diagnosis of the patients for whom HPG axis suppression was achieved with 3.75 mg and 7.5 mg LA dosages revealed that those requiring 7.5 mg LA for suppression were found to have higher mean body Wt, BMI and BMI-SDS values and also elevated mean baseline LH, estradiol and peak stimulated LH levels at the time of diagnosis (Table 1). Among the patients with successful suppression at a dose of 3.75 mg LA, suppression was achieved with a mean dose of $0.11 \pm 0.03$ mg/kg, whereas in the patients for whom 3.75 mg dose was not adequate for suppression, the initially given dose of $0.08 \pm 0.02$ mg/kg (3.75 mg in total) was insufficient due to high body Wt,

**Table 1. Clinical and laboratory characteristics of patients treated with leuprolide acetate at doses of 3.75 mg vs 7.5 mg**

| | 3.75 mg LA (n = 195) | 7.5 mg LA (n = 25) | p value |
|---|---|---|---|
| Age at diagnosis (years) | 8.2 ± 1.0 | 8.3 ± 0.5 | 0.535 |
| Bone age (years) | 10.2 ± 0.9 | 10.3 ± 0.9 | 0.422 |
| Body weight (kg) | 32.1 ± 6.1 | 44.9 ± 7.1 | < 0.001 |
| BMI (kg/m$^2$) | 18.7 ± 3.3 | 27.5 ± 8.4 | < 0.001 |
| BMI-SDS | 1.1 ± 1.2 | 2.4 ± 1.2 | < 0.001 |
| Height (cm) | 135.2 ± 9.2 | 136.2 ± 10.0 | 0.172 |
| Height-SDS | 1.1 ± 1.2 | 1.1 ± 1.3 | 0.991 |
| Height-SDS for BA | -0.6 ± 1.0 | -0.5 ± 1.2 | 0.833 |
| Basal FSH (IU/L) | 4.5 ± 2.1 | 5.3 ± 2.5 | 0.121 |
| Basal LH (IU/L) | 1.2 ± 0.7 | 1.9 ± 1.2 | < 0.001 |
| Basal estradiol (pg/mL) | 30.6 ± 14.4 | 52.5 ± 9.1 | < 0.001 |
| Peak stimulated LH (IU/L) | 11.7 ± 5.0 | 16.7 ± 9.4 | < 0.001 |
| Age at six months post-treatment (years) | 8.7 ± 1.0 | 8.8 ± 0.5 | 0.546 |
| Bone age at six months post-treatment (years) | 10.5 ± 1.2 | 10.6 ± 1.2 | 0.624 |
| Height-SDS at six months post-treatment | 1.1 ± 1.2 | 1.1 ± 1.3 | 0.991 |
| Basal LH at six months post-treatment (IU/L) | 0.3 ± 0.3 | 0.3 ± 0.3 | 0.848 |
| Basal estradiol at six months post-treatment (pg/mL) | 12.5 ± 2.5 | 13.4 ± 3.2 | 0.434 |
| Age at 12 months post-treatment (years) | 9.2 ± 1.0 | 9.3 ± 0.5 | 0.485 |
| Bone age at 12 months post-treatment (years) | 11.0 ± 1.3 | 11.2 ± 1.2 | 0.626 |
| Height-SDS at 12 months post-treatment | 1.0 ± 1.2 | 1.0 ± 1.2 | 0.866 |
| Basal LH at 12 months post-treatment (IU/L) | 0.2 ± 0.2 | 0.2 ± 0.2 | 0.824 |
| Basal estradiol at 12 months post-treatment (pg/mL) | 10.3 ± 1.6 | 10.4 ± 1.8 | 0.386 |

BMI-SDS: body mass index-standard deviation scores, LH: luteinising hormone, LA: leuprolide acetate, FSH: follicle stimulating hormone, BA: bone age

**Table 2. Factors affecting treatment dosage based on univariate logistic regression analysis**

| Variables | Odds ratio | 95% CI | | p value |
|---|---|---|---|---|
| Body weight ≥36.2 kg | 1.619 | 1.330 | 1.914 | < 0.001 |
| BMI ≥20.7 kg/m$^2$ | 1.941 | 1.515 | 2.488 | < 0.001 |
| BMI-SDS ≥1.64 | 2.165 | 1.735 | 2.690 | < 0.001 |
| Basal LH ≥1.5 IU/L | 1.084 | 0.898 | 1.309 | 0.401 |
| Basal estradiol ≥41 pg/mL | 1.004 | 0.995 | 1.014 | 0.330 |
| Peak stimulated LH ≥15.7 IU/L | 1.240 | 0.742 | 1.726 | 0.421 |
| Pubertal stage (advanced vs early) | 2.516 | 0.877 | 7.215 | 0.020 |

BMI-SDS: body mass index-standard deviation scores, CI: confidence interval, LH: luteinising hormone

and suppression was only achieved when these patients received a dose of 7.5 mg LA (0.16 ± 0.03 mg/kg).

ROC curves were used to determine the threshold levels for the factors which may affect the dose that achieved pubertal suppression. The best threshold values that differentiated the two doses (3.75 mg/28 days vs 7.5 mg/28 days LA) were 36.2 kg for body Wt (AUC = 0.934, p = 0.0001, sensitivity 100%, specificity 66.7%), 20.7 kg/m$^2$ for BMI [area under the curve (AUC) = 0.964, p = 0.0001, sensitiviy 94%, specificity 74%], +1.64 for BMI-SDS (AUC = 0.914, p = 0.0001, sensitivity 100%, specificity 71.2%), 1.5 IU/L for basal LH (AUC = 0.710, p = 0.0004, sensitivity 68%, specificity 67%), 41 pg/mL for basal estradiol (AUC = 0.898, p = 0.0001, sensitivity 100%, specificity 68%) and 17.6 IU/L for peak stimulated LH (AUC = 0.710, p = 0.0006, sensitivity 68%, specificity 67%) in ROC analysis. Age did not differ between the two different dose populations (8.2 ± 1.0 vs 8.3 ± 0.5). Univariate analysis indicated Wt, BMI and BMI-

SDS above the defined thresholds, as well as advanced stage of puberty were associated with higher dose of LA for effective treatment ($p < 0.001$, $< 0.001$, $< 0.001$, 0.02, respectively) (Table 2). However, thresholds for basal LH, estradiol and stimulated LH peak did not differentiate between the two doses of LA since they were insignificant in the univariate analysis. Since Wt and BMI-SDS were related factors, these factors were not used together in multiple regression analysis but tested in separate regression models. Multiple logistic regression showed that thresholds for BMI-SDS and Wt were significant to differentiate the two doses of LA ($p < 0.001$) (Table 3, 4), whereas thresholds for basal LH, estradiol and stimulated peak LH did not differentiate the two dose groups and thus could not be used to assess dose of LA required to suppress puberty.

## Discussion

In this study we showed that LA treatment at a dose of 3.75 mg/28 days was effective in suppresing the HPG axis in the majority (88.6%) of girls with iCPP, while suppression was achieved in the remaining 11.4% of cases with a dose of 7.5 mg/28 days. Studies from Europe and Brazil have shown that suppression of the HPG axis can be achieved in 85-96% of the cases using a dose of 3.75 mg/28 days LA (7,9,22,23) which is consistent with our findings. Studies carried out in the United States report higher LA doses of at least 7.5 mg/monthly for HPG suppression (24,25). In Japan, Tanaka et al (26) compared doses of 10, 30 and 90 µg/kg in 36 children with CPP (90 µg/kg being roughly equal to 3.75 mg LA) and concluded that the minimum suppressive dose of LA was 30 µg/kg, which is one tenth of the US recommendations and much lower than the dose of 3.75 mg/28 days.

Recently, use of three-monthly LA depot preparations in pediatric patients appeared in the literature (27). The dose difference in the use of LA depot formulations between United States and Europe was reported to persist in this report. In a French study of 40 cases with CPP, a three-monthly dose of 11.25 mg provided suppression of GnRH-stimulated gonadotropin levels (28). In a study from the United States, Fuld et al (29) compared three doses of LA (LA 7.5 mg/month, 11.25 mg/3 months and 22.5 mg/3 months) in 54 patients with CPP, and showed that the dose of 22.5 mg/3 months provided a better suppression of LH levels in comparison to a dose of 11.25 mg/3 months. However, these last two doses did not differ in their effect on other parameters studied which were growth velocity, progression of BA or estradiol levels. Mericq et al (30) compared the same three doses of LA in 14 children and recommended the use of high-dose LA depot formulations in cases with a body Wt of more than 30 kg, although LA depot formulation at a dose of 11.25 mg/3 months also provided sufficient (75%) pubertal suppression.

One major constraint in the published studies is that they were carried out in small populations of children. What is more, many studies analyzed mixed populations with respect to sex, involving both girls and boys, and etiology which included both idiopathic and organic cases. The GnRHa dose required to suppress the HPG axis may differ between girls and boys, and also between CPP cases of organic or idiopathic etiology. In addition most studies comparing monthly vs three monthly preparations did not include LA at a dose of 3.75 mg/28 days.

There is one study from the USA which included monthly 3.75 mg LA and compared it with 7.5 mg/month and 11.25 mg/3 months LA. In that study Badaru et al (27) showed that in patients on treatment with LA using a dose of 3.75 mg/month and 11.25 mg/3 months had peak stimulated LH and FSH levels higher than those using a dose of 7.5 mg/month (the mean depot LA-stimulated LH was $1.30 \pm 0.74$, $1.73 \pm 0.99$ and $2.13 \pm 1.41$, with doses of 7.5 mg/month, 3.75 mg/month, and 11.25 mg/3 months, respectively). However, the authors underlined that clinically significant elevation to merit dose escalation was observed only in a small number of patients. In addition, serum estrogen levels did not differ between the three dose regimens.

In the current study a large homogenous population of girls with iCPP was analyzed to see if pubertal suppression can be achieved with lower monthly doses of LA. The suppressive dose of LA and factors that may impact on its

**Table 3. Factors affecting treatment dose based on multivariate logistic regression analysis (first model)**

| Variables | Odds ratio | 95% CI | | p value |
|---|---|---|---|---|
| BMI-SDS ≥1.64 | 2.846 | 1.268 | 6.324 | < 0.001 |
| Pubertal stage (advanced vs early) | 2.247 | 0.382 | 26.263 | 0.489 |

BMI-SDS: body mass index-standard deviation scores, CI: confidence interval

**Table 4. Factors affecting treatment dose based on multivariate logistic regression analysis (second model)**

| Variables | Odds ratio | 95% CI | | p value |
|---|---|---|---|---|
| Body weight ≥36.2 kg | 2.134 | 1.646 | 3.116 | < 0.001 |
| Pubertal stage (advanced vs early) | 3.212 | 0.525 | 14.284 | 0.365 |

CI: confidence interval

effectiveness were also investigated. It was hypothesized that such an analysis may help predict the dose of LA that can suppress puberty and avoid high doses of LA thus avoiding the associated adverse effects. The current study suggests that LA at a dose of 3.75 mg/28 days effectively suppresses HPG axis in most girls with iCPP. A comparison of the two populations (pubertal suppression by LA 3.75 mg/28 days vs 7.5 mg/28 days) showed that there was significant difference between them in several clinical and laboratory parameters such as body Wt, BMI, basal LH, estradiol, and peak stimulated LH on the initial GnRH test. As would be expected, higher GnRHa doses may be required for pubertal suppression in cases at advanced stages of puberty. Similarly, the patients who required dose escalation had higher baseline LH and estradiol levels as well as higher peak LH in the GnRH test. We showed that the most significant factors indicating a need for LA at a dose of 7.5 mg/28 days were body Wt ≥36.2 kg, and BMI-SDS ≥1.64.

In general, the use of high dose of GnRHa may have two important consequences. Firstly, oversuppression of puberty using a high dose of GnRHa may carry the risk of suppression of growth. Secondly, extensive pubertal suppression may affect bone mineral density (BMD) adversely, since long-term oversuppression of estrogen may decrease bone mineral accrual. In addition, higher doses would increase treatment costs excessively. It is well-known that one major purpose of GnRHa treatment is to increase the final height potential. Thus it may seem contradictory to suggest that oversuppression of puberty may adversely affect growth. Studies investigating long-term effects of GnRHa treatment have shown the expected deceleration in BA advancement as well as suppression of puberty increasing final height (31). However, these studies did not address the relation between height gain and the dose of GnRHa used to suppress puberty.

Mitamura et al (32) studied 24 hour gonadotropin and sex steroid profile in 17 girls (5-11.5 years) and showed that a diurnal rhythm of gonadotropins was present in all subjects including those aged 5-6 years. Also one third of their prepubertal subjects had elevated early morning stradiol. They suggested that preparation for the onset of female puberty may begin in 5- to 6-year-old girls. Lampit et al (33) compared GnRHa therapy with and without mini dose estrogen in a small number of patients. They showed that during GnRH agonist therapy a mini-dose of estrogen effectively maintained normal prepubertal growth without acceleration of bone maturation for at least 24 months, whereas growth velocity may decrease in those receiving GnRHa alone.

Currently it is not clear whether over suppression of the HPG axis would do more harm than good in terms of growth, since this issue is not specifically addressed. Moreover it is not known whether such an oversuppression, even if it decreased growth velocity, would also affect final height adversely. Unfortunately, long-term results of high dose LA (7.5 mg/28 ds or higher) are scarce, and no study has compared long term height gain with low versus high dose LA. Extensive suppression of growth may be unwanted. Thus studies are required to specifically address these issues.

Puberty is the critical period for bone development and accrual of peak bone mass (34). Approximately half of peak bone mass is acquired during puberty (35). Postmenopausal decrease in BMD, as well as reduction of BMD in premenopausal adults using GnRHa treatment is attributed to hypo-estrogenism. GnRHa therapy for CPP is also suggested to create a hypo-estrogenic condition which may have a negative impact on bone mass (35). There are contradictory reports to that effect in children. Some studies report a decrease in BMD in children using GnRHa therapy, whereas in others no difference was shown during treatment (36,37). Most studies were carried out using 3.75 mg/28 days of LA. Oversuppression of HPG axis with 7.5 mg/28 days or higher doses may have a greater negative impact on accrual of bone mineral. There is a need for long-term, randomized trials investigating the impact of high dose of LA on bone health in children with CPP.

Another disadvantage of unnecessary high-dose LA treatment is that it is costly. Healthcare costs have been increasing globally in the last decades, and there is an increasing pressure worldwide to reduce costs and improve efficiency, while maintaining quality. Thus expensive treatments without added benefit to health is an issue of consideration.

The present study had several advantages in terms of its sample size and choice of patient population. It also provided an analysis of factors that may affect the suppressive dose of LA in a large sample of 220 girls with iCPP, and provides a strategy based on body Wt in the choice of the initial dose of LA for pubertal suppression. Another advantage was the use of the gold standard GnRH test to assess pubertal suppression.

### Study Limitations

Dose titration was not carried out in this study. LA was used at a dose of 3.75 mg initially and 7.5 mg subsequently in those with inadequate suppression. This approach does not provide information on minimum effective dose for successful suppression.

## Conclusion

Monthly injections of LA (3.75 mg/28 days) was an effective treatment in terms of HPG axis suppression in the majority of girls with iCPP. This treatment option was also more cost-effective than an initial high dose of 7.5 mg/28 days dose. A higher initial dose may be preferred in patients with a body Wt ≥36 kg or BMI-SDS ≥1.6 for effective suppression of the HPG axis although these patients would require closer clinical follow-up. Further studies comparing long term impact of different doses of GnRHa on growth and bone health are required.

### Ethics

**Ethics Committee Approval:** The study were approved by the Ethics Committee of Hacettepe University (approval number: GO 19/453-41).

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

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

### Authorship Contributions

Surgical and Medical Practices: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, Concept: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, Design: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, Data Collection or Processing: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, İrem İyigün, Dicle Canoruç, Alev Ozon, Analysis or Interpretation: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, Nazlı Gönç, Alev Ozon, Literature Search: Doğuş Vurallı, Ayfer Alikaşifoğlu, Nurgün Kandemir, Nazlı Gönç, Alev Ozon, Writing: Doğuş Vurallı, Ayfer Alikaşifoğlu, İrem İyigün, Dicle Canoruç, Alev Ozon, Nazlı Gönç, Nurgün Kandemir.

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# The Significance of Thiol/Disulfide Homeostasis and Ischemia-modified Albumin Levels in Assessing Oxidative Stress in Obese Children and Adolescents

**Eda Mengen[1], Seyit Ahmet Uçaktürk[1], Pınar Kocaay[1], Özlem Kaymaz[2], Salim Neşelioğlu[3], Özcan Erel[3]**

*[1]Ankara City Hospital, Children's Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey*
*[2]Ankara University Faculty of Medicine, Depatment of Statistics, Ankara, Turkey*
*[3]Yıldırım Beyazıt University Faculty of Medicine, Department of Biochemistry, Ankara, Turkey*

### What is already known on this topic?

Although different mechanisms are proposed for the pathogenesis of complications associated with obesity, the most widely accepted hypothesis is that adipose tissue inflammation plays a critical role.

### What this study adds?

Chronic inflammation due to oxidative stress induced by impaired metabolic parameters in metabolically unhealthy obese (MUO) children caused impairment in thiol redox homeostasis. Our data suggested that the degree of oxidant imbalance in obese children worsened as obesity and metabolic abnormalities increased. It is hypothesized that thiol/disulfide homeostasis and high serum ischemia-modified albumin levels may be reliable indicators of oxidant-antioxidant status in MUO children.

## Abstract

**Objective:** There is an association between obesity and several inflammatory and oxidative markers in children. In this study, we analyzed thiol/disulfide homeostasis and serum ischemia-modified albumin (IMA) levels for the first time in order to clarify and determine the oxidant/antioxidant balance in metabolically healthy and unhealthy children.

**Methods:** This study included obese children and healthy volunteers between 4-18 years of age. The obese patients were divided into two groups: metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO). Biochemical parameters including thiol/disulfide homeostasis, and IMA concentrations were analyzed.

**Results:** There were 301 recruits of whom 168 (55.8%) were females. The obese children numbered 196 (MHO n = 58 and MUO n = 138) and healthy controls numbered 105. No statistically significant difference could be found in ages and genders of the patients among all groups ($p > 0.05$, for all). Native thiol (SH), total thiol (SH + SS), and native thiol/total thiol (SH/SH + SS) ratio were statistically significantly lower in the MUO group than the control group ($p < 0.001$, $p = 0.005$, and $p = 0.005$; respectively). Disulfide (SS), disulfide/native thiol (SS/SH), disulfide/total thiol (SS/SH + SS) and IMA levels were statistically significantly higher in the MUO group than the control group ($p = 0.002$, $p < 0.001$, $p < 0.001$, and $p = 0.001$, respectively).

**Conclusion:** Chronic inflammation due to oxidative stress induced by impaired metabolic parameters in MUO children caused impairment in thiol redox homeostasis. Our data suggested that the degree of oxidant imbalance in obese children worsened as obesity and metabolic abnormalities increased. It is hypothesized that thiol/disulfide homeostasis and high serum IMA levels may be reliable indicators of oxidant-antioxidant status in MUO children.

**Keywords:** Obesity, children and adolescents, thiol/disulfide homeostasis, ischemia-modified albumin

**Address for Correspondence:** Eda Mengen MD, Ankara City Hospital, Children's Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey
**Phone:** +90 312 596 96 46 **E-mail:** drmengen@hotmail.com **ORCID:** orcid.org/0000-0003-1597-8418

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## Introduction

Childhood obesity is one of the most important health problems of the 21st century (1). This is a global problem and continuously affects urban populations of many low and middle-income families and the prevalence is increasing at an alarming rate. A recent study proposed that 1.48 billion adults in the world are overweight, 502 million adults are obese, and 180 million children are overweight or obese (2). There has been a dramatic increase in the prevalence of overweight and obesity in the adult Turkish population, reaching figures as high as 30-40% (3).

Although different mechanisms are proposed for the pathogenesis of complications associated with obesity, the most widely accepted hypothesis is that adipose tissue inflammation plays a critical role, and oxidative stress (OS) appears in obese individuals (4). OS is the loss of the normal homeostatic balance between reactive oxygen species (ROS) and antioxidant substances. OS is toxic to cells by causing membrane lipid peroxidation and membrane damage (5). Thiols are important antioxidants and play a role in non-enzymatic elimination of ROS. Thiol/disulfide homeostasis is necessary for some detoxification mechanisms. Previous studies have reported parameters of thiol/disulfide homeostasis which include native thiol, total thiol, and disulfide concentrations and disulfide/native thiol, native thiol/total thiol, and disulfide/total thiol ratios (6,7,8,9,10,11,12,13). Dynamic thiol/disulfide homeostasis plays a key role in antioxidant protection, detoxification, signal transduction, apoptosis, regulation of enzymatic activity, the function of some transcription factors and some cellular signalling mechanisms (14,15). Moreover, dynamic thiol/disulfide homeostasis has been implicated in the pathogenesis of many disorders (16,17,18,19,20,21, 22,23,24,25).

Ischemia-modified albumin (IMA) is produced through the modification of albumin by ROS produced as a result of ischemic episodes (26). High IMA concentrations have been used to predict cardiovascular risk in obese children and to evaluate subclinical vascular disease in patients with diabetes mellitus (26,27).

The aim of this study was to evaluate antioxidant status in obese children with a focus on both markers of thiol/disulfide homeostasis and IMA. This is the first study to measure both thiol/disulfide homeostasis and serum IMA concentrations in metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) children in order to clarify and determine their roles in oxidant/antioxidant balance. In addition, the effect of obesity in metabolically unhealthy children on biomarkers of OS was investigated.

## Methods

### Study Design and Patient Selection

This case-control study was conducted in Ankara Pediatric Hematology and Oncology Training and Research Hospital between May-2018 and July 2018 and included obese children and healthy controls aged between 4-18 years. Exclusion criteria for obese patients were the presence of any hepatic, renal, cardiac, autoimmune, infectious, musculoskeletal or malignant diseases, taking any vitamin supplementation, or drug use that might lead to obesity and the presence of any chromosomal, endocrine or genetic syndromes. The control group included 105 healthy children without any known chronic or acute disease. The control group consisted of sex- and age-matched healthy subjects who were of normal weight for age. In addition, none of the control group had insulin resistance (IR), impaired fasting glucose, dyslipidemia or hypertension.

Obese patients were divided into two groups. Subjects who did not have dyslipidemia, impaired fasting glucose, IR, hepatosteatosis or hypertension were accepted as MHO and those who had at least one of these conditions were accepted as MUO, as previously described (28). Clinical and laboratory findings of the obese and control groups were compared.

Weight measurements were performed with the subjects dressed in thin clothes and without shoes, using an electronic weighing device with the SECA 274 Stadiometer (Hamburg, Germany) with 1 mm accuracy. Height measurements were performed with the Ayrton® Stadiometer (5322 Frost Point Prior Lake, MN 55372, USA), sensitive to a 0.1 cm difference in an upright position with bare feet. Body mass index (BMI) was calculated with the standard formula: [weight (kg) / height$^2$ (m$^2$)]. For statistical evaluation, BMI-standard deviation score (BMI-SDS) was used. Patients whose BMI-SDS were $>2$ were accepted as obese (29). The BMI-SDS values were calculated using the reference values developed by Neyzi et al (30).

Routine physical examinations were performed in all obese subjects and controls. Puberty was assessed in all obese subjects and controls by Tanner staging. In girls, stage 2 breast development and in boys 4 mL testis volume were accepted as indicating the start of puberty (31,32).

Blood pressure measurement was performed from the right arm in the sitting position after 15 minutes of resting, using a mercury sphygmomanometer (ERKA, Germany). If the blood pressure was above the 95th percentile according to age, gender and height, two more measurements were

obtained. Hypertension was accepted if two of the three measurements were at or above the 95th percentile (33).

### Laboratory Analysis

Blood samples were obtained after 8-10 hours of fasting. Fasting plasma glucose (FPG), fasting plasma insulin, alanine aminotransferase, aspartate aminotransferase, total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured. Low-density lipoprotein cholesterol (LDL-C) concentrations were measured using the Friedewald formula (34). Serum glucose and lipid profile measurements were performed using the Roche modular system/Integra 800 device and kit (Mannheim, Germany). FPG between 100-125 mg/dL was accepted as "impaired fasting glucose" (35). A TC concentration of ≥200 mg/dL, TG ≥150 mg/dL, HDL-C ≤35 mg/dL and LDL-C ≥100 mg/dL was accepted as dyslipidemia (36).

Fasting insulin, thyroid-stimulating hormone (TSH) and free T4 (fT4) concentrations were measured using enzymatic immunoassay method using a Beckman Coulter DXI 800 device (Brea, CA, USA). Reference values for Beckman Coulter TSH and fT4 kits used in our hospital were 0.7-5.69 μIU/mL for TSH and 0.65-1.06 ng/dL for fT4. IR was calculated using the Homeostasis Model Assessment of fasting IR (HOMA-IR) method with the following formula: FPG (mmol/L) x fasting insulin (mIU/mL)/22.5 (37). The HOMA-IR cut-off value for prepubertal subjects was taken as 2.5 and for pubertal subjects as 4 (38).

Hepatosteatosis was evaluated in our radiology clinic with upper abdominal ultrasonography using a Toshiba Xaria I Style Ultrasound device (Tokyo, Japan). Liver ultrasound findings were staged as follows: normal liver appearance (no hepatosteatosis), mild (stage 1), moderate (stage 2) and severe hepatosteatosis (stage 3) (39).

### Measurement of Serum Ischemia-modified Albumin

Blood samples were obtained using anticoagulant-free tubes and centrifuged for five minutes at 3500 rpm. Serum aliquots for measuring IMA blood concentrations were pipetted into Eppendorf tubes and stored at -80 °C until testing. Serum IMA concentrations were measured by a colorimetric method described by Bar-Or et al (40) and results were reported as absorbance units (ABSU).

### Measurement of Thiol/Disulfide Homeostasis Parameters

Blood samples were obtained between 8 a.m. and 10 a.m. after 8-10 hours fasting. The samples were then centrifuged at 1500 rpm for 10 minutes. Separated serum samples were immediately frozen and stored at -80 °C until analyzed. All thiol/disulfide parameters were studied in the same samples. Serum concentrations of native and total thiol and ratios of disulfide, and native and total thiol were determined by a spectrophotometric method using an automatic clinical chemical analyzer (Roche, Cobas 501, Mannheim, Germany) as previously described by Erel and Neselioglu (41).

### Ethical Statements

The study was approved by Ankara Children's Hematology Oncology Training and Research Hospital's Ethics Committee (approval number: 2018-70). The study was performed in accordance with the ethical rules based on the principles of the Helsinki Declaration. Written informed consent forms were obtained (when appropriate) from the parents and the children.

### Statistical Analysis

A sample size calculation was performed considering detection of 0.20 effect size, $\alpha = 0.05$ and a power of 88.0% using variance analysis (one-way ANOVA). The result of the power analysis showed that the minimum number of patients required was 303. Data obtained from this study were analyzed using Statistical Package for Social Sciences for Windows, version 23.0 (IBM Inc., Armonk, NY, USA) (42). Frequency distributions and percentages were given for categorical variables. For continuous variables, assumption of normality was tested by visual (histogram and probability plots) and analytic methods (Kolmogorov-Smirnov/Shapiro-Wilk test). Descriptive statistics were presented as mean ± standard deviation or median and interquartile ranges for continuous variables as appropriate. Equality of variances was controlled with the Levene test. One-way ANOVA was used to measure the difference among three groups if parametric test conditions were met, and the Bonferroni test among *post hoc* tests was used to make binary comparisons. Kruskal-Wallis test was used when parametric test conditions were not met. Student's t-test was used to determine whether a difference existed between two groups when parametric test conditions were met and Mann-Whitney U tests was used when conditions were not met. Chi-square ($\chi^2$) test was used for the analysis of categorical variables. For the MUO group, cut-off values of native thiol, total thiol, disulfide, disulfide/native thiol, disulfide/total thiol, native thiol/total thiol and IMA concentration were determined by using Receiver Operating Characteristic (ROC) curve analysis. Significance level of the tests was accepted to be $p < 0.05$.

## Results

This study included 301 children, of whom 168 (55.8%) were female. Within the group, 138 (45.85%) were MUO, 58 (19.3%) were MHO and there were 105 (34.9%) healthy volunteers. The obese group consisted of 70.4% MUO and 29.6% MHO. No statistically significant difference could be found in ages and genders of the patients among all groups ($p > 0.05$, for all). BMI-SDS, HOMA-IR, and concentrations of glucose, insulin, TC, LDL-C and TSH were higher in the obese children than in the controls. The demographic, clinical and laboratory characteristics of the participants are displayed in Table 1.

Obese patients had lower native thiol (SH) and total thiol (SH + SS) concentrations, and a lower native thiol/total thiol (SH/SH + SS) ratio than controls ($p < 0.001$, $p = 0.002$ and $p = 0.013$, respectively). In addition obese patients had increased disulfide (SS) concentrations and higher disulfide/native thiol (SS/SH) and disulfide/total thiol (SS/SH + SS) ratios than the controls ($p = 0.005$, $p < 0.001$ and $p < 0.001$, respectively). Moreover, serum IMA concentration was significantly higher in the obese group ($p < 0.001$). Thiol/disulfide homeostasis parameters and comparison of IMA concentrations between the control and the obese groups are given in Table 2.

Native thiol (SH) and total thiol (SH + SS) concentrations and native thiol/total thiol (SH/SH + SS) ratio were statistically significantly lower in the MUO group than the control group ($p < 0.001$, $p = 0.005$ and $p = 0.005$, respectively). Disulfide (SS) and IMA concentrations and disulfide/native thiol (SS/SH) and disulfide/total thiol (SS/SH + SS) ratios were statistically significantly elevated in the MUO group compared with the control group ($p = 0.002$, $p < 0.001$, $p < 0.001$ and $p = 0.001$, respectively). Comparison of dynamic thiol/disulfide homeostasis parameters and IMA concentrations among the groups are given in Table 3.

All patients with a BMI-SDS > 3 were categorized as a subgroup and were investigated separately for the study parameters. This subgroup included 14 patients from the MHO and 24 patients from the MUO groups. Interestingly, all parameters were similar in both groups ($p > 0.05$, for all; Table 4).

ROC curve analysis was performed for the MUO group for native thiol, total thiol, disulfide, disulfide/native thiol, disulfide/total thiol, native thiol/total thiol and IMA. The values for sensitivity and specificity are shown in Table 5.

## Discussion

Evidence of OS due to obesity in adults and more recently, evidence in children, has appeared over the last few years (43). Obesity creates pro-oxidant conditions that promote the development of comorbid diseases. Energy imbalance leads to storage of excess energy in adipocytes, resulting in both hypertrophy and hyperplasia. These processes are related to abnormalities of adipocyte function, especially mitochondrial stress and impaired endoplasmic reticulum function (44,45). OS can also be induced by adipocyte-associated inflammatory macrophages (46). There is a close association between obesity, chronic low-level inflammation and OS. In addition, the dysfunction of adipocytokines secreted by adipose tissue and induced by OS, act synergistically in metabolic abnormalities associated with obesity. Evaluation of oxidative status has been reported to aid in identification of patients with high risk of complications (43).

Thiol-disulfide balance has vital importance and the new method developed by Erel and Neselioglu (41) is capable of measuring both separate variables and providing an overall picture of thiol-disulfide balance while allowing both individual and integral evaluations. Until now, many studies have evaluated oxidant-antioxidant status and reported various results for obese children. However, to the best of our knowledge, no previous study has reported thiol/disulfide homeostasis in MUO and MHO children. Elmas et al (47) first evaluated thiol/disulfide homeostasis in obese children, reporting that antioxidant parameter levels were low in obese patients, while pro-oxidant parameters were elevated. In contrast to Elmas et al (47) we measured thiol/disulfide homeostasis in MHO and MUO children for the first time in order to investigate differences in oxidant/antioxidant balance between these two different groups of obese children.

In our study, the concentrations of native thiol and total thiol and native thiol/total thiol ratio were lower while disulfide concentration and disulfide/native thiol and disulfide/total thiol ratios were higher in obese children than the healthy control group. This suggests a shift in thiol/disulfide homeostasis towards disulfide production. Oxidant parameters were high and anti-oxidant parameters were low in obese children. When obese children were divided into MUO and MHO groups, parameters of OS in the MUO group were higher compared to the healthy control group. Interestingly, there was also no difference between the MUO and MHO groups. Development of chronic inflammation due to OS indicated by metabolically impaired parameters in obese children has been shown to lead to disruption

**Table 1. Demographic, clinical and laboratory characteristics of participants**

| | Control group (n = 105) | | MUO group (n = 138) | | MHO group (n = 58) | | p value | p value[a] | p value[b] | p value[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) | | | | |
| Age (years) | 11.62 ± 3.13 | 11.60 (4.50) | 12.80 ± 2.81 | 13.11(3.89) | 11.99 ± 3.30 | 11.90 (5.33) | 0.609 | | | |
| Sex (female) | 57 (54.3 %) | | 77 (55.8 %) | | 34 (58.6 %) | | 0.867** | | | |
| BMI-SDS | 0.24 ± 0.78 | 0.30 (1.54) | 2.59 ± 0.55 | 2.45 (0.77) | 2.42 ± 0.57 | 2.15 (0.57) | < 0.001* | < 0.001[a] | < 0.001[b] | 0.284[c] |
| Fasting blood glucose (mg/dL) | 88.03 ± 6.62 | 90 (10) | 98.01 ± 8.00 | 98 (11) | 92.67 ± 4.79 | 93 (7) | < 0.001* | 0.001[a] | < 0.001[b] | < 0.001[c] |
| Insulin (mcIU/mL) | 6.83 ± 3.13 | 6.30 (5.01) | 16.45 ± 9.54 | 13.74 (10.04) | 9.55 ± 3.38 | 9.08 (5.13) | < 0.001* | < 0.001 a | 0.049[b] | < 0.001[c] |
| HOMA-IR | 1.43 ± 0.71 | 1.27 (1.08) | 4.14 ± 2.71 | 3.53 (2.47) | 2.24 ± 0.83 | 2.13 (1.22) | < 0.001* | < 0.001 a | 0.030[b] | < 0.001[c] |
| TC (mg/dL) | 143.09 ± 25.86 | 139 (43) | 171.80 ± 34.56 | 171 (48.5) | 156.90 ± 22.46 | 157 (35) | < 0.001* | < 0.001 a | 0.014[b] | 0.004[c] |
| TG (mg/dL) | 94.26 ± 27.29 | 79 (49) | 140.48 ± 69.09 | 122 (87.75) | 93.16 ± 22.78 | 93.5 (29.25) | < 0.001* | < 0.001[a] | 0.99[b] | < 0.001[c] |
| HDL-C (mg/dL) | 52.43 ± 9.15 | 47 (7) | 46.28 ± 9.89 | 44.5 (12.25) | 51.28 ± 9.40 | 49 (12.25) | < 0.001* | < 0.01[a] | 0.99[b] | 0.001[c] |
| LDL-C (mg/dL) | 72.81 ± 17.35 | 74.20 (27.80) | 98.76 ± 26.62 | 95.90 (32.75) | 83.90 ± 14.76 | 87.70 (22.08) | < 0.001* | < 0.001[a] | 0.006[b] | < 0.001[c] |
| ALT (U/L) | 15.80 ± 6.08 | 13 (7) | 24.38 ± 16.09 | 18.50 (15) | 17.74 ± 12.49 | 16 (7.25) | < 0.001* | < 0.001[a] | 0.99[b] | 0.003[c] |
| TSH (µIU/mL) | 2.54 ± 1.04 | 2.50 (1.40) | 3.15 ± 1.71 | 2.70 (1.77) | 3.35 ± 2.11 | 2.58 (2.22) | 0.002 | 0.011[a] | 0.007[b] | 0.99[c] |
| fT4 (ng/dL) | 0.89 ± 0.12 | 0.89 (0.15) | 1.52 ± 7.59 | 0.87 (0.16) | 0.88 ± 0.12 | 0.88 (0.17) | 0.564 | | | |
| SBP (mmHg) | 102.57 ± 10.77 | 100 (20) | 111.77 ± 17.62 | 110 (20) | 102.53 ± 16.49 | 100 (20) | < 0.001* | < 0.001[a] | 0.99[b] | < 0.001[c] |
| DBP (mmHg) | 63.86 ± 10.61 | 60 (15) | 73.22 ± 14.16 | 70 (20) | 65.26 ± 9.62 | 65 (10) | < 0.001* | < 0.001[a] | 0.99[b] | < 0.001[c] |

*Significance in analysis of variance (comparison among three groups). **chi-square test.

