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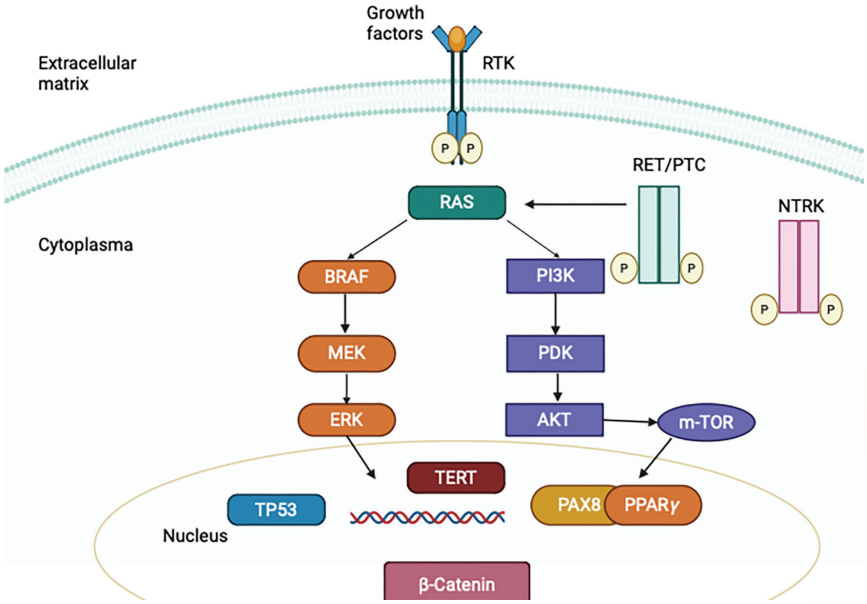

Pathogenesis of Thyroid Cancer with Particular Emphasis on the Role of Anoikis

Akın Kağızmanlı et al.

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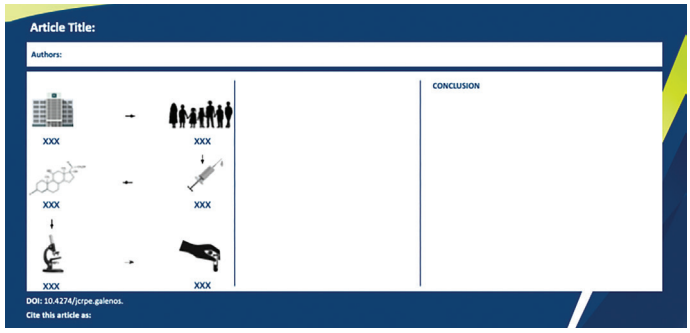

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J Clin Res Pediatr Endocrinol 2026;18(3):374-384

Pathogenesis of Thyroid Cancer with Particular Emphasis on the Role of Anoikis

Gözde Akın Kağızmanlı[1,2], Selen Kum Özşengezer[2], Korcan Demir[1], Zekiye Altun[2]

[1]Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye
[2]Dokuz Eylül University, Oncology Institute, Department of Basic Oncology, İzmir, Türkiye

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ABSTRACT

Thyroid cancer (TC) is the most prevalent malignancy of the endocrine system, and its incidence has been increasing worldwide in recent years. Although it generally has a favorable prognosis, aggressive forms, such as anaplastic TC, are associated with high mortality rates. Cell-microenvironment interactions largely influence the progression and metastatic behavior of TC however, the precise mechanisms underlying these processes remain inadequately understood. One critical factor influencing metastasis is anoikis, a form of programmed cell death triggered by the detachment of cells from the extracellular matrix. Resistance to anoikis allows tumor cells to escape apoptosis, survive in circulation, and metastasize to distant organs. Given this pivotal role in metastasis, anoikis resistance represents a key area of study in understanding cancer progression. This review will discuss the molecular mechanisms and pathogenesis of TC, with a focus on the role of anoikis resistance in metastatic spread and thus its potential as a therapeutic target.

Keywords: Thyroid cancer, anoikis, metastasis, apoptosis, extracellular matrix

Introduction

Thyroid cancer (TC) is the most prevalent malignancy of the endocrine system, and in recent years its incidence has been increasing worldwide (1,2). While it can affect both sexes, approximately 75% of TC cases occur in women. TC is most commonly diagnosed in individuals in their early 50s but can occur at any age. Notably, TC is the most common malignancy among adolescents and young adults aged 16-33 years (1). In the pediatric population specifically, TC constitutes over 6% of all childhood cancers, with its occurrence has demonstrated a marked rise over the past four decades (3,4).

TCs originate from the follicular and parafollicular C cells of the thyroid gland and are histologically divided into three main categories (1,5). The most prevalent among them, accounting for over 95% of all TC cases, are differentiated thyroid carcinomas (DTCs) derived from follicular epithelial cells. These include papillary TC (PTC), follicular TC (FTC), and Hurthle cell TC (1). PTC generally progresses slowly and is associated with a favorable

Corresponding Author: Gözde Akın Kağızmanlı, MD, Dokuz Eylül University Faculty of Medicine, Division of Pediatric Endocrinology; Dokuz Eylül University, Oncology Institute, Department of Basic Oncology, İzmir, Türkiye
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prognosis, although it frequently presents with multifocal lesions and regional lymph node metastases due to a tendency for lymphatic spread (6). In contrast, FTC demonstrates a more invasive growth pattern and is often characterized by capsule formation. This type is more commonly seen in adults and is rare in childhood. Cases with only capsular invasion typically have a favorable prognosis, whereas vascular invasion may lead to metastases in distant organs. Hurthle cell tumors, a rare subtype of DTCs, account for 5% of all cases. These tumors are more frequently seen in men, typically present as encapsulated masses, and predominantly occur in elderly individuals (2,6,7). Notably, compared to adult patients, children and adolescents with DTCs are more prone to regional lymphatic spread, extension beyond the thyroid capsule, and distant metastases, despite often responding well to treatment (8).

In contrast to DTCs, poorly DTCs (PDTCs) represent a more aggressive cancer type, also originating from follicular cells. Histopathologically and biologically, they demonstrate characteristics intermediate between well-differentiated carcinomas (e.g., PTC and FTC) and anaplastic carcinoma. Undifferentiated tumors arising from follicular cells are termed anaplastic TC (ATC), the rarest (<1%) type, and are characterized by a highly aggressive course and a poor prognosis (1,5). Medullary TC (MTC), which differs from these other subtypes, originates from parafollicular C cells and constitutes approximately 2-3% of all TCs. While 70% of MTC cases occur sporadically, 30% are associated with Multiple Endocrine Neoplasia Type 2 (MEN2) syndrome and are inherited in an autosomal dominant manner (1,6,7,9).

Significant advances have been made in understanding the molecular and clinical characteristics of TC, yet important gaps remain, particularly in identifying the factors that drive differences in aggressiveness and metastatic potential among TC subtypes. Anoikis resistance, a process that allows tumor cells to escape apoptosis after detachment from the extracellular matrix (ECM), is an important mechanism in metastasis. However, its involvement in TC metastasis is not well understood, and studies about this phenomenon are limited (10,11,12,13). This aim of this article is to review the current understanding of the molecular pathways involved in TC progression, with a focus on anoikis resistance, its role in metastasis and its potential as a therapeutic target.

Pathophysiology and Molecular Mechanisms of Thyroid Cancer

The pathogenesis of TC is a complex process influenced by genetic and environmental factors, including iodine deficiency or excess, exposure to ionizing radiation, and chronic inflammatory conditions (14). The development of TC involves numerous molecular genetic and epigenetic alterations that interfere with fundamental biological functions, such as cell division, proliferation, and regulation of apoptosis (2,7).

At the molecular level, several genetic changes are observed during TC progression, including somatic mutations, gene amplifications, copy number alterations, and chromosomal rearrangements. These changes are regarded as traditional tumorigenic triggers that drive uncontrolled cancer cell growth and dissemination (7,14). Moreover, TC exhibits intratumoral heterogeneity, characterized by the presence of genetically, phenotypically, and behaviorally diverse cell populations within a single tumor. This heterogeneity arises from genetic and epigenetic factors differences between tumor subclones, such as copy number changes, point mutations, gene rearrangements, and epigenetic modifications. Genomic instability and the progressive accumulation of genetic alterations further contribute to the development of new subclones, increasing tumor complexity and promoting the transition from early-stage tumors to more aggressive cancers (15).

Epigenetic mechanisms, such as DNA methylation, play an important role in TC. DNA methylation can suppress gene expression, thereby altering cellular behavior and contributing to tumor development. The interplay between genetic alterations and epigenetic modifications reinforces the complexity of TC tumorigenesis (7). In recent years, non-coding RNAs (ncRNAs) have also been identified as important molecules that play a regulatory role in TC. ncRNAs are involved in the post-transcriptional regulation of gene expression and imbalances in these molecules may affect cancer pathogenesis. In particular, microRNAs (miRNAs) may contribute to cancer progression by modulating oncogenes or tumor suppressor genes in TC (2,16).

Genetic Alterations in Thyroid Cancer

Over the past three decades, rapid advances in genome sequencing technologies have facilitated a deeper understanding of the molecular mechanisms underlying TC. Although TC was considered a disease with a relatively low somatic mutation burden, driver mutations triggering cancer development have been identified in more than 90% of cases (7). The most frequently observed genetic alterations are point mutations in the *BRAF* and *RAS* genes, which are often followed by *RET/PTC* rearrangements. Less common mutations include alterations in *PIK3CA, TP53, TSHR, PTEN, GNAS,* and *CTNNB1* genes, all of which may contribute to tumorigenesis (14). Notably, oncogenic gene fusions are much more prevalent in pediatric TC, present in 50-60% of cases, compared to only 15% in adults. Conversely, point mutations are less frequent in pediatric cases, observed in approximately 30% of pediatric TCs, compared with nearly 70% in adults (4).

The *BRAF* gene belongs to the serine-threonine kinase family and regulates the expression of genes involved in cell proliferation, differentiation, and apoptosis through the MAPK/ERK signaling pathway. The most frequently reported mutation is *BRAF-V600E*, which is associated with increased invasion, tumor growth,

and loss of cell differentiation, particularly in PTC (2,4,7). *BRAF* mutations are found in up to 60% of adult PTC cases but are significantly rarer in pediatric PTC, identified in only about 25% of cases, indicating age-related differences in the molecular drivers of TCs (3).

RAS gene mutations are among the most common and important genetic alterations, after *BRAF* mutations (17). RAS functions as a G-protein that is inactive when bound to GDP and becomes active upon binding to GTP. There are three main isoforms of the *RAS* gene: *HRAS*, *KRAS,* and *NRAS*. Mutations in the *NRAS* gene, which primarily affect the PI3K/AKT pathway, are frequently implicated in thyroid tumorigenesis. However, these mutations may also activate the MAPK/ERK signaling pathways, in addition to PI3K/AKT. While *RAS* mutations are predominantly observed in FTCs, they have also been identified in other TC types, including PTC and PDTC (14). The frequency of *RAS* mutations varies between subtypes, occurring in 10-20% of PTC cases and 40-50% of FTC cases. However, *RAS* mutations are notably rare in pediatric TCs (18).

Another critical genetic alteration that may play a role in the pathogenesis of TC is the rearrangement of the *RET* proto-oncogene. The *RET* gene is expressed in neuroendocrine C cells and its mutations are primarily associated with MTC (7). However, *RET/PTC* rearrangements have been implicated in PTC carcinogenesis by leading to uncontrolled activation of MAPK/ERK and PI3K-AKT pathways (17). Notably, the prevalence of *RET/PTC* rearrangements differs between adult and pediatric cases, occurring in 5-15% of adult sporadic PTCs but rising to 25-65% in pediatric cases (19).

PAX8/PPARγ fusion protein is one of the most common genetic alterations in PTC after *RAS* mutations and has been associated with invasive tumors occurring at a younger age. This fusion protein has also been detected in follicular variant PTC, follicular adenoma, and Hurthle cell carcinoma (20). Furthermore, recent studies have highlighted the higher prevalence of telomerase reverse transcriptase promoter mutations in aggressive and undifferentiated tumors. These mutations, observed across all histological subtypes of TC, underscore their critical role in TC progression (Figure 1) (7,21).

Meanwhile, most cases of MTC are caused by mutations in the *RET* proto-oncogene. These mutations can occur as inherited germline mutations in MEN2A and MEN2B syndromes or arise sporadically. Mutations in *HRAS, KRAS,* and *NRAS* are responsible for a smaller number of sporadic MTC cases (7,22).

Signaling Pathways in Thyroid Cancer

Disruptions in the MAPK/ERK and PI3K/AKT signaling pathways play a critical role in the molecular pathogenesis of TC (7). The MAPK/ERK pathway, one of the key signaling cascades involved in TC development, regulates cell proliferation, survival, and tumor progression. Genetic alterations, including mutations in *ALK, NTRK1, NTRK3,* and *EIF1AX*, which activate the MAPK/ERK pathway, have been reported in cases where *BRAF* and *RAS* mutations are absent. This pathway is particularly significant in the development of PTC (22). *BRAF-V600E* mutation activates the MAPK/ERK signaling pathway and interacts with ECM proteins in the thyroid tumor microenvironment. This affects the viability, motility, and adhesion properties of cancer cells. This process is regulated through paracrine and autocrine signaling mechanisms between cancer cells and stromal cells, with these interactions in the tumor microenvironment leading to cancer progression (2).

PI3K/AKT pathway, another critical signaling cascade, plays a pivotal role in the development of FTC. It is activated by mutations in *PIK3CA* and *AKT1* genes or inactivation of the *PTEN* gene, in addition to *RAS* mutations and *PAX8/PPARγ* fusion protein (16). Furthermore, the PI3K/AKT pathway contributes to TC pathogenesis by influencing secondary molecular pathways, including WNT-β-catenin, hypoxia inducing factor 1-α, FOXO3 and Nuclear Factor Kappa B (NF-κB) (2).

The progression of TC and its transformation into poorly differentiated TC or ATC is driven by additional mutations affecting key signaling pathways. Notably, disruptions in p53 and Wnt/β-catenin signaling pathways play an important role in this transformation. The NF-κB signaling pathway also contributes by regulating proliferative and anti-apoptotic mechanisms in TC cells, enhancing tumor survival and malignancy (7).

Relationship Between Thyroid Cancer and Anoikis

Cellular Mechanisms of Anoikis and Its Role in Cancer

Anoikis is a form of programmed cell death triggered by the detachment of cells from the ECM. This term, which means “homelessness” in Greek, refers to the inability of cells to survive if they lose their connection with the ECM. Integrin receptors, which mediate cell-ECM interactions, not only establish a physical connection to the cytoskeleton but also regulate various cellular processes, including cell migration, proliferation, and survival, by transmitting signals from the ECM to the cell (23,24).

Anoikis serves as a crucial defense for the organism by preventing the reattachment and uncontrolled growth of misplaced cells within the ECM, thus maintaining tissue homeostasis (25). When anoikis is dysregulated, cells may survive or proliferate in inappropriate environments, a hallmark of cancer cells that facilitates metastases to distant organs (26).

Intrinsic and Extrinsic Pathways of Anoikis

Anoikis is a process initiated by the activation of caspases and leads to the activation of endonucleases, DNA fragmentation,

and cell death. This process is orchestrated through the two main pathways of apoptosis, the intrinsic and extrinsic pathways (Figure 2). Bcl-2 family proteins play a central role in both pathways and this family is divided into three main groups consisting of anti-apoptotic proteins such as Bcl-2, Bcl-XL, Mcl-1, pro-apoptotic proteins such as Bax, Bak and Bok, and pro-apoptotic proteins with BH3 domain-only proteins, such as Bid, Bad, Bim, Bmf, Noxa, and Puma (23,27).

The intrinsic pathway is activated by intracellular stress factors, such as DNA damage or endoplasmic reticulum stress, leading to mitochondrial dysfunction. During this process, pro-apoptotic Bax and Bak proteins are transported from the cytosol to the outer mitochondrial membrane, where they oligomerize and form pores that increase mitochondrial permeability. This leads to the release of cytochrome-C, which combines with apoptosis protease activation factor and caspase-9 to form a complex called the apoptosome. The apoptosome initiates apoptosis by activating caspase-3 (23,28). BH3-domain proteins, particularly Bid and Bim, play a crucial role in the intrinsic pathway of the anoikis. They are rapidly activated when cells detach from the ECM, facilitating the oligomerization of Bax and Bak, which in turn promotes the release of cytochrome C from the outer mitochondrial membrane. Furthermore, other BH3-only proteins, such as Bad, Bik, Bmf, Noxa, Puma, and Hrk indirectly promote Bax-Bak oligomerization by inhibiting the anti-apoptotic effects of Bcl-2 (29).

The extrinsic pathway is initiated by ligand binding to death receptors belonging to the tumor necrosis factor (TNF) receptor superfamily, such as Fas, TNFR1, and TRAIL receptor-1 and -2. The death-inducing signaling complex (DISC) is formed as a result of this binding. Caspases 8 are activated as a result of DISC's interactions with adaptor proteins, such as Fas-associated death domain protein. Once released into the cytoplasm, active caspase-8 activates effector caspases such as caspase-3, caspase-6, and caspase-7, leading to cell death (23,30). Furthermore, the Bid protein is cleaved and activated by caspase-8, which starts the release of mitochondrial cytochrome-C and apoptosome formation. This mechanism establishes a functional link between extrinsic and intrinsic pathways (31). Detachment of cells from the ECM increases the expression of Fas and Fas ligands. These changes illustrate the important role of the extrinsic pathway in anoikis (32). Furthermore, alteration of cell shape may also lead to activation of Fas receptors and initiation of extrinsic anoikis (33). Anti-apoptotic signals contributing to anoikis resistance are summarized in Table 1.

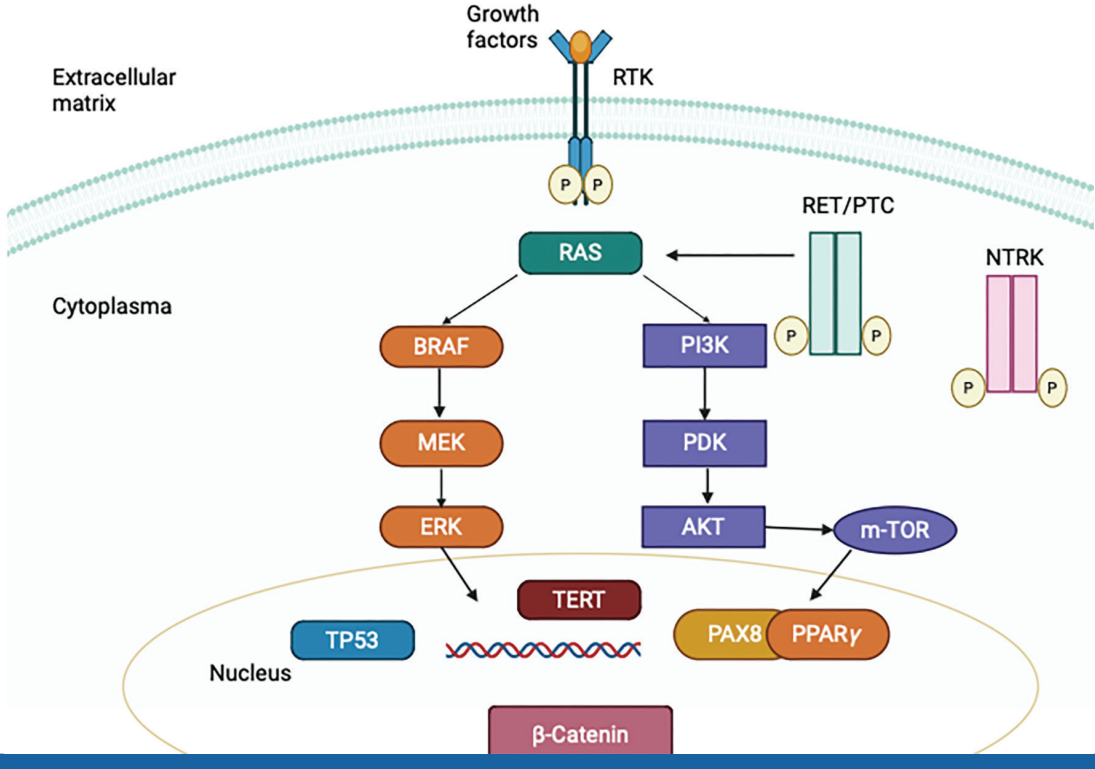

Figure 1. Molecular pathogenesis of thyroid cancer. Adapted from Prete et al. (7)
RTK: receptor tyrosine kinase, RAS: rat sarcoma virus (RAS protein family), RAF: rapidly accelerated fibrosarcoma, ERK: extracellular signal-regulated kinase, PI3K: phosphoinositide 3-kinase, PDK: phosphoinositide-dependent kinase, AKT: protein kinase B, mTOR: mechanistic target of rapamycin, NTRK: neurotrophic tyrosine receptor kinase, RET: rearranged during transfection, TERT: telomerase reverse transcriptase, TP53: tumor protein p53, PAX8: paired box gene 8, PPARγ: peroxisome proliferator-activated receptor gamma

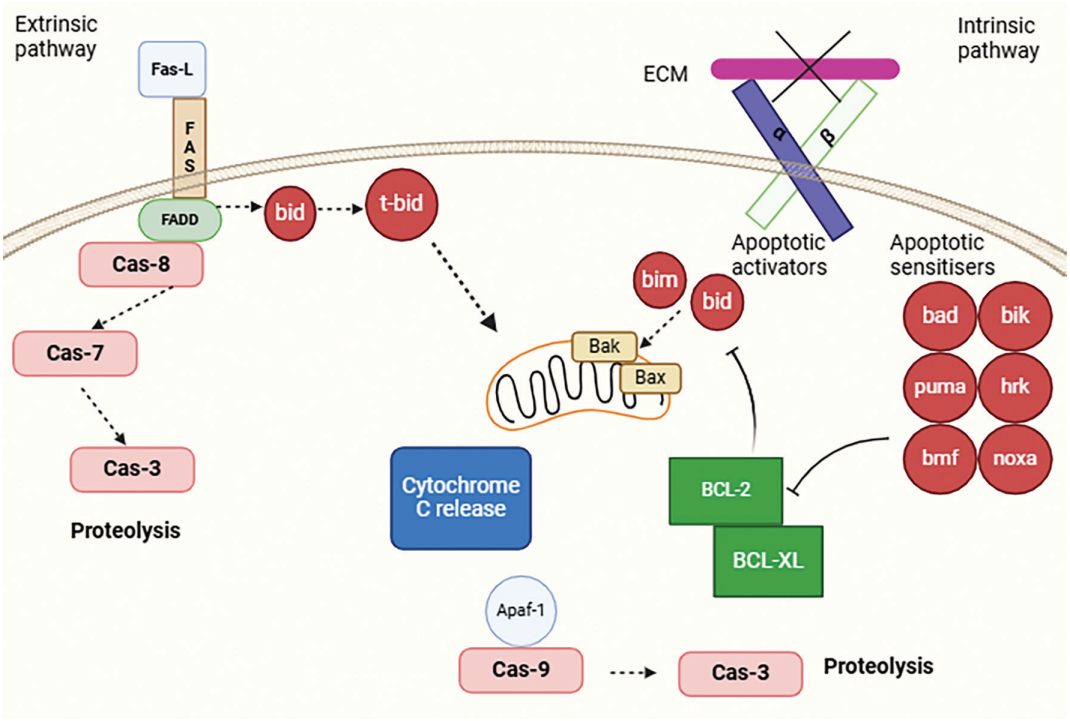

Figure 2. Activation of anoikis mechanism. Adapted from Taddei et al. (27)
ECM: extracellular matrix, FasL: Fas ligand, FADD: Fas-associated death domain protein, Bid: truncated Bid, Bax: BCL-2-associated X protein, Bcl-2: B-cell lymphoma 2, Bcl-XL: B-cell lymphoma-extra large, Apaf-1: apoptotic protease activating factor 1

Protection Against Anoikis Under Physiological Conditions

Epithelial cells are protected against anoikis when they bind to ECM proteins under physiological conditions. Studies have shown that integrins suppress apoptosis and transmit cell survival signals through cell-ECM interactions (34). Various integrin subunits (α5β1, αvβ3, α1β1, α6β1) activate different signaling pathways that promote cell survival. These pathways include key signaling cascades such as focal adhesion kinase (FAK), Src kinase, integrin-linked kinase (ILK), PI3K/AKT, and MAPK/ERK. FAK, which is rapidly activated after contact with ECM, regulates cell survival, proliferation, and motility. The PI3K/AKT pathway prevents cell death by inhibiting the activation of caspases and phosphorylating pro-apoptotic proteins (23). In addition, ligand-independent activation of several growth factor receptors, including vascular endothelial growth factor receptor, hepatocyte growth factor receptor, platelet-derived growth factor receptor, and epidermal growth factor receptor, can be triggered by ECM interaction. This activation activates the MAPK/ERK and PI3K/AKT pathways and transduces signals that inhibit cell death (23,35).

During cell migration, cells may temporarily lose their attachment to the ECM. Mesenchymal motility is the process by which cells migrate using specific adhesion points, driven by proteolysis of the ECM. During this type of migration, cell-ECM interactions and activation of growth factor receptors transduce survival-promoting signals by triggering the PI3K pathway (36). Another form of migration, ameboid motility, allows cells to move rapidly without proteolysing the ECM and without forming adhesion points. This process occurs by supporting cells that make poor contact with the ECM by transmitting survival signals through Rho family GTPases (23).

Cell-cell contacts play a crucial role in cell survival. Cadherins act as fundamental membrane proteins that provide binding between cells and interact with integrins and growth factor receptors to transmit survival signals to the cell. E-cadherin blockade triggers anoikis, while β-catenin overexpression increases anoikis resistance in epithelial cells. N-cadherin transduces survival signals via the PI3K/AKT pathway (37). Other cell surface molecules such as P- and L-selectin are also involved in this process, activating the FAK, Src, PI3K/AKT, and MAPK/ERK pathways, thus ensuring cell survival (38,39). Inhibition of the PI3K/AKT pathway, which transduces survival signals, impairs energy metabolism and decreases ATP levels in cells that are separated from the ECM. This may lead to an increase in reactive oxygen species (ROS) and trigger cell death. However, autophagy may act as a temporary survival mechanism under these conditions. During autophagy, cells generate energy by breaking down their components and can survive until they reattach to the ECM (40).

Table 1. Overview of anti-apoptotic mechanisms contributing to anoikis resistance in thyroid cancer

Mechanism/pathway	Key molecules	Mechanism of action in anoikis resistance
Intrinsic apoptotic pathway inhibition	Bcl-2, Bcl-XL, Mcl-1	Prevents mitochondrial cytochrome-C release and caspase-9 activation.
Extrinsic apoptotic pathway suppression	Fas, FasL, FADD, Caspase-8	Inhibits caspase-8-mediated apoptosis and maintains Bid in an inactive form.
PI3K/AKT pathway activation	PI3K, AKT, PTEN (loss)	Suppresses pro-apoptotic proteins and promotes survival under ECM-detached conditions.
MAPK/ERK pathway activation	BRAF (V600E), MEK, ERK	Enhances cell proliferation and survival, suppresses pro-apoptotic signaling, and supports EMT induction.
NF-κB pathway activation	NF-κB	Induces transcription of anti-apoptotic genes (e.g., Bcl-2).
Integrin-mediated survival signaling	αvβ3, α5β1, FAK, Src, ILK	Maintains survival via ECM-independent activation of AKT/MAPK signaling.
Growth factor receptor activation	VEGFR, HGF-R, PDGFR, EGFR	Activates PI3K/AKT and MAPK/ERK pathways via ligand-independent signaling, enhancing survival.
Hypoxia response and ROS adaptation	HIF-1α, NADPH oxidase, ROS, NF-κB, p53	Promotes EMT and survival under oxidative stress, suppresses apoptosis via redox modulation.
EMT	Snail, Twist, ZEB1/2, N-cadherin, Vimentin	Suppresses E-cadherin, promotes mesenchymal traits, enhances motility, invasion, and anoikis resistance.
Cadherin switching and β-catenin signaling	E-cadherin (downregulated), N-cadherin (upregulated), β-catenin	Reduces intercellular adhesion and promotes survival during ECM detachment.
Metabolic reprogramming (Warburg effect)	GLUT1, LDHA, HIF-1α	Maintains ATP levels by increasing glycolysis during ECM detachment.
Autophagy activation	Beclin-1	Provides temporary survival through metabolic stress adaptation.

ECM: extracellular matrix, FAK: focal adhesion kinase, Src: Src family kinases, ILK: integrin-linked kinase, PI3K: phosphoinositide 3-kinase, AKT: protein kinase B, NF-κB: nuclear factor kappa B, VEGFR: vascular endothelial growth factor receptor, HGF-R: hepatocyte growth factor receptor, PDGFR: platelet-derived growth factor receptor, EGFR: epidermal growth factor receptor, EMT: epithelial-mesenchymal transition, ROS: reactive oxygen species, GLUT1: glucose transporter 1, LDHA: lactate dehydrogenase A, HIF-1α: hypoxia-inducible factor 1-alpha, Bcl-2: B-cell lymphoma 2, Bcl-XL: B-cell lymphoma-extra large, Mcl-1: myeloid cell leukemia 1, Fas: Fas cell surface death receptor, FasL: Fas ligand, FADD: Fas-associated death domain protein, PTEN: phosphatase and tensin homolog, BRAF: B-Raf proto-oncogene: serine/threonine kinase, MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, ERK: extracellular signal-regulated kinase, αvβ3: alpha v beta 3 integrin, α5β1: alpha 5 beta 1 integrin, ATP: adenosine triphosphate, NADPH: nicotinamide adenine dinucleotide phosphate, p53: tumor protein p53, ZEB1/2: zinc finger E-box binding homeobox 1/2

Anoikis Resistance in Cancer Cells

In normal cells, anoikis functions as a vital mechanism for elimination of abnormal cells and maintenance of tissue homeostasis. In contrast, cancer cells can develop resistance to anoikis, enabling them to evade apoptosis when detached from the ECM. This resistance plays a critical role in cancer progression and metastatic spread. By avoiding anoikis through various molecular and cellular reprogramming processes, cancer cells survive at the primary tumor site and may metastasize to distant organs. These resistance mechanisms involve several key processes such as changes in integrin profile, epithelial-mesenchymal transition (EMT), constitutive activation of survival signaling pathways, and metabolic adaptations, including the Warburg effect (see below) and autophagy (23,41).

One of the most important mechanisms enabling cancer cells to survive despite detachment from the ECM is changes in the expression profile of integrins. These changes allow cancer cells to acquire resistance to anoikis and survive both in the early stages of oncogenic transformation and during metastatic colonization. For instance, the overexpression of integrin subunits such as αvβ3 and α5β1 leads to activation of signaling pathways that promote cancer cell survival. Molecules, such as FAK and Src kinase are activated through integrins, triggering PI3K/AKT and MAPK/ERK signaling pathways (Figure 3). These pathways transmit signals that promote cell survival, inhibit caspase activation, and lead to inactivation of apoptotic proteins. Furthermore, these signals facilitate metastatic spread by increasing the motility of cancer cells (23,41,42).

EMT is another physiological process characterized by epithelial cells reorganizing their cytoskeleton, losing connections, and acquiring a motile phenotype. In cancer cells, EMT has been shown to enable detachment from neighboring cells, resistance to anoikis, migration to distant tissues, and invasion. During EMT, the expression of cell-cell adhesion molecules, like E-cadherin, is suppressed while mesenchymal markers, such as vimentin, fibronectin, and N-cadherin are activated, reducing ECM dependence and enabling metastasis (43). EMT-inducing transcription factors (Snail, Twist, NF-κB, ZEB1/2) activate the PI3K/AKT pathway in cancer cells, suppress pro-apoptotic proteins, and protect against cell death (Figure 3) (27). Hypoxia-inducible factors (HIFs), particularly HIF-1, trigger EMT in hypoxic

tumor microenvironments by increasing Twist and NF-κB activation and promoting Snail expression. Furthermore, HIF-1 activates the MAPK/ERK pathway, leading to the degradation of pro-apoptotic proteins and increasing anoikis resistance (44).

Resistance to anoikis in cancer cells is closely associated with the reprogramming of apoptotic mechanisms despite the loss of integrin signaling. These cells evade apoptosis by overexpression of anti-apoptotic proteins or suppression of pro-apoptotic proteins. The PI3K/AKT pathway, a critical survival signaling cascade, is activated via integrins and growth factor receptors, ensuring cell survival (27,45). Resistance to anoikis is further enhanced when negative regulators of this pathway, such as PTEN, are inactivated (23,27). In addition, Src family kinases and ILK strengthen resistance to anoikis, promoting ECM-independent survival, invasion, and metastasis (46).

The regulation of anoikis resistance in cancer cells through increased oxidative stress and hypoxia plays a critical role in tumor survival, proliferation, and metastatic spread. Chronic ROS production enhances survival signaling and suppresses apoptotic pathways by activating enzymes, including NADPH oxidase, leading to sustained PI3K/AKT and MAPK/ERK pathway activity (Figure 3). Redox-sensitive transcription factors, such as NF-κB, HIF-1α, and p53, also contribute to anoikis resistance (47). The continuous generation of ROS leads to the inhibition of enzymes involved in apoptotic processes, such as PTEN and PTP-1B, through oxidative modification of phosphatases. Increased ROS levels also activate antioxidant responses, enabling cancer cells to persist even without ECM attachment (23,40).

Cancer cells reprogram their metabolic pathways to sustain survival under ECM-deprived conditions. Unlike normal cells, which rely on oxidative phosphorylation for energy production, cancer cells shift their metabolism to glycolysis, a process known as the Warburg effect. Upon detachment from the ECM, glucose uptake, glycolysis, and mitochondrial respiration typically decrease, leading to ATP depletion and energy deficiency. However, cancer cells compensate by maintaining energy production through glycolysis, achieved by increasing glucose uptake and activating signaling pathways, such as PI3K/AKT and HIF-1α (23,41). In addition, cancer cells activate the autophagy pathway to manage metabolic stress. During autophagy, these cells maintain energy balance and suppress apoptotic signaling. By integrating metabolic reprogramming and autophagy, cancer cells effectively adapt to hostile environments, ensuring survival and facilitating metastatic progression (41).

Cancer progression to malignancy consists of a series of steps, including carcinogenesis, sustained proliferative

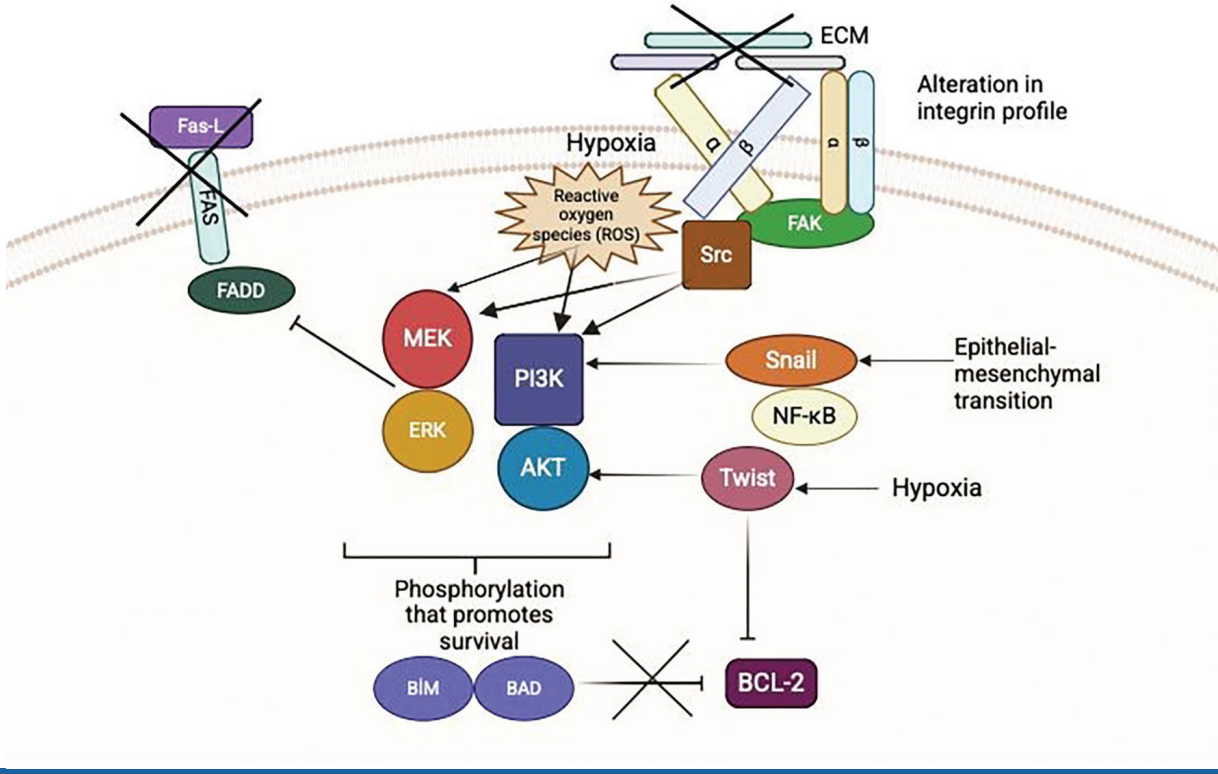

Figure 3. Molecular mechanisms of anoikis resistance in cancer cells. Adapted from Taddei et al. (27)
ECM: extracellular matrix, FAK: focal adhesion kinase, Src: Src family kinases, PI3K: phosphoinositide 3-kinase, AKT: protein kinase B, ERK: extracellular signal-regulated kinase, NF-κB: nuclear factor kappa B, BCL-2: B-cell lymphoma 2, ROS: reactive oxygen species, HIF: hypoxia-inducible factor

signaling, the formation of a hypoxic tumor environment, angiogenesis, lymphangiogenesis, interaction with the tumor microenvironment, ECM migration and invasion, intravasation into the bloodstream, survival in circulation, extravasation, metastatic niche preparation, and proliferation in distant organs (48). Resistance to anoikis plays a critical role in many of these processes. Therefore therapeutic approaches targeting anoikis resistance hold important potential for halting cancer progression (23).

Role of Anoikis in Thyroid Cancer

TC subtypes exhibit significant differences in biological behavior and therapeutic response. PTC is the most common subtype and is typically associated with a favorable prognosis (1). However, 15-50% of PTCs exhibit local invasion and regional lymph node metastases. In contrast, FTC is more invasive and tends to metastasize to distant organs at a reported rate of 20%. ATC is the most aggressive subtype and exhibit metastases to distant sites in over 98% of cases. These differences in metastatic characteristics make TC an informative model for exploring the underlying mechanisms of metastasis (1,10).

Investigation of cell death processes is crucial for understanding the molecular mechanisms driving metastasis in TC. Among these processes, apoptosis is the most extensively studied (49-51). According to existing research, one of the most prevalent molecular changes in TC is the activation of the PI3K/AKT signaling pathway, which promotes cancer cell survival by triggering anti-apoptotic signals. Furthermore, overexpression of Bcl-2 family proteins plays an important role in the suppression of apoptotic processes (49). Specifically, mutations in the p53 tumor suppressor gene, which are prevalent in ATC, affect the operation of apoptotic processes, which in turn leads to a more aggressive phenotype of cancer cells (52).

Anoikis resistance is essential for TC cells to detach from the ECM and survive during the metastatic process. Although studies into anoikis resistance in TC are limited, it is recognized as a critical factor for tumor progression and metastasis (10-12,53). Research has shown that TC cells increase their invasive capabilities through EMT. Metastatic TCs are characterized by overexpression of mesenchymal markers, such as osteopontin and vimentin (54). Signaling pathways that directly regulate EMT include integrin, Notch, TGF-β, NF-κB, and PI3K. Some of these pathways have been found to play an important role in PTC progression (55). Furthermore, the activation of the PI3K/AKT signaling pathway plays a critical role in TC progression. Nuclear AKT activation has been identified in TC cells invading surrounding tissues, highlighting its importance in facilitating metastasis (56).

Although studies of the mechanisms by which TC cells develop resistance to anoikis are limited, this area has garnered significant research interest. Recent findings highlight various molecular pathways and cellular interactions that contribute to anoikis resistance in TC. A study reported that TC cells detached from the ECM communicate with each other through gap junctions to transmit anti-apoptotic signals. In this process, the overexpression of the Connexin 43 gap junction protein enhanced the survival capacity of cancer cells during metastasis. In particular, sustained activation of the AKT signaling pathway in cells carrying the *BRAFV600E* mutation has been shown to develop resistance to anoikis. Furthermore, inhibition of gap junction-mediated intercellular communication (GJIC) suppressed AKT activation and led to apoptosis in cells with high PTEN expression. These findings suggest that GJIC could serve as a potential therapeutic target in the treatment of TC (53). In another study, the effects of integrin β4 on PTK were investigated and it was found that β4 expression was significantly increased in PTC compared to normal thyroid tissues. This upregulation was associated with extrathyroidal extension, lymph node metastasis, and higher tumor stage. Moreover, it has been proposed that integrin β4 enhances the capacity of circulating tumor cells to metastasize, and this impact is linked to the activation of the PI3K, MAPK/ERK, and NF-κB signaling pathways. In addition, prior *in vitro* studies have demonstrated that tumor cells' resistance to invasion, migration, and anoikis is enhanced when β4 expression is upregulated, whereas these functions are diminished when β4 is suppressed (57,58).

Zhao et al. (11) investigated the role of cell cycle checkpoint kinase 2 (CHK2) protein on tumor progression and metastasis in PTC cells. CHK2 is a tumor suppressor kinase that maintains genomic integrity in response to DNA damage. The study reported that CHK2 levels were high in PTC tissues but significantly reduced in lymph node metastases. These findings suggest that CHK2 is associated with tumor aggressiveness, particularly lymph node metastasis. Moreover, CHK2 was found to suppress anoikis resistance through the PRAS40 signaling pathway. PRAS40 is an AKT kinase-dependent substrate that promotes cell growth and survival by regulating mTORC1 activity. It was observed that suppression of CHK2 prevented anoikis by increasing p-PRAS40 levels in a p53-independent manner. In summary, CHK2 appears to regulate anoikis via PRAS40, presenting a potential therapeutic target for cancer treatment (11). Pan et al. (59) found that anoikis resistance in PTC could be inhibited by miR-363-3p targeting integrin α6. This study highlighted the role of miR-363-3p in the metastatic process and its clinical potential in preventing metastasis (59). These findings supported earlier research demonstrating the dual functions of miRNAs as pro- and anti-metastatic agents in metastatic processes, including invasion, cell migration, anoikis resistance, and EMT (60,61).

A study investigating anoikis-related genes in patients with TC identified six critical genes, *EZH2, PRKCQ, CD36, INHBB, TDGF1,*

and *MMP9*. Functional analyses demonstrated their significant roles in tumor progression, cell migration, and cancer-related signaling pathways. It has been found that *EZH2* enhances cell motility by weakening the connection of cells to the ECM, thereby promoting anoikis resistance. *PRKCQ* strengthens anoikis resistance by activating the MAPK/ERK pathway through its kinase activity. Blocking *CD36* eliminates anoikis resistance by reducing integrin sequestration. *INHBB* has been shown to inhibit cell migration by suppressing anoikis resistance through the TGF-β signaling pathway. *TDGF1* has been shown to increase both anoikis resistance and invasion potential and *MMP9* has been shown to trigger anoikis by degrading intercellular binding components and acting on the epithelium. The contribution of these genes to anoikis resistance was elucidated in detail and receiver operating characteristic curves confirmed that these six genes are a strong indicator for prognosis prediction in patients with PTC. According to the risk scores, patients were divided into high and low-risk groups; immunological analyses revealed that plasma cells, T cells, and macrophages were more prevalent in tumors in the high-risk group, whereas CD8+ T cells and NK cells were found at lower levels. These findings support the critical role of the immune microenvironment in tumor development and its relationship with anoikis resistance (62). On the other hand, a proteomic study by Saharat et al. aimed to identify the key proteins involved in anoikis resistance in TC cell lines and found that overexpression of tumor susceptibility gene 101 (TSG101) protein played a critical role in TC cells with high metastatic potential. It has been suggested that TSG101 is involved in cellular processes such as vesicle biogenesis, endocytosis, membrane transport, and receptor recycling. Overexpression of TSG101 may contribute to the retention of death receptors via endocytosis, leading to anoikis resistance (10). TSG101 has been implicated as an oncogenic factor in various cancers, including breast, prostate, and hepatocellular carcinoma. Suppression of TSG101 has been shown to arrest the cell cycle in these cancers, suggesting its potential as a therapeutic target (63,64,65). These findings suggest that TSG101 plays a pivotal role in regulating anoikis resistance in TC and may serve as a molecular therapeutic target against metastatic TC (10).

In conclusion, the evidence produced by these studies show that anoikis resistance plays a critical role in tumor progression and metastasis in TC. Molecules such as TSG101, CHK2, integrin β4, and miR-363-3p have emerged as potential therapeutic targets, especially in metastatic TC. Moreover, the genes *EZH2, PRKCQ, CD36, INHBB, TDGF1,* and *MMP9* appear to play important roles in anoikis resistance and tumor progression. These findings offer potential advances in the treatment of metastatic TC by providing a promising foundation for the development of new strategies targeting anoikis resistance.

Footnotes

Authorship Contributions

Concept: Gözde Akın Kağızmanlı, Selen Kum Özşengezer, Korcan Demir, Zekiye Altun, Design: Gözde Akın Kağızmanlı, Selen Kum Özşengezer, Korcan Demir, Zekiye Altun, Literature Search: Gözde Akın Kağızmanlı, Selen Kum Özşengezer, Zekiye Altun, Writing: Gözde Akın Kağızmanlı, Zekiye Altun.

Conflict of Interest: One author of this article, Korcan Demir, is a member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, he was not involved in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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Clinical and Genetic Characterization of Noonan Syndrome in a Colombian Pediatric Cohort

Silvia C. Martínez Rueda[1], Maria del Pilar Montilla[1], Carolina Baquero[2], Susana Gómez[1], Maria Victoria Lopera[1], Nora Alejandra Zuluaga[1,3], Adriana Carolina Forero[1,3], Gustavo Giraldo[2], Nicolás Pineda Trujillo[4], Juan Camilo Martínez[1], Paola Durán Ventura[5], Juan Manuel Alfaro[1]

[1]Universidad de Antioquia Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Medellín, Colombia
[2]Hospital Pablo Tobón Uribe, Clinic of Genetics, Medellín, Colombia
[3]Hospital San Vicente Fundación, Clinic of Pediatric Endocrinology, Medellín, Colombia
[4]Universidad de Antioquia Faculty of Medicine, Department of Genetics, Medellín, Colombia
[5]Fundación Cardioinfantil and Endociencia, Department of Pediatric Endocrinology, Bogotá, Colombia

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ABSTRACT

Objective: Describe the clinical manifestations and genetic variants of Noonan syndrome (NS) in a Colombian pediatric population and to identify the genes most frequently associated with specific phenotypic features.

Methods: A retrospective observational study was conducted on patients under 18 years of age diagnosed with NS between 2013 and 2023. Clinical and molecular data were collected from medical records across several hospitals in Colombia. Molecular confirmation was achieved in all included patients through next generation sequencing-based clinical exome sequencing. However, parental samples were not available for segregation analysis in all cases. Descriptive statistical analyses were performed using R version 4.3.1 to evaluate demographic, clinical, and genetic variables.

Results: Among the 45 patients included (21 females, 24 males; mean age at diagnosis 7.5±5.2 years), pathogenic variants were identified across 13 genes, with *PTPN11* being the most frequent (26/45, 57.8%), followed by *PPP1CB* and several less frequent genes including *SOS1, SOS2, LZTR1, RAF1, MAP2K1, KRAS, NRAS, BRAF, CBL, NF1,* and *SHOC2*. The most common phenotypic features were congenital heart defects (80%, 36/45), predominantly pulmonary stenosis (31.1%, 14/45), short stature (66.7%, 30/45), and learning difficulties (51.1%, 23/45). Seven patients developed neoplasms. Juvenile myelomonocytic leukemia was the most frequent neoplasm and occurred mainly in patients with *PTPN11* variants. Ten patients (22.2%) received growth hormone therapy.

Corresponding Author: Silvia C. Martínez Rueda, MD, Universidad de Antioquia Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Medellín, Colombia

E-mail: criss_0704@hotmail.com **ORCID:** orcid.org/0009-0001-1563-4319

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Conclusion: This study represents the first genetically characterized Colombian cohort for NS. *PTPN11* was the predominant gene identified, particularly the p.(Asn308Asp) variant. These findings reiterate the genetic heterogeneity of NS in Colombian children and the importance of early molecular diagnosis to guide clinical management. Multidisciplinary follow-up is essential due to the risks of growth failure, cardiac anomalies, and malignancies.

Keywords: Noonan syndrome, *PTPN11*, RASopathy

What is already known on this topic?

Noonan syndrome (NS) is a RASopathy characterized by distinctive facial features, short stature, congenital heart defects, particularly pulmonary valve stenosis, and various other anomalies. Complications may include growth retardation, bleeding disorders, lymphatic abnormalities, and an increased risk of malignancies, especially in individuals with variants in the *PTPN11* gene.

What this study adds?

In this Colombian pediatric population, variants in *PTPN11* were the most frequent, with p.(Asn308Asp) variant being the most common. Congenital heart defects were present in 80% (36/45) of patients, most commonly pulmonary valve stenosis. Short stature was observed in 66.7% (30/45) of cases. Pathogenic variants were identified across 13 genes, with *PTPN11* being the most frequent. Less frequent variants involved *PPP1CB, SOS1, SOS2, LZTR1, RAF1, MAP2K1, KRAS, NRAS, BRAF, CBL, NF1,* and *SHOC2,* highlighting the genetic heterogeneity of NS in this cohort.

Introduction

Noonan syndrome (NS) is a common genetic disorder with an estimated incidence of 1 in 2,000 to 2,500 live births (1). It is characterized by three cardinal features: distinctive facial features; postnatal short stature; and congenital heart disease. Additional manifestations may include cryptorchidism, delayed puberty, bleeding disorders, lymphedema, thoracic abnormalities, skin disorders, varying degrees of learning difficulties, and a predisposition to myeloproliferative disorders (2,3), reflecting the marked clinical heterogeneity of the syndrome.

Early descriptions of features now associated with NS date back to 1883, when Koblinski reported a patient with a congenital neck deformity later termed pterygium colli. However, NS was more clearly delineated in 1968, when Jacqueline Noonan described nine patients with shared features, including pulmonary valve stenosis and extracardiac anomalies (4,5), thereby establishing NS as a distinct syndrome affecting both sexes, with normal karyotypes and familial inheritance (3,6,7).

NS belongs to the group of developmental disorders known as RASopathies (8), which result from pathogenic variants affecting components or regulators of the RAS/mitogen-activated protein kinase (MAPK) pathway. This pathway plays a central role in cell proliferation, differentiation, and survival (8,9,10).

NS can be inherited in an autosomal dominant or autosomal recessive manner or occur sporadically. Inherited cases predominantly show maternal transmission, whereas *de novo* cases are more commonly of paternal origin. This paternal predominance reflects the well-established higher mutation rate associated with continuous spermatogonial stem cell divisions, together with positive selection in the male germline, whereby activating variants, particularly those affecting the RAS/MAPK pathway, may undergo clonal expansion with advancing paternal age (4,11,12).

PTPN11 was the first gene identified in association with NS and remains the most frequently affected (4,13,14). Since then, more than 20 genes involved in the RAS/MAPK pathway have been associated with NS and related disorders (2,9,10,14). Pathogenic variants in *PTPN11* account for approximately 50% of cases (14,15), followed by variants in *SOS1, RAF1, RIT1*, and *KRAS*, whereas variants in genes such as *NRAS, BRAF, SHOC2, CBL,* and *LZTR1* are less frequent. *LZTR1* is the only gene associated with both autosomal dominant and autosomal recessive inheritance patterns (9,16), whereas *SPRED2* is the only gene inherited exclusively in an autosomal recessive manner. Overall, a molecular diagnosis can be established in approximately 70% to 80% of patients (17,18).

Studies from different populations have demonstrated considerable phenotypic and genetic heterogeneity in NS. However, data from Latin American pediatric populations, particularly from Colombia, remain scarce. Therefore, the aim of this study was to characterize the clinical manifestations and genetic findings of a Colombian pediatric cohort with NS.

Methods

A retrospective, observational, descriptive study was conducted by screening medical records with ICD-10 codes Q871 (Syndromes of congenital malformations primarily associated

with short stature) and Q878 (Other specified congenital malformation syndromes, not elsewhere classified) from 2013 to 2023. The study was conducted at Research and Ethics Committee of Pablo Tobón Uribe Hospital (HPTU), approved under protocol 2022.088 (date: 08.11.2022); San Vicente Fundación Hospital (HSVF), approved under act number 12-2023 (date: 24.04.2023); and other healthcare centers where pediatric endocrinology consultations are performed. Clinical records were reviewed, and only patients with a molecularly confirmed diagnosis of NS were included. Genetic testing was performed using next-generation sequencing (NGS)-based clinical exome methodology on peripheral blood samples. This approach employed targeted capture to selectively enrich coding regions (exons), which represent approximately 1% of the genome but contain the majority of known disease-causing variants. A total of 7,233 clinically relevant genes were analyzed. Bioinformatic analysis included the detection of single nucleotide variants, small insertions and deletions (indels), and copy number variants. Variant interpretation was performed according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines, integrating molecular findings with the patient's clinical phenotype. Family segregation analysis was not performed because parental samples were unavailable. Authorization was obtained from the ethics committee of the aforementioned institutions. For patients not affiliated with these institutions, informed consent was obtained and signed by their legal representatives.

All patients were clinically diagnosed by geneticists according to the van der Burgt criteria (19), and the diagnosis was molecularly confirmed by NGS-based clinical exome testing. Only patients under 18 years of age were included. Patients with a suggestive phenotype but without confirmatory genetic studies were excluded.

Extracted variables included demographic data, molecular findings, prenatal and postnatal history, auxological measurements, craniofacial features, musculoskeletal abnormalities, learning difficulties, endocrine manifestations, malignancies, and congenital heart disease.

Statistical Analysis

A descriptive analysis was conducted. Absolute and relative frequencies were calculated for qualitative variables, while measures of central tendency and dispersion were obtained for quantitative variables. Clinical characteristics were visualized using a lasagne plot. All data were processed using R statistical software, version 4.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 45 patients were evaluated (21 females, 24 males), with an average age at diagnosis of 7.5±5.2 years. Pathogenic variants were identified in 13 genes. Variants in *PTPN11* were the most frequent, present in 57.8% of cases (26 of 45 patients). The most common variant was c.922A>G; p.(Asn308Asp), identified in 11 of these 26 patients. The second most commonly affected gene was *PPP1CB*, observed in 6.7% of cases, with the variant c.146C>G; p.(Pro49Arg) being the most frequent in this group (Table 1). No novel variants were identified. Segregation analysis was not available; therefore, the *de novo* status of the identified variants could not be determined.

There were 21 females and 24 males (Table 2). A maternal history of NS was present in 11.1% (5/45) of cases, occurring more frequently in patients with variants in *PTPN11* and *SOS1*. Regarding prenatal history, chylothorax was identified in 6.7% of cases, predominantly among those with *PTPN11* variants. In terms of postnatal history, prematurity was observed in 15.6 % (7/45) of cases. Being small for gestational age was noted in 11.1% (5/45) of cases, commonly associated with *PTPN11* and *KRAS* variants.

The most common characteristics observed in this cohort included congenital heart disease, low-set ears, short stature, downward-slanting palpebral fissures, short neck, learning difficulties, and hypertelorism, each present in more than 40% (18/45) of cases (Figure 1).

The most common cardiac defect observed was pulmonary stenosis, occurring in 31.1% (14 of 45 patients) of cases, followed by atrial septal defect in 22.2% (10 of 45 patients), and mitral regurgitation in 13.3% (6 of 45 patients) (Table 3).

Seven patients in this cohort developed neoplasms. Among them, three female patients were diagnosed with juvenile myelomonocytic leukemia (JMML). Two unrelated patients carried the same PTPN11 variant, c.1507G>A; p.(Gly503Arg). At the time of diagnosis, all three of these girls were under five years of age.

Two cases involved central nervous system neoplasms (glioma and astrocytoma), both harboring the *PTPN11* variant c.922A>G; p.(Asn308Asp). In addition, two patients with neuroendocrine tumors each carried the *PPP1CB* variant c.146C>G; p.(Pro49Arg).

Discussion

Genetic Findings and Diagnostic Considerations

We report the clinical and molecular characteristics of a cohort of 45 Colombian children and adolescents with NS, molecularly confirmed through clinical exome sequencing. This study represents one of the few pediatric NS cohorts reported from

Table 1. Detected pathogenic and likely pathogenic variants in the cohort

Gene	Frequency n (%)	Variant count	Nucleotide change	Protein change	Transcript (NM)
BRAF	1 (2.2)	1	c.1455G>T	p.(Leu485Phe)	NM_004333.6
CBL	1 (2.2)	1	c.1228-2A>G	p.?	NM_005188.4
KRAS	2 (4.4)	1	c.178G>A	p.(Gly60Ser)	NM_033360.4
		1	c.40G>A	p.(Val14Ile)	NM_004985.5
LZTR1	2 (4.4)	1	c.410C>T	p.(Ala137Val)	NM_006767.4
		1	c.2090G>A	p.(Arg697Gln)	NM_006767.4
MAP2K1	1 (2.2)	1	c.364A>G	p.(Asn122Asp)	NM_002755.4
NF1	1 (2.2)	1	c.4267A>G	p.(Lys1423Glu)	NM_000267.3
NRAS	1 (2.2)	1	c.38G>A	p.(Gly13Asp)	NM_002524.5
PPP1CB	3 (6.7)	2	c.146C>G	p.(Pro49Arg)	NM_002709.3
		1	c.548A>C	p.(Glu183Ala)	NM_002709.3
PTPN11	26 (57.8)	11	c.922A>G	p.(Asn308Asp)	NM_002834.5
		2	c.1507G>A	p.(Gly503Arg)	NM_002834.5
		2	c.124A>G	p.(Thr42Ala)	NM_002834.5
		1	c.1493G>T	p.(Arg498Leu)	NM_002834.5
		1	c.181G>A	p.(Asp61Asn)	NM_002834.5
		1	c.1510A>G	p.(Met504Val)	NM_002834.5
		1	c.218C>T	p.(Thr73Ile)	NM_002834.5
		1	c.417G>C	p.(Glu139Asp)	NM_002834.5
		1	c.781C>T	p.(Leu261Phe)	NM_002834.5
		1	c.1519G>C	p.(Gly507Arg)	NM_002834.5
		2	c.846C>G	p.(Ile282Met)	NM_002834.5
		1	c.188A>G	p.(Tyr63Cys)	NM_002834.5
		1	c.923A>G	p.(Asn308Ser)	NM_002834.5
RAF1	2 (4.4)	1	c.786T>G	p.(Asn262Lys)	NM_002880.4
		1	c.770C>T	p.(Ser257Leu)	NM_002880.4
SHOC2	1 (2.2)	1	c.4A>G	p.(Ser2Gly)	NM_007373.4
SOS1	2 (4.4)	1	c.3145A>T	p.(Thr1049Ser)	NM_005633.4
		1	c.1297G>A	p.(Glu433Lys)	NM_005633.4
SOS2	2 (4.4)	1	c.1532C>T	p.(Ala511Val)	NM_006939.4
		1	c.800T>A	p.(Met267Lys)	NM_006939.4

No novel variants were identified. Segregation analysis was not available; therefore, *de novo* status could not be determined

Colombia and provides valuable data from an underrepresented Latin American population.

PTPN11 was the most frequently affected gene, identified in 57.8% (26/45) of cases, consistent with previous reports indicating it accounts for approximately 50% of NS cases (20,21,22). Prior studies have shown that up to 86% of pathogenic *PTPN11* variants cluster in exons 3, 8, and 13, as reported in the first Brazilian cohort (13). In our cohort, the most frequent variant was c.922A>G; p.(Asn308Asp). This variant has also been reported as the most prevalent in a cohort of 26 individuals with NS followed at a reference center in southwest Colombia (22,23), as well as in other populations, representing approximately 20% of cases in Europe, the United States, Argentina (15), and Brazil (13), and 11% in southern India (16). These findings suggest a shared distribution of this variant across different populations; however, a recurrent mutational event cannot be excluded.

NS is characterized by significant phenotypic variability (20) ranging from isolated anomalies, such as pulmonary valve stenosis, to subtle facial features, short stature, or multiple congenital anomalies. Due to phenotypic overlap with other conditions and age-related changes in craniofacial characteristics,

Table 2. Family, prenatal, and postnatal history of the patients according to the affected gene

Characteristics	All (n=45)	*KRAS* (n=2)	*LZTR1* (n=2)	*NRAS* (n=1)	*PTPN11* (n=26)	*RAF1* (n=2)	*SOS1* (n=2)	*SOS2* (n=2)
Demographics								
Female:Male	21:24	1:1	1:1	1:0	12:14	2:0	0:2	1:1
Age at diagnosis, years (mean ± SD)	7.5±5.2	4.1±4.2	11.9±4.5	12.0	7.1±5.4	2.7±1.5	3.5±0.47	10.4±10.3
Family history								
Consanguinity, n (%)	3 (6.7)	-	-	-	1 (3.8)	-	-	2 (100)
Mother with Noonan syndrome, n (%)	5 (11.1)	-	-	-	4 (15.4)	-	1 (50)	-
Father with short stature, n (%)	1 (2.2)	-	-	-	1 (3.8)	-	-	-
Mother with short stature, n (%)	5 (11.1)	-	-	-	4 (15.4)	-	1 (50)	-
Prenatal history								
Prenatal chylothorax, n (%)	3 (6.7)	-	-	-	1 (3.8)	-	1 (50)	-
Prenatal pleural effusion, n (%)	1 (2.2)	-	-	-	1 (3.8)	-	-	-
Hydrops fetalis, n (%)	1 (2.2)	-	-	-	1 (3.8)	-	-	-
Prenatal cystic hygroma, n (%)	2 (4.4)	-	-	-	1 (3.8)	1 (50)	-	-
Postnatal history								
Prematurity, n (%)	7 (15.6)	1 (50)	1 (50)	-	3 (11.5)	1 (50)	1 (50)	-
SGA, n (%)	5 (11.1)	1 (50)	-	-	4 (15.4)	-	-	-
Postnatal pleural effusion, n (%)	1 (2.2)	-	-	-	-	1 (50)	-	-
Postnatal chylothorax, n (%)	1 (2.2)	-	-	-	-	1 (50)	-	-

Only genes with more than one clinically relevant observation or selected genes of interest are shown
SD: standard deviation, NS: Noonan syndrome, SGA: small for gestational age

Table 3. Cardiac manifestations according to the affected gene (number of patients)

Cardiac manifestation	All (n=45)	*BRAF* (n=1)	*CBL* (n=1)	*KRAS* (n=2)	*LZTR1* (n=2)	*MAP2K1* (n=1)	*PPP1CB* (n=3)	*PTPN11* (n=26)	*RAF1* (n=2)	*SHOC2* (n=1)	*SOS1* (n=2)	*SOS2* (n=2)
Pulmonary stenosis	14	-	-	-	-	-	-	12	-	-	1	-
Atrial septal defect	10	-	-	1	-	-	-	8	-	1	-	-
Mitral regurgitation	6	1	1	-	-	-	2	-	1	-	1	-
Hypertrophic cardiomyopathy	5	-	-	-	-	-	-	2	2	1	-	1
Ventricular septal defect	5	-	-	-	-	-	-	2	1	-	-	1
Arrhythmia	2	-	-	1	-	-	-	-	1	-	-	-
Ventricular extrasystole	2	-	-	1	-	-	-	-	1	-	-	-
Bicuspid aortic valve	2	-	-	-	-	-	-	2	-	-	-	-
Tricuspid regurgitation	2	-	-	-	-	-	2	-	-	-	-	-
Patent ductus arteriosus	1	-	-	-	-	-	1	-	-	-	-	-
Cardiomyopathy (unspecified)	1	-	-	-	-	-	-	1	-	-	-	-
Tetralogy of Fallot	1	-	-	-	1	-	-	-	-	-	-	-

establishing a definitive diagnosis based solely on clinical criteria is often challenging (15). In this context, genetic analysis plays an important role in confirming the diagnosis and may guide clinical follow-up and therapeutic decisions.

Facial Features

Distinctive facial features are more common in patients with *PTPN11* variants and typically include an inverted triangular facial shape (9,10), low-set ears with posterior rotation and a thickened helix, downward-slanting palpebral fissures (9,24),

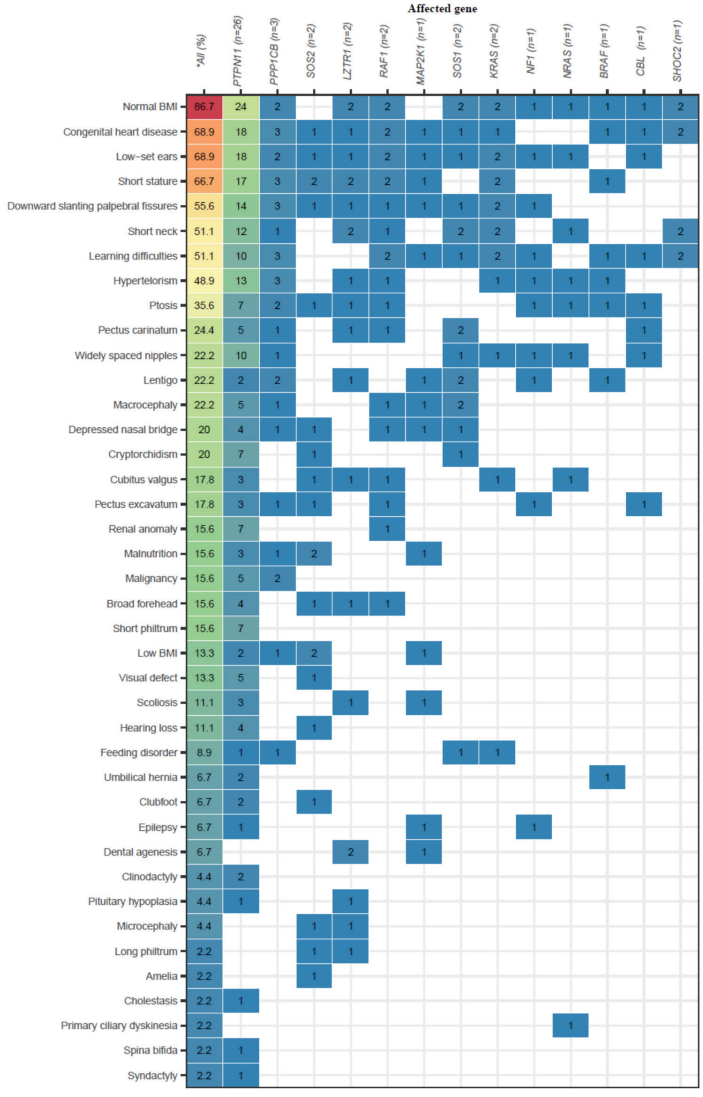

Figure 1. Clinical characteristics of the studied patients according to the affected gene
BMI: body mass index

hypertelorism (10), ptosis, arched eyebrows, a depressed nasal bridge with a bulbous nasal tip, a long and deep philtrum, high peaks of the upper lip, a low anterior hairline in a "W" shape, a high-arched palate, malar hypoplasia, and a short or webbed neck (4,25). These facial characteristics may vary with age, becoming more evident during puberty and less pronounced in adulthood (26,27,28).

In this Colombian cohort, low-set ears were observed in 68.9% (31/45) of patients, a short neck in 51% (23/45), hypertelorism in 48.9% (22/45), ptosis in 35.6% (16/45), macrocephaly in 22.2% (10/45), a depressed nasal bridge in 20% (9/45), and a wide forehead with a short nasolabial fold in 15.6% (7/45). This phenotype has been more frequently associated with *PTPN11* variants and is consistent with findings reported in other NS populations, showing similar frequencies (10).

Cardiac Findings

Congenital heart defects occur in up to 80% of individuals with NS, with pulmonary valve stenosis being the most common anomaly, reported in 25% to 71% of cases, followed by atrial septal defects in 4% to 57% of cases (29). Hypertrophic cardiomyopathy, often presenting as asymmetric septal hypertrophy, is observed in approximately 20% of patients (29). Electrocardiographic abnormalities have been reported in up to 87% of cases, including wide QRS complexes with a predominant negative pattern in the left precordial leads, left-axis deviation, and giant Q waves (26,29,30)

PTPN11 and *SOS1* variants are more frequently associated with pulmonary valve stenosis, whereas *RAF1* and *RIT1* variants are more commonly linked to hypertrophic cardiomyopathy. In our cohort, pulmonary valve stenosis was the most common cardiac defect, occurring in 31.1% of patients (14/45), of whom 12 carried *PTPN11* variants. Atrial septal defects were identified in 22.2% of patients (10/45), with *PTPN11* variants present in 8 of the 10 affected individuals (29). These findings are consistent with other NS cohorts, in which pulmonary valve stenosis is the most frequently reported congenital heart defect (10,31).

Short Stature and Growth Hormone Therapy

Short stature occurs in up to 80% of individuals with NS, typically presenting with normal prenatal growth (25). Its pathophysiology is multifactorial and may involve growth hormone deficiency, growth plate abnormalities, and mild growth hormone resistance (28,32,33). This resistance is believed to occur at the post-receptor level, primarily through impaired STAT5B signaling, and is more pronounced in individuals with *PTPN11* variants due to alterations in the SHP-2 protein, which plays a key role in growth hormone signaling (29,34).

Genes in the RAS-MAPK pathway also play a role in the development of the hypothalamic-pituitary axis, which may result in structural and functional alterations in RASopathies (29,35). A higher prevalence of short stature has been reported in patients with NS carrying *PTPN11*, *RAF1*, and *KRAS* variants compared to those harboring *SOS1* variants (28,36). In our cohort, short stature was observed in 66.7% of patients and was noted mainly among patients carrying variants in *PTPN11, PPP1CB, SOS2, LZTR1, RAF1,* and *MAP2K1*, whereas variants in *SOS1, NF1, NRAS, CBL,* and *SHOC2* were not linked to this feature.

Somatropin therapy was administered to 10 patients in this cohort, nine of whom received treatment due to a confirmed diagnosis of growth hormone deficiency. Growth hormone therapy has been approved by the U.S. Food and Drug Administration for use in patients with NS since 2007, at a dose of 0.066 mg/kg/day (37). However, it has not been approved by the European Medicines Agency (37,38,39).

In our cohort, none of the patients receiving somatropin therapy harbored variants typically associated with hypertrophic cardiomyopathy, such as *RAF1* or *RIT1,* and no cardiac complications related to the treatment were observed during follow-up. Although growth hormone therapy remains controversial in NS, particularly in patients with variants linked to hypertrophic cardiomyopathy or high-risk *PTPN11*-related neoplasms (37,38,40), our findings align with previous reports suggesting that treatment may be considered after five years of age, when the risk of hypertrophic cardiomyopathy and lymphoproliferative neoplasms is lower.

Skeletal and Other Systemic Findings

Skeletal deformities, such as pectus carinatum and pectus excavatum, are reported in up to 95% of patients with NS. Valgus deformity of the elbow occurs in approximately 50% of cases, clinodactyly in 30%, and thoracic scoliosis in 13% (31).

Other associated manifestations include hemorrhagic diathesis (65%) (3), altered spermatogenesis (60-80%), renal anomalies (10%), cryptorchidism (80% of males) (3), peripheral lymphedema (<20%), hearing deficits (10-25%), and delayed puberty (approximately two years) (6). In our cohort, these complications were observed at lower frequencies. Learning difficulties, reported in up to 50% of NS patients (25), were present in 51.1% of our cohort and were most commonly associated with variants in *PTPN11, PPP1CB, RAF1,* and *KRAS*.

Neoplasms

Individuals with NS have an increased risk of malignancy, estimated to be 3.5 times higher than that of the general population (40). Some studies report an 8.1-fold increased risk for JMML and solid tumors, including brain tumors, neuroblastoma, and rhabdomyosarcoma (39). Specific variants, such as *PTPN11*, c.218C>T; p.(Thr73Ile), *PTPN11* codon 61 variants, and *KRAS* p.(Thr58Ile), have been associated with predisposition to myeloproliferative disorders (13).

In addition to JMML, two patients in our cohort developed central nervous system tumors, one glioma and one astrocytoma, both harboring the PTPN11 c.922A>G; p.(Asn308Asp) variant. Brain tumors have been reported in patients with NS, particularly in association with PTPN11 variants, although they remain uncommon. Previous reports have described glial and glioneuronal tumors, including low-grade gliomas, dysembryoplastic neuroepithelial tumors, and astrocytomas, in patients with PTPN11-related NS. Notably, the p.(Asn308Asp) variant identified in our two patients has also been reported in patients with NS and different types of brain tumors. However, given the small number of affected patients in our cohort, no causal or genotype-specific association can be established (11).

In addition, two patients in our cohort with the PPP1CB c.146C>G; p.(Pro49Arg) variant developed neuroendocrine tumors. PPP1CB is a recognized RASopathy-associated gene; however, the relationship between germline PPP1CB variants and neuroendocrine tumors has not been clearly established. Therefore, although this finding is noteworthy, the small number of affected patients and the retrospective nature of our study preclude establishing a causal or genotype-specific association. Further studies are required to determine whether neuroendocrine tumors represent part of the neoplastic spectrum associated with PPP1CB-related NS.

In our cohort, seven patients developed neoplasms, three of whom were diagnosed with JMML. Two shared the *PTPN11* c.1507G>A; p.(Gly503Arg) variant, while the third carried the *PTPN11* c.218C>T; p.(Thr73Ile) variant. None of the three patients with JMML received growth hormone therapy prior to the diagnosis of JMML. All were under five years of age at diagnosis of JMML and were monitored through regular clinical evaluations and complete blood counts, in accordance with published surveillance recommendations (40).