[a]Significance between control group and MUO group (pairwise comparison).

[b]Significance between control group and MHO group (pairwise comparison.

[c]Significance between MUO and MHO groups (pairwise comparison).

SD: standard deviation, IQR: interquartile range, MUO: metabolically unhealthy obese, MHO: metabolically healthy obese, BMI-SDS: body mass index-standard deviation score, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, TG: triglyceride, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, ALT: alanine aminotransferase, TSH: thyroid-stimulating hormone, fT4: free T4, SBP: systolic blood pressure, DBP: diastolic blood pressure

of thiol redox homeostasis. Our data suggest that the increased pro-oxidant status in obese children worsens with metabolic abnormalities.

Many previous studies have found increased pro-oxidant status in obese individuals, similar to our study. Vehapoğlu et al (48) found that antioxidant capacity was significantly lower in prepubertal obese children. Karamouzis et al (49) demonstrated that loss of normal oxidant-antioxidant homeostatic balance led to increased OS with decreased antioxidant capacity in obese prepubertal and adolescent girls. Paltoglou et al (50) found that childhood obesity was associated with aseptic inflammation and OS. In another study investigating the changes in the oxidant/antioxidant homeostasis in obese children with and without metabolic syndrome, a significant impairment in the oxidant/antioxidant balance was reported in those with metabolic syndrome (51). Another study also showed that children were more susceptible to OS than adults and the authors suggested that this was probably due to the incomplete development of the antioxidant system (52). The results of our study were consistent with those reported in studies on obesity and excessive OS. We suggest that thiol/disulfide homeostasis in MUO children may be a reliable indicator of oxidant-antioxidant status.

An increased degree of obesity has been shown to predict increased metabolic risk in obese children and adolescents (53). When compared to their moderately obese peers, the severely obese are at greater risk for adult obesity, early atherosclerosis, hypertension, type 2 diabetes,

**Table 2. Thiol/disulfide homeostasis parameters and comparison of ischemia-modified albumin between the control group and all obese patients**

| | Control group (n = 105) | | Obese group (n = 196) | | p value |
|---|---|---|---|---|---|
| | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) | |
| Native thiol (μmol/L) | 455.43 ± 34.60 | 450.40 (42.55) | 437.98 ± 41.97 | 442.65 (56.93) | < 0.001 |
| Total thiol (μmol/L) | 492.48 ± 36.77 | 492.00 (48.80) | 477.29 ± 43.34 | 480.85 (62.95) | 0.002 |
| Disulphide (μmol/L) | 17.69 ± 6.23 | 18.05 (11.63) | 19.65 ± 4.50 | 19.59 (6.05) | 0.005 |
| Disulphide/native thiol, % | 3.92 ± 1.44 | 3.83 (2.52) | 4.52 ± 1.11 | 4.51 (1.52) | < 0.001 |
| Disulphide/total thiol, % | 3.61 ± 1.28 | 3.71 (2.17) | 4.13 ± 0.93 | 4.14 (1.28) | < 0.001 |
| Native thiol/total thiol, % | 92.50 ± 2.77 | 92.35 (3.73) | 91.74 ± 1.86 | 91.73 (2.56) | 0.013 |
| IMA (ABSU) | 0.57 ± 0.06 | 0.56 (0.07) | 0.61 ± 0.11 | 0.62 (0.12) | < 0.001 |

Significance between control and obese group (Student's t-test). IQR: interquartile range, ABSU: absorbance units, IMA: ischemia-modified albumin, SD: standard deviation

metabolic syndrome, fatty liver disease and premature death (54). In our study, there was no differences in thiol/disulfide homeostasis parameters and IMA between the MUO and MHO children with >3 BMI-SDS. This result may be significant for metabolic risk that may develop in metabolically healthy but seriously obese children and adolescents. It would be necessary to undertake longer term studies of the seriously obese but metabolically healthy children in order to determine if they will progress to the unhealthy group and what the natural history of this progression in terms of thiol/disulfide parameters might be.

Many previous studies assessed serum IMA levels in adult obese patients and found correlations between some anthropometric and laboratory measurements. Piva et al (55) and Kazanis et al (56) reported that serum IMA concentration was significantly elevated in obese adults and overweight/obese postmenopausal women and this was associated with OS. In addition, both studies found an association between serum IMA concentration and BMI. Baysal et al (26) investigated studied serum IMA in obese children for the first time and found higher levels in children with metabolic syndrome. Similarly, in our study, serum IMA concentration was higher in the obese group than the control group although we could not detect a difference between the MUO and MHO groups. We found positive correlations between serum IMA concentration and BMI, fasting blood glucose, insulin, and HOMA-IR levels.

The IMA variable has the highest AUC value of the parameters examined for predicting MUO and at a cut-off value of 0.665 ABSU the sensitivity and specificity were 74% and 66%, respectively. Both native thiol concentration and total thiol values had high specificity at the identified cut-offs (439.2 μmol/L and 477.5 μmol/L, respectively) suggesting that they are likely to differentiate between MUO and healthy individuals. The sensitivity and specificity of the disulfide parameter, relative to the cut-off value (23.18 μmol/L), were 79% and 43%, respectively. Here, the sensitivity was good suggesting that it may be useful in distinguishing between high values and possibly unhealthy patients.

### Study Limitations

An important limitation is that this study is cross-sectional. Furthermore, thiol/disulphide parameters were not compared with other enzymatic and non-enzymatic OS parameters.

## Conclusion

In this study, we showed that thiol/disulfide homeostasis, one of the important parameters of OS, is impaired in MUO children. Our data prove that the increased oxidant status in obese children is related to the metabolic abnormality. Measurable increases in OS may be the basis of obesity-related comorbidities. Reducing chronic inflammation and OS levels in childhood can prevent subsequent metabolic disorder as well as increased cardiovascular morbidity and mortality in adulthood. Our study provides an idea about these issues; however, future in-depth studies are warranted.

**Table 3. Comparison of dynamic thiol/disulfide homeostasis and ischemia-modified albumin between the control, metabolically unhealthy obese and metabolically healthy obese groups**

| | Control group (n = 105) | | MUO group (n = 138) | | MHO group (n = 58) | | p value | p value[a] | p value[b] | p value[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) | | | | |
| Native thiol (µmol/L) | 455.43 ± 34.60 | 450.40 (42.55) | 435.45 ± 41.20 | 440.75 (56.15) | 444.01 ± 43.52 | 449.70 (58.68) | 0.001* | < 0.001[a] | 0,235[b] | 0.500[c] |
| Total thiol (µmol/L) | 492.48 ± 36.77 | 492.00 (48.80) | 475.39 ± 43.15 | 478.80 (59.78) | 481.82 ± 43.86 | 485.35 (55.75) | 0.006* | 0.005[a] | 0,344[b] | 0.959[c] |
| Disulphide, (µmol/L) | 17.69 ± 6.23 | 18.05 (11.63) | 19.97 ± 4.52 | 20.16 (5.87) | 18.90 ± 4.42 | 18.19 (5.54) | 0.003* | 0.002[a] | 0.460[b] | 0.560[c] |
| Disulphide/native thiol, % | 3.92 ± 1.44 | 3.83 (2.52) | 4.62 ± 1.10 | 4.55 (1.41) | 4.30 ± 1.11 | 4.29 (1.69) | < 0.001* | < 0.001[a] | 0.165[b] | 0.317[c] |
| Disulphide/total thiol, % | 3.61 ± 1.28 | 3.71 (2.17) | 4.21 ± 0.92 | 4.17 (1.18) | 3.95 ± 0.93 | 3.95 (1.44) | < 0.001* | < 0.001[a] | 0.162[b] | 0.340[c] |
| Native thiol/total thiol, % | 92.50 ± 2.77 | 92.35 (3.73) | 91.58 ± 1.84 | 91.66 (2.37) | 92.11 ± 1.87 | 92.10 (2.88) | 0.006* | 0.005[a] | 0.855[b] | 0.386[c] |
| IMA (ABSU) | 0.57 ± 0.06 | 0.56(0.07) | 0.61 ± 0.12 | 0.62 (0.14) | 0.61 ± 0.09 | 0.63 (0.11) | 0.001* | 0.001[a] | 0.021[b] | 0.99[c] |

*Significance in analysis of variance (comparison among three groups).

[a]Significance between control group and MUO group (pairwise comparison).

[b]Significance between control group and MHO group (pairwise comparison).

[c]Significance between MUO group and MHO group (pairwise comparison).

IQR: interquartile range, IMA: ischemia-modified albumin, ABSU: absorbance units, MUO: metabolically unhealthy obese, MHO: metabolically healthy obese, SD: standard deviation

**Table 4. Comparison of dynamic thiol/disulfide homeostasis and ischemia-modified albumin between subgroups with body mass index-standard deviation score > 3 from both the metabolically healthy obese and metabolically unhealthy obese groups**

| | MHO group (BMI-SDS > 3) (n = 14) | MUO group (BMI-SDS > 3) (n = 24) | p value |
|---|---|---|---|
| Native thiol (µmol/L) | 420.08 ± 41.70 | 417.34 ± 33.25 | 0.825 |
| Total thiol (µmol/L) | 461.19 ± 43.29 | 455.68 ± 35.66 | 0.673 |
| Disulphide (µmol/L) | 20.55 ± 4.45 | 19.17 ± 4.26 | 0.356 |
| Disulphide/native thiol, % | 4.93 ± 1.12 | 4.60 ± 1.03 | 0.371 |
| Disulphide/total thiol, % | 4.47 ± 0.93 | 4.20 ± 0.86 | 0.376 |
| Native thiol/total thiol, % | 91.06 ± 1.86 | 91.60 ± 1.72 | 0.376 |
| IMA (ABSU) | 0.61 ± 0.85 | 0.64 ± 0.10 | 0.299 |

Significance between MHO (BMI-SDS > 3) and MUO (BMI-SDS > 3) group (Student's t-test).

IMA: ischemia-modified albumin, ABSU: absorbance units, MHO: metabolically healthy obese, MUO: metabolically unhealthy obese, BMI-SDS: body mass index-standard deviation score

**Table 5. The cut-off values of parameters in predicting metabolically unhealthy obese group**

| | Native thiol | Total thiol | Disulfide | Disulfide/ Native thiol (%) | Disulfide/ Total thiol (%) | Native thiol/ Total thiol (%) | IMA |
|---|---|---|---|---|---|---|---|
| **Cut-off value** | 439.2[L] | 477.5[L] | 23.185[S] | 5.1025[S] | 4.63[S] | 90.7395[L] | 0.665[S] |
| **Sensitivity (%)** | 51 | 52 | 79 | 71 | 71 | 71 | 74 |
| **Specificity (%)** | 74 | 71 | 43 | 46 | 46 | 46 | 66 |
| **AUC (95% CI)** | 0.611 [0.54;0.68] | 0.590 [0.52;0.66] | 0.561 [0.49;0.64] | 0.582 [0.51;0.66] | 0.582 [0.51;0.66] | 0.582 [0.51;0.66] | 0.719 [0.66;0.78] |
| **p** | 0.003 | 0.016 | 0.103 | 0.029 | 0.029 | 0.029 | < 0.001 |

[S]Smaller test result indicates more positive test, [L]Larger test result indicates more positive test.

AUC: Area under curve, CI: confidence interval, IMA: ischemia-modified albumin

### Ethics

**Ethics Committee Approval:** The study was approved by Ankara Children's Hematology Oncology Training and Research Hospital's Ethics Committee (approval number: 2018-70).

**Informed Consent:** Written informed consent forms were obtained (when appropriate) from the parents and the children.

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

### Authorship Contributions

Surgical and Medical Practices: Eda Mengen, Seyit Ahmet Uçaktürk, Pınar Kocaay, Concept: Eda Mengen, Seyit Ahmet Uçaktürk, Pınar Kocaay, Design: Eda Mengen, Seyit Ahmet Uçaktürk, Pınar Kocaay, Data Collection or Processing: Eda Mengen, Seyit Ahmet Uçaktürk, Pınar Kocaay, Analysis or Interpretation: Özlem Kaymaz, Salim Neşelioğlu, Özcan Erel, Literature Search: Eda Mengen, Seyit Ahmet Uçaktürk, Pınar Kocaay, Salim Neşelioğlu, Özcan Erel, Writing: Eda Mengen.

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# Prevalence of Obesity and Metabolic Syndrome in Children with Type 1 Diabetes: A Comparative Assessment Based on Criteria Established by the International Diabetes Federation, World Health Organisation and National Cholesterol Education Program

**Özlem Yayıcı Köken[1], Cengiz Kara[2], Gülay Can Yılmaz[3], Hasan Murat Aydın[4]**

*[1]University of Health Sciences Turkey, Dr. Sami Ulus Training and Research Hospital, Clinic of Pediatric Neurology, Ankara, Turkey*
*[2]İstinye University Faculty of Medicine, Department of Pediatrics, İstanbul, Turkey*
*[3]Mardin State Hospital, Clinic of Pediatric Endocrinology, Mardin, Turkey*
*[4]Ondokuz Mayıs University Faculty of Medicine, Department of Pediatric Endocrinology, Samsun, Turkey*

### What is already known on this topic?

Some studies have reported the prevalence of obesity and metabolic syndrome (MetS) in pediatric patients with type 1 diabetes (T1D). There are no such data from Turkey. In T1D patients diagnosis of MetS at early ages is critical to manage and prevent macrovascular complications. Nevertheless, identifying the presence of MetS in T1D is difficult and it is not clear which criteria are most suitable for accurate identification.

### What this study adds?

The prevalence of obesity and MetS in children with T1D in our region is reported. MetS prevalence was 10.5%, 8.5% and 13.5% according to International Diabetes Federation (IDF), World Health Organisation (WHO) and National Cholesterol Education Program (NCEP) criteria, respectively. Also, this study comparatively assesses the widely accepted and used diagnostic criteria for MetS established by IDF, WHO and NCEP. Using IDF criteria seems more suitable because obesity is a prerequisite and they include accepted criteria for childhood.

## Abstract

**Objective:** To determine the prevalence of obesity and metabolic syndrome (MetS) in children and adolescents with type 1 diabetes (T1D) and to compare the widely accepted and used diagnostic criteria for MetS established by the International Diabetes Federation (IDF), World Health Organisation (WHO) and National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATPIII).

**Methods:** We conducted a descriptive, cross sectional study including T1D patients between 8-18 years of age. The three sets of criteria were used to determine the prevalence of MetS and findings compared. Risk factors related to MetS were extracted from hospital records.

**Results:** The study included 200 patients with T1D (52% boys). Of these, 18% (n=36) were overweight/obese (body mass index percentile ≥85%). MetS prevalence was 10.5%, 8.5% and 13.5% according to IDF, WHO and NCEP criteria, respectively. There were no statistically significant differences in age, gender, family history of T1D and T2D, pubertal stage, duration of diabetes, hemoglobin A1c levels and daily insulin doses between patients with or without MetS. In the overweight or obese T1D patients, the prevalence of MetS was 44.4%, 38.8% and 44.4% according to IDF, WHO and NCEP-ATPIII criteria, respectively.

**Conclusion:** Obesity prevalence in the T1D cohort was similar to that of the healthy population of the same age. Prevalence of MetS was higher in children and adolescents with T1D compared to the obese population in Turkey. The WHO criteria include microvascular complications which are rare in childhood and the NCEP criteria do not include a primary criterion while diagnosing non-obese patients according to waist circumference as MetS because the existence of diabetes is considered as a direct criterion. Our study suggests that IDF criteria which allows the diagnosis of MetS with obesity and have accepted criteria for the childhood are more suitable for the diagnosis of MetS in children and adolescents with T1D.

**Keywords:** Type 1 diabetes, metabolic syndrome, double diabetes, prevalence

**Address for Correspondence:** Özlem Yayıcı Köken MD, University of Health Sciences Turkey, Dr. Sami Ulus Training and Research Hospital, Department of Pediatric Neurology, Ankara, Turkey
**Phone:** +90 530 762 42 00 **E-mail:** drozlemkoken@gmail.com **ORCID:** orcid.org/0000-0003-2112-8284

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## Introduction

Type 1 diabetes (T1D) is a chronic disease characterized by absolute insulin deficiency due to immune-mediated destruction of pancreas beta cells. T1D has been associated with a leaner phenotype in the past. However, the number of obese patients with T1D is increasing, mirroring the global increase in obesity prevalence (1,2). Metabolic syndrome (MetS), also called insulin resistance syndrome, comprises a cluster of diagnostic criteria including abdominal obesity, type 2 diabetes (T2D) and cardiovascular risk factors such as hypertension, dyslipidemia and nephropathy (3). Determining the frequency of MetS in patients with T1D requires the determination of coexisting obesity, hypertension and, dyslipidemia in these patients. Yet, studies in pediatric T1D patients to determine the prevalence of obesity and MetS are scarce.

In the T1D population, while the incidence of microvascular complications is decreasing owing to intensive diabetes management, macrovascular complications are now more commonly seen, a finding considered to be associated with the increasing incidence of obesity and MetS (4,5,6,7,8). This has led to the realisation that diagnosis of MetS and obesity is essential if continued improvements in the quality and duration of life in children and adolescents with T1D is to be achieved. For the diagnosis of MetS in childhood and adolescence, the International Diabetes Federation (IDF) criteria are globally accepted. The World Health Organization (WHO) and the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATPIII) criteria used for the diagnosis of MetS in the adult population can also be used for children and adolescents, with some modifications. The aim of this study was to determine the prevalence of obesity and MetS in a large cohort of children and adolescents with T1D, and to compare widely accepted and used criteria for the diagnosis of MetS.

Ethics committee approval was received for this study from the Local Ethics Committee of the Faculty of Medicine, Samsun Ondokuz Mayıs University (2014-354).

### Patients and Data Documentation

A descriptive, cross sectional study was conducted, comprising a total of 200 T1D patients between 8-18 years of age who were followed up for at least six months. Clinical and laboratory data were obtained from the patients' medical records, including age, sex, anthropometric measurements, duration of diabetes, daily insulin dose (IU), degree of metabolic control based on mean annual hemoglobin A1c (HbA1c) values, comorbidities and treatments. History of T1D and/or T2D in first- and second-degree relatives was recorded. Laboratory results were collected which included HbA1c, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride concentrations. Average IU per kg of body weight was calculated for the intensive insulin treatment group. Briefly, the total IU over three randomly selected days from the previous month was collected and mean daily dose was divided by body weight. The patients were evaluated for complications of diabetes and accompanying diseases. Existence of hypertension, prehypertension, microalbuminuria, retinopathy and neuropathy in addition to thyroid and coeliac disease were recorded. Patients who had other types of diabetes including T2D, maturity onset diabetes of the young and secondary diabetes were excluded from the study.

Height, weight and waist circumference (WC) were measured and body mass index (BMI) was calculated. Overweight and obesity were defined as a BMI ≥85$^{th}$ and ≥95$^{th}$ percentile, respectively (9).

The existence of hypertension was defined as a positive history of antihypertensive medicine or average blood pressure measurements above the 95$^{th}$ percentile of Turkish pediatric age measurements determined by Tümer et al (10). Values between 90 and 95$^{th}$ percentile were accepted as prehypertension while values below the 90$^{th}$ percentile were accepted as normal. The amount of albumin measured in appropriately collected 24-hour urine samples was used to determine the existence of nephropathy. Albumin less than 30 mg in 24 hours was considered negative and more than 30 mg was considered positive for albuminuria. The existence of pathologic changes indicating retinopathy in ophthalmoscopy were recorded. The patients were questioned for coeliac disease and the scans were completed using laboratory tests when necessary.

Total daily insulin dosage for the last three months was obtained by randomly selecting three days from the patient records. Mean daily dose (IU/kg) was calculated by dividing total daily IU to patient weight (kg).

In order to establish the degree of metabolic control, mean HbA1c level, measured over the past year, was calculated. The patients were divided into three metabolic control groups based on mean annual HbA1c: good, HbA1c < 7.5%; moderate, HbA1c 7.5-9%, and poor control, HbA1c > 9% (1).

The patient groups whose anthropologic and clinical data were recorded at first presentation, the third month of follow-up and the last routine clinic visit consisted mainly of patients who had ketoacidosis at presentation and patients who had just begun treatment. For this reason, data obtained after the onset of disease and at the third month of

treatment were accepted as baseline values. The evaluation at the last routine clinic visit was deemed to represent outcomes under treatment.

WHO and NCEP-ATPIII diagnostic criteria were applied to the study cohort to determine the prevalence of MetS in addition to the IDF MetS criteria (2005) for children and adolescents. WHO defines MetS as glucose intolerance, impaired glucose tolerance or diabetes mellitus, and/or insulin resistance, along with two or more of the following: high blood pressure (≥140/90 mmHg); hypertriglyceridemia (≥150 mg/dL); and/or low HDL cholesterol (<35 mg/dL in men and <39 mg/dL in women); central obesity (waist/hip ratio >0.9 in men and >0.85 in women); and/or a BMI >30 kg/m$^2$ and microalbuminuria (urinary albumin excretion rate ≥20 µg/min or albumin/creatine ratio ≥30 µg/mg) (11). According to the NCEP-ATPIII definition, a subject has MetS if they meet three or more of the following criteria: abdominal obesity (WC ≥102 cm in men and ≥88 cm in women); hypertriglyceridemia (≥150 mg/dL); low HDL cholesterol (<40 mg/dL in men and <50 mg/dL in women); high blood pressure (>130/85 mmHg); and/or high fasting glucose (>110 mg/dL) (12). Children over eight years of age were eligible for the study because there were well-defined criteria to diagnose MetS in children aged six to 10 years and above 10 years (13). According to the IDF definition of MetS in children a subject has MetS if he or she is between 6 to 10 years of age and has obesity defined as having a WC >90$^{th}$ percentile. If the age is between 10 to 16 years, a subject has MetS if he or she has a WC value >90$^{th}$ percentile (or adult cut-off, if lower) and has two or more of the following criteria: hypertriglyceridemia (≥150 mg/dL); low HDL cholesterol (<40 mg/dL); high blood pressure percentile for age, sex and height; and/or raised fasting glucose (>100 mg/dL). Since WHO and NCEP-ATPIII criteria were formulated for adults, for the purposes of this study these criteria were modified for use in our study cohort by applying pediatric percentiles All children with T1D were assumed to have impairment of glucose tolerance and fasting high blood sugar. Impaired glucose tolerance, impaired fasting glycemia or existence of T2D, which are part of the mentioned criteria were accepted as positive for our T1D patient group. Patients were examined for the existence of either of the two remaining criteria. Dyslipidemia was accepted as a HDL concentration <50 mg/dL and triglyceride >150 mg/dL. Pediatric percentiles were used for estimation of hypertension, WC and BMI (9). Patients with MetS according to the IDF criteria were compared in terms of demographic and clinical data.

### Statistical Analysis

Data analyses were performed by using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, United States). Whether the distribution of continuous variables were normal or not was determined by Kolmogorov-Smirnov test. Levene test was used for the evaluation of homogeneity of variances. Unless specified otherwise, continuous data were described as mean ± standard deviation (SD) for normal distributions, and median (range) for skewed distributions. Categorical data were described as number of cases (%).

Statistical analysis differences in normally distributed variables between two independent groups were compared by Student's t-test, Mann-Whitney U test were applied for comparisons of the not normally distributed data. While the differences in normally distributed variables among more than two independent groups were analyzed by one-way ANOVA, otherwise, Kruskal-Wallis test was applied for comparisons of the not normally data. When the p value from one-way ANOVA or Kruskal-Wallis test statistics were statistically significant post-hoc LSD or Conover's non-parametric multiple comparison test were used to know which group differ from which others.

## Results

The study group consisted of 200 T1D patients with a mean age of 13.8 ± 2.8 years, duration of diabetes 4.6 ± 3.3 years. More than half (52%) of the patients were male and the majority (87%) pubertal. Mean HbA1c was 8.40 ± 1.63% and mean IU was 0.87 ± 0.26 U/day. In the family history, T1D and T2D were present in 17.5% and 44% of the patients, respectively. Only three patients were using an insulin pump, and all the remaining patients (n = 197) were on multiple insulin injections. Metabolic control (mean annual HbA1c) across the whole cohort was good in 26.5%, moderate in 37% and poor in 36.5%. Of the 200 patients with T1D, 19 (9.5%) were overweight and 17 (8.5%) were obese.

Prevalence of MetS in the whole study cohort was 10.5%, 8.5% and 13.5% according to IDF, WHO and NCEP-ATPIII criteria, respectively. Figure 1 shows a Venn diagram of the numbers of patients with MetS diagnosis according to different diagnostic criteria for whole group. However, in the 36 overweight/obese T1D patients, the prevalence of MetS was 44.4%, 38.8% and 44.4% according to IDF, WHO and NCEP-ATPIII criteria, respectively.

Table 1 depicts the clinical characteristics of MetS positive versus negative T1D patients according to IDF criteria. There were no statistically significant differences in age, gender,

family history of T1D, pubertal stage, duration of diabetes, HbA1c levels and daily IU between patients with or without MetS but the difference was significant concerning family history of T2D and clinical and laboratory components of MetS. LDL-cholesterol and triglyceride concentrations were significantly elevated in patients with MetS ($p < 0.001$). When demographic and clinical data of patients with and without MetS according to WHO and NCEP-ATPIII criteria were evaluated, similar results to IDF criteria were obtained (data not shown).

As shown in Table 2, BMI-SD score (SDS) values of all patients increased during intense insulin treatment following diagnosis according to the IDF, WHO and NCEP-ATPIII criteria. The BMI-SDS values of the group diagnosed with MetS according to IDF, WHO and NCEP-ATPIII criteria, were significantly greater than the non-MetS group in all three evaluations. All cases diagnosed as MetS according to all three sets of criteria had BMI values above the 50$^{th}$ percentile at the diagnosis of T1D.

**Table 1. Comparison of the demographic and clinical findings of type 1 diabetes patients with and without metabolic syndrome according to International Diabetes Federation criteria (10)**

| Characteristics<br>T1D (n = 200) | MetS positive<br>n = 21 (10.5%) | MetS negative<br>n = 179 (89.5%) | p value |
|---|---|---|---|
| Gender (male) (n, %) | 10 (47.6) | 94 (52.5) | 0.671 |
| Age (years) (mean ± SD) | 13.7 ± 3.3 | 13.8 ± 2.8 | 0.995 |
| Pubertal status (n, %) | | | |
| Prepubertal | 4 (19) | 22 (12.3) | 0.488 |
| Postpubertal | 17 (81) | 157 (87.5) | |
| Family history (n, %) | | | |
| T1D | 3 (14.3) | 32 (17.9) | 1 |
| T2D | 13 (61.9) | 75 (41.8) | 1 |
| Duration of diabetes (mo) | 57.3 ± 45.8 | 54.9 ± 39.2 | 0.949 |
| Waist circumference (cm) (mean ± SD) | 82.9 ± 10.8 | 67.9 ± 7.7 | < 0.001 |
| Waist circumference SDS (mean ± SD) | 2.2 ± 0.6 | 0.3 ± 0.97 | < 0.001 |
| Insulin dose (U/kg) (mean ± SD) | 0.9 ± 0.2 | 0.9 ± 0.3 | 0.932 |
| Status of metabolic control (HbA1c %) (n, %) | | | |
| Good (≤7.5) | 4 (19) | 49 (27.4) | |
| Moderate (7.5-9.0) | 8 (38.1) | 66 (36.9) | 0.684 |
| Poor (≥9.0) | 9 (42.9) | 64 (35.8) | |
| Existence of acanthosis (n, %) | 5 (23.8) | 6 (3.4) | 0.002 |
| Comorbidities (n, %) | | | |
| Prehypertension | 2 (9.5) | 5 (2.8) | < 0.001 |
| Hypertension | 14 (66.7) | 10 (5.6) | < 0.001 |
| Dyslipidemia | 12 (21.1) | 45 (78.9) | 0.004 |
| Microalbuminuria | - | 8 (4.5) | 1.000 |
| Existence of additional disease (n, %) | | | |
| Thyroid autoantibody positivity | 3 (14.3) | 25 (14) | 1.000 |
| Thyroid disease | 1 (4.8) | 19 (10.6) | 0.701 |
| Coeliac disease | - | 4 (2.2) | 1.000 |
| HbA1c levels (mean ± SD) | | | |
| Recent year | 8.8 ± 1.4 | 8.6 ± 1.5 | 0.374 |
| At the most recent control | 8.6 ± 1.5 | 8.4 ± 1.6 | 0.439 |
| Lipid profile (mean ± SD) | | | |
| Triglyceride (mg/dL) | 130.1 ± 60.4 | 85.2 ± 42.0 | < 0.001 |
| HDL cholesterol (mg/dL) | 55.4 ± 15.4 | 62.1 ± 15.6 | 0.050 |
| LDL cholesterol (mg/dL) | 106.2 ± 20.3 | 77.0 ± 24.7 | < 0.001 |

IDF: International Diabetes Federation, MetS: metabolic syndrome, T1D: type 1 diabetes, T2D: type 2 diabetes, SD: standard deviation, SDS: SD score, HbA1c: hemoglobin A1c, HDL: high density lipoprotein, LDL: low density lipoprotein

## Discussion

There is no data on the prevalence of obesity in children and adolescents with T1D in Turkey. In our study group, comprising 200 children with T1D between 8 and 18 years, total prevalence of overweight and obesity was found to be 18%. The prevalence of overweight and obesity in schoolchildren and adolescents from different regions of our country has been reported in the range of 12.8-20.2% (14,15,16,17,18). Accordingly, the present study has shown that obesity prevalence of our T1D cohort was similar to that of the general population in Turkey. Studies from other countries have indicated that the increased incidence of overweight and obesity in T1D population mirrors what happens in the general population (19,20,21,22,23). Although the traditional belief is that patients with T1D are normal or thin, overweight and obesity figures in these patients have been found to increase in parallel with the normal population. Intense insulin therapy and weight gain due to the anabolizing and lipogenic effect of insulin are thought to be responsible for the increase. Additionally, the change in nutrition habits and shift to sedentary life style which are probably responsible for a global increase in overweight and obesity have also affected the young population with T1D. At present, T1D patients are more obese compared to the past, which has been associated with intense insulin treatment. The Epidemiology of Diabetes Interventions and Complications study revealed that the incidence of obesity in T1D patients has significantly increased due to widespread intense insulin treatment following Diabetes Control and Complications Trial (23,24,25). Some authors indicated that being female was a risk factor for a higher BMI-SDS six years after diabetes onset (26). All of our patients had gained weight at the time of diagnosis, third month of follow up and the last visit. Since the patients probably lost weight before diagnosis, the BMI-SDS values at the third month follow up and the last visit were evaluated and a striking weight gain was present. This reflects the effect of intense insulin treatment. All the patients gained weight but weight gain was more pronounced in the MetS positive group. Another striking point is that the BMI and BMI-SDS values in the MetS positive group were significantly higher than the MetS negative group and this was present from diagnosis up until the study period.

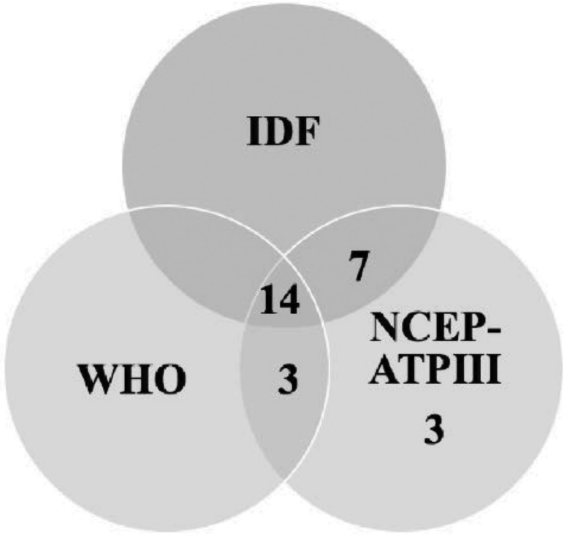


**Figure 1.** The numbers of patients with metabolic syndrome diagnosis based on different diagnostic criteria

*IDF: International Diabetes Federation, WHO: World Health Organisation, NCEP-ATPIII: National Cholesterol Education Program-Adult Treatment Panel III*

**Table 2. Changes in body mass index-standard deviation score values over time among metabolic syndrome positive and negative groups according to International Diabetes Federation, World Health Organisation and National Cholesterol Education Program-Adult Treatment Panel III criteria**

| Time | Criteria | BMI-SDS | | p value |
|---|---|---|---|---|
| | | MetS (+) | MetS (-) | |
| At time of diagnosis | IDF | 0.3 ± 1.6 | -0.8 ± 1.7 | 0.004 |
| | WHO | -0.1 ± 1.4 | -0.7 ± 1.7 | < 0.001 |
| | NCEP-ATPIII | 0.4 ± 1.8 | -0.8 ± 1.7 | < 0.001 |
| At third month of follow up | IDF | 1.0 ± 0.3 | -0.7 ± 1.1 | 0.003 |
| | WHO | 0.8 ± 1.6 | 0.03 ± 1.4 | < 0.001 |
| | NCEP-ATPIII | 1.1 ± 1.3 | -0.4 ± 1.4 | < 0.001 |
| During study period | IDF | 1.3 ± 0.8 | -0.1 ± 1.1 | < 0.001 |
| | WHO | 1.1 ± 0.8 | -0.1 ± 1.1 | < 0.001 |
| | NCEP-ATPIII | 1.2 ± 1.0 | -0.15 ± 1.0 | < 0.001 |

BMI-SDS: body mass index-standard deviation score, MetS: metabolic syndrome, IDF: International Diabetes Federation, WHO: World Health Organisation, NCEP-ATPIII: National Cholesterol Education Program-Adult Treatment Panel III

Data on MetS prevalence in patients with T1D is controversial. The prevalence of MetS was evaluated using three sets of recognized criteria and in 200 T1D children; it was 10.5% according to IDF, 8.5% according to WHO and 13.5% according to NCEP-ATPIII. Although there are no studies that report the prevalence of MetS in children and adolescents with T1D in Turkey, MetS seems to be more common in the T1D population when compared to the obese population. The results of the small number of studies comparing the prevalence of MetS in T1D children and adolescents vary between countries. In a study where 115 T1D patients between 5-16 years of age were investigated, MetS prevalence was found to be 13.2% according to IDF. The researchers found a significantly low incidence of MetS for that study population (27,28). Pinhas-Hamiel et al (20) reported a 7.1% prevalence of MetS in 326 T1D patients whose median age was 18.5 years.

The prevalence of MetS in overweight and obese children and adolescents in Turkey has been reported to vary between 20%-38% according to WHO, IDF and NCEP-ATPIII criteria (29,30,31). In our study, the incidence of MetS in overweight and obese patients was 41.7% according to IDF, 38.3% according to WHO and 47.2% according to NCEP-ATPIII criteria which is somewhat higher than has previously been reported but may simply reflect the secular trend in increasing prevalence of overweight and obesity.

Although it is widely accepted that it is necessary to diagnose MetS in the early stages of T1D, the main problem is the inadequacy of the criteria for the diagnosis of MetS in T1D patients. Use of the NCEP-ATPIII criteria resulted in the highest prevalence in our study (13.5%) since NCEP-ATPIII allows diagnosis of MetS by any three positive out of five criteria, with no mandatory prerequisites. Since diabetes is accepted as positive, two out of the remaining four criteria are enough and these criteria are already present in patients diagnosed using IDF criteria. As such, the MetS positive group determined by NCEP-ATPIII includes both the IDF criteria group and the group which did not meet the WC criterion, that is they are not obese according to WC, but are positive for two other criteria. The WC of the group which meets NCEP-ATPIII criteria but not the IDF criteria is below 90$^{th}$ percentile while the two criteria they met were any two of hypertriglyceridemia, low HDL or hypertension. Since there are no primary criteria in NCEP-ATPIII and it evaluates the WHO dyslipidemia criteria as two distinct criteria, it encompasses all cases diagnosed using WHO and IDF.

When the results of our study were evaluated according to IDF criteria, it was evident that the incidence of MetS was higher in the group where T1D was accompanied by overweight and obesity when compared to those who do not have T1D but are overweight or obese. The incidence of MetS is similar in overweight and obese individuals independent of T1D, according to WHO criteria. This discrepancy is caused by differences in definitions of MetS. Impaired glucose tolerance or T2D is a prerequisite for MetS in the WHO criteria, so it is understood that MetS prevalence will not change if the criteria is actualized as T1D. On the other hand, obesity as determined by WC is a prerequisite for MetS while impaired glucose tolerance and T2D are secondary criteria. Since this criterion is met by all patients in addition to obesity in our study population, which consisted of T1D patients, the ratio was found to be high.

On the other hand, insulin resistance and/or diabetes are mandatory primary criteria for the WHO definition and our T1D patient group meet these criteria. The WHO criteria also include microalbuminuria, which has the highest sensitivity for MetS in adulthood. However, our study group, because of the patient age range, had the lowest prevalence for this criterion, since these complications had not yet occurred (32,33,34). In the study group, there were 25 patients whose BMI was below the 95$^{th}$ percentile while WC was above the 90$^{th}$ percentile, which led to the identification of more MetS using IDF and NCEP-ATPIII in T1D. All patients who were diagnosed positive using the WHO criteria are found to also meet NCEP-ATPIII.

The WHO criteria include microvascular complications, which are rare in childhood, and the NCEP criteria do not include a primary criterion while diagnosing non-obese patients according to WC as MetS because the existence of diabetes is considered as a direct criterion. Due to these reasons, these criteria do not seem to be useful for the diagnosis of MetS in children and adolescents with T1D. Using IDF criteria seems more suitable because obesity is a prerequisite and they include accepted criteria for childhood (11,12,13).

### Study Limitations

Our study has several limitations and strengths. The main limitation is the absence of accepted clinical and laboratory criteria for the diagnosis of MetS in children and adolescents with T1D. Thus existing criteria had to be modified for a pediatric population in order to determine the prevalence. On the other hand, using and comparing three different modified criteria is a strength of our study. A further strength of this study lies in the accuracy of data, which adds to the available information concerning the prevalence of MetS in children and adolescents with T1D.

## Conclusion

Overweight and obesity prevalence of the group with T1D was similar to that of the population of the same age group in Turkey, but the prevalence of MetS was found to be higher than that of the general population. Except for the components of MetS, the other clinical and laboratory parameters were not helpful for prediction. It has been observed that all children and adolescents with T1D gained weight under intense insulin treatment. However, weight gain was more prominent in the MetS positive group. It is clear that appropriate modification of the criteria is required for the early detection of MetS in children and adolescents with T1D. This study suggests that IDF criteria are more suitable for the diagnosis of MetS in children and adolescents with T1D.

### Ethics

**Ethics Committee Approval:** The Local Ethics Committee of Faculty of Medicine, Samsun Ondokuz Mayıs University (approval number: 2014-354).

**Informed Consent:** Written informed consent was obtained from the parents of the patients who participated in this study.

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

### Authorship Contributions

Surgical and Medical Practices: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Concept: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Design: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Data Collection or Processing: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Analysis or Interpretation: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Literature Search: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın, Writing: Özlem Yayıcı Köken, Cengiz Kara, Gülay Can Yılmaz, Hasan Murat Aydın.

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# Macular Variability in Children and Adolescents with Metabolic Syndrome: A Cross-sectional Study Examining the Associations with Anthropometric Measurements, Metabolic Parameters and Inflammatory Markers

**Hakan Öztürk[1], Bediz Özen[1], Gönül Çatlı[2], Bumin N. Dündar[2]**

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

### What is already known on this topic?

Obesity and metabolic syndrome (MetS) are capable of causing damage to several organ systems by triggering a chronic subclinical inflammatory process. In the eye this damage includes microangiopathic changes, retinal degeneration, optic nerve function impairment and damage in the choroid and macular regions. Optical coherence tomography may show early macular damage.

### What this study adds?

This is the first study to show that macular retinal thickness and macular retinal volume values decrease as body mass index-standard deviation scores (SDS) and waist circumference-SDS (WC-SDS) increase in obese children and adolescents with MetS, providing evidence of macular damage. The increases in neutrophil/lymphocyte ratio, the platelet/lymphocyte ratio and the systemic immune-inflammatory index may be markers of chronic inflammation in children with MetS, and are associated with macular damage.

## Abstract

**Objective:** Macular damage may be observed in obesity and metabolic syndrome (MetS), a condition which leads to chronic subclinical inflammation and affects most organ systems.
To investigate the association between macular variability and anthropometric measurements, metabolic parameters, and inflammatory markers in children and adolescents with MetS.
**Methods:** Two hundred and twenty eyes of 62 obese and 48 healthy children and adolescents were examined. Bilateral macular retinal thickness (MRT) and macular retinal volume (MRV) were measured in all subjects using optical coherence tomography. Associations between mean MRT and mean MRV and age, auxological measurements including body mass index standard deviation scores (BMI-SDS) and waist circumference-SDS (WC-SDS), metabolic parameters and inflammatory parameters including neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio and systemic immune-inflammatory index (SIII) were investigated.
**Results:** No statistically significant difference was observed between the groups in terms of age or sex distribution ($p > 0.05$). Mean MRT ($r = -0.326$, $p = 0.007$) and MRV ($r = -0.303$, $p = 0.007$) values in the obese group with MetS decreased as homeostasis model assessment-insulin resistance (HOMA-IR) values increased. SIII values were higher in obese groups, but particularly in obese subject with MetS, compared to the control group ($p = 0.021$). The decrease in mean MRT ($r = -0.544$, $p = 0.046$) and MRV ($r = -0.651$, $p = 0.031$) in the obese subjects with MetS was negatively correlated with NLR. Mean MRT and MRV decreased in all obese subjects as SIII increased ($p < 0.05$).
**Conclusion:** This is the first study to show that mean MRT and MRV values decrease as BMI-SDS, WC-SDS and HOMA-IR increase in obese children and adolescents with MetS. NLR and SIII may serve as markers of chronic inflammation in obese children with MetS associated with macular damage.
**Keywords:** Macular retinal thickness, macular retinal volume, metabolic syndrome, optical coherence tomography, pediatric obesity

**Address for Correspondence:** Bediz Özen MD, University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital, Clinic of Ophthalmology, İzmir, Turkey
**Phone:** +90 232 469 69 69 **E-mail:** bedizozen@yahoo.com **ORCID:** orcid.org/0000-0001-9020-3810

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## Introduction

The prevalence of obesity and metabolic syndrome (MetS), usually a complication of obesity, is increasing. MetS is capable of causing damage to several organ systems by triggering a chronic subclinical inflammatory process. In the eye this damage includes microangiopathic changes, retinal degeneration, optic nerve function impairment and damage in the choroid and macular regions (1,2). These changes can be identified in the early stages using optical coherence tomography (OCT). The layers of the eye can be visualized in a painless, rapid, and non-invasive manner with OCT (3). OCT is particularly used to visualize macular pathologies, such as diabetic macular edema and macular degeneration (4,5,6). The macula is the region of the eye which is critical for detailed vision and is of great importance to the sense of sight. A deleterious effect in the macular region can cause progressive or permanent vision impairment. The macula can also be damaged in obesity and MetS because of the accompanying chronic subclinical inflammation (7).

Complete blood count is an inexpensive and easily accessible test. The neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR) and systemic immune-inflammatory index (SIII), all of which are easily calculated from complete blood count, have been shown to indicate subclinical inflammation in several studies (8,9,10).

To the best of our knowledge, no previous studies have investigated the association between changes in macular retinal thickness (MRT) and macular retinal volume (MRV) with metabolic parameters nor with inflammatory markers in children with MetS. The purpose of this study was to use OCT to investigate variability in MRT and MRV between obese children and adolescents with and without MetS and healthy controls as evidence of macular damage. This study also evaluated associations between changes in macular retinal measurements and anthropometric measurements, metabolic parameters, pubertal stage, and NLR, PLR and SIII.

## Methods

This prospective observational study was undertaken after receipt of Institutional Medical Research Ethical Committee approval (2019/9-3). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Patients aged 10-18 years, with no history of ocular disease or surgery, with no neurological diseases, and with spherical values between -0.75 diopter (D) and +0.75 D were enrolled in the study and control groups. All children and their parents consented to participate.

Children with a history of ocular trauma and dense media opacities, use of systemic corticosteroids, with diabetes mellitus or any systemic disease, or with acute/chronic local/systemic infectious disease and those unsuitable for OCT measurement, were excluded.

One hundred and twenty-four eyes of 62 obese children and adolescents aged between 10.1 and 17.9 years who presented to İzmir Tepecik Training and Research Hospital Pediatric Endocrinology Clinic, Turkey, between February 2016 and February 2019 were included in the study. Controls consisted of the 96 eyes of 48 healthy children and adolescents aged between 10.0 and 17.8 years presenting during the same period. The controls were composed of healthy children who applied to the ophthalmology clinic for routine control and without obesity.

Demographic data for all groups were extracted from medical records. Body measurements, blood pressure values and pubertal stages were assessed by a pediatric endocrinologist. Pubertal stages were classified based on the Tanner system (11). Height was measured to the 0.1 cm nearest centimeter with a rigid stadiometer. All subjects were also weighed unclothed to the nearest 0.1 kg using a calibrated balance scale. Body mass index (BMI) was calculated using the standard formula; weight (kg)/height ($m^2$). Standard deviation scores (SDS) for weight, height and BMI were calculated based on reference values established for Turkish children (12). Obesity was diagnosed according to the World Health Organization criteria (13). Blood pressure was measured three times at 10 minute intervals following a rest period. Systolic and/or diastolic blood pressure values exceeding the 95$^{th}$ percentile were regarded as hypertensive (14). In the obese subjects complete blood count, blood glucose, insulin and serum lipids were measured from fasting venous specimens collected on the same day as anthropometric measurements were taken. Specimens were analyzed using Roche Hitachi Modular System (Mannheim, Germany). Insulin resistance (IR) assessed by homeostasis model assessment-IR (HOMA-IR) was calculated with the formula; fasting insulin (μIU/mL) × fasting glucose (mg/dL)/405 (15). MetS was diagnosed based on International Diabetes Federation criteria (16). The syndrome was defined as hypertriglyceridemia (>150 mg/dL), decrease in high density lipoprotein (HDL) (<40 mg/dL), blood pressure elevation (systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg), or glucose metabolism disorder in the presence of abdominal obesity in cases aged 10-16 years. Adult criteria were used for the diagnosis of MetS at the age of 16 years and over. Percentile curves established for Turkish children were used for waist circumference. A waist circumference of the ≥90$^{th}$ percentile

was regarded as central obesity (17). The online calculator program developed by Demir et al (18) was used for all auxological measurements and blood pressure evaluation.

Three groups were established – healthy control (group 1), MetS negative (MetS-) obese (group 2), and MetS positive (MetS+) obese (group 3).

All cases underwent extensive ocular examination by the same ophthalmologist. This included best corrected visual acuity, detailed anterior segment examination with slit-lamp biomicroscopy, intraocular pressure using a Goldman applanation tonometer, ocular motility examination and optic nerve and retina examination with a 90 D lens. One percent cyclopentolate hydrochloride (Sikloplejin - Abdi İbrahim İlaç Sanayi, İstanbul, Turkey) eye drops were administered three times at five minute intervals for pupillary dilation. Measurements were recorded using an automated refractor (Topcon KR-1, Topcon, Tokyo, Japan) 30 minutes after the final drop administration. The mean value of the three measurements was recorded.

Retinal thickness and volume in the macular region were measured using a Spectralis OCT device (Heidelberg Spectralis, Heidelberg Engineering, Heidelberg, Germany). All participants were asked to wait in a darkened room before measurement. All scans were carried out on a 20 x 20 degree cube with 49 raster lines at 120-µm intervals. Retinal thickness and volume in the macula were calculated automatically as the distance separating the vitreoretinal interface from the margin representing the junction of the photoreceptor inner and outer segments. The macula was divided automatically into three concentric 1-, 3- and 6-mm rings. Retinal thickness and volume were measured on these three rings in accordance with the Early Treatment Diabetic Retinopathy Study Groups macular map (19). The inner and outer rings were further divided into four quadrants (superior, temporal, inferior, and nasal) by two reticules.

The 1-mm ring was defined as the central circle, the 3-mm ring as the inner segment circle and the 6-mm ring as the outer segment circle. Ocular observation was performed automatically in real time. Measurements were taken from both eyes by two independent masked observers. In order to avoid diurnal fluctuations, all OCT images were taken between 10.00 and 12.00 hours. Total macular thickness and volume including all nine subfields were subjected to analysis.

The MRT and MRV values were subjected to statistical analysis and comparison between the groups. In addition, correlation analysis was performed between mean MRT and MRV values and age, pubertal stages, body measurements, systolic/diastolic blood pressures, fasting insulin, HOMA-IR, lipid values, NLR, PLR, and SIII. SIII, was calculated using the formula (platelet × neutrophil)/lymphocyte.

### Statistical Analyses

Statistical analysis was performed on Statistical Package for Social Sciences (SPSS 20.0; IBM, Chicago, Ill., USA) software. Normality of sample distribution was evaluated using the Kolmogorov-Smirnov test. Mean and standard deviation values were calculated for normally distributed data. Pearson correlation analysis was applied for normally distributed variables, and Spearman correlation analysis for non-normally distributed variables. A $p < 0.05$ was considered statistically significant.

## Results

One hundred and ten subjects took part in the study, divided into 62 obese patients and 48 healthy controls. In the whole cohort the ages ranged from 10 to 18 years. MetS was identified in 45.1 % (28/62) of the obese group, but in no members of the control group. Mean ages were 13.5±3.5 years in the control group (group 1, n = 48), 13.8±2.9 in the MetS- obese subjects (group 2, n = 34), and 14.1±3.3 in the MetS+ obese subjects (group 3, n = 28). No statistically significant difference was observed between the groups in terms of age, sex distribution, or pubertal stages ($p > 0.05$).

BMI-SDS was $3.0 \pm 0.4$ in the obese group and $0.5 \pm 0.4$ in the control group ($p < 0.001$). Mean fasting blood glucose values were within normal limits and not different between the obese and control groups (controls $81.7 \pm 8.7$ mg/dL, all obese patients $85.3 \pm 9.9$ mg/dL; $p = 0.65$). BMI-SDS and WC-SDS were significantly higher in the obese cases than in the control group ($p < 0.001$). Morning fasting insulin and HOMA-IR values were also significantly higher than in the controls (obese and control group fasting insulin $20.6 \pm 10.8$ vs $8.3 \pm 3.1$ mIU/mL, respectively, $p = 0.02$; HOMA-IR $4.7 \pm 2.7$ vs $1.8 \pm 0.8$, respectively, $p = 0.01$). There was no difference in serum lipid values between the two groups ($p > 0.05$). Although NLR and PLR were higher in obese cases than in the controls, the difference was not statistically significant ($p > 0.05$). SIII values were higher in both obese groups, and particularly in the MetS+ subjects, compared to the control group ($p = 0.021$). Comparison of clinical and laboratory data for the MetS- and MetS+ obese subgroups and the healthy control group are shown in Table 1.

Evaluation of central, inner circle and outer circle macular thickness and volume revealed no significant difference between the sexes or between the two eyes ($p > 0.05$). When the thickness and volume of the whole macular retinal

region was evaluated, no statistically significant difference was found between the groups (Tables 2, 3).

Investigation of the relationship between clinical and laboratory variables and MRT and MRV revealed no significant association between mean MRT and MRV and age, pubertal stage, BMI-SDS, WC-SDS, systolic/diastolic blood pressure, NLR, PLR or SIII in the control group. No significant difference was observed between age, pubertal stage, systolic/diastolic blood pressure, triglyceride, HDL cholesterol, or PLR in the MetS+ and MetS- obese groups ($p > 0.05$). MRT and MRV values decreased significantly as BMI-SDS and WC-SDS increased in the MetS- and MetS+ obese groups ($p < 0.05$). In contrast to the other groups, in the MetS+ obese group mean MRT ($r = -0.326$, $p = 0.007$) and MRV ($r = -0.303$, $p = 0.007$) values decreased significantly as HOMA-IR values increased. The decrease in MRT ($r = -0.544$, $p = 0.046$) and MRV ($r = -0.651$, $p = 0.031$) in the MetS+ obese group was also negatively correlated with NLR. Mean MRT and MRV decreased as SIII increased in all obese subjects ($p < 0.05$). The results of Pearson correlation analysis of MRT and MRV and clinical and laboratory data are shown in Table 4.

## Discussion

Obesity and MetS associated with chronic inflammation can affect most systems in the body (1,2,20). This chronic inflammation also has the potential to produce changes in the retina and macular layer (20). Indeed, previous

**Table 1. Clinical and laboratory characteristics of the study groups**

| Characteristics | Control (n = 48) | Obese group (n = 62) | | p* |
|---|---|---|---|---|
| | | MetS-/obese (n = 34) | MetS + /obese (n = 28) | |
| Gender (male/female) | 23/25 | 14/20 | 15/13 | 0.81 |
| Age (years) | 13.5 ± 3.5 | 13.8 ± 2.9 | 14.1 ± 3.3 | 0.99 |
| Puberty stage (pre-pubertal/pubertal) | 10/38 | 8/26 | 6/22 | 0.98 |
| BMI-SDS | 0.5 ± 0.4 | 2.6 ± 0.8 | 3.1 ± 1.0 | < 0.0001 |
| WC-SDS | 0.7 ± 0.9 | 2.2 ± 0.7 | 3.3 ± 1.4 | < 0.001 |
| Systolic BP (mmHg) | 105.1 ± 10.6 | 118.6 ± 10.1 | 128 ± 20.1 | 0.19 |
| Diastolic BP (mmHg) | 65.2 ± 10.1 | 70.0 ± 12.3 | 79.0 ± 18.9 | 0.28 |
| Fasting glucose (mg/dL) | 81.7 ± 8.7 | 86.7 ± 8.9 | 89.1 ± 10.5 | 0.65 |
| Fasting insulin (mIU/mL) | 8.3 ± 3.1 | 19.6 ± 9.8 | 26.7 ± 15.3 | 0.02 |
| HOMA-IR | 1.8 ± 0.8 | 4.6 ± 2.5 | 5.4 ± 3.1 | 0.01 |
| Triglycerides (mg/dL) | 132.3 ± 60.8 | 137.9 ± 82.3 | 149.9 ± 89.1 | 0.07 |
| LDL-cholesterol (mg/dL) | 92.3 ± 28.9 | 99.3 ± 35.8 | 121.5 ± 71.7 | 0.06 |
| HDL-cholesterol (mg/dL) | 46.6 ± 10.7 | 42.2 ± 10.9 | 36.9 ± 36.1 | 0.72 |
| NLR | 1.7 | 2.1 | 2.4 | 0.33 |
| PLR | 118.5 | 121.7 | 119.9 | 0.36 |
| SIII | 377.9 | 467.2 | 513.5 | 0.021 |

*p value is between control and obese groups.

BMI-SDS: body mass index-standard deviation score, BP: blood pressure, HDL: high density lipoprotein, LDL: low density lipoprotein, HOMA-IR: homeostasis model assesment-insulin resistance, MetS: metabolic syndrome, NLR: neutrophil/lymphocyte ratio, PLR: platelet/lymphocyte ratio, SIII: systemic immune-inflammatory index, SDS: standard deviation score, TG: triglycerides, WC: waist circumference

**Table 2. Macular retinal thickness in control and obese children with and without metabolic syndrome**

| Macular retinal thickness | Control (n = 40) | Obese group (n = 62) | | p* |
|---|---|---|---|---|
| | | MetS-/obese (n = 34) | MetS + /obese (n = 28) | |
| Central circle (µm) | 271.5 ± 87.7 | 270.7 ± 101.9 | 265.5 ± 108.8 | 0.456 |
| Inner circle (µm) | | | | |
| Superior | 356.5 ± 89.5 | 353.4 ± 90.8 | 350.7 ± 101.1 | 0.944 |
| Temporal | 343.7 ± 110.2 | 336.2 ± 113.0 | 335.2 ± 91.0 | 0.244 |
| Inferior | 352.5 ± 118.6 | 348.1 ± 135.8 | 339.2 ± 125.1 | 0.352 |

*p value is between control and obese groups.

MetS: metabolic syndrome

studies have shown that obesity reduces the thickness of the retinal nerve fiber layer in children and affects choroid tissue (21,22,23). Some studies have shown that obesity leads to changes in the macular layers in children. Although the methods and macular layers investigated differ in all these studies, it may nevertheless be concluded that obesity results in macular variability and damage in the pediatric age group (24,25,26). One animal study involving a rodent MetS and impaired glucose tolerance model demonstrated developmental retinal degeneration using microscopy and immunohistochemical methods (27).

To the best of our knowledge, ours is the first study to show macular changes in MetS + children and the relationship between that variability and metabolic and inflammatory

**Table 3. Macular retinal volume in control and obese children with and without metabolic syndrome**

| Macular retinal volume | Control (n = 48) | Obese group (n = 62) | | p* |
|---|---|---|---|---|
| | | MetS-/obese (n = 34) | MetS + /obese (n = 28) | |
| Central circle ($mm^3$) | 0.21 ± 0.69 | 0.21 ± 0.16 | 0.20 ± 0.19 | 0.433 |
| Inner circle ($mm^3$) | | | | |
| Superior | 0.56 ± 0.14 | 0.55 ± 0.17 | 0.54 ± 0.21 | 0.960 |
| Temporal | 0.54 ± 0.17 | 0.53 ± 0.15 | 0.49 ± 0.19 | 0.261 |
| Inferior | 0.55 ± 0.18 | 0.55 ± 0.21 | 0.54 ± 0.29 | 0.413 |
| Nasal | 0.55 ± 0.11 | 0.55 ± 0.17 | 0.53 ± 0.18 | 0.325 |
| Outer circle ($mm^3$) | | | | |
| Superior | 1.61 ± 0.22 | 1.59 ± 0.14 | 1.58 ± 0.23 | 0.076 |
| Temporal | 1.60 ± 0.51 | 1.60 ± 0.53 | 1.57 ± 0.69 | 0.465 |
| Inferior | 1.68 ± 0.34 | 1.63 ± 1.62 | 1.67 ± 1.20 | 0.474 |
| Nasal | 1.74 ± 0.40 | 1.72 ± 0.47 | 1.70 ± 0.60 | 0.401 |

*p value is between control and obese groups.

MetS: metabolic syndrome

**Table 4. Correlation analysis of macular retinal thickness and macular retinal volume with clinical and laboratory parameters in the study groups**

| | RP | Age | | Puberty stage | | BMI-SDS | | WC-SDS | | Sistolic BP | | Diastolic BP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (n = 48) | | r | p | r | p | r | p | r | p | r | p | r | p |
| | MRT | 0.020 | 0.901 | 0.051 | 0.752 | -0.061 | 0.709 | -0.151 | 0.079 | 0.071 | 0.662 | 0.082 | 0.616 |
| | MRV | 0.019 | 0.909 | 0.078 | 0.631 | -0.024 | 0.883 | -0.259 | 0.091 | 0.054 | 0.742 | 0.060 | 0.712 |
| MetS-/obese (n = 34) | MRT | -0.025 | 0.820 | 0.033 | 0.842 | -0.457 | 0.004 | -0.751 | 0.038 | -0.090 | 0.593 | -0.287 | 0.081 |
| | MRV | -0.041 | 0.807 | 0.023 | 0.893 | -0.455 | 0.004 | -0.459 | 0.047 | -0.070 | 0.676 | -0.274 | 0.096 |
| MetS + /obese (n = 28) | MRT | 0.027 | 0.934 | 0.067 | 0.773 | -0.563 | 0.003 | -0.511 | 0.033 | -0.299 | 0.112 | -0.321 | 0.098 |
| | MRV | 0.033 | 0.765 | 0.056 | 0.892 | -0.611 | 0.002 | -0.477 | 0.038 | -0.212 | 0.098 | -0.378 | 0.087 |

**Table 4. Continued**

| | RP | HOMA-IR | | TG | | HDL | | NLR | | PLR | | SIII | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (n = 48) | | r | p | r | p | r | p | r | p | r | p | r | p |
| | MRT | -0.099 | 0.089 | -0.077 | 0.832 | 0.036 | 0.870 | -0.065 | 0.650 | -0.069 | 0.821 | -0.072 | 0.689 |
| | MRV | -0.277 | 0.09 | -0.069 | 0.642 | 0.045 | 0.779 | -0.032 | 0.775 | -0.023 | 0.820 | -0.044 | 0.651 |
| MetS-/obese (n = 34) | MRT | -0.047 | 0.781 | 0.039 | 0.819 | -0.247 | 0.141 | -0.232 | 0.093 | -0.331 | 0.057 | -0.391 | 0.048 |
| | MRV | -0.029 | 0.867 | 0.061 | 0.721 | -0.237 | 0.158 | -0.091 | 0.211 | -0.301 | 0.061 | -0.601 | 0.024 |
| MetS + /obese (n = 28) | MRT | -0.326 | 0.007 | 0.041 | 0.811 | -0.222 | 0.221 | -0.544 | 0.046 | -0.298 | 0.088 | -0.581 | 0.037 |
| | MRV | -0.303 | 0.007 | 0.069 | 0.723 | -0.245 | 0.156 | -0.651 | 0.031 | -0.288 | 0.095 | -0.503 | 0.041 |

RP: retinal parameters, MRT: macular retinal thickness, MRV: macular retinal volume, MetS: metabolic syndrome, BMI-SDS: body mass index-standard deviation score, WC: waist circumference, BP: blood pressure, TG: triglycerides, HDL: high density lipoprotein, NLR: neutrophil/lymphocyte ratio, PLR: platelet/lymphocyte ratio, SIII: systemic immune-inflammatory index, HOMA-IR: homeostasis model assesment-insulin resistance

parameters. In the present study, mean MRT and MRV values decreased as BMI-SDS increased in the all obese groups ($p < 0.05$). In addition, negative correlation was found between BMI-SDS and both MRT and MRV values in MetS - obese and MetS + obese groups (Table 4). Negative correlation with BMI-SDS was determined only in the obese group. No correlation with BMI-SDS was observed in the control group. Thus mean MRT and MRV decreased as the proportion of adipose tissue contributing to body composition increased.

Increased adipose tissue in obese cases may cause chronic systemic inflammation and microvascular damage. Vascular endothelial damage, oxidative stress, and chronic inflammation can impair the permeability and supply of microvascular structures which in turn may leads to oxidative stress and hypoxia in tissues. In addition, changes in leptin and adipokine levels, adipose tissue dysfunction, and IR can also develop. The production of inflammatory cytokines and reactive oxygen species increases. Apoptosis and tissue necrosis may then be triggered. Studies have shown that oxidative stress may be an important factor in cell death (1,2,28,29,30). Our results demonstrate that increased adipose tissue appeared to affect MRT and MRV and resulted in thinning of the macula. NLR, PLR and SIII, which can be simply and inexpensively calculated from complete blood count, have been shown to indicate subclinical inflammation in several previous studies (8,31,32,33). Furuncuoglu et al (33) showed that NLR, PLR, and SIII are positively correlated with BMI in adults. Another study of 26,016 adult patients determined positive correlation between NLR and MetS and obesity-related anthropometric data (32). One study of obese children with sleep apnea syndrome, a condition capable of leading to chronic hypoxia, showed that NLR and PLR increased with obesity (34). No previous studies have investigated changes in NLR, PLR, and SIII in children with MetS and ours is the first study to investigate this in children and adolescents. Although these inflammatory markers were higher in all the obese cases in our study group compared to controls, the differences were not statistically significant. However, SIII values were higher in both obese groups, and particularly the MetS + subjects, than in the control group ($p = 0.021$). Ours is also the first study to investigate macular variability and metabolic parameters and inflammatory markers in children. In contrast to the other groups, in the obese group with MetS mean MRT and MRV were significantly negatively correlated with increasing HOMA-IR values. The decrease in mean MRT and MRV in the MetS + subjects was also negatively correlated with NLR. Mean MRT and MRV also significantly decreased as SIII increased in all our obese subjects.

### Study Limitations

There are a number of limitations to this study. Plasma levels of inflammatory mediators such as adiponectin, leptin, and interleukin-6 were not measured. However, NLR, PLR and SIII values have recently been shown to reflect chronic subclinical inflammation, were investigated easily and inexpensively. In addition, due to the cross-sectional nature of our study, we were unable to determine whether weight loss and a decrease in adipose tissue would produce any positive change in MRT and MRV, particularly in obese children with MetS. Long-term, prospective observational studies in which weight control is established and inflammation reduced would be needed to show if there was any improvement in markers of macular tissue health with successful management of obesity and MetS.

## Conclusion

In conclusion, this study demonstrated that mean MRT and MRV values decreased as BMI-SDS and WC-SDS increased in MetS + obese children and adolescents. Mean MRT decreased as HOMA-IR values, a marker of IR, increased in children with MetS. Increased SIII and NLR are associated with macular damage in MetS + children and may be useful markers of chronic inflammation. Further long-term observational studies with larger participant numbers are now needed to confirm the results of this study.

### Ethics

**Ethics Committee Approval:** This prospective observational study was undertaken after receipt of Institutional Medical Research Ethical Committee approval (2019/9-3).

**Informed Consent:** All children and their parents consented to participate.

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

### Authorship Contributions

Concept: Hakan Öztürk, Bediz Özen, Design: Hakan Öztürk, Bediz Özen, Gönül Çatlı, Data Collection or Processing: Bediz Özen, Hakan Öztürk, Gönül Çatlı, Analysis or Interpretation: Bediz Özen, Hakan Öztürk, Literature Search: Bediz Özen, Gönül Çatlı, Bumin N. Dündar, Writing: Bediz Özen, Hakan Öztürk.

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# Growth and Adult Height during Human Growth Hormone Treatment in Chinese Children with Multiple Pituitary Hormone Deficiency Caused by Pituitary Stalk Interruption Syndrome: A Single Centre Study

**Fengxue Wang, Jinyan Han, Zengmin Wang, Xiaohong Shang, Guimei Li**

*Shandong Provincial Hospital affiliated to Shandong University, Department of Pediatrics, Shandong, China*

### What is already known on this topic?

Pituitary stalk interruption syndrome (PSIS) is a kind of congenital disease associated with multiple pituitary hormone deficiency (MPHD). Human recombinant growth hormone (hGH) treatment is the optimal therapy for short stature in children with isolated GH deficiency and can effectively increase growth velocity (GV) to attain adult heights within the target range.

### What this study adds?

GVs during hGH treatment were similar amongst pre-pubescent males and females with MPHD caused by PSIS. The GV during the first year of hGH treatment is an effective predictor of future height outcomes in patients with MPHD caused by PSIS.

## Abstract

**Objective:** The aim was to assess growth velocity (GV) during human recombinant growth hormone (hGH) treatment of children with multiple pituitary hormone deficiency (MPHD) caused by pituitary stalk interruption syndrome (PSIS) and to analyze the characteristics of patients that attained normal adult heights.

**Methods:** Data from 74 (16 female) children with MPHD caused by PSIS with GH, thyroid stimulating hormone, gonadotropin and adrenocorticotropic hormone deficiencies were collected. Subjects were divided into groups: 12 pre-pubescent females (Female-Group) and 36 pre-pubescent males (Male-Group 1). The remaining 22 males were further sub-divided into two groups (Male-Group 2 and Male-Group 3) according to the initiation of gonadotropin replacement treatment, based on bone age and height.

**Results:** No differences in change in height standard deviation score (△HtSDS) and GV were observed at different time points of hGH treatment between the Female-Group and Male-Group 1 ($p > 0.05$). GV was significantly greater in the first year of hGH therapy than in subsequent years: Female-Group $p = 0.011$; Male-Group 1 $p < 0.001$; Male-Group 2 $p = 0.005$; and Male-Group 3 $p = 0.046$. Adult height was achieved by 23 (19 males and 4 females) patients. The total gain in height positively correlated with the GV during the first year ($r = 0.626$, $p < 0.001$).

**Conclusion:** GV during hGH treatment were similar amongst pre-pubescent males and females with MPHD caused by PSIS. GV during the first year of hGH treatment appears to be an effective predictor of final height in patients with MPHD caused by PSIS.

**Keywords:** Pituitary stalk interruption syndrome, growth velocity, human growth hormone treatment, adult height

## Introduction

Pituitary stalk interruption syndrome (PSIS) is characterized by the occurrence of a thin or absent pituitary stalk, hypoplasia of adenohypophysis, and ectopic neurohypophysis on magnetic resonance imaging (MRI) of the hypothalamo-pituitary region (1). It is a rare congenital disease associated with multiple pituitary hormone deficiencies (MPHD). MPHD, by definition, represents an impaired production of one or more anterior pituitary hormones in addition to growth hormone (GH) and is a chronic, lifelong condition (2,3,4,5).

PSIS as the most common cause of MPHD, was first reported by Fujisawa et al (6) in 1987. Children with

**Address for Correspondence:** Guimei Li MD, Shandong Provincial Hospital affiliated to Shandong University, Department of Pediatrics, Shandong, China
**Phone:** +86 130 3171 6996 **E-mail:** lgmusa2015@163.com **ORCID:** orcid.org/0000-0002-1784-4555

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PSIS typically present with growth reduction and delayed puberty, leading to significant distress to children and their families. Recombinant human GH (hGH) treatment is the optimal therapy for short stature in children with isolated GH deficiency (IGHD) and can effectively increase height velocity to attain adult heights within the target range (7,8). Whether the benefits of hGH treatment in patients with MPHD caused by PSIS are consistent with those observed in IGHD is currently unknown.

In this study, we assessed the growth velocity (GV) of patients with MPHD caused by PSIS who were administered hGH. We further analyzed the characteristics of patients that subsequently attained adult heights.

## Methods

### Patients and Grouping

In this retrospective study, data from patients diagnosed with PSIS during childhood and adolescence presenting during the time period from January 2008 to November 2018 in pediatric endocrine outpatients of Shandong Provincial Hospital were analyzed. Patients with confirmed GH, thyroid stimulating hormone (TSH), gonadotropin and adrenocorticotropic hormone (ACTH) deficiencies were included in the analysis. None of the patients had spontaneous puberty and puberty was induced *via* gonadotropin or sex hormone treatment. Pre-pubescent subjects at presentation were divided into the Female-Group and Male-Group 1. Additionally, patients were treated with hGH followed by gonadotropin, administered at the appropriate age and the male patients from this subgroup formed Male-Group 2. The final study group consisted of male patients who received initial hGH and gonadotropin treatment simultaneously and these were designated Male-Group 3.

### Ethics

The study was approved by the Medical Ethics Committee of Shandong Provincial Hospital, affiliated to Shandong University (approval number: 2019-053). Patients or their parents/guardians provided verbal consent for anonymized data to be collated and analyzed, approved by the ethics committee.

### Diagnostic Criteria

The diagnosis of PSIS was based on cranial MRI of the hypothalamus and pituitary gland. Imaging criteria for a diagnosis of PSIS included an absent or thin pituitary stalk, hypoplasia of the anterior pituitary gland, and ectopic location of the posterior pituitary. MRI scans were performed using a 3.0 T Scanner (Siemens, Erlangen, Germany) in the sagittal and coronal planes on T1 and T2 weighted imaging (slice thickness 3 millimeters).

Bone age (BA) was determined by left hand and wrist X-ray images according to the method of Greulich and Pyle. Hypophyseal hormone levels were investigated in each patient. The pituitary axis was examined using the following tests: (1) GH deficiency (GHD) was diagnosed in the absence of a significant peak in GH secretion after more than one stimulation test. Diagnosis was based on GH peak concentrations $<10$ ng/mL, following two independent GH provocation tests which were either an intravenous arginine test, 0.5 mg/kg; maximum dosage 30 mg and/or an oral levodopa test, 10 mg/kg; maximum dosage: 500 mg [arginine produced by Harbin Pharmaceutical Group Co., Ltd. in Harbin, Heilongjiang, China and levodopa produced by Aikanglide Pharmaceutical (Zhejiang Co., Ltd in Quzhou, Zhejiang, China). Patients received GH provocation tests on the basis of normal thyroid and adrenal functions (2). TSH deficiency was defined as a low serum free thyroxine (fT4) of $<12.0$ pmol/L (reference range: 12.0-22.0 pmol/L) with concomitantly normal or decreased serum TSH (reference range: 0.27-4.2 uIU/mL) (3). ACTH deficiency was assessed by either decreased serum cortisol (COR) levels in the morning (COR $<138$ nmol/L) or an impaired serum COR concentration increase (COR $<550$ nmol/L) during insulin-induced hypoglycemia with inappropriately low serum ACTH concentrations (4). Gonadotropin deficiency was based on the gonadotropin hormone-releasing hormone-stimulation test [triptorelin: 2.5 μg/kg administered as a subcutaneous injection at a maximum dosage of 100 μg with cut-off points for a blunted response of 2.8 mIU/mL for luteinizing hormone (LH) and/or 3.7 mIU/mL for follicle-stimulating hormone (FSH)]; or basal levels of FSH and LH below the sensitivity range of the assay ($<0.1$ mIU/mL) on the basis of delayed or absent pubertal development (9,10). Serum GH levels were measured using chemiluminescence assays (Cobas E170, Roche Diagnostics, Germany). Serum fT4, TSH, ACTH, COR, FSH and LH were measured using chemiluminescence assays (Siemens Healthcare Diagnostics, USA). All patients underwent the same testing protocol and all tests were performed after overnight fasting (5).