Diagnostic Implications

The diagnosis of NS is primarily based on clinical features, most commonly using the van der Burgt scoring system to identify patients for molecular testing (19). Confirmation requires molecular testing, which may include targeted RASopathy panels, clinical exome sequencing, or broader approaches, such as whole-exome or whole-genome sequencing, depending on clinical suspicion and test availability (27). Overall, our findings reinforce the importance of an integrated clinical and molecular approach for accurate diagnosis, risk stratification, and individualized management of patients with NS.

Study Limitations

This study has several limitations. Its retrospective design and the relatively small sample size may limit the generalizability of the findings. In addition, as patients were recruited from tertiary referral centers, a referral bias cannot be excluded. Nevertheless, the availability of comprehensive clinical records and molecular confirmation by clinical exome sequencing strengthens the reliability and internal consistency of the data.

Conclusion

This study provides the first genetically characterized pediatric cohort of NS from Colombia, highlighting its marked genetic and phenotypic heterogeneity. *PTPN11* was the most frequently affected gene, and congenital heart disease, short stature, and learning difficulties were the predominant clinical features. These findings emphasize the importance of early molecular diagnosis and multidisciplinary follow-up for optimal clinical management.

Ethics

Ethics Committee Approval: The study was conducted at Research and Ethics Committee of Pablo Tobón Uribe Hospital (HPTU), approved under protocol 2022.088 (date: 08.11.2022); San Vicente Fundación Hospital (HSVF), approved under act number 12-2023 (date: 24.04.2023).

Informed Consent: Authorization was obtained from the ethics committee of the aforementioned institutions. For patients not affiliated with these institutions, informed consent was obtained and signed by their legal representatives.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Silvia C. Martínez Rueda, Concept: Silvia C. Martínez Rueda, Carolina Baquero, Susana Gómez, Maria Victoria Lopera, Nora Alejandra Zuluaga, Adriana Carolina Forero, Gustavo Giraldo, Nicolás Pineda Trujillo, Juan Camilo Martínez, Paola Durán Ventura, Juan Manuel Alfaro, Design: Silvia C. Martínez Rueda, Maria del Pilar Montilla, Data Collection or Processing: Silvia C. Martínez Rueda, Carolina Baquero, Analysis or Interpretation: Silvia C. Martínez Rueda, Maria del Pilar Montilla, Carolina Baquero, Susana Gómez, Maria Victoria Lopera, Nora Alejandra Zuluaga, Adriana Carolina Forero, Gustavo Giraldo, Nicolás Pineda Trujillo, Juan Camilo Martínez, Paola Durán Ventura, Juan Manuel Alfaro, Literature Search: Silvia C. Martínez Rueda, Maria del Pilar Montilla, Carolina Baquero, Writing: Silvia C. Martínez Rueda, Maria del Pilar Montilla, Susana Gómez, Maria Victoria Lopera, Nora Alejandra Zuluaga, Adriana Carolina Forero, Gustavo Giraldo, Nicolás Pineda Trujillo, Juan Camilo Martínez, Paola Durán Ventura, Juan Manuel Alfaro.

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The 40th Minute Cortisol Measurement is the Key Time-Point in the Low-Dose Synacthen Stimulation Test: A Large, Assay-Specific Pediatric Validation Study

Buşra Gürpınar Tosun[1], Hazal Arıkan Gacemer[2], Didem Helvacıoğlu[1], Serap Turan[1], Abdullah Bereket[1], Tülay Güran[1]

[1]Marmara University Faculty of Medicine, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye
[2]Marmara University Faculty of Medicine, Department of Pediatrics, İstanbul, Türkiye

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ABSTRACT

Objective: Low-dose Synacthen stimulation test (LDSST) is widely used to assess central adrenal insufficiency (CAI). With the adoption of monoclonal antibody (mAb) cortisol immunoassays, lower basal and peak cortisol concentration thresholds require external validation under real-world clinical conditions. To externally validate previously defined LDSST sampling strategies, basal cortisol thresholds, and gray-zone cut-offs in a large real-world cohort using mAb immunoassays.

Methods: This single-center retrospective study analyzed 646 LDSSTs in patients with suspected CAI, measuring baseline, 40th, and 60th minute cortisol levels after administration of 1 µg of synthetic Adrenocorticotropic hormone. The diagnostic performance of single and combined sampling strategies and previously defined basal cortisol thresholds and grey-zone cut-offs were evaluated across different peak cortisol criteria.

Results: Cortisol measurement at 40th minute provided the most reliable single time-point assessment, with significantly fewer false-negative results than at 60th minute ($p<0.0001$). At the basal cortisol threshold of ≥6.5 µg/dL identified in our previous prospective study, specificity decreased from 68% to 57.5% ($p=0.21$) and sensitivity remained comparable (73.8% vs. 75.1%, $p=0.74$), while negative predictive value declined significantly from 91% to 78.4% ($p=0.02$) and positive predictive value increased to 53% in this retrospective cohort. Validation of basal cortisol gray-zone thresholds confirmed high diagnostic accuracy across different peak cortisol cut-offs.

Conclusion: This study provides robust real-world external validation of LDSST sampling strategies and basal cortisol thresholds. Cortisol measurement at the 40th minute, combined with assay-specific basal cortisol interpretation and a gray-zone framework, offers a practical approach for individualized clinical decision-making in suspected CAI.

Keywords: Adrenocorticotropic hormone, cortisol, children, low-dose Synacthen stimulation test, monoclonal antibody immunoassay, central adrenal insufficiency, external validation

Corresponding Author: Prof. Tülay Güran, Marmara University Faculty of Medicine, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye
E-mail: tulay.guran@marmara.edu.tr, tulayguran@yahoo.com **ORCID:** orcid.org/0000-0003-2658-6866
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What is already known on this topic?

The low-dose Synacthen stimulation test (LDSST), which is widely used for suspected central adrenal insufficiency, has adopted lower peak and basal cortisol cut-offs in monoclonal antibody immunoassays.

What this study adds?

A large-scale real-world external validation of the previously defined LDSST sampling strategies is provided. The clinical utility of cortisol measurement at the 40th minute and a basal cortisol threshold of 6.5 µg/dL was validated for routine pediatric practice despite real-world variability.

Introduction

The low-dose Synacthen stimulation test (LDSST) is considered a safe and widely used diagnostic tool for evaluating patients with suspected central adrenal insufficiency (CAI) (1,2,3). Compared to the standard-dose Synacthen stimulation test, LDSST has been shown to provide greater sensitivity, as supraphysiological stimulation in standard-dose testing may result in false-negative responses (2,3,4,5,6,7). The interpretation of LDSST results has evolved with advances in cortisol assay methodologies. The introduction of newer monoclonal antibody (mAb) immunoassays, which offer higher analytical specificity, has led to the adoption of lower peak cortisol cut-off values, most commonly 18 µg/dL (500 nmol/L), with subsequent studies suggesting even lower cut-offs, ranging from 12.6 to 15.7 µg/dL (8,9,10,11,12,13,14,15,16).

Morning basal serum cortisol concentrations are commonly used as an initial screening tool for the evaluation of adrenal function (17,18,19,20,21,22,23,24). Very low basal cortisol levels are suggestive of AI, whereas higher concentrations may indicate preserved hypothalamo-pituitary-adrenal (HPA) axis function (4,11,25,26,27,28,29). However, a substantial proportion of patients fall into an intermediate range, in which basal cortisol values alone are insufficient for a definitive diagnosis. In everyday clinical practice, particularly in pediatric care, logistical constraints, patient cooperation, and outpatient scheduling often preclude consistent sampling during the early morning hours, thereby limiting the practical utility of basal cortisol measurement. External validation in heterogeneous clinical populations is essential to confirm the generalizability of diagnostic strategies derived from controlled prospective settings (30,31). Moreover, data on predictive basal cortisol thresholds in children, especially those derived from mAb immunoassays, remain limited.

In our previously published prospective study using mAb cortisol immunoassays, optimal LDSST performance was achieved with cortisol sampling at the 40th minute, alone or in combination with that at the 60th minute (32). Morning basal serum cortisol concentrations were identified as predictors of LDSST outcomes, particularly in clinical settings where frequent post-stimulation sampling is technically challenging. Although these findings were derived under controlled and prospectively defined conditions, their applicability in routine clinical practice and heterogeneous patient populations has not been fully explored.

Therefore, the present study retrospectively evaluated a large cohort of pediatric patients who underwent LDSST for suspected CAI to externally validate previously defined optimal sampling strategies, basal cortisol cut-offs, and gray-zone thresholds across different peak cortisol criteria using mAb immunoassays. We aimed to evaluate whether the optimal sampling strategy and basal cortisol thresholds identified in our previous prospective study remained robust and clinically applicable under real-world conditions in a large retrospective pediatric cohort.

Methods

Patients and Methods

This single-center retrospective study evaluated all LDSSTs performed in our pediatric endocrinology clinic among patients aged 0-18 years with suspected CAI between November 2016 and February 2022. Owing to the retrospective design and longitudinal follow-up of patients, some individuals underwent repeat LDSSTs. In our cohort, repeat testing was primarily performed in patients with ongoing glucocorticoid exposure, evolving pituitary pathology, or when initial results were borderline or clinically incongruent.

LDSSTs were performed by pediatric endocrine nurses following standard pre-test instructions, including overnight fasting and temporary (24-48 hours) withholding of steroid therapy. Basal adrenocorticotropic hormone (ACTH) and cortisol samples were collected between 08:00 and 11:00 a.m. after an intravenous (IV) line was inserted. Plasma cortisol levels were measured 40 and 60 min after an IV bolus of 1 µg tetracosactide (Synacthen® alfasigma). The samples were analyzed using mAb immunoassays. During the study period, assays from different immunoassay platforms were used, including the Roche® Elecsys

Cortisol II assay and Beckman Coulter Access Cortisol assay (run on UniCel DxI analyzers). A positive LDSST, suggestive of AI, was defined as a stimulated peak serum cortisol concentration of <18 µg/dL (500 nmol/L). Previously published optimal testing conditions derived from a prospective cohort were applied to a large real-world dataset to assess their consistency and reliability (32). Diagnostic classifications based on a single time-point (0, 40, and 0, 60 minutes) and combined time-point (0, 40, and 60 minutes) sampling strategies were compared. In addition, basal cortisol thresholds previously identified in our prospective study to predict peak cortisol cut-off values defined in the literature for mAb immunoassays were assessed for their performance in the current cohort. Specifically, basal cortisol thresholds of 6.5 µg/dL for a peak cortisol cut-off of 18 µg/dL (500 nmol/L), 6.4 µg/dL for a peak cut-off of 15.7 µg/dL (433 nmol/L), and 6.2 µg/dL for a peak cut-off of <12.6 µg/dL (350 nmol/L) were evaluated (8,9,10,13). Basal cortisol gray-zone cut-offs previously identified in our prospective study were externally validated in the current cohort. Diagnostic performance metrics, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were calculated.

Studies were performed with the approval of the Clinical Research Ethics Committee of the Marmara University Faculty of Medicine (approval no: 09.2022.387, date: 04.03.2021).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism® version 10 (GraphPad Software Inc., San Diego, California, USA). Continuous variables are presented as the mean±standard deviation for normally distributed data. Categorical variables are expressed as counts (percentages). Pairwise comparisons between continuous variables were performed using the Student's t-test, and categorical variables were compared using the chi-square test. Paired categorical outcomes obtained from different sampling time points during the LDSST were compared using McNemar's test.

Receiver operating characteristic curve analysis was used to evaluate the diagnostic performance of cortisol levels at different time points during the LDSST. Previously defined basal morning cortisol cut-off values were applied, and their diagnostic performance was assessed by calculating sensitivity, specificity, and the area under the curve (AUC). PPV and NPV were calculated for the identified thresholds. At the basal cortisol threshold of 6.5 µg/dL, sensitivity, specificity, PPV, and NPV were calculated for the prospective and retrospective validation cohorts. Between-cohort differences in each metric were compared using tests for equality of proportions (chi-square or Fisher's exact test, as appropriate), with analyses restricted to the relevant denominator for each metric. All tests were two-sided, and a p value <0.05 was considered statistically significant. The association between basal serum cortisol and peak cortisol concentrations during the LDSST was examined using linear regression analysis for descriptive purposes.

Results

A total of 646 LDSSTs performed in 537 children (241 females, 44.9%) at different time points were retrospectively analyzed. The indications for LDSST included suspected CAI due to a sellar mass (n=28), history of pituitary or cranial surgery or radiotherapy (n=121), long-term or high-dose glucocorticoid use (n=102), and the presence of other pituitary hormone deficiencies (n=286). The mean age of the patients at the time of testing was 9.2±5.8 years (range: 0.0-21.1 years). Of the 646 LDSSTs performed, 212 (32.8%) failed. Basal and peak serum cortisol levels were significantly different in patients who failed and passed the LDSST (p<0.0001). Age at the time of testing, basal cortisol, ACTH, and peak cortisol levels according to the test outcome (passed or failed) are presented in Table 1.

Among the patients who passed the LDSST (n=434), basal cortisol concentrations ≥18 µg/dL (500 nmol/L) were observed in only 29 patients (6.7%). The majority of the patients (88.6%) had cortisol concentrations ≥18 µg/dL (500 nmol/L) for the first time at the 40th minute, and 4.7% at the 60th minute. Cortisol levels at the 0th,

Table 1. Morning basal ACTH and cortisol concentrations of the patients

	LDSST passed (n=434)*			LDSST failed (n=212)*			p value
	Mean	Median	Range	Mean	Median	Range	
Age (years)	8.7±6.0	9.2	0.0-20.0	10.4±5.2	11.6	0.0-21.1	0.0003
Plasma ACTH (pg/mL)	28.0±22.7	21.0	3.0-156	28.6±23.3	22.8	4.0-134	0.76
Basal serum cortisol (mAb) (µg/dL)	9.9±4.7	9.1	0.4-24.5	6.0±3.6	5.9	0.0-15.6	<0.0001
Peak serum cortisol (mAb) in LDSST (µg/dL)	24.1±5.1	23.1	18-59.9	13.2±4.9	15.1	0.3-17.9	<0.0001

*Adrenal insufficiency was defined as a stimulated cortisol level <18.0 µg/dL (500 nmol/L). Patients whose serum cortisol did not exceed 18 µg/dL (500 nmol/L) during LDSST were labelled as "failed" and those whose serum cortisol was ≥18 at any time point were labelled as "passed."
Cortisol µg/dL=0.036×nmol/L
LDSST: low dose Synacthen stimulation test

40th, and 60th minutes are illustrated in Figure 1a. The association between basal cortisol and peak cortisol levels were shown in Figure 1b. Regardless of the LDSST outcome, peak cortisol concentrations occurred at the 40th minute in 50.5% of patients (n=326) and at the 60th minute in 49.5% (n=318). Comparison of the two groups according to age, sex, LDSST outcomes, and clinical indications for testing showed that children who reached peak cortisol at the 60th minute were significantly younger than those peaking at the 40th minute (mean age 6.7±5.5 vs 11.7±4.9 years, p<0.0001). Sex distribution and overall test outcomes were similar between groups. However, the distribution of clinical indications differed significantly (p=0.023), with long-term/high-dose glucocorticoid exposure being more common among patients reaching peak cortisol at the 60th minute than among those peaking at the 40th minute (30.2% vs 21.5%, p=0.012).

In the model of a single time-point cortisol measurement after tetracosactide stimulation, 64.1% (n=414) of the tests had an adequate response at the 40th minute and 55.9% (n=361) at the 60th minute. When efficiency was calculated based on the overall LDSST results, the most specific time to test for a single cortisol measurement after stimulation was the 40th minute (specificity, 95.6%; AUC: 0.989) (Figure 2a). Cortisol assessment at the 40th minute resulted in significantly fewer false-negative classifications than that at the 60th minute (χ^2=31.5, p<0.0001). If single sampling had been performed 40 minutes after stimulation, 20 patients (4.6%) with adequate response at the 60th minute would have been misdiagnosed with AI. In addition, comparison between the 0, 40 minute and 0, 40 and 60 minute sampling strategies using McNemar's test demonstrated a significant improvement in diagnostic classification with the inclusion of the 60 minute measurement (χ^2=18.05, p<0.0001) (Figure 1b).

When a basal cortisol threshold of 6.5 µg/dL was applied, 416 children (64.4%) had basal cortisol levels ≥6.5 µg/dL, of whom 326 (78.4%) passed the LDSST, while 90 (21.6%) had a failed test. Among the 230 children with basal cortisol <6.5 µg/dL, 122 (53.1%) failed the LDSST and 108 (46.9%) passed. Using basal cortisol ≥6.5 µg/dL to predict a normal LDSST outcome yielded a sensitivity of 75.1%, specificity of 57.5%, and a NPV of 78.4% for excluding AI. At a basal cortisol threshold of 6.5 µg/dL, diagnostic performance differed between the initial prospective study and the current retrospective validation cohort. The sensitivity decreased from 68% in the prospective cohort to 57.5% in the retrospective validation cohort; however, this

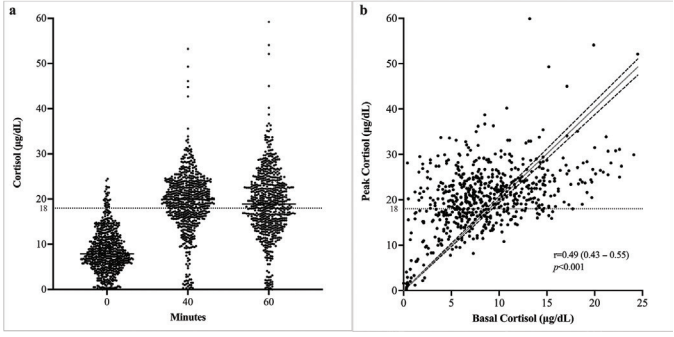

Figure 1. Cortisol responses during LDSST. (a) Serum cortisol levels at baseline, 40, and 60 minutes after tetracosactide administration. (b) Association between basal cortisol levels and peak cortisol responses during the LDSST
LDSST: low-dose Synacthen stimulation test

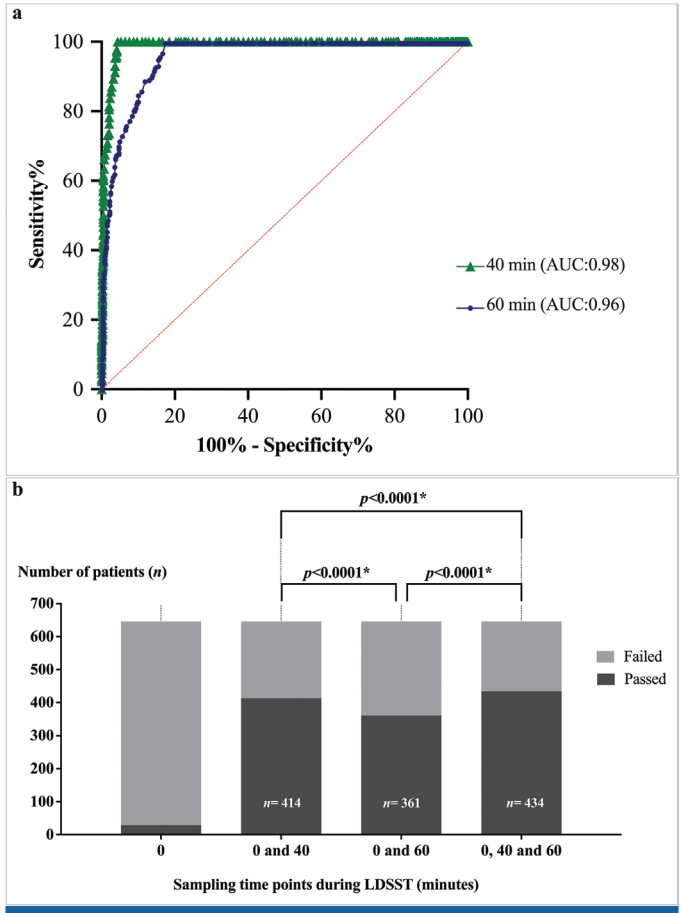

Figure 2. Diagnostic performance of cortisol measurements at different sampling time points during the LDSST. **(a)** Models of a single time point cortisol measurement after tetracosactide stimulation. **(b)** Stacked bar graph showing patients who passed the LDSST at different sampling time points, with a paired comparison of diagnostic classifications performed using McNemar's test
**Adrenal insufficiency was defined as a stimulated cortisol level <18 µg/dL (500 nmol/L).*
**Failed: Patients whose serum cortisol did not exceed 18 µg/dL (500 nmol/L) during LDSST were labelled as "failed."*
**Passed: Patients whose serum cortisol was ≥18 at any time point were labelled as "passed."*
**Cortisol µg/dL =0.036×nmol/L*
LDSST: low-dose Synacthen stimulation test, AUC: area under the curve

difference was not statistically significant (p=0.21). Specificity remained comparable between the two cohorts (73.8% vs. 75.1%, p=0.74). The PPV increased from 39.1% to 53%, whereas the NPV decreased significantly from 91% to 78.4% (p=0.02). Disease prevalence was higher in the retrospective validation cohort than in the prospective cohort (32.8% vs. 19.7%), which was reflected in the observed changes in predictive values. The diagnostic performance of predefined basal cortisol thresholds according to the different peak cortisol cut-offs are shown in Table 2.

Validation analyses were performed for the basal cortisol grey zone thresholds previously identified in our prospective study, in which basal cortisol values <2.5 µg/dL demonstrated 100% sensitivity and values >14.6 µg/dL demonstrated 100% specificity for passing the LDSST using a peak cortisol cut-off of 18 µg/dL (32). Basal cortisol values <2.5 µg/dL were observed in 58 tests, of which 45 failed and 13 passed the LDSST. Basal cortisol values >14.6 µg/dL were observed in 72 tests, of which 68 passed and four failed. The remaining 515 tests had basal cortisol values within the grey zone (2.5-14.6 µg/dL), including 352 passed and 163 failed tests. For basal cortisol <2.5 µg/dL, the sensitivity was 97.2% with a PPV of 77.6%. For basal cortisol >14.6 µg/dL, the specificity for excluding AI was 98.1%, with a NPV of 94.4%. Validation of basal cortisol gray-zone cut-offs according to different peak cortisol cut-offs during the LDSST are shown in Table 3.

Discussion

In this large, real-world cohort of pediatric patients evaluated for suspected CAI, we externally validated key findings from our previously published prospective study and demonstrated their applicability in routine clinical practice, in line with recommendations emphasizing the importance of external validation before the clinical implementation of diagnostic tests (30,33,34). Our results confirm that cortisol measurement at 40 minutes after LDSST provides the most reliable single time-point assessment, with superior diagnostic performance compared with the 60th minute and significantly fewer false-negative classifications.

The basal cortisol thresholds derived from the prospective cohort also showed consistent diagnostic behavior in the retrospective validation analysis. When the same basal cortisol threshold (≥6.5 µg/dL) was applied, sensitivity was largely preserved, whereas specificity and NPV were reduced, consistent with application of the same threshold under real-world conditions with higher disease prevalence and greater clinical heterogeneity (34,35,36). Lowering the peak cortisol cut-off was associated with a marked improvement in the NPV of basal cortisol, whereas sensitivity

Table 2. Diagnostic performance of basal cortisol thresholds according to different peak cortisol cut-off values during the LDSST

Adrenal insufficiency (peak cortisol concentration)	Basal cortisol threshold	Failed tests n (%)	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
<18.0 µg/dL (500 nmol/L)	6.5 µg/dL (179 nmol/L)	212 (32.8)	75.1	57.5	78.4	53.0
<15.7 µg/dL (433 nmol/L)	6.4 µg/dL (177 nmol/L)	123 (19.0)	73.0	68.3	90.7	37.3
<12.6 µg/dL (350 nmol/L)	6.2 µg/dL (171 nmol/L)	65 (10.1)	73.5	86.2	97.9	26.7

Basal cortisol thresholds were predefined based on previously published prospective data and existing literature derived from monoclonal antibody immunoassays
Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated without derivation of new cut-off values
LDSST: low dose Synacthen stimulation test

Table 3. Validation of basal cortisol grey-zone cut-offs according to different peak cortisol cut-offs during the LDSST

Adrenal insufficiency (peak cortisol concentration under)	Lower basal cortisol cut-off*	Specificity (%)	PPV (%)	Upper basal cortisol cut-off*	Specificity (%)	NPV (%)
<18.0 µg/dL (500 nmol/L)	2.5 µg/dL (69 nmol/L)	97.2	77.6	14.6 µg/dL (403 nmol/L)	98.1	94.4
<15.7 µg/dL (433 nmol/L)	2.7 µg/dL (75 nmol/L)	96.5	67.2	7.4 µg/dL (204 nmol/L)	76.4	91.6
<12.6 µg/dL (350 nmol/L)	2.4 µg/dL (66 nmol/L)	96.4	61.8	7.4 µg/dL (204 nmol/L)	93.8	98.8

*Represents basal cortisol values between the lower and upper thresholds
All thresholds were predefined based on prior prospective data and literature derived from monoclonal immunoassays
Diagnostic performance metrics were calculated without derivation of new cut-off values
PPV: positive predictive value (probability of adrenal insufficiency given a positive test result), NPV: negative predictive value (probability of a normal result given a negative test result), LDSST: low dose Synacthen stimulation test

remained relatively stable, consistent with Bayesian principles and the known dependence of predictive values on disease prevalence and spectral effects (36,37,38). These findings support the utility of basal cortisol as an effective rule-out tool for CAI and reinforce its role in clinical decision-making, particularly in settings where dynamic testing may be delayed, not readily available, or difficult to perform in children due to practical or clinical constraints (17,18,19,20,21,22,23,24,25,32,39,40).

An additional observation from the subgroup analysis was that patients reaching peak cortisol concentrations at the 60th minute were significantly younger than those reaching peak levels at the 40th minute, regardless of the LDSST outcome. This finding may reflect developmental differences in HPA responsiveness in younger children. Consistent with previous studies showing that younger children more frequently reach peak cortisol at later time points during ACTH stimulation, this pattern may be related to the relative immaturity of the HPA axis, leading to a delayed adrenal response (41,42,43). Despite this variability in the timing of the maximal cortisol response, the 40th minute remained the most informative single sampling time point in our cohort. However, delayed peak responses may be particularly relevant when interpreting LDSST results in very young children, especially preterm infants, neonates, or those younger than three years of age. Interestingly, long-term/high-dose glucocorticoid use was also more frequent among patients reaching peak cortisol at the 60th minute. Chronic glucocorticoid exposure may lead to partial suppression of the HPA axis, potentially resulting in a delayed adrenal response to ACTH stimulation.

Overall, this external validation demonstrates that, although the intrinsic diagnostic characteristics of the test are preserved, its rule-out performance reflects the realities of routine clinical practice rather than controlled research conditions (30,33,44). Importantly, the underlying diagnostic distribution of suspected CAI was similar between the prospective and retrospective cohorts. Although the age ranges were similar, the retrospective validation cohort had a significantly higher mean age, which may reflect routine pediatric endocrine practice and support the applicability of the proposed diagnostic strategy across a broader pediatric age spectrum. In addition, variability in test timing, pre-test steroid withdrawal, and execution under everyday care represents the real-world context in which the test is applied, reiterating the importance of validating diagnostic thresholds under pragmatic conditions (35,36,45).

Study Limitations

Our study had limitations including that it was a single-center study, which may have limited its generalizability. Secondly, although all cortisol measurements were performed using mAb-based immunoassays, two different assay platforms (Roche Elecsys Cortisol II and Beckman Coulter Access Cortisol) were used during the study period due to changes in laboratory contracts, which may have introduced some analytical variability. Finally, because of the retrospective design and clinical follow-up, some patients underwent repeated LDSSTs at different time points. However, this reflects routine clinical practice and did not materially alter the overall diagnostic performance of the proposed testing strategy.

The strengths of this study include its large sample size, inclusion of repeated tests reflecting everyday clinical workflows, and consistent use of mAb-based immunoassays. In a real-world validation study, pre-analytical and analytical variabilities can not be fully standardized. However, this reflects routine clinical practice and aligns with the primary aim of evaluating test performance under pragmatic conditions.

Conclusion

In conclusion, this study demonstrated the reliability of our initial diagnostic approach by validating its key findings in a large, real-world, but retrospective pediatric cohort. Thus, it appears that the proposed sampling strategy and cortisol threshold values provide clinicians with a robust framework for interpreting test results in daily practice. By using the optimal conditions defined in the prospective study as a reference, test results can be interpreted on an individual patient basis, allowing informed clinical decision-making, even under the variable conditions of routine care.

Ethics

Ethics Committee Approval: Studies were performed with the approval of the Clinical Research Ethics Committee of the Marmara University Faculty of Medicine (approval no: 09.2022.387, date: 04.03.2021).

Informed Consent: Due to the retrospective nature of the study, the requirement for informed consent was waived by the ethics committee.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Buşra Gürpınar Tosun, Hazal Arıkan Gacemer, Didem Helvacıoğlu, Concept: Buşra Gürpınar Tosun, Didem Helvacıoğlu, Design: Buşra Gürpınar Tosun, Serap Turan, Abdullah Bereket, Tülay Güran, Data Collection or Processing: Buşra Gürpınar Tosun, Hazal Arıkan Gacemer, Tülay Güran, Analysis or Interpretation: Buşra Gürpınar Tosun, Writing: Buşra Gürpınar Tosun, Tülay Güran.

Conflict of Interest: Two authors of this article, Abdullah Bereket and Serap Turan, are members of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, they were not involved in any stage of the editorial decision process for this manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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Myocardial Performance Index and Carotid Intima-Media Thickness in Children with Metabolically Healthy and Metabolically Unhealthy Obesity

Nazlican Civilibal Tang[1], Kazim Oztarhan[2], Helen Bornaun[3], Sumeyra Dogan[4], Ata Mert Civilibal[5]

[1]Washington University School of Medicine, Department of Pediatrics, Division of Rheumatology and Immunology, St Louis, Missouri, United States; University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital, Clinic of Pediatrics, Istanbul, Türkiye
[2]Istanbul University, Istanbul Faculty of Medicine; University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital, Department of Pediatric Cardiology, Istanbul, Türkiye
[3]University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital, Clinic of Pediatric Cardiology, Istanbul, Türkiye
[4]University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital, Clinic of Pediatric Radiology, Istanbul, Türkiye
[5]Bezmialem Vakif University Faculty of Medicine, Istanbul, Türkiye

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ABSTRACT

Objective: To compare the myocardial performance index (MPI) and carotid intima-media thickness (cIMT) of children who are metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) with children without obesity.

Methods: This study included obese patients between 6 and 17 years of age and age- and gender-matched healthy children. Two groups of obese patients were investigated: MUO and MHO.

Results: There were 62 obese (MUO=30; MHO=32) and 30 healthy controls included. Compared to controls, the MPI score and cIMT of both obese groups were significantly larger (p=0.004 and p=0.003, respectively). However, there was no significant difference in MPI and cIMT between the MUO and MHO groups. Moreover, there were independent associations between higher MPI and body mass index-standard deviation score (BMI-SDS) (β=0.312, p=0.002) and between higher cIMT and waist circumference-SDS (WC-SDS) (β=0.371, p=0.003).

Conclusion: The primary outcome of the study indicated that while both MPI and cIMT values were elevated in obese children compared to non-obese controls, there was no significant difference between MUO and MHO groups. This suggests that obesity itself, irrespective of metabolic health, is associated with increased cardiovascular risks. BMI-SDS and WC-SDS were useful markers for identifying children at cardiovascular risk in this cohort.

Keywords: BMI, childhood obesity, waist circumference

Corresponding Author: Nazlican Civilibal Tang MD, Washington University School of Medicine, Department of Pediatrics, Division of Rheumatology and Immunology, St Louis, Missouri, United States; University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital, Clinic of Pediatrics, Istanbul, Türkiye
E-mail: nazlicancivilibal@hotmail.com **ORCID:** orcid.org/0009-0008-8295-7119

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What is already known on this topic?

Obesity in children increases cardiovascular risk factors. Myocardial performance index (MPI) and carotid intima-media thickness (cIMT) are established measures of subclinical cardiovascular abnormalities. Body mass index (BMI) and, particularly, waist circumference (WC) have been associated with cardiometabolic risk and subclinical cardiovascular alterations in children with obesity. However, whether these cardiovascular abnormalities differ according to metabolic health status remains unclear.

What this study adds?

This study demonstrates that both metabolically healthy and metabolically unhealthy children with obesity have increased MPI and cIMT compared with non-obese controls, with no significant differences between the two obesity phenotypes. Higher MPI was independently associated with BMI-standard deviation score (SDS), whereas higher cIMT was independently associated with WC-SDS, suggesting that the degree of adiposity may contribute to early cardiovascular alterations irrespective of metabolic health status.

Introduction

Childhood obesity is a global problem leading to various endocrine, metabolic, and cardiovascular comorbidities (1). Changes in eating habits and a general decrease in physical activity associated with modern life-styles have made obesity an endemic disease in many societies.

Adults with obesity who do not exhibit risk factors for metabolic ill-health, such as dyslipidemia, insulin resistance (IR), and hypertension, are termed metabolically healthy obese (MHO). In contrast, those who have one or more of these risk factors are defined as metabolically unhealthy obese (MUO) (2,3). First described and investigated in adults with obesity, these phenotypes have also been extensively studied and confirmed in children and adolescents with obesity (3). Obesity plays an important role in the development of metabolic syndrome (MS). However, not all adults and children with severe obesity have MS or MUO.

The most common cardiovascular disorders (CVDs) due to obesity are increased left ventricular mass, myocardial dysfunction, and increased carotid intima-media thickness (cIMT) (4,5). These early indicators of subclinical CVD have been shown in both pediatric and adult obese cohorts (6,7,8,9). However, the impact of obesity-related conditions, such as IR, dyslipidemia, and arterial hypertension on the development of these CVDs is not yet clear in children.

In recent years, tissue Doppler imaging (TDI) has been more widely adopted to evaluate early changes in left ventricular systolic and diastolic function. Myocardial performance index (MPI) measured with TDI is less affected by age, heart rate, or preload compared to conventional pulsed wave Doppler (cPWD) echocardiography (10,11). The cIMT is a well-known marker of the atherosclerotic process and is a valuable indicator for long-term follow-up of children at high risk of atherosclerosis (12,13).

It has been widely shown that MPI and cIMT are increased in adults with obesity. However, to the best of our knowledge, there are few studies investigating the relationship of obesity-related metabolic factors with the increase in MPI and cIMT in children and adolescents. Therefore, the aim of this study was to compare MPI and cIMT of both MHO and MUO children and adolescents with healthy controls. A further aim was to evaluate the effects of obesity-related metabolic factors.

Methods

Study Design

This study included obese patients between 6 and 17 years of age in the pediatric endocrinology outpatient clinic of a single center. The patients with obesity were divided into two groups, MUO and MHO, based on the same criteria used for adults. The control group comprised age- and gender-matched healthy children and adolescents.

A post-hoc power analysis was conducted to ensure that our sample size was adequate for detecting significant differences in MPI and cIMT between MHO and MUO children, as well as non-obese controls. Based on previous studies in similar populations, an effect size (Cohen's d) of 0.5 was estimated for the cardiovascular measures. Using a two-sided alpha level of 0.05 and a desired power of 0.80, G*Power software calculations indicated that a minimum of 27 participants per group would be required to achieve sufficient statistical power.

This study was approved by the University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital Clinical Research Ethics Committee (approval no.: 2019/57, date: 22.03.2019). Written informed consent was obtained from the parents of patients and controls. The research related to human use has complied with all the relevant national regulations, and institutional policies and by the tenets of the Helsinki Declaration.

Patients with missing data, secondary obesity, any kind of chronic disease or systemic diseases, and use of medications known to alter blood pressure or lipid or glucose metabolism were excluded. Control subjects were selected from healthy children admitted to the hospital for mild illnesses with a BMI between the 25th and 75th percentile.

Height was measured to the nearest millimeter with a wall-mounted stadiometer, and weight was measured to the nearest 100 g by SECA digital scale with minimal clothes and without shoes. BMI was calculated by dividing the body weight in kilograms by height in meters squared. On the BMI reference curve, which was appropriate for Turkish children and adjusted for age and gender, those with BMI values at or above the 95th percentile were defined as "obese" (14). Waist circumference (WC) was measured using a nonelastic tape at the level of the umbilicus with the child standing and breathing normally. Waist measurements were evaluated using the percentile curves for WC for healthy Turkish children (15). Standard deviation scores (SDS) for BMI and WC were computed using the least mean squares (LMS) method and using the reference data for Turkish children (15,16).

Blood Pressure Measurements

Blood pressure (BP) measurements were made three times at 2-minute intervals on the right arm, in a seated patient, after at least five minutes of rest, by the auscultation method (ERKA®, Germany) with appropriate cuff size according to the age and constitution of the child. The last two BP measurements were averaged for analysis. Systolic BP (SBP) and diastolic BP (DBP) measurements of all patients were evaluated according to the American Academy of Pediatrics 2017 hypertension guideline and based on these data, we calculated the blood pressure SDS-score (16). SBP and/or DBP values that were >90th percentile were defined as elevated blood pressure, and values ≥95th percentile were defined as hypertension.

Biochemical Measurements

Glucose, insulin, triglycerides, total cholesterol, low-density lipoprotein (LDL)-cholesterol, and high-density lipoprotein (HDL)-cholesterol were measured in blood samples taken in the morning after a 12-hour fast. Blood glucose levels were measured by the glucose oxidase method and serum lipid profiles were measured using routine enzymatic methods. Insulin measurements were made using the immunofluorometric method (Modular E170 analyzer, Roche Diagnostics, Mannheim, Germany). The homeostasis model assessment of IR (HOMA-IR) was calculated to estimate IR using the following formula: [HOMA-IR=fasting plasma glucose (mg/dL) x fasting plasma insulin (μU/mL)/405] (6,9).

Assessment of Metabolic Status

The definition of MHO in children and adolescents is controversial and heterogeneous. There are two commonly used definitions for pediatric MS; modified National Cholesterol Education Program criteria and modified International Diabetes Federation criteria (17,18). Metabolic risk factors in both definitions are similar, but the cut-offs of the components are different. Moreover, the definitions are not clear for children under 10 years of age. For this reason, Damanhoury et al. (3) collaborated with 46 international experts and published a classification of the definitions of MHO and MUO in children in 2018. Based on this classification, standard MUO phenotype was defined as the presence of at least one of the following risk factors: SBP and/or DBP> 90th percentile; fasting blood glucose >100 mg/dL; HDL cholesterol <40 mg/dL, triglycerides >100 mg/dL (children <10 years) or >130 mg/dL (children >10 years). Individuals who met the obesity criteria but did not meet any of these criteria were considered to be MHO. Furthermore, following the suggestions of some earlier studies, we included IR, defined by HOMA-IR with thresholds >2.5 for prepubertal and >3.16 for pubertal (Tanner stage ≥2) participants, in our classification criteria (19,20). Detailed cut-off values and thresholds applied in this study, especially for age-specific thresholds are shown in Table 1.

Echocardiographic Measurements

Echocardiographic assessments were performed using the General Electric Medical Systems ViVid 7 Pro dimension

Table 1. Metabolic risk factor cut-off values used for classification of MUO

Parameter	Age group	Threshold
Systolic or diastolic BP	All ages	>90th percentile
Fasting blood glucose	All ages	>100 mg/dL
HDL cholesterol	All ages	<40 mg/dL
Triglycerides	<10 years	>100 mg/dL
Triglycerides	≥10 years	>130 mg/dL
HOMA-IR	Prepubertal	>2.5
HOMA-IR	Pubertal (Tanner ≥2)	>3.16

BP: blood pressure, HDL: high-density lipoprotein, HOMA-IR: homeostatic model assessment for insulin resistance, MUO: metabolically unhealthy obese

echocardiography device (GE Vingmed Ultrasound AS, Horten, Norway) equipped with TDI technology. All measurements were performed according to American Society of Echocardiography guidelines (21). Participants were examined in the left lateral position by the same experienced pediatric cardiologist blinded to clinical and laboratory outcomes. TDI was obtained from the apical four-chamber view, where the sample volume was placed on the septal and lateral sides of the mitral annulus.

For the calculation of MPI, systolic myocardial velocity (Sm), ejection time (ET), and isovolumetric contraction time (IVCT) as systolic parameters, and early (Em) and late (Am) diastolic velocities, the Em/Am ratio, and the isovolumetric relaxation time (IVRT) as diastolic parameters, were measured by TDI. We measured the IVRT from the end of the S-wave to the beginning of the E-wave and IVCT from the beginning of the first positive deflection after the Q-wave to the onset of the S-wave. ET was measured from the beginning to the end of the S-wave. The MPI (IVRT + IVCT/ET) was calculated to assess the LV global (systolic+diastolic) function. The results recorded from three cardiac cycles were averaged.

Vascular Assessment

The cIMT was measured using B-mode high-resolution ultrasonography (Toshiba Aplio 300 Ultrasound, Japan). Measurements were performed in the supine position, with the neck slightly hyperextended, and 1 to 2 cm proximal to the bifurcation of both common carotid arteries. The SDSs for cIMT were calculated using the LMS method and height-specific normative values (22).

Statistical Analysis

All statistical evaluations were performed using SPSS software, version 26.0 (IBM Inc., Armonk, NY, USA). The visual (histogram, probability plots) and analytic methods (Kolmogorov-Smirnov) were used to evaluate the distribution of continuous variables. Discrete variables are expressed as counts (percentage), continuous variables with normal distribution were calculated as mean±SD, and continuous variables with non-normal distribution as median. Differences in the means of MUO, MHO, and control subjects were initially tested by ANOVA. To identify specific group differences, post-hoc comparisons were conducted using the Tukey HSD test. Associations between variables were assessed by Pearson or Spearman's analysis, depending on the distribution type of the variable. The variables that showed a p value of 0.05 in the univariate analysis were tested in a stepwise linear regression analysis for the assessment of risk factors. A $p<0.05$ was considered statistically significant for all statistical evaluations.

Results

Of the 62 children with obesity included in the study, 32 (51.6%) were MHO and 30 (48.4%) patients had MUO. The control group consisted of 30 healthy normal-weight children. There were no significant differences between the groups regarding age and gender. Post-hoc comparisons showed that BMI, BMI-SDS, WC, WC-SDS, DBP, and DBP-SDS were similar in MUO and MHO groups, but significantly higher than the controls (Table 2). SBP, SBP-SDS, and HOMA-IR were significantly different between the three groups, while glucose and LDL levels were similar. Triglyceride was significantly higher and HDL lower in the MUO group compared to the other two groups.

When TDI and carotid ultrasonography findings were evaluated, the ET and Em/Am values of the three groups were significantly different, but the Sm, IVCT, Em, and IVRT were similar (Table 3). The MPI and cIMT means of obese groups were significantly higher than controls. However, there were no difference between the MPI and cIMT values obtained from the MUO and MHO patients.

All clinical and laboratory results were analyzed by univariate analysis to identify cardiometabolic risk factors affecting LV diastolic dysfunction (increase in MPI) and subclinical atherosclerosis (increase in cIMT) in patients with obesity (Table 4). Both MPI and cIMT were positively correlated with BMI-SDS, WC-SDS, SBP-SDS, and DBP-SDS. Moreover, MPI was found to be positively related to HOMA-IR, and cIMT was found to be negatively correlated with HDL. Finally, we identified an independent association between high MPI and BMI-SDS ($\beta=0.312$, $p=0.002$), and between cIMT and WC-SDS ($\beta=0.371$, $p=0.003$) in stepwise linear regression analysis (Table 5).

Discussion

This study showed that MPI and cIMT increased in children with both MUO and MHO and that BMI and WC were important predictors of these increases. Our findings reinforce the suggestion that the degree of obesity in children may be the important risk factor for increased MPI and cIMT and this is independent of metabolic abnormalities.

Left ventricular hypertrophy, systolic/diastolic dysfunction, and increased cIMT have been recognized as subclinical indicators of CVD in children and adults with obesity (9,13,23,24,25). Detection of cardiovascular structural and functional changes during the subclinical period in obese patients are important during clinical follow-up and in determining the prognosis. Studies into subclinical LV diastolic dysfunction and atherosclerosis in children with obesity are more limited than in adults. The strongest aspect of the present study is the comparison of MPI and cIMT measurements of children with and without

hypertension, hyperglycemia, IR, or dyslipidemia, that is MUO versus MHO, respectively.

In children and adults with obesity, structural and functional cardiac changes are frequently investigated using the cPWD echocardiography method (6,8). However, studies evaluating MPI using TDI in children with obesity are limited (11).

Obesity-related increased preload volume causes significant impairments in diastolic myocardial velocities (11,26). Similar to previous studies, we detected a significant increase in Am wave velocity and therefore a significant decrease in the Em/Am ratio. In addition to the low Em/Am ratio, we found that the MPI was significantly higher in the MUO and MHO groups compared to individuals without obesity. This generalized increase in both obese sub-groups was caused by the shortening of ET without significant changes in tissue Doppler-derived IVRT and IVCT.

Table 2. Demographic and anthropometric characteristics and laboratory findings of the study groups

	MUO (n=30)	MHO (n=32)	Control (n=30)	p value
Age (year)	13.3±2.5	13.2±3.1	12.3±2.3	0.160
Male n (%)	16 (53.3)	17 (53.1)	16 (53.3)	0.992
BMI (kg/m^2)	29.3±5.7	29.8±4.4	19.3±2.2**	<0.001
BMI-SDS	2.22±0.92	2.50±0.73	0.02±0.57**	<0.001
WC (cm)	97.9±13.8	98.1±12.9	76.8±8.9**	<0.001
WC-SDS	2.12±0.71	2.26±0.79	0.65±0.82**	<0.001
SBP (mmHg)	131.8±14.3	125.6±12.6	108.9±7.3	<0.001*
SBP-SDS	1.87±0.70	1.44±0.84	0.40±0.37	<0.001*
DBP (mmHg)	86.2±10.8	82.0±9.1	67.2±5.3**	<0.001
DBP-SDS	1.83±0.71	1.61±0.68	0.48±0.40**	<0.001
Triglycerides (mg/dL)	134.1±76.3**	101.9±42.1	96.0±22.0	0.013
HDL (mg/dL)	39.2±6.8 **	49.3±9.9	49.3±9.8	<0.001
LDL (mg/dL)	81.9±20.5	80.0±20.1	74.6±18.9	0.422
Glucose (mg/dL)	84.0±7.1	82.9±4.4	83.2±5.9	0.759
HOMA-IR	5.30±2.25	2.47±0.79	1.57±0.48	<0.001*

*The results of all groups were statistically different from each other
**The results were significantly different from the other two groups
MUO: metabolically unhealthy obese, MHO: metabolically healthy obese, BMI: body mass index, SDS: standard deviation scores, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, HDL: high density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: homeostatic model assessment for insulin resistance

Table 3. Comparison of tissue Doppler imaging and carotid ultrasonography findings

	MUO (n=30)	MHO (n=32)	Control (n=30)	p value
Systolic parameters				
Sm (cm/s)	9.90±1.01	9.83±1.05	9.76±1.12	0.717
ET (ms)	253.0±34.5	262.1±36.5	288.3±34.2	0.001*
IVCT (ms)	33.2±9.6	33.3±8.0	33.0±10.8	0.990
Diastolic parameters				
Em (cm/s)	18.0±4.2	18.0±4.0	17.5±2.7	0.818
Am (cm/s)	12.8±4.7	13.9±3.1	9.9±1.8**	<0.001
Em/Am ratio	1.50±0.35	1.32±0.23	1.81±0.24	<0.001*
IVRT (ms)	24.3±5.0	26.1±6.0	23.4±2.7	0.083
MPI	0.23±0.07	0.23±0.06	0.20±0.05**	0.004
cIMT (μm)	389.0±57.3	385.9±55.7	369.3±48.9**	0.003

*The results of all groups were statistically different from each other
**The results were significantly different from the other two groups
MUO: metabolically unhealthy obese, MHO: metabolically healthy obese, Sm: systolic myocardial velocity, ET: ejection time, IVCT: isovolumetric contraction time, Em: early diastolic velocity, Am: late diastolic velocity, IVRT: isovolumetric relaxation time, MPI: myocardial performance index, cIMT: carotid intima-media thickness

TDI-derived MPI is a relatively new parameter used to evaluate systolic and diastolic myocardial function. In addition, MPI reflects increased LV filling pressure and decreased left ventricular compliance (10,27,28). Our data are consistent with previous MPI studies in children with obesity (26,29,30,31).

The cause of this myocardial dysfunction remains unclear, although the severity and duration of obesity, chronic volume overload, IR, and hemodynamic and metabolic changes have all been implicated (32). LV systolic and diastolic dysfunction secondary to obesity has been associated with MS-related hypertension, dyslipidemia, and IR accompanying obesity. Some studies have shown that subclinical myocardial dysfunction identified by MPI was correlated with BMI and IR (11,29,31). In our study, MPI was significantly higher in both the MUO and MHO groups than in children without obesity, with no significant difference between the two obesity groups. MPI was positively correlated with both BMI-SDS (r=0.289, p=0.003) and WC-SDS (r=0.262, p=0.006), whereas stepwise linear regression analysis identified BMI-SDS as the only independent factor associated with higher MPI (β=0.312, p=0.002). These findings suggest that the degree of adiposity may contribute to subclinical myocardial dysfunction independently of metabolic health status. However, participants were not specifically categorized according to obesity severity; therefore, a direct comparison between obese and severely obese children could not be performed. Further studies specifically stratifying children according to the severity of obesity are needed to confirm this association.

Childhood obesity is associated with changes in cardiac structure and function, as well as various biomarkers of subclinical atherosclerosis such as cIMT. Similar to our results, elevated cIMT has been documented in numerous investigations involving children with obesity (13,33,34). This finding is indicative of the presence of subclinical atherosclerosis in patients with obesity, regardless of metabolic health. There is no consensus on the results regarding the increase in cIMT in patients with and without MS. Although some studies report higher cIMT in children with MS, other studies have reported that cIMT is not different between obese children with and without MS (13,35). In agreement with the studies of Zhao et al. (33) and Farello et al. (34), we found that cIMT values of obese groups were higher than controls, but cIMT was not different between obese groups with MUO or MHO. Therefore, we believe that obesity may be an important risk factor for increased cIMT even in the absence of metabolic abnormalities. The lack of difference in MPI and cIMT between MHO and MUO groups may be influenced by factors, such as the overall degree of obesity, genetic predispositions, lifestyle factors, and underlying subclinical inflammation that can affect cardiovascular outcomes regardless of metabolic health status.

In the present study, cIMT was positively correlated with BMI-SDS (r=0.239, p=0.010), WC-SDS (r=0.248, p=0.008), SBP-SDS, and DBP-SDS, and negatively correlated with HDL cholesterol. In stepwise linear regression analysis, WC-SDS was the only independent factor associated with higher cIMT (β=0.371, p=0.003). Similar to our findings, Sonmez et al. (13) reported an independent association between higher WC and increased cIMT in children with obesity. These findings support an association between the degree of adiposity, particularly central adiposity, and subclinical vascular alterations. However, because our

Table 4. Risk factors of myocardial performance index and carotid intima-media thickness

	MPI		cIMT	
	r	p value	r	p value
BMI-SDS	0.289	0.003	0.239	0.010
WC-SDS	0.262	0.006	0.248	0.008
SBP-SDS	0.185	0.039	0.194	0.032
DBP-SDS	0.195	0.031	0.222	0.017
HOMA-IR	0.237	0.011		
HDL			-0.210	0.022

Spearman's correlation analysis (only significant correlations shown)
MPI: myocardial performance index, cIMT: carotid intima-media thickness, BMI: body mass index, SDS: standard deviation scores, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostatic model assessment for insulin resistance, HDL: high density lipoprotein

Table 5. Independent predictors of myocardial performance index and carotid intima-media thickness in all obese patients

Dependent variable	Independent variable	β-coefficient	SE	p value
MPI	BMI-SDS	0.312	0.011	0.002
cIMT	WC-SDS	0.371	0.041	0.003

MPI: myocardial performance index, cIMT: carotid intima-media thickness, BMI: body mass index, SDS: standard deviation scores, WC: waist circumference

participants were not stratified according to obesity severity, this relationship should be interpreted as an association rather than evidence of a direct effect of obesity severity on cIMT.

BMI and WC are the most common anthropometric measures for predicting abdominal obesity. In recent years, an increasing number of studies support the use of WC instead of BMI in children with obesity (36,37). Besides, derived WC cut points for children to identify cardiovascular risk factors have been suggested in some countries (38,39).

Study Limitations

This study has some limitations. First, it was a cross-sectional study with a relatively small number of cases. Further prospective, long-term studies with a larger number of patients are needed to determine the effects of obesity on myocardial functions and atherosclerosis. Lack of obesity duration and weight status history were further limitations. Furthermore, participants were not stratified according to the severity of obesity, which limited our ability to directly assess differences in MPI and cIMT across different degrees of obesity.

Conclusion

The present study demonstrated increased MPI and cIMT as markers of subclinical diastolic dysfunction and atherosclerosis in children with obesity. The similarity of these two markers between MUO and MHO patients and the detection of an independent relationship between MPI and BMI-SDS, and cIMT and WC-SDS suggest that the degree of adiposity may be associated with MPI and cIMT independently of metabolic health status. Our results demonstrated the diagnostic value of MPI and cIMT for routine and widespread use in children with obesity due to their ease of application and reproducibility. Moreover, BMI and WC appear to be valuable and easy indicators of early CVD in children with obesity. Long-term multicenter prospective studies will provide better insight into early screening of cardiovascular risk factors in children with obesity.

Ethics

Ethics Committee Approval: This study was approved by the University of Health Sciences Türkiye, Kanuni Sultan Suleyman Training and Research Hospital Clinical Research Ethics Committee (approval no.: 2019/57, date: 22.03.2019).

Informed Consent: Written informed consent was obtained from the parents of patients and controls.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Nazlican Civilibal Tang, Concept: Nazlican Civilibal Tang, Ata Mert Civilibal, Design: Nazlican Civilibal Tang, Ata Mert Civilibal, Data Collection or Processing: Nazlican Civilibal Tang, Kazim Oztarhan, Helen Bornaun, Sumeyra Dogan, Analysis or Interpretation: Nazlican Civilibal Tang, Kazim Oztarhan, Literature Search: Nazlican Civilibal Tang, Kazim Oztarhan, Writing: Nazlican Civilibal Tang, Kazim Oztarhan, Helen Bornaun, Sumeyra Dogan, Ata Mert Civilibal.

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Gender Identity and Preferences in Children with Variations in Sex Development

Canice E. Crerand[1,2], Natalie M. Gallagher[3], Margaret P. Adam[4,5], Maria G. Vogiatzi[6], Elizabeth McCauley[5,7], Jennifer Hansen-Moore[1,2], Margarett Shnorhavorian[5,8], Patricia Y. Fechner[4,5], Anne-Marie E. Amies Oelschlager[5,9], Justin A. Indyk[2,10], V. Rama Jayanthi[11,12], Hailey M. Umbaugh[1], Rachel Horton[3], Shira Kahn-Samuelson[3], Grace Raber[3], Madeline McClinchie[1], Kristina R. Olson[3]

[1]Center for Biobehavioral Health, Nationwide Children's Hospital, Columbus, Ohio, United States
[2]Department of Pediatrics, The Ohio State University College of Medicine, Columbus, Ohio, United States
[3]Department of Psychology, Princeton University, Princeton, New Jersey, United States
[4]Department of Pediatrics, University of Washington, Seattle, Washington, United States
[5]Differences in Sex Development (DSD) Program, Seattle Children's Hospital, Seattle, Washington, United States
[6]Division of Endocrinology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, United States
[7]Department of Psychiatry, University of Washington, Seattle, Washington, United States
[8]Department of Urology, University of Washington, Seattle, Washington, United States
[9]Department of Obstetrics and Gynecology, University of Washington, Seattle, Washington, United States
[10]Section of Endocrinology and Metabolism, Nationwide Children's Hospital, Columbus, Ohio, United States
[11]Department of Pediatric Urology, Nationwide Children's Hospital, Columbus, Ohio, United States
[12]Department of Urology, The Ohio State University College of Medicine, Columbus, Ohio, United States

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ABSTRACT

Objective: To assess gender-typed preferences and gender identity in children with and without variations in sex developments (VSDs).

Methods: In this cross-sectional study, 78 children with VSDs (ages 3-12; mean age=7 years; 55% White, non-Hispanic) recruited through specialty clinics in the United States and 78 children without VSDs (ages 3-13; mean age=7 years; 55% White, non-Hispanic) recruited through university-based community databases completed assessments of gender-typed toy, clothing and peer preferences, continuous and categorical measures of gender identity, and perceived similarity to boys and to girls.

Results: Generally, children with and without VSDs did not differ in their gender development on 5 of 7 measures for each gender group. Children raised as girls who had VSDs had more masculine toy preferences, t(84.89)=3.421; p=0.001; d=0.698, and viewed themselves as more

Corresponding Author: Canice E. Crerand, Center for Biobehavioral Health, Nationwide Children's Hospital, Columbus, Ohio, United States
E-mail: canice.crerand@nationwidechildrens.org **ORCID:** orcid.org/0000-0003-0040-6396
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similar to boys, t(67.43)=2.994; p=0.004; d=0.648, than comparison children raised as girls. Boys with VSDs selected more masculine toys [t(55.17)=2.413; p=0.019; d=0.623], and responded in a more-masculine way on the continuous gender identity measure [t(38.40)=2.364; p=0.023; d=0.621], than did boys in the community comparison sample, though these effects, unlike the effects amongst girls, were not robust against corrections for multiple comparisons.

Conclusion: During early and mid-childhood, VSDs were not strongly associated with differences in gender development. Future longitudinal research on the gender development of youth with VSDs is necessary, particularly as they mature into adolescence.

Keywords: Disorders of sex development, gender identity, intersex

What is already known on this topic?

Gender development and relatedly, gender identity, are complex issues in the context of variations in sex development (VSD). Children with VSD appear to experience higher gender identity discordance or gender dysphoria relative to the general population. However, few studies have studied gender development in children with VSD using contemporary measures and appropriate comparison groups.

What this study adds?

This study of 78 children with VSD aged between 3 and 12 years compared their gender development to that of a comparison matched sample of youth without VSD. Few differences between groups were observed, and youth in both groups reported a range of both gender stereotypic and diverse identities and preferences. Results have implications for VSD care.

Introduction

Variations in sex development (VSDs) (1) include genital or reproductive differences (e.g., "atypical" genitalia), less common genetic karyotypes (e.g., 47,XXY, 45,X), and/or a combination of features (e.g., vulva and testes) that are either discordant with an individual's sex chromosomes (1) or less common in the general population. [Terminology is a complex issue in the context of variations in sex development, including historic use of stigmatizing terms to describe these variations, with controversies continuing today about use of terms such as "differences" or "disorders" of sex development or "intersex" across healthcare providers, patients, and families [Davies (2), 2015]. We use the term 'variations in sex development' and specific diagnosis names [Lin-Su et al. (3), 2015; e.g., congenital adrenal hyperplasia (CAH)], to avoid terms more often associated with these controversies. We report data including and separating data for females with CAH to reflect stakeholder preferences. Exact estimates of the frequency of VSDs range from 1.7 in 100 births (4) to 1.8 in 10,000 births (5) depending on the breadth of one's definition and inclusion or exclusion of particular conditions.

Parents of youth with VSDs often come to healthcare providers with questions about the likely course of their children's gender development. The aim of the current research was to provide contemporary answers about what gender development looks like in children with VSDs as compared to their peers without VSDs.

Today, "gender development" is understood to encompass many distinct aspects of a person's experience including their gender identity (i.e., whether they think of their gender category as a boy, girl, nonbinary, etc), gender presentation (i.e., whether they prefer clothing that is culturally-stereotyped as masculine, feminine, or androgynous), and gender role (i.e., for children, preferred play style or type) (6). Empirical data from children without (known) VSDs supports the notion that while, for example, gender identity is often correlated with gender-typed preferences, these do not always closely correspond with one another in stereotypical ways and can be fully dissociable in some children (7,8,9).

Due to the presence of less common variations in genitalia and/or the potential for discordances between physical characteristics and sex chromosomes, healthcare providers and parents of children with VSDs sometimes grapple with whether to initially raise a child with a VSD as a boy, a girl, or in a more gender neutral or open way [e.g., as a theybie, (2,10)]. This decision can also have implications for potential surgical or endocrine interventions, a controversial and debated issue in the context of VSDs (11).

Studies of individuals with VSDs suggest that some may experience more uncertainty about their gender identity or might experience more gender identity change than people without VSDs. For example, children with CAH who are initially raised as girls sometimes show higher rates of gender dysphoria (12,13) or identification as boys (14,15,16) than comparison groups, but other studies find no differences in gender identity (17,18). [When we use the term CAH for children raised as girls, we include 46,XX individuals with 21-hydroxylase deficiency,

17α-hydroxylase, or 11β- hydroxylase. The two 46,XY youth with CAH, both raised as boys, have 17α-hydroxylase].

Research on other aspects of gender development in children with VSDs is more limited. The literature that exists in this area has primarily focused on children with CAH who are raised as girls. The most robust effect reported in this literature suggests that, compared to girls without CAH, girls with CAH often show stronger preferences for toys that are culturally stereotyped as masculine (18,19, 20). Other studies have shown a parallel "masculinity bias" amongst girls with CAH in other domains: masculine-typed games (21), masculine playmates (22), and masculine-type clothing/dress-up (18). Much less is known about gender-typed preferences in children with other VSDs as systematic analyses and the use of a wide range of measures of gender expression and/or gender roles are rarer in this literature.

A further limitation of past work on gender development in children with VSDs is that much of it was completed 20 or more years ago. In the intervening time, societal ideas about gender have been shifting. Setting aside VSDs, more youth today are identifying as genders that differ from the ones they are assumed to have at birth. Large-scale and representative surveys and polls suggest that anywhere from 1.2 to 5% of adolescents and young adults identify as transgender and/or nonbinary (23,24,25). In contrast, as recently as 2011, estimates suggested numbers closer to 0.3% of the adult population identified as transgender (26).

In the present work, our aim was to investigate gender development, adopting measures of a diverse range of distinct aspects of gender, in a group of youth with VSDs and compare them to a group of youth without VSDs. Though we did not officially preregister a hypothesis, the study was designed to ask if children with VSDs showed more gender nonconformity than children without VSDs.

Methods

Participants and Procedures

Participants aged 3-12 years with variations in sex development were recruited from 3 children's hospitals: Seattle Children's Hospital (n=44); The Children's Hospital of Philadelphia (n=22); and Nationwide Children's Hospital (n=12) between October 7, 2019, and June 20, 2023 (M_{age}=7.14 years, SD_{age}=2.85 years). See Supplemental Material for additional recruitment details. The list of diagnoses of participants with VSDs is shown in Table 1. Demographics of both participant groups are included in Table 2.

Community comparison participants were recruited through one of two university databases of families who signed up to participate in child development research at the University of Washington and Princeton University. Each participant with a VSD was matched with a child raised as the same gender who was within five months of age at the time of testing (M_{age}=7.18 years, SD_{age}=2.91 years).

This research was approved through the IRBs of five testing locations. At least one parent had to provide informed consent, the child had to provide verbal assent, and the child had to complete the study assessment to be included in this analysis. This study approved by the Institutional Review Board of Princeton University (approval no.: 12940, date: 25.07.2020).

Child Measures

Categorical Gender Identity (7)

Children were asked if they were "a boy," "a girl," or "something else." Youth who selected "something else" then chose between "both," "neither," "it changes over time," and "I don't know."

Continuous Gender Identity (27)

In line with more recent conceptualizations of gender identity as a non-discrete aspect of human experience (28,29,30), children were given a non-categorical measure of gender identity. Children were shown a line on which they could indicate their gender from one end, described and labeled as "boy/man" (0) and the other end described and labeled as "girl/woman" (1), with a label in the middle indicating "in the middle means you feel like a mix of both". Children were told that "Some people feel they are a boy, some people feel they are a girl, and some people feel they are somewhere in between a boy and a girl. On the line below, move the slider to the place you think best shows how you feel on the inside." The slider appeared at the center of the slider, and they could move it in either direction.

Peer Preference (31)

Children saw eight trials, each featuring the photographs of two children. Six of the trials included one child who looked stereotypically like a girl and one child who looked stereotypically like a boy (the two remaining trials were filler trials featuring two children of the same gender, not used to compute scores; pairs within a trial were matched for race: six pairs included White children, one pair each were Black and Asian). Children were asked whom they would rather be friends with. Responses are reported as the proportion of times children selected the girl.

Toy and Clothing Preference (7,31)

Participants completed four trials selecting toys ("Which toy would you like to play with the most?") and four trials selecting clothing ("Which outfit would you like to wear?"). On each trial there were five possible selections presented in a random array. The items on each trial were selected based on pilot testing. Within each trial (i.e., within one set of five items), there was one item that the pilot (child) participants rated as especially masculine, one was seen as moderately masculine, one was

Table 1. Participant VSD variations and the gender assigned at birth

Gender-of-raising	Diagnosis	Sample size	Categorization
Boy	Hypospadias	6	Severe hypospadias
	Hypospadias with 87% 46,XY and 13% sex chromosome aneuploidy consisting of XXYY	1	
	Hypospadias, bifid scrotum, bilateral undescended testes	1	
	Klinefelter syndrome	4	Klinefelter syndrome + sex chromosome aneuploidy
	48,XXXY[4]/49,XXXXY[26]	1	
	Klinefelter syndrome + 21-hydroxylase deficiency	1	
	45,X/46,XY mosaicism with mixed gonadal dysgenesis	2	Mosaicism + mixed gonadal dysgenesis
	45,X/46,XY mosaicism	1	
	17α-hydroxylase deficiency (46,XY)	2	Other VSD
	Undescended testes	2	
	46,XY 5-alpha reductase deficiency	1	
	46,XY gonadal dysgenesis	1	
	46,XY partial gonadal dysgenesis due to a pathogenic variant in *MAP3K1*	1	
	Hypogonadotropic hypogonadism	1	
	47,XYY	1	
	Ovotesticular DSD	1	
	Ovotesticular DSD secondary to chimerism	1	
	Vanishing testes syndrome	1	
	46,XX male	1	
Girl	21-hydroxylase deficiency	18	46,XX CAH
	17α-hydroxylase deficiency (46,XX)	7	
	11-beta-hydroxylase deficiency	3	
	46,XX DSD due to a heterozygous pathogenic variant of *WT1* (gain-of-function)	1	46,XX nonsyndromic VSD
	46,XX female with UG (urogenital) sinus and posterior labial fusion	1	
	46,XX female with a UG, posterior fusion, duplicated vagina/cervix	1	
	46,XX female with clitoromegaly, posterior fusion, and UG sinus	1	
	46,XX isolated clitoromegaly	1	
	MRKH	1	
	CAIS	2	46,XY nonsyndromic VSD
	17 beta-HSD deficiency	1	
	46,XY 5-alpha reductase deficiency	1	
	46,XY DSD	1	
	46,XY partial gonadal dysgenesis with a urogenital sinus	1	
	PAIS	1	
	Turner syndrome	4	Turner syndrome
	Turner syndrome with mosaicism	3	

Categorization applies to presentation of data in Figure 2
VSD: variations in sex development; DSD: differences of sex development; MAP3K1: mitogen-activated protein kinase kinase kinase 1; CAH: congenital adrenal hyperplasia; WT1: Wilms tumor 1; UG: urogenital; MRKH: Mayer-Rokitansky-Küster-Hauser syndrome; CAIS: complete androgen insensitivity syndrome; HSD: hydroxysteroid dehydrogenase; PAIS: partial androgen insensitivity syndrome

Table 2. Parent-reported participant demographics, including reports of gender/sex history, current gender, and current pronouns

		Children with variation n=78	Community comparison n=78
Gender-of-raising	Boy	38.46%	38.46%
	Girl	61.54%	61.54%
Race and ethnicity	White, non-hispanic	55.13%	62.82%
	White, hispanic	6.41%	6.41%
	Asian, non-hispanic	8.97%	5.13%
	Black, non-hispanic	3.85%	2.56%
	Middle Eastern, non-hispanic	1.28%	0.00%
	Native American/Alaska native, non-hispanic	1.28%	0.00%
	Multiple race/ethnicity	12.82%	19.23%
	Incomplete or no response	10.26%	3.85%
Education of participating parent	Some schooling	1.28%	0.00%
	High school diploma	15.38%	1.28%
	Some college	28.21%	7.69%
	Bachelor's degree	21.79%	25.64%
	Advanced degree (MA, MD, PhD, etc)	28.21%	64.10%
	Non-response	5.13%	1.28%
Household income	Under $25,000/year	11.54%	0.00%
	$25,001-$50,000/year	12.82%	1.28%
	$50,001-$75,000/year	8.97%	8.97%
	$75,001-$125,000/year	21.79%	20.51%
	Over $125,001/year	39.74%	65.38%
	Non-response	5.13%	3.85%
Child's relationship to participating parent	Biological child	84.62%	98.72%
	Adopted child	8.97%	0.00%
	Grandchild	1.28%	0.00%
	Non-response	5.13%	1.28%
Informed of VSD before birth	No	74.36%	97.44%
	Yes	17.95%	1.28%
	Unknown (not birth parent)	5.13%	0.00%
	No response	2.56%	1.28%
Reported sex before child's birth	Binary aligned with gender in which child was raised	74.36%	84.62%
	Binary not-aligned with gender in which child was raised	2.56%	0.00%
	Was not told	10.26%	14.10%
	Unknown (not birth parent)	5.13%	0.00%
	Other	5.13%	0.00%
	Non-response	2.56%	1.28%
Sex on birth certificate	Binary aligned with gender in which child was raised	94.87%	98.72%
	Binary not-aligned with gender in which child was raised	2.56%	0.00%
	Other	0.00%	0.00%
	Non-response	2.56%	1.28%

Table 2. Continued

		Children with variation n=78	Community comparison n=78
Child's gender	Binary aligned with gender in which child was raised	94.87%	97.44%
	Binary not-aligned with gender in which child was raised	0.00%	0.00%
	Both	1.28%	1.28%
	Neither	1.28%	0.00%
	Other	0.00%	0.00%
	Non-response	2.56%	1.28%
Child's pronouns	Binary aligned with gender in which child was raised	93.59%	97.44%
	Binary not-aligned with gender in which child was raised	0.00%	0.00%
	They/them	2.56%	0.00%
	Mixed	1.28%	1.28%
	Non-response	2.56%	1.28%

VSD: variations in sex development; MA: Master of Arts; MD: Doctor of Medicine; PhD: Doctor of Philosophy

gender neutral, one was moderately feminine, and one was especially feminine. Participants' selections on each trial were initially coded as 1 (selecting most masculine) to 5 (selecting most feminine). Their scores on the four trials of each type were averaged (α_{toys}=0.88; $\alpha_{clothes}$=0.87) and then recoded to match the other measures such that the overall score ranged from 0 (most masculine) to 1 (most feminine). See Supplementary Material for more details on this task's piloting process and the variation in stimuli seen across participants.

Similarity to Boys and Girls (32)

Children were asked five questions about how similar they are to boys and five questions about how similar they are to girls (e.g., "How similar are you to boys/girls?", "How much do you look like boys/girls?", "How much do you act like boys/girls?"). Children indicated their responses on a 5-point pictorial scale coded from 0 (very different) to 5 (very similar). To compute overall scores, we calculated the average score of all of the boy items and an average score of all of the girl items (α_{boy}=0.89, α_{girl}=0.88). We then reverse-scored the boy items, to have high scores across all gendered measures represent similarity/identity with girl/feminine.

Parent Measures

Parents were asked: (a) whether they were told that their child had a variation in sex development before birth; (b) what sex they were told their child would have before birth; and (c) what sex was listed on their child's birth certificate. Parents were further asked about (d) their child's current gender and (e) which pronouns their child used in everyday life (he/him, she/her, they/them, other; parents also had an opportunity to provide further clarification).

Parents reported demographic information (see Table 1) and completed a measure of their children's gender identity and expression that are reported in the Supplementary Material.

Statistical Analyses

All analyses were conducted in R (R Foundation for Statistical Computing, Vienna, Austria), using the tidyverse, rstatix, psych, knitr, kableExtra, and gridExtra packages (33,34,35,36,37,38,39,40). Not every participant answered every question. In tables, we display non-response (for categorical measures) or total n (for continuous measures). When aggregating across items and measures (e.g., calculating similarity to girls across the five questions, calculating the gendered composite), non-response is omitted from the calculation. Data cannot be shared publicly due to privacy concerns; many participants have rare medical conditions and would be identifiable with minimal or no demographic information provided. Data will be shared when requested by researchers with approval of the authors' IRB and requester IRB with agreement to approved privacy protections.

Results

Children's Gender-related Responses

Overall children with and without VSDs did not show many significant differences in their gender development. Among children raised as boys, those with and without VSDs differed significantly on only two of seven measures. Throughout this paper we refer to participants in our study with the descriptor "raised as boys" and "raised as girls". We use this term to indicate that this was the gender they were raised as throughout most of

their childhood. A few participants may be better characterized as having another gender at the time of testing (see Table 2), however results are reported by socialized gender because: (1) there are not enough nonbinary youth for separate analysis; and (2) past literature typically reports results by socialized gender, allowing for comparison across current and past results.

Boys with VSDs selected more masculine toys [t(55.17)=2.413; p=0.019; d=0.623], and responded in a more-masculine way on the continuous gender identity measure [t(38.40)=2.364; p=0.023; d=0.621], than did boys in the community comparison sample, although it should be noted that neither effect was statistically significant if a Bonferroni correction for the seven tests was applied. The two groups did not differ in their clothing preference, peer preference, similarity to boys, similarity to girls, or likelihood of picking "boy" in response to the categorical gender identity question (all p>0.15). The distributions of these responses can be seen in Figure 1. Tables 3 and 4 contain summaries of the distributions and statistical comparisons.