### Height and Weight

Height was measured in centimeters (cm) in the morning by the same medical team. Height measurements were standardized for age and sex and expressed as standard deviation scores (HtSDS) relative to chronological age (CA) according to Growth Charts for Chinese Children and Adolescents (2009) (5,11). The GV during hGH treatment

was analyzed each year and was calculated in cm through the difference in previously recorded height. △HtSDS was calculated by the difference from the previously recorded HtSDS. The parental target height was calculated according to the following formula: ([height of the father + height of the mother] / 2) - 6.5 cm for girls and + 6.5 cm for boys, and also expressed as standard deviation scores (SDS) according to the Growth Charts for Chinese Children and Adolescents (2009) (11).

Weight was measured in kilogrammes (kg) in the morning, in the fasting state with no shoes and light clothes at each visit. Body mass index (BMI) was calculated using the standard formula of weight (kg) divided by height squared (meters). BMI values were transformed into BMI-SDS, based on Normative Values for Chinese Children and Adolescents (2009) (5,11) to adjust for the confounding effects of age and sex.

### Treatment and Follow-up

Patients received hormone replacement therapy according to their known hormone deficiencies. Hydrocortisone and L-thyroxine were immediately administered once ACTH and TSH deficiency were confirmed. The dosages of hydrocortisone were between 10-15 mg/m$^2$/day, oral administration, divided into two daily doses. The dosages of L-thyroxine were between 1.5-2.0 ug/kg/day, oral administration, qd. The dosages of hydrocortisone and L-thyroxine were adjusted to maintain the levels of fT4, COR, blood glucose and serum electrolytes within normal ranges. In patients with normal thyroid and adrenal hormone levels (including patients who were corrected with hydrocortisone and L-thyroxine) hGH was administered (hGH produced by ChangChun GeneScience Pharmaceuticals Co., Ltd in Changchun, Jilin, China). The dosage of hGH was between 0.10-0.15 IU/kg/day) and administered by daily injection 7 days/week. Puberty was initiated in 26 patients through the administration of gonadotropin; exogenous human chorionic gonadotropin (hCG) and/or urine-derived human menopause gonadotropin (hMG) therapy (hCG and hMG from Livzon Pharmaceutical Group Inc., in Zhuhai, Guangdong, China). For male patients the dosage of intramuscular (i.m.) hCG was 2000 IU, twice per week, whilst for female patients 75 IU i.m. hMG was administered twice a week. Boys had a pretreatment phase of hCG for three months. As serum testosterone (TO) reached normal values, hCG combined with hMG was administered to improve sexual development. If the TO concentration did not attain normal values, exogenous TO (TO undecanoate Catalent France Beinheim S.A. in France: 80-160 mg/day, oral administration) was given to induce puberty (12).

All patients were followed in outpatient clinic at three monthly intervals. Compliance with treatment was assessed at each visit, and patients underwent complete physical examinations by the same medical team, including height and weight measurements during and after treatment. Biochemical and hormone status were also assessed.

### Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences software (IBM SPSS for Windows, Version 25; IBM Corp., Armonk, NY, USA). The continuous data in our research were tested for normal distribution using Kolmogorov-Smirnov test, and found to be approximately normally distributed. The descriptive statistics of the quantitative variables were presented as means ± standard deviations (SD). Groups were compared using the Student's t-test. Pearson's correlation was used to assess the relationships between various parameters. The threshold for statistical significance was $< 0.05$.

## Results

In total, data from 74 patients (58 males and 16 females) was analyzed. Amongst these patients, 48 (36 males and 12 females) who received hGH treatment and other deficient hormones, excluding gonadotropin during the study period, were pre-pubescent. Pre-pubescent subjects at presentation were divided into the Female-Group ($n = 12$) and Male-Group 1 ($n = 36$). Additionally, 13 patients (10 males and 3 females) were treated with hGH followed by gonadotropin which was administered at the appropriate age to induce puberty. The 10 male patients in this latter group formed Male-Group 2. Lastly, 13 subjects (12 males and 1 female) received combined hGH and gonadotropin treatment at presentation. These 12 males were grouped into Male-Group 3.

### Growth Velocity of Pre-pubertal Patients (Female-Group and Male-Group 1) Treated with Human Growth Hormone Alone

CA, BA, BMI-SDS and HtSDS during hGH treatment were 8.51 ± 3.08 years, 4.91 ± 2.75 years, -0.20 ± 1.11 and -3.60 ± 1.76 in the Female-Group and 8.94 ± 3.42 years, 5.51 ± 3.22 years, 0.20 ± 1.35 and -2.97 ± 1.23 in the Male-Group 1. There were no differences in CA and BA, BMI-SDS and HtSDS at hGH treatments between pre-pubescent females (Female-Group) and males (Male-Group 1) ($p = 0.698$, $p = 0.653$, $p = 0.358$ and $p = 0.175$). The CA was significantly larger than the BA in both groups ($p = 0.006$ for Female-Group and $p < 0.001$ for Male-Group 1) (Figure 1A).

There were no differences in HtSDS at any point during hGH treatment (p = 0.292 and p = 0.157). For Female-Group patients, the GV was 11.63 ± 2.38 cm/y in the first year and 9.37 ± 1.48 cm/y in the second year. The GV of Male-Group 1 in the first year was 11.95 ± 2.62 cm/y compared to 9.83 ± 1.71 cm/y in the second year. No differences in GV at any time point during hGH treatment between prepubescent females (Female-Group) and males (Male-Group 1) (p = 0.710 for the first year and p = 0.410 for the second year) were observed. GV in the first year was higher than in the second year for both two groups (p = 0.011 for the Female-Group and p < 0.001 for the Male-Group 1) (Figure 1B). The △HtSDS1 for the Female- and Male-Group 1 were significantly higher than the △HtSDS2 values (1.21 ± 0.51 vs 0.59 ± 0.38 and p≤0.001 for Female-Group, 1.13 ± 0.57 vs 0.70 ± 0.47, p = 0.003 for Male-Group 1, respectively) (Figure 1C). Detailed information is shown in Table 1.

### Growth Velocity Male-Group 2 and Male-Group 3 Treated with hGH and Gonadotropin

Detailed information of CA, BA, height SDS and GV during hGH or gonadotropin treatment are shown in Tables 1 and 2. The CA following hGH treatment of the Male-Group 2 was larger than BA (11.24 ± 2.99 years vs 6.55 ± 3.82 years, p = 0.009). No differences in CA and BA at the initiation of hGH treatment between Male-Group 1 and Male-Group 2 were observed (p = 0.060 and p = 0.390, respectively). CA at the initiation of hGH treatment (hGH + gonadotropin treatment) of Male-Group 3 was larger than BA (17.42 ± 3.32 years vs 12.25 ± 1.37 years, p≤0.001). The CA of Male-Group 3 was significantly higher than that of Male-Group 1 and Male-Group 2 (both p≤0.001) which was also observed for BA values (both p≤0.001).

The GV during the first two years of hGH treatment in the Male-Group 2 were 13.37 ± 2.45 and 10.08 ± 2.16 cm/year. GV during the first two years of hGH (hGH + gonadotropin) treatment of Male-Group 3 were 10.68 ± 3.59 and 8.16 ± 2.03 cm/year. We observed no differences in GV during the first year of hGH treatment amongst the three groups (p = 0.132 between Group 1 and Group 2, p = 0.193 between Group 1 and Group 3, and p = 0.058 between Group 2 and Group 3). During the second year, no differences in GV were observed between Groups 1 and 2 (p = 0.701), but the GV of Group 3 was significantly lower than the other two male groups (p = 0.007 between Male-Group 1 and Male-Group 3, and p = 0.044 between Male-Group 2 and Male-Group 3).

GV during the first year of hGH treatment was higher than the second year for the three groups (p≤0.001 for Male-Group 1, p = 0.005 for Male-Group 2 and p = 0.046 for Male-Group 3, respectively) (Figure 1B). The differences in △HtSDS between the first and second year of hGH treatment for Male-Group 2 were also statistically significant (1.39 ± 0.58 vs 0.77 ± 0.41, p = 0.013). However, differences in the △HtSDS during the first two years of hGH treatment did not change in the Male-Group 3 (1.50 ± 0.79 vs 1.02 ± 0.76, p = 0.144) (Figure 1C).

**Table 1. Characteristics of patients with pituitary stalk interruption syndrome treated with human growth hormone**

| | | Female-Group (n = 12) | Male-Group 1 (n = 36) | Male-Group 2 (n = 10) | |
|---|---|---|---|---|---|
| | p* | (Mean ± SD) | (Mean ± SD) | (Mean ± SD) | p# |
| CA (years) at hGH onset | 0.698 | 8.51 ± 3.08 | 8.94 ± 3.42 | 11.24 ± 2.99 | 0.060 |
| BA (years) at hGH onset | 0.563 | 4.91 ± 2.75 | 5.51 ± 3.22 | 6.55 ± 3.82 | 0.390 |
| BMI-SDS at hGH onset | 0.358 | -0.20 ± 1.11 | 0.20 ± 1.35 | -0.02 ± 1.66 | 0.667 |
| HtSDS | | | | | |
| hGH treatment onset | 0.175 | -3.60 ± 1.76 | -2.97 ± 1.23 | -3.43 ± 2.67 | 0.435 |
| 1st year of hGH treatment | 0.292 | -2.39 ± 1.60 | -1.85 ± 1.14 | -2.04 ± 2.48 | 0.727 |
| 2nd year of hGH treatment | 0.157 | -1.98 ± 1.54 | -1.47 ± 0.86 | -1.69 ± 1.92 | 0.598 |
| △HtSDS | | | | | |
| △HtSDS1 | 0.668 | 1.21 ± 0.51 | 1.13 ± 0.57 | 1.39 ± 0.58 | 0.210 |
| △HtSDS2 | 0.467 | 0.59 ± 0.38 | 0.70 ± 0.47 | 0.77 ± 0.41 | 0.671 |
| GV (cm/year) | | | | | |
| 1st year of hGH treatment | 0.710 | 11.63 ± 2.38 | 11.95 ± 2.62 | 13.37 ± 2.45 | 0.132 |
| 2nd year of hGH treatment | 0.410 | 9.37 ± 1.48 | 9.83 ± 1.71 | 10.08 ± 2.16 | 0.701 |

*p: comparison between Female-Group and Male-Group 1; #p: comparison between Male-Group 1 and Male-Group 2.

CA: chronological age; BA: bone age, BMI: body mass index, SDS: standard deviation score, △HtSDS: differences in HtSDS from the former year, △HtSDS1: difference in HtSDS between the first year of human growth hormone (hGH) treatment and the onset of hGH treatment, △HtSDS2: differences in HtSDS between the second and first year of hGH treatment

The CA during the initiation of hGH + gonadotropin treatment of Male-Group 2 (13.39 ± 2.80) were significantly lower than those of Male-Group 3 (p = 0.007), but no differences in BA at the initiation of hGH + gonadotropin treatment between the groups were observed (p = 0.066). The GV in the first year of hGH + gonadotropin treatment in Group 3 was significantly higher than that of Group 2 (p = 0.041), which was similar for △HtSDS (p = 0.004).

### Characteristics of Patients Achieving Adult Height

In total, 23 patients (19 males) with PSIS reached adult height following hGH treatment. For male patients, 18/19 attained adult height > -2 SD, yet only seven reached a height above the 50th percentile (adult height ≥172.7 cm or adult HtSDS ≥0). For females, all patients (4/4) reached a normal adult height range and all were above the 50th percentile (adult height ≥160.6 cm or adult HtSDS > 0).

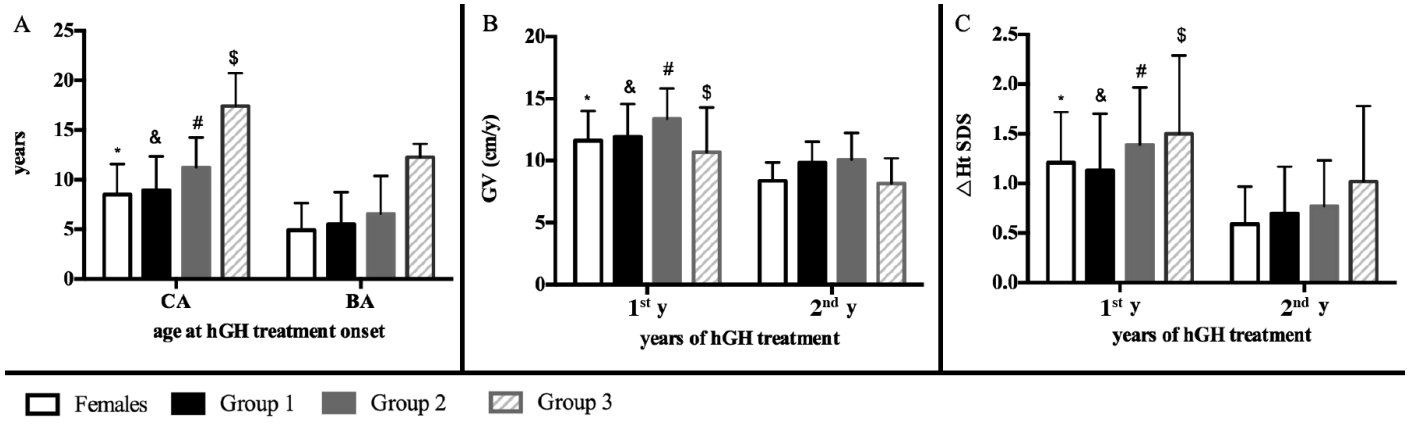


**Figure 1. A, B, C)** The age, growth velocity (GV) and △HtSDS at different points during hGH treatment. A) CA at the hGH treatment onset of four groups was significantly larger than their BA. *p = 0.006, &p≤0.001, #p = 0.009, $p≤0.001. B) GV in the first year during hGH treatment was significantly higher than in the second year. *p = 0.011, &p≤0.001, #p = 0.005, $p = 0.046. C) The difference in height SDS between the first year and the hGH treatment onset was higher than in the second year for Female-Group, Male-Group 1 and Male-Group 2. *p≤0.001, &p = 0.003, #p = 0.013, $p = 0.144

*GV: growth velocity, △HtSDS: height standard deviation score, hGH: human growth hormone, CA: chronological age, BA: bone age*

**Table 2. Characteristics of male patients with pituitary stalk interruption syndrome treated with both human growth hormone and gonadotropin**

| | Male-Group 2 (n = 10) Mean ± SD | Male-Group 3 (n = 12) Mean ± SD | p |
|---|---|---|---|
| CA (years) at hGH + gonadotropin onset | 13.39 ± 2.80 | 17.42 ± 3.32 | **0.007** |
| BA (years) at hGH + gonadotropin onset | 10.85 ± 2.00 | 12.25 ± 1.37 | 0.066 |
| BMI-SDS hGH + gonadotropin onset | 0.43 ± 1.53 | 0.33 ± 0.94 | 0.853 |
| Height SDS | | | |
| 1st year of hGH + gonadotropin treatment | -0.84 ± 1.96 | -3.60 ± 1.74 | **0.002** |
| 2nd year of hGH + gonadotropin treatment | -0.02 ± 1.14 | -2.09 ± 1.21 | **≤0.001** |
| △HtSDS | | | |
| △HtSDS1 hGH + gonadotropin treatment | 0.49 ± 0.62 | 1.50 ± 0.79 | **0.004** |
| △HtSDS2 hGH + gonadotropin treatment | 0.40 ± 0.75 | 1.02 ± 0.76 | 0.070 |
| Growth velocity (cm/year) | | | |
| 1st year of hGH + gonadotropin treatment | 7.93 ± 1.87 | 10.68 ± 3.59 | **0.041** |
| 2nd year of hGH + gonadotropin treatment | 8.10 ± 2.16 | 8.16 ± 2.03 | 0.947 |

p: comparison between Male-Group 2 and Male-Group 3; 1st/2nd year of hGH + gonadotropin treatment: first/second year of hGH combined with gonadotropin treatment.

CA: chronological age, BA: bone age, hGH: human growth hormone, BMI: body mass index, SDS: standard deviation score

The mean adult height was 168.5 ± 6.1 cm (HtSDS = -0.47 ± 1.11) for males and 164.0 ± 2.9 cm (HtSDS = 0.77 ± 0.49) for females. The parental target height was 170.1 ± 4.9 cm (HtSDS = -0.43 ± 0.82) for males and 160.8 ± 1.3 cm (HtSDS = 0.26 ± 0.03) for females. The mean age at initiation of hGH treatment in females was 10.4 ± 0.8 years and 14.4 ± 3.5 years in males. The mean BA at initiation of hGH treatment in females was 8.4 ± 0.8 years and 9.9 ± 3.5 years for males. Mean HtSDS at hGH treatment onset was -3.11 ± 1.86 for males and -1.75 ± 0.23 for females, respectively. The mean GV in the first year of hGH treatment were 11.0 ± 3.2 cm and 12.9 ± 1.9 cm for males and females, respectively. The mean total height gain was 23.9 ± 15.6 cm and 20.9 ± 4.9 cm for males and females, respectively.

A negative correlation was found between the total height gain and BA at hGH onset and between total height gain and height at hGH treatment onset ($r = -0.721$, $p < 0.001$; and $r = -0.822$, $p < 0.001$, respectively). Moreover, a positive correlation was observed between total height gain and GV in the first year of hGH treatment ($r = 0.626$, $p < 0.001$). Figure 2 graphically depicts these correlations.

## Discussion

Various anterior pituitary hormone deficiencies and clinical presentations are common in PSIS patients. To date, studies on the growth of children and adolescents with PSIS during the course of hGH treatment are sparse and continuous follow-up to adult age is rarely reported (5). In this retrospective study, measurements were performed in 74 patients with PSIS. Our analysis included long-term patient follow-up, performed at regular short intervals, by the same team of healthcare professionals. Despite certain limitations, the results provided various noteworthy observations. It has been reported that the addition of gonadotropins may affect GV in pubertal children (4), so male PSIS patients were divided into three groups on the basis of their gonadotropin treatment protocols.

All children short in stature can receive hGH treatment to decrease linear height deficits. The CA of PSIS patients receiving hGH treatment in this study were older than those of previous studies (13). Chinese parents are familiar with the idea of "delayed puberty", leading to delay in referral and older age at presentation in Chinese patients. A large number of children were from rural areas with an undeveloped economy, in which the attention to growth and development is low. These factors contribute to the older age of PSIS children and adolescents in our cohort. The BA of all the PSIS children in our study was lower than their respective CA, as previously described (4). GH deficiency leads to slow bone growth and maturity, owing to delayed BA. The treatment effect at different time points was independent of the gender amongst pre-pubescent PSIS children in this study. This differed from previous studies (12) in which pre-pubescent boys had a greater response to hGH treatment than pre-pubescent girls with GHD who were small for their gestational age. Deficiencies in other pituitary hormones may weaken the responses of males, and the low number of patients may have contributed to the discrepancies. The BMI SDS of pre-pubescent boys tended to be higher than those of females, though the differences were not statistically significant. The increase in BMI SDS may have adverse effects on height growth in shorter male children, consistent with previous reports (14).

In previous studies on congenital IGHD patients, the GV in the first year of hGH therapy was 8.92 ± 2.99 cm for males and 8.17 ± 3.15 cm for females (15). The GV in the first year of hGH treatment was significantly higher than that of GHD patients ($p < 0.001$ for males and $p = 0.004$ for females), whilst the CA values were similar ($p = 0.170$ for males and $p = 0.272$ for females). The greater responses to

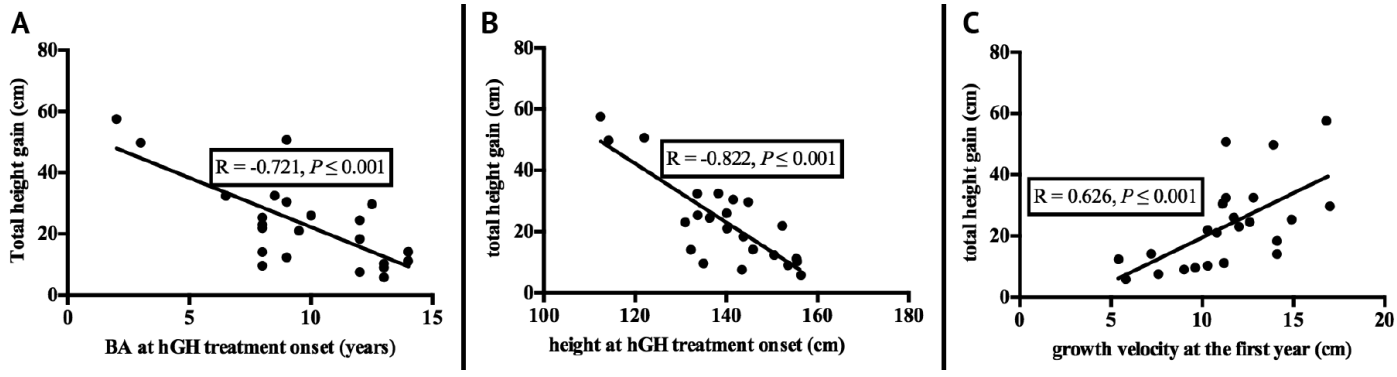


**Figure 2. A, B, C)** Correlation between total height gain and bone age, height and growth velocity at first year

*hGH: human growth hormone, BA: bone age*

hGH treatment in PSIS patients may be due to the higher sensitivity to hGH and/or severity of GH deficiency of these patients (16). The GV of patients in the Female-Group, Male-Group 1, Male-Group 2 and Male-Group 3 in this study were higher in the first year compared to the second year, consistent with previous studies and confirming that GHD children show faster linear growth during the initial stages of GH therapy (17) in addition to other studies on congenital MPHD (4).

Although the mean CA and mean BA of Male-Group 3 were both higher than those of the other two male groups, the GV during the first two years of hGH treatment was similar, which may be explained by the induction of puberty. The induction of puberty leads to a growth spurt which may explain the response to hGH treatment observed.

In previous GHD studies, only one third of patients reached normal adult height in response to hGH treatment (15). The duration of hGH treatment, age at initiation of hGH treatment and ethnicity may contribute to the discrepancies between this and previous studies. Although girls and boys had similar responses to hGH at different time points pre-puberty, females achieved better adult height than males in this study. The younger age at the initiation of hGH treatment of females and small sample size may explain this enhanced treatment effect.

A positive correlation between total height gain and GV in the first year of hGH treatment was in accordance with previous studies (18). The total height gain in the first year of hGH is an effective predictor of future height outcomes (19,20,21,22). Previous studies on GHD also indicate the importance of early treatment with hGH for IGHD patients (23,24). The negative relationship of BA and total height gain in this study also reflects a similar phenomenon in patients with PSIS and the total height gain may be greater if patients receive hGH at an earlier stage. It has been suggested that this is because early initiation of hGH permits a longer duration of treatment and larger gains in height (24) although height gains in the second year of therapy with hGH are consistently less impressive than those achieved in the first year of therapy.

### Study Limitations

The uneven number of male and female patients and differences in duration of hGH treatment may have influenced the results. Of particular note there were only four females in this study who attained adult height. It is not possible to reach a definite conclusion about the effect of hGH in female PSIS patients and the number of female patients is too low to compare with male patients reliably In addition data regarding the sexual development of PSIS patients was not collected and so analysis of the effect of hGH and gonadotropin therapy on pubertal development in PSIS patients was not performed. These limitations should be addressed in future studies.

## Conclusion

Males and females with MPHD caused by PSIS had a similar GV during hGH treatment before puberty. The GV during the first year of hGH treatment can predict future height outcomes for patients with MPHD caused by PSIS. PSIS patients may attain normal adult heights following hGH treatment.

### Ethics

**Ethics Committee Approval:** The study was approved by the Medical Ethics Committee of Shandong Provincial Hospital, affiliated to Shandong University (approval number: 2019-053).

**Informed Consent:** Patients or their parents/guardians provided verbal consent for anonymized data to be collated and analyzed. The authors are grateful to all the children and their parents for participating in this study.

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

### Authorship Contributions

Medical Practices: Fengxue Wang, Jinyan Han, Zengmin Wang, Xiaohong Shang, Concept: Guimei Li, Design: Fengxue Wang, Guimei Li, Data Collection or Processing: Fengxue Wang, Jinyan Han, Zengmin Wang, Xiaohong Shang, Analysis or Interpretation: Fengxue Wang, Jinyan Han, Guimei Li, Literature Search: Zengmin Wang, Xiaohong Shang, Writing: Fengxue Wang, Jinyan Han.

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# Effects of 5-Hydroxymethylfurfural on Pubertal Development of Female Wistar Rats

**Selin Elmaoğulları[1], Elçin Kadan[2], Elvan Anadol[3], Ayris Gökçeoğlu[4], Semra Çetinkaya[1], Gül Fatma Yarım[4], Seyit Ahmet Uçaktürk[5], Zehra Aycan[1]**

*[1]University of Health Sciences Turkey, Dr. Sami Ulus Children Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey*
*[2]University of Health Sciences Turkey, Gülhane Training and Research Hospital, Clinic of Pathology, Ankara, Turkey*
*[3]Gazi University, Laboratory Animal Breeding and Experimental Researches Center, Ankara, Turkey*
*[4]Ondokuz Mayıs University Faculty of Veterinary Medicine, Department of Biochemistry, Samsun, Turkey*
*[5]University of Health Sciences Turkey, Ankara Children Diseases Hematology and Oncology Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey*

### What is already known on this topic?

5-Hydroxymethylfurfural (HMF) is an organic compound that is present at high amounts in processed foods and foodstuffs as a result of heating, roasting, frying and toasting. Data on potential genotoxic, mutagenic, carcinogenic, DNA-damaging, organotoxic and enzyme inhibitory effects of HMF and its metabolites are conflicting. To the best of our knowledge there are no published data about the effects of HMF on pubertal development.

### What this study adds?

This is the first study of the effects of HMF on pubertal development. The results indicate that peripubertal exposure to HMF in high doses result in precocious puberty and decreased anti-Müllerian hormone levels in female Wistar rats.

## Abstract

**Objective:** 5-Hydroxymethylfurfural (HMF) is formed when sugars are heated in the presence of amino acids. HMF is naturally present in many foods. To investigate the toxic effects of HMF on the reproductive system of peripubertal rats.

**Methods:** In the study, 24 immature female Wistar rat were divided into three groups: control (CT) fed with no HMF; low dose fed with 750 mg/kg/day of HMF and high dose (HD) groups fed with 1500 mg/kg/day of HMF. All groups received these diets for three weeks from postnatal day (PND) 21. The vaginal opening (VO) was monitored daily and euthanasia occurred on PND 44. Gonadotropin, estradiol (E2), progesterone and anti-Müllerian hormone (AMH) concentrations were measured. Reproductive organ weights and ovarian follicle counts were compared.

**Results:** The HD HMF group had earlier VO. Higher mean luteinising hormone ($2.9 \pm 1.2$ vs $1.3 \pm 0.3$ mIU/mL) and mean E2 ($34.7 \pm 8.8$ vs $21.2 \pm 3.9$ pg/mL) and lower mean AMH ($2.7 \pm 0.5$ vs $4.7 \pm 0.7$ ng/mL) concentrations were found in the HD compared to the CT group. The HD group also had increased number of secondary atrophic follicles.

**Conclusion:** These results indicate that peripubertal exposure to HMF at HD result in precocious puberty and decreased AMH levels in female Wistar rats.

**Keywords:** Hydroxymethylfurfural, puberty, vaginal opening, anti-Müllerian hormone, rat

**Address for Correspondence:** Selin Elmaoğulları MD, University of Health Sciences Turkey, Dr. Sami Ulus Children Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey
**Phone:** +90 532 580 88 62 **E-mail:** ekerbicerselin@yahoo.com **ORCID:** orcid.org/0000-0003-4879-7859

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## Introduction

5-Hydroxymethylfurfural (HMF) is an organic compound produced by dehydration of fructose and glucose, through a non-enzymatic chemical reaction, in the presence of amino acids (1). The presence of HMF reduces protein digestibility and decreases the nutrition quality of foods. The concentration of HMF is widely used as a parameter to asses honey freshness and appropriate storage conditions (2). It is also ubiquitous in the human diet and is present in high concentrations in processed foods and foodstuffs as a result of heating, roasting, frying and toasting (3). HMF concentration is greater than 1 g/kg in dried fruits, caramel products and some fruit juices and up to 6.2 g/kg in instant coffee (4). It is also present in cigarette smoke, beer and medical products like parenteral solutions containing glucose and pharmaceutical syrups containing fructose (5,6,7,8). Additionally, HMF is used industrially in the production of polymers, surfactants, solvents, pharmaceuticals and plant protection agents (9).

Daily consumption of HMF from diet is estimated to be between 30-150 mg and safe levels of HMF consumption have not been clearly defined (7,10). While the effect of HMF on human health has long been the subject of research, it is not yet clear if HMF represents a potential health risk for humans by dietary exposure. There are conflicting data on potential genotoxic, mutagenic, carcinogenic, DNA-damaging, organotoxic and enzyme inhibitory effects of HMF and its metabolites (11,12,13,14). In terms of carcinogenic effect, HMF derivatives were found to cause hepatocarcinoma and increase skin tumor initiating activity in mice (15). Zhang et al (16) also showed that orally administered HMF in thermolyzed sucrose in rats initiates intestinal aberrant crypt foci formation and causes an increase in both number and size of these lesions in a dose dependent manner. However, in another murine study no evidence of intestinal aberrant crypt formation with HMF or its derivate was reported (17). The US National Toxicology Program (NTP) study of the toxicology and carcinogenesis of HMF in rats and mice, the most comprehensive study on toxic effects of HMF to date, revealed increased incidences of lesions of the olfactory and respiratory epithelium of the nose in rats and mice, and increased incidence of liver cancer in female mice after two years administration of oral HMF. The same study also revealed change in duration of estrous cycles and proportion of regular cycles which may point to possible fertility problems (4). Exposure of children to HMF has increased with changing eating habits in the last decades. No data on the possible toxic effects of HMF on pubertal development has been reported to date. Thus, the aim of this study was to evaluate whether peripubertal exposure to high levels of HMF had any effect on pubertal timing, reproductive organ growth, hormone levels and ovarian follicular development.

## Methods

This study was conducted in Gazi University Laboratory Animal Breeding and Experimental Researches Center (GÜDAM) and approved by Gazi University Local Ethics Committee for animal experiments (approval code: 17.025).

### Animals and Experimental Design

Twenty four Wistar albino rats, weaned on postnatal day (PND) 21, were divided into three equal sized groups (n = 8/group). The control (CT) group was given 5 mL/kg/day of tap water, the low dosage (LD) group was given 750 mg/kg day and high dosage (HD) group was given 1500 mg/kg/day of HMF (Sigma 25 mg 5-hydroxymethylfurfural, W501808-25G-K) (4). The treatments were performed orally (gavage), once daily for six days/week, at the same hour (between 9:00 and 10:00 AM), until PND 44. The groups were kept in different cages under identical conditions (22-24 °C, 25-30 % humidity, 12 hour light-dark cycle with free access to water and food). Each rat was weighed on PND 21, 26, 33, 40 and 44 just prior to feeding.

### Analysis of Vaginal Opening (VO)

The rats were examined for VO for the assessment of sexual maturity every morning between 9:00 and 10:00 AM. The procedure was performed visually without using a surgical loupe. To compare time of puberty, VO was scored as no VO (0 points), VO between PND 39-44 (1 point) and VO between PND 33-38 (2 points). The scale steps were set by dividing the time period (PND 33-44) that rats had VO into two.

### Euthanasia

The animals were anesthetized by intramuscular xylazine and ketamine (5 and 45 mg/kg, respectively) and then euthanized by cardiac puncture on PND 44, 24 hours after the last dosage of HMF. Blood samples were collected with cardiac puncture on termination day. After centrifugation, serum samples were stored at -80 °C until the time of analysis of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P) and anti-Müllerian hormone (AMH) levels.

### Measurement of Uterus Length, Organ Weight and Assessment of Follicular Score

After euthanasia, uterus and ovaries were dissected with a limited gross necropsy focused on reproductive organs. Ovaries and uterus were weighed to the nearest 0.001 g

with an electric scale (Sartorius Research R200D Electronic Semi-Microbalance). Organ weight per 100 mg of final body weight (relative organ weight) were calculated. In macroscopic analysis, both cervix lengths and uterine corns were measured from fundus to cervix of uterus individually, and the results were recorded. Afterwards, length of the longer corn and the cervical length were added to estimate uterus length. The ovaries and uterus were fixed in 10% buffered formalin, serial sections of 5 µm were made from the mid part of the ovaries and they were stained with haematoxylin and eosin. Four sections were evaluated from each ovary. Follicular quantitative analysis was performed in equidistant sections. Number of follicles at different stages was counted and grouped as healthy secondary, atrophic secondary, healthy tertiary and atrophic tertiary follicles. The follicle was defined as: 'primary', if the follicle had one layer of follicular cells; 'secondary', if the follicle had two or more layers of follicular cells and was larger than primary follicles; 'tertiary', if the follicle had a flood filled antrum and was "atretic", if the follicle had degenerate oocyte and/or degenerate layers of the membrana granulosa present (18,19). The follicular growth phases are shown in Figure 1. All microscopic analyses were performed with x4, x10, x20, and x40 magnification as a blind test.

### Hormonal Assays

The serum concentration of FSH was determined using a commercial rat-specific enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, E-EL-R0391, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer's instructions. The sensitivity of the assay was 1.88 ng/mL. The serum concentration of LH was measured using a commercially available rat-specific ELISA kit (Elabscience, E-EL-R0026, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer's instructions. The sensitivity of the assay was 0.94 mIU/mL. The serum concentration of E2 was measured using a commercially available rat-specific ELISA kit (LSBio, LS-F13008, 2401 Fourth Avenue Suite 900, Seattle, WA, USA) according to the manufacturer's instruction. The sensitivity of the assay was 15.6 pg/mL. The serum concentration of P was measured using a commercially available rat-specific ELISA kit (MyBioSource Inc., MBS762170, San Diego, CA, USA) according to the manufacturer's instruction. The sensitivity of the assay was $<0.188$ ng/mL. The serum concentration of AMH was measured using a commercially available rat-specific ELISA kit (Elabscience, E-EL-R0640, Memorial Drive, Suite 216, Houston, Texas, USA) according to the manufacturer's instruction. The sensitivity of the assay was 0.1 ng/mL. All of these assays were performed concurrently in duplicate and a standard curve was established for assay. Inter- and intra-assay variations were $<10\%$.

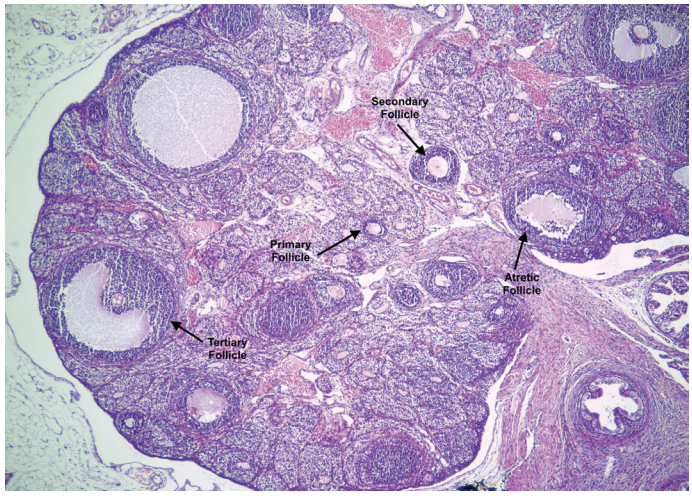


**Figure 1.** Ovarian photomicrograph from low dosage group showing growth phases of the follicles in x4

### Statistical Analysis

Statistical analysis of the data was performed with Statistical Package for the Social Sciences, version 20 (IBM Inc., Chicago, IL, USA) programme. Values were provided as mean ± standard deviation (minimum-maximum). Statistical significance was determined by Kruskal-Wallis one-way analysis of variance for multiple group comparisons and with the Mann-Whitney U test for two-group comparisons. Significance was accepted as $p<0.05$.

## Results

The study was completed with 23 animals, CT group (n = 8), LD group (n = 8) and HD group (n = 7) as one rat from the HD group died during the experiment from an unknown cause. The mean body weight of the rats was 42.5 ± 1.7 g on PND 21, at the beginning of the experiment. The mean body weight of the CT, LD and HD animals on PND 26, 33, 40 and 44 is given in Table 1. Although mean body weight differed among groups throughout the experiment, the difference was not significant between the groups at the end of the experimental period (PND 44).

Mean age at VO was PND 40 ± 3.2 (range 34-43) in the CT and 35.7 ± 2.7 (range 33-40) in the HD group. Three rats from the LD group did not have VO on termination day. The difference in time of VO was significant ($p=0.025$). The HD group had VO earlier than both the CT ($p=0.023$) and LD groups ($p=0.018$). According to the scale, VO seemed to be slightly delayed in the LD group compared to the CT group, however the difference was not significant (Table 2).

Serum FSH and P concentrations did not differ between the study groups. Serum LH concentrations were significantly higher in the HD group compared to the CT group ($p = 0.001$). However, there was no difference between serum LH concentration in the LD group and the CT group or between the HD group and the LD group. Serum E2 concentrations were increased in the HD group compared to the LD group ($p = 0.04$) and the CT group ($p = 0.01$). Serum AMH concentrations were significantly lower in the HD group compared to both the LD group ($p = 0.03$) and the CT group ($p = 0.01$) (Table 3).

The mean absolute and relative weight of ovaries and uterus lengths were not different between the groups. The mean absolute and relative uterine weight was increased in the HD group when compared to the CT group ($p = 0.037$ and $p = 0.005$ respectively). The mean number of healthy follicles also did not differ between the groups but the mean number of atrophic secondary follicles was increased in both the LD and HD groups ($p = 0.02$). Measurements of reproductive organs, numbers of follicles and hormone levels are shown in Table 3 and ovarian photomicrographs of each experimental group are shown in Figure 2.

## Discussion

The only study on reproductive and developmental toxicity of HMF was done by the US NTP concerning the toxicology and carcinogenesis of HMF in rats and mice. The study revealed that the duration of the estrous cycle was increased and that regular cycles were fewer in rats that were given 750 mg/kg/day or 1500 mg/kg/day of oral HMF for three months, starting from PND 42. These data indicated the potential of HMF to produce adverse effects in the reproductive system and for fertility (4). In the current study, 750 mg/kg/day or 1500 mg/kg/day of HMF was given orally to female rats starting on PND 21 for three weeks. Rats become sexually mature at the age of six weeks (20). To the best of our knowledge this is the first study investigating the effects of HMF on the reproductive system in sexually immature rats.

Although HMF is mostly present in high calorie foodstuffs, its direct effect on body weight and energy metabolism is controversial. In physiological analyses, redox metabolism is severely affected by HMF, while the effects on the energetics is less well established (21). We found no difference in mean final body weight between the CT and HMF groups. Zaitzev et al (22) reported no change in final body weight of rats receiving 40 mg/kg or 80 mg/kg of HMF for 11 months. The NTP study reported different results for different groups, loss in body weight of rats receiving HMF for three weeks or three months in doses exceeding 750 mg/kg/day and no change in body weight was reported in rats receiving HMF for two years at any dose (4). Heaton and Robinson (23) reported acceleration in body weight gain with 75-225 mg/kg of HMF for an unspecified duration, without giving a detailed description of nutrition conditions. However, it is not appropriate to compare these studies because of the different doses and durations of HMF consumption.

VO, a marker for pubertal onset in rodents, is caused by an apoptotic process in vaginal epithelial cells triggered by increased levels of estrogen. VO of rats of the same strain from different laboratories, or rats of the same strain and laboratory but from different litters, varies hugely. Mean VO time in Wistar rats was reported to range between $33.4 \pm 1.98$ and $41.6 \pm 3.7$ days, compatible with the mean VO of the CT group (24). VO in the HD group was also within the reported ranges but it was earlier than in the CT and LD groups. In addition, the E2 concentrations were higher in the HD group, which could be interpreted as high doses of HMF causing precocious puberty in female rats.

**Table 1. Mean body weight (g) of each experimental group on different postnatal days**

| Parameters | | | |
|---|---|---|---|
| **PND** | **CT (n = 8)** | **LD (n = 8)** | **HD (n = 7)** |
| **26** | $62.0 \pm 8.0$ | $55.3 \pm 5.8$* | $58.1 \pm 8.6$*, ** |
| **33** | $80.0 \pm 10.2$ | $74.6 \pm 7.8$* | $76.2 \pm 11.2$*, ** |
| **40** | $88.7 \pm 11.3$ | $87.4 \pm 9.2$* | $91.7 \pm 13.5$*, ** |
| **44** | $94.7 \pm 12.1$ | $96.8 \pm 10.2$ | $105.3 \pm 15.6$ |

*Significantly different ($p \leq 0.05$) from mean body weight of the CT.

**Significantly different ($p \leq 0.05$) from mean body weight of the LD.

PND: postnatal day, CT: control group, LD: low dosage group, HD: high dosage group

**Table 2. Vaginal opening time (in days) in different experimental groups**

| Parameter | Groups | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **CT** | | | | | | | | **LD** | | | | | | | | **HD** | | | | | | |
| **Subject** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| **PND** | 34 | 37 | 40 | 40 | 40 | 43 | 43 | 43 | 33 | 37 | 40 | 44 | 44 | - | - | - | 33 | 33 | 33 | 37 | 37 | 37 | 40 |
| **VO scale*** | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 2 | 1 |

*0: VO until the end of the experimental period, 1: VO between PND 39-44, 2: VO between PND 33-38.

PND: postnatal day, CT: control, LD: low dosage, HD: high dosage, VO: vaginal opening

Uterine weight increases as puberty progresses in rodents and this increase is associated with E2 levels. However, studies have reported that increased E2 levels my cause a decrease or no change in uterine weight of immature rats (25,26,27). These unexpected results were explained by altered sensitivity of the estrogen receptors in the uterus due to high E2 concentrations or due to the substance used in the experiment (27). In this present study, absolute and relative uterine weight was increased in the HD group and both LH and E2 concentrations were higher in the HD group compared to the CT and LD groups. It may be that HMF somehow activated the hypothalamo-pituitary system resulting in increased E2 concentrations, led to early VO and may also have caused an uterotrophic effect. Detailed physiological studies are needed to understand and explain the mechanism fully.

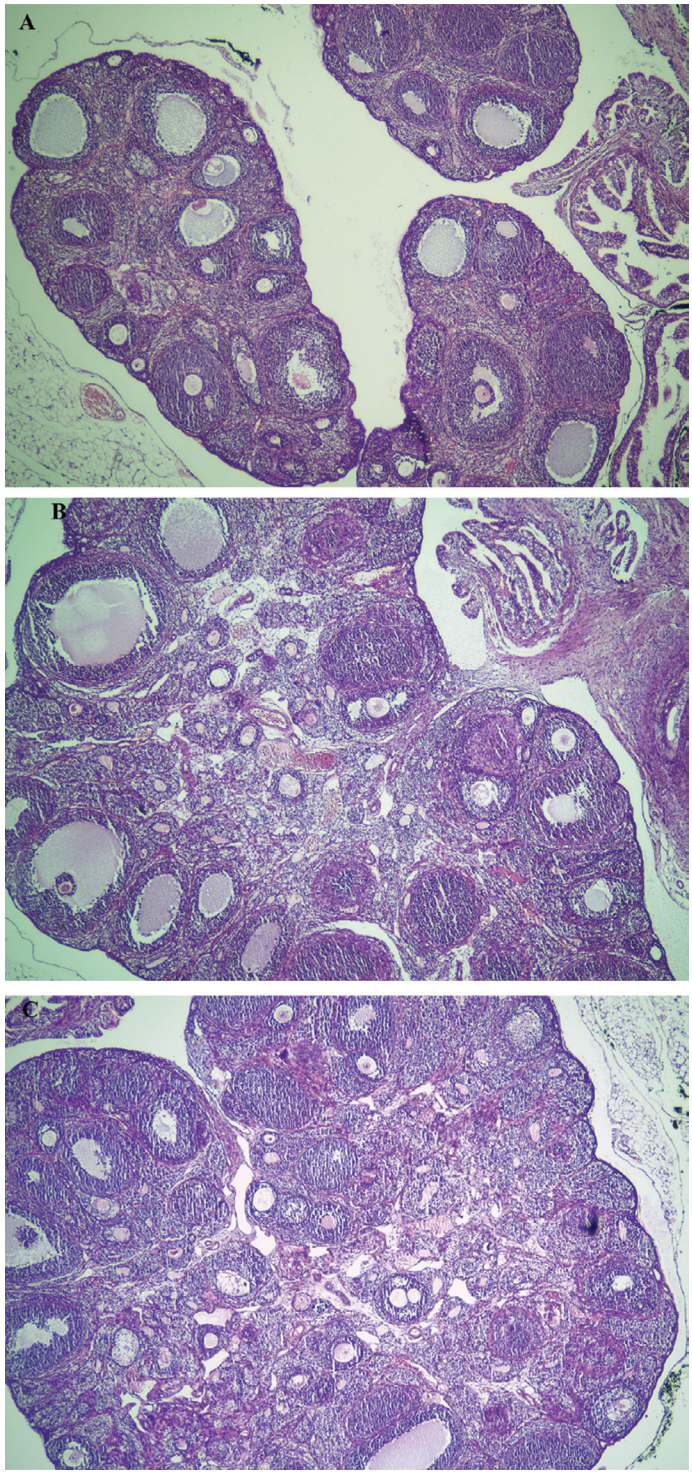


**Figure 2.** Ovarian photomicrographs of each experimental group in x4 magnitution. (A) Control group (B) low dosage group (C) high dosage group

Intense maturational changes in the hypothalamic-pituitary system are accompanied by an increase in gonadotropin response in the ovaries, resulting in development of gonadotropin related follicles. Measuring ovarian weight and microscopic examination are indispensable steps for female reproductive toxicology studies (28). In our study, absolute or relative ovarian weight did not vary between groups but number of atrophic secondary follicles was increased in the HMF groups compared to the CT group. Numerous atrophic follicles may be present at the normal peripubertal stage, before ovulation. As the rat matures and cyclicity is set after several cycles, the number of atrophic follicles decreases. Atrophic follicles are prominent in rats that are euthanized around PND 42 (28). However, ovarian toxicology studies have shown that an increased number of atrophic follicles was among the most common histopathologic features indicating ovarian detriment, even in rats at six weeks of age (29). As 3/8 LD rats did not have VO on necropsy day and 2/8 had VO the day before necropsy, an increased number of atrophic follicles in the LD group may be attributed to their immaturity, but the ovaries of the HD group seem to have been affected by HMF toxicity.

AMH is produced by growing ovarian follicles and reflects the antral follicle count (30). Rodent studies have shown that AMH has a critical role in initial follicle recruitment and selection of dominant follicles (31). Decrease in serum AMH correlates directly with the decrease in the number of growing follicles (32). In this study, AMH concentrations were significantly decreased in the HD group, which may indicate decreased ovarian reserve and HMF-related ovarian damage. Although the role of E2 in AMH expression is not clear, another possible cause for the decline in AMH concentrations may be increased concentrations of E2. Increased E2 has been shown to reduce the activation of AMH promoter in some *in vitro* studies (33,34,35). In contrast there are also studies supporting the opposite or showing that E2 has no direct effect on AMH (36,37,38). Thus, the relationship between AMH and E2 concentrations is still controversial.

**Table 3. Measurements of mean serum hormone concentrations, weight/length of reproductive organs and follicle counts of the study groups**

| Parameters (mean ± SD) | Groups | | |
|---|---|---|---|
| | CT (n = 8) | LD (n = 8) | HD (n = 7) |
| FSH (ng/mL) | 9.4 ± 1.9 | 10.1 ± 3.1 | 13.7 ± 3.6 |
| LH (mIU/mL) | 1.3 ± 0.3 | 2.2 ± 1.5 | 2.9 ± 1.2* |
| E2 (pg/mL) | 21.2 ± 3.9 | 20.1 ± 8.6 | 34.7 ± 8.8*, ** |
| Progesterone (ng/mL) | 10.1 ± 1.8 | 9.7 ± 1.6 | 11.2 ± 2.4 |
| AMH (ng/mL) | 4.7 ± 0.7 | 4.1 ± 0.8 | 2.7 ± 0.5*, ** |
| Absolute weight of ovaries (mg) | 58.6 ± 17.6 | 50.6 ± 11.6 | 60.2 ± 12.7 |
| Relative weight of ovaries (mg/%) | 59.4 ± 12.6 | 51.9 ± 9.0 | 57.4 ± 10.0 |
| Absolute weight of uterus (mg) | 208.5 ± 89.4 | 234.2 ± 115.7 | 368.3 ± 176.5* |
| Relative weight of uterus (mg/%) | 214.0 ± 77.4 | 242.5 ± 125.1 | 339.0 ± 141.0* |
| Uterus length (mm) | 27.6 ± 4.9 | 32.3 ± 7.1 | 32.0 ± 6.6 |
| Healthy secondary follicles (n) | 53.0 ± 16.4 | 77.8 ± 24.4 | 70.5 ± 21.3 |
| Atrophic secondary follicles (n) | 4 ± 1.6 | 6.1 ± 1.8* | 8.0 ± 4.0* |
| Healthy tertiary follicles (n) | 6.6 ± 2.6 | 8.5 ± 3.5 | 7.8 ± 3.8 |
| Atrophic tertiary follicles (n) | 2.1 ± 1.8 | 2.8 ± 1.2 | 2.8 ± 0.8 |
| Atrophic/total follicle (%) | 10.3 ± 7.0 | 9.8 ± 2.9 | 11.9 ± 3.8 |

*Significantly different (p≤0.05) from the control group.

**Significantly different (p≤0.05) from LD group.

FSH: follicle stimulating hormone, LH: luteinizing hormone, E2: estradiol, AMH: anti-Müllerian hormone, CT: control, LD: low dosage, SD: standard deviation

## Conclusion

HMF is present in numerous foodstuffs at high levels and peripubertal children have an increasing exposure to this potentially toxic metabolite with changing dietary habits. This is the first study of the toxic effects of HMF in peripubertal rats and it was shown that high doses of HMF given orally for three weeks caused early VO, an increased number of secondary atrophic follicles and decreased AMH concentrations. However, these results may not be directly related to humans given the experimental dosage and duration applied in this rat model. Therefore, there is a need for further studies to elucidate the mechanisms leading to these findings.

### Ethics

**Ethics Committee Approval:** Ethic board consent for the study was approved by the by Gazi University Local Ethics Committee for animal experiments (approval code: 17.025).

**Informed Consent:** Experimental rat study.

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

### Authorship Contributions

Surgical and Medical Practices: Elvan Anadol, Concept: Selin Elmaoğulları, Design: Selin Elmaoğulları, Semra Çetinkaya, Seyit Ahmet Uçaktürk, Zehra Aycan, Data Collection or Processing: Selin Elmaoğulları, Elçin Kadan, Elvan Anadol, Analysis or Interpretation: Elçin Kadan, Ayris Gökçeoğlu, Gül Fatma Yarım, Literature Search: Selin Elmaoğulları, Semra Çetinkaya, Seyit Ahmet Uçaktürk, Zehra Aycan, Writing: Selin Elmaoğulları.

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# Assessment of Bisphenol A Levels in Preschool Children: Results of a Human Biomonitoring Study in Ankara, Turkey

**İsmet Çok[1], Özlem Toprak İkidağ[1], Dilek Battal[2], Ayça Aktaş[2]**

*[1]Gazi University Faculty of Pharmacy, Department of Toxicology, Ankara, Turkey*
*[2]Mersin University Faculty of Pharmacy, Department of Toxicology, Mersin, Turkey*

### What is already known on this topic?

Bisphenol A (BPA) is an endocrine disrupting chemical and exposure to BPA is almost inevitable in daily life. Relationships between BPA exposure and various health risks have begun to be established. In different societies, BPA levels in young children seem to be higher in comparison with adolescents and adults.

### What this study adds?

This first study of biomonitoring in preschool children from Turkey is an important contribution to the limited information about childhood exposure to BPA in Turkey and the world. The magnitude of exposure of BPA by children, estimated daily intake was calculated first time for Turkish children in this study.

## Abstract

**Objective:** There is general concern regarding environmental chemical exposure and the impact it may have on human health. This is particularly important for vulnerable populations such as infants and children during critical periods of development. Bisphenol A (BPA) is an endocrine disrupting chemical used worldwide over the last 30 years in many consumer products. Evidence points to widespread human exposure to BPA. The aim of this study was to evaluate the exposure of Turkish preschool children to BPA.
**Methods:** This study was conducted as a preliminary investigation of BPA in urine, collected from 3-6 year old children living in Ankara. After spot urine samples were taken from preschool children, free BPA, β-D-glucuronide and total BPA were determined using high-performance liquid chromatography tandem mass spectrometry and adjusted by creatinine concentration.
**Results:** Preschool children from Ankara (n = 125; males n = 70, females n = 55; mean age: 4.50 ± 1.26) were recruited. BPA was detected in 76.8% of children from Ankara city, with urinary concentrations ranging from < limit of quantification to 18.36 µg/g creatinine. Total BPA levels were not statistically different between boys (1.26 µg/g creatinine) and girls (2.24 µg/g creatinine) ($p > 0.05$).
**Conclusion:** This study is an important contribution to the limited information about childhood exposure to BPA. The estimated daily BPA intake in this study is substantially lower than the European Food Safety Authority derived tolerable daily intake of 4 µg/kg BW/day.
**Keywords:** Bisphenol A, urine, children, liquid chromatography-mass spectrometry, Turkey

## Introduction

There is general concern regarding environmental chemical exposure and its impact on human health, but this is particularly important for vulnerable populations, such as infants and children during sensitive periods of development. In 1997 the leaders of the G8 countries stated, "*We acknowledge that, throughout the world, children face significant threats to health from an array of environmental hazards. The protection of human health remains a fundamental objective of environmental policies to achieve sustainable development. We increasingly understand that the health and well-being of our families depends upon a clean and healthy environment. Nowhere is this more true than in the case of children, who are particularly vulnerable to pollution*" (1). In addition, one of the biggest concerns of the World Health Organization (WHO) for children is exposure to chemicals during the

**Address for Correspondence:** İsmet Çok MD, Gazi University Faculty of Pharmacy, Department of Toxicology, Ankara, Turkey
**E-mail:** ismetcok@gmail.com **ORCID:** orcid.org/0000-0003-3128-677X
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intrauterine and childhood periods and associated health problems that arise later in life (2). In recent years, exposure to environmental pollution with chemicals known to act as endocrine disruptors (EDs) has been implicated in the incidence of many diseases and disorders.

Bisphenol A (BPA), with approximately 3.6 million tons annual global production (3), is an ED. The United States Environmental Protection Agency (EPA) estimates that more than 400,000 kilograms of BPA are leached into the environment every year (4). Due to the widespread use of BPA, over 90% of tested humans have detectable BPA, with the highest levels found in infants and children (5).

Childhood exposure to BPA occurs through specific exposure routes including mouthing, food intake, the use of BPA-containing products, inhalation, dermal contact and ingestion. Children are more susceptible to chemicals such as BPA than the general population due to their rapid development and increased food intake per kg body weight (2). BPA exposure has been linked to a range of adverse human health outcomes including decreased fertility, behavioural effects, disruption of endocrine function, altered development and increased prevalence of metabolic diseases (4,5). For example, relationships between BPA exposure and altered neurobehavioral outcomes including hyperactivity, attention problems, anxiety, and depression, in children have been reported by several human studies (6,7,8,9,10,11). Following the demonstration of a wide variety of adverse effects associated with BPA exposure in humans and laboratory animals over the last two decades, the Canadian Ministry of Health banned the import and marketing of infant feeding bottles made of polycarbonate in 2008, as BPA is used in the production process. In 2011, the European Union banned BPA use in the production of polycarbonate baby bottles and prohibited the sale and import of BPA-containing products that come in contact with food for children aged 0-3 (12). The same restrictions have been applied in Turkey since 2011.

Numerous studies estimate exposures to BPA using urinary biomonitoring. Most have focused on adults from different societies to quantify human exposure to BPA. These studies have shown large variations between participants and studies, but very limited data are available for young children (13,14). To our knowledge, data regarding human exposure to BPA in Turkey are scarce (15). The primary aim of this study was to quantify exposure of preschool children to BPA.

## Methods

### Chemicals and Reagents

All chemicals used were of analytical grade. BPA and creatinine standards were purchased from Sigma-Aldrich (St Louis, MO, USA). The isotope-labeled internal standards $^{13}C_{12}$-BPA (99%), creatinine-d3 and $^{13}C_{12}$-BPA β–D-glucuronide ($^{13}C_{12}$-BPA-GLU) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile (KGaA, Darmstadt, Germany) were used. Ammonium acetate was obtained from J. T. Baker (Phillipsburg, NJ, USA). Stock solutions of the BPA (100 ng/mL) and BPA-GLU (1000 ng/mL) were prepared in methanol and were stored at -20 °C. Deionized water (18.2 MΩ) treated with the Millipore (Simplicity, 185) Milli-Q water purification system (Elga Labwater Veolia, Anthony, France) was used for all aqueous solutions.

### Study Population

Urine samples were collected from preschool children (3-6 years old) between November 2015 and May 2016. Four day-care centers in Ankara, Turkey participated. Each parent completed a questionnaire about their children's dietary habits; exposure to BPA in their daily life, at home, and in the school environment; medical history; weight and height. The study was designed in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Approval for the study protocol was obtained from the Ethics Commission of Mersin University Clinical Research Ethical Committee in Mersin, Turkey (document number: 12.02.2015/37). Written informed consent was obtained from all parents of individual participants.

Each child provided a single spot urine sample collected in a 125 mL glass screw cap culture tube lined with polytetrafluoroethylene that had been previously cleaned with hexane. The sample was divided into aliquots. Urine samples from healthy children were collected in the evening, before a meal, to determine the spot urine concentrations of both free BPA and BPA-GLU.

The samples were kept cool until transportation to the study center and were immediately frozen at -20 °C until analysis.

### Sample Preparation

Sample preparation, chromatographic and mass spectrometric (MS) conditions were used as described previously (16). Briefly, $^{13}C_{12}$-BPA was used as a stable internal standard and added to the samples at the beginning

of the extraction. The BPA and BPA-GLU in 500 µL urine samples were purified by protein precipitation/dilution with 500 µL of acetonitrile and 50 µL of $^{13}C_{12}$-BPA and $^{13}C_{12}$-BPA-GLU. After protein precipitation, samples were centrifuged at 2250 rpm at 25 °C for 10 minutes. Total BPA values were calculated as reported previously. All other analytical data such as QA/QC assurance, matrix effects and data repeatability have been reported previously (16).

### Instrumental Analysis

Identification and quantification of free BPA and BPA-GLU were performed with an Agilent 1200 Series 6460 (Agilent Technologies, CA, USA) triple quadrupole MS with Jet-Stream atmospheric pressure electrospray ionization source and Mass Hunter data acquisition/Quantitation software. The HPLC system was equipped with a binary pump, vacuum degasser, low carryover autosampler and thermoregulated column compartment. Twenty microliters of the extract was injected onto an Agilent (Agilent Technologies, CA, USA) Pursuit 3 pentafluorophenyl propyl column (100 × 3.0 mm, 3 µm particle size). The mobile phases A and B consisted of 2 mM ammonium acetate in water and methanol respectively. The limits of detection for free BPA and BPA-GLU were 0.03 ng/mL and 0.10 ng/mL and the limits of quantification (LOQ) were 0.08 ng/mL and 0.33 ng/mL respectively. The tandem MS-MS was operated with negative electrospray ionization in the selected reaction monitoring (SRM) mode. Nitrogen was used as both curtain and collision gas. The monitored quantifier SRM transitions were 227.1 > 132.8 for free BPA, 403.1 > 113.1 for BPA-GLU, and 239.2 > 224.1 for $^{13}C_{12}$-BPA (internal standard).

### Creatinine Analysis in Urine

Both the free BPA and BPA-GLU values obtained in this study were corrected for creatinine. To assess the impact of creatinine adjustment on the total variance of spot urine samples, urine creatinine levels were analyzed using a modified method developed and validated for creatinine analysis by Park et al (17). Briefly, a 10 µL aliquot of urine was diluted with milli-Q water (1000-fold) and 100 µL (5 mg/L) of creatinine-d3 (internal standard, 5 mg/mL) was added. Creatinine was analyzed with LC-MS/MS in electrospray positive ionization mode and the SRM transitions monitored were 114.1 > 86.1 for creatinine and 117.2 > 89.2 for creatinine-d3. One microliter of the extract was injected onto an Agilent (Agilent Technologies, CA, USA) Zorbax SB-C18 chromatographic column (3 x 50 mm, 3.5 µm particle sizes). The mobile phases A (water) and B (methanol) both contained 2 mM ammonium acetate. The analysis for creatinine was achieved using isocratic conditions (80 % B).

### Estimated Daily Intake (EDI) Calculation

To understand the magnitude of BPA exposure in the children, EDI was calculated based on the assumption of urine excretion volumes of 0.4 L (ages 3-4 years) and 0.5 L (ages 5-6 years) for 24 hours for children (18). The daily exposure doses of BPA were estimated using the following equation:

$$\text{EDI (µg/kg bw/day)} = \frac{\text{Urinary BPA concentration (µg/L)} \times \text{Urinary output (L/day)}}{\text{Body weight (kg)}}$$

### Statistical Analysis

The statistical evaluations of the data were performed with Statistical Package for the Social Sciences, version 11.5 for Windows (IBM Inc., Armonk, NY, USA). Data were summarized as minimum, maximum, median, mean, geometric mean (GM), and standard deviation for total and each group. The normality of the data distribution was assessed with the Shapiro-Wilk test. The Mann-Whitney U test was used for multiple comparisons between groups. A p value less than 0.05 were accepted as statistically significant.

## Results

In this study, free BPA and glucuronide conjugate of BPA (BPA-GLU) were measured in 125 preschool children (55 females, mean age 4.42 ± 1.09 years and 70 males, mean age 4.56 ± 1.39 years) who lived in Ankara. Table 1 presents the distribution of the main characteristics of the study populations. Urinary total BPA concentrations (adjusted for creatinine) in females and males are presented in Table 2. Total BPA was determined in 76.8 % of the analyzed urine samples and BPA concentrations were equal to or above the LOQ of 0.08 ng/mL. Total urinary concentrations of BPA in Turkish preschool children ranged from LOQ-18.36 µg/g creatinine, with a mean concentration of 1.79 µg/g creatinine.

**Table 1. Population characteristics**

| | | Mean (min.-max.) |
|---|---|---|
| Age by sex (years) | Female (n = 55) | 4.42 (3-6) |
| | Male (n = 70) | 4.56 (3-6) |
| Total group | n = 125 | 4.50 (3-6) |
| Height (cm) | Female | 107 (78-126) |
| | Male | 109 (85-138) |
| Weight (kg) | Female | 18.28 (11-29) |
| | Male | 19.30 (12-36) |
| BMI (kg/m$^2$) | Female | 16.00 (12.15-23.99) |
| | Male | 16.21(11.81-26.67) |

BMI: body mass index, min.: minimum, max.: maximum

The mean concentrations of total BPA in female and male groups were 2.24 µg/g creatinine and 1.26 µg/g creatinine, respectively, and there was no statistically significant difference (p = 0.202). However, when the children were divided by age into < 4 years and > 4 years the mean BPA values of the < 4 years-old females were statistically higher than the males of the same age (p = 0.005) (Figure 1, Table 3).

For positive samples (values > LOQ) daily intakes ranged from 7 ng/kg bw/day to 2.916 ng/kg bw/day. The EDI for the preschool children was calculated as 35 ng/kg bw/day (GM) in this study. The mean EDI values were lower for the male group than the female group (Table 4), but this difference was not statistically significant (p > 0.05). For risk assessment, in 1993, the US EPA (19) and in 2006 the European Food Safety Authority (EFSA) (20) recommended 50 µg/kg bw/day dose as the tolerable daily intake (TDI) and reference dose for BPA exposure. The EFSA revised the TDI for BPA to 4 µg/kg bw/day in January 2015 (21). The

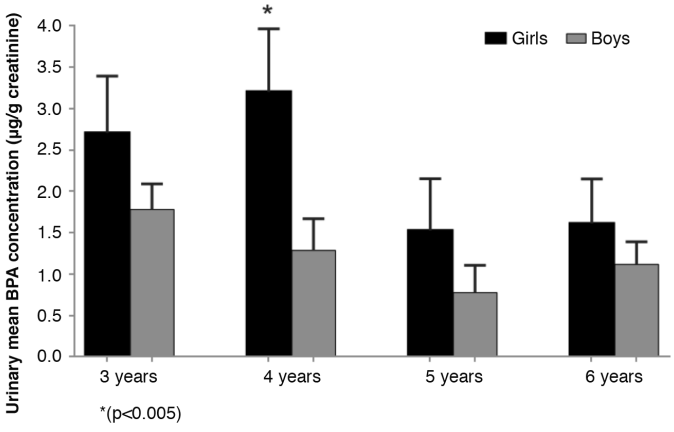


**Figure 1.** Urinary mean bisphenol A values obtained in age groups (µg/g creatinine)

GM and 95th percentile daily intakes of BPA determinated in both age groups and gender groups in this study were much lower than the guidelines established by the EFSA and US EPA (Table 4). This indicates that Turkish preschool childrens have a safe level of BPA exposure.

## Discussion

BPA is a high trade volume chemical because it is widely used in many consumer products and exposure is almost inevitable in daily life. In addition to being the first study to evaluate BPA exposure in preschool children in Turkey, the results of this study are important for providing basic data on BPA concentrations in the human population in Turkey. Studies assessing BPA exposure show that because of a dramatic increase in the use of BPA-containing products in daily life, BPA and its metabolites are present at detectable levels in nearly every person's blood, tissue and urine. In order to assess the exposure of humans to BPA, measurement of their urinary concentration of free species, in this case BPA, and target compound conjugates, in this case conjugated BPA, is essential (22,23). BPA in biological samples is found as both free and conjugated BPA. Among the conjugated BPAs, BPA-GLU is a sufficiently specific and stable compound that can be regarded as a biomarker to evaluate BPA exposure (16). Varying levels of BPA and BPA-GLU are detected in urine samples depending on nutrition and lifestyle.

Although there is a general concern about possible effects of exposure to environmental chemicals on human health, these concerns are especially important for susceptible groups such as babies and children, during critical stages of their development. One of the biggest concerns of the WHO regarding infants is health problems that will show

**Table 2. Urinary concentration of bisphenol A in Turkish preschool children (µg/g creatinine)**

| Total BPA | n | > LOQ[a] (%) | Min.-max. | Mean ± SD | Median | GM | 95% CI | p[b] |
|---|---|---|---|---|---|---|---|---|
| **Males** | 70 | 77 | LOQ-9.34 | 1.26 ± 2.16 | 0.50 | 0.81 | 0.51-1.77 | 0.2022 |
| **Females** | 55 | 76 | LOQ-18.36 | 2.24 ± 2.24 | 0.65 | 0.98 | 0.83-3.57 | |
| Σ | 125 | 77 | LOQ-18.36 | 1.79 ± 3.74 | 0.60 | 1.05 | 1.13-2.45 | |

[a]LOQ: limit of quantification, 0.33 ng/mL, [b]Mann-Whitney U test, GM: geometric mean, 95% CI: 95% confidence interval, BPA: bisphenol A, min.: minimum, max.: maximum, SD: standard deviation

**Table 3. Urinary bisphenol A values by age groups in females and males (µg/g creatinine)**

| Age | | Females | | | Males | | | p[a] |
|---|---|---|---|---|---|---|---|---|
| | | n | Median | Max. | n | Median | Max. | |
| < 4 years | 70 | 32 | 1.33 | 18.36 | 38 | 0.43 | 9.34 | < 0.005 |
| > 4 years | 55 | 23 | 0.30 | 16.6 | 32 | 0.56 | 8.89 | > 0.05 |

[a]Mann-Whitney U test.
max.: maximum

up later in life because of exposure to chemicals during the intrauterine and childhood periods. In particular, ED chemicals make important alterations in cellular pathways that provide a basis for these diseases (2). Hormones are the chemicals that regulate physiological homeostasis and functions of our body. These regimens are carried out in very small doses at the "picogram" level. Therefore, as a result of continuous exposure to EDs such as BPA, minor changes in hormone levels may cause major changes in biological function, particularly over the long term (2).

BPA is an ED (24) and is ubiquitous in the environment due to its widespread use in many consumer products globally over the past 30 years including in toys, baby bottles, plastic storage containers, heating containers for food and beverages, the lining of metal cans, medical equipment, consumer electronics and dental sealants, to give but a sample of the products containing BPA. A recent hypothesis states that BPA exposure may lead to many health risks (25), particularly obesity (26) and poor reproductive health (27). As exposure to this compound during a critical period, such as childhood, will provide a basis for exposure-related health problems, it is vital to determine the extent of BPA exposure in childhood both for the health of the individual and for future healthcare planning.

Numerous biomonitoring studies of children to quantifify childhood exposure to BPA during the last decade from different societies and different age groups have reported large variations between participants and studies. However, there are a limited number of studies of BPA exposure levels in preschool children. Huang et al (28) calculated the average global EDI of BPA for children based on the results of studies of children aged between 2-17 years from 18 nations between 2000 and 2016. The average global EDI for the children was 60.08 ng/kg bw/day in their study with the highest estimated child BPA daily intake found in Taiwan at 201.00 ng/kg bw/day, whereas Italy had the lowest with 15.34 ng/kg bw/day. The European Commission estimated the daily intake of BPA to be 0.4 µg/kg bw/day for adults, 1.2 µg/kg bw/day for children between 4 and 6 years, and 1.6 µg/kg bw/day for infants in EU countries (28). In our study, we detected half of the mean global value reported by Huang et al (28) (35 ng/kg bw/day).

**Table 4. Estimated daily intake of bisphenol A in Turkish preschool children (µg/kg bw/day)**

| | n | GM (95% CI) | 95th |
|---|---|---|---|
| Males | 70 | 0.031 (0.028-0.034) | 0.189 |
| Females | 55 | 0.042 (0.037-0.046) | 0.245 |
| Total | 125 | 0.035 (0.030-0.039) | 0.206 |
| Age 3 yrs | 35 | 0.038 (0.033-0.041) | 0.236 |
| Age 4 yrs | 35 | 0.046 (0.037-0.051) | 0.264 |
| Age 5 yrs | 25 | 0.024 (0.020-0.026) | 0.171 |
| Age 6 yrs | 30 | 0.036 (0.034-0.040) | 0.242 |

yrs: years, GM: geometric mean, 95% CI: 95% confidence interval

Due to anticipation that exposure of children might be particularly high, recent studies have determined the BPA exposure extent in preschool children in various countries. Some examples of these studies are summarized in Table 5, with a focus on studies assessing preschool children, similar to our study. These studies demonstrate that BPA levels tend to decrease with increasing age in almost every society. For example, in a Health Measures Survey conducted in Canada between 2007 and 2011, the youngest study group, consisting of children aged 6-8, had the highest BPA level (Table 5) (7). Similarly, the 3-5 year age group (GM 3.55 µg/L) had a higher urinary BPA concentration than the 6-8 (GM 2.72 µg/L), 9-11 (GM 2.22 µg/L), and 12-14 (GM 2.42 µg/L) year age groups in the German Environmental Survey for Children (29). These results indicate that younger people, particularly infants and children below the age of six, are subjected to greater exposure risk. Similar results were obtained in our study.

In this study, no significant associations between the consumption of various canned foods and beverages and BPA levels were found ($p > 0.05$) (Table 6). Urinary BPA levels of children consuming their food from heated plastic containers tended to be higher, but it was not statistically significant ($p > 0.05$). Dental materials made of BPA derivatives such as BPA-dimethacrylate and BPA-diglycidyl-dimethacrylate, have been used as an alternative to mercury amalgams in dentistry. Therefore, in this study, whether the children had white dental filling was also evaluated. Composite restorations were not associated with urinary BPA concentrations in our study ($p > 0.05$). Further, there were no statistical associations between BPA levels and the use of plastic materials and toys ($p > 0.05$).