Among children raised as girls, those with and without VSDs differed significantly on only two of seven measures, and these differences held even when a Bonferroni correction for seven tests was applied. Girls with VSDs had more masculine toy preferences [t(82.06)=3.538; p=0.001; d=0.722], and felt more similar to boys [t(73.30)=2.822; p=0.006; d=0.609], than community comparison girls. The two groups did not differ on the continuous gender identity measure, clothing preference, peer preference, similarity to girls, or likelihood of picking "girl" in response to the categorical gender identity question (all p>0.2). Tables 3 and 4 contain summaries of the distributions and statistical comparisons.

The only specific VSD diagnosis for which we had a large enough group for separate analysis was girls with CAH. We compared this group to 28 age-and-gender matched community comparison girls. As with the overall comparison, girls with CAH had significantly more masculine toy preferences than the community comparison girls [t(48.22)=4.314, p<0.001, d=1.153]; the two groups did not differ significantly in clothing preference, peer preference, continuous gender identity, similarity to girls, or categorical gender identity (all p>0.2). Unlike the overall comparison, the difference for similarity to boys was not significant [t(42.45)=1.327, p=0.192, d=0.383]. Full results comparing girls with CAH to community comparison girls can be seen in the Supplementary Material (Table S1).

To show the general results by diagnosis type, we computed a composite of all gender-relevant child measures. To include the gender identity category measure, we treat "girl" as 1, "boy" as 0, and all other answers ("both," "neither," "it changes," "I don't know") as 0.5. While these measures were selected because they represent a range of gender development constructs (e.g., identity, gender-typed preferences) that can be dissociable for individual children, past work suggests that these measures are often correlated (9). The seven measures formed a reliable composite (α=0.94) in which 0 represents the responses most stereotypically associated with boys and 1 represents the responses most stereotypically associated with girls. In Figure 2, we show the level of this composite variable for participants

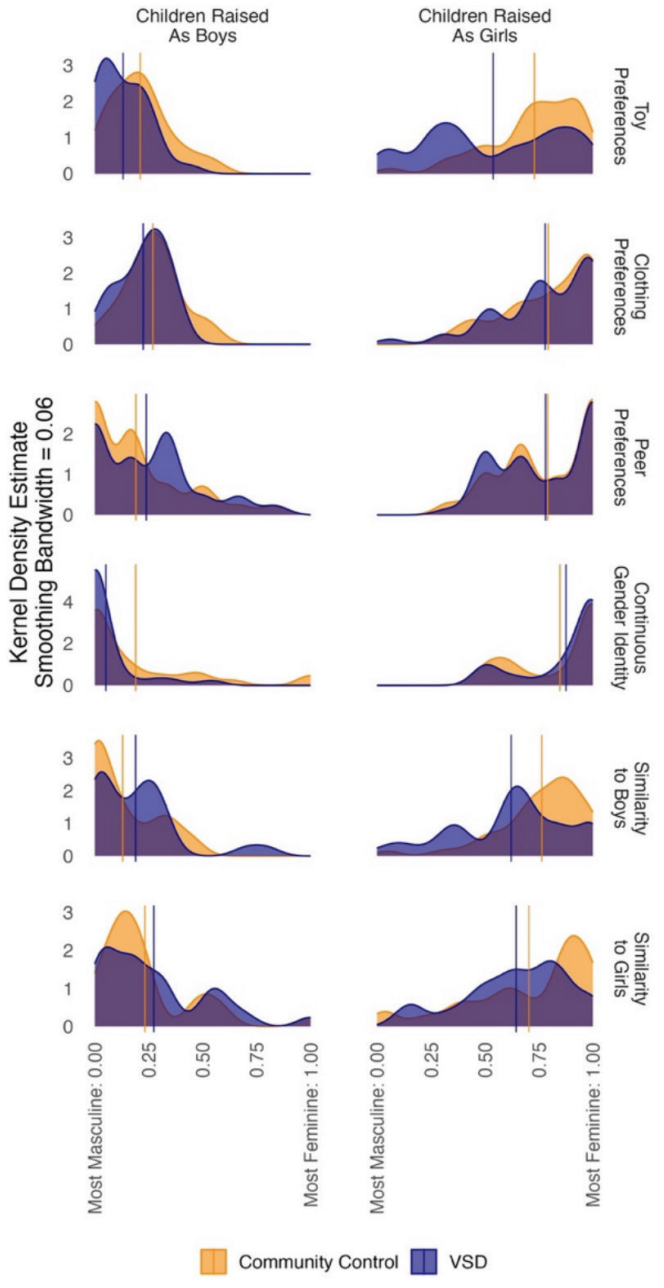

Figure 1. Distributions of responses on the primary gender measures. Each measure ranges from 0 (most masculine) to 1 (most feminine). Mean values for each group are indicated by vertical lines
VSD: variations in sex development

within each diagnostic category, including community comparison participants as their own subgroup. We present this data for exploratory purposes, rather than formal analyses, given the heterogeneity within groups and the small sample sizes.

Parent Report of Child History, Current Gender, and Current Pronouns

Among parents of children with VSDs, a chi-square test comparing those who said yes (17.95%) or no (74.36%) showed that parents were more likely not to have been told about their child's VSD prior to the child's birth [χ^2(df=1)=26.889, p<0.001, fei=0.61, one-sided 95% confidence interval (0.42,1)]. The majority of parents in both groups reported that: (a) they were told their child's natal sex before their child's birth; (b) the natal sex reported on their child's birth certificate aligns with the gender in which the child was raised; (c) their child's current gender is the same as the gender in which the child was raised and (d) their child's current pronouns reflect the gender in which the child was raised. Due to the infrequency of non-majority responses on these measures, we did not conduct statistical comparisons between the parents of children with VSDs and community comparison parents. Full responses can be seen in Table 2.

The results from the parent-reported gender measure converge with observations from the children themselves; full details are described in the Supplementary Material.

Discussion

Overall, we observed very few differences between a sample of children with a range of VSDs and children from a community

Table 3. Within-group means and standard deviations for continuous measures of gendered self-report

	Raised-as boys					Raised-as girls				
	Children with VSD		Community comparison		t-test	Children with VSD		Community comparison		t-test
Measure	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	
Toy preference	30	0.13 (0.11)	30	0.21 (0.14)	t(55.17)=2.413 **p=0.019** d=0.623	48	0.54 (0.31)	48	0.73 (0.21)	t(82.06)=3.538 **p=0.001** d=0.722
Clothing preference	30	0.22 (0.11)	30	0.27 (0.13)	t(56.49)=1.420 p=0.161 d=0.367	48	0.78 (0.22)	48	0.79 (0.20)	t(93.07)=0.351 p=0.727 d=0.072
Peer preference	30	0.24 (0.23)	29	0.19 (0.23)	t(56.99)=-0.810 p=0.442 d=-0.211	48	0.78 (0.22)	47	0.79 (0.21)	t(92.99)=0.233 p=0.816 d=0.048
Continuous gender identity	29	0.05 (0.13)	29	0.19 (0.29)	t(38.40)=2.364 **p=0.023** d=0.621	48	0.88 (0.18)	48	0.85 (0.20)	t(93.41)=-0.706 p=0.482 d=-0.144
Similarity to boys	28	0.19 (0.20)	29	0.13 (0.15)	t(50.90)=-1.282 p=0.206 d=-0.340	41	0.62 (0.27)	47	0.76 (0.20)	t(73.30)=2.822 **p=0.006** d=0.609
Similarity to girls	28	0.27 (0.25)	28	0.23 (0.23)	t(53.41)=-0.636 p=0.527 d=-0.170	41	0.64 (0.24)	48	0.70 (0.28)	t(86.99)=1.059 p=0.293 d=0.224

Each measure ranges from 0 (most stereotypically associated with boys) to 1 (most stereotypically associated with girls)
VSD: variations in sex development; N: sample size; M: mean; SD: standard deviation

Table 4. Categorical gender self-report

	Raised-as boys			Raised-as girls		
Response	Children with VSD	Community comparison	Chi-square	Children with VSD	Community comparison	Chi-square
Boy	93.33%	90.00%	χ^2 (df=1)<0.01 p>0.9 Cohen's w<0.001	2.08%	0.00%	χ^2 (df=1)<0.01 p>0.9 Cohen's w<0.001
Girl	0.00%	3.33%		85.42%	87.50%	
Both	0.00%	0.00%		2.08%	2.08%	
Neither	3.33%	0.00%		0.00%	0.00%	
It changes over time	0.00%	3.33%		4.17%	2.08%	
I don't know	3.33%	0.00%		4.17%	8.33%	
Non-response	0.00%	3.33%		2.08%	0.00%	

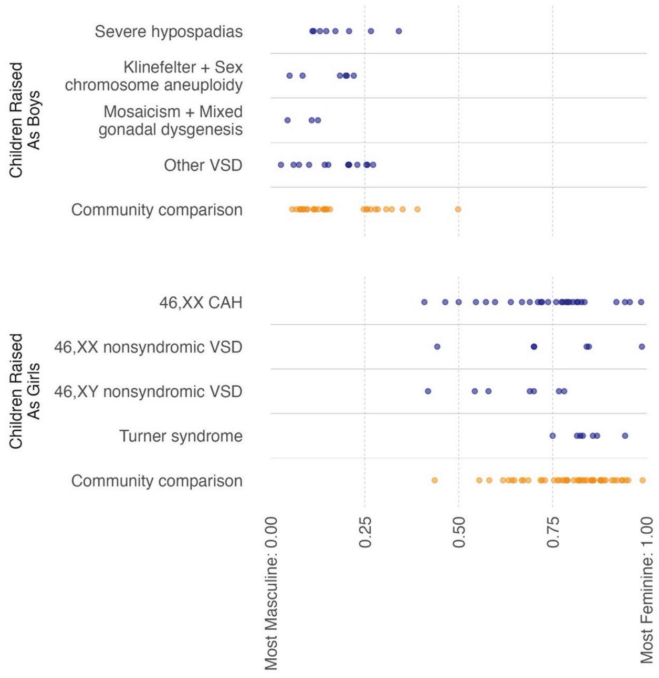

Figure 2. Composite gendered self-report within diagnostic subgroups. The vertical dashed lines show 0.25, 0.5, and 0.75 as reference points. Composite ranges from 0 (most masculine) to 1 (most feminine)

VSD: variations in sex development; CAH: congenital adrenal hyperplasia

sample in their gender development. Both groups showed patterns common in the literature for children who were raised as members of their gender group. We saw this pattern, that is a lack of significant difference between groups, across measures assessing distinct aspects of gender development, including gender identity (the categorical measure) and gender-typed preferences (e.g., clothing, peers).

A few differences emerged. Children raised as girls who had VSDs demonstrated more masculine toy preferences and indicated that they were more similar to boys on the similarity measure than the community sample of girls. The preference for more masculine toys amongst girls with VSDs, particularly those diagnosed with CAH, is consistent with past research (17,19,22). Boys with VSDs also had more masculine toy preferences than their matched community sample, and more masculine gender identities as indicated on a continuous measure of identity. These differences observed in the boys with VSDs are less well-documented, though some studies have noted similar findings, for example in a study of boys with hypospadias, the patients showed more masculine behavior than a comparison group (41). Given that we examined seven different dependent variables, and that a Bonferroni correction for that number of tests would not yield significant differences amongst the boys, and given less past literature documenting these effects, we urge some caution in interpreting them. Future research is needed to better understand this association with VSDs and masculine preferences/identity. If the results continue to replicate, including in larger samples, we would have greater confidence in these results.

In general, the primary take-away message appears to be considerable similarity in gender development across children with and without VSDs. Notably, we observed that while girls and boys tended to generally show gender-stereotypic responses, there was a wide range, such that some girls provided strongly feminine responses, and others provided more gender-neutral responses. We observed this wide range in youth with and without VSDs.

As broader cultural understandings of gender become more widely accepted, it will be interesting to ask whether more youth with VSDs adopt these more expansive terms for their own genders, and if they do so at higher rates than other children. Adolescence is a time in which gender may be especially salient. We are eager to examine what happens as these youth enter adolescence, a time when more of these changes in identity seem to be emerging in broader society. It is important to track children's gender development over time and to recruit samples of adolescents to further understand cultural impacts on gender development amongst contemporary youth with VSDs. Anecdotally, at some of our clinics we have begun to meet some youth who are, for example, opting to use they/them pronouns or identify as nonbinary. Whether this is at higher rates than amongst their peers is currently unknown.

Many children in our sample were first identified as having VSDs within the first year of life. Therefore, it is difficult to determine the causes of the couple of small differences we observed between children with VSDs and the community comparison sample. For most of these youth, their caretakers were aware of their VSD-related differences, making a strict separation of biological and social contributors to gender development difficult to discern. Further, this idea of being able to separate biology and social experience is overly simplistic, as gender development is affected by multiple interacting influences including biological, socio-cultural, and individual factors (42).

Notably, toy preferences for youth with VSDs were different relative to the comparison group, with children raised as girls who had VSDs demonstrating more masculine toy preferences. Of note, our VSD sample had a large number of girls with CAH. Prior research has observed more masculine toy preferences among girls with CAH which is often attributed to early (prenatal) androgen exposure that occurs in the context of CAH (18,19,22).

While gender development and identity in the context of VSD is complex, there are strategies and approaches that clinicians can use and model for families which can support healthy development for children with VSD, including open communication about their condition, asking about how the child feels about who they are, and creating a supportive and accepting environment for discussion of gender and related exploration. Further, clinicians can also offer resources (e.g., connections to family support organizations, allies) and support when caregivers and other important individuals in the child's life encounter difficulties accepting or understanding complexities around gender identity (43).

Some VSDs are associated with higher risks for gender dysphoria [e.g., 5-alpha-reductase-2 deficiency; (44)], while other diagnoses are associated with little gender identity change or distress [e.g., Klinefelter syndrome; (45)], or have variable rates of gender diversity and/or identity change [e.g., CAH; (12,15,17)]. Healthcare providers and parents of children with VSDs sometimes grapple with decisions about whether to initially raise a child with a VSD as a boy, a girl, or in a more gender neutral or open way and whether the selected gender will align with the child's later gender identity. Deciding whether to initially raise a child as a boy, girl, or another gender can also have implications for potential surgical interventions to remove gonads and/or modify the child's genitalia, a controversial and debated issue in the context of VSDs (11).

While our data suggest that overall, children with VSDs are similar to their peers on indices of gender development, VSD care providers can normalize the existence of gender variations across all children and offer education and guidance about appropriate support and resources. Further, our findings also highlight the importance of interdisciplinary, coordinated care for children with VSDs that incorporates psychologists and/or other psychosocial providers who can tailor education about gender development in the context of an individual child's VSD and offer strategies to support healthy psychosocial functioning and adaptation (42). For example, clinicians can educate parents and children over time about gender identity and its relationship to their VSD diagnosis and to other important factors, such as socio-cultural and individual factors which can affect gender development (46).

Study Limitations

Our study has several strengths, including rigorous assessment of gender development in children with VSDs and inclusion of an age and sex-matched comparison group. The study also used more updated measures of gender, including a more continuous and less discrete assessment of gender identity. Nonetheless, our findings are potentially limited by the cross-sectional design, heterogeneity of the VSDs sample, and modest sample size. Our study included a large age range from 3 to 12 years of age. While previous work has suggested that there are few developmental shifts on these types of measures in this age range (7), one still may wonder about that possibility in a VSD sample. The sample size was too small, especially within a single diagnostic category, to examine age differences. These are important topics for future work.

Our comparison group, while matched on age and gender, was not well-matched on parental education or socioeconomic status. Our most common finding, that the two groups did not differ on measures of gender, reduces this concern to some extent, but better matched groups would be useful in the future. Our findings underscore the need to evaluate gender development in youth with VSDs using larger samples and longitudinal designs that span the transition to adolescence and/or adulthood to further understand changes over time.

Conclusion

In this multi-site study of children with VSDs and an age-matched comparison group, our primary findings were similarities between groups with respect to gender development. We did observe some differences in toy preferences among youth with VSDs relative to the comparison group. Our findings have implications for clinical care, as these data may inform anticipatory guidance for families of children born with VSDs regarding what to expect about youth's gender during childhood. Additional longitudinal work is needed to better understand how gender development changes over time in children with DSD as they navigate adolescence and adulthood, as gender may be especially salient during these stages of development.

Ethics

Ethics Committee Approval: This study approved by the Institutional Review Board of Princeton University (approval no.: 12940, date: 25.07.2020).

Informed Consent: At least one parent had to provide informed consent, the child had to provide verbal assent, and the child had to complete the study assessment to be included in this analysis.

Footnotes

Authorship Contributions

Concept: Kristina R. Olson, Design: Kristina R. Olson, Data Collection or Processing: Canice E. Crerand, Margaret P. Adam, Maria G. Vogiatzi, Elizabeth McCauley, Jennifer Hansen-Moore, Margarett Shnorhavorian, Patricia Y. Fechner, Anne-Marie E. Amies Oelschlager, Justin A. Indyk, V. Rama Jayanthi, Hailey M. Umbaugh, Shira Kahn-Samuelson, Grace Raber, Madeline McClinchie, Kristina R. Olson, Analysis or Interpretation:Canice E. Crerand, Natalie M. Gallagher, Kristina R. Olson, Literature Search: Canice E. Crerand, Jennifer Hansen-Moore, Kristina R. Olson, Writing: Canice E. Crerand, Natalie M. Gallagher, Margaret P. Adam, Maria G. Vogiatzi, Elizabeth McCauley, Jennifer Hansen-Moore,

Margarett Shnorhavorian, Patricia Y. Fechner, Anne-Marie E. Amies Oelschlager, Justin A. Indyk, V. Rama Jayanthi, Hailey M. Umbaugh, Rachel Horton, Shira Kahn-Samuelson, Grace Raber, Madeline McClinchie, Kristina R. Olson.

Conflict of Interest: Co-author Dr. Fechner is performing industry sponsored clinical research studies using new medications in the treatment of congenital adrenal hyperplasia with Diurnal, Neurocrine Bioscience and Spruce Bioscience. She serves as a consultant for Neurocrine Biosciences. Maria Vogiatzi is performing industry sponsored clinical research trials for new treatments in CAH with Spruce and Neurocrine Biosciences, Adrenas therapeutics and Crinetics. She serves as a consultant for Spruce Bioscience, Crinetics and Eton Pharmaceuticals. The other authors report no conflicts of interests.

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Supplementary: https://d2v96fxpocvxx.cloudfront.net/cf9d60d6-523c-458a-a2e6-78728d3ffbb0/content-images/1ecb2907-358f-472d-85a3-765d62798eed.pdf

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Menstrual Health in Adolescents with Congenital Heart Disease

Demet Aygün Arı[1], Melis Pehlivantürk Kızılkan[1], İlker Ertuğrul[2], Aydın Adıgüzel[3], Mehmet Emre Arı[4], Tevfik Karagöz[2], Orhan Derman[1], Sinem Akgül[1]

[1]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, Ankara, Türkiye
[2]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Cardiology, Ankara, Türkiye
[3]University of Health Sciences Türkiye, Gülhane Training and Research Hospital, Clinic of Pediatrics, Division of Pediatric Cardiology, Ankara, Türkiye
[4]University of Health Sciences Türkiye, Ankara Etlik City Hospital, Clinic of Pediatrics, Division of Pediatric Cardiology, Ankara, Türkiye

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ABSTRACT

Objective: As life expectancy increases in patients with congenital heart disease (CHD), providing appropriate gynecological care has become more important. While the gynecologic and reproductive health concerns of adults with CHD are increasingly recognized, studies focusing on menstrual problems in adolescents with CHD are limited. The aim was to evaluate menstrual characteristics and related symptoms in adolescents with CHD.

Methods: Adolescents aged 12-21 years who had experienced menarche at least two years earlier were included. The CHD group was classified as mild, moderate, or severe, based on the European Society of Cardiology guidelines, and healthy adolescents served as controls. Menstrual histories were obtained from all participants. In the CHD group, additional clinical data (diagnosis, surgery/interventions, and medication use) were collected. Menstrual parameters were compared between the CHD and control groups, as well as across CHD subgroups.

Results: A total of 108 adolescents with CHD (41 mild, 42 moderate, 25 severe) and 76 healthy controls were enrolled. No significant differences were found between groups in terms of age at menarche, menstrual cycle length, period duration, or daily pad use ($p>0.05$). Dysmenorrhea severity and frequency were similar; however, school absenteeism due to dysmenorrhea was significantly higher in the control group ($p=0.037$). Physical premenstrual symptoms, particularly bloating, anxiety, and mood swings, were more frequent in controls ($p<0.05$). Menstrual education rates from healthcare professionals were low in both groups.

Conclusion: Our findings indicate that adolescents with CHD have menstrual characteristics comparable to those of healthy controls. However, despite these similarities, they may differ in their perception and tolerance of menstrual symptoms.

Keywords: Congenital heart disease, menstrual dysfunction, premenstrual symptom, premenstrual syndrome

Corresponding Author: Demet Aygün Arı, MD, PhD, Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, Ankara, Türkiye
E-mail: demetaygunari@gmail.com **ORCID:** orcid.org/0000-0002-8701-6085
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What is already known on this topic?

Females with congenital heart disease (CHD) may experience delayed menarche and higher rates of menstrual abnormalities, particularly in complex or cyanotic disease. Although reproductive health guidelines exist for adults with CHD, menstrual health in adolescents remains underexplored.

What this study adds?

Adolescents with CHD showed largely similar menstrual characteristics to healthy peers, despite subtle differences in pain and symptom perception. Antiplatelet/anticoagulant therapy tended to be associated with increased menstrual bleeding, highlighting the importance of individualized menstrual counseling in this population.

Introduction

Congenital heart disease (CHD), the most common major congenital anomaly, is a growing global health concern. Its prevalence is increasing, having been reported as 9.4 per 1,000 live births between 2010 and 2017 (1). Advances in treatment have markedly improved survival, with over 97% of affected children now reaching adulthood, depending on the severity of the lesion (2,3). As life expectancy rises, addressing broader health needs, including menstrual, sexual, and reproductive health, has become increasingly important (4).

Studies have shown that females with CHD tend to experience menarche later than the general population, particularly those with complex or cyanotic heart disease (5,6,7). Furthermore, primary amenorrhea and menorrhagia have been reported more frequently in these patients (6,8). While menstrual patterns in acyanotic patients appear similar to those of the general population, menstrual abnormalities are more commonly observed in patients who remain cyanotic until menarche (5).

In a study of 114 females aged 14-21 years, 83.3% reported at least one menstrual problem. While lesion complexity was not linked to problem frequency, those with more complex CHD were more likely to seek medical care (9).

Although guidelines exist for pregnancy and reproductive health in adults with CHD (10,11,12), gynecologic issues in adolescents remain underexplored (9). The American Heart Association advises obtaining a gynecological history after menarche and emphasizes age-appropriate counseling on sexual health, contraception, and pregnancy from early adolescence (13). Early identification of menstrual problems in this population is essential for timely intervention and appropriate counseling.

The aim of this study was to investigate menstrual problems specific to adolescents with CHD that may arise due to their underlying condition or related treatments.

Methods

Participants

This observational, cross-sectional study enrolled CHD patients presenting to a pediatric cardiology clinic over one year and healthy adolescents attending routine visits at the same hospital. Participants were 12-21 years old and at least two years post-menarche. Patients with syndromic heart disease, chronic conditions, or medications affecting menstruation were excluded.

Data Collection

The age, weight, height, and body mass index of all participants were recorded. A cardiac data form was completed for patients with CHD, and a menstrual data form was filled out for all participants. All procedures were conducted in accordance with the Declaration of Helsinki.

Cardiac Data Form

The CHD diagnosis of all patients was made by a pediatric cardiologist. CHDs were classified as mild, moderate, or severe based on the severity of the defect, according to the European Society of Cardiology guidelines (12), and were also categorized as cyanotic or acyanotic based on the presence of cyanosis. Electronic medical records were reviewed for cyanosis status and duration, history of interventions or surgery (biventricular or univentricular), and use of anticoagulants, antiplatelets, or other CHD-related medications. When determining the duration of cyanosis, patients were considered cyanotic until the age at which they underwent corrective surgery.

Menstrual Data Form

Menstrual history of the participants, included age at menarche, menstrual cycle duration (days), menstrual bleeding duration (days), daily menstrual management product count, degree of bleeding during their period which patients classified as light,

moderate, or heavy, and the presence of menstrual overflow. Light bleeding was defined as "needing to change pads/tampons a few times a day, and they're rarely soaked". Moderate bleeding as "needing to change pads/tampons every 3-4 hours, but they don't usually leak or overflow". Heavy bleeding as "having to change pads/tampons every 1-2 hours because they get completely soaked or sometimes passing large clots or have leakage onto my clothes or bedding".

Oligomenorrhea was defined as cycles >45 days within 2-3 years after menarche, or >35 days beyond 3 years (14). Menstrual bleeding lasting 2-7 days was considered normal, and ≥8 days as prolonged (15).

Participants were asked about dysmenorrhea, its severity (on a scale of 1-10), and any treatments received and their effectiveness. Additional questions addressed whether they had received professional information or care for menstrual problems, sexual activity, and contraceptive use. All participants received comprehensive sexual and menstrual counseling.

The presence of premenstrual symptoms was assessed. Physical symptoms questioned included joint or muscle ache, breast tenderness and pain, headache, a sensation of bloating or weight gain, and gastrointestinal symptoms. In addition, emotional symptoms were investigated, asking about anger, anxiety, depressed mood, mood swings, suddenly feeling sad or tearful, crying, irritability, tension, or sense of hopelessness, while behavioral symptoms questioned included decreased interest in usual activities, feelings of lethargy, fatigue, or reduced energy, changes in appetite, either overeating or craving a specific food, changes in sleep pattern (hypersomnia or insomnia), difficulty in concentrating, and feeling overwhelmed or out of control (16).

Participants who met the diagnostic criteria were diagnosed with premenstrual syndrome or premenstrual dysphoric disorder based on self-reported retrospective history. Premenstrual syndrome was diagnosed by the presence of at least one physical, emotional, or behavioral symptom that occurred in the final week before the onset of menses, began to improve within a few days after the onset of menses, and became minimal or absent in the week following menses, in each of the three previous menstrual cycles. Premenstrual dysphoric disorder was diagnosed when five or more symptoms were present during the same timeframe. At least one of the symptoms must be emotional, and at least one must be a behavioral or physical symptom. The symptoms must cause clinically significant distress and should not be better explained by another psychiatric or medical disorder, or attributable to the physiological effects of a substance (17,18).

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the national research ethics guidelines of Türkiye and with the Helsinki Declaration of 1975, as revised in 2008. This study was approved by the Ethics Committee of Hacettepe University (approval no.: GO 21/1221, date: 16.11.2021). Written informed consent was obtained from all participants and their parents.

Statistical Analysis

Analyses were conducted using IBM SPSS Statistics, version 27.0 (IBM Corp., Armonk, NY, USA). Continuous variables are summarized as mean±standard deviation or median (Q1-Q3), and categorical variables as frequencies and percentages. Group comparisons were performed using Student's t-test or one-way ANOVA when parametric assumptions were met, and Mann-Whitney U or Kruskal-Wallis tests otherwise. Normality was assessed with the Shapiro-Wilk test. Categorical data were analyzed with the Pearson chi-square test. Correlations were assessed using Spearman's rank correlation. A p value <0.05 was considered statistically significant.

Results

A total of 108 patients with CHD (41 with mild defects, 42 with moderate defects and 25 with severe defects) and 76 healthy adolescents were included in the study. The mean age was 16.32±2.40 years in the CHD group and 16.14±2.61 years in the control group (p=0.630). There were no differences in age, weight, height, and body mass index at admission between the CHD subgroups and the control group (Table 1).

There were also no significant differences between the CHD and control groups in terms of age at menarche, length or period or menstrual cycle, and number of menstrual products used per day (p>0.05 for all comparisons). When subgroups were compared, only the menstrual cycle duration was slightly longer in the moderate CHD group compared to the mild CHD group (p=0.013). There were no significant differences in the frequencies of oligomenorrhea and prolonged menstruation between the CHD and control groups (Table 1).

Menstrual blood loss did not differ between CHD and control groups (p=0.237). Menstrual overflow was significantly more frequent in controls compared to the CHD group and subgroups (p=0.002 and p=0.017, respectively). Details are shown in Table 1.

Of the 108 patients with CHD, 17 (15.7%) were using anticoagulants or antiplatelets. In the CHD group, patients using antiplatelet/anticoagulant therapy had a menstrual cycle length

Table 1. Menstrual data of the participants

	Mild CHD[a] (n=41)	Moderate CHD[b] (n=42)	Severe CHD[c] (n=25)	CHD total (n=108)	Control[d] (n=76)	Subgroups	Groups
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	p_1	p_2
Age	15.63±1.64	17.05±2.60	16.21±2.80	16.32±2.40	16.14±2.61	0.070	0.630
Weight	53.78±7.74	53.12±12.12	53.32±12.36	53.42±10.64	56.10±9.04	0.356	0.075
Height	161.22±6.68	158.90±6.12	160.36±8.58	160.12±6.97	162.10±5.88	0.083	**0.044**
BMI	20.78±3.38	20.96±4.13	20.64±3.88	20.82±3.77	21.33±3.33	0.792	0.342
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	p_1	p_2
Menarche age (years)	12.5 (11.75-13)	13 (12-13.5)	13 (11.75-13)	12.83 (12-13)	12.83 (12-13)	0.181	0.532
Length of menstrual cycle (days)	30 (28-30)	30 (30-33.5)	30 (30-31.5)	30 (30-30)	30 (28-30.75)	**0.013**[a-b]	0.383
Period length (days)	7 (5-7)	6 (5-7)	6 (4.5-7)	6 (5-7)	6 (5-7)	0.686	0.760
Number of pads per day	3 (3-4)	3 (2-5)	3 (2-5)	3 (2-4)	3 (2-4)	0.941	0.737
Dysmenorrhea score	6 (4.5-8)	5 (0-7)	6 (4-8)	6 (3.25-8)	6 (3.25-8)	0.241	0.527
	n (%)	n (%)	n (%)	n (%)	n (%)	p_1	p_2
Amount of blood loss Light/moderate Heavy	 33 (80.5%) 8 (19.5%)	 35 (83.3%) 7 (16.7%)	 22 (88%) 3 (12%)	 90 (83.3%) 18 (16.7%)	 58 (76.3%) 18 (23.7%)	0.582	0.237
Menstrual overflow No Rarely/yes	 28 (68.3%) 13 (31.7%)	 27 (64.3%) 15 (35.7%)	 19 (76%) 6 (24%)	 74 (68.5%) 34 (31.5%)	 35 (46.1%) 41 (53.9%)	**0.017**	**0.002**
Oligomenorrhea	1 (2.4%)	6 (14.3%)	5 (20%)	12 (11.1%)	5 (6.6%)		0.296
Prolonged menstruation	3 (7.3%)	6 (14.3%)	4 (16%)	13 (12.0%)	9 (11.8%)		0.968
Dysmenorrhea	37 (90.2%)	29 (69%)	22 (88%)	88 (81.5%)	65 (85.5%)	**0.043***	0.470
Treatment for dysmenorrhea No Acetaminophen NSAIDs	 17 (45.9%) 13 (35.1%) 7 (18.9%)	 12 (41.4%) 11 (37.9%) 6 (20.7%)	 9 (40.9%) 10 (45.5%) 3 (13.6%)	 38 (43.2%) 34 (38.6%) 16 (18.2%)	 34 (52.3%) 16 (24.6%) 15 (23.1)	0.645	0.187
School absenteeism	6 (16.2%)	9 (31%)	4 (18.2%)	19 (21.6%)	24 (36.9%)	0.099	**0.037**
Menstrual education	5 (12.2%)	5 (11.9%)	1 (4.0%)	11 (10.2%)	12 (15.8%)	0.490	0.258
Doctor visit for menstrual problems	7 (17.1%)	8 (19.0%)	6 (24.0%)	21 (19.4%)	21 (27.6%)	0.547	0.193

p_1: Comparison among mild CHD, moderate CHD, severe CHD, and control groups
p_2: Comparison between CHD (total) and control groups
*The frequency of dysmenorrhea in patients in the moderate CHD group was significantly lower than the others (p=0.043)
a: mild congenital heart disease, b: moderate congenital heart disease, c: severe congenital heart disease, d: healthy controls
CHD: congenital heart disease; IQR: interquartile range; NSAIDs: non-steroidal anti-inflammatory drugs; SD: standard deviation

of 30 (30-31.5) days, a period duration of 7 (5-7.5) days, and used 4 (3-5.5) menstrual products per day, whereas in those not using such medication, these values were 30 (30-30), 6 (5-7), and 3 (2-4), respectively. No significant differences in menstrual cycle length (p=0.724), period length (p=0.146), or number of daily menstrual products (p=0.084) were found between CHD patients who were and were not taking antiplatelet/anticoagulants. Prolonged menstruation was present in four (23.5%) patients who were using antiplatelet/anticoagulant medications, compared to nine (9.9%) patients who were not using these medications [odds ratio (OR)=2.80; 95% confidence interval (CI)=0.75-10.44; Fisher's exact p=0.122]. Among those using antiplatelet/anticoagulant therapy, bleeding was classified as heavy by four (23.5%) and light/moderate by 13 (76.5%) patients. In those not using such therapy, 14 (15.4%) had heavy bleeding, while 77 patients (84.6%) had light/moderate bleeding (OR=1.69; 95% CI=0.48-5.95; Fisher's exact p=0.478). Additionally, menstrual overflow occurred in eight patients (47.1%) using antiplatelet/anticoagulants, compared to 26 patients (28.6%) in the non-user group (OR=2.22; 95% CI=0.77-6.39; p=0.132).

In the CHD group, 88 patients (81.5%) reported dysmenorrhea and this number was 65 (85.5%) in the control group (p=0.470). When comparing the control group with the CHD subgroups, the frequency of dysmenorrhea was significantly lower in

the moderate CHD group than in the other groups (p=0.043); however, there was no significant difference in dysmenorrhea scores between the groups (p=0.241).

The rates of receiving treatment for dysmenorrhea and the types of medications used are presented in Table 1. Forty-five of the patients (90%) in the CHD group and 30 (96.8%) in the control group who used analgesics for dysmenorrhea had benefited from the treatment (p=0.258). Ten participants from each group reported using herbal tea for dysmenorrhea relief, with perceived benefit (p=0.589).

In the past year, 19 CHD patients (21.6%) and 24 controls (36.9%) reported school absenteeism due to dysmenorrhea (p=0.037), with no significant difference between CHD subgroups (p=0.099). Menstrual education was received by 11 CHD patients (10.2%) and 12 controls (15.8%) (p=0.258), and 21 CHD patients (19.4%) versus 21 controls (27.6%) consulted a healthcare professional for menstrual issues (p=0.193) (Table 1).

Only one participant in the control group admitted being sexually active. There were no patients using contraception in the CHD and control groups.

No significant differences were observed in the number of premenstrual symptoms between CHD and control groups (p=0.089) or among CHD subgroups (p=0.128) (Table 2). However, bloating, anxiety, and mood swings were more frequent in the control group than CHD group (p=0.013, p=0.022, and p=0.020, respectively), while other symptoms showed no significant difference. In addition, premenstrual syndrome and premenstrual dysphoric disorder prevalence was comparable between groups (p=0.304, p=0.940, respectively) (Table 3).

When CHD patients were divided into two groups, cyanotic and acyanotic, there were 28 patients with cyanotic CHD and 80 patients with acyanotic CHD. Seven (11.5%) of the 61 patients who underwent surgery had univentricular physiology, while the remaining 54 had biventricular physiology. Among the patients with cyanotic CHD, five (17.9%) had persistent cyanosis. The mean duration of cyanosis was 1.66±5.0 years. The mean age at menarche was 12.92±1.16 years in patients who remained cyanotic until menarche (n=6), and 12.77±1.46 years in those who were cyanotic at birth but underwent corrective surgery during childhood (n=22) (p=0.826). The menstrual cycle was significantly longer in the cyanotic CHD group compared to the acyanotic group (p=0.034), however, it remained within the normal range in all groups. The comparison of menstrual data based on cyanosis status is presented in Table 4. Spearman correlation analysis revealed no significant association between the duration of cyanosis and age at menarche (ρ=0.162, p=0.410, n=28), menstrual cycle length (ρ=0.021, p=0.915, n=28), menstrual period length (ρ=0.181, p=0.357, n=28), or the number of pads used per day (ρ=-0.084, p=0.671, n=28). Likewise, no significant correlations were found between cyanosis duration and the number of premenstrual symptoms (ρ=-0.029, p=0.885, n=28), physical (ρ=-0.254, p=0.192, n=28), emotional (ρ=0.030, p=0.881, n=28), or behavioral symptoms (ρ=0.082, p=0.677, n=28).

Discussion

This study evaluated menstrual characteristics and associated symptoms among adolescents with CHD and compared them to healthy peers. Contrary to our initial hypothesis, the findings revealed no significant differences between groups in terms of age at menarche, cycle length, duration of menstruation, or dysmenorrhea severity and frequency. These results suggest that the presence and severity of CHD may not substantially alter basic menstrual patterns in adolescents who have reached menarche.

One interpretation for these findings is that adolescents with CHD, particularly those with well-managed or surgically corrected lesions, may experience relatively stable overall health and endocrine function, mitigating potential menstrual disruptions. Moreover, consistent cardiology follow-up may promote better general health awareness and lifestyle regulation, which could buffer against menstrual irregularities. In a study of adolescents and adults with CHD, mean menarche age was 13 years in simple cases and 14.5 years in complex cases, both later than the general

Table 2. Comparison of premenstrual symptom counts

	Mild CHD (n=41)	Moderate CHD (n=42)	Severe CHD (n=25)	CHD Total (n=108)	Control (n=76)	Subgroups	Groups
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	p_1	p_2
Number of premenstrual symptoms	8 (4.5-10)	5.5 (2-10.25)	7 (2.5-11)	7 (3-10)	7.5 (4-11)	0.128	0.089
Number of physical symptoms	2 (1-3)	1 (0-3)	1 (0-2)	1 (0-2.75)	2 (1-3)	0.061	**0.049**
Number of emotional symptoms	2 (1-5)	3 (0-4.25)	3 (1-5.5)	3 (1-5)	3.5 (1.25-6)	0.197	0.057
Number of behavioral symptoms	3 (1-5)	2 (1-3.25)	2 (0-3.5)	2 (1-4)	2 (1-4)	0.107	0.603

p_1: comparison among mild CHD, moderate CHD, severe CHD, and control groups
p_2: comparison between CHD (total) and control groups
CHD: congenital heart disease; IQR: interquartile range

Table 3. Comparison of premenstrual symptoms between control and CHD groups

	Control (n=76)		CHD (n=108)		
	n (%)		n (%)		
	+	-	+	-	p value
Physical symptoms					
Abdominal pain	65 (85.5%)	11 (14.5%)	88 (81.5%)	20 (18.5%)	0.470
Joint/muscle ache	35 (46.1%)	41 (53.9%)	49 (45.4%)	59 (54.6%)	0.927
Breast tenderness	28 (36.8%)	48 (63.2%)	27 (25%)	81 (75%)	0.084
Breast pain	17 (22.4%)	59 (77.6%)	18 (16.7%)	90 (83.3%)	0.332
Headache	27 (35.5%)	49 (64.5%)	30 (27.8%)	78 (72.2%)	0.263
Bloating	33 (43.4%)	43 (56.6%)	28 (25.9%)	80 (74.1%)	**0.013**
GI symptoms	13 (17.1%)	63 (82.9%)	18 (16.7%)	90 (83.3%)	0.938
Emotional symptoms					
Anger	48 (63.2%)	28 (36.8%)	61 (56.5%)	47 (43.5%)	0.364
Anxiety	27 (35.5%)	49 (64.5%)	22 (20.4%)	86 (79.6%)	**0.022**
Depressed mood	40 (52.6%)	36 (47.4%)	43 (39.8%)	65 (60.2%)	0.085
Mood swings	42 (55.3%)	34 (44.7%)	41 (38%)	67 (62%)	**0.020**
Crying	31 (40.8%)	45 (59.2%)	37 (34.3%)	71 (65.7%)	0.366
Irritability	27 (35.5%)	49 (64.5%)	36 (33.3%)	72 (66.7%)	0.758
Tension	40 (52.6%)	36 (47.4%)	55 (50.9%)	53 (49.1%)	0.820
Sense of hopelessness	20 (26.3%)	56 (73.7%)	23 (21.3%)	85 (78.7%)	0.428
Behavioral symptoms					
Decreased interest in usual activities	20 (26.3%)	56 (73.7%)	34 (31.5%)	74 (68.5%)	0.449
Feelings of fatigue/lack of energy	48 (63.2%)	28 (36.8%)	61 (56.5%)	47 (43.5%)	0.364
Change in appetite	29 (38.2%)	47 (61.8%)	37 (34.3%)	71 (65.7%)	0.587
Food cravings	41 (53.9%)	35 (46.1%)	54 (50%)	54 (50%)	0.598
Sleep problems	18 (23.7%)	58 (76.3%)	31 (28.7%)	77 (71.3%)	0.448
Difficulty in concentrating	25 (32.9%)	51 (67.1%)	35 (32.4%)	73 (67.6%)	0.945
Feelings of being out of control	18 (23.7%)	58 (76.3%)	18 (16.7%)	90 (83.3%)	0.237
PMS	25 (32.9%)	51 (67.1%)	28 (25.9%)	80 (74.1%)	0.304
PMDD	8 (10.5%)	68 (89.5%)	11 (10.2%)	97 (89.8%)	0.940

CHD: congenital heart disease, GI: gastrointestinal, PMDD: premenstrual dysphoric disorder, PMS: premenstrual syndrome

Table 4. Comparison of menstrual data among cyanotic, acyanotic, and control groups

	Cyanotic CHD[a]	Acyanotic CHD[b]	Control[c]	
	Median (IQR)	Median (IQR)	Median (IQR)	p
Menarche age (years)	13 (12-13.38)	12.5 (12-13)	12.83 (12-13)	0.648
Length of menstrual cycle (days)	30 (30-38.75)	30 (28-30)	30 (28-30.75)	**0.034**[a-b]
Period length (days)	6.5 (5-7)	6 (5-7)	6 (5-7)	0.949
Number of pads per day	3 (2-5)	3 (2-4)	3 (2-4)	0.820

a: cyanotic congenital heart disease, b: acyanotic congenital heart disease, c: healthy controls
CHD: congenital heart disease, IQR: interquartile range

average of 12.8 years (8). Similarly, a study in adults showed that the average age at menarche was 12.5 years in the control group, whereas it was 13.1 years in women with acyanotic CHD, and 13.9 years in those who remained cyanotic until menarche (5). In a more comprehensive and recent study evaluating 1,593 patients, individuals with cyanotic and acyanotic CHD were compared without a control group. While the average age at menarche in the same population was reported as 13.15 years, it was found to be 13.3 years in patients with CHD and 13.5 years in those with cyanotic CHD, regardless of surgical status. This was attributed to an increased incidence of primary amenorrhea in the cyanotic CHD group. It was also noted that patients who remained cyanotic until menarche reached menarche at an age similar to those who were cyanotic at birth but had undergone surgical correction during childhood (6). In the present study, although the mean age at menarche was higher in the cyanotic CHD group compared to the acyanotic CHD and control groups, this was not statistically significant. Moreover, there was no difference in age at menarche between those who remained cyanotic until menarche and those who were cyanotic at birth but underwent surgical correction. No correlation was found between duration of cyanosis and age at menarche. However, since our study included only adolescents who had experienced menarche at least two years prior, we were unable to capture data from those with delayed menarche or primary amenorrhea, which may partly explain discrepancies with findings from adult studies.

Although the difference was not significant, oligomenorrhea was observed more frequently in the CHD group compared to the control group and was most common in the severe CHD group. A study evaluating adults with CHD without a control group reported that oligomenorrhea was common among women with CHD, although there was a lack of comparative data from the general population (6). It remains unclear whether these abnormal menstrual patterns represent a chronic anovulatory state, dysfunction of the hypothalamic-pituitary-ovarian axis, or disturbed uterine hemostasis secondary to chronic hypoxemia or erythrocytosis-induced hyperviscosity commonly seen in cyanotic CHD. Apart from the heart condition, multiple childhood surgeries may affect the biological processes that control the menstrual cycle (5,7,19).

The frequency of menstrual overflow was higher among controls compared to both the overall CHD group and its subgroups. However, given the subjective nature of this symptom and the observation that some participants who reported normal menstrual product usage and low bleeding volume still described overflow, this difference was not considered clinically meaningful. The prevalence of prolonged menstruation was comparable between the CHD and control groups, although the highest rate was noted in the severe CHD subgroup, without reaching statistical significance. When CHD patients using anticoagulant/antiplatelet medications were compared to those not receiving such treatment, trends toward longer bleeding duration, greater daily menstrual product use, and increased rates of prolonged menstruation, heavy menstrual bleeding, and menstrual overflow were observed. However, none of these differences were significant. Given the relatively small number of patients receiving these medications, these findings should be interpreted with caution, as the study may have been underpowered to detect potential differences. A previous study involving both adolescents and adults with CHD similarly reported higher rates of menorrhagia in patients with complex lesions, but found no significant association with warfarin use (8). While our findings suggest a possible link between medication use and increased menstrual volume and duration, they remain inconclusive, and further research with larger cohorts is needed to clarify these associations.

Interestingly, physical premenstrual symptoms, such as bloating, mood swings, and anxiety, were more frequently reported in the control group. This unexpected pattern raises questions about symptom perception and reporting, which may differ in adolescents with chronic conditions (20). One possible explanation is that adolescents with CHD, who often experience more serious health concerns, may underreport or place less emphasis on menstrual discomfort relative to their peers. Alternatively, they may have developed higher symptom tolerance or different coping strategies over time. However, these interpretations remain speculative, as pain perception, coping mechanisms, and quality of life were not directly assessed in this study. In this context, to the best of our knowledge, while previous studies have focused on menstrual problems in adolescents with CHD and on reproductive and sexual health in adult patients, no studies have specifically evaluated premenstrual symptoms other than dysmenorrhea. Our findings therefore suggest that the frequency of premenstrual symptoms, premenstrual syndrome, and premenstrual dysphoric disorder in adolescents with CHD is comparable to that of healthy peers.

The frequency and severity scores of dysmenorrhea were similar between the CHD and control groups. Similarly, the rates of receiving treatment and response to treatment did not differ significantly. However, school absenteeism due to dysmenorrhea was higher in the control group, which may further support our hypothesis concerning the potential underreporting or downplaying of menstrual symptoms by adolescents with CHD. It may also reflect differences in family or school-based support systems, individual pain thresholds, or prioritization of symptoms relative to overall health burdens. In a study evaluating menstrual problems in adolescents and young adults with CHD, dysmenorrhea was reported at a similar rate to that of the general population, consistent with our findings (9).

Despite these findings, the low rates of menstrual health education reported in both groups are concerning. This underscores the need for integrating reproductive health discussions into routine care, particularly for adolescents with chronic medical conditions like CHD, who may have unique risks or needs related to contraception, anticoagulation, or pregnancy counseling.

Study Limitations

This study has several limitations. The cross-sectional design precludes causal inference, and symptom data relied on self-report, which may be influenced by recall or reporting bias. Premenstrual symptoms, premenstrual syndrome or premenstrual dysphoric disorder were assessed based on retrospective self-report rather than prospective daily symptom recording, as recommended by diagnostic guidelines. Therefore, the findings may be subject to recall bias and should be interpreted with caution. Although prospective daily symptom tracking is considered the gold standard, its implementation in adolescent populations may be limited by adherence and feasibility challenges (21). The sample size, particularly in subgroup analyses by CHD severity and in patients receiving anticoagulant/antiplatelet therapy, was relatively small, limiting statistical power. As a result, some clinically meaningful differences, especially in bleeding-related outcomes, may not have been detected. Furthermore, psychosocial factors influencing menstrual experiences were not comprehensively assessed. Nevertheless, we believe our study to be the first to evaluate premenstrual symptoms, premenstrual syndrome, and premenstrual dysphoric disorder in adolescents with CHD, which adds to its significance.

Conclusion

In conclusion, our study demonstrated that adolescents with CHD exhibited menstrual characteristics similar to those of their healthy peers, though their perception of pain and symptoms may differ. Although not statistically significant, we observed that as the CHD becomes more complex, the age at menarche tends to be later, and the frequencies of oligomenorrhea and prolonged menstruation increased. In addition, the use of antiplatelet or anticoagulant therapy appears to be associated with increased menstrual bleeding. Therefore, while each patient should be evaluated individually, based on diagnosis, duration of cyanosis, and surgical history, we believe it is important that all CHD patients are educated on the characteristics of normal menstruation and on disease-specific risks, as this is an essential component of their overall care and follow-up.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Hacettepe University (approval no.: GO 21/1221, date: 16.11.2021).

Informed Consent: Written informed consent was obtained from all participants and their parents.

Footnotes

Authorship Contributions

Concept: Demet Aygün Arı, Melis Pehlivantürk Kızılkan, İlker Ertuğrul, Tevfik Karagöz, Orhan Derman, Sinem Akgül, Design: Demet Aygün Arı, Melis Pehlivantürk Kızılkan, İlker Ertuğrul, Tevfik Karagöz, Orhan Derman, Sinem Akgül, Data Collection or Processing: Demet Aygün Arı, İlker Ertuğrul, Aydın Adıgüzel, Mehmet Emre Arı, Tevfik Karagöz, Orhan Derman, Sinem Akgül, Analysis or Interpretation: Demet Aygün Arı, Melis Pehlivantürk Kızılkan, Aydın Adıgüzel, Mehmet Emre Arı, Sinem Akgül, Literature Search: Demet Aygün Arı, Mehmet Emre Arı, Writing: Demet Aygün Arı, Melis Pehlivantürk Kızılkan, Mehmet Emre Arı, Sinem Akgül.

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Investigating the Role of *LRG1* in Hepatosteatosis and Insulin Resistance in Obese Children

Berna Singin[1], Zeynep Donbaloğlu[1], Ebru Barsal Çetiner[1], Bilge Aydın Behram[1], Aynur Bedel[1], Sebahat Özdem[2], İkbal Özen Küçükçetin[2], Mesut Parlak[1], Hale Ünver Tuhan[1]

[1]Akdeniz University Hospital, Department of Pediatric Endocrinology, Antalya, Türkiye
[2]Akdeniz University Hospital, Department of Biochemistry, Antalya, Türkiye

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ABSTRACT

Objective: This study aimed to investigate the relationship between *leucine-rich alpha-2-glycoprotein 1* (*LRG1*), hepatosteatosis, and insulin resistance (IR) in obese children, and to evaluate the potential role of *LRG1* as a biomarker in these metabolic conditions.

Methods: A cohort of obese and non-obese children were enrolled. Obese subjects were further grouped by hepatosteatosis and IR status. Anthropometric measurements, biochemical parameters, and inflammatory markers including *LRG1*, adiponectin, and tumor necrosis factor-α (TNF-α) were evaluated. Associations between these markers and metabolic parameters were analyzed.

Results: The cohort consisted of 172 children, of whom 100 (58.1%) were obese and 72 were non-obese. Obese children had significantly higher body mass index (BMI), BMI standard deviation scores, waist and upper arm circumferences, triceps skinfold thickness, total and percentage body of fat (PBF), and elevated systolic blood pressure (SBP) and diastolic blood pressure (DBP) ($p<0.001$). Laboratory findings revealed elevated glucose, insulin, homeostatic model assessment of IR (HOMA-IR), alanine aminotransferase, triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and lower high-density lipoprotein cholesterol (HDL-C) in the obese group ($p<0.05$). *LRG1* levels did not differ by obesity or hepatosteatosis status but were significantly lower in those with IR ($p=0.03$). *LRG1* was negatively correlated with waist circumference, DBP, insulin, HOMA-IR, TG, TC, and LDL-C, and positively with HDL-C ($p<0.05$). Adiponectin showed inverse correlations with waist circumference, SBP, insulin, HOMA-IR, TC, LDL-C, TG, and a positive correlation with HDL-C ($p<0.05$).

Conclusion: Findings suggest *LRG1* may not serve as a direct biomarker for hepatosteatosis in obese children but was negatively associated with IR and dyslipidemia. These results highlight a complex role for *LRG1* in obesity-related metabolic dysfunction and support further longitudinal and pathophysiological studies.

Keywords: LRG1, obesity, hepatosteatosis, insulin resistance, children, adipokines

Corresponding Author: Assoc. Prof. Hale Ünver Tuhan, Akdeniz University Hospital, Department of Pediatric Endocrinology, Antalya, Türkiye
E-mail: halenvr@hotmail.com **ORCID:** orcid.org/0000-0002-7637-9630
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What is already known on this topic?

Leucine-rich alpha-2-glycoprotein 1 (*LRG1*) has emerged as an adipokine implicated in obesity-related metabolic dysfunction, particularly in adult populations. Adult studies have generally reported higher circulating *LRG1* levels in association with insulin resistance (IR), dyslipidemia, and cardiometabolic risk. The evidence regarding *LRG1* in pediatric obesity is limited, and its relationship with hepatosteatosis and IR in children remains unclear. Adiponectin is consistently linked to improved insulin sensitivity and a favorable metabolic profile, whereas tumor necrosis factor-α is a key proinflammatory cytokine associated with obesity-related low-grade inflammation. A combined assessment of adipokines/cytokines may provide a more comprehensive view of metabolic derangements in childhood obesity.

What this study adds?

This study is among the first to comprehensively evaluate *LRG1* in pediatric obesity with respect to hepatosteatosis and IR. The finding of lower *LRG1* levels in insulin-resistant children contrasts with adult data, suggesting distinct, age-specific pathophysiological mechanisms. The results imply that *LRG1* may act as more than an inflammatory marker and may be a complex metabolic regulator during growth and development.

Introduction

In the last decades, being overweight and obese have become recognized as major lifestyle-related health issues, ranking as the fifth leading cause of death globally according to recent statistics (1). Furthermore, research has indicated that low-grade chronic inflammation is a common symptom of these conditions and contributes significantly to the development of various physical issues and chronic illnesses, including cancer, diabetes, metabolic syndrome, cardiovascular diseases, and neurodegenerative disorders (1). Obesity is characterized by chronic systemic low-grade inflammation accompanied by deregulated circulating levels of adipokine and inflammatory markers (2).

Adipose tissue is recognized as a key endocrine organ that releases a variety of bioactive peptides, known as adipokines, many of which play a role in regulating overall energy balance and inflammation throughout the body (3). Several secretory molecules, including leptin, adiponectin, and retinol binding protein 4, have been identified in adipocytes (4,5,6). Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that plays a central role in obesity-related chronic low-grade inflammation and the development of insulin resistance (IR) (7). Disrupted expression, secretion, and function of these adipokines are linked to obesity, IR, and cardiovascular complications (3,7). However, the roles and identities of many other adipokines involved in obesity-related metabolic diseases are still largely unclear.

Adipokines have been found to exhibit both proinflammatory properties (e.g., leptin and resistin) and anti-inflammatory effects (e.g., omentin and adiponectin). However, it has been observed that adiponectin can be pro-inflammatory under certain conditions and in a tissue-dependent manner (8,9,10,11).

Leucine-rich alpha-2-glycoprotein 1 (*LRG1*), which was initially isolated from human plasma (12), is a member of a highly conserved protein family that contains the leucine-rich-repeat domains (13). In addition to regulating angiogenesis (13), variations in levels of *LRG1* have also been implicated in a number of diseases, including some cancers (13,14,15), arterial stiffness (16), heart failure (17), aging (18), and inflammatory disorders (19). However, the function and mechanisms of action of *LRG1* in metabolism remain unknown. *LRG1* has recently been recognized as anadipokine involved in metabolic regulation and inflammation. Experimental and clinical studies in adults have demonstrated that *LRG1* is secreted by adipose tissue and may contribute to obesity-related IR and hepatic lipid accumulation through mechanisms involving altered lipid metabolism and hepatocyte dysfunction. However, data regarding the role of *LRG1* in pediatric obesity are scarce, and its relationship with hepatosteatosis and IR during childhood remains poorly understood, which constituted the main rationale for the present study.

LRG1, either alone or in combination with other known factors, is considered a potential biomarker for inflammation and obesity. High levels of *LRG1* are positively correlated with obesity, while low plasma *LRG1* levels predict weight loss following surgery for obesity and metabolic diseases (20). Thus, elevated circulating and adipose tissue *LRG1* levels have been associated with increased body mass index (BMI), visceral adiposity, and waist circumference in obese individuals. Based on these findings and laboratory observations, it is hypothesized that *LRG1* contributes to increased fat storage by inhibiting the breakdown of fatty acids and promoting lipid production through the activation of sterol regulatory element-binding transcription factor 1. In addition, *LRG1* may facilitate hyperglycemia by reducing the expression of insulin receptor substrates (*IRS1* and *IRS2*) (21).

It has been suggested that *LRG1* may contribute to hepatosteatosis by enhancing *de novo* lipogenesis in the liver while inhibiting fatty acid oxidation (21). More specifically, it has been demonstrated that increased *LRG1* levels in the bloodstream, produced by adipocytes, can disrupt the function of hepatocytes, thereby playing a role in

the development of IR and hepatosteatosis (21). However, it should be noted that the majority of these findings have been derived from studies conducted in adult populations, and data regarding the role of *LRG1* in pediatric obesity and related metabolic complications remain limited.

In general, previous reports indicate that *LRG1* plays a role in the development of diabetes and obesity-related complications in adults. However, research on *LRG1* in the pediatric age group remains limited. This study aims to comprehensively elucidate the associations between *LRG1*, adiponectin, and TNF-α levels and a range of biochemical and clinical parameters in the pediatric and adolescent population, stratified by obesity status, presence of hepatosteatosis, and IR.

Methods

Study Design and Participants

This research was designed as a single-center, case-control study. Participants, aged between 6 and 18 years, were included. The obese group consisted of children diagnosed with obesity at the pediatric endocrinology outpatient clinic of our hospital. The control group consisted of healthy, non-obese volunteers of similar age and gender who visited the hospital for routine health checkups. Exclusion criteria for the obese group included chronic endocrine-related disease (e.g., Cushing's syndrome, hypothyroidism), obesity-related syndromes (e.g., Prader-Willi, Bardet-Biedl syndromes), other systemic diseases, or a history of medication use. The control group exclusion criteria were chronic systemic or endocrine diseases and obesity.

The clinical and laboratory features of the obese patients were compared to those of the control group. Obese individuals were categorized into two subgroups, hepatosteatosis (+) and hepatosteatosis (-), based on hepatobiliary ultrasound results and compared accordingly. In addition, obese individuals were classified into two further sub-groups, those with and without IR, and comparisons were made accordingly.

Ethical Considerations

Ethical approval was obtained from the Akdeniz University Faculty of Medicine Clinical Research Ethics Committee prior to the commencement of the study (protocol number: KAEK-340, date: 11.05.2022). The research was conducted in full compliance with the principles set forth in the Declaration of Helsinki and in accordance with ethical standards. Informed consent was obtained from the parents of all participants prior to their inclusion in the study.

Clinical Investigations and Anthropometric Measurements

Height was measured using a wall-mounted stadiometer, both while standing upright and during deep inspiration. BMI was calculated by dividing the weight in kilograms by the square of the height in metres (kg/m^2). Standard deviation scores (SDS) for height, weight, and BMI were determined using reference values for Turkish children (22).

Participants with a BMI above the 95th percentile for their age and sex, based on the reference values for Turkish children, were classified as obese and placed in the obese group. Those with a BMI between the 3rd and 85th percentiles were categorized into the non-obese group, excluding any overweight individuals. Waist circumference was measured with a tape measure at the level of the umbilicus, with the child standing upright and the abdomen exposed. Upper arm circumference was taken at the midpoint between the acromion and olecranon processes, with the elbow flexed at 90 degrees. All measurements were recorded in centimeters (cm) and analyzed. Triceps skinfold (TSF) thickness was measured with the arm hanging freely by the side of the body, using a caliper at the midpoint of the posterior surface of the upper arm. Waist-to-height ratio (WtHR) was calculated by dividing waist circumference by height, and participants were categorized as having central obesity when WtHR was ≥0.5. Bioelectrical impedance analysis, using the Tanita BC-418 device (Tanita, Tokyo, Japan), was employed to assess fat mass and the percentage of body fat (PBF). Blood pressure was measured following a validated protocol: systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded twice on the right arm after a 10-minute rest in a supine position, using a calibrated sphygmomanometer, and performed by one of the investigators. The average of the two readings was taken. Hypertension was defined as blood pressure values above the 95th percentile for height, age, and gender (23). Pubertal evaluation was conducted based on the Marshall and Tanner (24) classification.

Peripheral blood samples were obtained in the morning (between 8:00 and 9:00 a.m.) after a 10-hour fasting period. Fasting glucose levels were measured using the hexokinase method, while fasting insulin levels were assessed with the radioimmunoassay technique. IR was evaluated using the homeostatic model assessment of IR(HOMA-IR) formula: fasting insulin (μU/mL)×fasting glucose (mg/dL)/405. Participants with HOMA-IR values greater than 4 during the pubertal stage and greater than 2.5 in the prepubertal stage were classified as having IR (25).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a spectrophotometric method. Triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) levels were determined enzymatically with the DP Modular Systems (Roche Diagnostic Corp., Indianapolis, IN, USA). Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula when plasma TG levels were <400 mg/dL.

The measurement methods and kit details for adipokines are as follows;

LRG1: Serum samples were analyzed using the Enzyme-Linked Immunosorbent Assay (ELISA) method with a Elabscience brand kit (Elabscience Bionovation Inc, Houston, TX, USA) (Kit catalog no: E-EL-H6067). Intra-assay coefficient of variation (CV) was <10%, and the inter-assay CV was also <10%. The kit sensitivity was 0.38 μg/mL, and measurement linearity ranged from 0.65-40 μg/mL. The kit was stored at 2-8 °C until use.

Adiponectin: Serum samples were analyzed using the ELISA method with an Elabscience kit (Kit catalog no: E-EL-H6122). Intra-assay CV was <10%, and inter-assay CV was <10%. The kit sensitivity was 0.1 μg/mL, and measurement linearity ranged from 0.16-10 μg/mL. The kit was stored at 2-8 °C until use.

TNF-α: Serum samples were analyzed using the ELISA method with an Elabscience kit (Kit catalog no: E-EL-H0109). Intra-assay CV was <10%, and inter-assay CV was <10%. The kit sensitivity was 4.69 pg/mL, and measurement linearity ranged from 7.81-500 pg/mL. The kit was stored at 2-8 °C until use.

The diagnosis and grading of hepatosteatosis were performed using ultrasonographic imaging according to standard criteria assessing liver echogenicity, vascular structure visibility, and diaphragm clarity (26), with all examinations conducted by the same qualified radiologist.

Statistical Analysis

Measures of association for categorical variables were analyzed with chi-square and Fisher exact test. Skewed distributions of continuous variables in groups were compared by Wilcoxon-Rank Sum test. Pearson's correlation analysis was conducted to evaluate the relationships between *LRG1*, adiponectin, TNF-α, and clinical as well as biochemical parameters. All analyses were performed using STATA software, version 17.0 Basic Edition (StataCorp LLC, 4905 Lakeway Drive, College Station, TX 77845, USA). A p-value of <0.05 was considered statistically significant.

Results

The clinical and laboratory characteristics of obese and non-obese subjects are summarized in Table 1. The age, gender, and pubertal status were similar in the two groups. The BMI, SDS, waist and upper arm circumferences, TSF thickness, total body fat mass, PBF, and systolic and DBPs were significantly greater in the obese group (p<0.001). When laboratory parameters were investigated, fasting glucose, insulin, HOMA-IR, ALT, TG, TC and LDL-C levels were significantly higher in obese patients while HDL-C was lower (p<0.05). When comparing obese and non-obese patients, *LRG1* and adiponectin levels were similar (p=0.58; p=0.08; respectively), whereas TNF-α levels were significantly higher in the obese group p<0.001. In the control group, only two participants (2.8%) had a WtHR ≥0.5, whereas in the obese group, only three participants (3%) had a WtHR <0.5. Due to the highly unbalanced distribution, further statistical comparisons based on WtHR categories were not performed.

Out of the 69 patients with hepatosteatosis, 46 (66.7 %) had grade 1, 18 (26.1 %) had grade 2, and 5 (7.2 %) had grade 3 hepatosteatosis. Comparisons of the clinical and laboratory characteristics of obese subjects with and without hepatosteatosis are shown in Table 2. In patients with and without hepatosteatosis, similar age, gender, and pubertal status were observed. The BMI, SDS, waist and upper arm circumferences, and total body fat mass were significantly greater in patients with hepatosteatosis (p<0.05). Insulin, HOMA-IR, and TG levels were significantly higher in those with hepatosteatosis while HDL-C was lower (p<0.05). No statistically significant differences were observed in *LRG1*, adiponectin, and TNF-α levels among those with or without hepatosteatosis.

In the total cohort, IR was present in 66 patients, whereas 106 patients had normal HOMA-IR values when pubertal status was taken into consideration. Comparison of the clinical and laboratory characteristics of subjects with and without IR are shown in Table 3. Although gender distribution and pubertal status were comparable between patients with and without IR, the mean age of patients exhibiting IR was significantly higher (p=0.0005). The BMI, SDS, waist and upper arm circumferences, TSF thickness, total body fat mass, PBF, and systolic and DBPs were significantly greater in the IR group (p<0.001). When laboratory parameters were analyzed, fasting glucose, insulin, HOMA-IR, ALT, TG, TC and LDL-C levels were significantly greater patients with IR, while HDL-C was lower (p<0.05) in IR group. *LRG1* and adiponectin levels were significantly lower, while TNF-α levels were significantly higher in patients with IR (p=0.03, p=0.008, and p=0.004, respectively).

Correlation analysis revealed several significant associations between serum biomarkers and clinical parameters in obese subjects (Table 4). Serum *LRG1* levels showed significant negative correlation with waist circumference (r=-0.220, p=0.028), DBP (r=-0.201, p=0.045), insulin (r=-0.288, p=0.004), HOMA-IR (r=-0.267, p=0.007), TG (r=-0.283, p=0.004), TC (r=-0.269, p=0.007), and LDL-C (r=-0.307, p=0.002). Conversely, LRG1 was positively correlated with HDL-C (r=0.332, p=0.0007).

Adiponectin levels were negatively correlated with waist circumference (r=-0.247, p=0.013), SBP (r=-0.208, p=0.038), insulin (r=-0.281, p=0.005), HOMA-IR (r=-0.264, p=0.008), TG (r=-0.260, p=0.009), TC (r=-0.208, p=0.038), and LDL-C (r=-0.240, p=0.016); while a positive correlation was observed with HDL-C (r=0.270, p=0.007).

Table 1. The clinical and laboratory characteristics of obese and non-obese subjects

	Obese subjects (n=100)	Non-obese subjects (n=72)	p value
Age (year)	12.1±3.2	11.3±2.7	0.08
Male (%)	55.2	44.8	0.57
Pubertal (%)	58.5	41.5	0.85
BMI (kg/m²)	32.3±6.4	18±2.4	**<0.001**
BMI-SDS	2.9 (2.6-3.4)	-0.13 (-0.985-0.395)	**<0.001**
Waist circumference (cm)	93.8±13.6	61.9±7.2	**<0.001**
Upper arm circumference (cm)	32.5±4.8	21.8±3.1	**<0.001**
TSF thickness (mm)	22.1±7.3	7.2±3.9	**<0.001**
Fat mass (kg)	32.5±14.9	7.9±3.2	**<0.001**
PBF (%)	38.3 (35.7-42.7)	21.5 (17.25-23.8)	**<0.001**
SBP (mmHg)	120±12	100±11	**<0.001**
DBP (mmHg)	80±10	65±8	**<0.001**
Glucose (mg/dL)	88± 7	85±6	**0.008**
Insulin (uIU/mL)	26±16	8±4	**<0.001**
HOMA-IR	5.6±3.6	1.7±0.8	**<0.001**
ALT (U/L)	24±14	14±9	**<0.001**
AST (U/L)	22±8	22±6	0.74
TG (mg/dL)	110±55	67±25	**<0.001**
TC (mg/dL)	164±28	155±25	**0.04**
LDL-C (mg/dL)	100±24	87±20	**<0.001**
HDL-C (mg/dL)	48±11	60±14	**<0.001**
LRG1 (µg/mL)	84.9±53.9	94.6±63.1	0.58
TNF-α (pg/mL)	44.7±27.8	24.4±18.1	**<0.001**
Adiponectin (µg/mL)	14.4±9.2	17.2±11.2	0.08

Data are given as mean±SD, median (IQR 25-75 percentile) or n (%). Bold values indicate statistical significance (p<0.05).
BMI: body mass index, BMI-SDS: standard deviation score of body mass index, TSF: triceps skinfold, PBF: percentage of body fat, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR, homeostasis model assessment of insulin resistance, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TG: triglycerides, LDL-C: low density lipoprotein cholesterol, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TNF- α: tumor necrosis factor-α, *LRG1: leucine-rich alpha-2-glycoprotein 1,* IQR: interquartile range

Table 2. Comparison of the clinical and laboratory characteristics of obese subjects with and without hepatosteatosis

	Hepatosteatosis (+) group (n=69)	Hepatosteatosis (-) group (n=31)	p value
Age (year)	12±0.4	11±0.5	0.28
Male (%)	33.3	29.1	0.67
Pubertal (%)	85.5	77	0.31
BMI (kg/m²)	33.2±0.83	30.2±0.84	**0.03**
BMI-SDS	3.1 (2.7-3.5)	2.8 (2.5-3)	**0.01**
Waist circumference (cm)	97±2	87±2	**<0.01**
Upper arm circumference (cm)	33±0.6	31±0.8	**0.01**
TSF thickness (mm)	22.7±0.9	20.8±1.2	0.22
Fat mass (kg)	35±2	27±2	**0.01**
PBF (%)	39.3 (36.3-44.1)	37.5 (34.8-41)	0.09
SBP (mmHg)	119±1	116±2	0.09
DBP (mmHg)	77±1	76±2	0.79
Glucose (mg/dL)	88±1	88±1	0.98
Insulin (uIU/mL)	28.5±2	19.5±1.9	**0.007**
HOMA-IR	6.2±0.5	4.3±0.5	**0.01**
ALT (U/L)	26±2	20±2	0.06
AST (U/L)	22±1	22±2	0.99
TG (mg/dL)	118±7	91±6	**0.02**
TC (mg/dL)	163±3	164±5	0.89
LDL-C (mg/dL)	99±3	100±5	0.91
HDL-C (mg/dL)	47±1	52±2	**0.01**
LRG1 (µg/mL)	80.1±5.7	95.6±11.8	0.18
TNF-α (pg/mL)	43.9±2.9	46.5±6.4	0.67
Adiponectin (µg/mL)	13.6±0.9	16.1±1.9	0.21

Data are given as mean±SD, median (IQR 25-75 percentile) or n (%). Bold values indicate statistical significance (p<0.05).
BMI: body mass index, BMI-SDS: standard deviation score of body mass index, TSF: triceps skinfold, PBF: percentage of body fat, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TG: triglycerides, LDL-C: low density lipoprotein cholesterol, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TNF- α: tumor necrosis factor-α, *LRG1: leucine-rich alpha-2-glycoprotein 1,* IQR: interquartile range

No statistically significant correlations were found between TNF-α and any of the measured clinical or laboratory parameters.

Discussion

This study comprehensively examined the metabolic effects of childhood obesity and the roles of biomarkers, including *LRG1*, adiponectin, and TNF-α in this context. Our findings demonstrated significant metabolic disturbances in obese children. Specifically, there were marked increases in BMI and waist circumference, as expected, together with elevated insulin, HOMA-IR, TG, and LDL-C in combination with significant decreases in HDL-C and adiponectin levels. These results align with previous studies reporting the central role of obesity in the development of metabolic syndrome and IR (21,27).

Further stratification revealed that children with hepatosteatosis and IR exhibited more pronounced metabolic abnormalities, confirming that hepatic steatosis and IR are critical determinants of the clinical progression of obesity. These findings highlight

Table 3. Comparison of the clinical and laboratory characteristics of obese subjects with and without insulin resistance

	IR (+) group (n=66)	IR (-) group (n=106)	p value
Age (year)	12.7±0.3	11.2±0.3	**0.0005**
Male (%)	41.4	58.6	0.563
Pubertal (%)	40.1	59.9	0.299
BMI (kg/m^2)	33.7±0.8	21.7±0.6	**<0.001**
BMI SDS	3 (2.7-3.5)	0.4 (-0.5-2.5)	**<0.001**
Waist circumference (cm)	98.2±1.5	69.4±1.4	**<0.001**
Upper arm circumference (cm)	33.7±0.5	24.5±0.5	**<0.001**
TSF thickness (mm)	23.6±0.9	11.1±0.7	**<0.001**
Fat mass (kg)	36.2±1.9	13.5±1	**<0.001**
PBF (%)	39.9 (36.3-45.6)	23.6 (19.6-34.1)	**<0.001**
SBP (mmHg)	119±1	105±1	**<0.001**
DBP (mmHg)	78±1	68±1	**<0.001**
Glucose (mg/dL)	89.1±0.8	84.7±0.6	**<0.001**
Insulin (uIU/mL)	32.8±1.8	9.3±0.4	**<0.001**
HOMA-IR	7.2±0.4	2±0.1	**<0.001**
ALT (U/L)	26.8±1.9	15±0.8	**<0.001**
AST (U/L)	22±1	21.7±0.6	0.802
TG (mg/dL)	116.3±7.1	76.5±3.5	**<0.001**
TC (mg/dL)	166.7±3.1	155.8±2.6	**0.008**
LDL-C (mg/dL)	101.7±2.7	89.9±2.2	**0.001**
HDL-C (mg/dL)	47.3±1.4	57±1.3	**<0.001**
LRG1 (µg/mL)	76.7±5.8	96.6±6.1	**0.03**
TNF-α (pg/mL)	45±3.5	30.7±2.2	**0.004**
Adiponectin (µg/mL)	12.9±1	17.2±1.1	**0.008**

Data are given as mean±SD, median (IQR 25–75 percentile) or n (%). Bold values indicate statistical significance (p<0.05).
BMI: body mass index, BMI-SDS: standard deviation score of body mass index, TSF: triceps skinfold, PBF: percentage of body fat, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, ALT: alanine aminotransferase, AST: Aspartate aminotransferase, TG: triglycerides, LDL-C: low density lipoprotein cholesterol, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, TNF- α: tumor necrosis factor-α, *LRG1: leucine-rich alpha-2-glycoprotein 1,* IQR: interquartile range

Table 4. The relationship between LRG1, TNF-α, adiponectin and clinical and laboratory parameters in the obese subjects

	LRG1	TNF-α	Adiponectin
Age (years)	p=0.2537 r=-0.115	p=0.2194 r=0.124	p=0.0839 r=-0.174
BMI SDS	p=0.3459 r=-0.095	p=0.4617 r=0.074	p=0.2952 r=-0.106
Waist circumference (cm)	p=0.0281* r=-0.220	p=0.8257 r=-0.022	p=0.0131* r=-0.247
Upper arm circumference (cm)	p=0.2698 r=-0.111	p=0.3860 r=0.088	p=0.1508 r=0.145
TSF thickness (mm)	p=0.7227 r=-0.036	p=0.2756 r=0.110	p=0.1621 r=-0.141
Fat mass (kg)	p=0.2342 r=-0.120	p=0.4819 r=0.071	p=0.1225 r=-0.155
PBF (%)	p=0.8019 r=-0.025	p=0.8125 r=0.024	p=0.7756 r=-0.029
SBP (mmHg)	p=0.0614 r=-0.188	p=0.4335 r=0.079	p=0.0379* r=-0.208
DBP (mmHg)	p=0.0446* r=-0.201	p=0.9630 r=-0.005	p=0.0929 r=-0.169
Glucose (mg/dL)	p=0.9114 r=0.011	p=0.5052 r=0.067	p=0.8831 r=-0.015
Insulin (uIU/mL)	p=0.0037* r=-0.288	p=0.4568 r=0.075	p=0.0046* r=-0.281
HOMA-IR	p=0.0074* r=-0.267	p=0.4584 r=0.075	p=0.0080* r=-0.264
ALT (U/L)	p=0.1455 r=-0.147	p=0.2073 r=-0.127	p=0.4072 r=-0.084
AST (U/L)	p=0.7748 r=-0.029	p=0.1803 r=-0.135	p=0.9020 r=-0.012
TG (mg/dL)	p=0.0043* r=-0.283	p=0.7863 r=-0.027	p=0.0090* r=-0.260
TC (mg/dL)	p=0.0068* r=-0.269	p=0.7801 r=-0.028	p=0.0382* r=-0.208
LDL-C (mg/dL)	p=0.0019* r=-0.307	p=0.8452 r=-0.020	p=0.0161* r=-0.240
HDL-C (mg/dL)	p=0.0007* r=0.332	p=0.5220 r=0.065	p=0.0066* r=0.270

*Statistically significant correlation
BMI-SDS: standard deviation score of body mass index, TSF: triceps skinfold, PBF: percentage of body fat, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TG: triglycerides, LDL-C: low density lipoprotein cholesterol, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol

the importance of early intervention in childhood obesity to prevent metabolic complications (21).

Regarding *LRG1* protein levels, our results differ from several reports in the literature. While prior studies have suggested a positive correlation between *LRG1* and inflammation, positioning *LRG1* as a biomarker for cardiometabolic diseases (13,21,27), our study found significantly lower *LRG1* levels in insulin-resistant individuals, with negative correlations observed between *LRG1* and waist circumference, insulin, and HOMA-IR. This discrepancy may be attributed to physiological and metabolic differences unique to the pediatric and adolescent populations. In contrast to findings in adult studies, *LRG1* levels may serve as a negative marker during childhood and adolescence. Therefore, further studies are needed to investigate the longitudinal changes of *LRG1* levels and their relationship with metabolic parameters.

This apparent discrepancy might also reflect the pleiotropic and context-dependent functions of *LRG1*. In adults, *LRG1* has been associated with metabolic deterioration, including obesity-related hepatosteatosis and IR (21), whereas other findings suggest that *LRG1* may promote insulin sensitivity and suppress inflammation (27). In pediatric populations, particularly during puberty, a period characterized by hormonal fluctuations, rapid adipose tissue expansion, and transient IR, the expression and function of *LRG1* may be regulated differently. Moreover, the tissue-specific expression of *LRG1* and its interaction with transforming growth factor beta signaling could vary between developmental stages, potentially explaining contrasting findings in different age groups (13,21). These observations highlight the need for further age-stratified and mechanistic studies to delineate the precise role of *LRG1* in metabolic regulation during growth and maturation. On the other hand, the secretion of *LRG1* from different tissues such as adipose tissue, liver, and immune cells may complicate the biological interpretation of measured serum levels. Therefore, future studies should be supported by translational approaches evaluating tissue-specific expression of *LRG1*.

Methodological variations and biological heterogeneity also contribute to differences in findings. Variability in ELISA assay sensitivity and specificity, along with genetic and environmental factors, may account for the heterogeneity in *LRG1* levels. Although TNF-α levels were elevated in obese subjects, no significant correlation with *LRG1* was observed, suggesting that LRG1 might act through molecular mechanisms distinct from classical inflammatory markers. This indicates that *LRG1* may have functions beyond being an inflammatory biomarker in obesity and metabolic diseases.

Consistent with previous research, our findings concerning adiponectin showed decreased levels and negative correlations with obesity and IR markers. Given the role of adiponectin in enhancing insulin sensitivity and its anti-inflammatory and cardioprotective properties, the reduced adiponectin levels in obesity likely contribute to the development of metabolic risk (28,29). The lack of significant correlations between TNF-α and metabolic parameters reinforces the concept that inflammatory processes in obesity involve a complex interplay of multiple cytokines, and a single biomarker cannot fully capture the clinical picture. In pediatric patients, Ultrasonography (USG) is a useful non-invasive method for detecting hepatic lipid accumulation but USG is not capable of a definitive assessment of hepatic inflammation (30). In the present study, as in many previous pediatric reports, a substantial proportion of patients with hepatosteatosis were classified as grade 1, a stage in which inflammatory changes may not yet be fully established. Therefore, TNF-α levels may not differ significantly between groups, and other proinflammatory cytokines may likewise not exhibit the alterations commonly described in adult populations.

The lower circulating *LRG1* levels observed in obese children compared with adult populations may reflect age-related and developmental differences in adipokine regulation. During childhood and adolescence, metabolic and inflammatory pathways are dynamically regulated, and compensatory mechanisms may prevent the overt activation of pathways commonly observed in adults with long-standing obesity. Moreover, pubertal status, shorter disease duration, and differences in adipose tissue distribution may contribute to the distinct *LRG1* profile observed in pediatric patients. These findings suggest that the role of *LRG1* in obesity-related metabolic dysfunction may be both age dependent and evolve over time.

Study Limitations

This study has some limitations that should be considered. The case-control design limits the ability to infer causality, and the relatively small sample size, particularly in subgroup analyses, may affect the statistical power. In addition, as the study was conducted at a single center, the generalizability of the findings may be limited. Biomarker measurements were performed at a single time point, which may not fully capture temporal variations. Although efforts were made to control for confounding factors, the possibility of residual confounding cannot be completely ruled out. Furthermore, the difference in pubertal stage distribution between the obese and non-obese groups may have introduced potential bias, as pubertal status may influence metabolic parameters. The markedly unbalanced distribution of WtHR categories limited the feasibility of subgroup analyses based on central obesity. Although liver biopsy is considered the gold standard for the diagnosis of nonalcoholic fatty liver disease, its invasive nature limits its routine use in pediatric populations and therefore, ultrasonographic evaluation was used in the present study as this was ethically more acceptable, but should be considered a limitation.

Conclusion

The present study suggests that the role of *LRG1* in childhood obesity may differ from that in obese adults. This highlights the necessity for age-specific evaluations of *LRG1* as a clinical biomarker and will require longitudinal studies with larger cohorts. Furthermore, given the complex nature of obesity and metabolic syndrome, multi-parameter analyses including adiponectin, TNF-α, and a wider range of hormonal and adipokine biomarkers will be necessary for a more comprehensive understanding of the biological functions of *LRG1* at different ages and pubertal stages.

Ethics

Ethics Committee Approval: Approval was obtained from the Akdeniz University Faculty of Medicine Clinical Research Ethics Committee prior to the commencement of the study (protocol number: KAEK-340, date: 11.05.2022). The research was conducted in full compliance with the principles set forth in the Declaration of Helsinki and in accordance with ethical standards.

Informed Consent: Informed consent was obtained from the parents of all participants prior to their inclusion in the study.

Footnotes

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authorship Contributions

Surgical and Medical Practices: Berna Singin, Zeynep Donbaloğlu, Ebru Barsal Çetiner, Bilge Aydın Behram, Aynur Bedel, Mesut Parlak, Hale Ünver Tuhan, Concept: Berna Singin, Hale Ünver Tuhan, Design: Berna Singin, Hale Ünver Tuhan, Data Collection or Processing: Berna Singin, Zeynep Donbaloğlu, Ebru Barsal Çetiner, Bilge Aydın Behram, Aynur Bedel, Mesut Parlak, Hale Ünver Tuhan, Analysis or Interpretation: Berna Singin, Sebahat Özdem, İkbal Özen Küçükçetin, Hale Ünver Tuhan, Literature Search: Berna Singin, Mesut Parlak, Hale Ünver Tuhan, Writing: Berna Singin, Hale Ünver Tuhan.

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Effects on Muscle Mass and Strength in Children with Newly Diagnosed Type 1 Diabetes Mellitus

Hazal Canbaz Özdemir[1], Mesut Parlak[2]

[1]University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Türkiye
[2]Akdeniz University Faculty of Medicine Hospital, Department of Pediatric Endocrinology, Antalya, Türkiye

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ABSTRACT

Objective: Children with type 1 diabetes mellitus (T1DM) are at risk for reduced muscle mass and strength, which may be influenced by insulin deficiency. Although insulin is known to regulate muscle metabolism, data on its effects in newly diagnosed pediatric patients are limited. To describe changes in muscle mass and muscle strength after insulin treatment in children newly diagnosed with T1DM.

Methods: This was a prospective analysis of hospitalized children with newly diagnosed T1DM and age, sex-matched outpatient healthy controls between 2020 and 2021. The primary outcome was muscle strength, muscle mass measured at diagnosis, and three and six months after insulin initiation in patients with T1DM, compared with age- and sex-matched healthy controls. Total body muscle mass were assessed using bioelectrical impedance analysis and muscle strength was measured by handgrip dynamometry.

Results: The study included 36 children with newly diagnosed T1DM and 43 matched controls. Baseline muscle mass did not differ significantly between T1DM patients and controls ($p=0.73$), but muscle strength was significantly lower in the T1DM group ($p=0.001$). During follow-up, both muscle mass and muscle strength increased significantly within the T1DM group compared with baseline ($p<0.001$ for both). No significant correlations were found between muscle parameters and biochemical markers.

Conclusion: Insulin treatment in children with newly diagnosed T1DM was associated with improvements in muscle mass and strength during early follow-up. However, the honeymoon (partial remission) period was not specifically assessed; therefore, its potential contribution to these improvements could not be determined. Regular glycemic control and insulin therapy may contribute to delaying or mitigating complications related to impaired muscle development. Longitudinal studies are warranted to explore the long-term musculoskeletal outcomes of insulin therapy in pediatric T1DM.

Keywords: Type 1 diabetes mellitus, muscle strength, muscle mass

Corresponding Author: Prof. Mesut Parlak, Akdeniz University Faculty of Medicine Hospital, Department of Pediatric Endocrinology, Antalya, Türkiye
E-mail: mesutparlak@akdeniz.edu.tr **ORCID:** orcid.org/0000-0002-3550-1425

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What is already known on this topic?

Children with newly diagnosed type 1 diabetes mellitus (T1DM) often present with reduced muscle strength and altered body composition at diagnosis. Insulin therapy improves metabolic control and may have anabolic effects on muscle tissue. Previous studies have evaluated metabolic and anthropometric changes after insulin initiation, but data on longitudinal changes in both muscle mass and strength in pediatric T1DM remain limited.

What this study adds?

This study is the longitudinally evaluate both muscle mass and muscle strength in children with newly diagnosed T1DM after the initiation of insulin therapy. We demonstrate significant improvements in muscle strength and body composition parameters within six months of treatment, providing novel evidence for the anabolic effects of insulin beyond metabolic control. Our findings highlight the importance of early intervention and comprehensive follow-up for musculoskeletal health in pediatric T1DM patients

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by insulin deficiency due to destruction of pancreatic β-cells (1). Insulin plays a key role in glucose uptake, glycogenesis, glucose oxidation, and protein synthesis in skeletal muscle, primarily through the protein kinase B (Akt/PKB) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) pathways (2). Insulin deficiency leads to hyperglycemia and increases the risk of various complications (3). In individuals with T1DM, muscle mass, muscle strength, and bone mass are adversely affected due to impaired insulin action. Notably, muscle mass and strength have been shown to be negatively correlated with glycemic control, as reflected by hemoglobin A1c (HbA1c) levels (4).

The musculoskeletal system comprises approximately 40% of total body weight and 50-75% of total body protein content. Techniques such as dual-energy X-ray absorptiometry (DXA), bioelectrical impedance analysis (BIA), and magnetic resonance imaging (MRI) are commonly used to evaluate adiposity and muscle mass distribution in pediatric and adolescent populations. Among these, BIA is widely utilized owing to its safety, non-invasiveness, affordability, reproducibility, and rapid output.

Muscle function in children can be efficiently assessed using a hand dynamometer, which measures maximal isometric grip strength of the forearm (5). Grip strength serves as a useful indicator of general health status, protein reserves, and nutritional status (6).

Although reduced muscle mass and strength have been linked to cardiovascular and metabolic diseases, impaired bone health, sarcopenia, and osteoporosis in children with T1DM, there is limited evidence about changes in these parameters following insulin therapy. To the best of our knowledge, no previous study has prospectively evaluated alterations in muscle mass and strength after the initiation of insulin therapy in pediatric patients with newly diagnosed T1DM. The aim of this study was to assess the effects of starting insulin treatment on muscle strength, fat mass, and muscle mass in children and adolescents recently diagnosed with T1DM and to explore the relationships between these outcomes and various biochemical parameters.

Methods

Newly diagnosed T1DM patients and age- and sex-matched healthy controls, aged 5-18 years, were enrolled. Exclusion criteria included any history of neuropathic or orthopedic disorders, current medication use that could affect muscle function, or prior upper extremity surgery. The healthy control group was evaluated only once at baseline due to logistical limitations and because repeated assessments were not considered ethically appropriate in healthy children. “Newly diagnosed diabetes” referred to children who presented with diabetic ketoacidosis and were subsequently diagnosed with T1DM at first presentation, with no prior diabetes treatment (glucose >200 mg/dL, ketonemia/ketonuria, venous pH <7.3 or bicarbonate <15 mmol/L). Diagnosis of T1DM was also based on the presence of anti-glutamic acid decarboxylase (anti-GAD), anti-insulin, or anti-islet cell antibodies. Laboratory evaluations were performed on the day of hospital admission. In patients, anthropometric measurements, BIA, and handgrip dynamometry were performed after metabolic stabilization, during the diabetes education period, specifically on the fifth day of hospitalization. Total body muscle mass were assessed using BIA (Tanita MC-780, Tanita Corp., Tokyo, Japan) and muscle strength was measured by isometric handgrip dynamometry using a GRIP-D dynamometer (Takei, Tokyo, Japan).

Ethical Considerations

This study was approved by the Akdeniz University Faculty of Medicine Ethics Committee (approval no: KAEK-157, date: 19.02.2020). Written informed consent, in accordance with the

Declaration of Helsinki, was obtained from all participants and/or their legal guardians before inclusion in the study.

Clinical Evaluation

Height, weight, and body mass index (BMI) were recorded, and standard deviation scores (SDS) were calculated using age- and sex-specific reference values for Turkish children (7). BMI was calculated as weight (kg) divided by height squared (m^2). Pubertal status was assessed according to the Marshall and Tanner (8) staging system and categorized as prepubertal (stage 1) or pubertal (stages 2-5).

Body Composition Assessment

Total body muscle mass and fat mass were evaluated using BIA with the Tanita MC-780 analyzer (Tanita Corp., Tokyo, Japan). All measurements were performed by a single trained clinician to ensure consistency. Participants were instructed to fast for at least one hour prior to the test, void their bladder, and wear lightweight clothing. During the measurement, individuals stood barefoot on the device platform while grasping the hand electrodes with both hands.

Muscle Strength Assessment

Muscle strength was measured using a GRIP-D hand dynamometer (Takei, Tokyo, Japan), which evaluates isometric grip strength. Three consecutive measurements were obtained from the dominant hand, positioned with the thumb over the other fingers, and the average value was used for analysis. In the T1DM group, both BIA and dynamometer assessments were conducted at baseline (day five of hospitalization), and at three and six months following diagnosis. In the control group, these measurements were performed only at baseline.

Biochemical Assessment

In the T1DM group, venous blood samples were collected at diagnosis, and at the two follow-up time points. The following parameters were analyzed: serum calcium, phosphorus, aspartate aminotransferase (AST), alanine aminotransferase (ALT), hemoglobin A1c (HbA1c), thyroid-stimulating hormone (TSH), free thyroxine (FT4), fasting blood glucose, and hemoglobin levels. In the control group, these parameters were measured once at baseline. Comparisons between groups were performed for all relevant variables.

Statistical Analysis

Statistical analyses were performed using SPSS, version 23.0 (IBM Corp., Armonk, NY, USA). Categorical variables are presented as numbers and percentages. Comparisons between groups were performed using the chi-square or Fisher's exact test for categorical variables, and the Student's t-test or Mann-Whitney U test for continuous variables, as appropriate based on data distribution. The Friedman test was used to compare repeated measures that did not follow a normal distribution. Pearson's or Spearman's correlation analyses were used to assess relationships between continuous variables depending on their distribution. Normality of continuous variables was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests, supported by visual inspection of histograms and Q-Q plots. Parametric tests were applied to normally distributed variables, whereas non-parametric tests were used when normality assumptions were not met. Sex-based and pubertal subgroup analyses were considered exploratory; therefore, no formal correction for multiple comparisons was applied, and these results should be interpreted with caution. A two-sided p-value<0.05 was considered statistically significant.

Results

A total of 36 children with newly diagnosed type 1 diabetes mellitus (T1DM) (mean age: 9.99±3.02 years) and 43 age- and sex-matched healthy controls (mean age: 10.32±2.70 years) were enrolled. There were no significant differences between the two groups in terms of age, sex distribution, pubertal stage, height, weight, BMI, BMI SDS, fat mass, or muscle mass (Table 1).

At baseline, muscle strength was significantly lower in the T1DM group [median 10.4 N (5-25.5)] compared with controls [median 15.8 N (6.2-31.9); p=0.001]. Fasting blood glucose levels were significantly higher in the T1DM group. Serum calcium and phosphorus levels were also significantly lower in patients with T1DM (both p<0.001), while other laboratory parameters did not differ between groups (Table 1). During follow-up, significant improvements were observed in anthropometric, metabolic, and musculoskeletal parameters in the T1DM group. Fat mass increased from 6.65 kg (2.0-20.2) at baseline to 8.25 kg (2.2-22.3) at three months and 8.25 kg (2.3-28.3) at six months (p<0.001). BMI increased from 17.5±3.28 kg/m^2 at baseline to 18.57±3.28 kg/m^2 at six months (p=0.004). Glycemic control improved significantly following insulin initiation, with HbA1c and fasting blood glucose levels decreasing at both three and six months (p<0.001 for both). Muscle strength increased significantly from baseline to six months, representing an approximately 40-50% improvement. Similarly, muscle mass increased from a median of 23.8 kg (10.9-55.5) at baseline to 25.6 kg (11.7-61.5) at three months and remained stable at six months [24.9 kg (1.5-58.8); p<0.001] (Table 2). Sex-based analyses showed that boys had higher muscle strength at baseline (p=0.030); however, no significant sex differences were observed at three or six months. Both sexes demonstrated significant within-group improvements in muscle strength and muscle mass over time (p<0.001) (Table 3). At six months, pubertal children had higher muscle strength (21.15±6.39 N vs 11.9±3.72 N; p<0.001) and muscle mass (36.55±10.25 kg vs 20.76±6.18 kg; p<0.001) compared with prepubertal children. From baseline to six months, muscle

strength increased by +5.72 N in pubertal patients and +3.49 N in prepubertal patients (p=0.014), while muscle mass increased by +3.61 kg vs +1.81 kg, respectively (p=0.038) (Table 4). Finally, muscle strength showed strong positive correlation with muscle mass at all time points (r=0.867-0.932; p<0.001), supporting the close relationship between structural and functional muscle parameters.

Discussion

The novelty of the present study lies in the simultaneous longitudinal assessment of both muscle mass and muscle strength in children with newly diagnosed T1DM, providing functional insight beyond body composition alone. At diagnosis, muscle mass and fat mass were comparable between the T1DM and control groups. However, muscle strength was already

Table 1. Baseline characteristics, laboratory findings, muscle mass, and body composition levels in T1DM and control groups

Parameters	T1DM group (n=36)	Control group (n=43)	p value
Age (years)	9.99±3.02	10.32±2.7	0.613
Gender (M/F) n (%)	19/17 (52.8/47.2)	21/20 (48.8/51.2)	0.727
Pre-pubertal/pubertal	19/17	20/23	0.579
Height (cm)	141.69±20.43	140.03±17.1	0.695
Weight (kg)	35.85 (14- 82.9)	34.1 (18.2-78.7)	0.821
BMI (kg/m^2)	16.75 (13.2-25.4)	17.3 (13.5-27.7)	0.705
BMI SDS	-0.38 (-1.92-1.96)	-0.52 (-1.92-1.91)	0.894
Muscle mass (kg)	23.8 (10.9-55.5)	22.3 (13.4-51.6)	0.738
Fat mass (kg)	6.65 (2-20.2)	7.1 (3-26.1)	0.327
Muscle strength (Newton)	10.4 (5-25.5)	15.8 (6.2-31.9)	**0.001***
Fasting blood glucose (mmol/L)	14.85 (11.27-16.60)	5.00 (2.94-6.22)	**<0.001***
Calcium (mmol/L)	2.33±0.14	2.48±0.10	**<0.001***
Phosphorus (mmol/L)	1.22±0.30	1.59±0.22	**<0.001***
ALT (U/L)	13 (7-52)	15 (9-51)	0.085
Hemoglobin (g/L)	135.8±12.4	131.3±10.1	0.080
TSH (UIu/mL)	2.16 (0.71-4.62)	2.24 (0.65-4.93)	0.867
FT4 (ng/dL)	1.14 (0.8-3.7)	1.22 (0.82-1.4)	0.668

*p<0.05
Statistical tests: Mann-Whitney U and Pearson chi-square tests were used, median values were given
T1DM: Type 1 diabetes mellitus, BMI: body mass index, sds: standard deviation score, ALT: alanine aminotransferase, TSH: thyroid stimulating hormone, FT4: free thyroxine

Table 2. Parameters in the T1DM patient group by months

Parameters	0th month	3rd month	6th month	p value
Muscle strength (N)	10.4 (5-25.5)^{a,b}	13 (5.3-31.5)^{c}	14.8 (7.4-34.2)	**<0.001**
Fat mass (kg)	6.65 (2-20.2)^{a,b}	8.25 (2.2-22.3)	8.25 (2.3-28.3)	**<0.001**
Muscle mass (kg)	23.8 (10.9-55.5)^{a,b}	25.6 (11.7-61.5)	24.9 (1.5-58.8)	**<0.001**
BMI (kg/m^2)	(17.5±3.28)^{a,b}	18.06±3.2	18.57±3.28	**0.004**
BMI SDS	(-0.2±1.1)^{a,b}	0.05±1.03	0.15±0.99	**0.021**
HbA1c	(13.68±2.59)^{a,b}	6.94±1.31	7.15±0.97	**<0.001**
Fasting blood glucose (mmol/L)	14.85 (11.27-16.59)^{a,b}	6.11 (3.55-16.48)	7.41 (3.61-15.21)	**<0.001**
Height (cm)	(141.69±20.43)^{a,b}	(142.89±20.05)^{c}	144.28±19.94	**<0.001**
Weight (kg)	35.85 (14-82.9)^{a,b}	36.25 (15.6-81.7)^{c}	37.4 (17.3-81.3)	**<0.001**

p<0.05, ^{a}0-3 month, ^{b}0-6 month, ^{c}3-6 month
Statistical tests: ANOVA, Friedman tests were used and median values were given
T1DM: type 1 diabetes mellitus, N: Newton, BMI: body mass index, SDS: standard deviation score, HbA1c: glycosylated haemoglobin

Table 3. Parameters in the T1DM girls and boys by months

Parameters	Girl (n=17)	Boy (n=19)	p value
Muscle strength (N) 0th month	8.4 (5-18.5)[a,b]	12.8 (5.7-25.5)[a,b]	**0.030**
3rd month	11.2 (5.3-20.1)[c]	14.5 (6.5-31.5)[c]	0.087
6th month	13.8 (7.4-23.4)	15.9 (7.6-34.2)	0.114
p value	**<0.001**	**<0.001**	
Fat mass (kg) 0th month	6.5 (2.5-16.2)[a,b]	7.3 (2-20.2)[a,b]	0.684
3rd month	7.4 (3.8-17.4)	8.7 (2.2-22.3)	0.707
6th month	8 (4.2-24.8)	8.3 (2.3-28.3)	0.851
p value	**<0.001**	**<0.001**	
Muscle mass (kg) 0th month	19 (10.9-36.1)[a,b]	25.6 (11.9-55.5)[a,b]	0.061
3rd month	21.9 (11.7-41.3)	29.9 (13.5-61.5)	0.087
6th month	22.1 (11.5-38.6)	31.1 (13-58.8)	0.100
p value	**<0.001**	**<0.001**	
BMI (kg/m²) 0th month	(16.79±2.71) [b]	18.25±3.65	0.187
3rd month	17.34±2.65	18.71±3.57	0.204
6th month	17.68±2.59	19.35±3.69	0.129
p value	**0.024**	0.060	
BMI sds 0th month	(-0.32±1.03)[b]	-0.1±1.18	0.559
3rd month	0.04±0.88	0.07±1.18	0.937
6th month	0.08±0.7	0.2±1.2	0.706
p value	**0.042**	0.233	
HbA1c 0th month	(14.26±2.69)[a,b]	(13.15±2.45)[a,b]	0.205
3rd month	7.05±1.17	6.85±1.45	0.644
6th month	7.11±0.97	7.18±1.01	0.828
p value	**<0.001**	**<0.001**	

p<0.05; [a](0-3 months), [b](0-6 months), [c](3-6 months)
T1DM: type 1 diabetes mellitus

significantly reduced in T1DM group. In contrast to previous pediatric studies that primarily focused on body composition, the present study demonstrated that muscle strength is already reduced at diagnosis but improves in the short term in parallel with muscle mass following insulin therapy (4,9,10,11). These findings extend the existing pediatric literature by providing functional evidence supporting the anabolic role of insulin beyond changes in body composition. Although percentage-based muscle and fat mass measures could allow a more precise interpretation of compositional changes independent of weight gain, these data were not systematically recorded during the initial data collection period. Therefore, analyses were limited to prospectively collected absolute values to avoid methodological

Table 4. Parameters in the T1DM pre-pubertal and pubertal stages by months

Parameters	Pre-pubertal (n=19)	Pubertal (n=17)	p value
Muscle strength (N) 0th month	(8.51±2.72)[a,b]	(15.44±5.07)[a,b]	**<0.001**
3rd month	(10.14±3.67)[c]	(18.46±5.62)[c]	**<0.001**
6th month	11.9±3.72	21.15±6.39	**<0.001**
p value	**<0.001**	**<0.001**	
Fat mass (kg) 0th month	4.8 (2-14.1)[a,b]	8.5 (5.1-20.2)[b]	**0.001**
3rd month	6.1 (2.2-18.7)	10 (6-22.3)	**0.003**
6th month	6 (2.3-18.2)	10.9 (6.3-28.3)	**0.002**
p value	**<0.001**	**0.010**	
Muscle mass (kg) 0th month	(18.95±5.8)[a,b]	(32.95±9.43)[a,b]	**<0.001**
3rd month	20.72±6.32	36.29±10.84	**<0.001**
6th month	20.76±6.18	36.55±10.25	**<0.001**
p value	**<0.001**	**<0.001**	
BMI (kg/m²) 0th month	16.46±3.01	18.79±3.21[a,b]	**0.031**
3rd month	16.88±2.86	19.38±3.12	**0.017**
6th month	17.32±2.9	19.96±3.2	**0.014**
p value	0.124	**0.015**	
BMI SDS 0th month	-0.1±1.19	-0.32±1.03	0.559
3rd month	0.24±1.06	-0.15±1	0.268
6th month	0.26±1.05	0.01±0.093	0.455
p value	0.155	0.059	
HbA1c 0th month	(13.67±2.3)[a,b]	(13.68±2.95)[a,b]	0.987
3rd month	7.33±1.26	6.52±1.26	0.063
6th month	7.42±1.01	6.85±0.87	0.084
p value	**<0.001**	**<0.001**	

p<0.05, [a](0-3 month), [b](0-6 month), [c](3-6 month)
Statistical tests: Mann-Whitney U, ANOVA and Friedman tests were used
BMI-SDS: body mass index-standard deviation score, HbA1c: glycosylated haemoglobin

bias. Importantly, the concurrent and significant improvement in muscle strength suggests a true functional recovery rather than a passive increase secondary to weight gain, supporting a functional anabolic contribution of insulin therapy to skeletal muscle. The absence of longitudinal follow-up in the healthy control group represents an important limitation when interpreting these findings. Healthy controls were evaluated only at baseline and soage- and growth-related physiological increases in muscle mass and strength over the 6-month period could not be directly accounted for. Therefore, the observed

longitudinal improvements should be interpreted as within-patient changes following insulin initiation rather than direct comparisons with normal growth trajectories.

Age-related and sex-related differences in body composition are well documented. Boys typically have higher muscle mass and lower fat mass than girls (12). Consistent with this, boys in our cohort had higher muscle mass, but both sexes showed significant increases in muscle and fat mass following insulin therapy. Notably, a significant increase in BMI and BMI SDS was observed only in girls at six months, a finding that parallels previous reports of greater weight gain in adolescent girls with T1DM (10,13,14). Unlike prior pediatric studies that primarily focused on weight or fat mass changes, our findings highlight sex-specific differences in the pattern of musculoskeletal response to insulin therapy. When analyzed by pubertal status, both muscle and fat mass were significantly greater in pubertal children at all time points. Furthermore, increases in these parameters were more pronounced in pubertal patients compared to prepubertal peers, supporting the known anabolic effects of insulin and the additional contribution of pubertal hormones such as androgens (15,16,17). Although BMI was higher in pubertal children, BMI SDS did not significantly differ, suggesting alignment with age- and sex-specific normative data. This may reflect increased bone mass and epiphyseal closure during puberty.

Few studies have assessed muscle strength in children with T1DM. In our cohort, muscle strength was significantly lower at diagnosis compared to controls, corroborating previous reports (18,19,20), though some studies have shown no difference (21). Following insulin therapy, muscle strength improved significantly in both sexes. While boys had higher baseline strength, this difference was not sustained after treatment, possibly due to greater relative gains in girls or pubertal effects (22). Pubertal patients demonstrated greater improvements in both muscle mass and strength compared to prepubertal patients, supporting the role of pubertal hormones and insulin in promoting musculoskeletal development (23). We found strong positive correlations between muscle strength and both muscle and fat mass across all time points. Impaired muscle function in T1DM is reported to be multifactorial, involving metabolic, hormonal, and neuromuscular mechanisms (19,24,25,26,27,28). Although calcium and phosphorus levels were lower in the T1DM patients, they remained within normal ranges and were not correlated with muscle strength, suggesting that other mechanisms may contribute to the observed improvements. We identified a moderate negative correlation between HbA1c and both muscle mass and strength at the third month of treatment, highlighting the impact of glycemic control on muscle health. The slight increase in HbA1c observed at six months may reflect adolescent insulin resistance or challenges in adherence. These findings suggest that improved glycemic control may contribute to favorable musculoskeletal outcomes in pediatric T1DM. While adult studies have similarly linked better glycemic control with increased skeletal muscle mass (29), conflicting results have also been reported (30), highlighting the complexity of this relationship.

Study Limitations

This study has several limitations. First, the control group was evaluated only at baseline, which precluded longitudinal comparisons of muscle strength and muscle mass trajectories between patients and healthy peers. As a result, normal age- and growth-related increases in musculoskeletal parameters among healthy children could not be accounted for, and the observed longitudinal improvements in the T1DM group may partly reflect physiological growth in addition to treatment-related effects, potentially leading to an overestimation of the insulin effect. Second, bone mass, an important contributor to total body weight and BMI was not assessed, which may limit the interpretation of body composition changes. The absence of bone-related measurements restricts the ability to fully differentiate between lean tissue accumulation and skeletal growth, particularly during puberty. The honeymoon (partial remission) period was not specifically assessed; therefore, its potential influence on changes in muscle mass and strength during early follow-up could not be determined. In addition, serum calcium levels were measured only once in the control group, preventing comparative analysis of dynamic changes over time. Third, physical activity levels, nutritional status, and detailed insulin regimen characteristics (such as insulin dose adjustments) were not included in the analyses. These factors are known to influence muscle mass, fat mass, and muscle strength and may have affected the magnitude and interpretation of the observed relationships. Finally, the relatively small sample size and short follow-up duration may limit the generalizability of our findings. Future studies involving larger cohorts with extended follow-up and comprehensive skeletal and lifestyle assessments are warranted to validate and expand upon these results.

Conclusion

Children and adolescents with newly diagnosed T1DM exhibited reduced muscle strength at diagnosis, despite similar muscle and fat mass compared to healthy controls. Our findings suggest that the first six months of insulin therapy, by restoring glycemic control, may contribute to anabolic processes associated with improvements in both muscle mass and muscle strength, particularly during early treatment. These results highlight the potential importance of optimizing insulin therapy not only for metabolic control but also for musculoskeletal health. Longitudinal monitoring of muscle function and body

composition in pediatric T1DM may help identify patients at risk for sarcopenia, osteoporosis, and metabolic complications, and may support early preventive strategies.

Ethics

Ethics Committee Approval: This study was approved by the Akdeniz University Faculty of Medicine Ethics Committee (approval no: KAEK-157, date: 19.02.2020).

Informed Consent: Written informed consent, in accordance with the Declaration of Helsinki, was obtained from all participants and/or their legal guardians before inclusion in the study.

Footnotes

Authorship Contributions

Concept: Hazal Canbaz Özdemir, Mesut Parlak, Design: Mesut Parlak, Data Collection or Processing: Hazal Canbaz Özdemir, Analysis or Interpretation: Hazal Canbaz Özdemir, Mesut Parlak, Literature Search: Hazal Canbaz Özdemir, Writing: Hazal Canbaz Özdemir, Mesut Parlak.

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Association Between Gastrointestinal Symptoms and Sleep Habits in Children with Metabolic Dysfunction-Associated Steatotic Liver Disease: A Cross-Sectional Study

Behzat Özkan[1], İshak Abdurrahman Işık[2], Atike Atalay[2], Nazlı Sivil[2], Memduha Sarı[2], Arzu Aras[3], Coşkun Fırat Özkeçeci[3], Hicran Altın[4], Mehmet Özen[5], İsmail Topal[6], Ulaş Emre Akbulut[2]

[1]University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Türkiye
[2]University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Pediatric Gastroenterology Hepatology and Nutrition, Antalya, Türkiye
[3]University of Health Sciences Türkiye, Antalya City Hospital, Clinic of Pediatric Gastroenterology Hepatology and Nutrition, Antalya, Türkiye
[4]University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Pediatrics, Antalya, Türkiye
[5]University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Family Medicine, Antalya, Türkiye
[6]Trabzon University Faculty of Medicine, Department of Pediatrics, Trabzon, Türkiye

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ABSTRACT

Objective: Reduced sleep quality in children has been associated with obesity and fatty liver disease. However, there are no studies evaluating the impact of gastrointestinal symptoms on sleep quality in children with metabolic dysfunction-associated steatotic liver disease (MASLD). The aim of this cross-sectional study was to investigate the prevalence of gastrointestinal symptoms and sleep disturbances in children with MASLD and to examine the association of gastrointestinal symptoms with sleep disturbances.

Methods: Anthropometric and biochemical examinations were performed on children aged 8-18 years, including children with MASLD and matched healthy controls for comparison. The Hepatic Steatosis Index (HSI) and Non-Alcoholic Fatty Liver Disease Fibrosis Score (NFS) were calculated for children with MASLD. Sleep disturbances were assessed using the Child Sleep Habits Questionnaire (CSHQ), and gastrointestinal symptoms were assessed using the Gastrointestinal Symptom Rating Scale (GSRS). Logistic regressions were used to examine factors associated with sleep quality.

Results: A total of 176 children, consisting of 84 (47.7%) with MASLD and 92 healthy controls were included. Of these 123 (69.9%) had sleep disturbances. In unadjusted analysis, sleep disturbances were significantly more common in the MASLD group (89% vs 57%, $p<0.001$). However, in the final multivariate regression model adjusting for metabolic confounders, the independent predictors of sleep disturbances were low family income [odds ratio (OR)=9.56, 95% confidence interval (CI)=2.80-32.57], total GSRS score (OR=1.15, 95% CI=1.06-1.24), and Homeostatic

Corresponding Author: Prof. Behzat Özkan, University of Health Sciences Türkiye, Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Türkiye
E-mail: ozkan.behzat@gmail.com **ORCID:** orcid.org/0000-0002-9153-8409
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Model Assessment of Insulin Resistance (OR=1.51, 95% CI=1.01-2.27). The presence of MASLD lost statistical significance after adjustment (p=0.554). NFS, a marker of fibrosis risk, was associated with both sleep disturbances and gastrointestinal symptoms.

Conclusion: This study showed that sleep disturbances and gastrointestinal symptoms are more common in children with MASLD, and that gastrointestinal disturbances are significantly associated with sleep disturbances. Furthermore, the results suggest that sleep disturbances may be more common in children with MASLD who have a higher estimated risk of liver fibrosis. Children with MASLD should be evaluated not only for liver health but also for extrahepatic conditions, including sleep and gastrointestinal disorders.

Keywords: Children, gastrointestinal symptoms, metabolic dysfunction-associated fatty liver disease, obesity, sleep quality

What is already known on this topic?

Gastrointestinal disorders and sleep disorders are more common in obese individuals than in the normal population. Some children with obesity will develop metabolic dysfunction-associated steatotic liver disease (MASLD).

What this study adds?

This study demonstrated a high prevalence of sleep disturbances and gastrointestinal symptoms in children with MASLD. Gastrointestinal symptoms contributed to sleep disturbances in children with MASLD. Liver fibrosis risk was associated with sleep disturbances, particularly sleep anxiety and sleep duration.

Introduction

Sleep, a vital component of quality of life, plays a critical role in children's physical growth, neurological development, and behavioral maturation (1). Sleep disorders in children may lead to numerous issues, including daytime sleepiness, diminished neurocognitive performance, and poor academic achievement (2). It is estimated that 10-20% of children experience sleep disorders severe enough to require medical intervention (3). Obese children are at higher risk of developing sleep disorders, particularly obstructive sleep apnea (4,5). Obesity also exerts both direct and indirect effects on gastrointestinal functions. In these individuals, central adiposity increases intra-abdominal pressure, contributing to a wide range of problemsthat may include gastroesophageal reflux and gallstone formation because of metabolic dyslipidemia (6). Moreover, in obese individuals with metabolic dysfunction-associated steatotic liver disease (MASLD), alterations in proinflammatory adipokine and cytokine release, disruption of the liver-brain-gut neural axis and gut-liver crosstalk, and changes in gut microbiota are frequently associated with functional disorders, like irritable bowel syndrome (7).

Insufficient sleep, delayed sleep onset, and poor sleep quality are common in adults with functional gastrointestinal disorders (8). Similarly, children with functional gastrointestinal disorders also exhibit prevalent sleep disturbances, and those with sleep problems report more severe abdominal pain (9). In obese adults, gastrointestinal symptoms have been shown to correlate with sleep disorders (10). However, no studies have investigated the relationship between sleep disturbances and gastrointestinal symptoms in children with MASLD. To address this, the aims of the present study were: (i) to determine the frequency of gastrointestinal symptoms and prevalence of sleep disorders in children with MASLD, (ii) to examine the association between gastrointestinal symptoms and sleep disturbances in this population, and (iii) to investigate if and how how hepatic steatosis severity correlated with both sleep problems and gastrointestinal symptoms.

Methods

Study Design and Ethical Aspects

This study was a cross-sectional, analytical investigation with a control group. The research was conducted among overweight or obese children and adolescents aged 8-18 years and a healthy sex and age-matched control group with a normal body mass index (BMI), no known disease, normal liver function tests and liver ultrasound (US). The study protocol was approved by the Antalya Training and Research Hospital Scientific Ethics Committee for Medical Research (date: 05.12.2024, approval no.: 19/21) prior to commencement. Written and verbal informed consent was obtained from both participating children and their parents prior to enrollment. The study was conducted in accordance with the Declaration of Helsinki regarding human and animal rights and local laws and regulations.

Participants

The study included overweight or obese children and adolescents aged 8-18 years who were referred to our

department with suspected liver disease. Based on the most recent NAFLD Nomenclature consensus group guidelines (11), patients were divided into two groups, those with MASLD and those without MASLD, using laboratory and ultrasound findings as sub-group inclusion criteria. Children were excluded from study participation if they had an infectious or autoimmune liver diseases (hepatitis A, B, C, infectious mononucleosis, autoimmune hepatitis), metabolic liver diseases (Wilson's disease, alpha-1 antitrypsin deficiency, cystic fibrosis), other chronic conditions (e.g., bronchial asthma, chronic renal failure, oncologic diseases), endocrine-related obesity (hypercortisolism, hypopituitarism, hypothyroidism, hypothalamic-pituitary injury), medications and substance use (use of medications that affect body weight, blood pressure, or metabolism, such as glucocorticoids, psychiatric medications, anticonvulsants-smoking and/or alcohol consumption), and refusal to provide informed consent by parents and/or patients over 16 years of age. The control group consisted of age- and sex-matched children who were examined at the same hospital's pediatric outpatient clinics for mild physical symptoms, such as a common cold, rhinitis and had no chronic medical or psychiatric conditions.

Socio-demographic Data Form

This form was designed to collect socio-demographic information about the children and their families. The form included details such as, participants' age, gender, family's monthly income and parental education levels.

Anthropometric Measurements

Measurements of body weight and height were taken when the children were wearing light clothing and were barefoot. BMI was calculated using the standard formula: weight (kg)/ height^2 (m). According to the World Health Organization, overweight was defined as BMI standard deviation score (SDS) >1, and obesity as BMI SDS >2 (12).

Liver Ultrasonography

Routine B-mode US investigation was performed by a single radiologist with more than 10 years of experience in US imaging. US images of the liver were obtained using subcostal and intercostal planes. The requested fasting time was 6 hours. Hepatic steatosis was diagnosed based on known US characteristics, including increased parenchymal echogenicity, hepatorenal echo contrast, impaired visualization of the diaphragm line and intrahepatic portal vein wall, and deep attenuation of the liver parenchyma (13).

Biochemical Analyses

Blood samples were collected from participants after a 12-hours overnight fast. Routine biochemical analyses including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), albumin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, glucose and insulin, all of which were measured by standard clinical laboratory techniques. The homeostasis model assessment of insulin resistance [HOMA-IR=insulin (mU/L) glucose (mmol/L)/22.5] was calculated using serum concentrations of glucose and insulin (14).

Hepatic İndexes

Different indirect hepatic markers were calculated by taking into account the necessary criteria and applying accepted formulas (15). The Hepatic Steatosis Index (HSI) and Non-Alcoholic Fatty Liver Disease Fibrosis Score (NFS) were chosen as proxy markers for hepatic steatosis in this population (16). The HSI was obtained using the established formulas and includes the ALT/AST ratio, BMI, presence of diabetes mellitus and sex, and was developed to detect hepatic steatosis. The NFS identifies individuals at risk for significant liver fibrosis due to steatosis using factors such as age, BMI, presence of diabetes, AST/ALT ratio, platelet count, and albumin, again using the established formula. These indices were selected as they reflect different aspects of MASLD: the HSI primarily estimates the degree of hepatic steatosis, while the NFS estimates the risk of associated fibrosis, incorporating broader metabolic factors.

Gastrointestinal Symptom Rating Scale (GSRS)

The GSRS is a measurement tool used to assess gastrointestinal symptoms in individuals, originally developed by Revicki et al. (17) in 1998. Its validity and reliability for the Turkish population were established by Turan et al. (18) in 2017, confirming its applicability. The scale consists of 15 items scored on a 7-point Likert scale (ranging from "no discomfort" to "very severe discomfort") and is divided into five subdomains: reflux (items 2 and 3), abdominal pain (items 1,4, and 5), indigestion (items 6,7,8, and 9), diarrhea (items 11,12, and 14), and constipation (items 10,13, and 15). Each item evaluates the patient's self-reported gastrointestinal symptoms over the past week. The total score ranges from 0 to 105, with higher scores indicating more severe gastrointestinal symptoms. In the adaptation study, the scale demonstrated high internal consistency, with a Cronbach's alpha coefficient of 0.82 (18).

Child Sleep Habits Questionnaire (CSHQ)

This questionnaire was originally developed by Owens et al. (19) in 2000 as a 45-item tool, with a subsequent short form consisting of 33 functional items. Its Turkish adaptation has been validated, demonstrating a Cronbach's alpha coefficient of 0.78 (20). The CSHQ comprises eight subscales: bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night wakings, parasomnias, sleep-disordered breathing, and daytime sleepiness. Parents retrospectively evaluate their child's sleep

patterns over the past week. Items are scored on a 3-point scale: usually (behavior occurring 5-7 times/week)=3, sometimes (2-4 times/week)=2, and rarely (never or once weekly)=1. Six items are reverse-scored. The clinical cutoff is set at 41 points, with higher scores indicating clinically significant sleep problems, while a total score ≤41 suggests no significant sleep disturbances.

Sample Size

G Power software version 3.1.9.7, was used with an alpha of 0.05 (two-sided) and 95% power. We calculated the sample size based on the mean difference between two independent groups (obese group and healthy). The means and standard deviations of sleep problems in children were taken from an earlier similar study (obesity case 43.62±8.26, healthy control 39.89±5.19) (21) as we used the CSHQ in this earlier research. The required sample size was a minimum of 82 participants in each group.

Statistical Analysis

Statistical evaluations were made using Statistical Package for the Social Sciences (SPSS) for Windows, version 26.0 (IBM Corp., Armonk, New York, USA). The normality of the distribution was evaluated by the Kolmogorov-Smirnov test. To compare the data obtained from the MASLD groups and control groups who participated in the study, the independent sample t-test was used for normally distributed data and the Mann-Whitney U test was used for non-normally distributed data. Categorical data were compared using the chi-square test. Descriptive statistical values including mean and standard deviation were derived for continuous data, and median and interquartile range (IQR) were calculated for non-parametric data. To evaluate the relationship between the sociodemographic data and scale scores of the MASLD groups and controls, Pearson's correlation analysis was used for parametric data, and Spearman's correlation analysis was used for non-parametric data. The association between several factors and sleep disturbance was investigated using logistic regression after adjusting for confounding factors, then analyzed using three models. Model 1 was adjusted for demographic characteristics (gender, age, family income status) and key metabolic confounders (BMI SDS and HOMA-IR score). Model 2 was adjusted for all variables in Model 1 plus the presence of MASLD. Model 3 was adjusted for all variables in Model 2 plus the total GSRS score.

Results

A total of 176 participants, including 84 (47.7%) children with MASLD and 92 healthy control, who presented to the Antalya Training and Research Hospital, Clinic of Pediatric Gastroenterology between 01 January 2025 and 30 August, 2025, were included in the study (Figure 1).

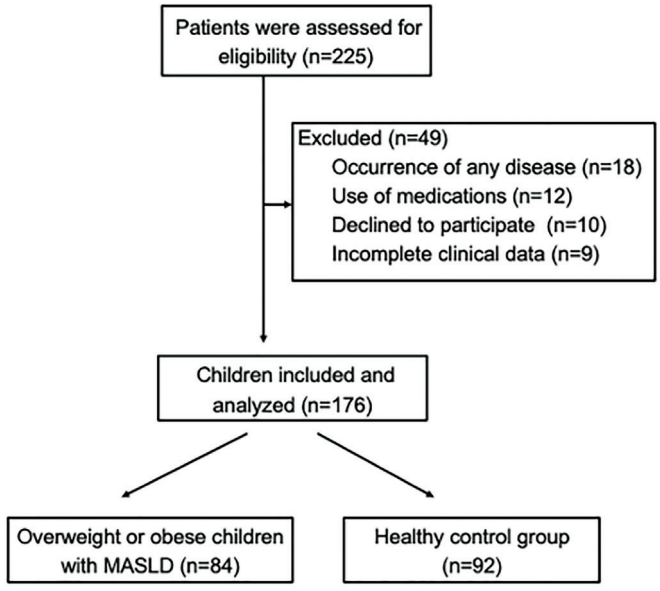

Figure 1. Number of children evaluated for enrollment, included in the study, and excluded

Characteristics of Participants

The participants' demographic characteristics, anthropometric measurements, and laboratory parameters are shown in Table 1. The median age of the children in the study was 13.5 years (Range 8-18 years), and 47.2% were girls. There was no significant difference between the MASLD and control groups in terms of age, gender, education level, marital status of parents or family income status ($p>0.05$). Laboratory parameters including ALT (14.0 vs. 30.0, $p<0.001$), AST (20.0 vs. 27.0, $p<0.001$), GGT (13.0 vs. 24.0, $p<0.001$), ALP (125.0 vs. 161.0, $p=0.005$), HOMA-IR (1.37 vs. 3.48, $p<0.001$), and triglyceride (70.0 vs. 103.0, $p<0.001$) levels were higher in children with MASLD.

A Comparison of Scales Between the Children with MASLD and Control Groups

Children with MASLD demonstrated significantly higher total GSRS scores compared to the healthy controls [$p=0.001$, odds ratio (OR)=1.081]. Based on GSRS results (Table 2), symptoms of reflux, abdominal pain, indigestion, diarrhea, and constipation were all significantly more prevalent in the MASLD group. Table 2 also shows a comparison of the CSHQ scores between the children with MASLD and the control groups. The CSHQ scores revealed that the total scale score ($p<0.001$, OR=1.111), along with the subscale scores for bedtime resistance, sleep duration, sleep anxiety, night waking, parasomnias, and sleep-disordered breathing, were all statistically significantly worse in children with MASLD.

A total of 123 participants (69.9%) had clinically significant sleep disturbances according to the CSHQ. Sleep disturbances were present in 75 children (89.3%) with MASLD, compared to 48 children (52.2%) in the control group (p<0.001).

Factors Associated with Sleep Disturbances

The relationship between potential risk factors and sleep disturbances was re-evaluated using multivariable logistic regression to adjust for key metabolic confounders, including BMI SDS and HOMA-IR. The results of the adjusted analyses are presented in Table 3. In the initial model adjusted for demographic and metabolic factors only (Model 1), low family income [adjusted OR (aOR)=6.72, 95% CI 2.19-20.62, p=0.001] and higher BMI SDS (aOR=1.59, 95% CI 1.11-2.28, p=0.011) were significantly associated with sleep disturbances. When MASLD status was added to the model (Model 2), low family income remained a strong predictor (aOR=6.87, 95% CI 2.24-21.01, p=0.001), but the presence of MASLD itself was not statistically significant (aOR=3.67, 95% CI 0.55-24.58, p=0.181). In the final, fully adjusted model (Model 3), which also included gastrointestinal symptom severity, low family income (aOR=9.56, 95% CI 2.80-32.57, p<0.001), higher GSRS total score (aOR=1.15 per point, 95% CI 1.06-1.24, p<0.001), and higher HOMA-IR (aOR=1.51, 95% CI 1.01-2.27, p=0.045) emerged as independent factors associated with sleep disturbances. The presence of MASLD was not a significant predictor in this model (aOR=0.51, 95% CI 0.05-4.81, p=0.554).

Correlation Between Digestive Symptoms and Sleep Disturbances in Children with MASLD

The analysis revealed distinct correlation patterns between the symptom scales and liver disease indices. No correlation was

Table 1. Socio-demographics characteristics and laboratory parameters children with MASLD and healthy controls

Parameters	Healthy controls (n=92)	Children with MASLD (n=84)	p value
Gender, female, n (%)	44 (47.8%)	39 (46.4%)	0.881
Age, years, mean±SD	13.3±2.5	13.7±2.6	0.813
Child's schooling status, n (%)			
Primary school	22 (23.9)	15 (17.9)	0.398
Secondary school	44 (47.8)	38 (45.2)	
High school	26 (28.3)	31 (36.9)	
Marital status of parents, n (%)			
Married	82 (89.1)	76 (90.5)	0.768
Divorced	10 (10.9)	8 (9.5)	
Family income status, n (%)			
Good	10 (10.9)	9 (10.7)	0.811
Average	54 (58.7)	53 (63.1)	
Poor	28 (30.4)	22 (26.2)	
BMI, kg/m^2, mean±SD	19.8±3.3	32.7±6.1	<0.001*
BMI z score, median (IQR)	0.07 (-0.83-0.58)	2.85 (2.39-3.25)	<0.001*
ALT, U/L, median (IQR)	14.0 (11.0-17.0)	30.0 (21.0-54.75)	<0.001*
AST, U/L, median (IQR)	20.0 (16.0-23.0)	27.0 (20.0-36.75)	<0.001*
GGT, U/L, median (IQR)	13.0 (11.0-16.0)	24.0 (18.0-32.25)	<0.001*
ALP, U/L, median (IQR)	125.0 (98.0-184.0)	161.0 (104.0-249.0)	0.005*
Albumin, g/dL, mean±SD	4.3±0.5	4.1±0,4	0.265
Total cholesterol, mg/dL, mean±SD	138.1±28.9	153.3±33.8	0.001*
Triglyceride, mg/dL, median (IQR)	70.0 (59.5-90.0)	103.0 (77.7-150.5)	<0.001*
HDL cholesterol, mg/dL, mean±SD	55.1±10.8	44.5±11.0	<0.001*
LDL cholesterol, mg/dL, mean±SD	77.7±21.8	88.0±26.8	0.006*
HOMA-IR, median (IQR)	1.37 (1.10-1.92)	3.48 (2.60-6.05)	<0.001*

*Statistically significant
BMI: body mass index, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyltransferase, ALP: alkaline phosphatase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, HOMA-IR: homeostesis model assessment-estimated insulin resistence, MASLD: metabolic dysfunction-asociated steatotic liver disease, SD: standard deviation, IQR: interquartile range.

Table 2. A comparison of gastrointestinal symptom rating scale and child sleep habits questionnaire scores between the children with MASLD and healthy controls

	Children with MASLD (n=84)	Healthy controls (n=92)	Odds ratio	95% Confidence interval	p value
CSHQ, mean (SD)					
Bedtime resistance	10.8 (2.7)	9.6 (2.5)	1.190	1.059-1.338	0.003*
Sleep-onset delay	1.8 (0.8)	1.6 (1.3)	-	-	0.217
Sleep duration	5.4 (1.8)	4.3 (1.2)	1.364	1.149-1.618	0.001*
Sleep anxiety	6.3 (1.7)	5.1 (1.9)	1.327	1.129-1.561	<0.001*
Night wakings	4.7 (1.4)	3.8 (1.1)	1.603	1.259-2.041	<0.001*
Parasomnias	11.3 (3.1)	8.6 (2.4)	1.404	1.221-1.616	<0.001*
Sleep-disordered breathing	5.0 (1.4)	3.5 (1.2)	1.672	1.344-2.081	<0.001*
Total score	54.9 (10.7)	43.8 (10.1)	1.111	1.071-1.153	<0.001*
GSRS, mean (SD)					
Reflux	3.9 (1.2)	2.9 (0.9)	1.209	1.046-1.397	0.004*
Abdominal pain	7.6 (2.2)	5.1 (1.6)	1.238	1.120-1.369	0.001*
Indigestion	9.7 (2.3)	6.5 (2.1)	1.174	1.085-1.271	0.003*
Diarrhea	6.4 (1.9)	3.8 (1.1)	1.479	1.239-1.765	<0.001*
Constipation	5.9 (1.8)	4.3 (1.3)	1.215	1.078-1.370	0.001*
Total score	33.6 (9.6)	22.6 (6.2)	1.081	1.047-1.115	0.001*

*Statistically significant
CSHQ: child sleep habits questionnaire, GSRS: gastrointestinal symptom rating scale, MASLD: metabolic dysfunction-associated steatotic liver disease, SD: standard deviation

Table 3. Logistic regression analysis of factors associated with sleep disturbances in children

Variable	Model 1 aOR (95% CI)	p value	Model 2 aOR (95% CI)	p value	Model 3 aOR (95% CI)	p value
Age	1.024 (0.867-1.209)	0.782	1.022 (0.863-1.209)	0.804	0.974 (0.804-1.178)	0.783
Gender (Male)	0.896 (0.379-2.116)	0.802	0.999 (0.414-2.410)	0.997	1.120 (0.407-3.082)	0.826
Family Income (Low)	6.721 (2.191-20.620)	0.001	6.867 (2.244-21.012)	0.001	9.555 (2.803-32.569)	<0.001
BMI z-score	1.591 (1.112-2.277)	0.011	1.142 (0.628-2.078)	0.664	1.602 (0.791-3.244)	0.191
HOMA-IR	1.446 (0.998-2.096)	0.051	1.350 (0.931-1.956)	0.114	1.514 (1.010-2.271)	0.045
MASLD (Presence)	-	-	3.665 (0.547-24.580)	0.181	0.507 (0.053-4.812)	0.554
GSRS Total Score	-	-	-	-	1.146 (1.063-1.236)	<0.001

Model 1: Adjusted for age, gender, family income, BMI z-score, and HOMA-IR
Model 2: Adjusted for all variables in Model 1 plus the presence of MASLD
Model 3: Adjusted for all variables in Model 2 plus the total gastrointestinal symptom rating scale (GSRS) score
aOR: adjusted odds ratio, CI: confidence interval, MASLD: metabolic dysfunction-associated steatotic liver disease, GSRS: gastrointestinal symptom rating scale

found between the total GSRS score and the HSI, and among the GSRS subgroups, only reflux showed a significant link to HSI. Similarly, the total CSHQ score was not correlated with HSI, with only its sleep-onset delay and sleep duration subscales demonstrating a significant, albeit weak, relationship. In contrast, while the total GSRS and total CSHQ scores also showed no correlation with the NFS, specific subscales suggest a different story. For NFS, positive correlations were identified with the GSRS subgroups of reflux and diarrhea. Furthermore, a broad range of sleep issues were associated with NFS, including bedtime resistance, sleep-onset delay, sleep duration, sleep anxiety, night wakings, and parasomnias. The strength of these correlations was mostly weak, except for the relationships between NFS and both sleep duration and sleep anxiety, which were of moderate strength (Table 4).

Discussion

The results of our study suggest that children with MASLD had a higher prevalence of sleep disturbances and gastrointestinal symptoms compared to healthy children. However, after adjusting for key metabolic confounders, including BMI and HOMA-IR, the strongest independent risk factors for sleep disturbance were low family income, increased gastrointestinal

Table 4. Correlation between hepatic steatosis index and NAFLD fibrosis score with CSHQ and GSRS scores in children with MASLD

	Hepatic steatosis index		NAFLD fibrosis score	
	r	p value	r	p value
CSHQ				
Bedtime resistance	0.035	0.374	0.220	0.025*
Sleep-onset delay	0.211	0.027*	0.210	0.031*
Sleep duration	0.250	0.011*	0.301	0.003*
Sleep anxiety	0.103	0.177	0.361	<0.001*
Night wakings	0.074	0.250	0.208	0.032*
Parasomnias	0.013	0.453	0.247	0.014*
Sleep-disordered breathing	0.017	0.438	0.016	0.446
Total score	0.081	0.233	0.097	0.196
GSRS				
Reflux	0.260	<0.008*	0.291	0.004*
Abdominal pain	0.046	0.339	0.017	0.440
Indigestion	0.008	0.472	0.144	0.101
Diarrhea	0.154	0.081	0.204	0.034*
Constipation	0.003	0.489	0.000	0.997
Total score	0.042	0.351	0.110	0.166

Data are presented as Spearman's correlation coefficient (r_s) and corresponding p values
*Statistically significant
CSHQ: child sleep habits questionnaire, GSRS: gastrointestinal symptom rating scale, MASLD: metabolic dysfunction-associated steatotic liver disease

symptom severity, and higher HOMA-IR. NFS, which reflects liver fibrosis risk, was significantly associated with multiple subdimensions of both sleep disturbances and gastrointestinal symptoms in children with MASLD, whereas the relationships with the HSI were more limited. Our findings demonstrate that the clinical implication of MASLD extends beyond the liver and is intertwined with sleep and gastrointestinal health, with these extra-hepatic manifestations being more strongly linked to underlying metabolic dysfunction and socioeconomic factors than to the hepatic diagnosis alone.

A meta-analysis by Yu et al. (22) reported that individuals with NAFLD commonly experience sleep disorders, primarily short sleep duration. A recent large-scale study also identified a strong association between sleep disorders and MASLD (23). In line with these reports, we observed a strong unadjusted association between MASLD and sleep disturbances. However, after adjustment for BMI and HOMA-IR, this association was attenuated and lost statistical independence. This suggests that the link between MASLD and poor sleep may be largely mediated by the underlying obesity and insulin resistance that characterize MASLD, rather than hepatic steatosis per se. Although not directly measured in our study, the pro-inflammatory state and gut dysbiosis associated with obesity and insulin resistance (24) are known to enhance systemic cytokines (TNF-α, IL-1β, IL-6), which may cross the blood-brain barrier, trigger neuroinflammation, and potentially disrupt sleep-regulating pathways (25,26).

A key finding from our adjusted analysis (Model 3) was that low family income, gastrointestinal symptom severity (GSRS score), and HOMA-IR each emerged as independent risk factors for sleep disturbance. The strong and independent association of the gastrointestinal symptom score with sleep problems suggests a potential direct link between gut discomfort and sleep disturbance in children, irrespective of a MASLD diagnosis. Gastrointestinal discomfort, particularly reflux, is common in individuals with MASLD and obesity (7,27). Symptoms such as nocturnal reflux, abdominal pain, or bloating can directly interrupt sleep, prolong sleep onset latency, and reduce sleep depth. The significantly higher "night wakings" and "sleep onset delay" subscale scores in our MASLD group support this mechanism. Furthermore, increased gastrointestinal symptoms and chronic abdominal discomfort are thought to be associated with a shift toward sympathetic dominance in the autonomic nervous system (28). Although we did not assess autonomic function, such a shift, if present, could theoretically exacerbate symptoms and simultaneously suppress parasympathetic activity, which is crucial for initiating and maintaining sleep, thereby potentially contributing to sleep disorders (29).

The persistent and strong association between low family income and sleep disturbances across all our regression models underscores the profound influence of socioeconomic status as a key social determinant of health. This relationship likely operates through multiple interconnected pathways. Firstly, financial strain can create a chronically stressful home environment, elevating hormones responsive to stress, like cortisol, that disrupt circadian rhythms and sleep architecture. Secondly, socioeconomic constraints often translate into suboptimal sleep conditions, including crowded living spaces, noise pollution, and lack of a consistent bedtime routine. Thirdly, lower income is frequently associated with poorer dietary quality, characterized by high consumption of processed foods, which can negatively impact both sleep quality and MASLD progression. Therefore, low family income should not be viewed merely as a demographic variable but as a marker of broader environmental and psychosocial adversity that was correlated with sleep health in our cohort.

The analysis revealed distinct correlation patterns between the symptom scales and the two hepatic indices. While the total scores of CSHQ and GSRS did not correlate with either index, the subscale analysis provided more nuanced insights. The HSI, primarily reflecting the degree of fat accumulation, showed limited and weak correlations with only a few specific subscales

(sleep-onset delay, sleep duration, and reflux). In contrast, the NFS, which estimates the risk of advanced fibrosis and encapsulates a broader profile of metabolic dysfunction (including age, BMI, and diabetes), demonstrated significant correlations with a wider array of both sleep disturbances (bedtime resistance, sleep-onset delay, sleep duration, sleep anxiety, night wakings, and parasomnias) and gastrointestinal symptoms (reflux and diarrhea). The strength of these correlations was notably higher for sleep anxiety and sleep duration. This divergence suggests that the extra-hepatic manifestations in sleep and gastrointestinal domains may be more closely linked to the underlying metabolic burden and disease progression risk (as approximated by fibrosis risk scores) than to the presence of hepatic steatosis. This interpretation aligns with the existing literature. A meta-analysis reported that obstructive sleep apnea is associated with progressive hepatic fibrosis (30), and a recent study showed a link between sleep duration and hepatic steatosis (31). Zong et al. (32) found that later bedtimes were associated with the degree of liver fibrosis, suggesting that circadian disruption may adversely affect metabolic hormones and promote fibrosis. Furthermore, our finding that NFS correlated with sleep disturbances, as well as with gastrointestinal symptoms like reflux and diarrhea, could indicate that advancing disease severity (fibrosis risk) may exacerbate extra-hepatic manifestations by amplifying systemic inflammation.

Our findings align with the understanding of pediatric MASLD as a manifestation of a broader metabolic dysfunction. Although the diagnosis of MASLD itself was not an independent sleep risk factor after metabolic adjustment, the cluster of conditions that include obesity, insulin resistance, gastrointestinal symptoms, and sleep disturbances frequently co-exist. The strong independent associations of gastrointestinal symptoms and insulin resistance with sleep problems highlight potential direct mechanistic pathways beyond hepatic steatosis. Furthermore, the fact that the severity of liver disease, as reflected by the NFS, showed broader and stronger correlations with both sleep and gastrointestinal symptoms, suggests that the systemic burden of the underlying metabolic disorder may be greater in those with a higher risk of disease progression. This pattern of associations reinforces the clinical recommendation that a child presenting with obesity and MASLD should be evaluated within a multidisciplinary framework that proactively screens for and manages sleep disturbances, gastrointestinal symptoms, and insulin resistance, addressing the root metabolic and socioeconomic contributors to improve overall health and quality of life.

Study Limitations

The strengths of our study include: (i) well-characterized patient and control groups, (ii) good socio-demographic matching between groups, (iii) the use of valid and reliable scales (CSHQ, GSRS), and (iv) comprehensive statistical analyses examining relationships through multivariate analyses. However, the limitations of the study can be listed as follows. First, the cross-sectional design may limit our ability to establish direct causal relationships between variables. Second, the CSHQ and GSRS scores are based on parent- or self-reports, which, due to participants' subjective perceptions, could introduce bias. Moreover, the simultaneous use of these subjective instruments assessing the same recall period (past week) raises the possibility of common method bias, which could inflate the observed associations between gastrointestinal symptoms and sleep disturbances. Third, we did not collect data on hypertension, a component of metabolic syndrome that could influence both sleep architecture and gastrointestinal motility. Fourth, pubertal stage was not assessed using standardized methods (e.g., Tanner staging). Although age was included as a covariate in all analyses and was similar between groups, the potential confounding effect of pubertal hormonal changes on our outcomes cannot be ruled out. Fifth, the diagnosis and grading of hepatic steatosis relied on ultrasonography, which is operator-dependent. Although all scans were performed by a single experienced radiologist to minimize inter-observer variability, we did not formally assess intra-observer reliability, which is a methodological limitation. Sixth, the absence of an obese control group without MASLD limits our ability to determine whether the observed associations with sleep and gastrointestinal symptoms are specific to MASLD pathophysiology or are related to obesity more broadly. Future studies including this control group would help clarify the unique contribution of hepatic steatosis and metabolic dysfunction.

Clinical Implications and Future Directions

Our findings carry implications for the management of children with MASLD. First, they promote a shift from a purely hepatocentric view to a holistic, multi-system assessment. We suggest that routine clinical evaluation of children with MASLD should incorporate screening for sleep disturbances and gastrointestinal symptoms. Particular attention should be paid to patients from lower socioeconomic backgrounds and those with pronounced insulin resistance or gastrointestinal symptoms, as these factors were independently associated with sleep problems. Second, management strategies should be equally comprehensive. Addressing sleep hygiene, treating specific gastrointestinal symptoms, notably refluxand diarrea, and intensifying efforts to improve insulin sensitivity through lifestyle interventions could be associated with improvements in both quality of life and metabolic health outcomes in these children. A proposed screening algorithm to identify children with MASLD at high risk for sleep disturbances, based on our key findings, is presented in Figure 2.

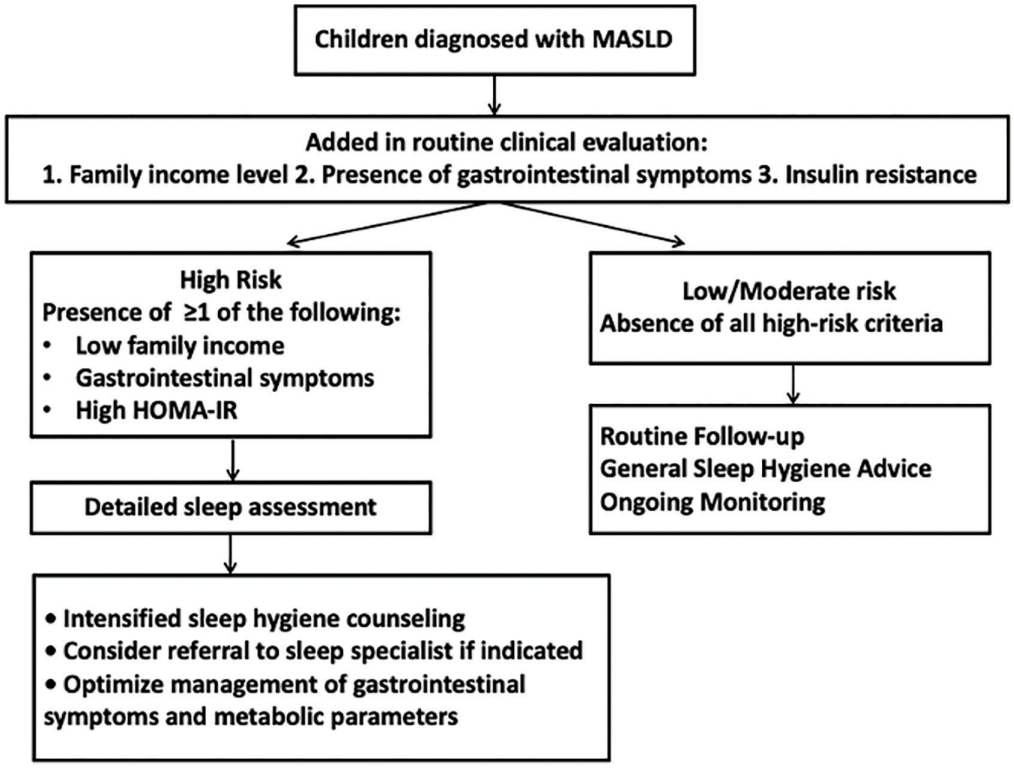

Figure 2. Proposed clinical screening algorithm for identifying children with metabolic dysfunction-associated steatotic liver disease who are at high risk for sleep disturbances

Conclusion

This study demonstrated a high prevalence of sleep disturbances and gastrointestinal symptoms in children with MASLD. After adjusting for key metabolic confounders, sleep disturbances were independently associated with low family income, gastrointestinal symptom severity, and insulin resistance. The risk of liver fibrosis, as indicated by the NFS, was further associated with a broader range of sleep and gastrointestinal symptoms. These cross-sectional findings indicate that in children with MASLD, sleep problems show a stronger association with underlying metabolic dysfunction, especially insulin resistance, and concurrent gastrointestinal distress than with MASLD. Therefore, the clinical management of children with MASLD should adopt a holistic approach that includes routine screening for sleep disturbances and gastrointestinal symptoms, with particular attention to those from lower socioeconomic backgrounds and/or with evidence of insulin resistance. Future longitudinal studies are needed to examine the impact of managing sleep, gastrointestinal symptoms, and insulin resistance on the progression of MASLD and to investigate underlying mechanisms, that may include cytokine profiles, gut microbiota, and autonomic nervous system function.

Ethics

Ethics Committee Approval: The study protocol was approved by the Antalya Training and Research Hospital Scientific Ethics Committee for Medical Research (date: 05.12.2024, approval no.: 19/21) prior to commencement.

Informed Consent: Informed consent in this study was taken from all participants.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: İshak Abdurrahman Işık, Atike Atalay, Nazlı Sivil, Memduha Sarı, Arzu Aras, Coşkun Fırat Özkeçeci, Hicran Altın, Ulaş Emre Akbulut, Concept: Behzat Özkan, Ulaş Emre Akbulut, Design: Behzat Özkan, Mehmet Özen, İsmail Topal, Ulaş Emre Akbulut, Data Collection or Processing: Behzat Özkan, İshak Abdurrahman Işık, Atike Atalay, Nazlı Sivil, Memduha Sarı, Arzu Aras, Coşkun Fırat Özkeçeci, Hicran Altın, Ulaş Emre Akbulut, Analysis or Interpretation: Behzat Özkan, İshak Abdurrahman Işık, Atike Atalay, Nazlı Sivil, Memduha Sarı, Arzu Aras, Coşkun Fırat Özkeçeci, Hicran Altın, Mehmet Özen, İsmail Topal, Ulaş Emre

Akbulut, Literature Search: Behzat Özkan, İshak Abdurrahman Işık, Atike Atalay, Nazlı Sivil, Memduha Sarı, Arzu Aras, Coşkun Fırat Özkeçeci, Hicran Altın, Mehmet Özen, İsmail Topal, Ulaş Emre Akbulut, Writing: Behzat Özkan.