A few studies have determined the BPA exposure level of individuals in Turkey. In 2014, mean urinary BPA values were 0.61 µg/g creatinine in 200 people from Mersin city (15). In a further study, BPA amounts were quantified for 26 female children aged 4-8 years having the endocrine condition Idiopathic Central Precocious Puberty (ICPP) and 21 healthy controls. The average BPA concentration was 1.62 µg/g creatinine in the healthy group, whereas this value was 8.34 µg/g for the ICPP group (30). As a result, the estrogenic effects of BPA may be an etiologic factor for ICPP. Similarly, a study was performed on newly diagnosed ICPP patients ($n = 42$; mean age $7.4 \pm 0.68$ years) and peripheral

**Table 5. Urinary bisphenol A concentrations in children from different countries**

| Country | Years | n | Ages (years) | Urinary BPA concentrations | References |
|---|---|---|---|---|---|
| Canada | 2007-2009<br>2009-2011 | 590 | 6-8 | 1.8 µg/L GM | 7 |
| China | 2014-2017 | 253 controls<br>215 ADHD | 6-12 | Controls (6-9 ages) 1.58 µg/L GM<br>ADHD (6-9 ages) 3.44 µg/L GM | 33 |
| Germany | 2007-2008 | 137<br>145 | 3-5<br>6-8 | 3.55 µg/L GM<br>2.72 µg/L GM | 29 |
| South Korea | 2001-2006<br>2016 | 164 | 3-5<br>7-9 | 0.76 µg/g creatinine median<br>0.61 µg/g creatinine median | 34 |
| USA | 2001-2010 | 408<br>401<br>318 | 3<br>5<br>7 | 7.4 µg/L mean<br>5.4 µg/L mean<br>5.8 µg/L mean | 35 |
| USA | 2004-2014 | - Non-hispanic white: 200<br>- Non-hispanic black: 96 | 1-8 | (White) 2.7 µg/L GM<br>(Black) 3.2 µg/L GM | 36 |
| Spain | 2011-2012 | 60<br>59 | 5-8<br>9-11 | 2.33 µg/g creatinine GM<br>1.72 µg/g creatinine GM | 37 |
| Australia | 2012-2013 | 64 boys<br>36 girls | 2-3 | (Boys) 3.09 µg/L GM<br>(Girls) 2.17 µg/L GM<br>2.72 µg/L GM | 13 |
| Portugal | 2014-2015 | 70 | 4-11 | 1.87 µg/g creatinine median | 14 |
| Turkey | 2015-2016 | 70 boys<br>55 girls | 3-6 | (Boys) 0.81 µg/g creatinine GM<br>(Girls) 0.98 µg/g creatinine GM | Çok et al. (this study) |

BPA: bisphenol A, GM: geometric mean; ADHD: attention deficit hyperactivity disorder

precocious puberty (PPP) patients (n = 42; mean age 7.4 ± 0.61) between August 2012 and July 2013 in Ankara. Urinary median BPA levels were 10.60 µg/g creatinine for these ICPP patients and 10.15 µg/g creatinine for PPP patients and 10.91 µg/g creatinine for control group (31).

In a very recently completed study, BPA was detected in 100% of 40 maternal urine samples (GM; 0.12 µg/L), their 1–2-month-old infant urine samples (GM; 0.13 µg/L) and breast milk (GM; 0.12 µg/L). However, these BPA concentrations were relatively low compared to previous studies (32). In another recent study, urinary BPA levels of 50 children with type 1 diabetes mellitus and of 50 healthy children, all aged between 5 and 18 years, were measured using HPLC (38). In this study, urinary BPA levels of children with type 1 diabetes mellitus and healthy children were found to be 27.71 ± 17.53 µg/g creatinine and 25.37 ± 17.89 µg/g creatinine respectively. These values are somewhat higher than the values found in our study and other previous studies. This may be due to the HPLC method used to determine urinary BPA levels. Since HPLC is not a low-precision chromatographic method for determining BPA levels, it is not currently preferred by researchers to determine low BPA levels in biological materials.

### Study Limitations

We believe that the present study makes an important contribution to the limited information about exposure to BPA during childhood. Although 125 children from Ankara were included in this study, this number is not sufficient for this type of population biomonitoring study. However, our results might be evaluated as preliminary finding for Turkish children. In order to provide a better understanding of exposure to BPA, studies on a larger population are needed and daily exposure levels from different sources should be determined.

## Conclusions

From a global perspective, the average BPA exposure for children is much higher than in adult populations. Although regulations have attempted to prevent BPA exposure of food origin in infants and children, the extensive use of BPA in other commodities places humans in an environment with abundant levels of this chemical. Increasing knowledge of BPA-related toxicity in children indicates that exposure to BPA leads to health risks, especially in sensitive developmental periods. The determination of the exposure to BPA through biomonitoring, specifically in children, is very

**Table 6. Bisphenol A-specific questionnaire data of the study population, mean ± standard deviation**

| | n | % | BPA-total (µg/g creatinine) |
|---|---|---|---|
| **FAST FOOD CONSUMPTION HABIT** | | | |
| None | 11 | 8.8 | 1.63 ± 2.63 |
| 1 meal/month | 67 | 53.6 | 1.94 ± 3.77 |
| 1-3 meals/month | 39 | 31.2 | 2.04 ± 3.83 |
| 3-5 meals/month | 8 | 6.4 | 1.63 ± 2.01 |
| **PLASTIC COATED FOOD PRODUCT PURCHASE** | | | |
| Never | 27 | 21.6 | 2.44 ± 0.86 |
| Sometimes | 86 | 68.8 | 1.47 ± 2.76 |
| Always | 12 | 9.6 | 2.92 ± 3.83 |
| **HEATING FOOD WITHIN A PLASTIC CUP IN MICROWAVE** | | | |
| Yes | 10 | 8 | 2.88 ± 5.58 |
| No | 115 | 92 | 1.72 ± 3.05 |
| **CONSIDERING INSTRUCTIONS FOR PACKAGING AND LABELLING** | | | |
| Sometimes | 40 | 32.0 | 2.11 ± 3.63 |
| Always | 82 | 65.6 | 1.70 ± 3.19 |
| Never | 3 | 2.40 | 1.99 ± 2.87 |
| **EXISTENCE OF WHITE DENTAL FILLING** | | | |
| Yes | 11 | 8.80 | 0.62 ± 0.91 |
| No | 114 | 91.2 | 1.93 ± 3.43 |
| **QUALITY OF THE PURCHASED PLASTIC** | | | |
| I wouldn't purchase low priced products | 25 | 20.0 | 2.33 ± 4.71 |
| I wouldn't mind | 24 | 19.2 | 1.22 ± 1.46 |
| I would purchase well-known brands | 32 | 25.6 | 2.65 ± 4.31 |
| **FEATURE OF THE PURCHASED TOY** | | | |
| Mostly metal toys | 11 | 8.8 | 2.22 ± 3.43 |
| Mostly wooden toys | 8 | 6.4 | 1.70 ± 1.12 |
| Mostly plastic toys | 106 | 84.8 | 1.82 ± 3.45 |
| **WASHING THE PLASTIC FOOD CONTAINERS IN A DISHWASHER** | | | |
| Yes | 55 | 44.0 | 2.78 ± 3.31 |
| No | 75 | 56.0 | 1.94 ± 4.04 |
| **CANNED FOOD CONSUMPTION** | | | |
| Yes | 10 | 8.0 | 1.70 ± 3.19 |
| No | 115 | 92.0 | 2.94 ± 3.77 |
| **CANNED BEVERAGES CONSUMPTION** | | | |
| Yes | 78 | 62.4 | 2.74 ± 1.80 |
| No | 47 | 37.6 | 1.67 ± 2.87 |
| **STORAGE OF FOOD IN PVC CONTAINERS** | | | |
| Sometimes | 33 | 26.4 | 2.41 ± 3.13 |
| Always | 92 | 73.6 | 2.65 ± 2.96 |
| Never | - | - | - |

BPA: bisphenol A

important for public health in all countries. This first study of biomonitoring in preschool children from Turkey should be followed by other studies from large communities from various provinces. Future studies should focus on children living in different provinces and extended follow-up of those exposed to high concentrations of BPA during critical

developmental periods should be undertaken to evaluate the likely long-term impact of these EDs. It is believed that the results of this study will enhance awareness not only for BPA, but also for other chemical compounds, amongst health care professionals with an empahsis on pre-school aged children.

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### Ethics

**Ethics Committee Approval:** The study was approved by the Ethics Commission of the Mersin University Clinical Research Ethical Committees (protocol number: 12.02.2015/37).

**Informed Consent:** Written informed consent was obtained from the childrens' parents.

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

### Authorship Contributions

Concept: İsmet Çok, Design: İsmet Çok, Sample Collection and Analysis: Dilek Battal, Özlem Toprak İkidağ, Ayça Aktaş, Analysis or Interpretation: Özlem Toprak İkidağ, Dilek Battal, İsmet Çok, Literature Search. Özlem Toprak İkidağ, Writing: İsmet Çok.

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# Evaluation of Turner Syndrome Knowledge among Physicians and Parents

**Berna Eroğlu Filibeli[1], Nesrin Havare[2], Huriye Erbak Yılmaz[3], Jülide Gülizar Yıldırım[4], Gönül Çatlı[5], Bumin N. Dündar[5]**

*[1]University of Health Sciences Turkey, Tepecik Training and Research Hospital, Clinic of Pediatric Endocrinology, İzmir, Turkey*
*[2]University of Health Sciences Turkey, Tepecik Training and Research Hospital, Clinic of Pediatrics, İzmir, Turkey*
*[3]Katip Çelebi University, Atatürk Traning and Research Hospital, Clinic of Biochemistry, İzmir, Turkey*
*[4]Katip Çelebi University Faculty of Health Sciences, Department of Public Health Nursing, İzmir, Turkey*
*[5]Katip Çelebi University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey*

### What is already known on this topic?

Turner syndrome (TS) is one of the most commonly observed chromosomal abnormalities, estimated at around 1 in 2500 live births. To the best of our knowledge, there are no studies related to the incidence of TS in Turkey. Nevertheless, in a multicenter study carried out in 2013-2014, 842 patients with TS between 0-18 ages were examined retrospectively in 35 different centers, and the average diagnosis age was determined as 10.2 ± 4.4 years. It is thought that TS is diagnosed at a later age in Turkey.

### What this study adds?

This study shows that physicians do not have adequate knowledge of TS. Poor knowledge about TS may increase diagnosis delays. The education program about TS should be revised and implemented to address this problem at the medical faculty and post-graduate levels.

## Abstract

**Objective:** Turner syndrome (TS) is one of the most common chromosomal abnormalities and an important cause of short stature and infertility due to ovarian failure in females. The aim was to evaluate the knowledge of TS among physicians and parents of children with TS and to enhance awareness about this subject.

**Methods:** One hundred and forty physicians were included in the study. The study population comprised 37 pediatricians (26.4%), 15 gynecologists (10.7%), 88 family physicians (62.9%) and 30 parents who had daughters with a diagnosis of TS. Two separate questionnaires were administered to evaluate TS knowledge of physicians and parents.

**Results:** According to the self-reports of physicians, 49% had insufficient knowledge of TS, while 15.7% indicated that they had no knowledge of TS. The mean percentage of correct answers was 50.71 ± 16.17% for all physicians. When the entire group of physicians was considered, 67.1% of them did not know the approximate incidence of TS, while 14.3% of them incorrectly indicated that TS was a condition that was seen in boys. The mean percentage of correct answers among parents was 68 ± 15%, and there was no difference between the mothers' and fathers' correct answer rates (p = 0.063). The majority of parents was not aware of TS-associated diseases and increased malignancy risk in TS.

**Conclusion:** Physician knowledge of TS was poor and that there is a need for continued education about TS at the medical faculty and post-graduate levels.

**Keywords:** Turner syndrome, knowledge levels, questionnaire, education

**Address for Correspondence:** Berna Eroğlu Filibeli MD, University of Health Sciences Turkey, Tepecik Training and Research Hospital, Clinic of Pediatric Endocrinology, İzmir, Turkey
**Phone:** +90 232 469 69 69 **E-mail:** bernafilibeli@gmail.com **ORCID:** orcid.org/0000-0002-2696-0195

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## Introduction

Turner syndrome (TS) is a sex chromosome abnormality in females, characterized by partial or complete loss of one of the X chromosomes (1). In nearly half of patients, it can be diagnosed in infancy with the presence of typical clinical findings. While a limited number of patients with TS are diagnosed with short stature during childhood, the rest of them are diagnosed with primary amenorrhea in adolescence (2). Through early diagnosis and appropriate treatment of these patients (growth hormone treatment, estrogen replacement, training and psychological support), they have a chance to participate in academic and social life, and they also achieve nearly normal adult height, bone density and sexual development. Several studies have focused on diagnosing TS earlier (3,4,5). Chronic complications may be prevented by earlier diagnosis and initiating treatment at birth or during infancy. Additionally, parents can deal with the situation more easily with early TS diagnosis (6).

Although TS is common, the exact incidence of TS in Turkey is unknown, and awareness regarding this issue may be inadequate. Patients with TS are diagnosed late in Turkey. Therefore, in this study, we aimed to evaluate the TS knowledge and awareness levels of physicians and parents of children with TS.

## Methods

This descriptive study was a questionnaire survey. The researchers designed two questionnaires; one to be administered to physicians volunteers (n = 140) and the other to parents (n = 30). The questionnaire for the physicians was developed based on the current literature, guidelines, and expert opinions (7,8). The questionnaire for the parents was developed based on family information flyers from the internet and expert opinions (TS: a guide for families https://turnersyndromefoundation.org/wp-content/uploads/2017/08/New-Turner-Syndrome-Guide-for-Families-Patricia-Reiser-CFNP-and-Marsha-Davenport-MD.pdf and http://nhfv.org/wp-content/uploads/2016/02/Turner-Syndrome-A-Guide-for-Families.pdf). This study included pediatricians, gynecologists, family physicians and parents whose children were diagnosed with TS. Ethics committee approval for the surveys was obtained from the Katip Çelebi University Local Ethics Committee (date: 16 June 2016, ethics approval number: 194). The physicians and parents in question were informed about the questionnaire, and surveys were performed face-to-face by NK after the informed consent form had been signed. An attempt was made to word all questions in a neutral manner. All response data of the participants were analyzed anonymously. No incentives were provided to the respondents.

The first physician survey comprised 18 multiple-choice questions. The first four questions assessed the physicians' specialties, proficiency regarding TS knowledge, number of years working in their profession and the institutions that they work for. The other 14 questions included TS epidemiology, clinical findings, diagnosis, treatment and follow-up recommendations. There were 19 "yes/no" questions in the survey designed for the parents. The first few questions concerned the demographic features (age, gender and education status) of the parent, and the remaining questions concerned the diagnosis, treatment and follow-up of TS. The patients' age at diagnosis was obtained from medical records. The answers were evaluated as correct or incorrect by researchers. Examples of the questionnaires applied to both physicians and parents are given in the supplementary documents (Supplementary 1, 2).

### Statistical Analysis

The sample size was calculated according to the estimated size within the sampling universe using the formula referred to as 'the formula to estimate the number of individuals for a known sample and width of population' (9).

The analysis of physician survey data was carried out using Statistical Package for the Social Sciences, version 22.0, program (IBM Inc., Armonk, NY, USA), and the percentage distribution was performed using the chi-square test. Descriptive statistics for family surveys, however, were presented as frequency, percentage, average, standard deviation, median, minimum, maximum and range values. In the analysis of differences between measurement values of the two groups, the Mann-Whitney U test or the independent sample t-test were used according to the distribution. A significance test for the difference in two proportions and a Pearson chi-square test were used. A $p < 0.05$ was considered statistically significant.

## Results

A total of 140 physicians working at training and research hospitals, state hospitals, university hospitals and primary care clinics (PCCs) and 30 parents whose children had been diagnosed with TS were included in the study. Of the physicians, 62.9% were family physicians, 26.4% were pediatricians and 10.7% were gynecologists. A total of 50.7% of physicians were working at training and research hospitals, 15% at universities, 3.6% at state hospitals and 30.7% at PCCs and 62.9% of them had been working for 10 years or less.

### Physician Knowledge of Turner Syndrome

Thirty-five percent of physicians self-reported that their knowledge level of TS was adequate, 49.3% indicated that their knowledge was insufficient and 15.7% reported having no knowledge of TS. When all the physicians were considered, the rate of correct answers was 50.71 ± 16.17%. The percentages of correct answers among the 88 family physicians, 37 pediatricians and 15 gynecologists were 46.08 ± 16.51, 56.2 ± 19.02 and 58.3 ± 20.4, respectively.

Responses to several questions related to the frequency and findings of TS are presented in Table 1. For the question concerning chromosomal abnormality, the pediatricians' accurate answer rate was higher than that of other specialties (p = 0.023). The question that referred to the type of hypogonadism was answered incorrectly by 72.1% of all physicians; however, 53.3% of gynecologists answered it correctly.

Gynecologists had the highest accurate answer rate related to fertility and malignancy questions (p = 0.028); 64.3% of physicians mistakenly thought that the intelligence level of patients with TS was low. Pediatricians were significantly more well-informed regarding this issue (p = 0.018). Approximately 63.6% of the physicians gave incorrect answers to the question regarding estrogen and growth hormone treatment (Table 2).

### Knowledge of Parents About Turner Syndrome

Thirty parents whose children were diagnosed with TS participated in the study. The mean age of girls with TS was 58.8 ± 50.95 months. The median diagnostic age was 66 months (1-168 months). The parents' percentage of correct answers was 68 ± 15%, and no significant difference was found between mothers and fathers (mothers 74%, fathers 63%; p = 0.063). The rate of correct responses among parents was higher than that of physicians, but the difference was not significant. Parent responses to several questions regarding TS are presented in Table 3.

The median correct response rate of primary school graduates was 74% (range, 37-84%), and the median correct response rate of high school or university graduates was 66% (range, 32-89%). There was no significant difference between the parents according to their educational status (p = 0.690). However, the median (range) age at diagnosis was significantly younger in children of parents who graduated from high school [21 months (1-120) vs. 90 months (1-168); p = 0.008].

**Table 1. Numbers and percentages of item responses on the survey of physician knowledge about Turner syndrome frequency and findings**

| | Number of correct answers (%) | | | p |
|---|---|---|---|---|
| | Gynecologists (n = 15) | Pediatricians (n = 37) | Family physicians (n = 88) | |
| Frequency | 6 (40%) | 15 (40.5%) | 25 (28.4%) | 0.345 |
| Gender | 12 (80%) | 31 (83.3%) | 77 (87.5%) | 0.690 |
| Chromosomal abnormality | 13 (86.7%) | 35 (94.6%) | 65 (73.9%) | **0.023** |
| Hypergonadotropic or hypogonadotropic hypogonadism | 8 (53.3%) | 12 (32.4%) | 19 (21.6%) | **0.031** |
| Physical examination findings | 9 (60%) | 23 (62.2%) | 33 (37.5%) | **0.022** |
| Most common finding: short stature | 9 (60%) | 26 (70.3%) | 43 (48.9%) | 0.084 |

**Table 2. Number and percentages of the item responses on the survey of physician knowledge about diseases associated with Turner syndrome**

| | Number of right answers (%) | | | p |
|---|---|---|---|---|
| | Gynecologists (n = 15) | Pediatricians (n = 37) | Family physicians (n = 88) | |
| The most common cardiovascular disease | 6 (40%) | 21 (56.8%) | 31 (35.2%) | 0.083 |
| Fertility | 10 (66.7%) | 10 (27%) | 33 (37.5%) | **0.028** |
| Intelligence level | 3 (20%) | 20 (54.1%) | 27 (30.7%) | **0.018** |
| TS and malignancy | 10 (66.7%) | 14 (37.8%) | 27 (30.7%) | **0.027** |
| TS and osteoporosis coexistence | 13 (86.7%) | 23 (62.2%) | 56 (63.6%) | 0.192 |
| Definitive diagnosis | 12 (80%) | 33 (89.2%) | 70 (79.5%) | 0.427 |
| *SHOX* gene mutation | 5 (33.3%) | 15 (40.5%) | 25 (28.4%) | 0.413 |
| Estrogen and growth hormone treatment | 6 (40%) | 12 (32.4%) | 33 (37.5%) | 0.826 |

**Table 3. Number and percentages of the item responses on the survey of parent knowledge about Turner syndrome**

| | Mother's correct answers n (%) | Father's correct answers n (%) | p |
|---|---|---|---|
| Genetic disorder | 16 (94.1%) | 11 (84.6%) | 0.565 |
| Only girls are affected | 14 (82.4%) | 9 (69.2%) | 0.666 |
| Prenatal diagnosis possibility | 12 (70.6%) | 8 (61.5%) | 0.705 |
| The most common finding | 13 (76.5%) | 11 (84.6%) | 0.672 |
| Chromosome abnormality | 14 (82.4%) | 11 (84.6%) | 1.0 |
| Pregnancy probability | 14 (82.4%) | 7 (53.8%) | 0.123 |
| Normal intelligence | 13 (76.5%) | 10 (76.9%) | 1.0 |
| Congenital heart disease possibility | 11 (64.7%) | 7 (53.8%) | 0.821 |
| Definitive treatment | 9 (52.9%) | 4 (30.8%) | 0.399 |
| Short stature can be treated with growth hormone treatment | 13 (76.5%) | 9 (69.2%) | 0.698 |
| Risk of cancer | 5 (29.4%) | 2 (15.4%) | 0.427 |
| Risks of diabetes mellitus, thyroid and celiac disease | 8 (47.1%) | 4 (30.8%) | 0.465 |
| Growth hormone treatment can prevent infertility | 12 (70.6%) | 5 (38.5%) | 0.165 |
| Estrogen treatment can prevent osteoporosis | 11 (64.7%) | 9 (69.2%) | 1.0 |
| Radiological evaluation for renal disease is necessary | 14 (82.4%) | 8 (61.5%) | 0.242 |
| Psychiatric evaluation is required | 14 (82.4%) | 9 (69.2%) | 0.66 |
| Clinic follow up is important | 14 (82.4%) | 8 (61.5%) | 0.242 |

## Discussion

TS is one of the most commonly observed chromosomal abnormalities with an incidence of 1 in 2500 live births and effects nearly 1.5 million women in the world (2,10,11). There are no studies related to the incidence of TS in Turkey. Nevertheless, in a multicenter study carried out in 2013-2014, 842 patients with TS between 0-18 ages were examined retrospectively in 35 different centers, and the average diagnosis age was determined as $10.2 \pm 4.4$ years (12). In a study carried out in England, it was estimated that there were 12,500 TS cases; however, it is known that there are approximately 1000 cases in TS support associations and expert hospital clinics. This means that a large number of cases cannot be diagnosed and do not receive medical care (13). Compared to developed countries, it is thought that TS is diagnosed at a later age in Turkey. This is most likely due to the lower awareness level of Turkish physicians. For this reason, we aimed to investigate TS knowledge and awareness levels of physicians and parents whose children were diagnosed with TS. In the survey, only just over half ($50.71 \pm 16.17\%$) of all questions were correctly answered by physicians. It is not possible to compare our results with previous ones because, to the best of our knowledge, no study on this topic has been published in the past in Turkey or in other countries.

The question related to short stature, the most common finding in TS, was not answered properly by 51.1% of family doctors and 29.7% of pediatricians. The growth curve and monitoring of children are important in primary care. The reason for the insufficient knowledge level of physicians may be that they do not encounter such patients because there is no referral chain system. When there is no referral chain, it is difficult for family physicians to maintain health care services, and this situation forms the weakest point of the family medicine practice. In the study by Kringos (14), this situation was given as one of the most important factors why Turkey ranks in the poor category for primary care health services. The final adult height of TS patients is positively related to a younger age at diagnosis and the duration of growth hormone treatment (15). Primary care physicians missing short stature will result in late diagnosis and insufficient benefit from growth hormone treatment for all children who would have benefitted, including girls with TS. When physicians are asked about the intelligence level of children with TS, 64.3% of them answered incorrectly. The inadequate knowledge level of physicians about this issue can cause children diagnosed with TS to be guided in a wrong way and perhaps lead to their exclusion from society. Although patients with TS tend to have some problems with mathematics, these can be overcome with additional time and adequate education. The overall education level of women with TS is equal to or better than that of the overall female population (16). When educational and psychological support is commenced early in TS, it can help academic success and social integration.

The question about high gonadotropin levels in TS was answered more correctly by gynecologists, compared to the physicians in other specialties. This shows that undiagnosed and late-diagnosed girls sought the care of gynecologists with a primary amenorrhea complaint. Although family physicians and pediatricians had inadequate knowledge regarding fertility, 66.7% of gynecologists answered the question correctly. This could be explained by the fact that patients diagnosed with TS consult them for infertility treatment. Most women with TS will be infertile; however, pregnancy has been achieved with oocyte donation and *in vitro* fertilization (17).

Today, many diseases can be diagnosed with simple scanning programs, and in this way, more significant complications can be prevented. The standard approach for cardiac evaluation in TS is echocardiography and four extremity blood pressure measurements that should be performed on every patient at the time of diagnosis (18,19). Even if echocardiography is normal, every patient should be evaluated with magnetic resonance imaging as soon as it is feasible without the need for general anesthesia (7,20). Physicians responded to the question related to cardiovascular disease incorrectly 58.6% of the time. A lack of knowledge can cause late-diagnosed cardiovascular system diseases and increased mortality. There is an increased risk of gonadoblastoma in patients with TS carrying Y chromosome fragmentation, and it is known that remocal of streak gonads are performed by obstetricians and gynecologists. It was not surprising that gynecologists were more knowledgeable than other physicians in terms of the combination of TS and malignancy.

The TS knowledge level of physicians was determined to be unsatisfactory when compared with the knowledge of TS parents. As families research TS in a detailed way after diagnosis, it is not wrong to expect their knowledge level to be higher. Parents want to obtain all information related to the disease since TS is an unknown disease in society and therefore there is an increased level of concern in families. Parents responded with 90% correct answers to the question about TS being a genetic disease. We can imply that this chronic condition led to desperation in families, which increased their solution-oriented quests. Nevertheless, parent knowledge was not sufficient in relation to the diseases accompanying TS and parents should be informed by specialists about this. In a study performed on children with chronic disease, it was reported that there was an important effect of the relationship between the families of hospitalized children and the nurses conducting the care of the sick children. However, the physicians were not well informed about the problems regarding psychosocial adjustment (21,22). Meeting the psychosocial and educational requirements, as well as the medical requirements, of the population affected by chronic disease will increase the childrens' and families' quality of life in both the acute and follow-up periods. Additionally, it will positively affect communication between health personnel and families.

The awareness level of primary care family physicians, pediatricians and gynecologists should be enhanced in the areas of early diagnosis and treatment to decrease mortality and morbidity in patients with TS. Our study has revealed troubles related to this issue. It has been shown that major social campaigns are effective in the renewal of knowledge for both families with sick children and physicians, and there is an apparent increase in the early diagnosis of diseases (16). Education programs must be regularly applied to staff by experts in issues such as the requirements of ill children and their parents, and all staff members must be offered counselling services in healthcare organizations. Qualified staff must be employed to apply psychological support, social orientation, and special programs for sick children and their families in healthcare organizations. Therefore, the results show that education programs should be maintained following graduation as well. New early diagnosis strategies should be developed to overcome the delay of treatment in patients with TS.

### Study Limitations

The limitation of this study is the relatively small number of physicians and parents of girls with TS. Accordingly, generalizations from these findings to the total population of physicians and families with children diagnosed with TS must be made cautiously.

## Conclusion

This study indicates that the knowledge about TS of physicians (especially family physicians) was insufficient, although TS is a relatively common disease. To prevent late diagnosis, increased complications and inadequate treatment in patients with TS, post-graduation education programs for physicians should be increased, and the referral chain of the patient must be applied in the health system. The parents' answers showed that they were worried about TS and the associated problems (short stature, infertility, etc.) and they sought information about TS from clinicians, brochures and the internet. Intermittent information and training programs should be organized for families with TS. Cooperation between the physicians and parents provides

better follow-up for these children and better control of the accompanying conditions related to TS.

### Ethics

**Ethics Committee Approval:** The study protocol was approved by the Katip Çelebi University Ethical Committee (approved number: 16/06/2016-194).

**Informed Consent:** The physicians and parents in question were informed about the questionnaire, and surveys were performed face-to-face by NK after the informed consent form had been signed.

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

### Authorship Contributions

Medical Practices: Berna Eroğlu Filibeli, Nesrin Havare, Huriye Erbak Yılmaz, Gönül Çatlı, Bumin N. Dündar, Concept: Bumin N. Dündar, Design: Bumin N. Dündar, Data Collection or Processing: Nesrin Havare, Huriye Erbak Yılmaz, Jülide Gülizar Yıldırım, Analysis or Interpretation: Jülide Gülizar Yıldırım, Berna Eroğlu Filibeli, Gönül Çatlı, Bumin N. Dündar, Literature Search: Berna Eroğlu Filibeli, Nesrin Havare, Gönül Çatlı, Bumin N. Dündar, Writing: Berna Eroğlu Filibeli, Nesrin Havare, Gönül Çatlı, Bumin N. Dündar.

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**Supplementary 1.** The questionnaire for physicians about Turner syndrome

*Please select the right option according to you*

**Descriptive information**

1. Your branch?
   a. Pediatrician
   b. Gynecologists
   c. Family physicians
2. How would you describe your level of knowledge about Turner syndrome?
   a. Adequate
   b. Insufficient
   c. No idea
3. How many years have you practiced in your profession?
   a. < 1 year
   b. 1-5 years
   c. 5-10 years
   d. Over 10 years
4. What is the type of institution you work with?
   a. University hospital
   b. Training and research hospital
   c. State hospital
   d. Primary care clinics

**Knowledge questions**

5. What is the frequency of Turner's syndrome?
   a. 1/1500-1/2500
   b. 1/10,000 -1/15,000
   c. 1/100,000
   d. 1/1000,000
6. In which sex type is Turner syndrome seen?
   a. Women
   b. Men
   c. In both
7. What is the chromosomal anomaly in Turner's syndrome?
   a. 22q11 deletion
   b. 45 XO
   c. 45 XXY
   d. *PTPN11* gene mutation
8. Which of the following is true in Turner syndrome?
   a. Hypogonadotropic hypogonadism
   b. Normogonadotropic hypogonadism
   c. Hypergonadotropic hypogonadism
9. Which of the following is not a physical characteristic of Turner syndrome?
   a. Cubitus valgus
   b. High-arch palate
   c. Syndactyly
   d. Edema of the hands and feet in the infant
10. What is the most common finding in Turner syndrome?
    a. Webbed neck
    b. Widely spaced nipples
    c. Short stature
    d. Madelung deformity
11. What is the most common cardiovascular disease seen in Turner syndrome?
    a. Ventricular septal defect
    b. Hypoplastic left heart syndrome
    c. Bicuspid aortic valve
    d. Atrial septal defect
12. Which of the following is false regarding fertility in patients with Turner syndrome?
    a. Spontaneous pregnancy can be achieved in most patients.
    b. 99% of patients are infertile.
    c. In the case of pregnancy, the risk of aortic dissection and/or rupture is high.
    d. Oocyte cryopreservation should be performed before the first sign of puberty.

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13. Which of the following is true about the intelligence of children with Turner syndrome?

a. General intelligence can be slightly delayed compared to the normal population.

b. There is serious mental retardation.

c. Intelligence is normal.

14. Which of the following statements is correct?

a. There is a lower risk of malignancy than in the normal population.

b. In patients with Y chromosome or Y chromosome fragmentation, prophylactic gonadectomy should be performed due to the risk of gonadal tumor.

c. The risk of endometrial cancer decreases in patients receiving estrogen therapy.

d. The incidence of multiple melanocytic nevi is lower than in the general population.

15. Which of the following is wrong related to Turner syndrome and osteoporosis?

a. The risk of osteoporosis is increased in Turner syndrome patients.

b. After 18 years of age, bone density should be measured.

c. Estrogen treatment increases bone mass.

d. Patients should be prohibited from exercising.

16. Which of the following is the definitive diagnosis of Turner syndrome?

a. Pelvic ultrasonography

b. Karyotype analysis

c. Clinical findings

d. Growth hormone stimulation test

17. Which of the following is associated with *SHOX* gene mutation in Turner syndrome?

a. Delayed puberty

b. Short stature

c. Germ cell defect

d. Streak gonad

18. Which of the following is false about treatment in Turner syndrome?

a. It is possible for patients to reach adult height with growth hormone treatment.

b. Induction and continuity of puberty is provided by estrogen treatment.

c. Growth hormone and estrogen treatment increases bone mass.

d. Estrogen therapy has no effect on stature.

**Supplementary 2.** The Turner syndrome questionnaire for the parents

*Please select the right option according to you.*

Age/Gender:

Education status: a. Primary school, b. High school-university

**Knowledge questions**

1. Turner syndrome is a genetic disease.
   a) Yes
   b) No
2. Turner syndrome is only seen in girls.
   a) Yes
   b) No
3. Turner syndrome can be diagnosed during pregnancy.
   a) Yes
   b) No
4. The most important finding of Turner syndrome is short stature.
   a) Yes
   b) No
5. In Turner syndrome, sex chromosomes are normal in number and structure.
   a) Yes
   b) No
6. Children with Turner syndrome are unlikely to have children.
   a) Yes
   b) No
7. In Turner syndrome, the intelligence levels of children are low.
   a) Yes
   b) No
8. In Turner syndrome, congenital heart diseases can be seen.
   a) Yes
   b) No
9. There is no definitive treatment for Turner syndrome.
   a) Yes
   b) No
10. In Turner syndrome, short stature can be treated with growth hormone replacement.
   a) Yes
   b) No
11. In Turner syndrome, some cancers are more common than in normal populations.
   a) Yes
   b) No
12. Diabetes mellitus, thyroid and celiac diseases are more common in patients with Turner syndrome.
   a) Yes
   b) No
13. Growth hormone treatment in Turner syndrome is effective in preventing infertility.
   a) Yes
   b) No
14. Estrogen treatment in Turner syndrome prevents osteoporosis.
   a) Yes
   b) No
15. Children with Turner syndrome should be examined for renal diseases.
   a) Yes
   b) No
16. Patients with Turner syndrome should receive psychiatric support.
   a) Yes
   b) No
17. Patients with Turner syndrome do not need to be under medical supervision in adulthood.
   a) Yes
   b) No

CASE REPORT

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# Hypophosphatasia: A Novel Mutation Associated with an Atypical Newborn Presentation

**Roger Esmel-Vilomara[1], Susana Hernández[2], Ariadna Campos-Martorel[3], Eva González-Roca[4], Diego Yeste[3], Félix Castillo[2]**

*[1]Hospital Universitari Vall d'Hebron, Clinic of Pediatrics, Barcelona, Spain*
*[2]Hospital Universitari Vall d'Hebron, Clinic of Neonatology, Barcelona, Spain*
*[3]Hospital Universitari Vall d'Hebron, Clinic of Pediatric Endocrinology, Barcelona, Spain*
*[4]Hospital Clínic de Barcelona, Clinic of Immunology, Barcelona, Spain*

### What is already known on this topic?

Hypophosphatasia is a rare Mendelian disease affecting bone metabolism through loss-of-function mutations in the gene encoding the tissue non-specific isoenzyme of alkaline phosphatase. To date, 381 different mutations have been described. The clinical expression of the disorder varies widely and can even differ between patients with the same mutation.

### What this study adds?

This report describes a novel mutation that has not been described in the literature to date. Also described is an atypical neonatal presentation of the syndrome, with mild phenotype, which is different to the classic neonatal form. However, it could also be an early diagnosis of the childhood form, which has better prognosis.

## Abstract

Hypophosphatasia, a rare genetic disease affecting bone metabolism, is characterized by decreased activity of tissue non-specific alkaline phosphatase (TNAP). The gene encoding TNAP *(ALPL)* has considerable allelic heterogeneity, which could explain different degrees of enzyme activity resulting in a wide clinical variability. We report the case of a preterm newborn in whom a corneal opacity was detected at birth. Blood tests performed to investigate this finding showed low alkaline phosphatase concentrations. The corneal opacity disappeared within a week but alkaline phosphatase remained persistently low. With persistently decreased levels of alkaline phosphatase, upon suspicion of hypophosphatasia, plain radiography detected changes suggestive of rickets. Sequencing of the *ALPL* gene revealed a heterozygous variant that has not been described in the literature to date. Our patient's condition may be an atypical neonatal form of the syndrome, with a mild phenotype, very different from the classic neonatal form, which can lead to severe skeletal disease and respiratory failure. However, it could also be an early diagnosis of the childhood form, which is associated with a better prognosis.