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Mucocutaneous Findings and Endocrinopathies in Children with Turner Syndrome: A Cross-Sectional Study

Selami Aykut Temiz[1], Filiz Cebeci[2], Aslıhan Çiçekli[1], Begüm Işık[3], Sevil Savaş Erdoğan[4], Selma Emre[5], Hilal Kaya Erdoğan[6], İsa An[7], Sezgi Sarıkaya Solak[8], Sevilay Kılıç[9], Eda Öksüm Solak[10], Arzu Ataseven[1], Beray Selver Eklioğlu[11], Suna Kılınç[12], Mehmet Boyraz[13], Enver Şimşek[14], Tuğba Çetin Kontbay[15], Filiz Tütüncüler[16], Durmuş Doğan[17], Ülkü Gül Şiraz[18], Güllü Eren[19]

[1]Necmettin Erbakan University Faculty of Medicine, Department of Dermatology, Konya, Türkiye
[2]University of Health Sciences Türkiye, Kartal Dr. Lütfi Kırdar City Hospital, Clinic of Dermatology, İstanbul, Türkiye
[3]University of Health Sciences Türkiye, Yozgat City Hospital, Clinic of Dermatology, Yozgat, Türkiye
[4]University of Health Sciences Türkiye, Sultan 2. Abdulhamid Han Training and Research Hospital, Clinic of Dermatology, İstanbul, Türkiye
[5]Ankara Yıldırım Beyazıt University Faculty of Medicine, Department of Dermatology, Ankara, Türkiye
[6]Eskişehir Osmangazi University Faculty of Medicine, Department of Dermatology, Eskişehir, Türkiye
[7]Şanlıurfa Training and Research Hospital, Department of Dermatology, Şanlıurfa, Türkiye
[8]Trakya University Faculty of Medicine, Department of Dermatology, Edirne, Türkiye
[9]Çanakkale Onsekiz Mart University Faculty of Medicine, Department of Dermatology, Çanakkale, Türkiye
[10]Erciyes University Faculty of Medicine, Department of Dermatology, Kayseri, Türkiye
[11]Necmettin Erbakan University Faculty of Medicine, Department of Pediatric Endocrinology, Konya, Türkiye
[12]University of Health Sciences Türkiye, Zeynep Kamil Women and Children Diseases Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye
[13]Ankara Yıldırım Beyazıt University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Türkiye
[14]Eskişehir Osmangazi University Faculty of Medicine, Department of Pediatric Endocrinology, Eskişehir, Türkiye
[15]Şanlıurfa Training and Research Hospital, Clinic of Pediatric Endocrinology, Şanlıurfa, Türkiye
[16]Trakya University Faculty of Medicine, Department of Pediatric Endocrinology, Edirne, Türkiye
[17]Çanakkale Onsekiz Mart University Faculty of Medicine, Department of Pediatric Endocrinology, Çanakkale, Türkiye
[18]Erciyes University Faculty of Medicine, Department of Pediatric Endocrinology, Kayseri, Türkiye
[19]Necmettin Erbakan University Faculty of Medicine, Department of Public Health, Konya, Türkiye

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Corresponding Author: Assoc. Prof. Selami Aykut Temiz, Necmettin Erbakan University Faculty of Medicine, Department of Dermatology, Konya, Türkiye
E-mail: aykutmd42@gmail.com **ORCID:** orcid.org/0000-0003-4878-0045
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ABSTRACT

Objective: To investigate the frequency of skin, hair, nail, and mucosal findings in children with Turner syndrome (TS) and their associations with coexisting endocrinopathies.

Methods: A cross-sectional study was conducted with TS patients who were followed up by pediatric endocrinology and referred to the dermatology outpatient clinic. Data were collected using standardized dermatological examination forms, including demographic information, clinical features, presence of endocrinopathies, and medication usage. Statistical analysis was used to evaluate differences between groups and to analyze relationships between variables.

Results: The cohort consisted of 112 girls with TS. Skin and hair findings were detected in 86.6% of the patients, with melanocytic nevi (44.6%) and xerosis (41.1%) being the most commonly observed. Oral mucosal findings were observed less frequently (17.0%). Nail findings were detected in 63.4% of the cases, with leukonychia (15.2%) and subungual hyperkeratosis (14.3%) being the most prevalent. Older age, delayed diagnosis, longer follow-up duration, and lower body mass index were associated with an increased frequency of skin and hair findings ($p<0.05$). Moreover, the presence of coexisting endocrinopathies was significantly associated with skin and hair findings. Nail findings were significantly associated with longer follow-up duration ($p=0.002$), the presence of endocrinopathies ($p<0.001$), and comorbidities ($p=0.004$).

Conclusion: This study confirmed that skin, hair, and nail abnormalities are commonly observed in TS. The association of these findings with endocrinopathies suggests that systemic factors influence dermatological problems in TS. It is recommended to integrate dermatological evaluations into the routine endocrine and cardiological follow-up of children with TS.

Keywords: Turner syndrome, skin findings, endocrinopathies, melanocytic nevus, nail findings

What is already known on this topic?

Turner syndrome (TS) is characterized by a constellation of morphological clinical features, including short stature, webbed neck, low posterior hairline, and widely spaced nipples. Existing literature documents associations between TS and increased melanocytic nevi, halo nevi, ichthyotic changes, pilomatricoma, café-au-lait spots, and hypertrichosis. Endocrinopathies are common in Turner syndrome. The relationship between these endocrinopathies and cutaneous findings is not clear in the literature.

What this study adds?

Oral mucosal findings were present in 17% of TS children. The most frequent findings were geographic tongue, fungiform papilla hypertrophy, atrophic glossitis, and scrotal tongue. There are no known studies specifically examining oral mucosal findings in TS patients, making our study the only one on the subject. In this study, nail findings were seen in 63.4%, with leukonychia most frequently observed, followed by subungual hyperkeratosis and pincer nail, a characteristic TS finding. The significant association between skin and hair findings and concomitant endocrinopathies ($p=0.001$) observed in this study supports the hypothesis of an association.

Introduction

Turner syndrome (TS) is a genetic disorder that affects females and is caused by the complete or partial loss of one X chromosome and is one of the most common chromosomal anomalies (1). The syndrome is characterized by a range of morphological clinical features (stigmata), including short stature, webbed neck, low posterior hairline, and widely spaced nipples (1). Affected individuals frequently present with linear growth retardation, gonadal dysgenesis, hypothyroidism, osteoporosis, and skeletal, cardiovascular, and renal anomalies. Early-onset sensorineural hearing loss, as well as neurocognitive and psychosocial issues, are also common systemic manifestations (2).

Although skin, hair, nail, and mucosal changes are commonly seen in TS, these findings are often under-recognized during patient follow-up. In the largest multicenter cohort reported from Turkey, dermatological problems were reported in 21.8% of cases (3). Existing literature has documented associations between TS and increased melanocytic nevi, halo nevi, ichthyosiform changes, pilomatricomas, café-au-lait spots, and hypertrichosis (4,5,6,7). However, a comprehensive understanding of the relationship between these dermatological findings and coexisting endocrinopathies remains limited. Moreover, the increased predisposition to autoimmunity in TS may elevate the risk of conditions such as vitiligo and alopecia areata (8,9). Nevertheless, strong evidence-based data supporting these associations are lacking.

The aim of this study was to comprehensively evaluate skin, hair, nail, and mucosal findings in children and adolescents with TS followed in a pediatric endocrinology clinic and to investigate the potential associations between these findings and coexisting endocrinopathies. Enhancing the knowledge

base regarding these findings may contribute to improving diagnostic and therapeutic approaches for children with TS.

Materials and Methods

This study involved a cross-sectional assessment of cutaneous, hair, nail, and mucosal findings in girls diagnosed with TS, all of whom were followed by a pediatric endocrinologist and subsequently referred to the dermatology outpatient clinic. Findings were collected from nine tertiary care centres. Exclusion criteria included: age over 18 years, male sex, pregnancy, lactation, prior epilation, and use of oral contraceptives, corticosteroids, cyclosporine, or spironolactone within the preceding three months.

All participants underwent thorough dermatological evaluations performed by a senior dermatologist, using standardized examination forms and routine dermatological examination techniques. Pertinent clinical data, including age, age at diagnosis, follow-up duration, medication history, height, weight, body mass index (BMI), comorbidities, and endocrinopathies (diabetes, hyperlipidemia, hypertension, thyroid disease), were systematically recorded. Standardized methods were consistently employed for dermatological evaluations across all participants. Informed consent was obtained from all participants and their parents prior to enrollment. Ethical approval was granted by the Necmettin Erbakan University Non-Drug and Non-Medical Device Research Ethics Committee (approval no.: 2021/3023, date: 22.01.2021).

Statistical Analysis

Data analysis was performed using SPSS (Statistical Package for the Social Sciences), version 18.0 (SPSS Inc., Chicago, ILL, USA). Statistical methods were employed to assess intergroup differences and analyze relationships between variables. Descriptive statistics included frequencies (n) and percentages (%) for categorical variables, and means±standard deviations (SD) and interquartile ranges (1st-3rd quartile) for numerical variables. Categorical variable distributions were examined using Pearson's chi-square test and Fisher's exact test. For non-normally distributed numerical variables, comparisons between two independent groups were performed using the Mann-Whitney U test, and comparisons among more than two independent groups were conducted using the Kruskal-Wallis test. In cases where the Kruskal-Wallis test yielded significant results, post-hoc analysis was performed using the Mann-Whitney U test with Dunn-Bonferroni correction. A *p*-value of <0.05 was considered indicative of statistical significance for all analyses.

Results

This study encompassed 112 children and adolescents diagnosed with Turner syndrome. Comprehensive examinations were conducted to assess skin, hair, oral mucosa, and nail characteristics. Table 1 presents the distribution of demographic and clinical characteristics. Karyotype analysis revealed the presence of 45,X in 48.2% (n=54) of cases, mosaic X chromosome in 32.1% (n=36), and X chromosome anomalies in 7.1% (n=8). Comorbidities were evident in 39.3% (n=44) of the Turner syndrome cohort, with cardiopathy representing the most common comorbidity (15.2%, n=17). Growth hormone (GH) therapy constituted the predominant treatment modality, administered to 50.0% (n=56) of the patients.

Dermatological findings are summarized in Table 2. Skin and hair abnormalities were identified in 86.6% (n=97) of the cohort, with melanocytic nevus (44.6%, n=50), xerosis (41.1%, n=46), and keratosis pilaris (25.9%, n=29) representing the most

Table 1. Demographic and clinical characteristics of Turner syndrome cases

Variables		Results (n=112)
Age (years); (mean±SD; IQR)		13.33±4.60 (11-18)
Sex; n (%)	**Female**	112 (100.0)
Karyotype; n (%)	45,X Mosaicism X chromosome anomaly Unknown	54 (48.2) 36 (32.1) 8 (7.1) 14 (12.5)
BMI; (mean±SD)		23.11±18.90
Age at diagnosis (years); (mean±SD; IQR)		6.93±5.01 (1-11)
Follow-up duration (months); (mean±SD; IQR)		79.17±64.61 (24-120)
Comorbidities; n(%) Cardiopathy Obesity Kidney anomaly Mental retardation Celiac disease Hyperlipidemia Selective IgM deficiency Allergic rhinitis FMF Hypertension ALL		44 (39.3) 17 (15.2) 7 (6.3) 7 (6.3) 7 (6.3) 5 (4.5) 3 (2.7) 2 (1.8) 2 (1.8) 1 (0.9) 1 (0.9) 1 (0.9)
Endocrinopathies; n (%) GH deficiency Hyperthyroidism Hypothyroidism Diabetes Osteoporosis		74 (66.1) 40 (35.7) 26 (23.2) 16 (14.3) 6 (5.4) 2 (1.8)
Medication use[a] Growth hormone therapy n (%) Estrogen n (%) Thyroid medication n (%) Other n (%)		 56 (50.0) 25 (22.3) 15 (13.4) 13 (11.6)

Footnote: a) The medication categories are not mutually exclusive; some cases involve the use of more than one medication.
BMI: body mass index, FMF: Familial Mediterranean Fever, ALL: acute lymphocytic leukemia, GH: growth hormone, IQR: interquartile range

frequent findings. Oral mucosal findings were detected in 17.0% (n=19) of participants, with geographic tongue and fungiform papilla hypertrophy each observed in 3.6% (n=4). Nail findings were observed in 63.4% (n=71) of all cases, the most common ones were, leukonychia (15.2%, n=17), subungual hyperkeratosis (14.3%, n=16), and pincer nail (10.7%, n=12).

Table 3 shows the distribution of skin, hair, and oral mucosal findings in relation to demographic and clinical characteristics. The prevalence of skin and hair abnormalities exhibited statistically significant elevations in cases characterized by older age, delayed diagnosis, longer follow-up duration, and lower BMI (p<0.001, p=0.001, p=0.002, p=0.049, respectively). Moreover, cases with concomitant endocrinopathy demonstrated significantly increased prevalence of skin and hair abnormalities (p=0.001). Older age and lower BMI were statistically associated with the prevalence of oral mucosal findings (p=0.002, p=0.009, respectively).

Table 2. Distribution of dermatological findings in patients

Variables	Results (n=112)	Variables	Results (n=112)
Skin and hair findings; n (%)	97 (86.6)		
Melanocytic nevus	50 (44.6)		
Xerosis	46 (41.1)		
Keratosis pilaris	29 (25.9)		
Seborrheic dermatitis	27 (24.1)		
Pruritus	21 (18.8)		
Striae	18 (16.1)	**Nail findings; n (%)**	71 (63.4)
Acne	15 (13.4)	Leukonychia	17 (15.2)
Telogen effluvium	9 (8.0)	Subungual hyperkeratosis	16 (14.3)
Dysplastic nevus	7 (6.3)	Pincer nail	12 (10.7)
Atopic dermatitis	7 (6.3)	Unguis ıncarnatus	9 (8.0)
Hypopigmentation	7 (6.3)	Longitudinal ridging	8 (7.1)
Café-au-lait spots	7 (6.3)	Terry's nails	8 (7.1)
Psoriasis	6 (5.4)	Brittle nails	8 (7.1)
Hirsutism	5 (4.5)	Transverseridging	7 (6.3)
Pityriasis alba	5 (4.5)	onycholysis	6 (5.4)
Pityriasis versicolor	5 (4.5)	Pitting	5 (4.5)
Traction alopecia	4 (3.6)	Increased nail curvature	4 (3.6)
Urticaria	4 (3.6)	Trachyonychia	3 (2.7)
Eczema	3 (2.7)	Onychophagia	2 (1.8)
Congenital nevus	3 (2.7),	Onychomadesis	1 (0.9)
Hemangioma	3 (2.7)	Beau's lines	1 (0.9)
Alopecia areata	3 (2.7)	Koilonychia (Spoon nails)	1 (0.9)
Androgenetic alopecia	3 (2.7)	Muehrcke's lines	1 (0.9)
Hyperpigmentation	3 (2.7)	Mees' lines	1 (0.9)
Lymphedema	3 (2.7)	Lindsay's nails	1 (0.9)
Acanthosis nigricans	2 (1.8)	Congenital malalignment	1 (0.9)
Verruca (Wart)	2 (1.8)	Racquet nail	1 (0.9)
Halo nevus	1 (0.9)	Other	4 (3.6)
Skin tag	1 (0.9)		
Trichotillomania	1 (0.9)		
Herpes labialis	1 (0.9)		
Tinea pedis	1 (0.9)		
Keloid	1 (0.9)		
Other	11 (9.8)		
Oral mucosal findings; n (%)	19 (17.0)	**Affected nail location; n (%)**	
Geographic tongue	4 (3.6)	Hand	12 (10.7)
Fungiform papilla hypertrophy	4 (3.6)	Foot	29 (25.9)
Atrophic glossitis	3 (2.7)	Both hand and foot	30 (26.8)
Scrotal tongue	3 (2.7)		
Candidiasis	2 (1.8)		
Morsicatio buccarum	2 (1.8)		
Gingival hypertrophy	2 (1.8)		
Aphthous ulcer	2 (1.8)	**Number of affected nails; (mean±SD; IQR)**	6.81±6.75 (0.7-10.0)
Angular cheilitis	1 (0.9)		
Exfoliative cheilitis	1 (0.9)		
Gingival bleeding	1 (0.9)		
Lichen planus	1 (0.9)		

SD: standard deviation, IQR: interquartile range

Table 4 presents the distribution of nail findings in relation to demographic and clinical characteristics. Nail finding prevalence was significantly more common in patients with longer follow-up duration (p=0.002) and a higher prevalence of associated comorbidities and endocrinopathies (p=0.004, p<0.001, respectively). A significant difference was found in age distribution according to the affected nail location (p=0.044). Participants with hand nail findings only were younger than those with both hand and foot nail findings (p<0.05). In girls using medications, the prevalence of foot nail findings was significantly more common (p=0.014).

Discussion

This study evaluated the frequency of mucocutaneous findings in children with TS and associations with demographic and clinical variables. Skin and hair findings were detected in more than four fifths, while nail findings were observed in approximately two-thirds of patients. Oral mucosal findings were observed at a much lower frequency (17.0%). Our findings suggest that the mucocutaneous spectrum in this TS cohort was broad, and skin-hair and nail findings should be considered together with accompanying clinical features. The detrimental effects of endocrine disorders on the skin, hair, and mucosal

Table 3. Distribution of skin, hair, and oral mucosal findings according to demographic and clinical characteristics

	Skin & hair findings			Oral mucosal findings		
	Present	Absent	p value	Present	Absent	p value
Age (years); (mean±SD; IQR)	14.36±4.71 (12-17)	6.67±6.45 (1-12)	**<0.001***	16.42±4.61 (15-20)	12.70±5.59 (10-17)	**0.002***
Karyotype; n (%) 45,X Mosaicism X chromosome anomaly	 42 (77.8) 34 (94.4) 7 (87.5)	 12 (22.2) 2 (5.6) 1 (12.5)	0.096**	 9 (16.7) 6 (16.7) 0 (0.0)	 45 (83.3) 30 (83.3) 8 (100.0)	0.455**
BMI; (mean±SD; IQR)	21.05±4.47 (17.9-23.5)	36.38±49.78 (15.1-23.6)	**0.049***	22.85±4.06 (20.4-25.8)	23.16±20.68 (17.1-22.9)	**0.009***
Age at diagnosis (years); (mean±SD; IQR)	7.53±4.91 (3-12)	2.86±3.65 (0-5)	**0.001***	7.53±5.94 (1-12.5)	6.82±4.85 (2.5-11.0)	0.285*
Follow-up duration (months); (mean±SD; IQR)	84.99±64.15 (36-120)	39.29±54.41 (12-39)	**0.002***	90.06±62.28 (42-144)	77.18±65.15 (24-120)	0.407*
Associated comorbidity; n (%)	39 (88.6)	5 (11.4)	0.612**	10 (22.7)	34 (77.3)	0.191**
Associated endocrinopathy; n (%)	70 (94.6)	4 (5.4)	**0.001****	15 (20.3)	59 (79.7)	0.193**
Medication use; n (%)	76 (89.4)	9 (10.6)	0.190***	16 (18.8)	69 (81.2)	0.556***

n (%); mean±SD; IQR, *Mann-Whitney U test, **Pearson chi-square test, ***Kruskal-Wallis test
SD: standard deviation, IQR: interquartile range

Table 4. Distribution of nail findings according to demographic and clinical characteristics

	Nail findings			Affected nail location			
	Present	Absent	p value	Hand	Foot	Hand+Foot	p value
Age (years); (mean±SD; IQR)	14.27±5.32 (11-18)	11.71±5.75 (8-16)	0.068*	12.58±3.26 (11-15)	13.38±4.98 (10-17)	15.80±6.00 (12-20)	**0.044*****
BMI; (mean±SD; IQR)	21.27±4.38 (17.9-23.8)	26.28±30.69 (16.5-21.9)	0.064*	21.78±6.35 (17.2-23.1)	20.83±4.45 (17.2-23.9)	21.49±3.39 (18.7-24.1)	0.641***
Age at diagnosis (years); (mean±SD; IQR)	6.44±4.69 (1.5-7.0)	7.76±5.46 (1.0-12.5)	0.197*	8.00±3.43 (5-11)	5.90±4.83 (0.4-9)	6.37±4.95 (1-11)	0.454***
Follow-up duration (months); (mean±SD; IQR)	92.71±67.36 (38-150)	56.39±53.03 (15-78)	**0.002***	57.09±40.71 (36-72)	87.32±67.96 (24-150)	110.80±70.12 (60-162)	0.064***
Comorbidity; n (%)	35 (79.5)	9 (20.5)	**0.004****	4 (11.4)	18 (51.4)	13 (37.1)	0.170**
Endocrinopathy; n (%)	57 (77.0)	17 (23.0)	**<0.001****	9 (15.8)	24 (42.1)	24 (42.1)	0.850**
Medication use; n (%)	56 (65.9)	29 (34.1)	0.332**	12 (21.4)	25 (44.6)	19 (33.9)	**0.014****

n (%); mean±SD; IQR, *Mann-Whitney U test, **Pearson chi-square test, ***Kruskal-Wallis test
SD: standard deviation, IQR: interquartile range

tissues are well known (10,11). In Turner syndrome, frequently encountered thyroid disorders and growth retardation may also be more commonly associated with mucocutaneous findings (6,8). Indeed, in our study, the significantly higher frequency of skin–hair findings associated with the presence of an endocrinopathy (p=0.001) supports this observation.

While skin–hair findings were associated with older age, diagnostic delay, and presence of endocrinopathy; oral mucosal findings were more closely associated with older age and low BMI. Nail findings significantly increased, particularly in the presence of endocrinopathy and comorbidities, suggesting that nail changes may be one of the most sensitive dermatological indicators of systemic involvement in Turner syndrome.

Prevalence data regarding dermatological findings in Turner syndrome vary widely due to methodological differences. Studies based on medical record reviews report lower prevalence rates, while dermatology-focused studies using detailed physical examination or patient/parent reporting have shown rates of findings such as xerosis and melanocytic nevi reaching 70-80% (3,8,12). This discrepancy may be explained because mild or subclinical findings are not always recorded as “clinical problems” during routine follow-up visits. In the present study, the use of a standardized dermatological examination form by a dermatologist likely contributed to the higher detection rates. Furthermore, the differences may also be affected by referral bias and inter-center variability in assessment practices.

The most common skin finding in our study cohort was melanocytic nevi, observed in about half of the patients. Benign acquired nevi were most frequently seen, while dysplastic and congenital nevi were less common. Although previous reports have indicated an increased prevalence of halo nevi in Turner syndrome, this was found in only one case in our study (9). Earlier studies have shown that the average number of nevi is higher in individuals with TS compared to the general population (3,8). Considering that the total number of melanocytic nevi is an independent risk factor for melanoma, it would be appropriate to include regular nevus monitoring in the follow-up of individuals with TS. However, there is currently no evidence suggesting a significantly increased risk of melanoma in girls and women with TS (4,13,14,15).

Growth hormone (GH) therapy is a commonly used approach in the management of short stature in TS and was also the most frequently applied treatment in our study. Some reports have suggested that GH treatment may increase the number of nevi in TS (16,17,18). However, the causal nature of this relationship remains unclear and further studies are needed to determine whether this is due to growth axis abnormalities or the therapy itself.

The frequent occurrence of xerosis cutis and keratosis pilaris among the most common dermatological findings suggests that keratinization processes may be widely affected in TS. As Borroni et al. (8) reported, skin dryness in TS should not only be considered as non-specific xerosis but also as a manifestation of ichthyosis-like keratinization disorders . In a more recent study, 78.7% of individuals with TS reported complaints of dry, scaly, or flaky skin, with this being one of the factors most affecting quality of life (12). Nonetheless, the pathophysiological relationship between xerosis and TS has not yet been fully elucidated.

A large-scale cohort study conducted in Denmark showed that inflammatory skin diseases, such as psoriasis, atopic dermatitis, and seborrheic dermatitis, were reported at higher rates in individuals with TS compared to the general population (19). In the present study, the relatively high frequency of seborrheic dermatitis (24.1%) may be considered consistent with this literature, suggesting that GH and the insulin-like growth factor (IGF) axis affect sebaceous gland function and sebum production (20) However we were not able to test whether TS patients who received GH treatment had higher frequency of thes problems than those who did not receive GH treatment .

The detection of acne vulgaris in 13.4% of cases in our study suggests that this finding occurs at a relatively low frequency in TS, consistent with existing literature (21). This may be related to reduced sebum production, low peripheral androgen levels, and the suppression of testosterone and dihydrotestosterone levels due to hormone replacement therapy in TS patients (21,22).

Lymphedema is frequently observed at birth in Turner syndrome and tends to regress in most cases by the age of two. Therefore, its low detection rate (2.7%) in our study was an expected finding.

Among hair-related dermatological findings, telogen effluvium was the most common (8.0%). Hormonal imbalances, such as estrogen deficiency, thyroid dysfunction, and abnormalities in the growth axis may affect the hair follicle cycle and increase transition to the telogen phase. This finding indicates that complaints related to hair should be systematically questioned during dermatological evaluations in TS patients.

Oral mucosal findings were observed in 17.0% of TS cases in our study. The most common findings included geographic tongue, hypertrophy of fungiform papillae, atrophic glossitis, and fissured tongue. Data evaluating oral mucosal findings in a systematic manner in TS are limited; therefore, our findings contribute to the existing evidence of this association. The positive association of oral mucosal findings with age and their negative association with BMI are noteworthy. This relationship may be linked to secondary nutritional deficiencies and micronutrient deficits. Particularly, findings such as atrophic glossitis and angular cheilitis are classic indicators of micronutrient deficiencies.

However, due to the lack of biochemical assessments in our study, further investigations are needed to confirm these associations.

Nail findings were observed in 63.4% of children with Turner syndrome in our study. The most frequent nail finding was leukonychia, followed by subungual hyperkeratosis and pincer nails, which are considered characteristic of TS. The significantly higher frequency of nail findings in the presence of endocrinopathy and their strong association with comorbidities suggest that nail changes may be a sensitive marker of systemic disease burden in Turner syndrome.

Circulatory disorders, hormonal imbalances, and connective tissue anomalies commonly reported in TS may negatively affect keratin production and nail structure. Hypoplastic and dysplastic nails have also been reported to be associated with lymphedema in the literature (23). Moreover, the wide and short foot structure common in TS, lymphedema, and hyperextension of the big toe may predispose individuals to mechanical trauma due to increased shoe pressure, potentially contributing to the development of leukonychia, subungual hyperkeratosis, and pincer nails (24).

In the present study, the observation that nail findings were more frequent in cases with a longer follow-up duration, and that patients with only fingernail involvement were younger than those with both hand and foot involvement, suggests that nail findings may be related to cumulative systemic and mechanical effects over time. In light of these findings, we believe that nail examination should not be neglected in the dermatological evaluation of individuals with Turner syndrome and should be considered a potential marker of systemic involvement.

Study Limitations

The main limitation of this study is the lack of a control group, which prevents direct comparison of the observed mucocutaneous findings with their frequencies in the general population. In addition, the cross-sectional design does not allow for causal inference between dermatological findings and endocrinopathies.

Endocrinopathies and comorbidities were recorded based on clinical diagnoses established by pediatric endocrinology. The presence of thyroid autoantibodies, hormone levels, IGF-1 measurements, and/or dynamic endocrine tests were not analyzed within the scope of this study. Thus, findings such as the higher reported frequency of hyperthyroidism compared to hypothyroidism may reflect differences in clinical records or classification rather than true prevalence. Considering that short stature in TS is often due to functional impairment of the growth axis rather than biochemical GH deficiency, some cases recorded as "GH deficiency" may instead reflect growth failure.

Endocrinopathies were not analyzed according to subtypes. The presence of multiple endocrine disorders in many cases may have introduced confounding effects. The complexity resulting from this and the lack of sufficient statistical power for subgroup analyses limited the ability to isolate specific associations between dermatological findings and individual endocrine conditions. Lastly, the absence of biochemical evaluations related to nutritional status and possible micronutrient deficiencies, such as iron, B-complex vitamins, and zinc, limited the interpretation of the association between low BMI and oral mucosal findings.

Conclusion

Endocrinopathies in individuals with TS were associated with an increase in the frequency of dermatological findings, particularly those involving the skin, hair, and nails. Therefore, in addition to routine endocrinology and cardiology follow-ups, regular dermatological evaluations may be appropriate for the optimal care of girls, adolescents and women with TS.

Ethics

Ethics Committee Approval: Ethical approval was granted by the Necmettin Erbakan University Non-Drug and Non-Medical Device Research Ethics Committee (approval no.: 2021/3023, date: 22.01.2021).

Informed Consent: Informed consent was obtained from all participants and their parents prior to enrollment.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Concept: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Design: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Begüm Işık, Sevil Savaş Erdoğan, Selma Emre, Hilal Kaya Erdoğan, İsa An, Sezgi Sarıkaya Solak, Sevilay Kılıç, Eda Öksüm Solak, Arzu Ataseven, Beray Selver Eklioğlu, Suna Kılınç, Mehmet Boyraz, Enver Şimşek, Tuğba Çetin Kontbay, Filiz Tütüncüler, Durmuş Doğan, Ülkü Gül Şiraz, Güllü Eren, Data Collection or Processing: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Begüm Işık, Sevil Savaş Erdoğan, Selma Emre, Hilal Kaya Erdoğan, İsa An, Sezgi Sarıkaya Solak, Sevilay Kılıç, Eda Öksüm Solak, Arzu Ataseven, Beray Selver Eklioğlu, Suna Kılınç, Mehmet Boyraz, Enver Şimşek, Tuğba Çetin Kontbay, Filiz Tütüncüler, Durmuş Doğan, Ülkü Gül Şiraz, Güllü Eren, Analysis or Interpretation: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Begüm Işık, Sevil Savaş Erdoğan, Selma Emre, Hilal Kaya Erdoğan, İsa An, Sezgi Sarıkaya Solak, Sevilay Kılıç, Eda Öksüm Solak, Arzu Ataseven, Beray Selver Eklioğlu, Suna Kılınç, Mehmet Boyraz, Enver Şimşek, Tuğba Çetin Kontbay, Filiz Tütüncüler, Durmuş Doğan, Ülkü Gül Şiraz, Güllü Eren, Literature Search: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli, Writing: Selami Aykut Temiz, Filiz Cebeci, Aslıhan Çiçekli.

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Social Cognition in Adolescents with Gender Dysphoria and Congenital Adrenal Hyperplasia: A Preliminary Investigation of Biological vs. Experiential Gender Effects

Begüm Yuluğ-Taş[1], Burcu Özbaran[2], İpek İnal-Kaleli[2], Nurhak Doğan[3], Arzu Jalilova[4], Samim Özen[5], Sezen Köse[2], Damla Gökşen[5], Şükran Darcan[5], Tezan Bildik[2]

[1]University of Health Sciences Türkiye, İzmir Tepecik Education and Research Hospital, Clinic of Child and Adolescent Psychiatry, İzmir, Türkiye
[2]Ege University Faculty of Medicine, Department of Child and Adolescent Psychiatry, İzmir, Türkiye
[3]UT Health Science Center at Houston, Department of Psychiatry and Behavioral Sciences, Houston, TX 77030, USA
[4]Acıbadem City Hospital, Clinic of Child and Adolescent Pediatric Endocrinology, İzmir, Türkiye
[5]Ege University Faculty of Medicine, Department of Child and Adolescent Pediatric Endocrinology, İzmir, Türkiye

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ABSTRACT

Objective: To explore hormonal and neurodevelopmental influences on social cognition among individuals with gender dysphoria (GD), congenital adrenal hyperplasia (CAH), and typically developing (TD) controls.

Methods: Participants included individuals with GD, CAH, and TD individuals. Social cognition was assessed using the Faces Test (FT), Reading the Mind in the Eyes Test (RMET), and Unexpected Outcomes Test (UOT). Psychiatric comorbidities were evaluated via the Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children, depressive symptoms using the Children's Depression Inventory, autistic traits with the Autism Spectrum Screening Questionnaire, and Attention-Deficit/Hyperactivity Disorder (ADHD) symptoms through the ADHD Rating Scale.

Results: Participants included 34 GD, 29 CAH, and 35 TD. Psychiatric diagnoses were significantly more prevalent in the GD group, with major depressive disorder (64.7%) and ADHD (50%) being the most common ($p<0.001$). TD participants showed moderately better performance on RMET ($p=0.003$) and UOT ($p<0.001$) compared to GD and CAH, while CAH individuals scored lower on FT ($p=0.046$). Regression analyses revealed depressive symptoms ($B=-0.105$, $p=0.004$) and CAH status ($B=-2.221$, $p=0.003$) predicted RMET scores, while GD ($B=-3.232$, $p=0.022$) and CAH ($B=-7.974$, $p<0.001$) predicted lower UOT performance. FT regressions were nonsignificant.

Conclusion: Findings highlight the interplay of hormonal and psychosocial factors in social cognition, reiterating the need for nuanced, context-sensitive approaches to supporting social functioning and well-being in gender-diverse youth.

Keywords: Androgen exposure, congenital adrenal hyperplasia, gender dysphoria, neurodevelopmental disorders, social cognition, theory of mind

Corresponding Author: Begüm Yuluğ-Taş, MD, University of Health Sciences Türkiye, İzmir Tepecik Education and Research Hospital, Clinic of Child and Adolescent Psychiatry, İzmir, Türkiye
E-mail: yulugbegum@gmail.com **ORCID:** orcid.org/0000-0001-7289-4355
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What is already known on this topic?

It is well-established that prenatal androgens affect social cognition skills. Additionally, individuals with gender dysphoria often exhibit deficits in social cognition, with accompanying psychiatric comorbidities influencing these impairments.

What this study adds?

A key strength of this study lies in its approach to social cognition skills, examining them from both perceptual and cognitive perspectives while incorporating a neurodevelopmental framework. The homogeneity of the participant groups represents a positive aspect; however, the crosssectional design and small sample size impose limitations on the generalizability of the findings.

Introduction

"Sex" is a phenotype influenced by chromosomes, reproductive anatomy, and sex steroids (1). Sex differentiation occurs during early intrauterine stages and concludes in the first days of life (2). Gender identity is the personal acceptance of one's gender, independent of genetic or societal influences (3), while gender dysphoria (GD) refers to the incongruence between assigned sex at birth and experienced gender (4). The development of GD has been linked to genetic (5), hormonal (6), and neurobiological factors (7). Although not directly associated with congenital adrenal hyperplasia (CAH), a condition involving prenatal androgen exposure (8), testosterone exposure is linked to reduced theory of mind skills, such as understanding emotions and empathy (9), which are also impaired in individuals with GD.

Gender identity is a core aspect of personal and social identity (10), while social cognition involves mental processes related to social interactions (11). Social cognitive skills are measured by theory of mind, which includes social cognition (interpreting others' behavior) and social perception (awareness of others' mental states through observable information) (12). Impairments in these skills are characteristic of autism (13) and may also be influenced by sex-related factors, as well as other conditions such as anorexia nervosa (14) and Attention-Deficit/Hyperactivity Disorder (ADHD) (15).

Testosterone affects emotions, behaviors, and cognition, with high levels influencing social interactions (16). A 2017 study found a negative relationship between prenatal testosterone levels and social interaction skills (17). Despite not having hormonal imbalances, individuals with GD show more significant social-cognitive differences compared to the general population (18), leading to social rejection, bullying, and discrimination (19). A 2011 study found that 72% of individuals with GD, with no psychiatric co-occuring disorders, experienced suicidal thoughts (20). The prevalence of autism is higher in those with GD, with 6.4% of children, 7.8% of adolescents, and 5.5% of adults diagnosed with GD also having autism (21). The reasons for this co-occurrence remain unclear, with theories such as the hyper-masculinized brain theory and social exclusion being discussed (22).

Thus, considering the complex interaction of neurodiversity, gender and hormonal influences, the aim of the present study was to explore how different hormonal and neurodevelopmental pathways might intersect with social cognition outcomes in adolescence. Specifically, we compared three groups: (1) adolescents assigned female at birth diagnosed with GD, presumed to have developed under typical hormonal conditions but experiencing significant gender-related psychosocial stress; (2) adolescents with CAH, exposed to elevated prenatal androgens but without GD; and (3) adolescents assigned female at birth, developing under typical hormonal conditions without GD or CAH. Rather than viewing these groups as strict representations of "biological" versus "experiential" gender factors, we approached them as differing developmental contexts in which social cognition may be shaped. Our primary aim was to examine group-level differences in theory of mind performance and autistic traits, and to explore whether any observed differences could be explained by affective, neurodevelopmental, or other variables such as depressive symptoms, attention, and autistic features.

Methods

Participants and Study Design

The cross-sectional study was conducted in collaboration with the Departments of Child and Adolescent Psychiatry and Pediatric Endocrinology at Ege University, following ethical standards outlined in the Declaration of Helsinki and approved by the Ege University Faculty of Medicine Clinical Research Ethics Committee (approval no: 22-12T/56, date: 10.06.2021). All children and their parents were informed about the study, and written informed consent, or verbal assent when written consent was not feasible, was obtained.

Participants, aged 11-18, were selected as this age range is optimal for diagnosis and scale reliability. All participants were assigned female at birth. Exclusion criteria included developmental delay, neurological conditions, and intellectual disabilities.

GD Group

Participants were recruited from the clinical registry of individuals who had previously presented to the child and adolescent psychiatry clinic and disclosed gender-related distress. From this registry, eligible individuals were invited to participate. During the intake interview, six no longer met Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria for GD and were excluded. An additional nine participants identified as nonbinary were also excluded due to the study focus on androgen exposure and male gender identity (i.e., assigned female at birth with current male identification). GD diagnoses were confirmed through structured clinical interviews conducted by a certified child and adolescent psychiatrist and confirmed by a senior child and adolescent psychiatrist with over 30 years of clinical experience and extensive expertise in gender-related care and neurodevelopmental disorders. All participants had normal hormonal profiles, confirmed via Pediatric Endocrinology evaluation.

CAH Group

The comparison group consisted of individuals with CAH who were recruited through pediatric endocrinology outpatient clinics and had no reported GD. Individuals with a diagnosis of autism were excluded from both the GD and CAH groups to avoid confounding effects when assessing autistic traits.

Typically Developing Comparison Group

A third comparison group included females, assigned female at birth, with age-appropriate secondary sex characteristics, no endocrine abnormalities, and no medical or neurodevelopmental conditions. Participants were recruited through advertisements posted throughout Ege University and via snowball sampling. All applicants underwent endocrinological screening to confirm the absence of hormonal abnormalities, and the absence of psychiatric disorders was verified through the Kiddie-Schedule for Affective Disorders and Schizophrenia (K-SADS-PL) clinical interview.

Measures

Sociodemographic Data Form (SDF): The SDF was developed to gather information about the sociodemographic characteristics of the groups. It included variables such as the age and educational levels of the participants and their parents.

Kiddie-Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL): This form is used to evaluate lifetime comorbid psychopathologies in children (23). If diagnostic symptoms are identified during the initial interview, an additional evaluation checklist is administered. The presence and severity of positive findings are determined based on the clinician's, family's, and participant's input. The standardization of the form has been conducted for Turkish children (24).

Global Assessment Scale (GAS): The GAS evaluates variables such as the level of illness, social and occupational functioning, and coping mechanisms for adverse conditions, providing a measure of the individual's overall well-being and functionality. Developed by the creators of the K-SADS, the scale assigns a score between 0-100 based on the clinician's assessment, as described in the literature. A higher score indicates better overall well-being and functionality (25).

Faces Test (FT): The FT, developed by Ekman et al. (26) in 1972, evaluates social perception skills by measuring participants' ability to recognize emotions (26). The test involves showing participants 60 photographs depicting six basic facial expressions (happiness, sadness, anger, disgust, surprise, fear). Participants are asked to identify the emotion represented in each photograph, one at a time, without time restrictions. Responses are scored on a 0-1 scale and compared with a control group. The Turkish version of the FT has been validated and standardized (27).

Reading the Mind in the Eyes Test (RMET): RMET aims to measure the ability of the participant to recognize emotions and infer the meaning beyond them by observing the eyes in a photograph (28). Initially developed by Baron-Cohen et al. (28) in 1997, it was revised and improved in 2001 to assess the social perception component of social cognition as identifying the emotional state of another person, solely from the eyes, without the additional cues provided by the rest of the face, presents a significant challenge. It consists of 28 photographs showing only the eye region, each accompanied by four response options. The goal is to observe subtle aspects of an individual's theory of mind. The Turkish version has been validated and standardized (29).

Unexpected Outcomes Test (UOT): The UOT is designed to assess participants' social cognitive theory of mind abilities by presenting 12 short scenarios. In each scenario, participants are asked to infer what the individuals depicted might be feeling or thinking based on the context (30). The responses to the questions are scored between 0 and 2, with a total score of 24. If a participant answers incorrectly three consecutive times, the test is terminated. Research has demonstrated that the UOT correlates well with other measures of social cognition skills (31). The Turkish version of the UOT has been used in Turkish samples, with high inter-rater reliability reported (32).

Children's Depression Inventory (CDI): The CDI is a self-assessment scale developed to evaluate depressive symptoms in childhood. It is intended for children aged 6-17 and is based on the Beck Depression Inventory. The scale was originally developed by Kovacs (33) and has been standardized for Turkish

children (34).

Autism Spectrum Screening Questionnaire (ASSQ): The ASSQ consists of 27 statements and is used as a screening tool to identify and initiate early intervention for Autism Spectrum Disorder (ASD). A parent-report form initially designed to screen for Asperger's syndrome in school-age children, it was later renamed under the broader category of autism and recognized as a reliable screening tool in assessing autistic traits (35). Köse et al. (36) have established the validity and reliability of the scale in the Turkish population with a cut-off of 16 or higher being considered highly indicative of ASD in distinguishing clinical groups from controls.

ADHD Rating Scale: This scale comprises 41 items across four subdomains, designed to assess ADHD and its two frequent comorbidities, Oppositional Defiant Disorder and Conduct Disorder (CD) in alignment with its description and criteria outlined in the DSM-IV (37). Responses are filled out by the caregivers and scored on a scale from 0 to 3. The standardization of the scale for Turkish participants was conducted by Ercan et al. (38).

Procedure

All participants and their parents were interviewed in person, and sociodemographic data, including ages and educational levels, were collected. Comorbid psychiatric disorders were assessed using the K-SADS-PL by a certified child and adolescent psychiatrist. IQ was informally assessed through clinical interviews, developmental milestone reviews, and academic performance (e.g., school grades), offering a general understanding of cognitive abilities.

The GAS was used to evaluate the impact of comorbid psychiatric symptoms on functionality. Social cognition skills were assessed using the FT, RMET, and the UOT. Furthermore, participants completed the CDI, while parents filled out the ASSQ and the ADHD Rating Scale.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS version 25.0 (IBM Inc., Armonk, NY, USA). The normality of the data was assessed using the Shapiro-Wilk test, histograms, and Skewness-Kurtosis coefficients. Differences in CDI, ASSQ, ADHD scales, RMET, FT, and UOT scores among the GD, CAH, and TD groups were evaluated using the Kruskal-Wallis test. Post-hoc pairwise comparisons between groups were performed with Dunn's test. Nominal data were compared using the Pearson chi-square test. Bonferroni correction was applied for all cases of multiple comparisons. Pairwise comparisons were conducted only for variables with statistically significant group differences in the omnibus test ($p<0.05$). No post-hoc tests were performed for non-significant results. The relationships between the dependent variables (RMET, FT, and UOT total scores) and predictors such as GD and CAH diagnosis, and other social cognition factors that differed between groups were analyzed through linear regression. All statistical tests were two-tailed, with significance set at $p<0.05$.

Results

The mean age of the participants were 14.98±2.05 years. There were no statistically significant differences between sub-group participants' ages or between the ages of their respective parents ($p>0.05$). A difference in maternal education was observed ($p=0.001$). In the pairwise comparisons with Bonferroni correction, it was found that this discrepancy was due to the higher observed prevalence of tertiary education ($p=0.012$) and lower observed prevalence of primary education than expected ($p=0.001$) among mothers of the TD subgroup. The observed difference in paternal education ($p=0.030$) revealed no statistically significant differences between the groups after Bonferroni correction (Table 1).

The proportion of psychiatric diagnoses in the GD group were significantly higher compared to the CAH group ($p<0.001$). Major Depressive Disorder (MDD) was diagnosed in 64.7% of the GD group, followed by ADHD (50%). All psychiatric diagnoses are summarized in Table 2, with generalized anxiety disorder, social anxiety disorder, and separation anxiety disorder identified through the K-SADS-PL being grouped under the category of "Anxiety Disorders".

The GD group received the lowest total score on the GAS, while the controls received the highest score ($p<0.001$). The CDI total score ($p<0.001$) and the Inattention Subscale of the ADHD Scale ($p=0.004$) were significantly higher in the GD group compared to the other groups ($p<0.001$). The GD group also had higher scores in the ASSQ compared to TD ($p=0.003$). The psychiatric diagnoses, psychiatric symptomatology and related scales are summarized in Table 3.

Among the social cognition tests, significant differences were observed in the RMET scores ($p=0.003$). Pairwise comparisons showed that the TD group had higher scores compared to both the GD and CAH groups, with no significant difference between GD and CAH. The FT test also showed a significant difference ($p=0.046$), with the CAH group scoring lower than the TD group (Supplementary Table 1). However, there were no significant differences between groups on the six FT subscales, which correspond to the basic emotions of surprise, happiness, anger, sadness, disgust, and fear. Moreover, there were marked differences in the UOT test between the groups ($p<0.001$), with both GD and CAH groups scoring lower than the TD group, but no significant difference between GD and CAH (Table 4).

Three separate linear multiple regression models were analyzed to control for variables that differed between groups and might explain the variations in social cognition test results for RMET, FT, and UOT. The GD and CAH groups were dummy-coded, and ASSQ, CDI, and Inattention scores were included in all three models to account for autistic traits, depressive symptoms, and inattention, which could potentially impact social cognition. The first model for RMET yielded significant results with $F(5.92)=4.441$, $p=0.001$, $R^2=0.194$, and an Adjusted R^2 of 0.151. CDI scores (B=-0.105, p=0.004, CI=-0.175 to -0.034) and CAH diagnosis (B=-2.221, p=0.003, CI=-3.669 to -0.773) were identified as significant negative predictors of RMET scores. In contrast, the second model for the FT was not significant, with $F(5.92)=1.694$, $p=0.144$, $R^2=0.084$, and an Adjusted R^2 of 0.035. The third model for UOT was again significant with $F(5.92)=8.144$, $p<0.001$, $R^2=0.307$, and an Adjusted R^2 of 0.269. For UOT, being in the CD group (B=-3.232, p=0.022, CI=-5.988 to -0.476) and the CAH group (B=-7.974, p<0.001, CI=-10.542 to -5.405) were significant negative predictors. The significant results for these two models are summarized in Table 5.

Table 1. Sociodemographic data of the participants with GD, CAH, and TD

	GD (n=34)	CAH (n=29)	TD (n=35)	H/χ^2	p value
Participant age	15.59±1.54	16.34±2.66	14.91±1.77	4.677	0.096
Maternal age	45.97±6.13	42.72±6.45	44.23±5.81	4.012	0.135
Maternal education, n (%) Primary Secondary Tertiary	15 (44.1) 6 (17.6) 13 (38.2)	15 (51.7) 4 (13.8) 10 (34.5)	2 (5.7) 7 (20) 26 (74.3)	19.199	**0.001**
Paternal age	50.24±6.55	46.03±7.23	48.06±6.20	4.540	0.103
Paternal education, n (%) Primary Secondary Tertiary	15 (44.1) 5 (14.7) 14 (41.2)	9 (31) 5 (17.2) 15 (51.7)	4 (11.4) 12 (34.3) 19 (54.3)	10.649	**0.030**

GD: gender dysphoria, CAH: congenital adrenal hyperplasia, TD: typically developing

Table 2. Psychiatric diagnoses and psychiatric symptomatology across GD, CAH and TD groups

	GD (n=34)	CAH (n=29)	TD (n=35)	H/χ^2	p value
Psychiatric disorder (n, %)				10.708*	**0.001**
No	3 (8.8)	13 (44.8)	35 (100)		
Yes	31 (91.2)	16 (55.2)	0 (0)		
ADHD					
No	17 (50)	2 (86.2)	35 (100)		
Yes	17 (50)	4 (13.8)	0 (0)		
MDD					
No	12 (35.3)	25 (86.2)	35 (100)		
Yes	22 (64.7)	4 (13.8)	0 (0)		
OCD					
No	34 (100)	28 (96.6)	35 (100)		
Yes	0 (0)	1 (3.4)	0 (0)		
Anxiety disorder					
No	27 (79.4)	21 (72.4)	35 (100)		
Yes	7 (20.6)	8 (27.6)	0 (0)		
BPD					
No	33 (97.1)	29 (100)	35 (100)		
Yes	1 (2.9)	0 (0)	0 (0)		

*TD group was excluded from the analysis as the inclusion criteria for the TD group was the absence of any psychiatric disorders
GD: gender dysphoria, CAH: congenital adrenal hyperplasia, TD: typically developing, ADHD: attention-deficit/hyperactivity disorder, MDD: major depressive disorder, OCD: obsessive compulsive disorder, BPD: bipolar disorder

Although the CAH (androgen-exposed) group had significantly lower scores on the FT in group comparisons, regression analysis did not yield a significant overall model. While androgen exposure emerged as a statistically significant individual predictor (B=-3.096, p=0.030), this should be interpreted cautiously, as the model itself did not reach significance, [F(5.92)=1.694, p=0.144] summarized in Supplementary Table 1.

Discussion

The present study revealed significant findings, suggesting a potential impact of hormonal and experiential variations on social cognition. First, individuals in the CAH group demonstrated significant social cognition deficits on the RMET and UOT tests, suggesting a potential biological influence of prenatal androgen exposure. Although a statistically significant group difference was found in the total score of the FT, this result should be interpreted with caution, as none of the emotion-specific subscales showed significant group differences, and FT performance was not predicted by any group or clinical variables in regression analyses. Taken together, these findings suggest that individuals with CAH may experience difficulties in both affective and cognitive aspects of theory of mind, specifically in interpreting and predicting social outcomes and attributing complex emotional states to others, while basic emotion recognition abilities remain intact.

Second, the GD group exhibited deficits in social perception and cognitive theory of mind abilities as evidenced by their lower scores in UOT and RMET compared to the TD group. However, the key finding and a potential explanatory link is that depressive symptoms emerged as a significant predictor of RMET performance. Although GD status was not itself a predictor, the GD group had elevated depressive symptoms, suggesting that their lower RMET scores may be attributable to affective distress rather than gender identity alone. Notably, the TD group

Table 3. Psychiatric symptomatology across GD, CAH and TD groups

	GD (n=34)	CAH (n=29)	TD (n=35)	H/χ^2	p	p^1	p^2	p^3	
GAS	82.79±7.804	91.72±4.487	97.43±3.509	57.577	**<0.001**	**0.001**	**<0.001**	**0.001**	**3>2>1**
CDI	23.44±10.16	15.9±8.78	14.89±6.65	15.565	**<0.001**	**0.004**	**0.001**	**1.000**	**1>2, 1>3**
ASSQ	12.5±9.67	8.21±6.38	5.69±5.06	11.669	**0.003**	**0.282**	**0.002**	**0.335**	**1>3**
ADHD scale score									
Inattention	8.82±6.96	4.07±4.61	4.37±3.94	11.075	**0.004**	**0.006**	**0.029**	**1.00**	**1>2, 1>3**
Hyperactivity	5.36±6.1	2.66±3.65	3.17±3.0	3.113	0.211				
Opposition-defiance	6.41±5.84	3.48±3.6	4.54±4.02	4.068	0.131				
Conduct problems	2.09±5.96	0.38±0.98	0.29±0.71	4.884	0.087				

1: GD, **2:** CAH, **3:** TD, p^1: GD-CAH pairwise comparison, p^2: GD-TD pairwise comparison, p^3: CAH-TD pairwise comparison
GD: gender dysphoria, CAH: congenital adrenal hyperplasia, TD: typically developing, ADHD: attention deficit hyperactivity disorder, GAS: global assessment scale, CDI: children's depression inventory, ASSQ: autism spectrum screening questionnaire

Table 4. Differences in social cognition (affective and cognitive theory of mind) in GD, CAH and TD groups

	GD (n=34)	CAH (n=29)	TD (n=35)	H	p value	p^1	p^2	p^3	
RMET	19.91±3.127	19.38±3.234	21.69±2.564	11.520	**0.003**	1.000	**0.036**	**0.004**	**1<3, 2<3**
FT Total Score	44.29±5.562	42.48±6.18	45.69±4.801	6.151	**0.046**	0.403	0.942	**0.040**	2<3
Surprise	14.62±3.402	13.66±4.194	15.86±2.658	5.418	0.067				
Happiness	9.21±1.225	9.28±0.882	9.46±0.886	0.370	0.831				
Anger	6.94±1.347	6.55±1.723	6.83±1.272	0.929	0.628				
Sadness	6.5±1.619	6.31±1.815	7.0±1.534	3.532	0.171				
Disgust	6.65±1.368	6.28±1.533	6.31±1.183	1.451	0.484				
Fear	0.15±0.359	0.1±0.31	0.17±0.453	0.324	0.850				
UOT	19.50±4.98	15.52±7.37	23.46±1.482	45.916	**<0.001**	0.084	**<0.001**	**<0.001**	**1<3, 2<3**

1: GD, **2:** CAH, **3:** TD, p^1: GD-CAH pairwise comparison, p^2: GD-TD pairwise comparison, p^3: CAH-TD pairwise comparison
GD: gender dysphoria, CAH: congenital adrenal hyperplasia, TD: typically developing, RMET: reading the mind in the eyes test, FT: faces test, UOT: unexpected outcomes test

Table 5. Two separate models of multiple linear regression analysis for reading the mind through the eyes (RMET) and the unexpected outcomes test (UOT)

	B	Std. Error	Beta	t	p value	95% CI	
						LL	UL
Model 1 (RMET)							
GD	-0.758	0.782	-0.117	-0.968	0.335	-2.312	0.796
CAH	-2.221	0.729	-0.328	-3.046	**0.003****	-3.669	-0.773
CDI	-0.105	0.036	-0.316	-2.935	**0.004****	-0.175	-0.034
ASSQ	0.004	0.046	0.01	0.089	0.929	-0.087	0.095
Inattention	-0.034	0.062	-0.062	-0.544	0.588	-0.157	0.089
Model 3 (UOT)							
GD	-3.232	1.388	-0.26	-2.329	**0.022***	-5.988	-0.476
CAH	-7.974	1.293	-0.616	-6.166	**<0.001*****	-10.542	-5.405
CDI	-0.046	0.063	-0.073	-0.735	0.464	-0.172	0.079
ASSQ	0.02	0.081	0.026	0.241	0.81	-0.142	0.181
Inattention	-0.104	0.11	-0.1	-0.945	0.347	-0.322	0.114

*p<0.05; **p<0.01; ***p<0.001
Model 1 (RMET): F(5,92)=4.441, p=0.001, R^2=0.194, Adjusted R^2=0.151
Model 3 (UOT): F(5,92)=8.144, p<0.001, R^2=0.307, Adjusted R^2=0.269
GD: gender dysphoria, CAH: congenital adrenal hyperplasia, CDI: children's depression inventory, ASSQ: autism spectrum screening questionnaire, LL: lower limit; UL: upper limit.

consistently outperformed both the CAH and GD groups across all social cognition measures, highlighting the complex interplay of hormonal, neurodevelopmental, and societal influences on social cognition.

Neurodevelopmental Perspective

This study attempted to compare two neurodevelopmentally diverse groups, those with experiential (GD) or hormonal (CAH) variations in gender norms, who are known to face challenges in social cognition, with a comparison group exhibiting typical neuroendocrine development. Neurodevelopment is strongly shaped by endocrine status in conditions such as CAH, but it also follows its own trajectory in individuals without endocrine differences, as seen in the GD group. As such, we also sought to interpret the factors influencing social cognition abilities, approaching the social cognition construct from both a social perception and a social cognitive ability perspective, while also identifying neurodevelopmental parameters that could potentially influence these skills, such as neurodevelopmental disorders, traits, and comorbid psychiatric conditions.

To that end, the three groups were first compared regarding their psychiatric disorders, as well as ADHD and autistic traits. The GD group had significantly more psychiatric disorders, such as MDD and ADHD-inattention, which is in line with the extant literature (39). The GD group also exhibited more autistic traits in group comparisons.

The rationale for assessing neurodevelopmental disorders, traits, and psychiatric conditions was straightforward, as challenges in social cognition skills are often difficult to separate and interpret independently from other executive functions (40). This approach facilitated the disentanglement of which observed differences are more likely driven by hormonal biology and which reflect broader neurodevelopmental or psychiatric processes. For instance, the ASSQ, which was utilized in our study to identify autistic traits, does not directly or indirectly measure social cognition but does capture social difficulties in autism. However, the social challenges identified by this scale may arise from a range of factors, with theory of mind deficits being only one possible contributor. As such, the ASSQ was included in the regression models to control for trait-level neurodevelopmental variation. Beyond autistic traits alone, psychiatric comorbidities such as MDD and anxiety disorders also affect social cognition. A review suggested that individuals with depression tend to exhibit a mood-congruent bias toward social stimuli, meaning they interpret cues in a way that aligns with their depressive mood, and face difficulties in the cognitive aspects of theory of mind (41). In addition, anxiety and depressive symptoms are reported to influence the relationship between autistic symptomology and social cognition and adjustment in children diagnosed with ASD, further emphasizing the role of comorbidities in social functioning (42).

Biological Influences: The Role of Androgens

Building on the neurodevelopmental framework, the deficits observed in the CAH group provide insights into how prenatal androgen exposure impacts social cognition. Secondary analyses comparing social cognition across groups revealed that the TD comparison group outperformed both the CAH and GD groups on the RMET and UOT tests, and only the CAH group on the FT test. These findings align with existing literature (43) and are particularly interesting given the role of androgens in emotional processing and aggression (44).

While these results suggest significant differences between groups, further investigation into psychiatric comorbidities, such as depression and inattention identified during preliminary comparisons, was necessary. In regression analyses controlling for inattention, depression, and autistic traits, membership of the CAH group emerged as a significant predictor. This may reflect the biological effects of androgens on social cognition and emotional processing. However, the lack of formal IQ testing in this study means that the observed differences could also be related to the cognitive challenges associated with CAH, highlighting the need for further research using standardized IQ assessments or other cognitive measures.

An alternative explanation for CAH as a predictor in the RMET and UOT is the direct effect of androgens on social cognition (45). Research has shown that individuals with higher fetal testosterone levels score lower on the RMET, which tests theory of mind (46). This test assesses social perception skills, crucial for understanding and adapting to others' perspectives (47). As emotion recognition and social perception are closely related (48), our findings further support the role of hormonal influences in shaping social cognition.

These findings highlight the hormonal pathway affecting neurodevelopmental outcomes and that endocrine processes may influence social cognitive development.

The GD "Experience"

The biological effects of androgens on social cognition provide one perspective, but the GD group's results highlight how affective, neurodevelopmental, and experiential factors may also influence social perception and theory of mind abilities. Notably, while the GD group scored significantly lower than the TD group on the RMET in group comparisons, GD status did not emerge as a significant predictor in the regression model. Instead, CDI scores were a negative predictor of RMET performance in the model. This suggests that the lower social cognition scores in the GD group may be more influenced by depressive symptoms than by autistic traits or GD *per se*, implying that the issue is more about "dysphoria" than "gender" (41). An alternative perspective has been proposed by other studies, suggesting that performance on the RMET is negatively associated with autistic traits and may be indirectly related to the severity of GD (51). However, it is particularly important to note that this study did not account for depressive symptoms. In contrast, our findings indicate that when depression is controlled for, the association between autistic traits and RMET performance becomes non-significant, offering an alternative interpretation of the underlying mechanisms. The RMET does not solely assess theory of mind abilities; it also involves broader psychological processes such as cognitive speed, attention, motivation, and facial expression processing. This aligns with previous studies reporting that, even in the absence of pronounced deficits on the RMET, individuals with depression often exhibit overall impairments in social cognition (50). Furthermore, there is evidence suggesting that depressive symptoms negatively affect social cognitive performance (41).

This distinction is important for a group already facing marginalization, as difficulties in social cognition may further hinder social integration and peer relationships (51). Addressing depressive symptoms in adolescents with GD may reduce social anxiety, making it easier for them to connect with typically developing peers (52).

From a developmental standpoint, gender identity and social cognition evolve in parallel throughout childhood and adolescence. A central feature of theory of mind is the ability to distinguish between external appearance and internal reality, which is also relevant for navigating identity and societal expectations (53). While none of the participants met the diagnostic criteria for autism, their ASSQ scores suggested a greater presence of autistic traits. Prior research has investigated whether features such as intense interests or perspective-taking difficulties, often seen in autism, may interact with the development of GD (54). Indeed, autistic traits have been found to be overrepresented in individuals with GD, pointing to a complex, but not necessarily causal, relationship between gender diversity, neurodivergence, and social cognition (21).

However, our regression analyses suggest that these traits alone do not account for the observed differences in social cognition. Specifically, GD status was a significant predictor of UOT performance even when depressive symptoms, inattention, and autistic traits were controlled for. This distinguishes UOT from RMET, where depressive symptoms explained the variance. Given that the UOT taps into pragmatic theory of mind, such as inferring how beliefs guide future actions, RMET primarily measures social perceptual abilities in the affective ToM domain and FT targets emotion recognition, the altered response in UOT may reflect a unique cognitive-emotional adaptation shaped by lived experience in gender-diverse individuals.

In contrast to the CAH group, where social cognition differences may reflect hormonal factors, the GD group's performance pattern may have been more strongly influenced by the psychosocial context. The finding that both RMET and UOT were predicted by CAH reinforces the role of biological variables, while the specific association of the UOT with GD may suggest an experiential component, as both neurodivergence traits and depressive symptoms were accounted for in the regression model. Although the present cross-sectional study did not measure interactions with social norms directly, and as such can not infer causality, the results raise important questions about how long-term navigation of societal expectations might shape social cognition in ways that are not captured by trait-level neurodevelopmental metrics (55).

The contrast with CAH is of particular importance, as endocrine status appears to modify developmental trajectories in CAH, whereas in GD, divergent pathways seem to arise primarily through psychosocial and neurodevelopmental mechanisms in the absence of hormonal variation. Collectively, these findings highlight that social cognition is shaped through both biological and experiential influences, which may converge or remain distinct depending on the context.

For example, individuals with GD may process social scenarios differently, albeit not incorrectly, based on their personal histories and identity development. This could explain altered performance on UOT, which involves anticipating others' behavior in ambiguous contexts. The interpretation of this altered performance in UOT therefore, might be simply that, an alteration, rather than a deficit. As such, these results highlight the limitations of conventional social cognition tasks, which often rely on majority-norm assumptions and may not fully capture the breadth of human diversity. Labeling social cognition responses as "right" or "wrong" may reflect biases rooted in societal norms, marginalizing minority populations like those with GD or autism (56).

This perspective aligns with emerging neurodiversity frameworks, which argue that cognitive divergence, including that seen in GD, autism and many others, should not be pathologized but understood as reflecting different, equally valid ways of engaging with the world. Autistic individuals, for example, often interpret the world in ways that diverge from conventional norms (57). Similarly, binary thinking embedded in societal norms may not align with the experiences of those with GD as they may interpret and respond to social situations in ways shaped by their unique experiences with gender identity and social marginalization.

Rather than expecting neurodiverse individuals to adjust to rigid standards of "correct" social cognition, it may be more constructive for neurotypical society to develop greater cognitive flexibility. This shift can foster more inclusive environments that reduce stigma, promote understanding, and support diverse modes of social engagement. By framing differences in social cognition as contextually shaped rather than inherently impaired, we may move toward a more compassionate and accurate understanding of human variation.

Study Limitations

A key strength of this study lies in its approach to social cognition skills, examining them from both perceptual and cognitive perspectives while incorporating a neurodevelopmental framework. The homogeneity of the participant groups represents a positive aspect. However, the cross-sectional design and small sample size impose limitations on the generalizability of the findings.

The present study refers to androgen exposure but does not measure the degree or duration of such exposure. While categorical differences were observed, the precise extent of these differences remains undetermined. In addition, the sample was limited to individuals assigned female at birth, and no comparison group assigned male at birth was included. This limits the ability to generalize findings across sexes with different physiological androgen exposure. Moreover, the absence of a control group with a male gender identity but without GD prevents disentangling the effects of gender identity from those of dysphoria itself. Consequently, the observed effects cannot be attributed solely to androgen exposure or dysphoria, although the findings offer meaningful insights into social cognition in individuals with XX chromosomes who experience atypical gender development.

Furthermore, while the GD and CAH groups were recruited from clinical settings, the TD comparison group was drawn from the general population through school and community advertisements. This difference in recruitment sources may introduce sampling bias, particularly in terms of psychiatric morbidity or access to care. Although structured psychiatric interviews (K-SADS-PL) were used to ensure diagnostic consistency, the context of referral remains a potential confounder.

Social cognition is heavily influenced by cognitive abilities, including intelligence. The lack of a formal IQ assessment represents a significant limitation of this study, particularly when considering the potential impact of steroids on cognitive development, especially in the CAH group (58). Although clinical evaluations of intelligence were conducted, they were not formalized. Given the established role of intelligence as a predictor of social cognition, larger-scale studies incorporating formal IQ measurements and integrating these into explanatory models are necessary.

As such, this study does not offer a definitive explanation or modeling of social cognition but rather a springboard for larger-scale studies and future research that integrate hormonal and neurodevelopmental factors in this field.

Conclusion

This study highlights the distinct yet interconnected roles of biological and societal influences on social cognition. By addressing both the biological challenges faced by CAH individuals and the societal pressures experienced by GD individuals, tailored interventions can foster improved social integration and well-being for these populations.

Social cognition skills are far more complex than they may seem, influenced by multifactorial interactions. In our study, prenatal androgen exposure represented by the CAH group emerged as the primary factor disrupting social cognition skills. The presence of social deficits in the CAH group, despite the absence of autistic traits, suggests a biological influence. In contrast, in the GD group, social perception skills may be influenced by accompanying conditions such as depression and neurodevelopmental traits, such as ADHD comorbidity that was present in 50% of our GD group, while cognitive abilities -social or otherwise- appear to be shaped by their unique way of perceiving and experiencing the world.

Individuals with CAH are typically monitored by a multidisciplinary treatment team from early developmental stages, which may contribute to their lower rates of psychiatric comorbidities. In contrast, GD individuals often seek mental health services later in adolescence, after attempting to navigate and adapt to a world that struggles to accept differences. This delayed access to support may result in higher levels of psychiatric diagnoses.

Therefore, it is important to adopt not only a diagnostic but also a transdiagnostic approach when addressing these cases. These findings underscore the need for mental health interventions that address both biological and societal influences on social cognition, with tailored approaches for CAH and GD populations that emphasize early support and societal acceptance.

Ethics

Ethics Committee Approval: The ethics committee approval was obtained from the Ege University Faculty of Medicine Clinical Research Ethics Committee (approval no: 22-12T/56, date: 10.06.2021).

Informed Consent: All children and their parents were informed about the study, and written informed consent, or verbal assent when written consent was not feasible, was obtained.

Prior Presentation: A portion of this study was presented at the 32nd National Child and Adolescent Mental Health and Diseases Congress in May, 10-13, 2023, İstanbul, Türkiye.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Begüm Yuluğ-Taş, Burcu Özbaran, İpek İnal-Kaleli, Nurhak Doğan, Arzu Jalilova, Samim Özen, Sezen Köse, Damla Gökşen, Şükran Darcan, Tezan Bildik, Concept: Begüm Yuluğ-Taş, Burcu Özbaran, Arzu Jalilova, Design: Begüm Yuluğ-Taş, Burcu Özbaran, Data Collection or Processing: Begüm Yuluğ-Taş, Burcu Özbaran, Arzu Jalilova, Samim Özen, Sezen Köse, Analysis or Interpretation: Begüm Yuluğ-Taş, İpek İnal-Kaleli, Nurhak Doğan, Literature Search: Begüm Yuluğ-Taş, Burcu Özbaran, İpek İnal-Kaleli, Nurhak Doğan, Sezen Köse, Damla Gökşen, Şükran Darcan, Tezan Bildik, Writing: Begüm Yuluğ-Taş, Burcu Özbaran, İpek İnal-Kaleli, Nurhak Doğan.

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Supplementary Table 1: https://d2v96fxpocvxx.cloudfront.net/c2f7718d-0796-4c18-a6d4-3c339e748b23/content-images/711d3f36-3dc8-43f2-835c-3b0cc64fe216.pdf

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ORIGINAL ARTICLE

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BMI-SDS Changes During GnRHa Therapy in 150 Girls with Idiopathic Central Precocious Puberty: Follow-up Through Final Height

Didem Helvacıoğlu, Büşra Gürpınar Tosun, Sefa Öğe, Serap Turan, Tülay Güran, Belma Haliloğlu, Zehra Yavaş Abalı, Abdullah Bereket

Marmara University Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Türkiye

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ABSTRACT

Objective: To evaluate longitudinal changes in body mass index-standard deviation score (BMI-SDS) in girls with central precocious puberty (CPP) treated with gonadotropin-releasing hormone analogues from treatment initiation to final adult height.

Methods: This retrospective study included 150 girls with idiopathic CPP treated with leuprolide acetate and followed to final adult height. BMI-SDS was assessed at treatment initiation, at 1 year of therapy, at treatment completion, and at final adult height. Patients were categorized according to BMI-SDS at the time of diagnosis as underweight, normal weight, overweight (OW), or obese (OB). BMI-SDS was evaluated at predefined time points and examined within baseline weight groups, and transitions between BMI-SDS categories were analyzed across the follow-up period. In addition to baseline weight status, participants were categorized as small for gestational age (SGA) or appropriate for GA (AGA) based on birth weight for gestational age.

Results: In normal-weight girls, BMI-SDS increased significantly during the first treatment year and then declined toward final height, with no difference between baseline and final height. BMI-SDS remained stable in those OW or OB at treatment initiation. BMI-category distribution changed over follow-up (overall p=0.014), OW+OB prevalence increased during treatment (48.6%→56.6%) and decreased by final height (45.3%) (baseline vs final p=0.533). By final height, OB increased (p=0.0076) and OW decreased (p=0.006), while normal-weight prevalence did not differ from baseline (p=0.098). BMI-SDS was lower in SGA than AGA at baseline, year 1, and treatment completion (p=0.04, p=0.04, and p=0.01, respectively), but not at final adult height (p=0.6). In multivariable analysis, baseline BMI-SDS was inversely related to ΔBMI-SDS (treatment end-baseline) (β=-0.174, 95% confidence interval -0.291 to -0.057; p=0.004).

Conclusion: In this large cohort of girls with idiopathic CPP followed through final adult height, BMI-SDS showed no sustained increase at final height across baseline weight groups, and the SGA-AGA differences observed earlier were not maintained. Baseline BMI-SDS was the key independent determinant of ΔBMI-SDS (treatment end-baseline), with lower baseline values predicting greater increases.

Keywords: Central precocious puberty, gonadotropin-releasing hormone analog, body mass index, long-term follow-up

Corresponding Author: Prof. Abdullah Bereket, Marmara University Faculty of Medicine, Department of Pediatric Endocrinology; Prof. Dr. Asaf Ataseven Hospital, İstanbul, Türkiye

E-mail: abereket@marmara.edu.tr **ORCID:** orcid.org/0000-0002-6584-9043

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What is already known on this topic?

Studies on the effect of gonadotropin-releasing hormone analogues treatment on weight and body mass index-standard deviation score (BMI-SDS) in idiopathic central precocious puberty (CPP) have reported conflicting results (increased, unchanged, or decreased BMI during therapy). Most did not account for baseline BMI-SDS category or birthweight status when evaluating BMI-SDS changes. Data on BMI-SDS trajectories through final adult height remain limited.

What this study adds?

This is one of the largest idiopathic CPP cohorts with follow-up to final adult height, stratified by pretreatment BMI-SDS and birthweight status. BMI-SDS showed no sustained increase at final height across baseline weight groups, and early small for gestational age or appropriate for gestational age differences were not maintained. Baseline BMI-SDS was the key independent predictor of ΔBMI-SDS when considering differences between treatment end and baseline values.

Introduction

Nutritional status during childhood plays an important role in pubertal timing and progression and may explain a substantial proportion of the variability in pubertal onset (1,2,3,4). In particular, overnutrition and obesity have been associated with earlier pubertal development, while impaired fetal growth and neonatal thinness have also been linked to earlier pubertal maturation (2). Central precocious puberty (CPP) is defined as the premature activation of the hypothalamic-pituitary-gonadal (HPG) axis before the age of 8 years in girls and 9 years in boys, leading to early secondary sexual characteristics, accelerated growth velocity, and advanced bone maturation (5). Gonadotropin-releasing hormone analogues (GnRHa) have been used in the treatment of CPP since the early 1980s and are currently considered the standard therapy. GnRHa therapy is generally safe and well tolerated. The most frequently reported adverse effects include local or systemic hypersensitivity reactions and mild hypoestrogenic symptoms, such as hot flushes, headache, and nausea (6,7).

Despite the well-established efficacy of GnRHa in controlling pubertal progression, its effects on body weight and body mass index (BMI) remain controversial. Some studies have reported an increase in body weight during GnRHa treatment (8,9), whereas, other studies have demonstrated no significant change in BMI standard deviation score (SDS) (10,11) or have even reported a decrease in BMI during therapy (12,13). Furthermore, most studies did not take the effect of initial pretreatment BMI category or birthweight status on changes in BMI-SDS during GnRHa therapy into account. Finally, data evaluating BMI-SDS trajectories extending to final adult height are limited.

Therefore, we aimed to evaluate longitudinal changes in BMI-SDS from treatment initiation through treatment completion and into final adult height in girls with idiopathic CPP treated with GnRHa. In addition, we investigated the effects of BMI-SDS at the beginning of therapy and being born small for gestational age (SGA) on BMI trajectories during GnRHa treatment.

Methods

Medical records of 150 girls diagnosed with CPP who were treated with GnRHa therapy using leuprolide acetate and followed until reaching final adult height at Marmara University Pediatric Endocrinology Clinic were retrospectively reviewed. The study was approved by the Marmara University Faculty of Medicine Department Research Ethics Committee (approval no.: 09.2022.486, date: 08.09.2023). All eligible participants and their parents provided informed consent.

CPP was defined as progressive breast development starting before 8 years of age, associated with accelerated growth (>6 cm/year) and skeletal maturation (advanced by at least one year) (3) and biochemical evidence of HPG-axis activation, defined as either basal luteinizing hormone (LH) >0.6 mIU/mL or a GnRH-stimulated peak LH ≥5 mIU/mL, as previously accepted in the literature and consensus statements (6,14). Uterine length >34 mm and ovarian volume >2 cm^3 were taken as supportive criteria (6,15). Fasting venous blood samples were obtained from all girls in the morning for follicle stimulating hormone, LH and estradiol (E2) measurements using chemiluminescent methods (Beckman Coulter Diagnostics, CA, USA).

Patients with organic causes of CPP or comorbid conditions known to affect BMI or pubertal development were excluded from this study. All patients received depot leuprolide acetate at a dose of 3.75 mg intramuscularly every 28 days. Height and weight were measured using a Harpenden stadiometer (Holtain Ltd. Crosswell, Crymych, Pembs, UK) and a calibrated digital scale (Seca GmbH. KG, Hamburg, Germany). BMI was calculated as weight divided by height squared (kg/m^2), and BMI-SDS were calculated using national reference data (16,17,18). Patients were classified according to baseline BMI-SDS into three weight

groups: normal weight (NW; BMI-SDS ≥-1 and <+1), overweight (OW; BMI-SDS ≥+1 and <+2), and obese (OB; BMI-SDS ≥+2). No patients had a BMI-SDS <-1 at treatment initiation. No structured lifestyle intervention (diet or supervised exercise program) was prescribed as part of the treatment protocol. Bone age was assessed by left-hand radiography according to the Greulich and Pyle method (19). Age at menarche and auxological measurements at menarche were recorded when available from the medical records. Final adult height was defined as a growth velocity of less than 1 cm/year together with a bone age of at least 15 years, in accordance with previously published criteria (20). BMI-SDS was evaluated at four predefined time points: treatment initiation, the end of the first year of treatment, treatment completion, and final adult height. Birth weight and gestational age data were obtained from medical records, and SGA was defined as a birthweight <-2 SD for gestational age (21). The primary outcome measure was the longitudinal change in BMI-SDS assessed at the four predefined time points. BMI-category distributions were compared across follow-up using paired categorical methods. In addition, BMI-SDS trajectories were analyzed according to baseline weight status and birthweight status to identify potential modifiers of BMI-SDS changes.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Normality of continuous variables was assessed using the Shapiro-Wilk test, and data are presented as mean±SD. Within-group changes in BMI-SDS over time were analyzed using repeated-measures ANOVA. Comparisons between independent groups were performed using the Independent Samples t-test or Mann-Whitney U test, as appropriate. Changes in BMI-category prevalence (underweight/normal/overweight/obese and OW+OB) across treatment initiation, treatment completion, and final adult height were analyzed using Cochran's Q test and post-hoc pairwise comparisons using McNemar's test with exact p values. A p value <0.05 was considered statistically significant. Baseline predictors of change in BMI-SDS (ΔBMI-SDS; end of treatment minus baseline) were assessed using multiple linear regression.

Results

A total of 150 girls with CPP who completed GnRHa treatment and reached final adult height were included in the analysis. The mean age at treatment initiation, completion of treatment, age of menarche and at final height were 8.0±1.2, 10.8±0.6, 12.4±1.2, and 18.6±2.4 years, respectively. At treatment initiation, most girls were of normal weight (n=77, 51.3%), while 57 girls (38.0%) were classified as overweight and 16 (10.7%) as obese. Although no girls were underweight at baseline, 18/150 (12.0%) were classified as underweight at final height, reflecting downward transitions during follow-up, primarily from baseline normal weight (n=15) and less commonly from baseline overweight (n=3) (Figure 1).

No patient required dose escalation during therapy. The mean duration of GnRHa treatment was 2.8±1.2 years. The mean age at treatment initiation differed significantly between the NW, OW, and OB groups (8.1±1.1, 8.1±1.0, and 7.1±1.8 years, respectively; p=0.01), whereas bone age at treatment initiation was comparable (9.6±1.6, 9.9±1.6, and 9.5±2.0 years, respectively; p=0.70). Compared with normal-weight girls, obese girls exhibited a more advanced bone age relative to chronological age and greater stature at treatment initiation. First-year growth velocity SDS did not differ significantly between baseline BMI groups, 0.4 (-0.5 to 0.8) in normal-weight girls, 0.2 (-0.6 to 0.7) in overweight girls, and 0.1 (-0.8 to 1.6) in obese girls (p=0.8). The baseline auxological characteristics of the cohort according to BMI category are summarized in Table 1.

When all patients were evaluated together, BMI-SDS changed significantly over time (p=0.001), with higher values at treatment completion compared with baseline (p=0.009), followed by a significant decrease toward final adult height (p=0.006). BMI-SDS at final adult height did not differ from baseline (p=0.13). When BMI-SDS changes were evaluated according to pre-treatment BMI categories, in the normal-weight group, BMI-SDS increased during the first year of treatment and remained elevated at treatment completion (p <0.01), followed by a significant decrease toward final adult height, resulting in comparable BMI-SDS with baseline. In contrast, BMI-SDS remained stable throughout treatment and follow-up in both the OW and OB groups, with no significant overall change across the four time points (p=0.4 and p=0.3, respectively) (Tables 2 and 3).

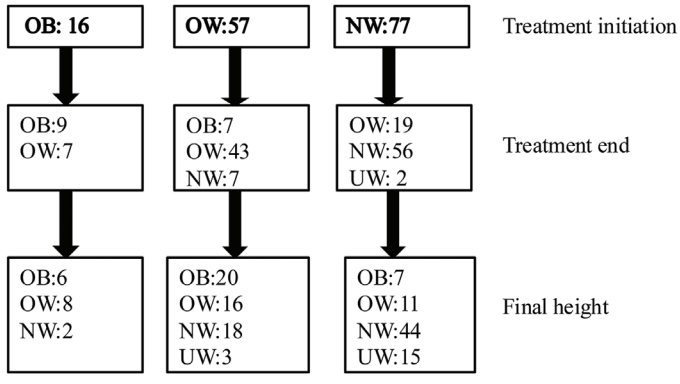

Figure 1. Transitions of BMI-SDS categories from treatment initiation to end of treatment and final adult height (n=150)
NW: normal weight, UW: underweight, OB: obese, OW: overweight, BMI-SDS: body mass index-standard deviation score

Table 1. Baseline clinical and hormonal characteristics by BMI-SDS category

Variable	All patients (n=150)	Normal weight (n=77)	Overweight (n=57)	Obese (n=16)	p value
Age at treatment (years)	8.0±1.2	8.1±1.1	8.1±1.0	7.1±1.8	**0.01**
Height SDS	0.9±1.2	0.7±1.3	1.2±1.0	1.4±1.1	**<0.0001**
Weight SDS	1.1±1.0	0.5±0.8	1.7±0.5	2.5±0.6	**<0.0001**
BMI-SDS	0.9±0.8	0.3±0.5	1.5±0.3	2.2 ±0.1	**<0.0001**
Bone age	9.7±1.6	9.6±1.6	9.9±1.6	9.5±2.0	0.7
BA-CA	1.6±1.3	1.4±1.0	1.6±1.7	2.4±0.6	**0.04**
Baseline LH (IU/L)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.3 (0.1-0.5)	0.9
Peak LH (IU/L)	5.5 (5.0-7.0)	6.4 (4.8-8.3)	5.7 (4.2-9.6)	7.4 (3.7-10.2)	0.7
Peak LH/FSH	0.7±0.6	0.7±0.7	0.6±0.5	0.6±0.2	0.6
Uterine length (mm)	34.0±7.2	33.0±10.0	34.4±7.8	33.0±5.6	0.4
Uterine volume (cc)	4.0±3.7	4.0±3.7	4.2±4.1	3.3±1.5	0.7

Data are presented as mean±SD or median (IQR), as appropriate
SD: standard deviation, BMI-SDS: body mass index-standard deviation score, IQR: interquartile range, LH: luteinizing hormone, FSH: follicle-stimulating hormone

Table 2. Changes in BMI-SDS and height-SDS from baseline to final height

	Baseline	Year 1	Treatment end	Final height	p value
All patients					
BMI-SDS	1.0 (0.8-1.2)	1.1 (1.0-1.3)	1.2 (0.9-1.3)	0.8 (0.6-1.1)	**0.001**
Height SDS	0.9 (0.8-1.2)	1.0 (0.9-1.2)	0.5 (0.3-0.7)	-0.02 (-0.3-0.2)	**<0.001**
Normal weight					
BMI-SDS	0.3 (0.3-0.6)	0.5 (0.3-0.7)	0.5 (0.3-0.7)	0.3 (-0.3-0.6)	**<0.001**
Height SDS	0.8 (0.4-1.0)	0.8 (0.5-1.0)	0.3 (0.1-0.5)	-0.4 (-0.6– -0.02)	**<0.001**
Overweight					
BMI-SDS	1.5±0.2	1.5±0.4	1.5±0.4	1.4±1.3	0.4
Height SDS	1.2 (0.8-1.6)	1.3 (1.0-1.7)	0.7 (0.3-1.2)	0.3(-0.05-0.6)	**<0.001**
Obese					
BMI-SDS	2.2±0.1	2.0±0.2	2.1±0.4	1.9±1.0	0.3
Height SDS	1.5 (0.8-2.3	1.6 (1.1-2.3)	0.9 (0.3-1.7)	0.1 (-0.7-0.8)	**<0.001**
SGA					
BMI-SDS	0.6 (-0.5-1.4)	0.8 (-0.1-1.6)	0.6 (-0.3-1.1)	1.0 (-0.1-1.9)	0.2
Height SDS	1.0 (-0.6-2.4)	0.9 (-0.7-2.3)	0.1 (-1.0-1.5)	-0.7 (-1.8-0.5)	**0.002**

Data are presented as mean±SD or median (IQR), as appropriate
SD: standard deviation, BMI-SDS: body mass index-standard deviation score, IQR: interquartile range, SGA: small for gestational age

Table 3. BMI-SDS changes over time: pairwise p values and overall four-time-point comparison by weight group

Comparison	Normal weight (n=77)	Overweight (n=57)	Obese (n=16)	All patients (n=150)
Baseline ↔ 1st year	**p=0.006** ↑	p=0.4	p=0.059	p=0.067
1st year ↔ End of treatment	p=0.3	p=0.5	p=0.7	p=0.074
Baseline ↔ End of treatment	**p=0.001** ↑	p=0.3	p=0.1	**p=0.009** ↑
End of treatment ↔ Final adult height	**p=0.008** ↓	p=0.2	p=0.4	**p=0.006** ↓
Baseline ↔ Final adult height	p=0.2	p=0.3	p=0.1	p=0.1

BMI-SDS: body mass index-standard deviation score

Figure 1 demonstrates bidirectional BMI-category transitions during follow-up. Although some girls who were overweight/obese at baseline shifted to a lower category by final height, a subset of girls with baseline normal weight transitioned to overweight/obesity. Thus, the prevalence of overweight/obesity (OW+OB) varied across treatment initiation, treatment end, and final height (overall p=0.014), increasing from 73/150 (48.6%) at baseline to 85/150 (56.6%) at treatment end (p=0.029) and decreasing to 68/150 (45.3%) at final height (p=0.012 vs treatment end), while baseline and final-height prevalences were not different (p=0.533). The normal-weight proportion decreased during treatment from 77/150 (51.3%) to 63/150 (42.0%) (p=0.013) and was 64/150 (42.7%) at final height (baseline vs final p=0.098). When examined separately, overweight prevalence increased numerically during treatment [57/150 (38.0%) to 69/150 (46.0%)] but did not reach statistical significance (baseline vs treatment end p=0.081), and decreased by final height [35/150 (23.3%); treatment end vs final p<0.001; baseline vs final p=0.006]. Obesity prevalence did not change during treatment [16/150 (10.7%) at both baseline and treatment end; p=1.00] but increased at final height [33/150 (22.0%); treatment end vs final p=0.006; baseline vs final p=0.0076]. Underweight was absent at baseline and remained rare at treatment end [2/150 (1.3%); baseline vs treatment end p=0.50] but increased at final height [18/150 (12.0%); treatment end vs final p<0.001]. In the multivariable model, girls who started treatment with a lower BMI-SDS experienced a larger increase in BMI-SDS during follow-up (ΔBMI-SDS=end of treatment minus baseline) (β=-0.174, 95% CI -0.291 to -0.057; p=0.004) (Table 4).

In the SGA group, no girls were obese at baseline (9 normal weight, 4 overweight). BMI-SDS was lower in SGA than appropriate for GA (AGA) at baseline, year 1, and treatment completion (p=0.04, p=0.04, and p=0.01, respectively), but not at final adult height (p=0.6). Within the SGA group, BMI-SDS remained stable over follow-up, with no significant changes observed between year 1, treatment completion, and final adult height (all p>0.05) (Tables 5 and 6).

Table 4. Baseline multivariable linear regression model for predictors of ΔBMI-SDS during treatment (end of treatment-baseline)

Independent variables	β (95% CI)	p value
Age at treatment initiation (years)	-0.005 (-0.239 to 0.229)	0.9
Baseline BMI-SDS	**-0.174 (-0.291 to -0.057)**	**0.004**
Baseline height SDS	0.026 (-0.064 to 0.117)	0.5
Baseline BA-CA (years)	-0.002 (-0.030 to 0.026)	0.8
Birth weight SDS	0.218 (-0.137 to 0.574)	0.2
Treatment duration (years)	-0.029 (-0.230 to 0.173)	0.7

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

Table 5. Clinical data at baseline in SGA and AGA patients

	SGA (n=13)	AGA (n=137)	p value
Birth weight SDS	-3.2±1.0	0.01±0.9	**<0.0001**
Age at treatment (years)	8.2±0.8	8.0±1.2	0.6
Height SDS	1.0 (-4.7-2.0)	1.0 (0.2-1.6)	0.1
Weight SDS	0.6 (-0.3-1.6)	1.2 (0.5-1.9)	0.06
BMI-SDS	0.5 (-0.2-1.3)	1.0 (0.4-1.6)	**0.04**
Bone age	9.9±1.4	9.7±1.6	0.6
BA-CA	1.7±1.2	1.6±1.4	0.8
Baseline LH (IU/L)	0.3 (0.1-0.8)	0.2 (0.1-0.6)	0.7
Peak LH (IU/L)	6.4+1.8	7.8+7.6	0.6
Peak LH/FSH	0.7+0.3	0.6+0.6	0.7
Uterine length (mm)	36.0±6.8	33.1±7.6	0.3
Uterine volume (cc)	8.0±6.7	3.7±3.2	0.8

BMI-SDS: body mass index-standard deviation score, SGA: small for gestational age, AGA: appropriate for gestational age, LH: luteinizing hormone, FSH: follicle-stimulating hormone

Table 6. BMI-SDS at baseline, year 1, treatment completion, and final height in SGA and AGA subgroups

	BMI-SDS SGA (n=13)	BMI-SDS AGA (n=137)	p value
Baseline	0.5±0.8	1.0±0.8	**0.04**
Year 1	0.59±0.83	1.06±0.83	**0.04**
Treatment end	0.55±0.72	1.10±0.84	**0.01**
Final height	1.01±1.07	0.77±1.56	0.6

BMI-SDS: body mass index-standard deviation score, SGA: small for gestational age, AGA: appropriate for gestational age

Discussion

In this longitudinal cohort of 150 girls with CPP who received GnRHa therapy and were followed until attainment of final adult height, we characterized BMI-SDS trajectories across treatment and post-treatment periods. Childhood obesity has been linked to earlier pubertal milestones, including earlier onset of breast development and pubic hair growth (22,23). At treatment initiation, nearly half of our cohort had excess weight (overweight+obesity: 48.6%), consistent with other CPP series. Palmert et al. (24) reported prevalences of overweight and obesity of 48% and 26%, respectively, and Arrigo et al. (12) likewise noted a substantial burden of excess weight among CPP patients. The absence of underweight patients at baseline supports the well-established association between greater adiposity and earlier pubertal activation. In our cohort, girls who were obese at treatment initiation commenced GnRHa at a younger chronological age than their normal-weight or overweight peers, despite similar absolute bone ages. This pattern suggests greater skeletal advancement relative to age (higher BA-CA) at presentation, consistent with reports that

obesity is associated with taller stature and advanced skeletal maturation for chronological age (25).

We evaluated treatment-related BMI-SDS changes stratified by weight status at initiation. The observed increase in BMI-SDS at treatment completion in the overall cohort was mainly attributable to changes in the normal-weight group in the first year of treatment, whereas BMI-SDS remained stable in patients who were overweight or obese at treatment initiation. However, at final height BMI-SDS was comparable to BMI-SDS at the beginning of GnRHa therapy in the overall cohort as well as in the subgroups of BMI categories. Categorical analyses added nuance to the continuous BMI-SDS findings: although OW+OB prevalence at final height was similar to baseline (p=0.533), BMI-category distribution changed over follow-up (overall p=0.014), reflecting bidirectional transitions across BMI-SDS cut-offs.

Most studies have not followed participants through adult height and have reported either increased BMI-SDS during treatment (26,27) or no change (28,29). Among the studies which reported data on final/near-final height (20,30,31,32,33,34,35,36), only four of them categorized the subjects according to their BMI-SDS prior to the commencement of the GnRHa therapy (30,31,32,33). Sinthuprasith et al. (30) reported in 58 subjects that BMI-SDS increased significantly at the first and second treatment years in the overweight/obesity group, whereas in the normal-weight group the increase was observed only during the first year. At near-final height, however, BMI-SDS did not differ significantly from baseline in either group. Arcari et al. (31) similarly noted BMI-SDS increases during treatment in normal-weight girls at both year 1 and year 2, whereas overweight girls showed a significant increase only at year 1, and obese girls showed no significant change. Notably, in the subgroup followed to final height (n=60), BMI-SDS decreased significantly at final height compared with treatment initiation. Similar findings were reported by Bruzzi et al. (32) who investigated 57 girls. These authors found that BMI-SDS increased during GnRHa therapy in normal-weight subjects, but not in overweight or obese subjects. Importantly, when near-final or final height was reached, BMI-SDS returned toward pretreatment values across the cohort.

In the study of Lee et al. (33), 35 patients followed to final height showed no significant BMI-SDS change across follow-up within the normal-weight (n=26) or overweight/obese (n=9) subgroups; BMI-SDS increased significantly only during the first year in normal-weight girls, with no further change between years 1 and 2, and no difference between overweight and obese groups during treatment. Our study adds several important elements to the literature. First, we report one of the largest single-center idiopathic CPP cohorts with follow-up to final adult height, with final-height data available for the entire treated cohort (n=150), whereas earlier reports included smaller final-height subsets [e.g., Arcari et al. (31) reported adult-height data for n=60 of 117; Bruzzi et al. (32) analyzed n=57]. Second, we present BMI-SDS at predefined longitudinal time points and complement continuous BMI-SDS analyses with paired categorical analyses of BMI-category redistribution/transition over follow-up (Figure 2), addressing the clinically relevant question of who moves between normal weight, overweight and obesity despite stable group-level BMI-SDS. Our observations in this large cohort followed through adult height are in agreement with the latter two studies and indicate that BMI-SDS increases in normal-weight girls during treatment, particularly in the first year, remains higher until treatment completion, and then declines after GnRHa discontinuation, returning toward baseline by adult height, whereas BMI-SDS tends to remain stable in girls who are overweight or obese at baseline.

Thus, the evidence suggests that changes in BMI during GnRHa therapy are strongly modified by baseline weight status. One proposed explanation for this observation is that apparent BMI-SDS changes may reflect altered linear growth rather than

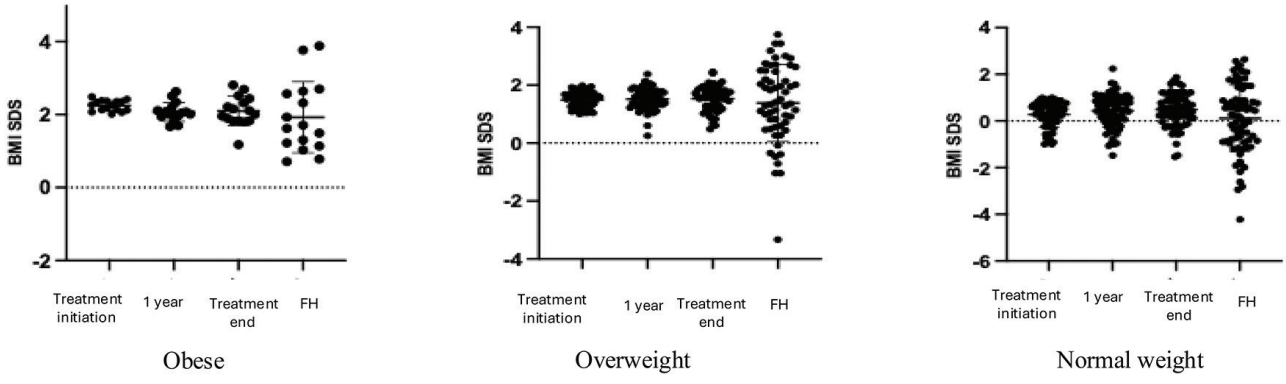

Figure 2. Changes in BMI-SDS over time according to baseline weight status
BMI-SDS: body mass index-standard deviation score

true adiposity gain, because pubertal suppression can reduce growth velocity and thereby increase BMI even when weight gain is modest (37,38). Differences in growth response by baseline adiposity may also modulate this pattern; for example, an obese/overweight subgroup showed a smaller decline in height SDS for chronological age during treatment compared with normal-weight peers in one cohort, which could attenuate BMI-SDS increases (39). However, growth velocity SDS did not differ significantly in our cohort, refuting this hypothesis. It was also proposed that E2 influences body composition and may promote fat-mass accrual during normal pubertal progression. Suppression of gonadal steroid production with effective GnRHa therapy may therefore blunt this pubertal, steroid-related increase in adiposity, potentially contributing to the relatively stable BMI-SDS observed in girls who were overweight at baseline. Nonetheless, this explanation does not fully account for the transient BMI-SDS increase observed in normal-weight girls during treatment in our cohort (40). Among the more likely explanations, given the known association between adiposity and precocious puberty in girls, some studies note that families of overweight/obese children may improve diet and physical activity during therapy, potentially limiting additional weight gain.

Another plausible explanation is that established excess adiposity may be maintained by biological mechanisms of weight-regulation (the "settling-point" framework), which can be relatively resistant to short-term change. In this model, adiposity-related signals (e.g., leptin/insulin) engage hypothalamic appetite–expenditure circuits and trigger compensatory responses that oppose sustained weight loss, potentially contributing to the relative BMI-SDS stability observed in girls with baseline overweight/obesity (41). In multivariate analyses, our finding that lower baseline BMI-SDS predicted a larger increase in BMI-SDS during treatment (β=-0.174; p=0.004) aligns with Wolters et al. (42) and Vuralli et al. (43) who likewise reported an inverse association between baseline BMI-SDS and on-treatment ΔBMI-SDS. Together, these data suggest that BMI-SDS changes during GnRHa therapy are largely determined by starting adiposity, rather than treatment duration or other auxological factors.

Puberty tends to start slightly earlier and may progress with a slightly faster tempo in girls born SGA compared with those born AGA (44). In our cohort, chronological age did not differ between the SGA and AGA groups. Evidence concerning longitudinal changes in BMI-SDS during GnRHa therapy stratified by birth size is limited. In a retrospective cohort followed to near-final/final height, Cho et al. (45) compared girls with idiopathic CPP born SGA (n=19) versus AGA (n=50) and reported no significant between-group differences in BMI-SDS across follow-up to completion of treatment. In our cohort, BMI-SDS was lower in SGA than AGA at treatment initiation, year 1, and treatment completion, but this difference was no longer evident at final adult height.

Study Limitations

BMI-SDS is a practical longitudinal marker but does not directly reflect body composition. Detailed measures of fat and lean mass (e.g., DXA) and metabolic outcomes were not available. Dietary intake and physical activity, as well as socioeconomic variables, were not systematically recorded, and no structured diet or supervised exercise program was prescribed as part of the treatment protocol. Therefore, residual confounding related to unmeasured lifestyle changes cannot be excluded. In addition, an untreated early puberty/control group was not available, so causal attribution of BMI-SDS and BMI-category changes specifically to GnRHa therapy is limited. Finally, subgroup analyses, particularly the SGA subgroup, and menarche-related anthropometric data were limited by small numbers or incomplete availability. Thus, non-significant findings should be interpreted cautiously given the risk of Type II error.

Conclusion

In this cohort of 150 girls with idiopathic CPP treated with leuprolide acetate, which is one of the largest series with follow-up to final adult height, BMI-SDS increased during therapy but returned toward baseline by adult height. This finding is reassuring regarding the long-term weight trajectory after completion of GnRHa treatment in idiopathic CPP. This pattern was mainly observed in normal-weight girls, whereas BMI-SDS remained stable in those overweight or obese at baseline. Our study also provides rare data stratified by birthweight category (SGA vs AGA), showing that early between-group differences in BMI-SDS were not sustained at final height.

Baseline BMI-SDS emerged as the key independent predictor of change in BMI-SDS during treatment, highlighting the importance of baseline weight status.

Ethics

Ethics Committee Approval: The study was approved by the Marmara University Faculty of Medicine Clinical Research Ethics Committee (approval no.: 09.2022.486, date: 08.09.2023).

Informed Consent: All eligible participants and their parents provided informed consent.

Footnotes

Authorship Contributions

Concept: Didem Helvacıoğlu, Serap Turan, Tülay Güran, Belma Haliloğlu, Zehra Yavaş Abalı, Abdullah Bereket, Design: Didem Helvacıoğlu, Büşra Gürpınar Tosun, Sefa Öğe, Serap Turan, Tülay Güran, Belma Haliloğlu, Zehra Yavaş Abalı, Abdullah Bereket,Data Collection or Processing: Didem Helvacıoğlu, Büşra Gürpınar Tosun, Sefa Öğe, Analysis or Interpretation: Didem Helvacıoğlu, Abdullah

Bereket, Literature Search: Didem Helvacıoğlu, Abdullah Bereket, Writing: Didem Helvacıoğlu, Abdullah Bereket.

Conflict of Interest: Two authors of this article, Abdullah Bereket and Serap Turan, are member of the Editorial Board of the Journal of Clinical Research in Pediatric Endocrinology. However, they were not involved in any stage of the editorial decision process for this manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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Early Childhood Obesity: Multifactorial Influences with a Prominent Familial Contribution

İsmail Hakkı Akbeyaz[1,2], Yasemin Akın[1], Berkin Berk Akbeyaz[1], Saygın Abalı[3]

[1]University of Health Sciences Türkiye, Kartal Dr. Lütfi Kırdar City Hospital, Clinic of Pediatrics, İstanbul, Türkiye
[2]Marmara University Pendik Training and Research Hospital, Department of Pediatrics, Division of Pediatric Neurology, İstanbul, Türkiye
[3]Acıbadem Mehmet Ali Aydınlar University Faculty of Medicine, Department of Pediatrics, Division Pediatric Endocrinology, İstanbul, Türkiye

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ABSTRACT

Objective: Early childhood obesity is an increasing public health problem with long-term consequences. Identifying risk factors is essential for effective preventative strategies. To evaluate prenatal, perinatal, and postnatal influences including: breastfeeding and nutritional practices; lifestyle and daily habits; household, sociocultural, and economic conditions; and parental anthropometric characteristics to better elucidate the determinants of obesity in early childhood.

Methods: This cross-sectional study included children aged 12-60 months, divided into children with obesity and non-obese controls. Standardized anthropometric assessments were performed in all participants. Parents completed a structured questionnaire capturing perinatal characteristics, infant feeding practices, dietary intake, medical history, lifestyle behaviors, and household-level factors.

Results: A total of 210 children (113 girls; 53.8%) were included, comprising 106 obese and 104 non-obese individuals, with comparable age between groups (3.4±1.3 vs. 3.3±1.3 years). Both maternal and paternal body mass index (BMI) values were significantly higher in the obese group, and maternal employment (27.4% vs. 5.8%) and maternal obesity (42.5% vs. 21.2%) were more prevalent. Maternal smoking during pregnancy (23.6% vs. 7.7%) and gestational diabetes (22.6% vs. 9.6%) were also more frequent. Feeding practices and current dietary patterns did not differ significantly between groups. In multivariable analysis, maternal employment (aOR 3.74), sibling obesity (aOR 2.21), and extended family obesity burden (aOR 1.19) independently predicted childhood obesity.

Conclusion: Familial obesity burden, including parents, siblings and extended family members, showed the strongest associations with obesity, highlighting the need for early identification of high-risk children. The implementation of family-centered and family-encompassing preventive strategies targeting physical activity, diet, and daily routines should be considered in these high-risk children.

Keywords: Obesity, early childhood, familial

Corresponding Author: Saygın Abalı, MD, Acıbadem Mehmet Ali Aydınlar University Faculty of Medicine, Department of Pediatrics, Division Pediatric Endocrinology, İstanbul, Türkiye

E-mail: abalisaygin@yahoo.com, saygin.abali@acibadem.edu.tr **ORCID:** orcid.org/0000-0001-6552-2801

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What is already known on this topic?

Globally, the prevalence of childhood overweight has risen steadily over the past three decades, with approximately 35 million children under five years of age classified as overweight by 2024.

Childhood obesity has a multifactorial etiology, involving the interplay of genetic susceptibility with perinatal, nutritional, socioeconomic, environmental, familial, and lifestyle factors.

What this study adds?

Familial obesity burden, that is obesity in parents, siblings and other family members, emerged as a prominent etiological factor, highlighting the importance of early recognition of genetically and environmentally high-risk children. The implementation of family-centered and family-encompassing preventive strategies targeting physical activity, diet, and daily routines should be considered in these high-risk children. Sibling obesity and a greater burden of obesity among extended family members were independently associated with early childhood obesity. These findings highlight the importance of early identification of children at increased familial risk and family-centered preventive strategies targeting physical activity, diet, and daily routines.

Introduction

Childhood obesity in the preschool period has emerged as a major public health concern due to its strong tendency to persist into later childhood and adulthood, leading to long-term cardiometabolic and psychosocial consequences (1,2,3). Data from Türkiye reports an obesity prevalence of about 5% among children aged 0-84 months (4). Studies show that approximately one-third of obese preschoolers and nearly half of overweight school-aged children remain obese in adulthood (5).

The etiology of childhood obesity is multifactorial, encompassing genetic, perinatal, nutritional, socioeconomic, environmental, familial, and lifestyle determinants (1,2,6,7). Shifts in dietary habits, reduced physical activity, increased screen time, and broader lifestyle changes increasingly contribute to excess weight gain in young children (1,2,5). In early childhood, family structure, caregiving patterns, and shared household behaviors play a particularly influential role, as children are largely dependent on their immediate environment for nutrition, physical activity, and daily routines (2,3,5). Genetic susceptibility also plays a significant role, with studies indicating that more than half of the variance in obesity risk is attributable to heritable factors (6,7). Monogenic forms typically present with rapid, early weight gain and hyperphagia, and should be particularly considered in children younger than five years or when syndromic features are present (6,7).

Beyond genetic predisposition, the "first 1000 days" constitute a critical window in which early-life exposures, including maternal pre-pregnancy body mass index (BMI), gestational weight gain, and feeding practices, shape later obesity risk (8). Given this broad etiological spectrum, a comprehensive evaluation of modifiable early childhood risk factors will be essential for effective prevention efforts.

The aim of the present study was to evaluate prenatal, perinatal, and postnatal influences; breastfeeding and nutritional practices, lifestyle and daily habits; household, sociocultural, and economic conditions; and parental anthropometric characteristics to better elucidate the determinants of obesity in early childhood.

Methods

Study Design and Participants

This cross-sectional, case-control study included children aged 12-60 months who presented to the outpatient clinic over a six-month period. Children were classified as obese or non-obese controls according to World Health Organization BMI and weight-for-height standard deviation scores (SDS) criteria (BMI SDS >+2.0 and/or weight-for-height SDS > +2.0) (1,9). Children with chronic illness or on weight-affecting medications were excluded.

Written informed consent was obtained from parents or legal guardians. Ethical approval was obtained from the University of Health Sciences Türkiye, Kartal Dr. Lütfi Kırdar City Hospital, Scientific Research Evaluation and Support Board on 29 January 2016, under application number 89513.307/1009/531 and decision number 112.

Data Collection

Standardized anthropometric measurements of all patients were performed by the principal investigator with an infantometer and a stadiometer, both sensitive to 0.1 cm. Weight was measured using an electronic scale (Seca GmBh, Hamburg, Germany) (sensitive to 5 g). The BMI of each patient was calculated as weight (kg) divided by height (m) squared (kg/m^2). SDS for all anthropometric measurements were calculated according to Turkish reference standards (9,10,11). Children with a BMI ≥+2

SDS were classified as obese. Weight-for-height was calculated as 100×[patient's weight (kg)/ideal weight at the 50th percentile for the child's length-age (kg)], and a weight-for-height value >120% was defined as obesity (12). Waist circumference SDS was derived from reference data for Turkish children (13). Maternal and paternal weights and heights were measured and BMI values were recorded during outpatient visits. Data were collected using a structured questionnaire administered by the investigator to parents. The survey captured information across several domains, including demographic characteristics, perinatal history, feeding practices, medical history, lifestyle behaviors, and socioeconomic and household factors.

Perinatal information included maternal weight gain, smoking during pregnancy, gestational diabetes mellitus (GDM), delivery mode, gestational age, birth weight SDS, Neonatal Intensive Care Unit (NICU) admission, phototherapy, and neonatal hypoglycemia. Feeding and nutritional data covered exclusive and total breastfeeding duration, infant formula use, vitamin D supplementation, and the child's intake of sugary beverages, fast food, ultra-processed foods, packaged snacks, and high-carbohydrate foods. Medical history included infection-related hospitalizations (>24 hours), antibiotic use, nebulizer therapy usage, and vaccination status. Socioeconomic and household characteristics assessed household income, parental education, maternal employment, primary caregiver, passive smoking exposure, family history of obesity, and household size. Lifestyle factors included daycare attendance, outdoor play or physical activity, child temperament and habits and daily screen time.

Statistical Analysis

Statistical analyses were performed using NCSS 2007 and SPSS, version 25.0 (IBM Inc., Armonk, NY, USA). Group comparisons used independent t-tests, Mann-Whitney U tests, and chi-square tests as appropriate. The dependent variable was obesity status (obese vs. control). Variables associated with obesity at $p<0.2$ in univariate analyses were entered into a multivariable logistic regression model using a backward stepwise method. Results are expressed as adjusted odds ratios (aOR) with 95% confidence intervals (CI), and $p<0.05$ was considered statistically significant.

Results

Of the study included 210 children, comprising 106 (50.5%) obese and 104 non-obese participants. The two groups were comparable with respect to age (3.4±1.3 vs. 3.3±1.3 years, p=0.398) and gender (girls comprised 53.8% in both groups, p=0.992). All anthropometric indices, including height SDS, weight SDS, BMI SDS, weight-for-height (%), weight-for-height SDS, and waist circumference SDS, are presented in Table 1.

Familial Characteristics

Familial characteristics are summarized in Table 2. The annual per-capita household income was significantly higher in the obese group (2502.5±1533.4 vs 1943.7±1317.5 USD, p=0.005), whereas household size was significantly smaller (4.1±1.1 vs 4.9±1.6 persons, $p<0.001$). Passive smoking exposure did not differ between groups (p=0.231). Parents of obese children had higher educational attainment. Fathers had higher education levels (p=0.028), mothers also had higher education levels (p=0.022) and maternal employment was substantially more frequent in the obese group (27.4% vs. 5.8%, $p<0.001$).

A family history of obesity emerged as a prominent determinant of childhood obesity. Parents of obese children exhibited significantly higher BMI values, with both paternal BMI (p=0.023) and maternal BMI (p=0.009) exceeding those observed in the control group. Consistently, maternal obesity was markedly more prevalent among obese children compared with controls (42.5% vs. 21.2%, p=0.002) although paternal obesity rates did not differ between groups (p=0.13). Furthermore, sibling obesity was significantly more frequent in the obese group (22.6% vs. 7.7%, p=0.005), and the number of obese individuals in the extended family (excluding parents and siblings) was also significantly higher ($p<0.001$), indicating a strong familial clustering of obesity (Table 2).

Table 1. Demographic and anthropometric characteristics

	Obese group	Control group	p value
Age, years	3.4±1.3	3.3±1.3	0.398*
Female n (%)	57 (53.8%)	56 (53.8%)	0.992#
Height SDS	1.2±1.1	0.2±0.8	**<0.001**+
Weight SDS	3.3±1.1	0.1±0.8	**<0.001**+
Body mass index SDS	3.6±1.0	0.0±1.0	**<0.001**+
Weight-for-Height (%)	141.9±17.0	100.0±8.1	**<0.001**+
Weight-for-Height SDS	3.6±1.0	-0.1±0.9	**<0.001**+
Waist circumference SDS	4.6±2.6	0.3±1.5	**<0.001**+

*Independent t-test, #Chi-square test, +Mann-Whitney U test
SDS: standard deviation score

Table 2. Familial characteristics			
	Obese Group	**Control Group**	**p value**
Annual per capita household income, USD	2502.5±1533.4	1943.7±1317.5	**0.005**$^{+}$
Household size, persons	4.1±1.1	4.9±1.6	**<0.001**$^{+}$
Passive smoking exposure n (%) None Indoor smoking Outdoor smoking	24 (22.6) 27 (25.5) 55 (51.9)	32 (30.8) 18 (17.3) 55 (51.9)	0.231$^{\#}$
Father's education, years	9.8±2.9	8.9±3.0	**0.028**$^{+}$
Father's BMI	28.0±4.5	26.7±3.5	**0.023**$^{+}$
Father with obesity n (%)	31 (29.2)	20 (19.2)	0.126$^{\#}$
Father with diabetes n (%)	4 (3.8)	0 (0.0)	0.135$^{\#}$
Mother's education years	9.5±3.3	8.4±3.2	**0.022**$^{+}$
Maternal employment n (%)	29 (27.4)	6 (5.8)	**<0.001**$^{\#}$
Mother's BMI	28.6±6.1	26.8±3.6	**0.009**$^{+}$
Mother with obesity n (%)	45 (42.5)	22 (21.2)	**0.002**$^{\#}$
Mother with diabetes n (%)	9 (8.5)	2 (1.9)	0.068$^{\#}$
Sibling with obesity n (%)	24 (22.6)	8 (7.7)	**0.005**$^{\#}$
Number of obese individuals in extended family (except parents and sibling) **Median (IQR)**	2.7±2.0 2 (1-5)	1.6±1.9 1 (0-3)	**<0.001***

*Independent t-test, +Mann-Whitney U test, #Chi-square test
USD: United Stated Dollars; BMI: body mass index, IQR: Interquartile range

Perinatal and Birth History

Perinatal and birth characteristics are presented in Table 3. Maternal gestational weight gain did not differ between groups (p=0.254). In contrast, maternal smoking during pregnancy was significantly more frequent in the obese group (23.6% vs. 7.7%, p=0.003), and the prevalence of GDM was also higher among mothers of obese children (22.6% vs. 9.6%, p=0.018).

Delivery mode, prematurity rates, and gestational age were comparable between groups. Although birth weight SDS was significantly higher in the obese group (p=0.0037), weight-for-gestational-age categories (small for gestational age, appropriate for gestational age, large for gestational age) did not differ significantly. Rates of NICU admission and phototherapy were similar but neonatal hypoglycemia was more frequently observed in obese children (11.3% vs. 1.9%, p=0.014).

Nutritional History and Lifestyle Factors

The proportion of children who had ever been exclusively breastfed did not differ significantly between groups (p=0.090). The duration of exclusive breastfeeding and total breastfeeding duration were slightly shorter in the obese group, although these differences were not statistically significant (p=0.074 and p=0.080, respectively). Infant formula use was significantly more commonly among obese children (72.6% vs. 57.7%, p=0.033), while the age at first formula introduction was similar between groups.

Current dietary habits, including ultra-processed food intake, fast-food consumption frequency, number of weekly fast-food meals, and high-carbohydrate food intake, did not differ significantly between groups.

Regarding medical history, hospitalization due to infection tended to be more frequent among obese children, although was not significant (p=0.058). No significant differences were observed in doctor visits due to infection, nebulizer therapy, vaccination status, or infantile colic severity.

Lifestyle factors showed notable differences. The distribution of primary caregivers differed significantly between groups (p<0.001), with mothers serving less frequently as primary caregivers in the obese group. Daycare attendance was significantly higher among obese children (27.4% vs. 11.5%, p=0.004), and physical activity was reported less frequently in this group (86.8% vs. 96.2%, p=0.031). Daily screen time did not differ between groups (p=0.871) (Table 4).

Multivariable Logistic Regression Analysis

To identify independent predictors of childhood obesity, a multivariable logistic regression model was constructed (Table 5). In the adjusted analysis, maternal employment (aOR 3.74, 95% CI 1.29-10.84, p=0.015), sibling obesity (aOR 2.21, 95% CI 1.08-4.51, p=0.030), and history of extended family members with obesity (aOR 1.19, 95% CI 1.01-1.41, p=0.038) emerged

as independent predictors of childhood obesity, indicating both intergenerational and household-level influences. Other familial, perinatal, and lifestyle variables were not independently associated with obesity in the multivariable model (Figure 1).

Discussion

This study provides insight into the determinants of obesity specifically during early childhood, a developmental period in which family environment and caregiving patterns may exert a stronger influence than individual lifestyle choices. Children aged 12-60 months were evaluated to examine the associations of infant feeding and nutritional practices, socioeconomic and household characteristics; lifestyle behaviors and daily habits; and familial obesity burden with early childhood obesity.

Several studies conducted in Türkiye have examined these risk factors. In a study by Kondolot et al. (14) investigating obesity risk among school-aged children, higher socioeconomic status, irregular eating patterns, insufficient physical activity, and a family history of obesity were identified as key contributors. These results highlight the pivotal role of familial obesity burden, socioeconomic context, and modifiable lifestyle-related factors in the development of obesity, beginning as early as childhood, and our results are consistent with this (14).

Familial and Environmental Characteristics

The association between socioeconomic status and childhood obesity is complex and may differ according to a country's level of economic development (15,16,17). In Türkiye, higher socioeconomic status has been associated with an increased risk of childhood obesity in several studies (14,15,16,17,18,19), whereas an inverse socioeconomic gradient, with a greater burden of obesity among socioeconomically disadvantaged groups, is more commonly observed in high-income countries.

Parental educational attainment and employment status may also influence childhood obesity risk. In the present study, both maternal and paternal education levels were significantly higher in the obese group. In addition, maternal employment was more frequent among families of obese children. This observation is consistent with previous reports indicating a higher prevalence of childhood obesity in households where primary caregivers are engaged in full-time employment. Importantly, maternal employment should not be interpreted as a causal or maternal-specific risk factor. Rather, it likely reflects broader family dynamics, including contributing to higher family socioeconomic status, caregiving arrangements, time constraints, and reliance on convenience foods, all of which may influence early childhood eating behaviors and activity patterns (18,20,21,22).

Perinatal and Birth History

Maternal factors before and during pregnancy are consistently highlighted as important determinants of childhood obesity risk (23). GDM is a significant risk factor for childhood obesity. A study found that GDM doubles the probability of childhood overweight and obesity at ages 2-5 years (24). In our cohort, GDM was more frequent in the obese group in univariate analyses; however, GDM did not remain an independent predictor in multivariate models.

Table 3. Perinatal and birth history

	Obese group	Control group	p value
Maternal weight gain kg	14.96±7.50	13.88±6.10	0.254*
Smoking in pregnancy n (%)	25 (23.6)	8 (7.7)	**0.003#**
Gestational diabetes n (%)	24 (22.6)	10 (9.6)	**0.018+**
Delivery mode n (%) Cesarean section Spontaneous vaginal	72 (67.9) 34 (32.1)	62 (59.6) 42 (40.4)	0.267#
Prematurity n (%)	11 (10.4)	8 (7.7)	0.662#
Gestational age, weeks	38.7±2.2	38.9±1.3	0.059*
Weight for gestational age n (%) SGA AGA LGA	6 (5.7) 75 (70.8) 25 (23.6)	8 (7.7) 80 (76.9) 16 (15.4)	0.301#
Birth weight SDS	0.50±1.45	-0.05±1.26	**0.004***
NICU admission n (%)	29 (27.4)	18 (17.3)	0.114#
Phototherapy n (%)	24 (22.6)	20 (19.2)	0.662#
Neonatal hypoglycemia n (%)	12 (11.3)	2 (1.9)	**0.014#**

*Independent t-test, +Mann-Whitney U test, #Chi-square test
SGA: small for gestational age; AGA: appropriate for gestational age; LGA: large for gestational age

Maternal smoking during pregnancy was significantly found to be more common in the obese group in univariate analyses. This is consistent with evidence showing that maternal smoking during pregnancy increases the risk of childhood overweight, independent of maternal pre-pregnancy BMI and genetic predisposition (25).

Children born large or small for gestational age, as well as those born preterm, have an increased risk of later obesity, particularly in the presence of maternal factors or rapid postnatal catch-up growth. Consistent with this, higher birth weight SDS was associated with obesity in univariate analyses in our study, although, once again, it did not remain an independent predictor in multivariable analysis (26,27,28,29,30).

Nutritional History and Dietary Intake

Breastfeeding is widely recognized as a potent protective mechanism against early-onset obesity (31,32). Increased duration of breastfeeding is often linked to a decreased risk of overweight later in life (33,34). Total breastfeeding duration tended to be lower in the obese group, although the difference

Table 4. Nutrition and lifestyle characteristics

	Obese group	Control group	p value
Infants exclusively breastfed at any time, n (%)	54 (50.9)	66 (63.5)	0.090#
Duration of exclusive breastfeeding, months	3.74±2.72	4.38±2.45	0.074*
Total duration of breastfeeding, months	13.65±8.89	15.78±8.66	0.080*
Use of infant formula, n (%)	77 (72.6)	60 (57.7)	**0.033#**
Age at first infant formula introduction, months	3.52±3.84	4.05±4.50	0.468*
Current nutrition and dietary practices			
Ultra processed food intake (portions per day) None Rare-1/day ≥2/day	 9 (8.5) 25 (23.6) 72 (67.9)	 6 (5.8) 26 (25.0) 72 (69.2)	0.739#
Fast food consumption n (%) None Occasional Frequent	 43 (40.6) 21 (19.8) 42 (39.6)	 16 (32.7) 66 (23.1) 22 (44.2)	0.493#
Fast-food meals per week	0.89±1.23	0.92±1.26	0.874*
High-carbohydrate foods (portions per day)	1.94±0.94	1.90±0.91	0.758*
Medical history			
History of hospitalization due to infection n (%)	25 (23.6)	38 (36.5)	0.058#
Doctor visits due to infection n/year	5.47±4.07	5.79±4.12	0.576*
Any nebulizer therapy n (%) None At hospital At home	 42 (39.6) 32 (30.2) 32 (30.2)	 42 (40.4) 36 (34.6) 26 (25.0)	0.658#
Vaccination status (schedule-compatible) n (%)	103 (97.2)	98 (94.2)	0.477#
Infantile colic history n (%) None Mild Severe	 38 (35.8) 41 (38.7) 27 (25.5)	 46 (44.2) 32 (30.8) 26 (25.0)	0.392#
Lifestyle factors			
Primary caregiver n (%) Mother Nanny Grandmother Single parent	 65 (61.3) 10 (9.4) 29 (27.4) 2 (1.9)	 88 (84.6) 2 (1.9) 12 (11.5) 0 (0.0)	**<0.001#**
Daycare attendance n (%)	29 (27.4)	12 (11.5)	**0.004#**
Physically active n (%)	92 (86.8)	100 (96.2)	**0.031#**
Screen time, hours per day	2.94±2.15	2.99±2.03	0.871*

*Independent t-test, +Mann-Whitney U test, #Chi-square test

Table 5. Multivariable logistic regression analysis of factors associated with childhood obesity

Variable	Adjusted OR	95% CI	p value
Maternal employment	3.74	1.29-10.84	**0.015**
Neonatal hypoglycemia	2.91	0.54-15.6	0.212
Sibling obesity	2.21	1.08-4.51	**0.030**
Gestational diabetes	2.11	0.83-5.36	0.115
Mother with diabetes	2.01	0.31-13.1	0.460
Daycare attendance	1.72	0.69-4.30	0.243
Maternal obesity	1.62	0.77-3.43	0.208
NICU admission	1.29	0.55-3.05	0.560
Extended family history of obesity	1.19	1.01-1.41	**0.038**
Mother's BMI, kg/m^2	1.04	0.97-1.12	0.287
Paternal obesity	1.09	0.64-1.87	0.756
Father's education, years	1.04	0.91-1.18	0.561
Mother's education, years	1.03	0.92-1.16	0.621
Father's BMI, kg/m^2	1.02	0.94-1.11	0.611
Household size, persons	0.80	0.60-1.06	0.116

BMI: body mass index, OR: odds ratio, CI: confidence interval, NICU: neonatal intensive care unit

was not significant, a trend consistent with evidence supporting a protective role of breastfeeding against childhood obesity. However, no significant associations were observed with exclusive breastfeeding or its duration (35,36).

The association between infant formula feeding and childhood obesity remains controversial. Early formula use has been linked to accelerated infant weight gain (37,38). Consistent with this, formula use was significantly more frequent (73% vs 58%) among obese children in our cohort.

In our cohort, fast-food consumption, weekly fast-food meal frequency, and ultra-processed food intake did not differ significantly between groups. Nevertheless, previous studies have consistently reported that higher consumption of ultra-processed products, such as packaged snacks, confectionery, and sugar-sweetened beverages, has been associated with increased obesity risk and unfavorable trajectories in BMI, fat mass index, and waist circumference throughout childhood (39,40). These foods are typically energy-dense and nutrient-poor, often displacing healthier dietary options and thereby promoting excess weight gain (41).

Medical History and Lifestyle Factors

A higher proportion of children in the obese group had a history of hospitalization due to infection compared with controls, although this difference was not statistically significant. Similarly, no significant between-group differences were observed in

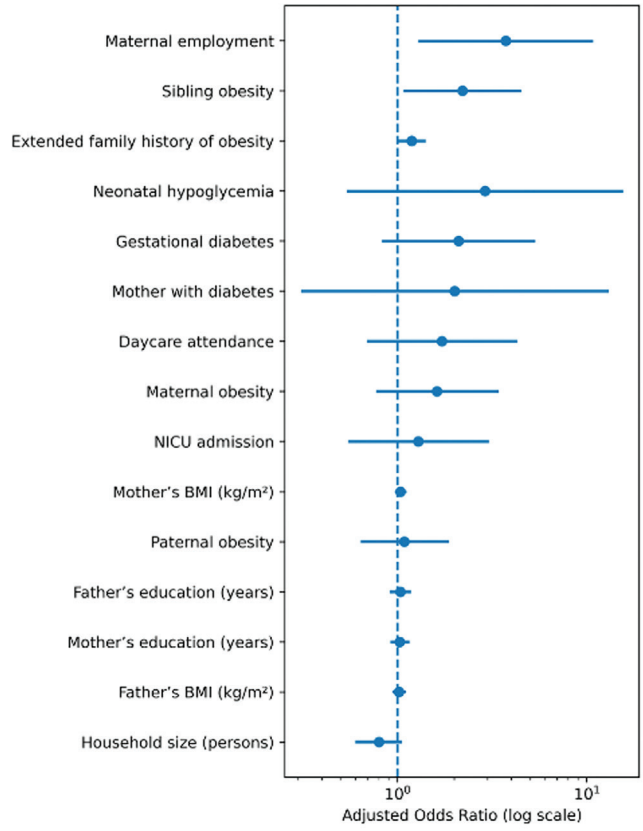

Figure 1. Multivariable logistic regression analysis of factors associated with childhood obesity Forest plot showing adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for factors associated with childhood obesity. Maternal employment, sibling obesity, and extended family history of obesity were independently associated with an increased risk of childhood obesity. The vertical reference line indicates an OR of 1.0. Variables with confidence intervals not crossing 1.0 are considered statistically significant
NICU: neonatal intensive care unit, BMI: body mass index

nebulizer therapy, vaccination status, or infantile colic severity. These findings suggest that early-life clinical exposures play a limited role in obesity development, consistent with previous studies reporting no clear association with later adiposity (42,43,44).

Children in the obese group were more often cared for by non-parental caregivers and attended daycare more frequently, consistent with evidence linking extra-familial childcare arrangements to childhood obesity. This association likely reflects broader contextual factors rather than caregiver-specific effects, and its impact appears heterogeneous across caregiving settings (45,46).

Physical activity is a well-established protective factor against childhood obesity (14). In our study, parent-reported physical activity was significantly lower among obese children compared with controls. Consistent with this finding, objective assessments

in the literature, including accelerometry-based studies, have demonstrated strong associations between lower activity levels and increased adiposity in preschool-aged children (47).

Screen time exposure, including use of mobile phones, tablets, and television, did not differ significantly between groups in our study. Although no association with screen time was observed in this study, previous literature suggests that excessive sedentary media exposure may contribute to obesity risk as children grow older (48,49).

Eating behaviors established in early childhood, beginning around three years of age and consolidated by school entry, are thought to confer a cumulative risk for later obesity, highlighting the importance of early-life interventions (50). In contrast, our findings indicated that early childhood obesity was more strongly associated with familial obesity burden than with lifestyle-related factors. In this age group, the home food environment likely represents the predominant dietary exposure shared across family members. Alternatively, these observations may suggest that genetic determinants exert a relatively greater influence on obesity development before five years of age, or a complex combination of genetic and social factors may be at play. Overall, our findings suggest that in early childhood, familial obesity burden may outweigh individual lifestyle behaviors as a determinant of obesity, underscoring the importance of family-centered preventive strategies initiated early in life.

Study Limitations

This study has several limitations that should be considered when interpreting the findings. First, physical activity and screen time were assessed using parent-reported measures rather than objective tools such as accelerometers or digital tracking devices, which may have attenuated true associations. Second, several potentially relevant factors, including parental dietary patterns, detailed household food environment, sleep duration and quality, and parental mental health, were not assessed and may have influenced obesity risk.

Although familial clustering of obesity was a prominent finding, the absence of genetic or polygenic risk analyses limits our ability to disentangle shared genetic susceptibility from shared environmental influences. Therefore, familial aggregation observed in this study likely reflects a combination of genetic predisposition and common household behaviors rather than a predominantly genetic effect.

Finally, the single-center, metropolitan setting and sociocultural characteristics of the study population may limit the generalizability of our findings to rural areas or populations with different socioeconomic or cultural backgrounds. These limitations highlight the need for future multicenter studies incorporating objective behavioral measures and genetic data.

Conclusion

Our findings were consistent with the hypothesis that early childhood obesity arises from the interaction between familial predisposition and modifiable environmental factors. Although multiple variables were associated with obesity in univariate analyses, maternal employment and a stronger familial burden of obesity were associated with substantially increased odds of obesity in this metropolitan Turkish cohort. Thus, preventive strategies should include early identification of high-risk children/families while targeting modifiable behaviors, including physical activity, dietary patterns, and household routines.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the University of Health Sciences Türkiye, Kartal Dr. Lütfi Kırdar City Hospital, Scientific Research Evaluation and Support Board (approval no.: 112, date: 29.01.2016).

Informed Consent: Written informed consent was obtained from parents or legal guardians.

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Authorship Contributions

Surgical and Medical Practices: İsmail Hakkı Akbeyaz, Saygın Abalı, Concept: İsmail Hakkı Akbeyaz, Yasemin Akın, Design: İsmail Hakkı Akbeyaz, Saygın Abalı, Data Collection or Processing: İsmail Hakkı Akbeyaz, Berkin Berk Akbeyaz, Saygın Abalı, Analysis or Interpretation: İsmail Hakkı Akbeyaz, Berkin Berk Akbeyaz, Saygın Abalı, Literature Search: İsmail Hakkı Akbeyaz, Yasemin Akın, Saygın Abalı, Writing: İsmail Hakkı Akbeyaz, Saygın Abalı.

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Subclinical Hypothyroidism in Children: Natural History, Risk Factors, and Outcomes

Nur Şeyma Zengin[1], Elif Sağsak[2], Seda Geylani Güleç[1]

[1]University of Health Sciences Türkiye, Gaziosmanpaşa Training and Research Hospital, Clinic of Pediatrics, İstanbul, Türkiye
[2]University of Health Sciences Türkiye, Gaziosmanpaşa Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye

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ABSTRACT

Objective: Subclinical hypothyroidism (SH) is defined as elevated thyroid-stimulating hormone (TSH) with normal thyroid hormone levels and typically presents without specific symptoms in children. Although treatment criteria exist, predictors of progression and treatment need remain uncertain.

Methods: To evaluate the natural course of mild SH, identify clinical conditions associated with elevated TSH, determine predictors of progression requiring levothyroxine, and assess growth outcomes. Records of children (3 months-18 years) with mild SH (TSH 5-10 mIU/L on ≥2 measurements) and ≥6 months of follow-up were retrospectively reviewed. Demographic, biochemical, anthropometric, etiological, and imaging data were analyzed. Children were categorized as idiopathic or as having associated clinical factors (autoimmune thyroiditis, iodine imbalance, obesity, or medication use). Outcomes were classified as euthyroid, persistent SH, or requiring treatment.

Results: During follow-up, 45 of the study cohort of 111 children (40.5%) became euthyroid, 49 (44.2%) remained subclinically hypothyroid, and 17 (15.3%) required levothyroxine. Idiopathic cases showed the most favorable course, with only 8.6% requiring therapy. Hashimoto's thyroiditis (HT) was the strongest predictor of progression (42.1% vs. 9.8% in non-HT). A baseline TSH>7.5 mIU/L increased treatment likelihood by ~3.5-fold. Growth parameters remained within normal limits, with no deterioration in untreated children.

Conclusion: Mild pediatric SH is generally benign and self-limiting, particularly in idiopathic cases. HT and higher baseline TSH levels are key predictors of progression, while growth remains stable. Management should be individualized based on underlying conditions, TSH severity, and autoimmune status.

Keywords: Natural history, risk factors, subclinical hypothyroidism

What is already known on this topic?

Subclinical hypothyroidism (SH) in children is a relatively uncommon but increasingly recognized condition, with variable natural history. Most cases are idiopathic and often show spontaneous resolution or stable course without progression.

Corresponding Author: Nur Şeyma Zengin, MD,, University of Health Sciences Türkiye, Gaziosmanpaşa Training and Research Hospital, Clinic of Pediatrics, İstanbul, Türkiye
E-mail: nszengin37@hotmail.com **ORCID:** orcid.org/0000-0001-7926-0064
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What this study adds?

This study describes one of the largest pediatric cohorts with mild SH from Türkiye, demonstrating its predominantly benign course. Higher baseline thyroid-stimulating hormone levels and Hashimoto's thyroiditis emerged as key factors associated with progression. Stable growth parameters across follow-up support existing evidence that untreated mild SH does not adversely affect growth.

Introduction

Subclinical hypothyroidism (SH) is defined by elevated serum thyroid-stimulating hormone (TSH) with normal thyroid hormone (tri-iodothyronine and thyroxine) levels and the absence of specific clinical signs (1,2). The normal range of TSH (approximately 0.4-0.5 to 4.0-5.0 µIU/mL) varies across assays, and mild SH, typically defined as TSH 4.5-10 mIU/L, encompasses most pediatric cases (3,4). Several descriptive terms are used in clinical practice to characterize mild TSH elevation. "Isolated hyperthyrotropinemia" refers to mild or transient increases in TSH levels without evidence of intrinsic thyroid dysfunction, whereas "primary compensated hypothyroidism" denotes early thyroid impairment that warrants closer clinical observation. Importantly, mild TSH elevation does not always indicate true thyroid disease; transient increases associated with obesity, fluctuations in iodine intake, or recovery from non-thyroidal illness may represent adaptive physiological responses rather than pathological conditions (5).

In children, SH arises in a wide range of clinical contexts, including autoimmune thyroiditis, iodine imbalance, congenital thyroid anomalies, genetic syndromes, medication effects, and idiopathic presentations (6,7). Idiopathic SH is characterized by mildly elevated TSH levels despite normal peripheral thyroid hormones and the absence of autoimmunity or identifiable secondary contributors (3). Most children are asymptomatic, and occasional nonspecific symptoms, such as dry skin, fatigue, or weight gain, are not diagnostic (8,9,10).

The natural history of SH differs between children and adults. Pediatric SH often remains stable or normalizes spontaneously, and progression to overt hypothyroidism (OH) occurs in a minority of cases (0-28%) (11,12,13).

Children with SH due to HT have a higher risk of deterioration (14,15), a risk that may further increase in chromosomal disorders such as Turner or Down syndrome (13). Among clinical and biochemical variables, baseline TSH is considered the strongest predictor of outcome (16).

Although OH impairs growth and neurocognitive development, the significance of untreated mild SH remains uncertain. Most evidence indicates no adverse effects on growth, bone maturation, or cognition, though subtle metabolic changes have been described (2,4).

Current recommendations favor observation in asymptomatic, antibody-negative children with TSH<10 mIU/L, and treatment in those with symptoms, goiter, autoimmunity, or rising TSH (4,10,17,18).

Given these considerations, the aim of the present study was to characterize the natural course of mild SH in a Turkish cohort, identify clinical conditions that accompany TSH elevation and may provide a background for SH, determine predictors of progression requiring treatment, and evaluate growth outcomes in affected children.

Methods

Study Design and Population

This single-center retrospective study included children aged 3 months to 18 years who presented to the Pediatric Endocrinology Outpatient Clinic of University of Health Sciences Türkiye, Gaziosmanpaşa Training and Research Hospital between 2016 and 2019.

Mild SH was defined as two TSH measurements of 5-10 mIU/L obtained ≥4 weeks apart, with normal free thyroxine (fT4) levels. Since SH is a biochemical state, conditions potentially influencing TSH, including obesity, genetic syndromes, medication use, and prior chemotherapy/radiotherapy, were not exclusion criteria. Patients with TSH>10 mIU/L, abnormal fT4, or known thyroid disease requiring treatment were excluded.

Study Procedure

The following variables were collected: date of admission, sex, presenting symptoms, age, history of regular medication use, comorbidities, use of iodized salt, family history of thyroid disease, height, body weight, physical examination findings, TSH, fT4, free triiodothyronine (fT3), anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-Tg) autoantibody status, urinary iodine concentration, and thyroid ultrasonography (USG) results. These variables were used to evaluate potential factors associated with mild SH, risk factors for requiring treatment, growth outcomes, and indications for therapy.

The etiological classification was made based on potential contributing factors described in the pediatric endocrinology literature; no causality was inferred. Conditions such as autoimmune thyroiditis, obesity, genetic syndromes, iodine deficiency, congenital thyroid malformations, medications

affecting thyroid function, and history of radiotherapy or chemotherapy were investigated.

For growth evaluation, height, weight, body mass index (BMI), and growth velocity were expressed as standard deviation scores (SDS) using the reference data of Neyzi et al. (19). Growth velocity SDS was calculated using the charts of Neyzi et al. (19) for girls aged 8-13 years and boys aged 10-15 years, and Baumgartner et al.'s (20) data for younger ages. Growth velocity could not be assessed in 37 patients due to levothyroxine initiation, epiphyseal closure, growth-affecting medications, Down syndrome, or prior chemotherapy.

Serum TSH, fT4, and fT3 were measured with immunoenzymatic assays on DXI 800 analyzers (Beckman Coulter Biyomedikal Ürünler San. ve Tic. Ltd. Şti., İçerenköy, İstanbul, Türkiye). Anti-TPO>9 IU/mL and anti-Tg>4 IU/mL were considered positive.

Urinary Iodine Concentration (UIC) was analyzed externally (Düzen Laboratory, Türkiye) using an ICP-MS system. Reference intervals were based on the World Health Organization (WHO) 2007 guideline, originally expressed in µg/L (21). After unit conversion, these correspond to the standard µg/L categories. UIC levels were therefore classified according to WHO 2007 interpretive criteria (21):<20 µg/L severe; 20-49 µg/L moderate; 50-99 µg/L mild; 100-199 µg/L adequate; 200-299 µg/L above requirements; and ≥300 µg/L excessive iodine levels.

Patient Follow-up and Treatment

After the diagnosis of SH (defined as elevated TSH and normal fT4 levels on at least two separate measurements) was confirmed, the date of diagnosis was designated as "Day 0." Following this, risk factors associated with the need for treatment, including autoimmune thyroiditis, goiter, higher baseline TSH levels, obesity, and chromosomal or autoimmune comorbidities, such as Down or Turner syndrome, were evaluated. In patients with goiter or strong risk indicators, thyroid USG was performed to assess gland size and echogenic heterogeneity suggestive of autoimmune thyroiditis.

In routine practice, patients without risk factors were advised to undergo clinical evaluation and TSH/fT4 measurement every 3-6 months, whereas those with goiter, autoimmune thyroiditis, higher baseline TSH levels (> 7.5-10 mIU/L), or other high-risk features were scheduled for re-evaluation within 1-3 months. However, due to the retrospective design of the study, variable appointment adherence, and differences in healthcare accessibility, follow-up intervals varied among patients. Therefore, only the initial (diagnostic) and the last available follow-up visits were included in the analysis, and patients with a follow-up duration of less than six months were excluded. The decision to initiate levothyroxine therapy was based on predefined biochemical and clinical indicators consistent with standard clinical practice. Specifically, TSH levels>10 mIU/L, a decline or downward trend in fT4, the presence of goiter, or clinical signs suggestive of OH were accepted as primary criteria for treatment initiation.

Ethical Considerations

This study was conducted as a specialty thesis in the Clinic of Pediatrics, University of Health Sciences Türkiye, Gaziosmanpaşa Training and Research Hospital. The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of University of Health Sciences Türkiye, Taksim Training and Research Hospital (approval no: 102, date: 24.07.2019). Due to the retrospective design of the study, informed consent was not required.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA). The distribution of continuous variables was assessed using the Shapiro-Wilk test. Descriptive statistics (mean, standard deviation, frequency) were calculated. Group comparisons for normally distributed variables were performed using the Student's t-test for two groups and one-way ANOVA for more than two groups, while differences between baseline and follow-up values were evaluated with the paired-samples t-test. Correlations between normally distributed parameters were assessed using Pearson correlation coefficients. The mean growth velocity SDS was compared with population reference values for healthy children using the one-sample t-test. Categorical variables were analyzed using the Pearson chi-square test or Fisher's exact test, and the Fisher-Freeman-Halton test was applied for larger contingency tables. The linear-by-linear association test was used to assess trends across ordered categories. To estimate the association between baseline TSH levels and treatment requirement, the odds ratio with 95% confidence interval (95% CI) was calculated. A p-value<0.05 was considered statistically significant.

Results

General Characteristics of the Patients

Among 175 patients initially identified with mildly elevated TSH, 111 were eligible after excluding those with follow-up<6 months or who were lost to follow-up. None had received thyroid-related treatment at admission. General characteristics of the 111 children with mild SH are shown in Table 1. A family history of thyroid disease was present in 49 patients (44.1%), most commonly thyroid nodules (46.9%), goiter (20.4%), autoimmune thyroiditis (12.2%), hypothyroidism (8.2%), thyroid cancer (2.0%), and unspecified thyroid disorders (10.2%).

Overall Follow-up Outcomes

During follow-up, 45 children (40.5%) became euthyroid, 49 (44.2%) remained subclinically hypothyroid, and 17 (15.3%) required levothyroxine. Of those requiring treatment, 10 (58.8%) were female and seven (41.2%) were male.

Treatment indications were isolated TSH>10 mIU/L (n=7; 41.2%), low fT4 (n=3; 17.6%), newly developed goiter (n=3; 17.6%), goiter accompanied by TSH>10 mIU/L (n=1; 5.9%), short stature (n=2; 11.8%), and weight loss with poor appetite (n=1; 5.9%) (Figure 1). Among treated children, one had valproic acid use (TSH>10 mIU/L) and one had prior chemotherapy/radiotherapy (low fT4).

Table 1. General characteristics of the patients

Variable	Mean±SD	Min-Max	n	%
Age (years)			111	
First visit	8.28±4.39	0.37-17.0		
Last visit	9.14±4.47	1.0-17.6		
Follow-up duration (years)	0.86±0.53	0.5-3.0	111	
Gender			111	
Female			59	53.2
Male			52	46.8
Family history of thyroid disease			111	
Absent			62	55.9
Present			49	44.1
Body weight SDS			111	
First visit	-0.06±1.31	-3.50-2.96		
Last visit	-0.09±1.34	-4.19-2.72		
Height SDS			111	
First visit	-0.46±1.28	-4.47-2.36		
Last visit	-0.45±1.31	-4.45-3.02		
BMI SDS			111	
First visit	0.24±1.18	-2.09-3.07		
Last visit	0.17±1.20	-2.09-3.11		
Growth velocity SDS	0.21±1.32	-3.38-4.27	74	

Growth velocity was evaluated in 74 untreated children after excluding those with factors that could affect growth (e.g., epiphyseal closure, growth-suppressing medications, Down syndrome, prior chemotherapy)
BMI: body mass index; SD: standard deviation, SDS: standard deviation score, n: number of patients

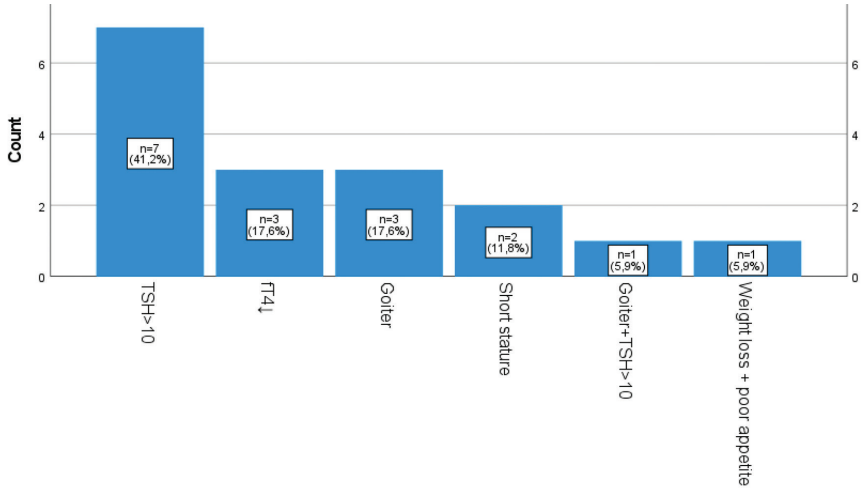

Figure 1. Reasons for initiation of treatment in the study cohort

Overall, 58.8% were treated due to biochemical deterioration, 35.3% due to clinical or structural findings, and 5.9% due to combined abnormalities.

Children requiring treatment had higher baseline TSH levels (p=0.012). Baseline TSH levels differed significantly among follow-up outcome groups (Kruskal-Wallis test, p=0.018), with post-hoc analyses demonstrating that this difference was primarily driven by higher baseline TSH levels in children who required treatment compared with those who became euthyroid (adjusted p=0.020). Although children requiring treatment tended to be older at diagnosis (p=0.049), post-hoc analyses did not confirm a statistically significant age difference between groups. Sex distribution, family history, BMI SDS, and UIC status did not differ significantly. Detailed baseline comparisons according to follow-up outcomes are provided in Table 2.

Potential Factors Contributing to the Etiology of SH

Among the 111 children with SH, no specific etiological factor was identified in 70 patients (63%). Factors contributing to the etiology included autoimmune thyroiditis (HT; n=19, 17.1%), low UIC (n=10; 9%), obesity (n=9; 8.1%), valproic acid use (n=5; 4.5%), and Down syndrome (n=4; 3.6%). In addition, one patient (0.9%) had a history of radiotherapy/chemotherapy, and one (0.9%) had congenital agenesis of the right thyroid lobe. Several children had overlapping factors, including HT with iodine deficiency (n=2), HT with valproic acid therapy (n=1), HT with obesity (n=3), and Down syndrome with valproic acid therapy (n=1). Therefore, the total number of etiology-contributing factors (n=119) exceeded the number of patients.

Comparison According to the Presence of Hashimoto's Thyroiditis

A total of 19 patients (17.1%) had HT, while 92 (82.9%) had no autoimmunity. Patients with HT were older at both baseline and final visits (p<0.001 for both), and female predominance was more pronounced (78.9% vs. 52.2%, p=0.013). Baseline TSH and follow-up duration were similar between groups (p=0.681 and p=0.853, respectively).