**Keywords:** Hypophosphatasia, mutation, newborn, alkaline phosphatase

## Introduction

Hypophosphatasia is a rare Mendelian disease affecting bone metabolism, in which activity of the tissue non-specific isoenzyme of alkaline phosphatase (TNAP) is decreased because of loss-of-function mutations in the gene encoding this isoenzyme (1,2). In Europe the incidence of severe forms has been estimated at 1/300,000 newborns. The prevalence of mild forms has not been reported, but is expected to be higher (3).

The clinical expression of hypophosphatasia varies widely, from death *in utero* with an unmineralized skeleton to problems with dentition in adult life, or an absence of symptoms (4). The severity of the disorder can even differ between patients with the same mutation (1). There are six

**Address for Correspondence:** Roger Esmel-Vilomara MD, Hospital Universitari Vall d'Hebron, Clinic of Pediatrics, Barcelona, Spain
**Phone:** +34-685248475 **E-mail:** roger.esmel@gmail.com **ORCID:** orcid.org/0000-0003-4449-3217

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forms of hypophosphatasia. Four are classified according to the age when bone disease becomes apparent: perinatal; infantile; childhood; and adult; with early-onset forms being the most severe. Odontohypophosphatasia exclusively causes dental manifestations. Some authors also include pseudohypophosphatasia, a rare infantile form with normal alkaline phosphatase values (4,5). The last type is benign prenatal hypophosphatasia, similar to the perinatal form in the age at presentation, but with a better prognosis and spontaneous clinical improvement (1).

We describe the case of an infant with an unusual presentation of hypophosphatasia diagnosed shortly after birth during investigation of an incidental clinical finding. A previously unreported mutation, found in the gene (*ALPL*) encoding TNAP, may be the cause of the patient's mild form of the disorder.

## Case Report

A late preterm male infant (34 + 3 weeks) was admitted to the minimal care neonatal unit for prematurity and infection risk, due to delivery five days after premature rupture of membranes. Cleft palate had been detected in the second trimester ultrasound study. The patient's family background included three paternal family members with cleft palate.

A corneal opacity was detected at birth. On blood testing to investigate this finding at five days after birth, the phosphate concentration was 8.2 mg/dL (normal value in preterm infants < 7.9 mg/dL) with normal calcium (9.6 mg/dL), vitamin D (25.4 ng/mL) and parathyroid hormone (19 pg/mL) but alkaline phosphatase was low at 52 IU/L (normal value > 75 IU/L). By one week of life, the corneal opacity had disappeared and the infant was asymptomatic.

In subsequent analyses, alkaline phosphatase remained low up to a maximum of 67 IU/L, with phosphate value below 8 mg/dL. The main potential causes of low alkaline phosphatase - hypothyroidism, anemia, low magnesium or zinc concentrations, vitamin D intoxication, low vitamin C, Wilson's disease, or intake of certain drugs - were ruled out. This led to a possible diagnosis of hypophosphatasia.

Radiographic studies on day eight of life revealed low radiological bone density. Subsequent radiography at 2.5 months of life (Figure 1) showed irregularities and widening of the radial and ulnar metaphyses, suggestive of rickets. A periosteal reaction in the femur with discrete metaphyseal widening was also observed.

Molecular genetic studies were performed using polymerase chain reaction amplification with specific primers. The sequencing reaction was carried out with the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, California, USA), capillary electrophoresis was performed on an ABI Prism 3730xl DNA Analyzer Sequencer (Applied Biosystems), and chromatograms were generated with the SeqPilot software (JSI Medical Systems, Ettenheim, Germany).

A heterozygous variant, c.1292T > A, was found in exon 11 of the *ALPL* gene, producing an amino acid change: p.(Val431Asp). This specific mutation, in the gene affected in hypophosphatasia, had not previously been reported (6). Bone densitometry of the lumbar spine was performed which showed a bone mineral density of 0.359 g/cm$^2$, consistent with normal reference values for Spain.

Clinical evaluation of first-degree relatives to investigate possible affected individuals within the family was negative, but the patient's mother showed a low plasma concentration of alkaline phosphatase (28 IU/L). She did not present stigmata of rickets or early tooth loss and her height was within the limits of normality. The gene encoding TNAP was analyzed to determine whether the mutation was *de novo* or inherited, and the same mutation was found in the mother.

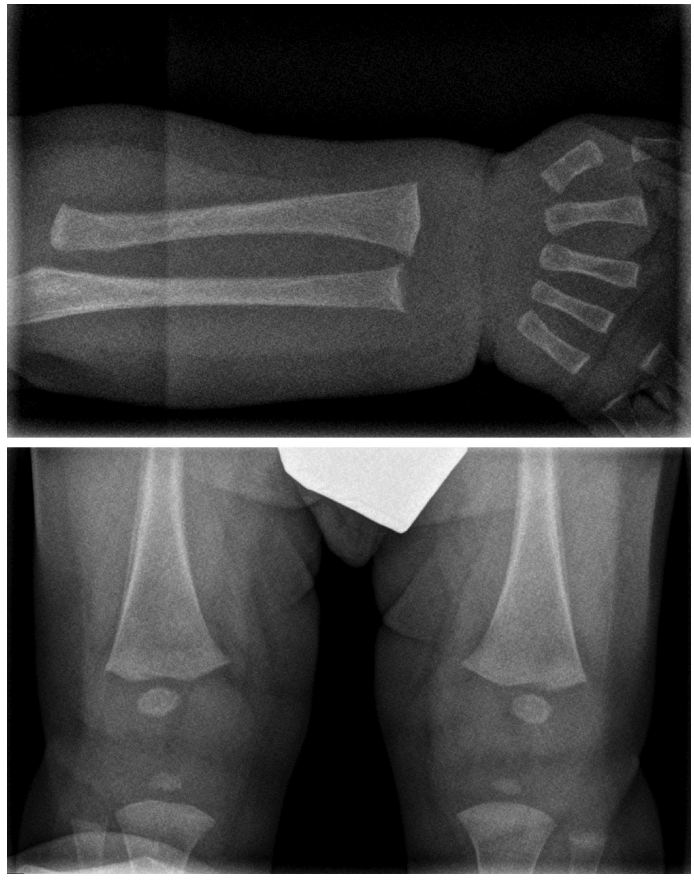

**Figure 1.** Radiographs at the time of the hypophosphatasia diagnosis

Currently, the patient is being monitored with periodic follow-up visits and is not receiving replacement therapy. Two years after diagnosis the alkaline phosphatase values remain low and are always < 70 IU/L. Radiographic studies (Figure 2) show signs of diffuse osteopenia with affected distal metaphysis of both ulnas with marked deflection, loss of trabecular density and slight significant widening on the left ulna. The rest of the metaphysis are enlarged with only a slight sclerotic line on the physis. There are no other skeletal findings.

Written informed consent was obtained from the patient's parents for publication of this case report and the accompanying images.

## Discussion

Hypophosphatasia is a rare inherited disease characterized by decreased alkaline phosphatase levels due to reductions in the tissue non-specific isoenzyme. Presumptive diagnosis of this disease is based on physical examination and consistent radiographic findings, in association with low concentrations of alkaline phosphatase in blood tests. The molecular diagnosis is established by sequencing the gene encoding TNAP (1).

The casual finding of corneal opacities in this patient was the starting point of the diagnosis, although the opacities had disappeared by the end of the first week of life. An association between hypophosphatasia and ocular signs of hypercalcemia, morphologically identical to band keratopathy, has been reported (7). However, the fast, spontaneous resolution of this finding suggests that it was not an abnormality related with the genetic disease.

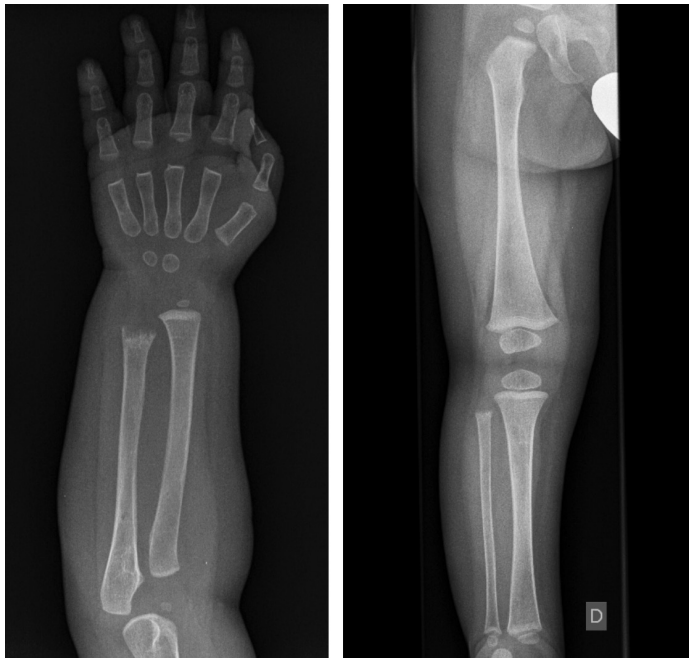


**Figure 2.** Radiographs at the age of 14 months

This case exemplifies an atypical presentation of the disorder, as the patient was asymptomatic at the time of the diagnosis, but had low alkaline phosphatase levels. Physical examination at birth provided no evidence of hypophosphatasia and radiographs only detected non-specific osteopenia. However, in subsequent examinations, the patient had the characteristic decrease in alkaline phosphatase activity and radiographic findings suggestive of hypophosphatasia. Imaging typically shows a generalized decrease in bone density and metaphyseal abnormalities in long bones, similar to those found in severe forms of rickets (1,2). However, unlike rickets, serum alkaline phosphatase levels are decreased in hypophosphatasia (1,4).

Reported evidence has indicated that the lower the alkaline phosphatase levels, the more severe are the manifestations of the disorder (1,8). The mutations in severe hypophosphatasia produce a protein that fails to reach the cell membrane, but instead, accumulates in the cis-Golgi apparatus and is then degraded in the proteasome without producing adequate enzymatic activity. However, mutations found in the mild forms produce enzymes that are, in part, properly located at the cell membrane and exhibit significant residual activity (9).

Our patient did not have very low levels of the enzyme, which suggests that the newly identified genetic variant may cause a mild phenotype. It may be an atypical, mild neonatal form, considering the age of presentation and absence of clinical features, despite the presence of radiographic abnormalities. Nonetheless, we cannot exclude that it may represent an early diagnosis of childhood hypophosphatasia, with a better prognosis than the neonatal forms. It cannot be considered as benign prenatal hypophosphatasia, as the diagnosis was made after birth and the patient had no visible abnormalities on prenatal ultrasound studies apart from the cleft palate.

The definitive diagnosis of hypophosphatasia is established by sequencing the *TNAP* gene, *ALPL*, located on chromosome 1p36.1-p34 (1,6), and subject to very strong allelic heterogeneity. To date, 381 different mutations have been described in *ALPL*, 70.6% of which are missense mutations (6). Allelic heterogeneity explains the different degrees of enzyme activity and the great variability observed in the clinical expression of the disease (1,3). In our case, a previously unidentified mutation, a heterozygous variant in exon 11 of *ALPL* (c.1292T > A) was detected in both the patient and his mother. This mutation produces

an amino acid change: p.(Val431Asp). Bioinformatic studies using SIFT (10) and Polyphen2 algorithms (11) predicted a harmful effect of the mutation on the structure or function of the protein. According to the American College of Medical Genetics and Genomics guidelines for the interpretation of sequence variants (12), the mutation would be considered probably pathogenic.

As to inheritance, severe forms of hypophosphatasia (perinatal and infantile) have an autosomal recessive transmission, whereas in mild forms, transmission can be recessive or dominant (2). Sporadic cases are rare. Our patient had a heterozygous mutation, suggesting dominance. The negative effects of some heterozygous mutations, resulting in TNAP activity from 20 to 40% of wild-type, explain the dominant transmission of hypophosphatasia. A mild phenotype is expected in the heterozygous state even if the mutation has a severe effect (1,2).

The dominant negative effect is corroborated by a previous missense mutation in the position of Val431. A mutation in the same allele -p.(Val431Ala)- leading to a change from valine to alanine, has been described in relation with odontohypophosphatasia (6). The clinical form of the condition in our patient remains to be defined, but as bone was affected on radiography, it is unlikely to be odontohypophosphatasia.

The severity of the condition can differ between patients showing the same mutation (1), and there is significant variability in the expected clinical features (3). In our patient, the mutation has a putative dominant inheritance; hence, relatives with the mutation might or might not develop mild hypophosphatasia and the patient's mother is still asymptomatic. It could be that this patient and his mother will have low alkaline phosphatase levels and harbor a recessive variant in the TNAP allele but never experience symptoms, in which case they could be considered carriers (1). The variability in the inheritance models and the variable penetrance complicates genetic counseling, although it could be of value in couples with an affected child (2,3).

Considering that the accumulation of the enzymatic substrates will be a key point in the future follow-up (1,4), to be able to predict any clinical manifestations of progression of the disease, the next step to confirm the phenotype would be the determination of the substrates of the enzyme.

Asfotase alfa, a recombinant human tissue non-specific alkaline phosphatase coupled to a deca-aspartate motif for bone targeting, has demonstrated efficacy for healing the skeletal manifestations of hypophosphatasia. In addition, it has been found to improve respiratory and motor function in perinatal and infantile forms, with a good safety profile (13). Given the age of the patient, the disease course should be monitored so that management can be adapted to the abnormalities that may occur.

A previously unidentified heterozygous variant located in exon 11 of the *ALPL* gene -c.1292T > A- is described as a cause of hypophosphatasia.

The case reported could be considered an atypical presentation of the neonatal form, with a mild phenotype and different from the classical neonatal form, or the childhood form with a very early diagnosis, which is associated with a better prognosis. The child's condition should be closely monitored, and if deemed appropriate, replacement therapy with asfotase alfa can be provided.

Persistently low levels of alkaline phosphatase should alert clinicians to hypophosphatasia. Definitive diagnosis can be achieved by genetic analyses.

### Ethics

**Informed Consent:** Written informed consent was obtained from the patient's parents for publication of this case report and the accompanying images.

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

### Authorship Contributions

Surgical and Medical Practices: Roger Esmel-Vilomara, Susana Hernández, Ariadna Campos-Martorel, Eva González-Roca, Concept: Roger Esmel-Vilomara, Susana Hernández, Ariadna Campos-Martorel, Diego Yeste, Félix Castillo, Design: Roger Esmel-Vilomara, Susana Hernández, Data Collection or Processing: Roger Esmel-Vilomara, Susana Hernández, Ariadna Campos-Martorel, Diego Yeste, Félix Castillo, Analysis or Interpretation: Roger Esmel-Vilomara, Susana Hernández, Ariadna Campos-Martorel, Diego Yeste, Félix Castillo, Literature Search: Roger Esmel-Vilomara, Susana Hernández, Writing: Roger Esmel-Vilomara.

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CASE REPORT

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# Aromatase Deficiency in Two Siblings with 46,XX Karyotype Raised as Different Genders: A Novel Mutation (p.R115X) in the *CYP19A1* Gene

**Samim Özen[1], Tahir Atik[2], Özlem Korkmaz[1], Hüseyin Onay[3], Damla Gökşen[1], Ferda Özkınay[2,3], Özgür Çoğulu[2,3], Şükran Darcan[1]**

*[1]Ege University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey*
*[2]Ege University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Genetics, İzmir, Turkey*
*[3]Ege University Faculty of Medicine, Department of Medical Genetics, İzmir, Turkey*

### What is already known on this topic?

The *CYP19A1* gene encodes the aromatase enzyme which catalyses the conversion of androgens to oestrogens. In cases with 46,XX karyotype, mutations in the *CYP19A1* gene can lead to disorders of sex development.

### What this study adds?

Two 46,XX sibling having the novel p.R115X (c.343 C > T) pathogenic variant in the *CYP19A1* gene and raised as different genders are presented. This variant was not associated with maternal gestational virilisation.

## Abstract

Aromatase deficiency rarely causes a 46,XX sexual differentiation disorder. The *CYP19A1* gene encodes the aromatase enzyme which catalyses the conversion of androgens to oestrogens. In cases with 46,XX karyotype, mutations in the *CYP19A1* gene can lead to disorders of sex development. Clinical findings in aromatase deficiency vary depending on the degree of deficiency. The effect of increased androgens, including acne, cliteromegaly and hirsutism, can be observed in mothers with placental aromatase deficiency. A decrease in maternal virilisation symptoms is observable in the postpartum period. It is rarely reported that there is no virilization in pregnancy. In this study, two 46,XX sibling having the p.R115X (c.343 C > T) novel pathogenic variant in the *CYP19A1* gene and raised as different genders, with no maternal virilisation during pregnancy, are presented. In conclusion, 46,XX virilised females should be examined in terms of aromatase deficiency once congenital adrenal hyperplasia has been excluded, even if there is no history of maternal virilisation during pregnancy.

**Keywords:** 46,XX disorder of sex development, aromatase deficiency, *CYP19A1* gene

## Introduction

Aromatase deficiency is a rare autosomal recessive disorder caused by mutations in the *CYP19A1* gene (1). The *CYP19A1* gene encodes the aromatase enzyme which catalyses the conversion of androgens to oestrogens. In the affected 46,XX cases, clinical findings in the neonatal period vary between mild cliteromegaly and complete labioscrotal fusion due to differences in exposure to increased androgens in the intrauterine phase. An increase in virilisation at puberty or the non-appearance of secondary sex characteristics, are the main clinical features in the late period. Affected 46,XY cases have normal prepubertal growth. Delayed epiphyseal closure, eunuchoid body structure and a decrease in bone mineral density can be observed in both sexes (2). This study presents a novel pathogenic variant in the *CYP19A1* gene in two 46,XX siblings raised as different genders.

**Address for Correspondence:** Tahir Atik MD, Ege University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Genetics, İzmir, Turkey
**Phone:** +90 232 390 12 30 **E-mail:** tahiratik@yahoo.com **ORCID:** orcid.org/0000-0002-1142-3872

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## Case Reports

### Case 1

A 14-year-old patient who had been raised as a male was brought to the pediatric endocrinology clinic for undescended testis and hypospadias. Although parental consanguinity was not reported to be present, the family history revealed that they were living in a village of only 500 inhabitants. The patient's mother, who was first pregnancy and primigravida, had no symptoms during pregnancy of excessive androgen production, such as hair loss, virilisation, or acne. On physical examination, height, weight, and phallus were measured to be 154.9 cm [standard deviation score (SDS): -2.5], 57 kg (SDS: -0.6), and 2 cm respectively. Breast tissue and palpable gonads were not detected. Prader stage 3, two urogenital openings and stage 2 pubic pilosity were also noted. On laboratory examination, bone age was 11 years. Gonadotropin concentrations were: follicle stimulating hormone (FSH) 70 mIU/mL (1.5-12.8 mIU/L); luteinizing hormone (LH) 30 mIU/mL (0.1-12 mIU/mL); free testosterone 0.9 pg/mL (0.8-1.4 pg/mL); and estradiol 22.9 pg/mL (7-60 pg/mL). Adrenocorticotropic hormone (ACTH), cortisol and 17-hydroxyprogesterone (17-OHP) were all found to be in the normal range. Pelvic ultrasonography (USG) revealed 19x14 mm right ovary and 15x12 mm left ovary and an absence of uterus. Karyotype was 46,XX and no variants were found in the *SRY* gene on fluorescence in situ hybridization (FISH) analysis. On laparoscopic examination normal-looking bilateral ovaries and a small uterus were observed. The biopsy findings of the right gonad were consistent with ovarian tissue and ovarian follicle cysts were observed. Sequence analysis of the *SOX9* gene revealed no mutation. Clinical and laboratory findings of the patient suggested aromatase deficiency and a novel homozygous nonsense p.R115X (c.343 C > T) pathogenic variant was found on Sanger sequencing of the *CYP19A1* gene (Figure 1A). Both parents were heterozygous for the same mutation (Figure 1C, 1D). There were no unusual clinical findings in the parents. The mutation found in the cases was predicted to be pathogenic by *in silico* analysis by Varsome program (https://varsome.com/).

The Institutional Council of Disorders of Sex Development decided that the case should be raised male on the ground of a more distinct male sexual identity. Salpingo-oophorectomy, hysterectomy and genitoplasty were performed. Intramuscular testosterone propionate and testosterone phenylpropionate treatments were administered with a 100 mg/month starting dose and gradually increased every six months. Oral estradiol hemihydrate treatment of 0.25 mg/day was initiated in the follow-up. At the age of 21, bilateral testicular prostheses were surgically implanted. During follow-up bone mineral densitometry showed early onset osteoporosis (L1-L4 bone mineral density Z score: -2,2) and oral calcium supplementation was given. Calcium, phosphorus, parathyroid hormone (PTH) and vitamin D concentrations were within normal limit. At the age of 22, weight, height, and phallus were measured to be 86.6 kg (SDS: 1.29), 173.5 cm (SDS: -0.43) and 7 cm respectively.

### Case 2

The eight year-old sibling of Case 1, who had been raised as a female. On physical examination, height and weight were 125.5 cm (SDS: -0.3), 22.3 kg (SDS: -0.9) respectively. Phallus was measured to be 1 cm. There were no palpable gonads, two urogenital openings and stage 1 pubic pilosity were also noted. Pubertal development was found to be stage 2 according to Prader score. Similar to the other sibling, the bone age of Case 2 was found to be retarded (5 years 9 months). FSH concentration was 22 mIU/mL

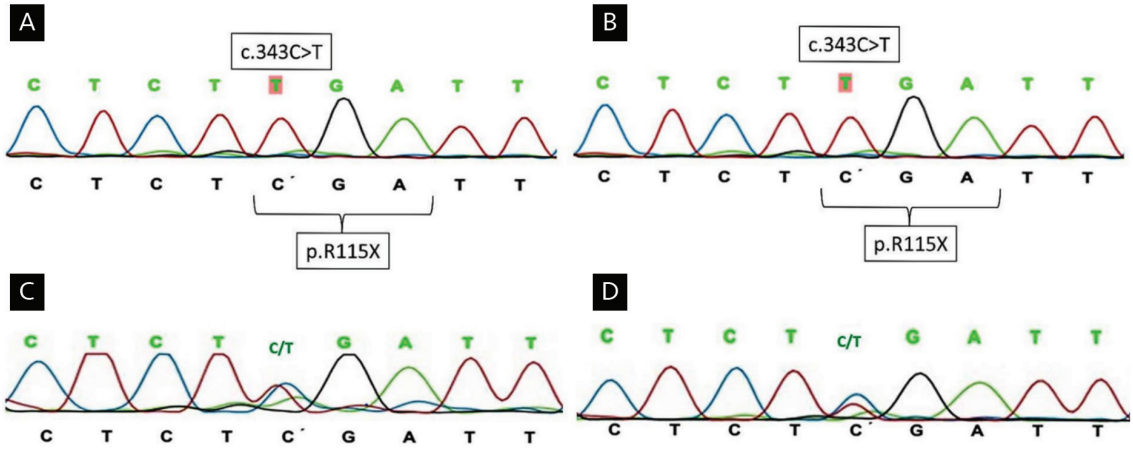


**Figure 1. A) (Case 1), B) (Case 2):** A novel homozygous nonsense pathogenic variant p.R115X (c.343 C > T) was detected in the *CYP19A1* gene sequence analysis. **C)** (Mother), **D)** (Father): The parents were heterozygous for the same mutation

(1.0-4.2 mIU/mL), LH: 30 mIU/mL (0.1-0.3 mIU/mL), free testosterone 0.2 pg/mL (0.15-0.6 pg/mL), estradiol: 5 pg/mL (N < 15). The patient had normal ACTH, cortisol and 17-OHP. Uterus and left ovary were not visualized on pelvic USG whereas a 12-mm right ovary was identified. Karyotype was found to be 46,XX. FISH analysis showed no variants in the *SRY* gene. The same homozygous pathogenic variant in the *CYP19A1* gene was also detected in this sibling (Figure 1B). The Institutional Council of Disorders of Sex Development recommended the case to be raised as a female, on the grounds that female sexual identity was more distinct. L1-L4 Z score was found to be -2.4 on bone mineral densitometry during the follow-up period. Calcium, phosphorus, PTH and vitamin D concentrations were within normal limits but oral intake of calcium was increased. At the age of 11, oral estradiol hemihydrate treatment was begun with 0.25 mg/day starting dose and was gradually increased every six months. At the age of 16, physical examination showed a weight of 58.7 kg (SDS: 0.28), height 160 cm (SDS: -0.44) and stage 5 puberty. Routine pelvic USG showed a uterus with the dimensions of 62x35 mm. The patient was treated with a combination of oestrogen and progesterone. After this treatment she had menarche and regular menstural cycles.

The parents of the patients were informed about the diagnosis and consent for laboratory analyses and publication were obtained.

## Discussion

Aromatase is a member of the cytochrome P450 superfamily that catalyses a hydroxylation reaction in which an oxygen atom is attached to an organic molecule (3). The human aromatase enzyme (P450C19) is the product of *CYP19A1* gene that converts androgens (P19) to oestrogens (P18) and is a microsomal enzyme responsible for oestrogen synthesis in all vertebrates (3,4). The enzyme-encoding gene is composed of 10 exons (5). Mutations in the *CYP19A1* gene lead to loss of enzyme function and decrease in oestrogen synthesis. Most of the reported mutations contain single base changes in exons (6,7). In the study, the *CYP19A1* gene sequence analysis detected homozygous novel nonsense p.R115X pathogenic variant in both siblings (Figure 1A, 1B). This nonsense mutation is predicted to be pathogenic using *in silico* analysis (MutationTaster) (8) and minor allele frequency data in several public databases including the NCBI dbSNPbuild141 (http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Project (http://www.1000genomes.org/) and the Exome Aggregation Consortium (http://exac.broadinstitute.org/).

Clinical findings in aromatase deficiency vary depending on the retained proportion of enzyme function. Due to the effect of increased androgens caused by placental aromatase deficiency, acne, cliteromegaly and hirsutism can be observed in mothers carrying affected fetuses. A decrease in the symptoms of maternal virilisation is observed in the postpartum period (9). Placental aromatase activity of as little as 1-2% is reported to be protective against maternal virilisation during pregnancy (10). In the family presented here, the mother had no symptoms of excessive androgen production during pregnancy. Enzyme activity was not studied in the patients nor their mother. Marino et al (11) reported that maternal virilisation was also absent in their three cases with *CYP19A1* mutations. During the follow-up period, phenotypic variability was determined among the affected patients. Two patients had a new mutation (c.574C > T). They found c.628G > A mutation in four of the six unrelated patients.

It has been reported that of 24 patients (12 males, 12 females) with proven *CYP19A1* deficiency, 70% of the females showed virilisation compatible with Prader stage 4-5, while males usually presented with metabolic problems and short stature (7,12). For affected female cases, variable phenotype, such as cliteromegaly due to increased androgen levels in the intrauterine phase or complete labioscrotal fusion can be observed. It has been suggested that in aromatase-deficient prepubertal girls, an amplification of FSH signalling might occur in the presence of high intra-ovarian androgen production and be responsible for the development of ovarian follicular cysts (3). On the other hand, hypoplastic ovaries rather than enlarged ovaries in aromatase-deficient females have rarely been reported. Lin et al (13) and Akçurin et al (14) reported a few cases of aromatase deficiency with hypoplastic ovaries and uterus. Lin et al (13) suggested that the streak ovaries may be an inherent manifestation of *CYP19A1* deficiency. Also, polycystic ovaries may appear in later periods depending on human chorionic gonadotropin stimulation. Cliteromegaly, hirsutism and acne can be seen in affected individuals with the non-appearance of secondary sex characteristics in the adolescence period (3). The studies have showed that loss of function mutations in the gene may result in various phenotypic changes, especially appearing in the pre-pubertal and pubertal period (11). In the study of Lin et al (13) it was demonstrated that human aromatase mutations may produce variable or "non-classic" phenotypes. They reported that low residual aromatase activity may be sufficient for the development of breast and uterus in adolescence, despite significant androgenization in the uterus. Such phenotypic variability can be further influenced by modifying factors such as

non-classical pathways of estrogen synthesis, variability in the core modifiers, or differences in androgen responses. The siblings presented in this study had been raised as different genders due to the appearance of their external genitalia and virilisation levels.

In aromatase deficiency, oestrogen replacement treatment regulates gonadotropin secretion, glucose metabolism and liver functions while reducing lipid and insulin levels (14,15). In our cases, lipid levels and glucose metabolism were found to be normal. However, decreased FSH and LH levels were observed with the oestrogen replacement treatment. Bone mineralisation and maturation are adversely affected in patients with aromatase deficiency. Oestrogen has positive effects on bone density by prolonging the life cycles of osteoblasts and osteocytes while reducing bone resorption (4). Osteoporosis was detected in both of our patients. Hormone replacement therapy was initiated and oral intake of calcium was increased as they were followed up.

In conclusion; 46,XX virilised cases should be examined in terms of aromatase deficiency after congenital adrenal hyperplasia has been excluded, even if there was no maternal history of virilisation during pregnancy. This should include *CYP19A1* mutation analysis. Early diagnosis of this disorder is of vital importance for gender selection and hormone replacement therapy.

### Ethics

**Informed Consent:** The parents of the patients were informed about the diagnosis and consent for laboratory analyses and publication were obtained.

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

### Authorship Contributions

Surgical and Medical Practices: Samim Özen, Tahir Atik, Özlem Korkmaz, Hüseyin Onay, Damla Gökşen, Ferda Özkınay, Özgür Çoğulu, Şükran Darcan, Concept: Samim Özen, Tahir Atik, Özlem Korkmaz, Hüseyin Onay, Şükran Darcan, Design: Samim Özen, Tahir Atik, Hüseyin Onay, Damla Gökşen, Ferda Özkınay, Özgür Çoğulu, Şükran Darcan, Data Collection or Processing: Samim Özen, Tahir Atik, Özlem Korkmaz, Hüseyin Onay, Analysis or Interpretation: Samim Özen, Tahir Atik, Özlem Korkmaz, Hüseyin Onay, Ferda Özkınay, Literature Search: Samim Özen, Tahir Atik, Özlem Korkmaz, Writing: Samim Özen, Tahir Atik.

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CASE REPORT

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# A Neurofibromatosis Noonan Syndrome Patient Presenting with Abnormal External Genitalia

**Esra Işık[1], Hüseyin Onay[2], Tahir Atik[1], Aslı Ece Solmaz[2], Samim Özen[3], Özgür Çoğulu[1,2], Şükran Darcan[3], Ferda Özkınay[1,2]**

*[1]Ege University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Genetics, İzmir, Turkey*
*[2]Ege University Faculty of Medicine, Department of Medical Genetics, İzmir, Turkey*
*[3]Ege University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, İzmir, Turkey*

### What is already known on this topic?

The Neurofibromatosis Noonan syndrome (NFNS) is a rare RASopathy syndrome characterized by phenotypic features of both neurofibromatosis type 1 *(NF1*) and NS. It occurs as a result of *NF1* gene mutations. Plexiform neurofibromas (PNFs) are seen rarely in NFNS patients.

### What this study adds?

A novel mutation in the *NF1* gene in a patient who presented with phenotypic features of both *NF1* and NS is reported. A PNF was present in this case and was responsible for an unusual genital phenotype. Thus PNF may be a rare cause of genital virilization in NFNS patients.

## Abstract

Neurofibromatosis Noonan syndrome (NFNS) is a rare RASopathy syndrome, resulting from *NF1* gene mutations. NFNS is characterized by phenotypic features of both neurofibromatosis type 1 *(NF1)* and Noonan syndrome. Plexiform neurofibromas (PNFs) are an unusual finding in NFNS. A seven year-old girl with typical clinical features of *NF1* was referred to our clinic due to short stature and abnormal genital appearance. Due to dysmorphic features, a clinical diagnosis of NFNS was considered in the patient and, following molecular analysis, revealed a novel heterozygous c.3052_3056delTTAGT (p.L1018X) variant in the *NF1* gene. Although evaluation for genital virilization, including karyotype and hormonal studies were normal, imaging studies revealed a diffuse genital PNF. Although PNFs are seen rarely in NFNS, this should be considered in the differential diagnosis of genital virilization in these patients to prevent unnecessary testing.

**Keywords:** Neurofibromatosis Noonan syndrome, *NF1* gene, abnormal external genitalia

## Introduction

Neurofibromatosis Noonan syndrome (NFNS) (OMIM 601321) is a rare RASopathy syndrome, first defined in four unrelated patients in 1985 by Allanson et al (1). These patients who were originally diagnosed as neurofibromatosis type 1 (NF1), also presented with clinical features of NS. NFNS patients show phenotypic features of both NF1 and NS. In subsequent years several cases showing clinical findings of both syndromes have been reported, thus introducing a new phenotypic syndrome; NFNS. NFNS has been shown to be due to heterozygous mutations in the *NF1* gene (2).

Neurofibromas are benign peripheral nerve sheath tumors and are classified as either dermal or plexiform neurofibromas (PNFs) (3). PNFs are usually congenital and originate from a bundle of fascicles or a large nerve plexus. PNFs can be seen in 25-50% of NF1 patients (4). However, they are more rarely seen in NFNS patients. It has been reported that in pediatric NF1 patients PNFs are most commonly localized in the head and neck region (4,5). Genital localization of PNFs has been reported rarely.

**Address for Correspondence:** Esra Işık MD, Ege University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Genetics, İzmir, Turkey
**Phone:** +90 232 390 14 42 **E-mail:** esrabadak36@gmail.com **ORCID:** orcid.org/0000-0003-0657-2408

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In this study, we report a young girl with NFNS and a genital PNF manifesting as abnormal external genitalia giving the impression of a disorder of sex development (DSD).

## Case Report

A seven year-old girl with typical clinical features of NF1 was referred to our clinic due to short stature and abnormal genital appearance. She was born at the 37$^{th}$ week of gestation with a birth weight and height of 2800 gr (10-25$^{th}$ centile) and 47 cm (10-25$^{th}$ centile), respectively. During the newborn period multiple café-au-lait spots and spina bifida were observed. Her neuromotor development was normal. At the age of three years an operation was performed for filum terminale lipoma. A family history revealed her father had similar clinical features.

On physical examination, weight, height and head circumference were measured as 18 kg (3-10$^{th}$ percentile), 106 cm (<3$^{rd}$ percentile), 54 cm (>97$^{th}$ percentile), respectively. She had macrocephaly, broad forehead, sparse eyebrows, depressed nasal bridge, hypertelorism, low set ears, deeply grooved philtrum, short and webbed neck, pectus excavatum, kyphoscoliosis, sacral hypertrichosis, multiple cafe-au-lait spots and axillary and inguinal freckling (Figure 1). On genital examination, abnormal external genitalia were observed. A genital structure resembling a phallus was measured as 3.5 cm. Genital appearance was evaluated as being Prader Stage 2. No Lisch nodule was detected via slit-lamp examination. Echocardiography was normal. Cranial magnetic resonance imaging (MRI) revealed a hamartoma.

Due to clinical features including macrocephaly, short stature, facial dysmorphism, webbed and short neck and pectus excavatum in addition to the typical findings of NF1, a clinical diagnosis of NFNS was considered in the patient. All coding exons and the flanking intronic regions of the *NF1* (NM_000267.3) and the *PTPN11* (NM_002834) genes were amplified by polymerase chain reaction and sequenced using Illumina MiSeq platform (Illumina Ltd., San Diego, USA). Molecular analysis revealed a novel heterozygous c.3052_3056delTTAGT (p.L1018X) variant in the *NF1* gene (Figure 2). In accordance with the American College of Medical Genetics recommendations (null variant, hot-spot region, variant not found in public databases) this variant has been predicted as pathogenic (6).

Laboratory tests for genital virilization, including karyotype (46,XX), thyroid function, follicle stimulating hormone, luteinizing hormone, estradiol, testosterone, 17-hydroxyprogesterone, 11-deoxycorticosterone, dehydroepiandrosterone sulphate, adrenocorticotropic hormone and cortisol concentrations and bone age were all normal (Table 1). MRI of the pelvis and external genitalia

**Table 1. Hormone concentrations of the patient at presentation**

| | |
|---|---|
| FSH (mIU/L) | 4.59 (1.0-4.2) |
| LH (mIU/L) | 0.53 (0.02-0.3) |
| Testosterone (ng/mL) | 0.1 (<0.2) |
| Estradiol (ng/mL) | <20 (<20) |
| 17-OH progesterone (ng/mL) | 0.2 (<2) |
| Cortisol (ng/dL) | 10.7 (6-18) |
| ACTH (µg/dL) | 36 (15-60) |
| DHEA-S (µg/dL) | 2 (<72) |

Normal values are shown in parantheses.