At follow-up, euthyroidism was achieved in 26.3% of HT patients and 43.5% of non-HT patients (p=0.048), while persistence of SH was comparable (31.6% vs. 46.7%, p=0.484). Levothyroxine therapy was required significantly more often in the HT group (42.1% vs. 9.8%, p=0.005) (Table 3). Consistently, HT was more

Table 2. Comparison of demographic and clinical characteristics between outcome groups

Variable	Normalized n=45 (%)	Stable n=49 (%)	Progressed n=17 (%)	p-value
Sex★				0.725
Female	31 (68.9%)	36 (73.5%)	12 (70.6%)	-
Male	14 (31.1%)	13 (26.5%)	5 (29.4%)	-
Age at diagnosis (years)	7.9±3.9	7.8±4.6	10.7±4.5	**0.049**
Follow-up duration (years)	0.89±0.42	0.79±0.54	0.99±0.76	0.365
Baseline TSH (mIU/L)	6.64±1.09	7.15±1.36	7.67±1.27	**0.012**
Baseline fT4 (ng/dL)	0.91±0.18	0.86±0.13	0.80±0.12	0.058
Family history of thyroid disease★	24 (53.3%)	17 (34.7%)	11 (64.7%)	0.090
Hashimoto thyroiditis†	5 (11.1%)	6 (12.2%)	8 (47.1%)	**0.005**
Idiopathic etiology†	32 (71.1%)	32 (65.3%)	6 (35.3%)	**0.030**
Use of medications affecting thyroid function (VPA etc.) †	2 (4.4%)	2 (4.1%)	1 (5.9%)	1.000
Obesity†	2 (4.4%)	4 (8.2%)	3 (17.6%)	0.236
BMI SDS	0.24±1.07	0.08±1.27	0.26±1.33	0.766
Urinary iodine status (n=39)†	Normalized n=16 (%)	Stable n=19 (%)	Progressed n=4 (%)	0.823
Severe deficiency	0 (0%)	1 (5.3%)	0 (0%)	
Moderate deficiency	0 (0%)	1 (5.3%)	0 (0%)	
Mild deficiency	4 (25.0%)	2 (10.5%)	2 (50.0%)	
Optimal	8 (50.0%)	8 (42.1%)	2 (50.0%)	
Above requirement	2 (12.5%)	3 (15.8%)	0 (0%)	
Excessive	2 (12.5%)	4 (21.1%)	0 (0%)	

Continuous variables were analyzed using one-way ANOVA with Tukey HSD post-hoc test. Categorical variables were analyzed using Pearson's chi-square (★) or Fisher's exact test (†), depending on expected cell counts. Statistical significance was set at p<0.05. Bold values indicate significance
SH: subclinical hypothyroidism, TSH: thyroid-stimulating hormone, fT4: free thyroxine, BMI: body mass index, SDS: standard deviation score, VPA: valproic acid

frequent in the treatment group (47.1%) than in the euthyroid (11.1%) or persistent SH groups (12.2%) (p=0.005) (Table 2).

Among treated patients, indications differed by HT status. In the HT group (n=8), treatment was initiated for goiter (n=3), TSH>10 mIU/L (n=2), low fT4 (n=2), or combined goiter + elevated TSH (n=1). In the non-HT group (n=9), indications included TSH>10 mIU/L (n=5), low fT4 (n=1), short stature (n=2), and weight loss with poor appetite (n=1) (Table 3).

To isolate autoimmune effects, patients with HT plus additional etiological factors were excluded. Among isolated HT cases (n=13), 30.8% became euthyroid, 38.5% remained SH, and 30.8% required treatment. In idiopathic SH (n=70), these rates were 45.7%, 45.7%, and 8.6%, respectively (p=0.075).

Urinary Iodine Status

Urinary iodine was measured in 39 patients. The findings were: severe deficiency in one (2.6%), moderate in one (2.6%), mild in eight (20.5%), optimal levels in 18 (46.2%), levels indicating risk for iodine-induced hyperthyroidism in five (12.8%), and other adverse outcomes in six (15.4%) (Table 2).

Baseline TSH and Treatment Requirement

When patients were categorized by baseline TSH level, those with TSH>7.5 mIU/L had a significantly higher likelihood of requiring levothyroxine therapy compared with those whose baseline TSH was≤7.5 mIU/L (27.0% vs. 9.5%). This association remained significant in both Pearson's chi-square (p=0.015) and Fisher's exact test (p=0.024), and was further supported by the likelihood ratio test (p=0.019) and a significant linear-by-linear trend (p=0.016). Children with baseline TSH>7.5 mIU/L had approximately 3.5-fold higher odds of treatment initiation (odds ratio=3.55; 95% CI: 1.22-10.28) (Table 4).

Table 3. Comparison of HT and non-HT SH groups

Variable	HT group n=19	Non-HT group n=92	p-value
Age at first visit (years)★	**11.5±3.5**	7.6±4.3	**<0.001**
Age at last visit (years)★	**12.4±3.7**	8.5±4.3	**<0.001**
Female, n (%)†	**15 (78.9%)**	48 (52.2%)	**0.013**
Baseline TSH (mIU/L)★	7.14±1.27	7.00±1.29	0.681
Follow-up duration (years)★	0.88±0.60	0.85±0.52	0.853
Became euthyroid†	5 (26.3%)	40 (43.5%)	**0.048**
Persistent SH†	6 (31.6%)	43 (46.7%)	0.484
Required levothyroxine†	8 (42.1%)	9 (9.8%)	**0.005**
Main reasons for treatment	n=8 (%)	n=9 (%)	
TSH>10 mIU/L	2 (25.0%)	5 (55.6%)	-
Low fT4	2 (25.0%)	1 (11.1%)	-
Short stature	-	2 (22.2%)	-
Weight loss / poor appetite	-	1 (11.1%)	-
Goiter	3 (37.5%)	-	-
Goiter + TSH>10	1 (12.5%)	-	-

Continuous variables were analyzed using the Independent Samples t-test, categorical variables were analyzed using Pearson's chi-square test or Fisher's exact test when appropriate, p<0.05 was considered statistically significant
★: Independent-samples t-test; †: Pearson's chi-square test or Fisher's exact test, as appropriate
HT: Hashimoto's thyroiditis, SH: subclinical hypothyroidism, TSH: thyroid-stimulating hormone, fT4: free thyroxine, BMI: body mass index, SDS: standard deviation score

Table 4. Treatment requirement according to baseline TSH categories

Variable	TSH≤7.5 mIU/L n=74 (%)	TSH>7.5 mIU/L n=37 (%)	Total n=111 (%)	p/OR
No treatment, n (%)	67 (90.5%)	27 (73.0%)	94 (84.7%)	-
Treatment initiated, n (%)	7 (9.5%)	10 (27.0%)	17 (15.3%)	0.015[1]/0.024[2] OR=3.55 (1.22-10.28)
Total, n (%)	74 (66.7%)	37 (33.3%)	111 (100%)	-

[1]Pearson's chi-square test, [2]Fisher's exact test (used due to small expected cell counts). Likelihood ratio and linear-by-linear association tests also supported the results. Odds ratio (OR) with 95% confidence interval (CI) was reported. p<0.05 was considered statistically significant
TSH: thyroid-stimulating hormone

Changes in Thyroid Hormone Levels

In the whole cohort (n=111), TSH levels declined significantly from baseline to the final visit (p<0.001), while fT4 levels remained stable (p=0.605).

TSH Changes by BMI-SDS Classification

Of the 111 children, 8.1% were obese (n=9), 18.9% overweight (n=21), 71.2% normal weight (n=79), and 1.8% underweight (n=2). Baseline TSH did not differ between groups (p=0.614).

At the final visit, TSH differed significantly between BMI categories (p=0.012). Obese children had higher final TSH than normal-weight (p=0.036) and overweight peers (p=0.007). No difference was found between normal-weight and overweight groups (p=0.479).

During follow-up, TSH decreased significantly in normal-weight and overweight groups (both p<0.001), but not in obese (p=0.648) or underweight children (p=0.727) (Table 5). TSH normalization was more frequent in non-obese children.

Clinical Outcomes by BMI-SDS

In the outcome evaluation according to BMI-SDS groups, progression requiring levothyroxine therapy was observed in 12 of 79 normal-weight children (15.2%), 2 of 21 overweight children (9.5%), and 3 of 9 obese children (33.3%). However, the difference between groups was not statistically significant (p=0.282).

Growth Outcomes

No significant changes in weight SDS, height SDS, or BMI SDS were observed between baseline and follow-up (all p>0.05). Among untreated children (n=94), baseline and final anthropometric values were also similar.

Growth velocity in untreated patients (n=74) remained normal (mean SDS=0.21±1.32; median=0.16; p=0.172).

Discussion

The present study contributes to the limited evidence on the natural course and determinants of SH in childhood. Previous studies have reported a pediatric SH prevalence ranging from 1% to 9.5% (3,22,23). The widespread use of thyroid testing in routine pediatric care has increased incidental detections, creating uncertainty about which children require treatment and which can be safely monitored.

In this cohort, most children with mild SH either normalized or maintained stable thyroid function during follow-up. Only 15.3% eventually required levothyroxine therapy. When the analysis was restricted to the idiopathic subgroup, the treatment requirement decreased to 8.6%. This supports the suggestion that SH without identifiable pathology usually follows a benign course (22,24).

These observations agree with the findings of De Luca et al. (25), who showed decreasing TSH levels, stable fT4 levels, and no adverse clinical outcomes in children with idiopathic SH followed for two years. Taken together, these results support a conservative management approach, unless biochemical or clinical deterioration occurs.

In the present study, levothyroxine was initiated for markedly elevated TSH, low fT4, the development of goiter, or other clinically relevant findings, including short stature or weight loss with poor appetite, when judged to be potentially related to thyroid dysfunction. Only 11 of 111 children (9.9%) required treatment solely due to biochemical deterioration, which aligns with reports indicating that mild pediatric SH rarely requires therapy in the absence of autoimmunity or structural abnormalities (26). In the literature, the proportion of children with mild SH requiring medical treatment has been reported to range between approximately 2% and 12% (22,27). Our findings indicated that the natural course of SH varied according to the underlying etiology, although this suggestion requires additional data to validate it.

The etiological background of SH in childhood is diverse. In our cohort, no specific etiology was identified in 63 percent of patients, consistent with previous reports in which idiopathic cases constitute the majority (28). In the remaining patients, factors thought to predispose to SH included HT, iodine imbalance, obesity, and medication-related effects.

Table 5. Evaluation of baseline and final TSH means according to BMI groups

Variable	Underweight n=2 (1.8%)	Normal n=79 (71.2%)	Overweight n=21 (18.9%)	Obese n=9 (8.1%)	p[1]
Baseline TSH (mIU/L)	6.02±1.06	7.09±1.31	6.85±1.11	7.10±1.45	0.614
Final TSH (mIU/L)	6.70±1.03	5.48±2.17	4.66±2.04	7.70±3.91	**0.012**
p[2] (Paired t-test)	0.727	<0.001	<0.001	0.648	-

[1]One-way ANOVA (post-hoc: Tukey HSD), [2]Paired Samples t-test
Levene's test confirmed homogeneity of variances (p=0.353)
Bold values indicate statistical significance (p<0.05)
BMI: body mass index;TSH: thyroid-stimulating hormone;SDS: standard deviation score, CI: confidence interval

HT was the most common identifiable cause in our cohort (17.1%). It predominantly affected older and female patients, consistent with reported epidemiology (29,30,31). The clinical course was less favorable in children with HT. Treatment was required in 42.1% of children with HT compared with 9.8% of those without autoimmunity. Even when the comparison was restricted to isolated HT and idiopathic SH, progression remained more frequent in the HT group (30.8% vs. 8.6%). These findings align with multicenter studies reporting a higher risk of progression when SH coexists with thyroid autoimmunity (13,32,33,34). In HT positive children, goiter or biochemical decline were major determinants of treatment. In idiopathic SH, treatment was mainly initiated for rising TSH or growth related concerns. This pattern suggests that idiopathic SH generally follows a stable course, whereas in HT-positive children treatment decisions were more often driven by objective findings, such as goiter or biochemical deterioration.

Iodine status is an important component of thyroid physiology (35,36,37,38). Although some population studies have described a U shaped association betweenUIC and TSH (39), a recent meta analysis found inconsistent relationships in the mild to moderate deficiency range (40). In our cohort, severe iodine deficiency was uncommon, whereas mild to moderate deficiency was more frequent. However, as UIC was measured only once and in a relatively limited subset of patients, these findings should be interpreted with caution, as single measurements may reflect short-term dietary fluctuations rather than true, persistent deficiency. All children with iodine deficiency were advised to use iodized salt but only one patient with documented deficiency reported not consuming iodized salt. As UIC was not reassessed during follow-up, the effect of iodine supplementation on subsequent TSH trajectories could not be evaluated, representing an inherent limitation of the retrospective study design.

Obesity has been associated with higher TSH levels in numerous pediatric studies. The prevalence of SH ranges between 7-23% in obese children, whereas it remains around 2% in their normal-weight peers (41,42), and national data from the KNHANES VI similarly reported SH rates of 24.3% in obese and 12.8% in non-obese children (43). In our cohort, 27.9% of children were overweight or obese, which is consistent with previous reports (27,44). Although the mechanism underlying elevated TSH in obesity is not fully understood, increased leptin is known to stimulate Thyrotropin Releasing Hormone (TRH) and TSH secretion, while weight loss reduces both leptin and TSH levels (45,46). In our cohort, baseline TSH levels did not differ significantly across BMI groups; however, during follow-up, TSH elevation persisted in obese children, whereas significant declines were observed in normal-weight and overweight peers. Although treatment was required more frequently in obese children, this was not significant. These findings suggest that TSH elevation in obesity may reflect not only an adaptive response but, in some cases, early alterations in the hypothalamic-pituitary-thyroid axis (46,47,48,49).

The magnitude of TSH elevation at diagnosis serves as a surrogate for the intrinsic functional reserve of the thyroid axis. Although pediatric SH generally follows a benign and often reversible course, our findings show that children with baseline TSH greater than 7.5 mIU/L constitute a subgroup with a higher likelihood of deterioration. Recognizing this threshold may assist clinicians in identifying patients who require closer follow-up and earlier therapeutic consideration.

Growth and bone development were not adversely affected in our cohort, consistent with several previous studies. Longitudinal studies in untreated children with SH have similarly reported no significant changes in height SDS, BMI SDS, or growth velocity compared with healthy controls, suggesting that mild thyroid dysfunction does not interfere with growth regulation (12,24,27,50). In our cohort, mean growth velocity SDS was 0.21±1.32, which was comparable to the population mean, further supporting the suggestion that mild SH is unlikely to impair somatic growth or bone maturation in the short to medium term.

Overall, our findings support the growing body of evidence that idiopathic pediatric SH is often a benign and self-limiting condition, whereas SH associated with HT carries a higher likelihood of requiring treatment. Baseline TSH>7.5 mIU/L also appeared to be a predictive factor in our cohort. Management decisions should be individualized, taking into account the underlying etiology, the severity of biochemical abnormalities, and the presence of clinical features.

Study Limitations

This study has several limitations. First, its retrospective design resulted in heterogeneous follow-up intervals and variable follow-up durations, which may have influenced the estimation of progression or regression rates. Second, the overall follow-up period was relatively short, limiting the ability to fully characterize the long-term natural course of mild SH and its potential effects on growth. Future prospective studies with standardized and extended follow-up schedules are warranted.

A small number of patients presented with clinical factors known to influence TSH levels, such as genetic syndromes, use of medications affecting thyroid function, or a history of chemotherapy/radiotherapy. These cases were intentionally included to reflect real-world clinical practice. However, they may introduce potential confounding and should be considered when interpreting the results.

Finally, although predefined index and final visits were used to minimize bias arising from irregular appointment intervals, this approach while improving generalizability, limited the evaluation of time-dependent trends in thyroid function.

Conclusion

In this Turkish cohort, mild SH in children demonstrated a predominantly benign and stable course. Idiopathic cases showed the most favorable outcomes, whereas HT and higher baseline TSH levels were the main predictors of progression requiring treatment. Although TSH elevations tended to persist in obese children, no adverse effects on growth were observed. These findings support an individualized follow-up strategy based on underlying clinical conditions, baseline TSH severity, and autoimmune status. Larger prospective studies are needed to refine risk-based monitoring and management approaches.

Ethics

Ethics Committee Approval: The study was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of University of Health Sciences Türkiye, Taksim Training and Research Hospital (approval no: 102, date: 24.07.2019).

Informed Consent: Due to the retrospective design of the study, informed consent was not required.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: Nur Şeyma Zengin, Elif Sağsak, Concept: Nur Şeyma Zengin, Elif Sağsak, Seda Geylani Güleç, Design: Nur Şeyma Zengin, Elif Sağsak, Seda Geylani Güleç, Data Collection or Processing: Nur Şeyma Zengin, Elif Sağsak, Analysis or Interpretation: Nur Şeyma Zengin, Literature Search: Nur Şeyma Zengin, Writing: Nur Şeyma Zengin.

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Heterozygous TSHR Variants in Pediatric Idiopathic Subclinical Hypothyroidism: Association with a Variable and Compensated Thyroid Phenotype

Ayşe Yaşar[1], Murat Hakkı Yarar[2], Heves Kırmızıbekmez[1], Fatma Dursun[1]

[1]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye
[2]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Medical Genetics, İstanbul, Türkiye

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ABSTRACT

Objective: Subclinical hypothyroidism (SH) in childhood is frequently idiopathic and usually follows a benign course. Heterozygous loss-of-function variants in the thyrotropin receptor (*TSHR*) gene have emerged as a genetic cause of isolated hyperthyrotropinemia but data regarding long-term outcomes and management are limited.

Methods: We retrospectively evaluated children (aged 1-18 years) diagnosed with idiopathic SH. *TSHR* gene analysis was performed using targeted next-generation sequencing in patients selected based on neonatal TSH elevation, family history of SH, thyroid gland *in situ*, or persistence of SH after levothyroxine withdrawal. Clinical, biochemical, ultrasonographic, and treatment outcomes were compared between patients with and without *TSHR* variants.

Results: The cohort consisted of 51 patients followed for a mean of 4.9±2.6 years. Heterozygous *TSHR* variants were identified in 18 (35.2%), including one novel missense variant. Variant-positive patients generally showed a stable, compensated thyroid phenotype without progression to overt hypothyroidism. Levothyroxine therapy was discontinued in 6 of 11 initially treated patients after genetic diagnosis, with sustained biochemical stability. However, five patients required re-initiation of therapy due to rising TSH levels (15.7-27.8 mIU/L) or clinical symptoms. Lower thyroid volume standard deviation scores and the presence of additional clinical risk factors, such as small-for-gestational-age birth or developmental delay, were associated with treatment requirement. No patient developed overt hypothyroidism.

Conclusion: Heterozygous *TSHR* variants appear to contribute to the pathogenesis of pediatric idiopathic SH and are frequently associated with a stable, compensated thyroid phenotype. These findings support a conservative, individualized management strategy rather than routine levothyroxine therapy.

Keywords: Children, idiopathic subclinical hypothyroidism, *TSHR* heterozygous variants, long-term follow-up

Corresponding Author: Ayşe Yaşar, MD, University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye
E-mail: aseyasar@gmail.com **ORCID:** orcid.org/0000-0002-3335-7976

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What is already known on this topic?

Heterozygous thyrotropin receptor (*TSHR*) variants are an established genetic cause of non-autoimmune subclinical hypothyroidism in childhood. Available data suggest that many affected individuals exhibit partial thyroid-stimulating hormone (TSH) resistance with a generally mild and stable clinical course, although long-term outcome data in homogeneous pediatric cohorts remain limited.

What this study adds?

This study included a homogeneous pediatric cohort and demonstrated a high prevalence (35%) of heterozygous *TSHR* variants in a well-characterized idiopathic subclinical hypothyroidism cohort with long-term longitudinal data. Clinical features associated with treatment necessity emerged and support the concept that a subset of these patients may exhibit a compensated, mild TSH resistance phenotype requiring individualized management.

Introduction

Subclinical hypothyroidism (SH), also referred to as isolated hyperthyrotropinemia, is characterized by elevated serum thyroid-stimulating hormone (TSH) levels with normal free thyroxine (fT4) and free triiodothyronine (fT3) concentrations, with no clinical signs of hypothyroidism. The diagnosis is usually confirmed after at least two independent measurements of TSH (1,2). Most cases follow a stable course with a low risk of progression (1,3).

Genetic factors play a significant role in the pathogenesis of SH. Heterozygous variants in the thyrotropin receptor (*TSHR*) gene have been associated with isolated hyperthyrotropinemia (4). These variants have been reported in up to 30% of affected children and are often associated with neonatal TSH elevation and a positive family history (5). Long-term studies suggest that *TSHR*-related SH may represent a stable, compensated state with a recalibrated hypothalamic-pituitary-thyroid axis, often not requiring levothyroxine (LT4) therapy (5,6,7).

Loss-of-function *TSHR* mutations are the most common cause of TSH resistance (RTSH), with a phenotype ranging from severe congenital hypothyroidism (CH) in biallelic cases to mild, non-autoimmune SH in heterozygous individuals (8,9,10,11). Reported variant frequencies range from 11% to 29% among cohorts of patients with SH across different populations (8,9,12,13,14). The aim of this study was to evaluate the frequency, clinical characteristics, and long-term outcomes of heterozygous *TSHR* variants in children with idiopathic SH.

Methods

Study Design and Population

This retrospective study included children aged 1-18 years diagnosed with idiopathic SH and followed between 2014 and 2024. SH was defined as persistently elevated TSH (>5.5 mIU/L) with normal fT4 and fT3 on at least two measurements (15). Patients with autoimmune thyroid disease, thyroid agenesis or ectopy, syndromes, prior thyroid surgery, medications affecting thyroid function, obesity-related TSH elevation, follow-up <12 months, or incomplete data were excluded. Patients were categorized based on clinical presentation and screening history. Those referred through the national newborn screening program or with documented neonatal TSH elevation were initially evaluated for CH. However, only patients who did not fulfill criteria for permanent CH after LT4 withdrawal were included in the idiopathic SH cohort. Patients diagnosed later in childhood without evidence of neonatal TSH elevation were classified as having acquired SH.

The study was conducted in accordance with the Helsinki II Declaration and approved by University of Health Sciences Türkiye, Ümraniye Training and Research Hospital Scientific Research Ethics Committee (approval no: 459, date: 26.12.2024). Written informed consent for the use of anonymized clinical and genetic data was obtained from the parents or legal guardians of all participants. Baseline demographic characteristics, including age [categorized for descriptive purposes as 1-3 years (toddler), 3-10 years (childhood), and 10-18 years (adolescence)], sex, and pubertal status, as well as perinatal history (gestational age, birth weight, neonatal TSH elevation) and family history (thyroid disease, consanguinity), were recorded. Height, weight, and body mass index were measured at diagnosis and during follow-up visits at 6-12-month intervals and expressed as standard deviation scores (SDS) using national reference data (16). Pubertal staging was assessed according to the Tanner criteria (17). LT4 therapy was initiated in accordance with guideline recommendations in symptomatic patients or in asymptomatic individuals with TSH levels >10 mIU/L (18,19).

Laboratory and Imaging Assessment

Fasting blood samples were obtained between 08:00 and 10:00 A.M. Serum TSH, fT4, fT3, and thyroglobulin (Tg) were measured using an electrochemiluminescence immunoassay on a Cobas 8000 analyzer (Roche Diagnostics). Reference ranges were: TSH 0.35-4.75

mIU/L, fT3 2-4.4 ng/L, fT4 0.85-1.70 ng/dL, and Tg 3.5-77 ng/mL. Ultrasonography was performed by a pediatric radiologist using high-frequency linear transducers (4.8-11 or 5-14 MHz) on Toshiba Aplio 500/300 or Siemens Acuson S3000 systems. Thyroid volume, echogenicity, nodular structure, and thyroid location were evaluated.

Patient Selection for *TSHR* Gene Testing

Idiopathic SH was defined after thorough exclusion of all secondary causes. Genetic analyses for *TSHR* was performed in patients who fulfilled at least one of the following criteria:

(i) Neonatal screening history: Persistent elevation of TSH with normal fT4 after withdrawal of LT4 therapy in infants identified by the national newborn screening program for CH (withdrawal trial applied when LT4 dose <2 μg/kg/day) (20);

(ii) Family history: Presence of SH in a first-degree relative;

(iii) Thyroid gland *in situ*: Thyroid gland located *in situ* with normal or hypoplastic size on ultrasonography; or

(iv) LT4 withdrawal evaluation: In patients previously treated for idiopathic SH, thyroid function tests, thyroid autoantibodies, and thyroid ultrasonography were reassessed after LT4 discontinuation. Persistence of SH in two measurements taken ≥2 months apart was considered diagnostic. Re-initiation of LT4 therapy was not based solely on TSH elevation but was considered in the presence of sustained TSH levels >10 mIU/L on repeated measurements and/or clinical symptoms suggestive of hypothyroidism. Asymptomatic patients with stable TSH levels between 5-10 mIU/L and normal fT4 concentrations were monitored longitudinally without treatment. Treatment decisions were made based on overall clinical assessment rather than isolated biochemical findings (1).

Phenotypic Interpretation of Variant-Positive Cases

To improve clinical interpretation, variant-positive patients were further evaluated according to the degree of genotype-phenotype concordance. Cases were pragmatically classified as: (i) solved/likely RTSH phenotype, (ii) indeterminate phenotype, or (iii) unresolved phenotype. This classification was based on longitudinal biochemical profile, family segregation pattern, thyroid imaging findings, neonatal thyroid status, and overall clinical course. The proposed categorization was intended solely as a descriptive clinical framework to aid phenotypic interpretation and should not be considered a formal assessment of pathogenicity, as all identified variants were classified as variants of uncertain significance (VUS).

Genetic Analysis

TSHR gene analysis was performed using a targeted next-generation sequencing assay covering all coding exons and exon-intron boundaries. Sequencing was performed using the Illumina NovaSeq platform (Illumina, Inc., San Diego, CA, USA), and data were analyzed using a validated bioinformatics pipeline with alignment to the hg38 reference genome. Variants were classified according to American College of Medical Genetics and Genomics (ACMG) guidelines (21).

The panel also included genes associated with CH (*DUOX2*, *DUOXA2*, *TG*, *TPO*, *SLC5A5*, *IYD*, *PAX8*, *NKX2-1*). In the initial cohort, eight patients were identified as carrying heterozygous variants in genes other than *TSHR* (*DUOX2*, *PAX8*, and *TG*) and were excluded to minimize potential oligogenic effects and ensure a genetically homogeneous cohort. Variants with low analytical reliability, synonymous changes without predicted functional impact, and common benign variants were excluded. All identified *TSHR* variants were classified as VUS. This approach does not detect large genomic rearrangements or deep intronic variants; therefore, findings were interpreted in conjunction with clinical and biochemical data.

The cohortwas stratified based on *TSHR* sequencing results into variant-positive and no detectable *TSHR* variant. Comparative analyses were performed between the two groups in terms of clinical characteristics and biochemical parameters. The variant positive subgroup was further stratified into those either receiving or not receiving LT4 therapy as an initial management approach.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. Continuous variables were expressed as mean ± SD or median (interquartile range), as appropriate. Categorical variables were analyzed using the chi-square test. Comparisons between groups were performed using the Mann-Whitney U test. All tests were two-tailed, and a p value <0.05 was considered statistically significant.

Results

Study Population

A total of 51 patients with idiopathic SH were included (29 girls, 56.9%). The mean age at diagnosis was 7.9±4.1 years, but ranged from 1.2-16.6 years, and the mean follow-up duration was 4.9±2.6 years. At presentation, 17 patients were adolescent, 26 were in the childhood age-range, and eight were toddlers. At presentation 30 patients (~60%) were prepubertal. Most patients exhibited a standard perinatal history, with 70% being born at term and 80% exhibiting an appropriate birth weight for gestational age. Sixteen patients were referred through the national newborn

screening program and were considered to have congenital onset, whereas 35 were classified as having acquired SH based on thyroid function abnormalities detected during childhood, family history or non-specific symptoms. A history of neonatal TSH elevation was identified in 24 patients, of whom 16 were referred through the national newborn screening program, while in eight patients, neonatal TSH elevation was documented retrospectively from previous medical records during follow-up. Patients referred through the screening program were initially treated with LT4, and withdrawal trials were performed during follow-up. After withdrawal, therapy was re-initiated in four patients, who were subsequently classified as having permanent CH, while the remaining patients were considered to have idiopathic SH. Among the 35 patients initially classified as having acquired SH, 21 were followed without treatment, while 14 had received LT4 at presentation. Following withdrawal trials, therapy was re-initiated in six patients. *TSHR* variants were identified in three of these patients, and the reasons for re-initiation are detailed in the Discussion section. In the remaining three, therapy was re-initiated due to serum TSH levels >10 mIU/L. A first-degree family history of SH was present in 38 (74.5%) patients, and parental consanguinity was identified in nine (17.65%) cases.

Genetic Analysis of the *TSHR* Gene

The cohort was divided into *TSHR* variant-positive (Group 1, n=18) and no detectable *TSHR* variant (Group 2, n=33). Group 1 was also assigned into subgroups based on initial therapeutic approach, into treated (Group 1a, n=11) and untreated patients (Group 1b, n=7). The clinical characteristics of the 18 patients (35.2%) carrying a *TSHR* variant are summarized in Table 1. All variants were heterozygous. A total of nine distinct missense variants were detected in these patients. Eight variants had been previously reported in the literature (p. Asp474Glu, p. Thr62Ala, p. Pro162Ala, p. Gly245Ser, p. Pro68Ser, p. Glu506Lys, p. Arg519His, p. Ala593Val), whereas one (p. Ile117Met) was novel. The variants were distributed across the *TSHR* gene without evidence of a defined hot-spot region. Bioinformatic predictions indicated loss-of-function effects for all missense variants. The most frequent variant was p. (Thr62Ala), identified in five patients. Among the 18 variant-positive patients, 11 had a retrospectively reported family history of elevated TSH, whereas seven had no documented family history.

Clinical Course of Variant-Positive Patients

Four of the 18 variant-positive patients were referred through newborn screening (using a blood-spot TSH cut off of 5.5 mU/L), while the remaining cases were evaluated because of a family history or clinical suspicion. Although baseline TSH levels were higher in patients who initially received LT4 therapy (Group 1a, n=11; 10.6±4.71 mIU/L) compared with untreated patients (Group 1b, n=7; 7.39±1.86 mIU/L), this difference was not significant (p=0.181). Following identification of the *TSHR* variant, treatment withdrawal was attempted in all Group 1a patients under close biochemical and clinical surveillance. Five maintained a stable SH phenotype without further intervention. In five patients (Patients 2c, 3c, 7, 8, and 12), LT4 therapy was re-initiated after at least 6 months of follow-up due to persistently elevated TSH levels (mean 21.1±4.58 mIU/L) confirmed on repeated measurements and/or the development of clinical or structural features suggestive of impaired thyroid function. Several of these patients also exhibited additional clinical features, including thyroid hypoplasia, hyperthyrotropinemia related hyperprolactinemia and oligomenorrhea, a history of small for gestational age (SGA), or developmental delay and obesity. In patient no. 13, although LT4 therapy had been initiated at diagnosis, follow-up data were insufficient to clearly determine treatment status during follow-up, and therefore this patient was not included in the re-initiation group. All patients in Group 1b remained off therapy throughout follow-up, with TSH values consistent with euthyroidism or SH. In addition, anthropometric measures and thyroid function tests did not differ significantly between Groups 1a and 1b at diagnosis or at the final evaluation. Thyroid volume SDS was significantly lower in Group 1a compared with Group 2 (p=0.023). Among the 18 variant-positive patients, genotype-phenotype concordance showed marked heterogeneity. The multigenerational family cluster carrying the p.Thr62Ala variant (Patients 2c-6c) was considered the subgroup most strongly consistent with a "solved/likely RTSH phenotype" because of familial clustering and persistent non-autoimmune hyperthyrotropinemia. In contrast, several other cases were interpreted as having "indeterminate" or "unresolved" phenotypes due to spontaneous normalization of thyroid function, absence of segregation analysis, or atypical clinical findings not fully explained by heterozygous *TSHR* variants alone. In particular, patients who became euthyroid during follow-up (Patients 9a, 9b, and 10) were classified as having an "unresolved phenotype," since spontaneous biochemical normalization is not fully compatible with persistent genetically determined partial TSH resistance. Likewise, the two patients with thyroid hypoplasia were considered clinically atypical and interpreted as being closer to an "indeterminate phenotype," suggesting the possible contribution of additional genetic or non-genetic mechanisms beyond isolated heterozygous *TSHR* variation. These findings suggest that thyroid function remains stable in a substantial proportion of individuals with heterozygous *TSHR* variants, and the need for treatment varies according to the clinical characteristics.

Table 1. Clinical, genetic, and thyroid-related characteristics of patients carrying heterozygous *TSHR* variants

No.	Sex-age at diagnosis (years)	Follow-up duration (years)	Gen/transcript/ genomic loc.(hg38)	HGVS/ACMG	Baseline TSH* (mIU/L)	LT4 at diagnosis	Thyroid ultrasound	Highest TSH* (mIU/L) during follow-up	Clinical feature	LT4 during follow-up	Final thyroid status
1[a]	F-6.3y	3y	TSHR NM_000369.5 chr14:81143480 C>A exon 10	c.1422C>A p.Asp474Glu missense VUS	6.58	No	Normal	6.67	None	No	SH
1[b]	F-5.11y	10y	TSHR NM_000369.5 chr14:81143480 C>A exon 10	c.1422C>A p.Asp474Glu missense VUS	10.9	Yes	Normal	8.47	None	No	SH
2[c]	F-2.6y	4.3y	TSHR NM_000369.5 chr14:81062161 A>G exon 2	c.184A>G p.Thr62Ala missense VUS	14.8	Yes	Normal	20.4	Growth delay (SGA)	Yes	SH
3[c]	M-8.1y	9y	TSHR NM_000369.5 chr14:81062161 A>G exon 2	c.184A>G p.Thr62Ala missense VUS	13.6	Yes	Normal	15.7	None (preterm SGA)	Yes	SH
4[c]	F-5.3y	7y	TSHR NM_000369.5 chr14:81062161 A>G exon 2	c.184A>G p.Thr62Ala missense VUS	15.6	Yes	Normal	19.1	None	No	SH
5[c]	F-9.9y	4y	TSHR NM_000369.5 chr14:81062161 A>G exon 2	c.184A>G p.Thr62Ala missense VUS	13.6	Yes	Normal	7.35	None	No	SH
6[c]	F-13.1y	4y	TSHR NM_000369.5 chr14:81062161 A>G exon 2	c.184A>G p.Thr62Ala missense VUS	12.9	Yes	Normal	7.52	None	No	SH
7	F-3.11y	8y	TSHR NM_000369.5 chr14:81092547 C>G exon 6 rs121908863	c.484C>G p.Pro162Ala missense VUS	15.6	Yes	Hypoplastic	27.8	None	Yes	SH
8	F-15.11y	4y	TSHR NM_000369.5 chr14:81087987 A>G exon 4	**c.351A>G p.Ile117Met** missense VUS	9.56	Yes	Hypoplastic	25.5	Hyperprolactinemia/ oligomenorrhea	Yes	SH
9[a]	F-12.8y	4y	TSHR NM_000369.5 chr14:81139719 G>A exon 9 rs189506473	c.733G>A p.Gly245Ser missense VUS	10.9	No	Normal	4.42	None	No	Euthyroid

Table 1. Continued

No.	Sex-age at diagnosis (years)	Follow-up duration (years)	Gen/transcript/ genomic loc.(hg38)	HGVS/ACMG	Baseline TSH* (mIU/L)	LT4 at diagnosis	Thyroid ultrasound	Highest TSH* (mIU/L) during follow-up	Clinical feature	LT4 during follow-up	Final thyroid status
10	M-9.11y	6y	TSHR NM_000369.5 chr14:81062179 C>T exon 2 rs142063461	c.202C>T p.Pro68Ser missense VUS	11.1	No	Normal	4.95	None	No	Euthyroid
11	F-12y	7y	TSHR NM_000369.5 chr14:81143574 G>A exon 10 rs762048531	c.1516G>A p.Glu506Lys missense VUS	15.2	Yes	Normal	7.43	None	No	SH
12	M-12.5y	4y	TSHR NM_000369.5 chr14:81143836 C>T exon 10	c.1778C>T p.Ala593Val missense VUS	9.53	Yes	Normal	19	Obesity	Yes	SH
13	F-15.1y	4y	TSHR NM_000369.5 chr14:81092547 C>G exon 6 rs121908863	c.484C>G p.Pro162Ala missense VUS	10.5	Yes	Normal	18.5	None	-	SH
14	F-11.9y	4y	TSHR NM_000369.5 chr14:81143614 G>A exon 10 rs780018604	c.1556G>A p.Arg519His missense VUS	10.2	No	Normal	8.62	None	No	SH
15[a]	M-6.9y	2y	TSHR NM_000369.5 chr14:81062179 C>T exon 2 rs142063461	c.202C>T p.Pro68Ser missense VUS	9.1	No	Normal	10.1	Twin/none	No	SH
15[b]	M-6.9y	2y	TSHR NM_000369.5 chr14:81062179 C>T exon 2 rs142063461	c.202C>T p.Pro68Ser missense VUS	9.1	No	Normal	10.1	Twin/none	No	SH

Novel variants not reported in gnomAD v4.1.0 are indicated in bold. Suffixes: "[a]", index patient; "[b]", sibling; "c", cousin from the same extended family.
SH: Subclinical hypothyroidism; LT4: Levothyroxine; SGA: Small for gestational age; M: Male; F: Female; *: Highest TSH value recorded baseline and during follow-up

Comparison of Variant-Negative and Variant-Positive Patients

Clinical and laboratory characteristics of variant-positive patients are presented in Table 2. Overall, anthropometric measures and thyroid function remained stable from diagnosis to the final follow-up, and no clinically significant progression in thyroid volume was observed. Comparisons between the groups revealed generally similar biochemical and anthropometric profiles, indicating that heterozygous *TSHR* variants did not exert a significant impact on thyroid function or growth parameters. At the last follow-up, TSH levels were significantly higher in Group 1. Although the proportion of patients continuing LT4 therapy was greater in the variant-positive group, this difference did not reach statistical significance (Table 2). Although most demographic and clinical characteristics were comparable between groups, several significant differences were observed in patients carrying *TSHR* variants. The frequency of persistent neonatal TSH elevation was significantly lower in the variant-positive group (p=0.001). At the final follow-up, SH was significantly more common among variant positive patients compared with variant-negative individuals (p=0.015). In addition, a family history of thyroid disease was present in all patients with *TSHR* variants and was significantly more frequent than in the variant-negative group (p=0.030) (Table 3).

Discussion

This study provides comprehensive data on the prevalence, phenotype, and long-term clinical course of heterozygous *TSHR* variants in children with idiopathic SH. The detection of a 35% variant frequency, including one previously unreported variants, highlights the considerable genetic heterogeneity underlying *TSHR*-related thyroid dysfunction. The frequent occurrence of neonatal TSH elevation and a family history of thyroid disease, together with the predominance of diagnosis during childhood, suggests that *TSHR*-related mild thyroid

Table 2. Comparison of clinical, biochemical, and diagnostic characteristics between *TSHR* variant-positive and variant-negative patients at baseline and final follow-up

Parameter		Group 1 (*TSHR* variant +) Mean ± SD n=18	Group 2 (*TSHR* variant -) Mean ± SD n=33	p value
Presentation				
TSH-1 (0.66-4.75 mIU/L)		8.90±3.10	9.97±3.34	0.983
fT4-1 (0.78-1.31 ng/dL)		1.20±0.19	1.26±0.10	0.405
TSH-2 (0.66-4.75 mIU//L)		9.71±2.48	9.47±2.84	0.239
fT4-2 (0.78-1.31 ng/dL)		1.20±0.20	1.26±0.16	0.413
TG (3.5-77 ng/mL)		32.5±28.40	50.5±42.7	0.186
Weight SDS		0.06±1.27	-1.73±4.86	0.884
Height SDS		-0.03±1.27	0.08±1.19	0.598
BMI SDS		0.10±1.08	-0.91±1.82	0.838
Thyroid volume SDS		-0.47±1.09	-0.22±0.79	0.331
Last visit				
TSH (0.66-4.75 mIU/L)		8.29±3.87	5.97±3.28	**0.007**
fT4 (0.78-1.31 ng/dL)		1.21±0.18	1.17±0.17	0.076
fT3 (2.56-5.01 ng/dL)		4.29±0.79	4.41±0.43	0.855
Weight SDS		-0.04±1.45	-0.79±0.48	0.884
Height SDS		-0.12±1.96	-0.08±0.89	0.598
BMI SDS		0.10±1.03	-0.68±0.77	0.838
LT4, n (%)	Yes	5 (27.7)	7 (21.2)	0.181
	No	13 (72.2)	26 (78.8)	
Diagnosis, n (%)	CH	4 (22.2)	12 (36.3)	0.298
	ASH	14 (77.7)	21 (63.6)	

Continuous variables were compared using the Mann-Whitney U test; categorical variables were compared using the chi-square test. TSH-1 and fT4-1 represent thyroid function tests at diagnosis (baseline); TSH-2 and fT4-2 represent values at the last follow-up evaluation. Bold values indicate statistical significance (p<0.05)
TSH: thyroid stimulating hormone, fT4: free thyroxine, fT3: free triiodothyronine, TG: thyroglobulin, BMI: body mass index, SDS: standard deviation score, CH: congenital hypothyroidism, ASH: acquired subclinical hypothyroidism

dysfunction tends to become clinically apparent in the pediatric age group.

Previous studies have reported widely variable frequencies of *TSHR* loss-of-function variants, ranging from 4% to 52%. These cohorts consistently highlighted the prominence of a positive family history and the frequent identification of cases through newborn screening (5,8,12,13,22,23). In a cohort of 111 children with SH, Vigone et al. (8) identified 17 distinct *TSHR* variants in 34 patients, eight of which were novel; notably, 17 (50%) patients were detected through newborn screening and 27 (79%) had a family history of SH. Similarly, Tenenbaum-Rakover et al. (6) reported that five of 27 (18.5%) patients carrying *TSHR* variants had abnormal newborn screening results. In another study, Calebiro et al. (22) identified *TSHR* variants in 12% of children with non-autoimmune SH.

Across these reports, most affected individuals were diagnosed in early to mid-childhood, particularly between 6 and 12 years of age. This pattern indicates that *TSHR*-related mild thyroid dysfunction is typically recognized in the pediatric age group. These studies were conducted in heterogeneous cohorts that included various SH subtypes and diverse *TSHR* genotypic profiles. In contrast, our study focused exclusively on children with idiopathic SH who were simple heterozygous carriers of *TSHR* variants. Consequently, our cohort represents a more homogeneous and clinically refined population compared with many previously published series. The 35% variant prevalence we observed falls within the upper range of reported frequencies, likely to reflect this methodological specificity. Consistent with previous reports, we found that *TSHR*-related SH most commonly manifested during childhood, underscoring the importance of early recognition and long-term follow-up.

Interestingly, the rate of persistent neonatal TSH elevation was lower in the variant-positive group. This finding is not fully consistent with the expected congenital presentation. However, a direct causal relationship cannot be established due to the lack of segregation and functional validation. Therefore, these variants should be interpreted with caution and may be considered genetic modifiers contributing to phenotypic variability rather than primary disease-causing variants. The need for treatment in *TSHR* mutation-related SH remains a matter of debate (24). In children with genetically confirmed RTSH, particularly those older than 3 years who are asymptomatic and exhibit normal growth, treatment decisions should not rely solely on TSH levels (25). This condition is considered a compensated dysfunction arising from the adaptation of the hypothalamic-pituitary-thyroid axis to a new equilibrium, and pharmacological therapy is generally not recommended in most cases. In our cohort, LT4 therapy was discontinued in 45% of treated patients (5/11) following the identification of a *TSHR* variant.

Among patients in whom treatment was either not initiated or subsequently withdrawn, TSH levels ranged between 4.42 and 19.1 mIU/L, and no clinical signs of hypothyroidism were observed during two years of follow-up, even in the patient with the highest TSH value (Patient 4c). Although previous studies have reported discontinuation rates of up to 70% after reassessment, with TSH levels typically remaining within the 5-10 mIU/L range (6,8), our rates were lower. This discrepancy may be attributable to the higher baseline TSH levels observed in our cohort. As highlighted by Kara et al., (26) variability in the TSH set point in RTSH indicates that treatment decisions should be guided by clinical findings rather than biochemical thresholds alone (26). In keeping with this, re-initiation of L-T4 therapy in our cohort was based on an individualized assessment, considering features such as SGA history, hyperprolactinemia-related oligomenorrhea, obesity, and, in one patient, thyroid hypoplasia with marked TSH elevation (27.8 mIU/L).

Table 3. Comparison of demographic, clinical, and biochemical characteristics between the groups

Parameter	Category	Group 1, n (%)	Group 2, n (%)	p value
Sex	Female/male	13 (72.2)/5 (27.8)	16 (48.5)/17 (51.5)	0.102
Gestational age	<38/38-42 weeks	6 (40)/9 (60)	5 (16.1)/26 (83.9)	0.219
Birth weight	SGA/AGA	2 (13.3)/13 (86.7)	2 (6.45)/29 (93.5)	0.357
Consanguinity	Yes/no	6 (37.5)/10 (62.5)	3 (11.5)/23 (88.5)	0.086
Neonatal TSH elevation (persistent)	Yes/no/unknown	4 (22)/10 (56)/4 (22)	20 (61)/4 (12)/9 (27)	**0.001***
Pubertal status	Pubertal/prepubertal	10 (55.6)/8 (44.4)	11 (33.3)/22 (66.7)	0.123
Age at diagnosis	Adolescence/childhood/toddler	8 (44.4)/9 (50.0)/1 (5.6)	9 (27.3)/17 (51.5)/7 (21.2)	0.240
Final thyroid status	SH/euthyroid	15 (83.3)/3 (16.7)	16 (48.5)/17 (51.5)	**0.015**
Family history of thyroid disease	Yes/no	18 (100)/0 (0)	20 (74)/7 (26)	**0.030***

Categorical variables were analyzed using the Chi-square test. Bold values indicate statistical significance ($p<0.05$). Neonatal TSH elevation data were obtained from medical records when available and, in some cases, from parental reports. Persistent elevation was defined as TSH levels above the newborn screening cutoff confirmed on repeat testing and/or requiring initiation of L-T4 therapy during infancy
TSH: thyroid stimulating hormone, SGA: small for gestational age, AGA: appropriate for gestational age, SH: subclinical hypothyroidism

Radetti et al. (27) reported that patients in whom LT4 therapy was re-initiated were most often either compound heterozygotes or single heterozygous individuals with additional risk factors for thyroid dysfunction, such as being SGA. Similarly, Vigone et al. (8) suggested that L-T4 therapy may also be considered in single heterozygous patients belonging to special risk groups, including those born preterm, SGA, after multiple pregnancies, or conceived through assisted reproductive techniques (8). However, even in light of these studies, the presence of SGA alone should not be considered a definitive indication for treatment. Among the five cousins carrying the same variant in our cohort, two had a history of SGA and similar biochemical findings, suggesting a possible association between the variant and the phenotype. In particular, LT4 therapy in Patient 2c was re-initiated not only because of the history of SGA, but also due to a decline in height SDS observed during follow-up (from -2.09 to -2.48). The subsequent improvement in height SDS after treatment (-2.02) further supported this approach. In contrast, the remaining three variant-positive individuals remained clinically and biochemically stable and so conservative follow-up was preferred.

Although thyroid hypoplasia is not a typical feature of SH associated with *TSHR* variants, this finding was observed in two patients in our cohort. Patient no. 7 was referred through the newborn screening program with a preliminary diagnosis of CH and was initiated on LT4 therapy. Following a trial of treatment withdrawal, LT4 therapy was re-initiated due to persistent elevation of TSH levels. No variants were identified in other genes associated with CH. Segregation analysis could be performed only in this patient and demonstrated the presence of the same *TSHR* variant in the mother; this finding was considered supportive of a potential clinical effect of the variant. Patient no. 8, in contrast, was followed as having acquired SH. LT4 therapy was initially started but discontinued after the identification of a *TSHR* variant. During follow-up, the development of oligomenorrhea and hyperprolactinemia prompted the re-introduction of thyroxine replacement therapy. Following normalization of TSH levels after treatment, prolactin levels also normalized and regular menstrual cycles were restored. In our cohort, obesity was present in one of the patients in whom LT4 therapy was re-initiated. In this case, obesity had already been present prior to treatment withdrawal and did not develop during follow-up. It is well established that obesity in children is associated with mild elevations in TSH levels, which are generally considered adaptive and reversible. However, in the context of genetically confirmed RTSH, the coexistence of obesity may complicate the interpretation of thyroid function and, in selected cases, may raise concern for relative tissue-level thyroid hormone insufficiency. In this patient, the presence of relatively high TSH levels (≈19 mIU/L), together with obesity as an additional metabolic risk factor, contributed to the clinical decision to continue treatment.

These observations indicate that heterozygous *TSHR* variants may present with a broader and more heterogeneous clinical spectrum than traditionally expected. Previous studies have shown that even carriers of the same *TSHR* variant within the same family can exhibit markedly different TSH levels, along with notable fluctuations over time within the same individual. Collectively, these results suggest that treatment decisions should not be based solely on the presence of a genetic variant but rather should be guided by the individual clinical course and biochemical characteristics of each patient. Environmental factors (e.g., iodine intake, acquired thyroid disorders, and drugs) or other genetic modifiers (e.g., polygenic inheritance, polymorphisms in thyroid hormone pathway genes, and epigenetic factors) are probably responsible for such variability (28). Therefore, the atypical morphological and biochemical features observed in our patients may not be solely attributable to a single genetic variant. Further molecular and functional studies are needed to better elucidate these mechanisms. Tenenbaum-Rakover et al. (6) reported in their long-term follow-up of 27 children with *TSHR*-related SH and CH that, in heterozygous cases, the hypothalamic-pituitary-thyroid feedback set point remains appropriately maintained, resulting in a stable and mild clinical course. Similarly, Calebiro et al. (22) described a maximum TSH level of 17 mIU/L in a 2-month-old infant, whereas in our cohort the highest TSH concentration was 27.8 mIU/L, observed in a 5-year-old girl. This finding suggests that the phenotypic expression of *TSHR* variants may vary across age groups and that a compensated state can be preserved, even at higher TSH levels in some patients.

In patients heterozygous for *TSHR* mutations, SH is generally considered a compensated thyroid dysfunction with an appropriately adjusted pituitary-thyroid set point and does not usually require treatment (1). However, it may progress to a decompensated state, particularly in the presence of concomitant thyroid disease (7,9). Vigone et al. (8) reported that discontinuation of LT4 was not associated with adverse clinical or biochemical outcomes. Our findings are largely consistent with the existing literature. Among variant-positive patients, anthropometric measures, thyroid function tests, and thyroid volume remained stable from diagnosis to the final assessment, and none of the untreated or treatment-discontinued children developed clinical hypothyroidism. However, the re-emergence of treatment requirements in some patients, particularly those with higher TSH levels, lower thyroid volume SDS, a history of SGA birth, or additional clinical risk factors, suggests that a treatment-free approach may not be equally safe for all individuals. Therefore, treatment decisions in children carrying heterozygous *TSHR*

variants should be carefully individualized, incorporating thyroid volume and the overall clinical risk profile into a comprehensive assessment.

All identified variants were classified as VUS according to ACMG criteria, and no definitive conclusions regarding their pathogenicity can be drawn. Accordingly, identification of a heterozygous *TSHR* variant should not automatically be considered equivalent to a definitive molecular diagnosis of RTSH, particularly in the absence of segregation, functional, or longitudinal supportive evidence.

Study Limitations

The absence of segregation analysis and functional validation, as well as the lack of formal exclusion of macro-TSH, constitute important limitations of the study and restrict causal inference. However, the observed phenotypic consistency supports a potential contributory role. In addition, iodine status was not assessed biochemically but there was no evidence suggestive of iodine imbalance based on clinical history, and the study population was derived from a region considered iodine sufficient. Nevertheless, the absence of objective iodine measurements should also be acknowledged as a significant limitation. Despite these limitations, the prolonged follow-up and consistent biochemical stability observed in variant-positive individuals provide clinically meaningful evidence supporting a compensated, mild RTSH phenotype, rather than progressive thyroid dysfunction.

Conclusion

Heterozygous *TSHR* variants were frequently identified in children with idiopathic SH and were associated with a generally stable and compensated thyroid phenotype in a subset of patients. However, variant detection alone was insufficient to establish definitive RTSH in all individuals. While most patients could be managed conservatively, selected cases required individualized treatment decisions based on clinical course and biochemical severity.

Ethics

Ethics Committee Approval: The study was conducted in accordance with the Helsinki II Declaration and approved by University of Health Sciences Türkiye, Ümraniye Training and Research Hospital Scientific Research Ethics Committee (approval no: 459, date: 26.12.2024).

Informed Consent: Written informed consent for the use of anonymized clinical and genetic data was obtained from the parents or legal guardians of all participants.

Footnotes

Authorship Contributions

Concept: Ayşe Yaşar, Fatma Dursun, Design: Ayşe Yaşar, Fatma Dursun, Data Collection or Processing: Ayşe Yaşar, Fatma Dursun, Murat Hakkı Yarar, Analysis or Interpretation: Ayşe Yaşar, Fatma Dursun, Literature Search: Ayşe Yaşar, Fatma Dursun, Writing: Ayşe Yaşar, Fatma Dursun, Heves Kırmızıbekmez.

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CASE REPORT

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Novel *IGF1R* Variants in Short Stature: Lessons from Two Patients and Outcome of Growth Hormone Therapy

Mehmet Eltan[1], Hilal Sekizkardeş[1], Sezin Canbek[2], Murat Hakkı Yarar[2], Saygın Abalı[3], Zehra Yavaş Abalı[4]

[1]University of Health Sciences Türkiye, Sancaktepe Şehit Prof. Dr. İlhan Varank Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Türkiye
[2]University of Health Sciences Türkiye, Ümraniye Training and Research Hospital, Clinic of Medical Genetics, İstanbul, Türkiye
[3]Acıbadem Mehmet Ali Aydınlar University Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Türkiye
[4]Marmara University Pendik Training and Research Hospital, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye

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ABSTRACT

The growth hormone (GH)-insulin-like growth factor 1 (IGF1) axis is essential for the regulation of growth. IGF1 exerts its effects through the IGF1 receptor type 1 (IGF1R) that plays a pivotal role in fetal and postnatal growth. Pathogenic monoallelic *IGF1R* variants are known to cause pre- and postnatal growth restriction, often accompanied by normal or elevated serum IGF1 levels. Herein, the clinical and genetic characteristics of two cases with *IGF1R* novel variants, describing their growth patterns, endocrinological findings, and response to recombinant human GH (rhGH) therapy are presented. Case 1 was a 6.3-year-old boy, with birth weight of 2,500 g [-2.5 standard deviation score (SDS)] and a height of 101.5 cm (-3.2 SDS). Laboratory investigations revealed IGF1 and IGFBP3 levels of 117.8 ng/mL (0.9 SDS) and 4.55 µg/mL (1.3 SDS), respectively. Clinical exome sequencing (CES) identified a novel heterozygous c.3722+1G>A/p.(?) variant in the *IGF1R* (NM_000875.5) inherited from the mother. At 6.9 years of age, rhGH treatment was initiated at a dose of 0.035 mg/kg/day. The patient has been receiving rhGH for two years, achieving a height gain of +0.3 SDS per year, with an uneventful follow-up. Case 2 was a 3-year-old male with short stature and a history of being born small for gestational age (SGA) (-2.6 SDS). His height and weight were 70.0 cm (-2.1 SDS) and 8.8 kg (-1.1 SDS), respectively. He had a history of frequent respiratory infections. Pituitary hormone levels were normal, and he had no evidence of GH deficiency. CES revealed a novel heterozygous variant c.2275_2278 dup/p.(Ala760Glyfs*21) in the *IGF1R*. Identifying genetic causes of idiopathic short stature in SGA babies is important, as it facilitates more precise diagnoses, reduces unnecessary testing, and potentially enables targeted therapies. Our experience with rhGH therapy in one patient suggests a modest growth response, consistent with previous studies. However, elevated IGF1 levels during treatment highlight the importance of balancing therapeutic doses to optimize height gains without causing side effects.

Keywords: *IGF1R*, short stature, growth hormone, IGF1

Corresponding Author: Assoc. Prof. Zehra Yavaş Abalı, Marmara University Pendik Training and Research Hospital, Department of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye
E-mail: zehra_yavas@yahoo.com - zehrayavas14@gmail.com **ORCID:** orcid.org/0000-0002-8971-6181
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What is already known on this topic?

The growth hormone-insulin-like growth factor 1 (IGF1) axis is the major regulator of longitudinal growth. IGF1 and IGF2 act through the IGF receptor type 1 (IGF1R). Monoallelic *IGF1R* gene variants result in pre-and postnatal growth failure, developmental delay, and elevated serum IGF1 levels.

What this study adds?

Clinical and molecular genetic characteristics of two patients with short stature were described. Two novel IGF1R variants were reported, one splice-site and one nonsense.

Introduction

The growth hormone (GH)-insulin-like growth factor 1 (IGF1) axis plays a central role in human growth and metabolism (1). Among its components, the IGF1 receptor (IGF1R) mediates the effects of IGF1 and IGF2. These growth factors bind to IGF1R to promote growth both in fetal and postnatal life. Thus, dysfunctions in either the *IGF1* or *IGF1R* genes, may lead to pre- and postnatal growth restrictions (2). Structurally, IGF1R is akin to the insulin receptor and operates as a heterotetrameric transmembrane glycoprotein (3). Its alpha subunit ensures ligand binding, while the beta subunit facilitates intrinsic tyrosine kinase activity for signal transduction (4).

IGF1R (MIM*147370), located on chromosome 15q26.3, encompasses 21 exons. Pathogenic variants in this gene may occur in both monoallelic and biallelic forms, with the latter generally being associated with more severe clinical manifestations. Over 170 *IGF1R* variants have been documented, including missense/nonsense variants and gross deletions (5). Clinically, these variants are frequently associated with intrauterine growth restriction (IUGR), short stature, microcephaly, delayed bone age, developmental delay, and dysmorphic features, including a receding hairline, triangular face, long/smooth philtrum, thin upper lip, and full lower lip. However, the expression and severity of these traits can vary, and not all individuals present with all these features, reflecting the multifactorial nature of these characteristics. In adults with *IGF1R* defects, a thorough evaluation is essential, particularly concerning components of metabolic syndrome and hypogonadism (6).

Identifying *IGF1R* defects in short stature remains challenging because of the variability in patient selection and genetic methodologies. A comprehensive approach, integrating both single nucleotide variants (SNVs) and copy number variant (CNVs) analyses, is essential for accurate diagnosis (7). Rapid advances in high-throughput next-generation sequencing (NGS) techniques have facilitated the diagnosis of patients with short stature due to *IGF1R* defects. This has reclassified numerous cases previously defined as idiopathic short stature into distinct genetic disorders.

Herein, we present two patients with novel heterozygous *IGF1R* variants, detailing their clinical presentations and discussing the outcomes of recombinant human GH (rhGH) therapy in one patient.

Subjects and Methods

Clinical Evaluation

Height measurements were obtained using calibrated Harpenden stadiometers (Holtain, Crymych, UK), and weight was measured with standard equipment. Standard deviation scores (SDS) for all measurements were computed using growth charts specific to Turkish children (8). Small for gestational age (SGA) was defined as a birth weight (BW) or length more than two standard deviations below the mean for gestational age (9).

Hormonal Assays

Plasma GH levels were assessed using electrochemiluminescence immunoassay on a Roche Cobas platform (Roche Diagnostics İstanbul, Türkiye). IGF1 and IGF binding protein 3 (IGFBP3) were analyzed by immunoassays using the IMMULITE 2000 system (Siemens Healthcare İstanbul, Türkiye). IGF1 and IGFBP3 SDS were calculated using the online tool Child Metrics (10,11,12).

Molecular Analyses

After obtaining informed consent from the parents, DNA isolation was performed from peripheral blood samples under the standard protocols of the QIAAmp DNA Mini kit (Qiagen, Hilden, Germany). Sequencing was performed using the Illumina NextSeq platform, and each patient was read at least 20X depth in the clinical exome sequencing (CES) panel. Bioinformatic analyses and variant calling were performed using the Sophia-DDM-V5.08 bioinformatics analysis program (SOPHIA Genetics, Rolle, Switzerland). This test evaluates the coding regions and exon-intron boundaries in the relevant genes. During the analysis, only pathogenic (P), likely pathogenic (LP), or variants of unknown significance were reported according to current scientific knowledge based on the ClinVar database (13). This database is constantly updated, and the data in the report is as of the date the report was written (2024); changes are possible

in the future. The interpretation of the variants was based on the American College of Medical Genetics and Genomics (ACMG) 2015 guideline (14). To assess the population frequency of the variants, data from gnomAD, the 1000 Genomes Project, dbSNP, and ExAC were utilized (15). CNVs were also examined with this analysis. Segregation analyses were performed for the parents.

Case Presentations

Clinical and molecular genetic characteristics of two patients from two unrelated families are described.

Patient 1 (P1)

A 6.3-year-old boy was referred for evaluation of short stature. He was born at term with a BW of 2,500 g (-2.5 SDS), which confirmed a diagnosis of being SGA. Parents were unrelated. His neurodevelopmental milestones were normal for age, except for delayed walking, which he achieved at three years.

At the time of assessment, his height was 101.5 cm (-3.4 SDS), and his weight was 15.1 kg (-2.8 SDS). Body proportions were normal for his age, and head circumference (HC) was -1.8 SDS. Midparental height (MPH) was -1.7 SDS, and his mother was notably short (148.0 cm, -2.6 SDS). Pubertal examination was Tanner stage I. He had distinctive facial features, including a long philtrum and retro-micrognathia. The bone age assessment was aligned with his chronological age, and the skeletal survey was normal. The ophthalmological evaluation identified strabismus, for which a follow-up was recommended.

Laboratory results showed normal liver, renal, and thyroid function tests. His IGF1 and IGFBP3 levels were 117.8 ng/mL (0.95 SDS) and 4.55 µg/mL (1.3 SDS), respectively. GH stimulation test revealed a peak GH concentration of 14.4 ng/mL, ruling out GH deficiency. Chromosome analysis was 46,XY.

At 6.9 years of age, rhGH treatment was initiated at a dose of 0.035 mg/kg/day. The patient has been receiving rhGH for two years, achieving a height gain of +0.3 SDS per year, with an uneventful follow-up. Patient 1's mother was also evaluated for her short stature. The IGF1 and IGFBP3 levels were 251 ng/mL (-0.51 SDS) and 4.24 µg/mL, respectively. She had no evidence of insulin resistance, and her metabolic profile was normal. Clinical and laboratory findings of P1 at diagnosis and during rhGH treatment are summarized in Table 1 and Figure 1.

Patient 2 (P2)

A 3-year-old boy was referred for evaluation of growth failure. He was born at term with a BW of 2,300 g (-2.6 SDS). His perinatal history reported concerns about short femur length, though no definitive diagnosis was made at birth. His parents were unrelated.

Table 1. Clinical and laboratory findings of P1 at the time of diagnosis and during rhGH therapy

	At presentation	1st year of rhGH	2nd year of rhGH
Age, years	6.3	7.5	8.4
Height, SDS	-3.4	-3.1	-2.8
Height velocity, SDS	-	0.08	0.23
BMI, SDS	-0.62	-0.74	-0.9
Bone age, years	6.0	7.0	8.0
MPH SDS	-1.7		
Tanner stage	I	I	I
GH stimulation test (peak GH) (ng/mL)	14.4		
IGF1 (ng/mL) RR: 57.5-216.0	117.8	177.1	278.1
IGF1, SDS	0.95	2.42	5.0
IGFBP3 (µg/L) RR:1.5-6.0	4.55	4.39	4.24
IGFBP3, SDS	1.29	2.42	0.74
Fasting glucose (mg/dL)	82.0	80.0	83.0
Fasting insulin (µIU/mL)	4.1	4.0	4.3
Triglyceride (mg/dL)	NA	NA	36.0
LDL Cholesterol (mg/dL)	NA	NA	63.0

GH: growth hormone; BMI: body mass index; SDS: standard deviation score; MPH: midparental height; IGF1: insulin like growth factor 1, IGFBP3: insulin like growth factor binding protein 3; NA: not available; RR: reference range; rhGH: recombinant human growth hormone; P1: patient 1

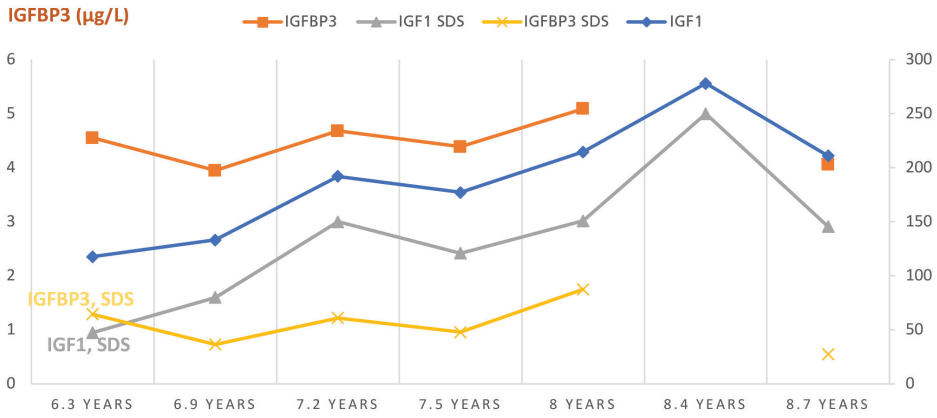

Figure 1. IGF1 and IGFBP3 levels of P1 while receiving GH treatment
IGF1: insulin like growth factor 1, IGFBP3: insulin like growth factor binding protein 3; P1: patient 1; GH: growth hormone; SDS: standard deviation score

At 1 year of age, his height was 70.0 cm (-2.1 SDS), weight was 8.8 kg (-1.1 SDS), and HC was 45.0 cm (-1.4 SDS). His mother was 158.0 cm (-0.87 SDS), his father was 166.0 cm (-1.65 SDS), and the MPH SDS was -1.25. The bone age corresponded to a chronological age of 3 to 6 months. Developmental milestones for gross motor and speech skills showed mild delays. Initial evaluations revealed normal IGF1 (88.53 ng/mL, -0.9 SDS) and IGFBP3 (4.2 ng/mL, -1.1 SDS). GH deficiency was ruled out with a glucagon stimulation test (peak GH: 8.0 ng/mL). The skeletal survey was normal.

At 2 years of age, his height velocity increased (+10.9 cm/year), however, height SDS remained below the expected range. At this time, his IGF1 level was elevated (164 ng/mL, 2.1 SDS), prompting further investigation for potential *IGF1R* variants.

His metabolic profile, including glucose (78 mg/dL), insulin (2.4 µIU/mL), triglyceride (35.5 mg/dL), and low density lipoprotein cholesterol (85 mg/dL), were normal.

The patient had recurrent respiratory infections, and selective immunoglobulin A deficiency was diagnosed, leading to a referral to clinical immunology for further evaluation.

At his most recent evaluation at 3.5 years old, his height and weight were 90.3 cm (-2.5 SDS) and 13.0 kg (-1.6 SDS), respectively. The arm span was 89.0 cm. The family was counseled about the possibility of initiating rhGH therapy.

The images of the patients and left-hand radiography are illustrated in Figure 2.

Molecular Genetic Results

Pedigree and Integrative Genomic Viewer of the NGS data are shown in Figure 3.

Analysis by CES in P1 revealed a heterozygous c.3722+1G>A/p.(?) variant in the *IGF1R* gene (NM_000875.5). This splice-site variant was classified as LP according to ACMG criteria (PVS1, PM2). This variant was classified as "deleterious" (MT, DANN, BayesDel) according to *in silico* prediction tools (16). For the splice-altering characteristics variant, SpliceAI is described as "Strong-Splice-altering" (17). This variant was not observed in the gnomAD (exomes and genomes) database (15). This *IGF1R* variant was also not reported in the HGMD professional database (November 2024) (5). Segregation analyses by Sanger sequencing revealed that his mother was also heterozygous for this variant.

In P2, the CES analysis identified a heterozygous c.2275_2278 dup/p.(Ala760Glyfs*21) variant in *IGF1R* (NM_000875.5), which is predicted to result in frameshift and premature termination. This variant was classified as LP according to ACMG criteria (PVS1, PM2) and was not observed in the gnomAD database (15). Once again, this *IGF1R* variant was also not reported in the HGMD professional database (November 2024) (5). Segregation analyses revealed that this variant was *de novo*.

Discussion

This report describes two rare cases of short stature due to novel heterozygous *IGF1R* variants, one involving a splice-site alteration and the other a frameshift variant. While the clinical features of these cases align with those previously reported, our findings contribute by documenting novel mutations and providing additional insights into growth response to rhGH therapy.

Although virtually all patients with monoallelic *IGF1R* variants present with pre- and postnatal growth restrictions, the extent varies remarkably. Walenkamp et al. (18) reported the detection of pathogenic *IGF1R* variants in approximately 2% of patients with short stature who were SGA. SGA-born cases represent a

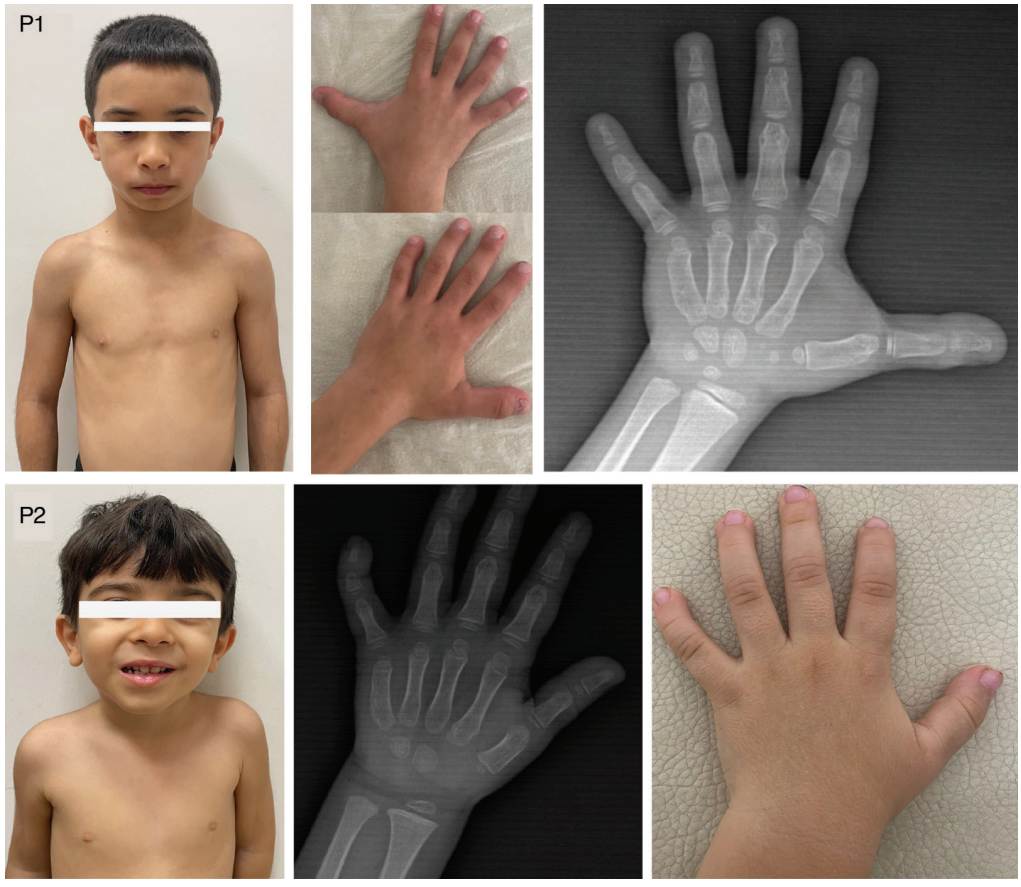

Figure 2. The pictures and left-hand radiography images of P1 and P2
P1: Long philtrum, retro-micrognathia, and clinodactly. The bone age was consistent with chronological age without any obvious pathological appearance
P2: Long philtrum, micrognathia, mildly low-set ears, and clinodactly. The bone age was consistent with chronological age with clinodactly
P1: patient 1; P2: patient 2

diverse group with varying clinical features. The reduced size at birth can be attributed to fetal, maternal, placental, and/or genetic factors. While many SGA babies achieve normal growth by the age of 2 years, about 15% remain below -2.0 SDS in height and continue to be short. Genetic factors have been identified in a limited number of short SGA children, notably having point mutations and deletions in the *IGF1* and *IGF1R* genes (19). Klammt et al. (20) reported eight SGA patients who were in the range of -1.5 to -3.5 SDS due to *IGF1R* variants. Both of our patients were born SGA and were unable to catch up with growth by two years of age. P2 had a history of short extremities in the prenatal evaluation, but later postnatal examination revealed proportionate short stature.

In the study of Gonc et al. (7), patients with short stature without GH deficiency having either a low BW or microcephaly were evaluated to detect *IGF1R* defects, and variants were detected in 14% of the cohort. Two *IGF1R* deletions and five heterozygous variants (one frameshift, four missense) were identified. All patients with *IGF1R* defects had a height, BW, and HC lower than -2.5 SDS, -1.4 SDS, and -1.36 SDS, respectively. IGF1 levels ranged from -2.44 to 2.13 SDS (7). Although the height SDS of P2 was similar, in our study, P1's height SDS at presentation was below -3.0 SDS.

Features described in cases with IGF1 resistance include mild facial dysmorphism (triangular face, brachycephaly, hypotelorism, low-set and prominent ears and micro-retrognathia), neurodevelopmental delay, and mild glucose intolerance (17). In our cases, some dysmorphic features, such as clinodactyly and micro-retrognathia, were also observed. Although the patients experienced delayed attainment of certain milestones, neuromotor development remained within normal limits. There were no abnormalities in carbohydrate metabolism. Given their prepubertal age, some clinical features may emerge later, and continuous monitoring will be necessary.