FSH: follicle-stimulating hormone, LH: luteinizing hormone, 17-OH: 17-hydroxyprogesterone, ACTH: adrenocorticotropic hormone, DHEA-S: sulfated dehydroepiandrosterone

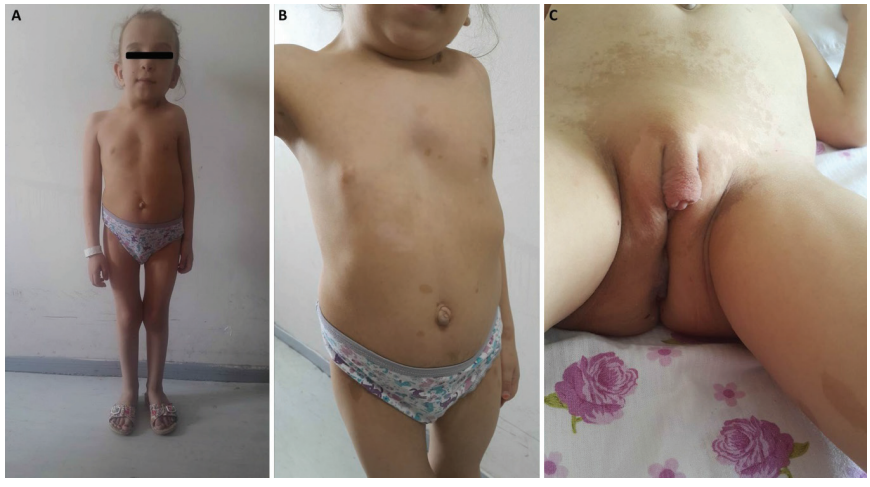


**Figure 1.** Phenotypic findings of the patient. A, B) Dysmorphic features: macrocephaly, broad forehead, sparse eyebrows, depressed nasal bridge, hypertelorism, low set ears, deeply grooved philtrum, short and webbed neck, pectus excavatum and multiple cafe-au-lait spots. C) Cliteromegaly

showed multiple PNFs filling the pelvic area with invasion of the sacral neural foraminae and subcutaneous region. Additionally, multiple neurofibromas were detected in the labium majus and the external genital area (Figure 3).

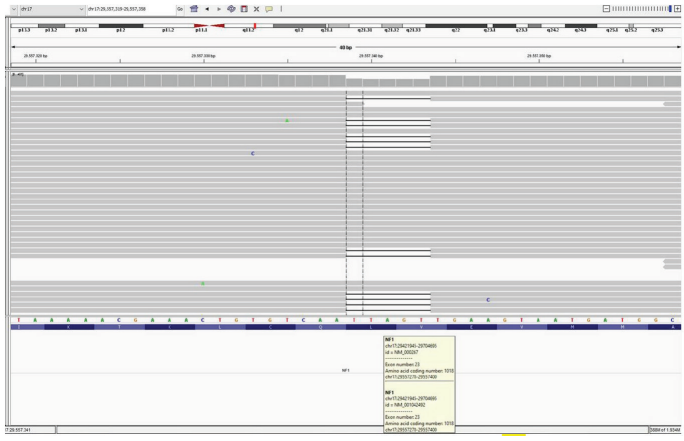

**Figure 2.** Next generation sequencing analysis in the patient demonstrating a heterozygous *NF1* mutation; c.3052_3056delTTAGT (p.L1018X)

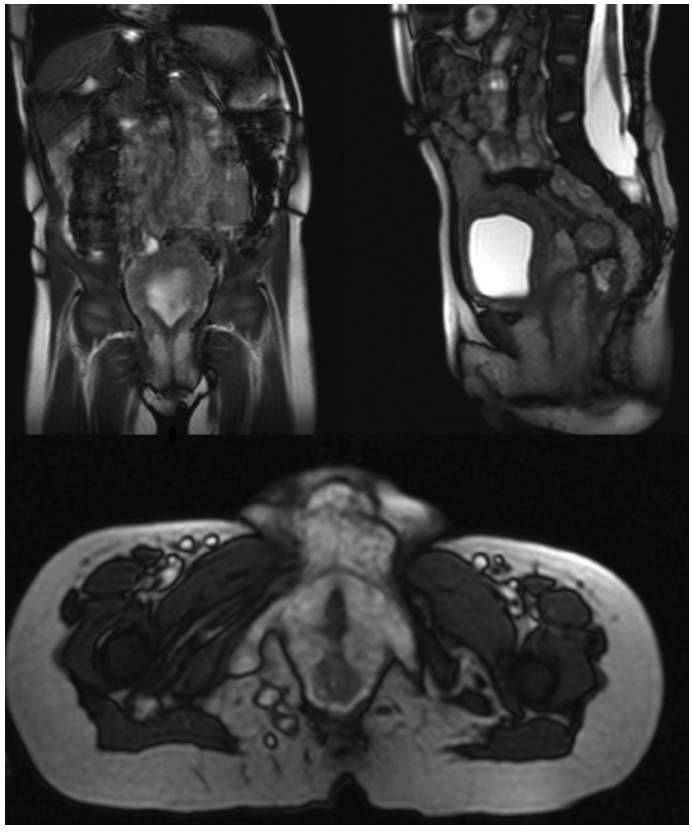

**Figure 3.** Magnetic resonance imaging of abdomen and external genitalia: multiple plexiform neurofibromas filling the pelvic area with invasion of the sacral neural foraminae and subcutaneous region, multiple neurofibromas in the labium majus and the external genital area

## Discussion

NS and NF1 are both included within the group of conditions known as the RASopathy syndromes. However, distinct clinical and genetic differences exist. The patient presented here had a number of features typical of both syndromes. NS is genetically heterogeneous. To date at least 14 genes, the most common being *PTPN11*, have been implicated in the etiology of NS (7). NF1 patients have characteristics different to NS while carrying *NF1* gene deletions or point mutations (2,8). To date, a number of cases, showing characteristic cardinal findings of both syndromes together with *NF1* gene mutations have been reported in the literature. Among NFNS patients, several patients were found to have mutations in both *PTPN11* and *NF1*. The majority of NFNS patients, however, have been reported to have only *NF1* mutations, without any detectable *PTPN11* mutation. Thus NFNS syndrome has been attributed to *NF1* mutations by most authors. In our patient, following sequencing of both *NF1* and *PTPN11* genes, the only pathogenic variant found was in *NF1* (9).

In NF1 patients mutations may appear anywhere throughout the entire gene. However, in the majority of NFNS patients, mutations have been localized within the GTPase-activating-protein (GAP) related domain (2). Consistent with this, the novel frameshift mutation observed in our patient was localized in the GAP related domain.

Disorders of sex development are classified into three groups (sex chromosome DSD, 46,XY DSD and 46,XX DSD) based on karyotype (10). In 46,XX DSD the genotype is 46,XX and the gonads present as ovaries; however, the external genitalia shows virilization (11). Virilization of external genitalia is rarely caused anything other than hormonal factors. Due to the abnormal appearance of the external genital organs in this case, a differential diagnosis for DSD was necessary. To exclude 46,XX DSD, hormonal tests were performed and they were all found to be normal. Subsequent imaging studies revealed a diffuse PNF which resulted in clitoral and labial enlargement in the patient. PNFs in external genital organs have rarely been described in NF1 patients (12,13) and to date there have been no reports of NFNS patients with PNFs invading the external genitalia. In the current literature, the hormonal analyses of all NF1 patients with genital abnormality have been found to be normal (12,13,14,15).

Neurofibromas are benign tumors, however their invasive nature and size may require surgical intervention. In almost 50 % of PNFs involving the genital area, surgical intervention has been required (4). Unfortunately surgical correction was not an option for our patient due to the extensive

involvement of the PNF in the pelvic area and the patient was started on sirolimus treatment, as this was seen as the most appropriate form of treatment.

Firstly, if a patient presents with the clinical features of both NS and NF1, NFNS should be considered. Secondly, in NFNS, as in NF1, genital PNF is an unusual clinical finding and could present as abnormal external genitalia giving the impression of a DSD. Imaging studies, prior to extensive hormonal work-up, should be performed to rule this out.

### Ethics

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

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

### Authorship Contribution

Concept: Esra Işık, Hüseyin Onay, Tahir Atik, Aslı Ece Solmaz, Samim Özen, Özgür Çoğulu, Şükran Darcan, Ferda Özkınay, Design: Esra Işık, Hüseyin Onay, Tahir Atik, Aslı Ece Solmaz, Samim Özen, Özgür Çoğulu, Şükran Darcan, Ferda Özkınay, Data Collection or Processing: Esra Işık, Hüseyin Onay, Tahir Atik, Aslı Ece Solmaz, Samim Özen, Analysis or Interpretation: Esra Işık, Hüseyin Onay, Tahir Atik, Aslı Ece Solmaz, Literature Search: Esra Işık, Aslı Ece Solmaz, Tahir Atik Writing: Esra Işık, Tahir Atik, Ferda Özkınay.

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CASE REPORT

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# An Unusual Presentation of Carney Complex

**Aydilek Dağdeviren Çakır[1], Hande Turan[1], Tiraje Celkan[2], Nil Çomunoğlu[3], Oya Ercan[1], Olcay Evliyaoğlu[1]**

*[1]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey*
*[2]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Hematology-Oncology, İstanbul, Turkey*
*[3]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pathology, İstanbul, Turkey*

### What is already known on this topic?

Carney complex (CNC) is a multiple neoplasia syndrome characterized by pigmented lesions of the skin and mucosa, cardiac, cutaneous and other myxomas, and multiple endocrine and non-endocrine tumors. Osteochondromyxoma (OMX) is an extremely rare myxomatous tumor of bone, affecting 1% of CNC patients.

### What this study adds?

CNC may present without typical findings such as pigmented skin lesions. This case presented initially with OMX although this diagnosis was delayed. The patient was subsequently diagnosed with large cell calcifying Sertoli cell tumor, leading to the final diagnosis of CNC. Clinicians should consider CNC if OMX is diagnosed.

## Abstract

Carney complex (CNC) is a multiple neoplasia syndrome, characterized by pigmented lesions of the skin and mucosa, cardiac, cutaneous and other myxomas and multiple endocrine and non-endocrine tumors. Most of the cases have an inactivating mutation in the *PRKAR1A* gene. Osteochondromyxoma (OMX) is an extremely rare myxomatous tumor of bone, affecting 1% of CNC patients. Large cell calcifying Sertoli cell tumor (LCCSCT) is a testicular tumor affecting more than 75% of males with CNC. Here, we report an atypical case of CNC without typical pigmented skin lesions, presenting with a bone based tumor as the first manifestation. Initial presentation was for a recurrent, locally invasive intranasal tumor without definite diagnosis. Further clinical developments during follow up, central precocious puberty and testicular tumor with calcification, led to the diagnosis of LCCSCT, a CNC-related tumor. Histopathologic examination of the intranasal tumor was re-evaluated with this knowledge and OMX was diagnosed. Coexistence of OMX and LCCSCT suggested CNC. Genetic analysis revealed a heterozygous non-sense p.Trp 224* (c.672G>A) in the *PRKAR1A* gene. In our case, the diagnosis of OMX was delayed, because it is extremely rare and little is known about this tumor. Thus the aim of this report was to alert other clinicians to consider CNC if OMX is diagnosed.

**Keywords:** Carney complex, osteochondromyxoma, large cell calcifying Sertoli cell tumor, central puberty precocious

## Introduction

Carney complex (CNC) is a rare multiple neoplasia syndrome with an autosomal dominant inheritance (1). However, approximately 30% of cases occur sporadically, as a result of *de novo* mutations (2). Most patients with CNC, have inactivating mutations in the protein kinase CAMP-dependent type-1 regulatory subunit alpha (*PRKAR1A*) gene. *PRKAR1A* may act as a tumor suppressor gene by regulating protein kinase A activity, which in turn can suppress or stimulate cell growth and differentiation (3).

CNC is characterized with spotty pigmentation of the skin, endocrinopathy and endocrine and nonendocrine tumors. Patients often have tumors of two or more endocrine glands, including adrenal cortex, the pituitary, thyroid, and gonads. This syndrome is associated with many other non-endocrine tumors, including cardiac myxomas, psammomatous melanotic schwannomas, breast myxomas, osteochondromyxomas (OMX) and abnormal pigmentation (lentiginious) or myxomas of the skin (2,4).

**Address for Correspondence:** Aydilek Dağdeviren Çakır MD, İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
**Phone:** +90 535 922 32 65 **E-mail:** aydi.dagdeviren@hotmail.com **ORCID:** orcid.org/0000-0001-7136-9241

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Here we report an atypical case of CNC. He had initially presented with an intranasal tumor, although the pathologic diagnosis was not clearly established. During follow-up, large cell calcifying Sertoli cell tumor (LCCSCT), a CNC related tumor, was detected in the testes and with this knowledge, the intranasal tumor was re-evaluated histopathologically and was found to be an OMX, a rare component of CNC. Coexistence of OMX and LCCSCT suggested CNC and a previously reported mutation, c.672G>A (p.Trp224*) was detected heterozygously in the *PRKAR1A* gene.

## Case Report

A 9-year-old male was referred to our pediatric endocrinology department because of partial empty sella on pituitary magnetic resonance imaging (MRI), detected during follow up of an intranasal tumor.

Patient clinical history revealed that he had been admitted to another hospital three years previously with the complaint of swelling around his right orbita. Orbital MRI had revealed an intranasal tumor filling the nasal sinuses. The tumor was invading and degrading the cribriform plate and orbita medial wall. This tumor was excised and osteochondroid tissue with osteoblasts, which suggested fibrous dysplasia histopathologically, was identified.

One year after the operation, at eight years of age, he presented with pubic hair. His physical examination revealed increased testicular volume. Central precocious puberty (CPP) was diagnosed with increased luteinizing hormone (LH) concentration and deranged LH to follicle stimulating hormone ratio on the gonadotropin releasing hormone (GnRH) test. GNRH analogue treatment was started. Cranial and pituitary MRI imaging was normal. Scrotal ultrasound (USG) revealed multiple bilateral macrocalcifications in the testes. At the age of nine years, he presented with difficulty in nasal breathing which was due to the recurrence of the intranasal tumor. Paranasal sinus computed tomography (CT) imaging revealed a lobulated, 22x25x28 mm mass with amorphous calcification, adjacent to the right frontal lobe, which extended to the base of the sphenoid sinus and which had destroyed surrounding tissues. Pituitary MRI was compatible with partial empty sella. At that point, the patient was referred to our clinic for endocrine evaluation and then followed up in conjunction with pediatric oncology and the otorhinolaryngology department. He was the first child of nonconsanguineous parents. His birth history was unremarkable and family history was not significant for tumor occurrence. On physical examination, his height was 146 cm [1.93 standard deviation (SD)], weight was 38.5 kg [1.25 SD] and body mass index was 18 kg/m$^2$ (0.23 SD). On his right lateral lumbar area, there were two domed, soft papules, the largest diameter of which was about 5 mm (Figure 1). Bilateral testes volumes were 6 cc and the stretched length of the penis was 7cm. General examination was otherwise normal. Endocrine evaluation showed adrenocorticotropic hormone (ACTH) deficiency, peak cortisol level was 13.4 µg/dL during a low dose ACTH stimulation test. All other pituitary hormone concentrations were normal, except gonadotropins which were prepubertal due to GnRH analogue treatment. Hydrocortisone replacement was started and the patient was operated for recurrent intranasal mass. Mesenchymal tumor containing chondroid component was reported by histopathology (Figure 2). Due to infiltration of the adjacent bone by the tumor (Figure 2A, 2B, 2D), focal osteoid-like matrix within the tumor (Figure 2C), recurrence of the tumor and the radiological findings, a diagnosis of osteosarcoma could not be excluded. Nevertheless, clinical findings during the follow-up were not compatible with osteosarcoma and it was decided to follow the patient closely without starting chemotherapy. The testes were re-evaluated with scrotal ultrasonography at around the same time. Numerous coarse parenchymal calcifications in both testes and a 6x5 mm hypoechoic, heterogeneous, solid lesion in the left testis were detected. Two months after the operation, cranial MRI revealed a 31x13 mm residual mass. Therefore, positron emission tomography/CT (PET/CT) scan was used to evaluate the malignancy potential of the lesion. $^{18}$F-FDG PET imaging revealed normal uptake value in the tumor and also in other parts of the body.

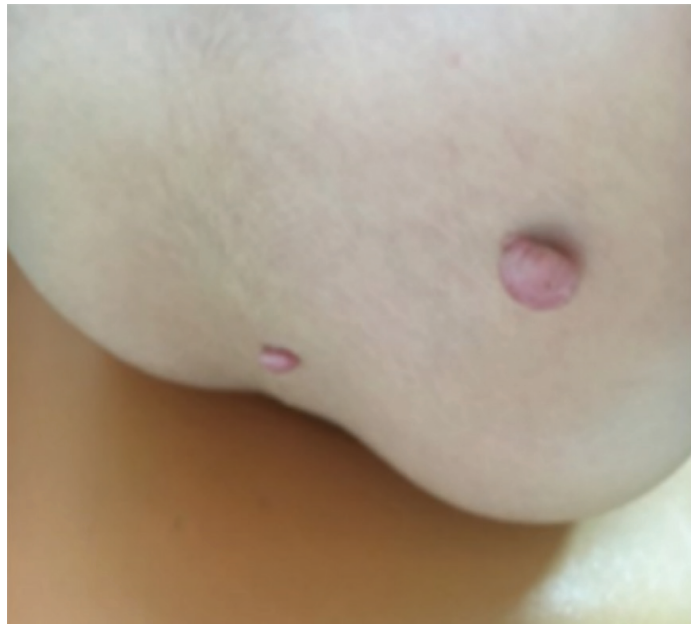

**Figure 1.** Cutaneous myxomas, the largest with a diameter of 5 mm

The patient was reoperated for the tumoral lesion in the nasal cavity and testicular biopsy was taken from the testicular lesions, considering that these lesions might be due to metastases of the primary disease. Histopathological examination of the intranasal lesion reported only granulation tissue and reactive bone formation were without tumoral tissue. However, testicular lesions were compatible with LCCSCT without malignant features (necrosis and mitosis) (Figure 3). The diagnosis of LCCSCT suggested the possibility of CNC. Robust histopathological diagnosis of the bone lesion was not clear, thus the specimens were re-evaluated for the possibility of OMX, which is known to be a rare CNC-associated bone tumor, most frequently located in the nasal sinuses and diaphyses of the long bones. Pathologic re-examination revealed osteoid and chondroid predominant lobular areas and focal mesenchymal spindle cells that suggested OMX. The patient was re-evaluated for fulfillment of the diagnostic criteria of CNC. The lesions on his back were cutaneous myxomas which are common components of CNC. Presence of OMX and LCCSCT, which are two major criteria, confirmed the diagnosis of CNC. Thus the patient was evaluated for other CNC-associated endocrinopathies, and growth hormone (GH) excess was detected. Pituitary MRI did not show any adenoma. Despite GH excess, growth velocity was not increased and other clinical manifestations related to GH excess were not found. No other endocrine dysfunction associated with CNC, such as hyperprolactinemia and hypercortisolemia, was detected. On the contrary, he had ACTH deficiency. For molecular confirmation, genetic analysis was performed and a heterozygous nonsense mutation, c.672G>A (p.Trp224*), was detected in the *PRKAR1A* gene. In this case, the malignancy potential of the LCCSCT was judged to be low by the histopathologist and the maximum diameter of the tumoral lesions was 6.7 mm. Therefore, the lesions were followed up with scrotal USG without intervention and the size of the tumoral lesions did not change during the subsequent 18 months follow-up. The patient was also screened for other CNC–associated tumors and no additional tumor was found.

## Discussion

CNC is a rare, multiple endocrine neoplasia syndrome with autosomal dominant inheritance. It is usually characterized by pigmented lesions of the skin and mucosal, cardiac, cutaneous and other myxomas, and multiple endocrine and non-endocrine tumors. To confirm the diagnosis, patients must meet at least two major criteria or one major and one supplemental criteria (5). In our case, CNC was diagnosed

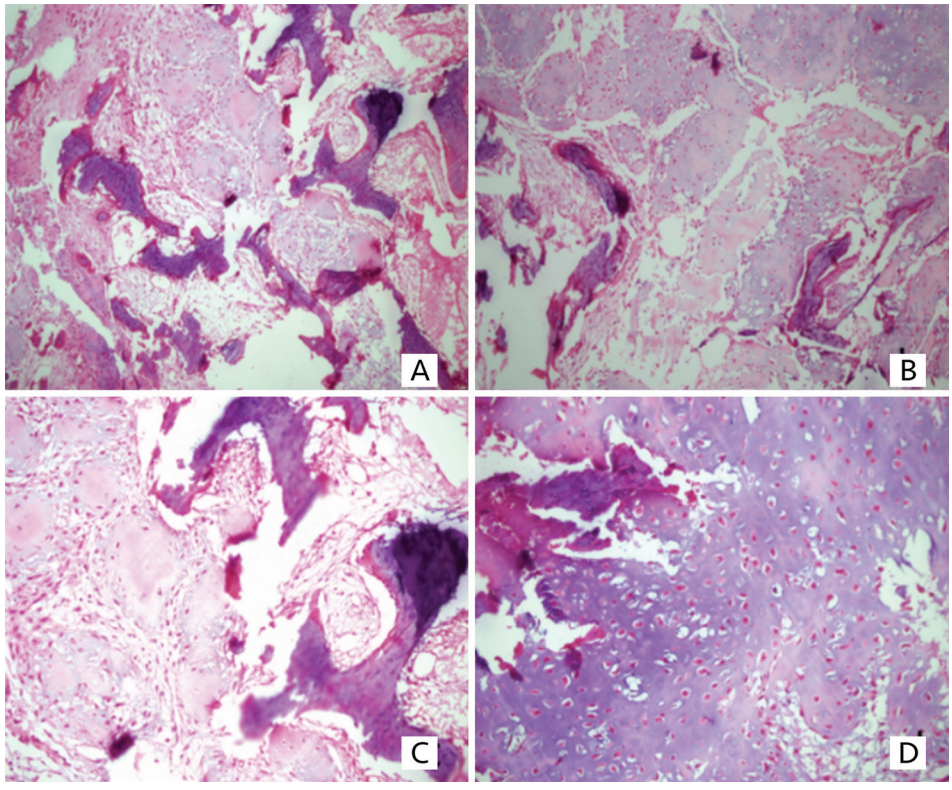


**Figure 2. A, B, C, D)** Tissue preparations of the nasal cavity-osteochondromyxoma: The tumor consists of lobular areas, with chondroid and predominantly osteoid cells. Focal mesencymal spindled cells are present (hematoxylin and eosin stain; magnification x200)

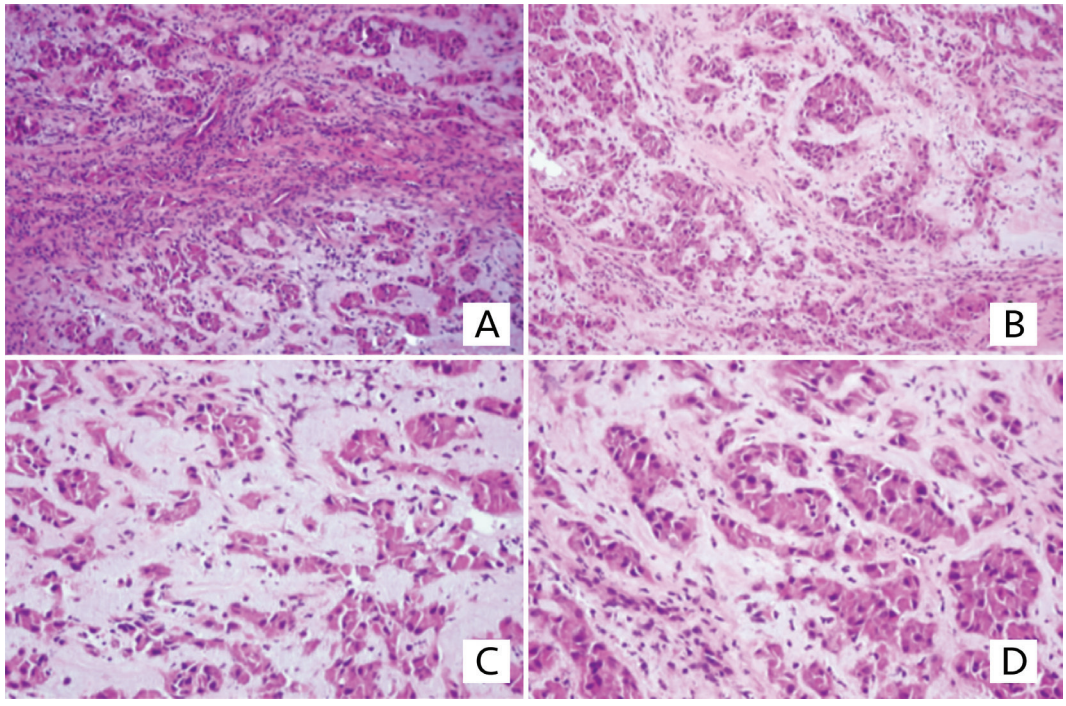


**Figure 3. A, B, C, D)** Tissue preparations of the testicular biopsy with large cell calcifying Sertoli cell tumor. The neoplastic cells form solid and hollow tubules and are immersed in a loose, myxoid matrix. The tumor is composed of large polygonal cells with abundant eosinophilic cytoplasm and eccentric nuclei (hematoxylin and eosin stain; magnification x100-200)

clinically with the coexistence of histologically proven OMX and LCCSCT as two major criteria and confirmed by genetic analysis. More than 80% of CNC patients develop spotty skin pigmentations, which typically appear early in life and may be located anywhere on the body, typically on the face, lips, genital area and mucosa (6). Pigmented skin lesions of CNC were not present in our case. Only two myxomatous lesions were detected as cutaneous manifestations.

CNC is characterized by endocrine overactivity. Primary pigmented nodular adrenocortical disease is the most common endocrine lesion and causes hypercortisolism (7). The adrenal imaging in this case were normal and he had hypocortisolism due to ACTH deficiency, which was associated with partial empty sella. Asymptomatic GH hypersecretion occurs in approximately 2/3 of patients, in most cases without imaging evidence of pituitary adenoma (5), as occurred in our patient.

In the case presented here the first manifestation of CNC was OMX, which is an extremely rare myxomatous tumor of bone, affecting 1% of CNC patients (8), but it is one of the 11 diagnostic criteria (5). Although he had presented with a bone tumor at the age of six, diagnosis of OMX was delayed by three years, because of the rarity of the tumor and the possibility of osteosarcoma. Awareness of OMX is poor due to the rarity of the tumor although diagnosis may be aided if there is a clinical suspicion of CNC of which the histopathologist is aware. Characteristically, OMX is a painless mass and may be unnoticed unless it enlarges and surrounds or invades vital structures. It may affect any bone, but is most frequently seen in the nasal sinuses and long bones of extremities (8). In our patient, the characteristic location of the tumor in the nasal sinus suggested OMX and it was subsequently proven histopathologically. Although OMX is a benign neoplasm, it can exhibit locally invasive characteristics (9). On imaging, OMX is well circumscribed, but can be destructive (8), as in our case. Osteosarcoma was considered in the differential diagnosis, because of the local invasive and destructive nature of the tumor and radiological findings. However, osteosarcoma was an unlikely diagnosis since no metastasis was observed during three years of follow-up. OMX has a good prognosis with complete excision, however local recurrence is very common with incomplete resection (8,9,10). Disease recurrence is therefore more likely at sites where complete resection is difficult, as in this case.

LCCSCTs, especially in children, present in association with CNC or Peutz-Jeghers syndrome (11). More than 75% of males with CNC may have LCCSCT (12). Malignancy is found in approximately 17% of patients, but is rare in young patients with bilateral tumors or in association

with a genetic syndrome (11,13). Malignant behaviour is associated with large size (>4 cm), necrosis, increased mitotic activity, atypia and vascular invasion (14,15). In our case, bilateral multiple nodular lesions were present. The largest diameter of nodules was 6.7 mm. There was no evidence of malignancy histopathologically. Tumors may lead to premature epiphyseal fusion and induction of CPP, due to increased aromatase activity (5,14). Our case also presented with CPP. At first, it was thought that CPP was related to excision of the intranasal tumor close to the sellar region, but then it was realized that CPP may be associated with LCCSCT. In light of the low malignancy risk of this tumor, only imaging surveillance was performed, as recommended (14).

In conclusion, a case of CNC is reported, presenting with undiagnosed OMX as the first manifestation. OMX is a very rare tumor, but should be considered in the differential diagnosis of local invasive, recurrent intranasal tumors. In addition the possibility of LCCSCT should be kept in mind in cases of calcified testis tumor presenting with CPP.

### Ethics

**Informed Consent:** Informed consent was taken from the parents of patient for publication.

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

### Authorship Contributions

Surgical and Medical Practices: Aydilek Dağdeviren Çakır, Hande Turan, Tiraje Celkan, Oya Ercan, Olcay Evliyaoğlu, Concept: Aydilek Dağdeviren Çakır, Olcay Evliyaoğlu, Design: Aydilek Dağdeviren Çakır, Olcay Evliyaoğlu, Data Collection or Processing: Aydilek Dağdeviren Çakır, Hande Turan, Tiraje Celkan, Analysis or Interpretation: Aydilek Dağdeviren Çakır, Oya Ercan, Olcay Evliyaoğlu, Literature Search: Aydilek Dağdeviren Çakır, Hande Turan, Writing: Aydilek Dağdeviren Çakır, Tiraje Celkan, Oya Ercan, Olcay Evliyaoğlu.

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# An Iranian Patient with Maroteaux Type Acromesomelic Dysplasia, Showing no Involvement of Distal Lower Limbs

**Hossein Moravej[1,2], Mozhgan Moghtaderi[1], Sara Mostafavi[3]**

*[1]Shiraz University of Medical Sciences, Neonatal Research Center, Department of Pediatrics, Shiraz, Iran*
*[2]Shiraz University of Medical Sciences, Department of Pediatric Endocrinology, Shiraz, Iran*
*[3]Shiraz University of Medical Sciences, Student Research Committee, Shiraz, Iran*

Dear Editor,

Acromesomelic dysplasia, Maroteaux type (AMDM) is an autosomal recessive form of acromesomelic dysplasia characterized by disproportionately short stature, shortening of the middle and distal segments of the limbs as well as vertebral involvement. AMDM is the result of a mutation in the natriuretic peptide receptor 2 (NPR2) genes which impairs skeletal growth (1,2,3).

A 2- years old boy, offspring of non-consanguineous parents and of a 2nd pregnancy, was referred to the endocrine and metabolic center of the Nemazee Hospital, located in southwestern Iran, for evaluation of short stature. The patient was born at 38 weeks of gestation by cesarean section and was healthy by Apgar scoring. Birth weight was 3100 g, length 45 cm and head circumference 35 cm. He had no dysmorphic features and general physical examination revealed no pathology. There was no satisfactory length gain after birth, as noticed by his parents. At the referral time at age 2 years the patient had a weight of 8200 g (-4 SD), a length of 71 cm [-4 standard deviation (SD)]. Head circumference was 48 cm (0.3 SD). Fingers of the hand were extremely short and broad with small nails; there was no redundant skin on the fingers (Figure 1). His feet and toes were normal. Frontal bossing, low set ears and wrist joint hyperflexibility were prominent features. All developmental milestones were within normal limits. His older sibling was of normal stature. Matental height was 156 cm (-1.6 SD) and the father was 163 cm (-1.9 SD) tall. His older sibling was of normal stature. None of the other family members were affected.

Radiographic findings showed curved radius, relatively short ulna, and broad metacarps with wide phalanges. The

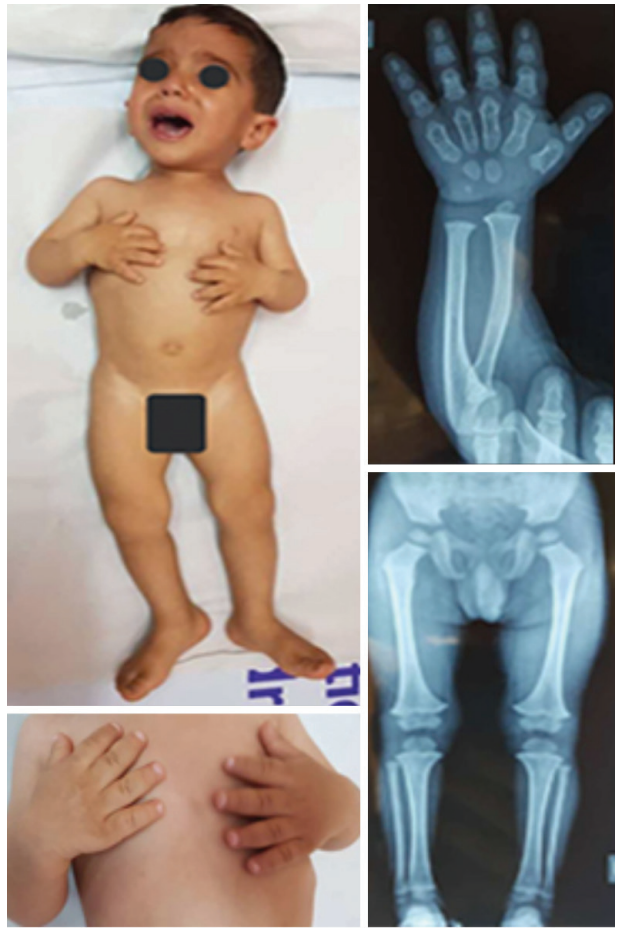

**Figure 1.** Clinical characteristics and radiographic features of the patient. Frontal bossing, low set ears and wrist joint hyperflexibility as well as short and broad fingers of the hand with small nails are noteworthy. Radiographic findings showed radial bowing with posterior dislocation, short lower end of the ulna as compared to the radius, and broad metacarps with wide phalanges

**Address for Correspondence:** Mozhgan Moghtaderi MD, Shiraz University of Medical Sciences, Neonatal Research Center, Department of Pediatrics, Shiraz, Iran
**Phone:** +90 98 71 36122218 **E-mail:** moghtadery@sums.ac.ir **ORCID:** orcid.org/0000-0002-9612-2429

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vertebrae were of normal size and showed no beaking. Iliac wings and metatarsal bones were normal (Figure 1). DNA was extracted from the peripheral blood by standard techniques and microsatellite analyses were performed. Whole exon sequencing test and mutation confirmation by direct Sanger screening were performed and evaluated by reference sequence, AMDM maps to 9p13.3. Cytogenic evaluation could not be performed in the parents and in the older sibling. Informed consent was obtained from his parents for this report.

The mutation of the case was displayed in NPR2 with cytogenic location of 9p13.3. This mutation overlaps with two diseases: firstly, autosomal dominant epiphyseal chondrodysplasia, miura type which is characterized by tall stature, long hands and feet with arachnodactyly, and secondly, short-rib thoracic dysplasia 5 with or without polydactyly (4,5). Both diseases have completely different clinical patterns and radiographic manifestations from AMDM.

In summary, considering the skeletal changes, radiological findings and sequence analysis of the mutation, this patient is the first AMDM case reported from Iran. The patient had severe short stature, but no obvious abnormality in the distal segment of his lower limb. We suggest that this patient may represent a new variant form of AMDM.

### Ethics

**Informed Consent:** The parents received oral and written information before signing a consent form.

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

### Authorship Contributions

Medical Diagnosis: Hossein Moravej, Concept: Hossein Moravej, Mozhgan Moghtaderi, Design: Hossein Moravej, Mozhgan Moghtaderi, Sara Mostafavi, Data Collection or Processing: Mozhgan Moghtaderi, Sara Mostafavi, Analysis or Interpretation: Hossein Moravej, Mozhgan Moghtaderi, Literature Search: Hossein Moravej, Mozhgan Moghtaderi, Sara Mostafavi, Writing: Hossein Moravej, Mozhgan Moghtaderi.

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The mistake has been made by author inadvertently. "c.386T > G" word in page 179, Abstract section, line 6 has been corrected as "c.386T > C".