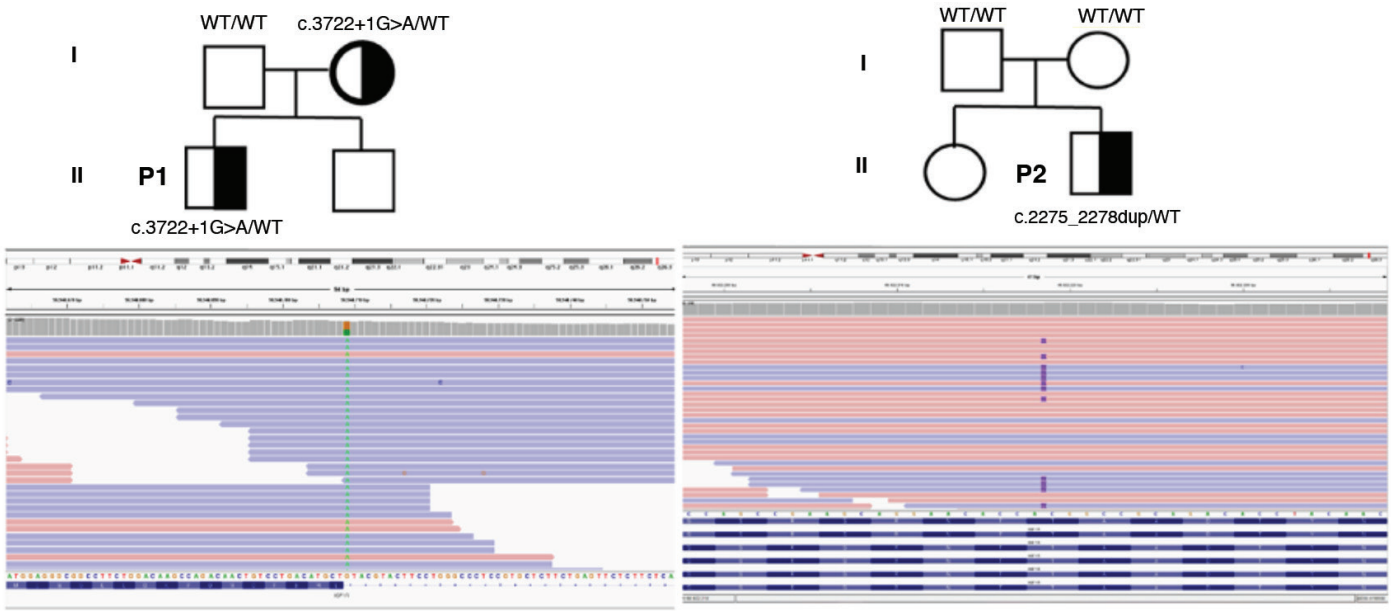

Figure 3. Pedigree and integrative genomics viewer (IGV) of the *IGF1R* gene variant in P1 and P2
The heterozygous (NM_000875.5) : c.3722+1G>A/p.(?) *IGF1R* variant in P1 and c. 2275_2278dup/p.(Ala760Glyfs*21) variant in P2. Segregation analysis was performed on the parents, while it was not conducted in clinically unaffected siblings. Future decisions regarding segregation analysis in siblings will be made based on their follow-up assessments
IGF1R: insulin-like growth factor 1 receptor; P1: patient 1; P2: patient 2

Cases of heterozygous *IGF1R* variants are typically characterized by IUGR, persistent postnatal growth failure, elevated serum IGF1 levels, and microcephaly (21). Our patients presented with moderately elevated IGF1 SDS values and did not exhibit microcephaly.

Most of the 170 variants identified in the *IGF1R* gene are missense/nonsense (n=107) and gross deletions (n=27). Splice-altering (n=9), small deletions (n=7), small indels (n=1), gross insertions (n=6), complex rearrangements (n=3), and regulatory region (n=3) variants have also been described (5). To date, splice-site variants (n=9) described are localized in the introns 1, 2, 3, 7, 8, 9, 10, and 18.

The c.3722+1G>A is a splice-site variant occurring at the donor site of intron 20. This variant is predicted to disrupt the normal splicing process, potentially leading to the exclusion of exon 20 from the mature mRNA transcript. Exon 20 encodes a portion of the tyrosine kinase domain of the IGF1R protein (catalytic domain of insulin receptor-like protein Tyrosine kinases), which is crucial for its signaling function. Alterations in this domain may impair the receptor's ability to transduce signals, affecting growth and development processes.

The *IGF1R* c.2275_2278dup/ p.(Ala760Glyfs*21) variant is a frameshift duplication located in exon 13 of the *IGF1R* gene (NM_000875.5). This exon encodes part of the extracellular fibronectin type III domain, which is critical for ligand binding and proper receptor function (22). Variants in specific domains (e.g., fibronectin type III or tyrosine kinase) often lead to growth failure and altered IGF1 signaling, as seen in patients with short stature due to *IGF1R* variants (23).

Previous studies have highlighted significant variability in height, IGF1 levels, and intrauterine growth in individuals with the same heterozygous *IGF1R* variants. This variability is likely due to the complex structure of *IGF1R* and its interaction with the insulin receptor, which modulates IGF1 signaling. Although growth is influenced by multiple factors beyond IGF1 signaling, genetic testing for *IGF1R* alterations is crucial for accurate diagnosis, particularly in cases exhibiting elevated IGF1 levels or an exaggerated IGF1 response to rhGH therapy (24,25).

The phenotype of short stature associated with *IGF1R* variants is not fully elucidated, and no approved therapy is currently available. However, effectiveness of rhGH treatment has been studied (18). In this report, P1 was treated with rhGH, and the height SDS gain was +0.3 at the first year and +0.6 SDS at the second year. Patients with heterozygous *IGF1R* variants may respond to rhGH treatment, aligning with previous reports. However, the response remains variable (26). Çelik et al. (27) concluded in their recent review that rhGH has a partial beneficial effect in cases with *IGF1R* defects, particularly when initiated early and administered long-term. Nearly half of the

patients achieved a height gain of more than 1 SDS over the long term (26). Walenkamp et al. (18) suggested that higher IGF1 levels may need to be tolerated during the treatment to achieve a clinically significant increase in height SDS due to the partial insensitivity to IGF1 in these patients. The reluctance to increase the rhGH due to elevated IGF1 SDS levels may have contributed to suboptimal treatment response in our case. Higher IGF1 levels after rhGH therapy may indicate *IGF1R* gene variants (18).

The correlation between *IGF1R* defects and clinical presentation remains unclear, and the wide phenotypic variability complicates the selection of patients for *IGF1R* gene sequencing. However, advances in NGS technologies have enabled massively parallel sequencing of multiple genes and genomic regions with high precision, significantly enhancing diagnostic yield. By capturing both coding and non-coding regions, NGS facilitates the comprehensive identification of pathogenic variants, including SNVs, small in/dels, and CNVs, thereby revolutionizing the diagnostic approach to genetically heterogeneous disorders (28).

In conclusion, uncovering the genetic causes of idiopathic growth failure is important, as it facilitates more precise diagnoses, reduces unnecessary testing, and potentially enables targeted therapies. Our experience with rhGH therapy in one patient suggests a modest growth response, consistent with previous studies. A +0.6 SDS gain over two years aligns with prior reports of partial responsiveness in *IGF1R* variant carriers. However, elevated IGF1 levels during treatment highlight the importance of balancing therapeutic doses to optimize height gains without causing side effects.

Ethics

Informed Consent: Written informed consent was obtained from the patient's parents for the publication of details of their medical case and any accompanying images.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: Mehmet Eltan, Hilal Sekizkardeş, Sezin Canbek, Murat Hakkı Yarar, Concept: Mehmet Eltan, Hilal Sekizkardeş, Sezin Canbek, Murat Hakkı Yarar, Saygın Abalı, Zehra Yavaş Abalı, Design: Mehmet Eltan, Zehra Yavaş Abalı, Data Collection or Processing: Mehmet Eltan, Hilal Sekizkardeş, Sezin Canbek, Murat Hakkı Yarar, Analysis or Interpretation: Mehmet Eltan, Sezin Canbek, Murat Hakkı Yarar, Literature Search: Mehmet Eltan, Saygın Abalı, Zehra Yavaş Abalı, Writing: Mehmet Eltan, Saygın Abalı, Zehra Yavaş Abalı.

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CASE REPORT

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A Case of Carney Complex with Pontine Glioma

Gülümay Vural Topaktaş[1], Emrullah Arslan[1], Tayfun Çinleti[2], Özlem Anlaş[3], Ebru Pala[4], Benay Turan[1], Eren Er[1], Bumin Nuri Dündar[5]

[1]University of Health Sciences Türkiye, İzmir City Hospital, Clinic of Pediatric Endocrinology, İzmir, Türkiye
[2]University of Health Sciences Türkiye, İzmir City Hospital, Clinic of Pediatric Genetic Diseases, İzmir, Türkiye
[3]University of Health Sciences Türkiye, İzmir City Hospital, Genetic Diseases Evaluation Center, İzmir, Türkiye
[4]University of Health Sciences Türkiye, İzmir City Hospital, Clinic of of Pathology, İzmir, Türkiye
[5]İzmir Katip Çelebi University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Türkiye

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ABSTRACT

Carney complex (CNC) is a rare autosomal dominant syndrome characterized by skin pigmentation abnormalities, endocrine tumors, and cardiac myxomas. This report presents an 11-year-old girl with a history of pontine glioma treated with chemotherapy and radiotherapy at 2.5 years of age, who presented with weight gain and short stature, along with syndromic features (multiple nevi around the mouth and nose, four café-au-lait spots, and bilateral clinodactyly of the fourth toes) identified during physical examination. Genetic testing revealed a novel pathogenic *PRKAR1A* variant, confirming the diagnosis of CNC. The patient was diagnosed with Cushing's syndrome due to unsuppressed cortisol levels observed in a high-dose dexamethasone suppression test. Pathological evaluation following unilateral adrenalectomy confirmed the presence of primary pigmented nodular adrenocortical disease. This case highlights the importance of recognizing the atypical course of CNC to prevent delays in diagnosis and treatment.

Keywords: Carney complex, pontine glioma, Cushing's syndrome, short stature

What is already known on this topic?

Carney complex is a rare autosomal dominant multiple neoplasia syndrome characterized by skin pigmentation abnormalities, endocrine tumors, cardiac myxomas, and primary pigmented nodular adrenocortical disease, which may cause adrenocorticotropic hormone-independent Cushing's syndrome.

What this study adds?

This case describes the coexistence of Carney complex and pontine glioma, an association not previously reported in the literature, and identifies a novel pathogenic *PRKAR1A* variant. It also highlights the phenotypic variability of Carney complex and the potential for subtle Cushing's syndrome to delay diagnosis.

Corresponding Author: Gülümay Vural Topaktaş, MD, University of Health Sciences Türkiye, İzmir City Hospital, Clinic of Pediatric Endocrinology, İzmir, Türkiye
E-mail: gulumayvural@hotmail.com **ORCID:** orcid.org/0000-0001-8996-1251
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Introduction

Carney complex (CNC) is a rare autosomal dominant genetic disorder, first described by Carney (1,2) in 1985. CNC is characterized by cutaneous pigmentation abnormalities, cardiac and cutaneous myxomas, endocrine gland neoplasms or hyperfunction, and schwannomas (1,2). Affected individuals may present with various endocrine manifestations, including primary pigmented nodular adrenocortical disease (PPNAD), growth hormone-secreting pituitary adenomas, prolactinomas, thyroid adenomas or carcinomas, and gonadal tumors, with two or more endocrine tumors often observed (3). The clinical presentation of CNC varies widely, ranging from multiple concurrent features to a single isolated manifestation. The components of CNC may manifest at any age, which may complicate the diagnosis in certain cases. PPNAD is a rare cause of adrenocorticotropic hormone (ACTH)-independent Cushing's syndrome (CS). ACTH-independent CS due to PPNAD has been found to be associated with CNC in 90% of cases (4). It represents the most common endocrine hyperactivity in CNC and occurs in approximately 25% of affected individuals. However, the incidence may be underestimated due to atypical and subclinical disease presentations. In autopsy studies conducted on patients with CNC, PPNAD was observed in nearly all cases (3,4,5).

In this article, we present a case of a patient with a history of pontine glioma who presented with short stature and obesity. Comprehensive evaluations led to a genetic diagnosis of CNC, followed by the identification of CS.

Case Report

An 11-year-old female patient presented with significant weight gain over the past six months and short stature noted over the preceding 1-2 years. Her medical history included a diagnosis of pontine glioma at the age of 2.5 years, identified during the evaluation of neurological symptoms, for which she underwent chemotherapy with temozolomide and radiotherapy (RT) at a total dose of 54 Gy. There was no consanguinity between the mother and the father. No history of pathological short stature or genetic disorders was reported in the family history. On physical examination she had a body weight of 48 kg [+0.98 standard deviation score (SDS)], a height of 136 cm (-1.6 SDS), and a body mass index of 25.95 (+2.06 SDS). Her pubertal development was compatible with Tanner stage 5. The patient, who had a reported age of menarche at 9.5 years, also reported regular menstruation. Clinical findings included proportional short stature, central obesity, multiple nevi around the mouth and nose, four café-au-lait spots with the largest (2.5×1.5 cm) located in the interscapular region. In addition, two smaller café-au-lait spots (<1×1 cm) were present on the posterior aspect of the right upper leg, and one spot (<1×1 cm) at the sacral level, and bilateral clinodactyly of the fourth toes (Figure 1). Given her early menarche potentially indicative of precocious puberty or rapidly progressing puberty, short stature, history of an intracranial mass, obesity, skin lesions, and syndromic features, further investigations were initiated with the preliminary consideration of a disorder from the rasopathy group.

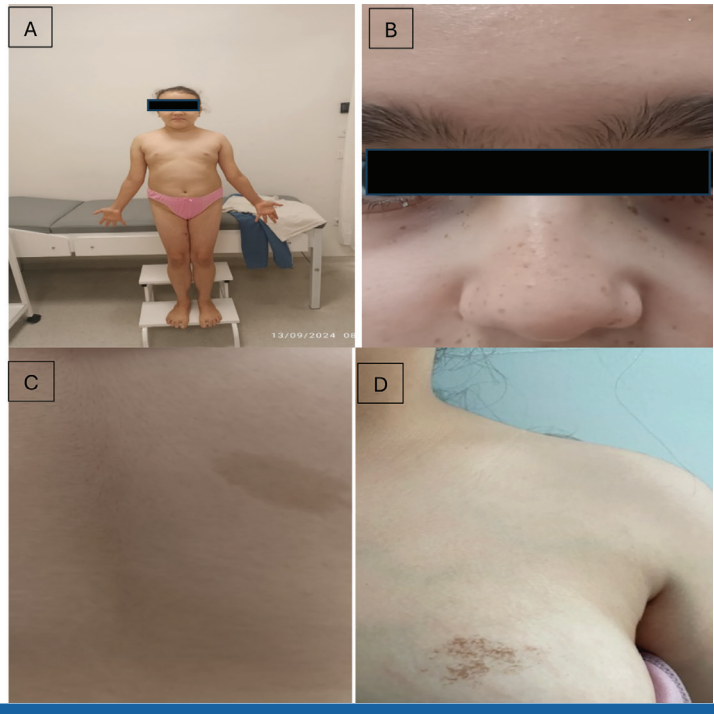

Figure 1. A) Central obesity, **B)** spotty black pigmentation on the cheeks and nose, **C)** café au-lait spot in the interscapular region, **D)** lentiginous skin lesion on the upper left breast

Biochemical tests conducted for the evaluation of short stature were within normal limits, and celiac tests were negative. Her insulin-like growth factor-1 level was low (72.1 ng/mL, -2.35 SDS), and bone age was consistent with 15 years (Table 1). Anterior pituitary hormone levels, assessed due to prior RT to the head and neck region were evaluated as appropriate for her age and pubertal stage.

Genetic analysis, including a broad-spectrum panel to encompass rasopathies, was performed. While CNC is not classified as a rasopathy, this panel was used because of overlapping clinical features. The genetic panel analysis identified a novel, heterozygous pathogenic variant, c.51dup (p.Cys18MetfsTer2), in the *PRKAR1A* gene (ENST00000589228). *PRKAR1A* variants are associated with autosomal dominant CNC type 1. This variant has a reported maximum population frequency of 0.0% in the gnomAD genome database.

The c.51dup (p. Cys18MetfsTer2) *PRKAR1A* variant, caused by a single-base insertion, is predicted to lead to a frameshift mutation and loss of function of the *PRKAR1A* protein. According to the American College of Medical Genetics and Genomics criteria and segregation analysis, this novel variant is classified as "pathogenic" based on PVS1, PM2, and PP1 scores. Segregation analysis confirmed the presence of this variant in the affected father, while it was absent in the mother. A detailed physical examination of the father revealed hyperpigmented macules on the lips as the sole clinical manifestation, with no additional abnormalities detected. The father was subsequently referred to adult endocrinology for comprehensive evaluation and further diagnostic screening. Subsequent to the genetic analysis, further communication with the family disclosed the presence of a previously unacknowledged paternal uncle who had undergone bilateral adrenalectomy and remained under ongoing medical surveillance for a confirmed diagnosis of PPNAD.

The patient underwent comprehensive screening for other components of CNC. Echocardiography did not reveal any evidence of cardiac myxomas, and on thyroid and pelvic ultrasonography there were no pathological findings reported. Due to her history of short stature and obesity, along with the well-established association between CNC, PPNAD, and ACTH-independent CS, further evaluation was conducted. Circadian cortisol rhythm analysis showed unsuppressed night-time serum cortisol levels, consistent with an impaired diurnal rhythm (5.29 µg/dL). Suppression tests, including overnight dexamethasone suppression and low-dose dexamethasone suppression, showed unsuppressed cortisol levels (4.02 µg/dL and 4.66 µg/dL, respectively Urinary free cortisol levels were within the reported reference range (16.6 µg/24 h; normal <37 µg/24 h). A high-dose Liddle test revealed a paradoxical increase in urinary free cortisol levels (Table 2).

Table 1. The patient's initial diagnostic tests performed because of presentation with obesity and short stature. Normal ranges are shown in brackets

Glucose	82 mg/dL (70-110)	**TSH**	2.56 mU/L (0.51-4.30)
Bun	8 mg/dL (5-18)	**fT4**	1.3 ng/dL (0.93-1.7)
Creatinine	0.41 mg/dL (0.39-0.73)	**FSH**	4.41 U/L (0.4-8.6)
Sodium	140 mmol/L (136-145)	**LH**	3.51 U/L (0.9-13.3)
Potassium	4.8 mmol/L (3.5-5.1)	**Estradiol**	53.6 ng/L
LDL	42 mg/dL (0-130)	**ACTH**	19.9 pg/mL
HDL	42 mg/dL (35-55)	**Cortisol**	13.2 µg/dL
Total cholesterol	173 mg/dL (92-200)	**IGF-1**	72.1 ng/mL (-2.35 SDS)
Triglyceride	54 mg/dL (0-150)	**Prolactin**	8 µg/L (4.79-23.3)
Urine	Density: 1019 pH: 5	**Insulin**	6.83 mU/L

BUN: blood urea nitrogen; LDL: low-density lipoprotein cholesterol; HDL: high-density lipoprotein cholesterol; TSH: thyroid stimulating hormone; fT4: free thyroxine; FSH: follicle stimulating hormone; LH: luteinizing hormone; ACTH: adrenocorticotropic hormone; IGF-1: insulin-like growth factor-1

Table 2. Results of the low and high dose dexamethasone suppression tests

	Low dose dexamethasone supression test		High dose (Liddle) dexamethasone test		
	Basal	**Final**	**Basal**	**Day 1**	**Final**
Plasma cortisol (µg/dL)	13.2	4.66	15.5	13.4	**6.47**
Free cortisol in urine (µg/24 h)	8.56	6.66	17.8	14.5	**26.6**

For the low-dose dexamethasone suppression test, plasma cortisol <5 µg/dL and urinary free cortisol <10 µg/day are considered normal; alternatively, urinary free cortisol should be suppressed by >50%. For the high-dose (Liddle) dexamethasone test, urinary free cortisol may decrease by approximately 90% from baseline, and serum cortisol measured the morning after the last dose is suppressed by >90% from baseline.

Dynamic contrast-enhanced computed tomography (CT) of the adrenal glands demonstrated a 6×5 mm lobulated lesion in the body of the left adrenal gland and a millimetric calcified focus in the medial limb of the right adrenal gland. The patient underwent unilateral adrenalectomy of the left adrenal gland Histopathological examination revealed multiple pigmented adrenocortical micronodules (most <1 mm), consistent with PPNAD (Figures 2 and 3).

No complications were observed after the operation and the patient remained under regular follow-up. Four months after unilateral adrenalectomy, the serum cortisol levels were measured and were 10.3 µg/dL at 08:00 AM and 2.35 µg/dL at 11:00 PM. The 24-hour urinary free cortisol level was 10.1 µg/24 h (normal <37 µg/24 h). At present, she has no additional complaints. However, ongoing follow-up is being conducted to monitor existing findings, evaluate the response to treatment, and identify the emergence of new manifestations. For endocrine complications of CNC, annual measurement of urinary free cortisol levels is planned for PPNAD monitoring. In cases of clinical suspicion, diurnal cortisol levels (with samples taken at 11:30 PM, 12:00 AM, 7:30 AM, and 8:00 AM), a dexamethasone suppression test (modified Liddle's test), and an adrenal CT examination will be conducted. Annual thyroid ultrasonography is recommended as part of routine surveillance in patients with CNC (3). If there is clinical suspicion for ovarian tumors, a suprapubic ultrasound will be performed. In cases of suspected gigantism and/or acromegaly, pituitary magnetic resonance imaging will be performed to evaluate for pituitary adenomas. In addition, a 3-hour oral glucose tolerance test and a 90-minute thyrotropin-releasing hormone stimulation test will be conducted, if necessary. Parental consent was obtained for publication of this case report.

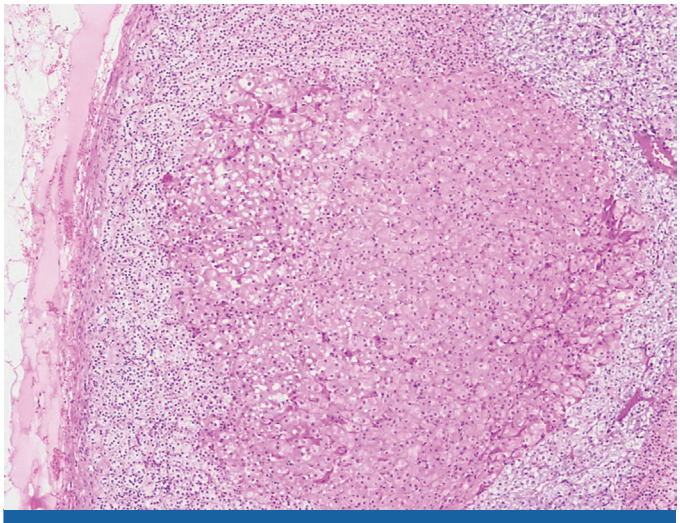

Figure 2. A micronodule (<1 cm) in the adrenal cortex, well-defined and composed of cells with eosinophilic cytoplasm (H&E stain, x100 magnification)
H&E, hematoxylin and eosin

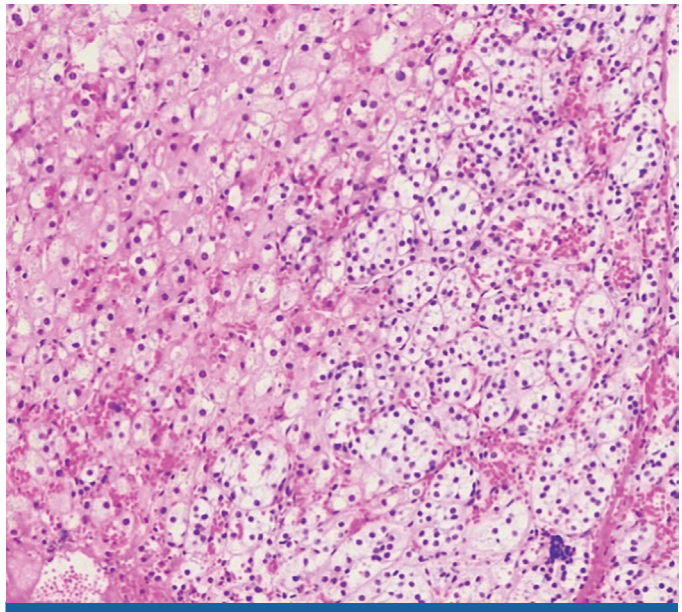

Figure 3. Cytoplasmic lipofuscin pigment in adrenal cortical micronodules (H&E stain, x200 magnification)
H&E, hematoxylin and eosin

Discussion

The presented case, who was referred to our clinic because of short stature and obesity but was subsequently diagnosed with CNC, is of interest because of the atypical features of CNC. To the best of our knowledge, this is report in the literature to describe the coexistence of pontine glioma and CNC. In addition, we suggest that in this case with precocious puberty and a history of cranial RT, the CS findings may have been overlooked due to their subtlety, which may explain her short stature. The very mild paternal CNC findings, despite harboring the same variant, also highlight the difference in expression. We suggest that because of the potential for atypical presentation, such as with short stature as in the presented case, it is beneficial to keep CNC in mind and at least investigate the additional findings of CNC. CNC is classified as a multiple neoplasia syndrome with an autosomal dominant inheritance pattern (1). Patients may present with the characteristic clinical features of CNC, but, as demonstrated in this case, the condition can also be identified in pediatric patients presenting to endocrinology clinics with nonspecific complaints, such as short stature although she had a history of cranial neoplasia. The clinical manifestations of CNC are highly variable, even among members of the same family (3).

Approximately 50% of CNC families reported in the literature have mutations in the *PRKAR1A* gene located on chromosome 17q22-24. The *PRKAR1A* gene, novel variants of which were identified in the patient and her father, encodes the regulatory subunit type 1α (R1α) of protein kinase A (PKA), a classical tumor suppressor protein. PKA is integral to numerous endocrine

signaling pathways. The R1α subunit inhibits PKA activity, and mutations in *PRKAR1A* may result in the production of a truncated, nonfunctional protein. This leads to increased PKA activity, resulting in intracellular signaling dysregulation and subsequently, endocrine hyperfunction or tumorigenesis (3,6,7).

Currently, approximately 62% of CNC cases have identifiable pathogenic *PRKAR1A* variants, with about 70% of these cases attributed to mutations in *PRKAR1A* (60% detected through sequencing and 10% via deletion-duplication analysis). In the remaining 30% of cases, the genetic basis remains unknown. Bertherat et al. (8), in the largest study to date, identified *PRKAR1A* mutations in 114 of 185 CNC families (62%), reinforcing the critical role of this gene in CNC pathogenesis.

Although universal diagnostic criteria for CNC have not yet been established, a revision was reported in 2001 that introduced major and minor diagnostic criteria, enhancing diagnostic sensitivity to approximately 98% (3). In the presented case, a comprehensive clinical evaluation and thorough patient history satisfied the diagnostic criteria, even prior to the confirmation of the diagnosis through genetic testing.

The association between CNC and CS has been well-documented. A study by Cazabat et al. (9) demonstrated that among CNC patients presenting with CS secondary to PPNAD, the frequency of identifying pathogenic *PRKAR1A* variants increased to 80%. CS is a significant and potentially life-threatening component of CNC. While CS can present with overt clinical symptoms, it may also manifest in an atypical manner, as observed in the presented case, where only mild Cushingoid features were present alongside disrupted diurnal cortisol rhythms. Diagnostic confirmation can be achieved using high-dose dexamethasone suppression testing, which would show cortisol non-suppression, or by identifying paradoxical increases in 24-hour urinary free cortisol levels.

A retrospective analysis conducted at the Mayo Clinic identified 37 patients with CNC. These patients were classified into four categories: classical CS (consistent clinical and laboratory findings); subclinical CS (absence of clinical symptoms but laboratory findings consistent with CS); possible CS (presence of some clinical findings with borderline results in one or more tests defining classical CS or adrenal pathological findings); and no CS (absence of both clinical and laboratory findings indicative of CS). Among the 17 patients diagnosed with classical CS, 15 underwent surgical intervention, including bilateral adrenalectomy (9 patients), subtotal adrenalectomy (2 patients), and partial unilateral adrenalectomy (4 patients), while two cases were diagnosed through autopsy reports. All patients who underwent bilateral adrenalectomy achieved complete remission. In the subtotal adrenalectomy group, remission was achieved in both patients, although one developed adrenal insufficiency. In the partial unilateral adrenalectomy group, two patients experienced recurrence of CS during follow-up and subsequently underwent total adrenalectomy. For the 12 patients with subclinical or possible CS, long-term follow-up without intervention, ranging from 1 to 36 years (mean: 10 years), demonstrated no progression to overt CS. Similarly, among the eight patients classified as having no CS, follow-up over 2 to 49 years (mean: 19.5 years) revealed no evidence of CS development. Notably, one patient remained untreated for nearly 30 years, while another exhibited spontaneous remission of CS. Furthermore, PPNAD was identified in two patients who exhibited no clinical features of CS, underscoring the potential for subclinical adrenal involvement in CNC (10). CS due to PPNAD must be treated to manage the consequences of cortisol overproduction. In rare cases, anti-cortisol medical treatments, such as ketoconazole or mitotane, have been used, but surgical interventions remain the primary approach currently (11,12) Although bilateral adrenalectomy remains the standard treatment for PPNAD, literature reports indicate heterogeneous clinical responses following unilateral adrenalectomy, with rare cases demonstrating sustained remission of CS for up to nine years postoperatively (10,13,14,15). These findings suggest the need to reconsider performing partial rather than total bilateral adrenalectomy in selected patients with CNC and CS.

In the presented case, considering the patient's young age, the presence of a 6×5 mm left adrenal lesion on imaging, and existing evidence suggesting relatively prolonged remission periods following unilateral adrenalectomy in selected cases, a comprehensive evaluation of potential clinical outcomes was conducted in consultation with the patient's family. Following a multidisciplinary consensus with the surgical team, unilateral adrenalectomy was deemed the most appropriate therapeutic approach. In our patient who underwent unilateral adrenalectomy, the most recent evaluation showed regression of CS findings, with no evidence of adrenal insufficiency.

Upon reviewing the existing literature, no documented association between CNC and pontine glioma was found. In the presented case, the pontine glioma has been considered a different presentation of CNC, which may be biologically reasonable given the tumor suppressor function of PRK. However, further research is needed to elucidate the potential relationship between CNC and intracranial masses, including pontine gliomas.

Previous studies have highlighted that CNC can exhibit clinical variability even among members of the same family and that the diagnostic process may be delayed for years. To expedite diagnosis, increasing awareness of CNC's multifaceted manifestations, obtaining a detailed medical history of patients and their families, and being vigilant about diagnostic pitfalls are essential. Notably,

the presence of unusual symptom combinations should prompt clinicians to consider rare endocrinopathy syndromes such as CNC (16). This case reinforces the importance of recognizing CNC as a rare but clinically challenging condition with systemic and endocrine manifestations. Early diagnosis, comprehensive screening for associated features, and appropriate management strategies are essential to mitigate potential complications and improve patient outcomes.

Conclusion

CNC represents a rare etiology of endocrine tumors. Although the majority of CNC cases develop CS at some stage, its atypical clinical course frequently results in delayed diagnosis. In patients presenting with ACTH-independent CS, PPNAD should be considered as a potential underlying cause. Given that CNC and its associated complications can manifest at any point during a patient's lifetime, comprehensive screening for PPNAD is mandatory in all cases. Furthermore, genetic testing and surveillance should be extended to family members of affected individuals to facilitate early or missed detection and mitigate the risk of life-threatening complications.

Ethics

Informed Consent: Parental consent was obtained for publication of this case report.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Gülümay Vural Topaktaş, Emrullah Arslan, Tayfun Çinleti, Özlem Anlaş, Ebru Pala, Benay Turan, Eren Er, Bumin Nuri Dündar, Concept: Gülümay Vural Topaktaş, Emrullah Arslan, Data Collection or Processing: Gülümay Vural Topaktaş, Emrullah Arslan, Bumin Nuri Dündar, Analysis or Interpretation: Gülümay Vural Topaktaş, Emrullah Arslan, Literature Search: Gülümay Vural Topaktaş, Emrullah Arslan, Tayfun Çinleti, Özlem Anlaş, Ebru Pala, Writing: Gülümay Vural Topaktaş, Emrullah Arslan, Özlem Anlaş, Bumin Nuri Dündar.

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CASE REPORT

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Novel *SOX9* Gene Variant Associated with Campomelic Dysplasia: Effects on Sex Phenotypes

Nanis Sacharina Marzuki[1], Hannie DH Kartapradja[1], Firman Pratama Idris[2]

[1]Eijkman Research Center for Molecular Biology, Research Organization for Health, National Research and Innovation Agency, Cibinong, Indonesia
[2]The Murdoch Children's Research Institute, the University of Melbourne, Melbourne, Australia

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ABSTRACT

Campomelic dysplasia (CD) is a rare autosomal dominant genetic disorder primarily caused by mutations in the *SOX9* gene. While this condition can affect multiple organ systems, it mainly influences skeletal and sexual development, leading to skeletal malformations and gonadal dysgenesis. We present two cases of CD diagnosed at an early age. Their clinical presentations were characteristic of this disorder, including bowing of the lower extremities, pretibial dimples, Pierre Robin sequence, and bilateral clubfoot. Both cases exhibited delays in motor skills and speech. The first case involved a 46,XY sex-reversed infant with a novel heterozygous *SOX9* gene substitution of p.Arg107Gly (NM_000346.4:c.319C>G). The second case involved a 1.5-year-old boy with typical male external genitalia carrying a heterozygous p.Ala116Val variant (c.347C>T) in the *SOX9* gene. Both variants were located in the high mobility group domain of the gene. Two variants, the novel p.Arg107Gly and the p.Ala116Var, in the *SOX9* gene were reported to be associated with CD. Despite being in the same domain, these variants lead to different sex phenotypes.

Keywords: Campomelic dysplasia, differences in sex development, 46,XY sex reversal, *SOX9* gene

What is already known on this topic?

Campomelic dysplasia is a rare genetic disorder caused by heterozygous variants in the *SOX9* gene. Its clinical manifestations involve various organ systems, primarily skeletal malformations and gonadal dysgenesis.

Corresponding Author: Nanis Sacharina Marzuki, MD, Eijkman Research Center for Molecular Biology, Research Organization for Health, National Research and Innovation Agency, Cibinong, Indonesia
E-mail: andi008@brin.go.id, sacharina99@gmail.com **ORCID:** orcid.org/0000-0003-1116-7669
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What this study adds?

This study identifies two variants in the high mobility group domain of the *SOX9* gene: p.Arg107Gly and p.Ala116Val. These variants were detected in two unrelated cases presenting classical campomelic dysplasia phenotypes while exhibiting opposite sex characteristics. The p.Arg107Gly variant is reported for the first time. Although p.Ala116Val is classified as a variant of uncertain significance according to American College of Medical Genetics and Association for Molecular Pathology guidelines, this report provides additional information on p.Ala116Val as a variant associated with campomelic dysplasia. To our knowledge, these are the first reported cases of campomelic dysplasia from our country.

Introduction

Campomelic dysplasia (CD; OMIM #114290) is a rare genetic condition primarily caused by mutations in the *SOX9* gene and following an autosomal dominant inheritance pattern. Due to the expression of the *SOX9* gene in different tissues, this disorder impacts skeletal development and has consequences for sexual development and other organ systems (1,2).

The *SOX9* gene encodes a transcription factor essential for the development of the skeleton. Genetic variations of *SOX9* impair its function, causing a variety of clinical outcomes, from significant malformations that can be fatal during the neonatal period to less severe forms that show subtler skeletal abnormalities (2-4).

In addition to its effects on skeletal tissues, about three-fourths of XY individuals with CD experience male-to-female sex reversal due to the impaired SOX9 function in the testis-determining pathway. This highlights the multifaceted effects of *SOX9* mutations and the complexity of their clinical presentations (2,4,5). This report presents two unrelated cases of CD, characterized by varying degrees of phenotypic features resulting from different mutations in the *SOX9* gene. These cases were our initial encounters with CD.

Case Report

The first case involved a two-week-old baby girl who presented with facial dysmorphism (Figure 1), macrocephaly, micrognathia, high-arched palate, laryngomalacia, bilateral bowing limbs, pretibial dimple, and bilateral congenital talipes equinovarus (CTEV). Her external genitalia exhibited typical female characteristics, lacking palpable gonads and having two openings. She was the fourth child of non-consanguineous parents, and no other family members showed similar symptoms. Initially, trisomy 18 was suspected, but her karyotype revealed 46,XY without any additional chromosomal abnormalities. Further molecular testing of the *SRY* and *AR* genes showed no pathological variants. However, we detected the novel heterozygous p.Arg107Gly [NM_000346.4:c.(319C>G)] variant in the *SOX9* gene leading to a diagnosis of CD (Figure 2). At follow-up, at 5 years and 8 months, her body weight and height were 16.6 kg [standard deviation score (SDS) -1.20] and 103.5 cm (SDS -1.85), respectively. She could already walk and speak but experienced delays in gross motor skills, poor concentration, and inconsistent responses to simple commands. She continues to receive therapy from a medical rehabilitation team. Her parents remained reluctant to undergo laparoscopic exploration to search for the presence of gonads in her abdomen.

The second case involved a 1.5-year-old boy brought to medical attention after his parents noticed similarities between his condition and the first case they had seen on social media. His birth weight was 3200 g, and his length at birth was 45 cm. His facial features included a large head, a broad forehead, retrognathia, a cleft palate, a flat face, and low-set ears (Figure 3A). At the time of examination, he weighed 9.4 kg (SDS -1.36) and measured 74 cm (SDS -3.06) in length. He displayed bowed lower extremities (Figure 3B), 11 ribs, and bilateral CTEV.

He had typical male external genitalia, with both testes in the scrotum. There were no similar phenotypic traits in other family members. Despite his physical deformities, he was an active

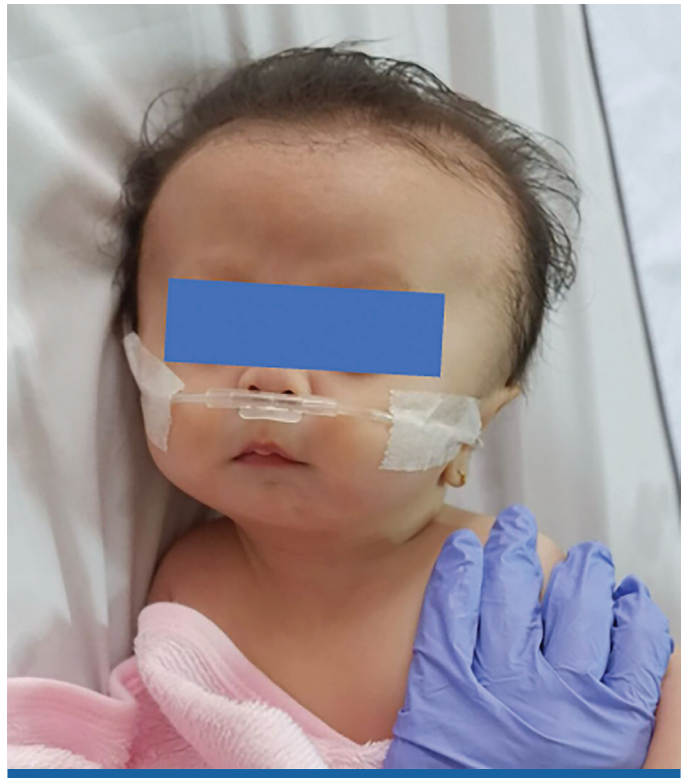

Figure 1. Facial appearances of the first case at 4 months old

child who could already walk and had started to speak, although he experienced delays in motor skills and speech. Molecular analysis revealed a heterozygous p.Ala116Val [NM_000346.4:c.(347C>T); ClinVar accession ID VCV001497400.5] variant in the *SOX9* gene (Figure 2).

Both identified *SOX9* variants are absent in gnomAD v.4.1.0, and subsequent *in silico* analysis with SIFT, CADD, MutationTaster, and Polyphen-2 software predicted that both are likely damaging. However, based on American College of Medical Genetics and Association for Molecular Pathology (ACMG-AMP) guidelines (6), we classified the p.Arg107Gly variant as likely pathogenic (PM1, PM2, PM5, and PP3) and p.Ala116Val as a variant of uncertain significance (VUS; PM2, PM1, and PP3).

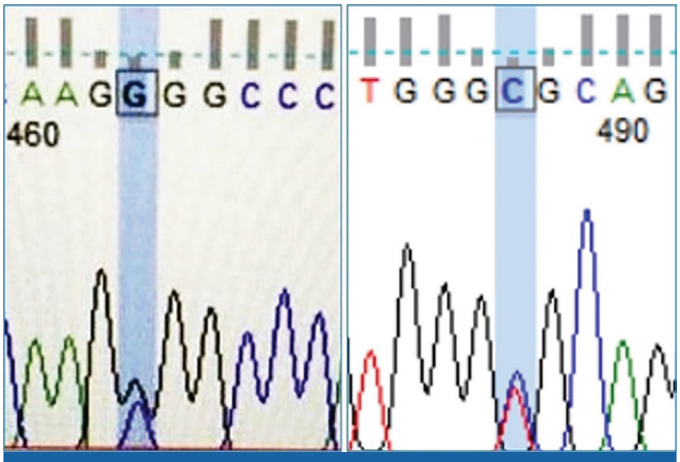

Figure 2. The electropherograms of the first (left) and second cases (right), which demonstrate heterozygous substitutions of p.Arg107Gly [NM_000346.4:c.(319C>G)] and p.Ala116Val [NM_000346.4:c.(347C>T)] in the *SOX9* gene

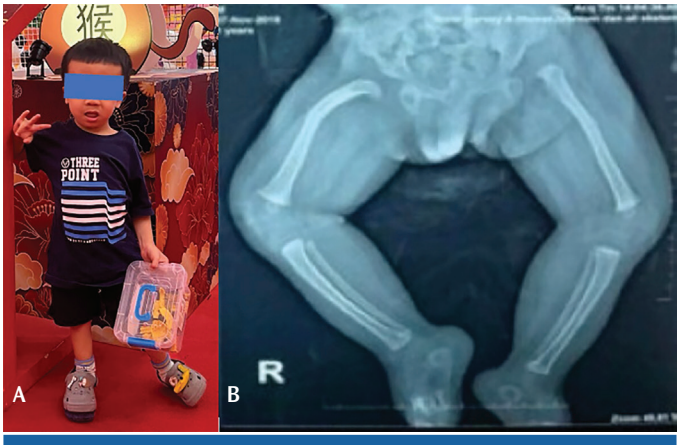

Figure 3. The second case, at 5.5 years old, his right foot was 3 cm shorter than the left one (A). The radiograph of the lower extremities in the second case shows bowing of the femurs, tibiae, and fibulae (B)

Molecular analysis for both cases was conducted using Sanger sequencing on all coding regions of the *SOX9* gene with previously published primers (7). Modifications were applied to Exon 3A (Forward 5'-CCTGATAAAAGGGGGCTGTCCAG-3'; Reverse 5'-GTGCTGCTGCTGCTCGCTGTA-3') and Exon 3B (Forward 5'-CAGGCGCACACGCTGACCAC-3'; Reverse 5'-CGGCCATCTTCGCCCTTCGT-3'). However, parental molecular analysis was not performed because DNA samples were unavailable.

Discussion

We documented two cases of CD, each presenting with different sex phenotypes. As these were our initial experiences with the condition, establishing the diagnosis was particularly challenging. We initially failed to recognize the simultaneous presence of bone deformities and male-to-female sex reversal, leading us to test for other genes. CD is a rare disorder, occurring in approximately 1 in 200,000 births. However, as demonstrated in our cases, it is likely underreported. It may often be misdiagnosed as other syndromes or skeletal dysplasia, or it might go undiagnosed because the severely affected cases may die in the perinatal period (1,2,8).

CD presents with variable symptoms, frequently including key features, such as the bowing of long bones, especially the femur and tibiae. These signs are often detected through prenatal ultrasounds (2,8), although they were not identified in our cases. Additional skeletal abnormalities include underdeveloped shoulder blades, 11 ribs, and a narrow chest. Individuals with this condition typically show distinctive facial features, including a flattened nasal bridge, micrognathia, glossoptosis, cleft palate, and airway obstruction associated with the Pierre Robin sequence (1,9). Our cases presented with these classic clinical manifestations, with varying degrees of motor and speech delay. However, of the cases presented herein were both aged almost six years at the most recent examinations.

Over 200 variants of the *SOX9* gene have been documented, mostly leading to diverse clinical presentations of CD (10). Multiple studies have failed to establish any genotype-phenotype relationships (11,12). Both *SOX9* variants identified in our cases were located within the high mobility group (HMG) domain of the gene. Meyer et al. (11) conducted a functional study of four missense mutations (p.Pro108Leu, p.Trp143Arg, p.Arg152Pro, and p.Pro170Arg) occurring in the HMG domain of *SOX9*, showing that these substitutions diminished or reduced DNA binding capability. These amino acid substitutions led to severe phenotypes and early death. Conversely, a sex-reversed 46,XY case carrying an HMG domain non-sense mutation, p.Gln117Ter, which led to an 80% loss of the protein structure, still survived at 12 years old (11).

Another notable feature of CD is XY sex reversal, as observed in the first case presented here. The sex reversal associated with CD is linked to gonadal dysgenesis since *SOX9* is involved in male sex determination (2). It is recognized that individuals with dysgenetic gonads and a Y chromosome are at significant risk for developing germ cell malignancy (13,14), and therefore, surgical removal before puberty is widely recommended. Consequently, we strongly advised the parents of the first case to consider a laparoscopic evaluation for the abdominal gonads. Nevertheless, the parents remained hesitant to approve any invasive procedures.

Despite being only nine amino acids apart, the *SOX9* variants in our cases located in the same domain resulted in different sex phenotypes. Adjacent reported missense variants in the HMG domain, the p.Lys106Glu (8) and p.Pro108Leu (11), have been associated with severe CD. One case involved a 46,XX fetus terminated at 21 weeks of gestation, and another a 46,XY sex-reversed infant who died at 6 months.

The p.Arg107Gly mutation likely causes protein conformation changes due to the amino acid switch from a positively charged hydrophilic residue to a non-polar hydrophobic residue. Conversely, the heterozygous p.Ala116Val mutation may result in less severe phenotypes since both amino acids involved in the transformation are non-polar and hydrophobic. Arg107 and Ala116 are located in helix-1 of *SOX9*, with the p.Arg107Ala variant located explicitly in the *SOX9* nuclear localization domain. The amino acid substitution in p.Arg107Ala may disrupt the nuclear import of *SOX9*, thereby impairing its function. However, functional analysis is needed to confirm this.

Furthermore, the helix regions of *SOX9* play a crucial role in DNA binding and bending, which is essential for *SOX9* regulatory activity. It was previously described that helix-1 contains key residues responsible for *SOX9*-mediated DNA bending (15). A change in the hydrophobic packing of a previously described variant, p.Phe154Leu, was shown to reduce DNA binding activity by 20-fold (16). However, the p.Ala116Val variant observed in this study would be less disruptive to this hydrophobic packing as alanine and valine residues share similar biochemical properties. Although the p.Ala116Val variant is currently classified as a VUS according to ACMG-AMP guidelines, this report may provide valuable information regarding its association with CD.

Two variants of the *SOX9* gene, p.Arg107Gly and p.Ala116Val, confirmed the diagnosis of CD in two children. The clinical presentation of CD may vary and may go unrecognized due to lack of clinical awareness. The specific location in the HMG domain and the alteration of biochemical properties may contribute to the clinical variations observed in these cases.

Ethics

Ethics Committee Approval: Ethical approval for the study was granted by the Health Research Ethics Committee, National Research and Innovation Agency (NRIA), No. 25/KE.03/SK/12/2023.

Informed Consent: Parental written informed consent was obtained before enrolling the cases.

Footnotes

Authorship Contributions

Medical Practices: Nanis Sacharina Marzuki, Concept: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Firman Pratama Idris, Design: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Data Collection or Processing: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Analysis or Interpretation: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Firman Pratama Idris, Literature Search: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Firman Pratama Idris, Writing: Nanis Sacharina Marzuki, Hannie DH Kartapradja, Firman Pratama Idris.

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CASE REPORT

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A Rare Presentation of HIST1H1E Syndrome with Short Stature and Multiple Pituitary Hormone Deficiencies

İlayda Altun, Elvan Bayramoğlu, Hasan Karakaş, Gökçe Velioğlu Haşlak, Mert Uçar, Hande Turan, Olcay Evliyaoğlu

İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Türkiye

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ABSTRACT

Histone Gene Cluster 1 Member E (HIST1H1E) syndrome is a rare autosomal dominant disorder resulting from a heterozygous variation in the *H1-4* gene located on chromosome 6p22.2. Mental retardation, recognizable facial features, skeletal abnormalities and overgrowth are the main clinical manifestations of this syndrome. A 17-year-old male presented to the pediatric endocrinology clinic due to short stature. He had a characteristic facial appearance with neurodevelopmental delay. Genetic analysis revealed a heterozygous pathogenic variation in the *H1-4* gene located on chromosome 6p22.2 which confirmed his diagnosis of HIST1H1E syndrome. This presentation was distinguished by the co-occurrence of short stature and obesity accompanying central hypothyroidism and growth hormone deficiency due to a hypoplastic pituitary gland, which is in marked contrast to the somatic overgrowth characterizing this syndrome. Furthermore, the presented case is the second patient reported with HIST1H1E syndrome with hyposecretion of multiple pituitary hormones caused by hypoplasia of the pituitary gland.

Keywords: Central hypothyroidism, growth hormone deficiency, hypoplastic pituitary gland, HIST1H1E, short stature

What is already known on this topic?

Mental retardation, recognizable facial features, overgrowth and skeletal abnormalities are the main clinical manifestations of Histone Gene Cluster 1 Member E (HIST1H1E) syndrome. Hypoplastic corpus callous, ventriculomegaly, small posterior fossa, partial decent of cerebellar tonsils were detected the common finding of brain magnetic resonance imaging of the patients diagnosed HIST1H1E syndrome.

What this study adds?

In our case, distinguished by the co-occurrence of short strature accompanying central hypothyroidism and growth hormone deficiency, which differs from the somatic overgrowth characterizing this syndrome. Pituitary hypoplasia has been reported in our case.

Corresponding Author: İlayda Altun, MD, İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Türkiye
E-mail: ilaydaauslu@gmail.com **ORCID:** orcid.org/0000-0002-2329-9223

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Introduction

Histones play a key role in dynamic packaging of nuclear DNA in chromatin, epigenetic modifications and the regulation of chromosome structure (1). Histone Gene Cluster 1 Member E (HIST1H1E), located at chromosome 6p22.2, a member of the *H1* gene family, encodes Histone H1.4, responsible for higher order chromatin structure. HIST1H1E syndrome (also known as Rahman syndrome, OMIM #617537) was first described as autosomal dominant intellectual disability syndrome and is associated with pathogenic frameshift variations in the C-terminal domain of the *H1-4* gene. It has been associated with a distinctive facial features (2) (micrognathia, deep-set eyes, down-slanting palpebral fissures, high hairline, hypertelorism, telecanthus), hypotonia, behavioral issues and mental problems (combinations of obsessive behaviors, anxiety/phobias, autistic spectrum disorder/traits, attention-deficit/hyperactivity disorder and others), cardiac and skeletal abnormality, cryptorchidism, abnormal dentition including dental erosions, thin enamel, crumbling teeth, and multiple dental caries, ectodermal abnormalities and hypothyroidism (3). Autism spectrum disorder and intellectual disability has been described as a component of the presentation in a patient with HIST1H1E syndrome (4).

HIST1H1E syndrome also serves as a mnemonic form for some of key features of the syndrome: H for hypotonia, I for intellectual disability, S for skeletal abnormalities, T for testicular and thyroid anomalies, H for heart anomalies and E for ectodermal features.

HIST1H1E syndrome was first described as an overgrowth syndrome. However, Takenouchi et al. (5) suggested that overgrowth is not a hallmark clinical feature of HIST1H1E syndrome. A few case reports (2,3,4) evaluating growth patterns in Rahman syndrome have also focused on height variability. We report a case of HIST1H1E syndrome in a patient with obesity, short stature and deficiency of multiple pituitary hormones.

Case Report

The male proband is the first child of healthy, consanguineous Turkish parents. He was born at 38 weeks, with a weight of 2,600 g [-1.78 standard deviation (SD)] and a length of 48.0 cm (-0.91 SD). Anthropometric measurements were appropriate for the gestational age. He stayed in the neonatal intensive care unit for a month due to hypotonia and respiratory distress. Cardiac problems, including secundum atrial septal defect and patent ductus arteriosus, were detected in the neonatal period. He was able to sit without support at the age of one year. He attained independent walking by the age of two years, and began to produce phrase-level speech after the age of three years. Cryptorchidism was detected and he had bilateral orchiopexy at 2 years of age. At 3 years of age, his weight was 20 kg (+2.08 SD), height 104 cm (+1.81 SD).

The patient demonstrated intellectual disability and neuromotor developmental delay. He had a history of afebrile seizure at the age of nine years. Brain magnetic resonance imaging (MRI) performed at the time showed focal hypoplastic corpus callosum. At the age of 17 years, he was referred to our tertiary pediatric endocrinology center with obesity and short stature. His body mass index (BMI) was 34.5 kg/m^2 with a weight of 87.6 kg (1.48 SD) and a height of 159.4 cm (-2.53 SD). His pubertal development was appropriate to his chronological age. He had a characteristic facial appearance with bitemporal narrowing, a high hairline, deep set eyes, hypertelorism and downslanting palpebral fissures. In addition, he exhibited problems of abnormal dentition, including dental erosions, crumbling teeth, thin enamel and multiple dental caries. His ectodermal issues included thin nails, sparse hair and hyperkerotosis. Informed consent was obtained from his parents and himself for publication of an image of the patient's face (shown in Figure 1). Skeletal anomalies included kyphoscoliosis and hallux deformity. The patient demonstrated moderate intellectual disability.

In the routine laboratory examination performed to assess the short stature of the patient, thyroid function tests were compatible with central hypothyroidism, with free thyroxine of 0.75 ng/dL [normal range (NR) 0.98-1.63], free triiodothyronine of 2.67 ng/dL (NR 2.56-5.01), thyroid-stimulating hormone of 3.2 mIU/L (NR 0.51-4.3). Furthermore, his insulin-like growth factor-1 (IGF-1) of 92 ng/mL [-2.91 SD score (SDS)] was low. Evaluation of other anterior pituitary hormones revealed: adrenocorticotropic hormone 38.3 pg/mL (NR 0-46); cortisol 14.4 µg/dL (NR 6.2-19.4); and prolactin (132 µg/L), all of which were normal. Gonadotropins, luteinizing hormone (LH) 8.96mU/mL and follicle stimulating hormone (FSH) 10.2 mU/mL were at the upper limit of the reference range but total testosterone levels (265 ng/dL) were not compatible with hyogonadism. His hypothyroidism was managed with levothyroxine 50 µg daily. Growth hormone (GH) deficiency was demonstrated in the GH stimulation tests performed after euthyroidism was achieved; peak GH in an L-Dopa stimulation test was 2.51 ng/mL and the peak GH in a clonidine stimulation test was 1.53 ng/mL. Wrist X-ray reported normal bone age consistent with the chronological age of the patient of 17 years. GH stimulation testing served a diagnostic rather than therapeutic purpose as part of adult endocrine evaluation because growth plates were closed. Therefore, the purpose and clinical utility of this functional assay was explained to the patient and his family before proceeding. A brain and sellar MRI showed pituitary hypoplasia. On the basis of these findings, the patient was diagnosed with central hypothyroidism and GH deficiency. Bone mineral density was measured at 1.11 g/cm^3 (1.9 SDS), which is within the NR (6).

Laboratory investigations for obesity were performed. These showed a high fasting glucose (105 mg/dL) with concurrent

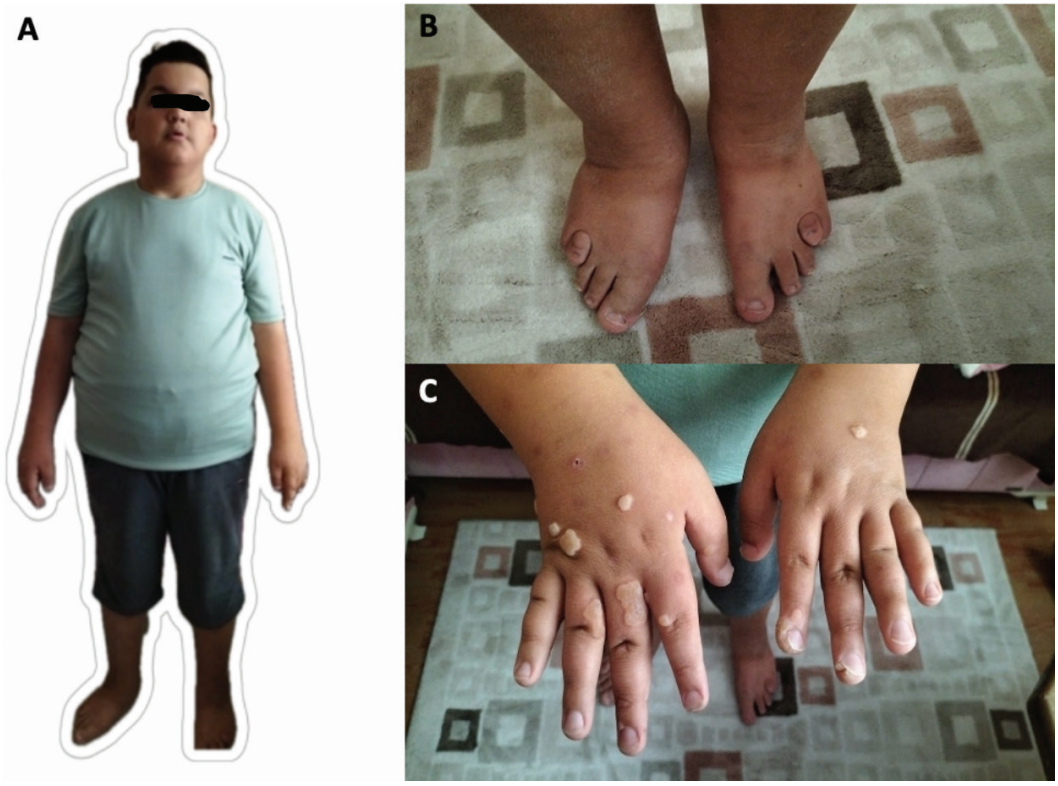

Figure 1. A) Distinctive external features including characteristic facial appearance, obesity, and short stature. **B)** Skeletal anomalies including hallux deformity. **C)** Ectodermal anomalies including hyperkeratosis

insulin of 13.4 mU/mL, C-peptide of 2.72 ng/mL, Homeostatic Model Assessment of Insulin Resistance of 3.5 and HbA1c of 28.96 mmol/mol (4.8%). Impaired glucose tolerance was demonstrated on glucose tolerance test with a 1 hour postprandial level of 168 mg/dL and a 2 hour postprandial level of >140 mg/dL. Total cholesterol was 216.8 mg/dL with triglyceride 252 mg/dL, low-density lipoprotein 147 mg/dL and high-density lipoprotein 30.4 mg/dL. Grade 2 fatty liver disease was detected by abdominal ultrasound. The patient was treated with metformin.

Conventional G-banding cytogenetic analysis demonstrated a normal karyotype of 46,XY. He subsequently underwent whole exome sequencing (WES) incorporating copy number variant analysis. WES was performed using the Roche KAPA HyperExome capture kit (Roche Sequencing Solutions, Pleasanton, CA, USA) and sequenced on the Illumina NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA) platform. Library preparation was conducted according to the manufacturer's protocols. Detected variants were annotated and interpreted using multiple population and disease databases, including ClinVar, OMIM, PubMed, and *in silico* tools. For pathogenicity classification, the Illumina BaseSpace Variant Interpreter, InterVar, VarSome, Franklin by Genoox, and other relevant resources were used, in accordance with the 2015 American College of Medical Genetics and Genomics (ACMG)-Association for Molecular Pathology guidelines. A *de novo* pathogenic frameshift variant in the *H1-4* gene: c.430dupG; p.(Ala144Glyfs*52) (OMIM 142220, NM_005321.3), located on chromosome 6p22.2 was found and was classified as pathogenic based on ACMG criteria. The analysis was guided by Human Phenotype Ontology terms, including short stature (HP:0004322), panhypopituitarism (HP:0000829), obesity (HP:0001513), and intellectual disability (HP:0001249).

Discussion

Although HIST1H1E syndrome was initially reported in 2017 as typically presenting with overgrowth and intellectual disability, recent studies have suggested that overgrowth may not be a hallmark feature of the syndrome (4). In the present report, a patient with a *de novo* truncating variant of the *H1-4* gene, exhibiting intellectual disability and the characteristic facial and skeletal features of HIST1H1E syndrome was described. However, in contrast to the typical presentation, the patient displayed marked decreased height over time and eventually short stature. Further investigation into the etiology of the short stature revealed multiple pituitary hormone deficiencies. Moreover, he also presented with obesity and impaired glucose tolerance. These findings suggest the clinical heterogeneity of the syndrome may be broader than previously reported in the literature.

Zhao et al. (3) systematically reviewed genotype-phenotype relationships based on data from a relatively large 52 patient cohort with HIST1H1E, and found 23 frameshift variants. These findings demonstrated that likely gene-disrupting variants in *H1-4* contribute to phenotypic heterogeneity. Variants in *H1-4* have frequently been associated with a specific subtype of neurodevelopmental disorders. Although the phenotypes of patients are complex, some clinical presentations that are common to most patients are observed, including developmental delay (50/52), prominent forehead (40/52), high hairline (45/52), downward slanting palpebral fissures (35/52), hypertelorism (37/52), and cryptorchidism (17/25), which were all exhibited by the patient in our case report. Furthermore, Zhao et al. (3) also reported that the 430 dupG;_p.(Ala144Glyfs*52) variant was most commonly detected among the 52 patients reviewed, which was also the variant detected in the patient we present.

The reported height SD range of patients with Ala144 frameshift variants has been very variable, ranging between -1.74 SD and +3.65 SD, but has not included clinical short stature. We wish to highlight the co-occurrence of short stature in the presented case with height ≤-2 SD. To the best of our knowledge, only one other case of HIST1H1E syndrome with short stature has been described by Tanabe et al. (7) with height ≤-2 SD.

Endocrine problems have been reported in several different case reports, including hypothyroidism. To date there has been no differential diagnosis between central hypothyroidism and primary hypothyroidism. Burkardt et al. (8) reported 30 unrelated individuals with frameshift *H1-4* variants. Twelve of them had 430 dupG; p.(Ala144Glyfs*52) variant which was the same variant found in our case and 25% were reported to have hypothroidism (8).

Zhao et al. (3) reported 4 (4/52) unrelated individuals diagnosed as HIST1H1E syndrome who had hypothyroidism and two of them had 430 dupG;_p.(Ala144Glyfs*52) variant.

Burkardt et al. (8) and Zhao et al. (9) reported highly variable brain MRI findings. Hypoplastic corpus callosum, ventriculomegaly, small posterior fossa, and partial descent of cerebellar tonsils were the most common MRI findings. Pituitary hypoplasia has been reported in one case who had a *de novo* c.360_361insA, p.(Ala123Glyfs*73) variant associated with hypothyroidism (3,8).

Tanabe et al. (7) reported first case of HIST1H1E syndrome with hyposecretion of multiple pituitary hormones, including central hypothroidism, GH deficiency and hypogonadotropic hypogonadism. The presented case is only the second case of HIST1H1E syndrome with short stature and multiple pituitary hormone deficiency (central hypothyroidism and GH deficiency) reported, after that of Tanabe et al. (7).

Interestingly, the current case had normal height SDS early in life (+1.81 at 3-years old) with short stature developing later, so that by nine years of age his height was -2.53 SDS. In the literature, there are cases where the birth length was normal, but there was a loss of height later in life (3). Although the mechanism for the development of short stature has not been clarified, in our case, it is thought that short stature developed due to central hypothyroidism and GH deficiency. This hypothesis is supported by the data from Tanabe et al. (7) whose case report described a patient with HIST1H1E syndrome with GH deficiency and hypothyroidism and who had short stature (6).

Given that the FSH and LH levels in the presented case were at the upper limit of the reference range (10) according to pubertal levels, the patient should be monitored for potential development of hypergonadotropic hypogonadism, as this may reflect an early compensatory response to declining testicular function. Although hypogonadotropic hypogonadism has been reported in association with HIST1H1E syndrome previously (7), to date, there have been no documented cases of hypergonadotropic hypogonadism.

Although obesity is not the main component of the Ala144 frameshift variants, the presented case was obese, with impaired glucose tolerance associated with hypercholesterolemia. Ahmed et al. (11) reported the first case of HIST1H1E syndrome with raised BMI SD and diabetes mellitus. The weight SD range of patients with Ala144 frameshift variants reported by Zhao et al. (3) were between -0.88SD and +3.3SD. A raised BMI may increase the risk of glucose metabolism problems with hypercholesterolemia.

H1.4 is a structural part of chromatin involved in the control of DNA compaction, DNA replication, recombination, repair and gene expression regulation. We would like to highlight that dysfunction or abnormality of the *H1-4* gene may cause neurodevelopmental dysfunction, because of the important structural and regulatory roles of histones. A *H1-4* pathogenic frameshift c430. Dup; p.(Ala144glyfs*52) may cause defects in pituitary gland organogenesis. In recent years, genome-wide methylation array analysis has allowed researchers to detect and investigate epigenetic features of diseases. Ciolfi et al. (12) reported that a clinically relevant proportion of the genes containing hypomethylated regions in HIST1H1E variants are predominantly expressed in brain and encode different subunits essential for synaptic function, synaptic plasticity, and cognitive function. HIST1H1E-mediated regulation of chromatin compaction may contribute to neurogenesis defects (12). A better understanding of site-specific functions of variants is needed to clarify the connection between histone proteins and pituitary gland development.

Conclusion

HIST1H1E syndrome is a neurodevelopmental syndrome which may be recognizable through characteristic facial features. The generally described phenotypic features facilitate current knowledge regarding the spectrum of HIST1H1E variants, but skeletal overgrowth, initially believed to be a phenotypic characteristic of HIST1H1E, does not appear to be an essential feature. The syndrome may present with short stature, especially if the patients are only diagnosed at older ages, which appears to develop over time in a very few cases. Multiple pituitary hormones deficiency should be kept in mind in *H1-4* pathogenic frameshift variations. Additional investigations are required to elucidate the biological mechanisms underlying the pheotypic features of frameshift variants of HIST1H1E.

Ethics

Informed Consent: Informed consent was taken from the parents of patient for publication. Consent was obtained from the parents for publication of an image of the patient's image.

Footnotes

Authorship Contributions

Surgical and Medical Practices: İlayda Altun, Elvan Bayramoğlu, Hasan Karakaş, Olcay Evliyaoğlu, Concept: İlayda Altun, Elvan Bayramoğlu, Olcay Evliyaoğlu, Design: İlayda Altun, Elvan Bayramoğlu, Olcay Evliyaoğlu, Data Collection or Processing: İlayda Altun, Analysis or Interpretation: İlayda Altun, Elvan Bayramoğlu, Hasan Karakaş, Gökçe Velioğlu Haşlak, Mert Uçar, Hande Turan, Olcay Evliyaoğlu, Literature Search: İlayda Altun, Writing: İlayda Altun, Elvan Bayramoğlu, Olcay Evliyaoğlu.

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CASE REPORT

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X-linked Osteoporosis due to PLS3 Pathogenic Variant: Case Report on Zoledronic Acid Treatment in Siblings

María Camila Velandia-Avendaño[1], María Paula Sarmiento-Ramón[2]

[1]Pediatric Resident, Universidad Industrial de Santander, Bucaramanga, Colombia
[2]Pediatric Endocrinologist, Fundación Oftalmológica de Santander, Floridablanca, Colombia

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ABSTRACT

Osteoporosis in children is a rare condition, often associated with genetic factors. Monogenic forms of osteoporosis linked to the X chromosome are often related to mutations in the gene encoding plastin 3 (PLS3). PLS3 is a protein involved in actin bundle formation in the cytoskeleton. We present two brothers with recurrent peripheral fractures and vertebral compression fractures, both associated with low bone mineral density (BMD). The patients shared the same deletion (c.589_590) in *PLS3* on Xq23, which was confirmed by next-generation sequencing. They were treated with zoledronic acid, calcium, and vitamin D, showing optimal improvement in BMD, a reduction in bone fractures, and enhanced quality of life.

Keywords: Bone diseases, Juvenile osteoporosis, PLS3, Zoledronic acid

What is already known on this topic?

Deficiency of PLS3 caused by pathogenic variants will lead to early-onset osteoporosis and bisphosphonate therapy has proven effective in reducing fracture recurrence.

What this study adds?

Our finding expanded the phenotypic spectrum, supporting the theory of scoliosis as a potential phenotype in the patients with PLS3 pathogenic variants.

Corresponding Author: María Camila Velandia-Avendaño, MD, Pediatric Resident, Universidad Industrial de Santander, Bucaramanga, Colombia
E-mail: mcamilavel@gmail.com **ORCID:** orcid.org/0000-0002-9809-0993
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Introduction

Osteoporosis is a skeletal disorder characterized by reduced bone mass and impaired bone microarchitecture, leading to increased bone fragility and a higher risk of fractures (1). Pediatric osteoporosis is rare. The diagnosis is established when bone mineral density (BMD) is below a Z-score of -2, accompanied with a significant fracture history, defined as two or more long bone fractures before the age of 10 years, or three or more before the age of 19 years. Notably, the presence of vertebral compression fractures alone is sufficient for diagnosis, even if BMD is within the normal range (1).

Osteoporosis can be classified as primary or secondary. Primary forms are caused by genetic conditions (1), with osteogenesis imperfecta being the most common, occurring in approximately 1 in 15,000 to 20,000 births (2). Over the past decade, pathogenic variants in at least 20 different genes have been identified as potential causes of childhood-onset primary osteoporosis (1). In contrast, secondary osteoporosis is typically associated with chronic systemic diseases or long-term use of medications, such as extended glucocorticoid therapy (3).

X-linked osteoporosis due to pathogenic variants in the plastin 3 *(PLS3)* gene was first described in 2013 and remains an extremely rare condition, with an estimated prevalence of fewer than 1 in 1,000,000 individuals (1). The *PLS3* gene encodes plastin-3, an actin-bundling protein that plays a critical role in cytoskeletal dynamics and bone mineralization. Loss of *PLS3* function has been associated with increased bone fragility, reduced BMD, and recurrent fractures beginning in childhood (4).

Bisphosphonate therapy has demonstrated efficacy in improving BMD and reducing fracture incidence in individuals with X-linked osteoporosis caused by *PLS3* pathogenic variants. Clinical studies have shown significant increases in BMD and vertebral body remodeling after 10 months to 2 years of treatment. Early diagnosis and timely intervention are essential to alter the disease course and prevent progression of osteoporosis (4,5,6).

We report two cases of X-linked osteoporosis caused by a *PLS*3 deletion (c.589_590) on Xq23, confirmed through next-generation sequencing. Both patients were treated with zoledronic acid, calcium, and vitamin D, resulting in marked improvement in BMD, reduced incidence of fractures, and enhanced quality of life.

Case Report

Patient 1

A 12-year-old boy was referred to pediatric endocrinology due to multiple fractures resulting from mild trauma. His first fracture occurred at age 3 years, involving the left elbow, after falling from a tricycle. At age 5 years, he fractured his left humerus after falling from standing height. A third fracture occurred at age 8 years, affecting the right scapula after a collision with a friend while playing. Subsequently, he fractured his left wrist at 10 years old and experienced another fracture of the left humerus at 11 years of age, again due to low-impact trauma.

No complications were noted during birth. He was born at term with appropriate neonatal adaptation, a birth weight of 3500 grams, and a birth length of 51 cm. There is no history of hypoglycemia or jaundice. The patient's neurodevelopment was normal. The mother's height is 177 cm, and the father's height is 172 cm. Accordingly, the mid-parental target height was calculated at 181 cm, with a target height range of 176-186 cm, which corresponds approximately to ±1.9 standard deviation (SD) according to the Colombian C3REF growth reference (5).

On physical examination, all the vital signs were within normal limits. There were no signs of blue sclerae, tooth abnormalities or hypermobility. Anthropometric evaluation showed a weight of 88.6 kg, a height of 178 cm (1.9 SD; C3REF), and a body mass index (BMI) of 27.96 kg/m^2 (+2.32 SD; OMS). Body proportions were harmonious, with no evidence of disproportion (normal upper/lower segment ratio and arm span for height, where assessed). Pubertal evaluation revealed Tanner stage G4P4, with a testicular volume of 15 mL. A notable finding was scoliosis on musculoskeletal examinations. Other systems were unremarkable.

Studies to rule out secondary causes of osteoporosis were performed and yielded normal results, as summarized in Table 1. The evaluation excluded renal and parathyroid disorders, thyroid dysfunction, glucocorticoid excess, hypogonadism, growth hormone deficiency, homocysteinuria, and chromosomal abnormalities, supporting a diagnosis of primary osteoporosis.

Renal and urinary tract ultrasound was normal, and the spine X-ray showed a left-convex scoliotic deviation at T12 and L4 of 18 degrees and at T6 and T22 of 11 degrees with a convexity of 9 degrees. Molecular analysis via clinical exome sequencing identified a hemizygous frameshift variant in the *PLS3* gene: NM_005032.5:c.589_590delTT (p.Leu197GlufsTer39). This variant consists of a two-nucleotide deletion (TT) in exon 6, resulting in a premature stop codon 39 amino acids downstream from the leucine at position 197. The predicted outcome is a truncated protein or, alternatively, non-sense-mediated mRNA decay, aligning with loss-of-function as a disease mechanism in *PLS3*-related disorders.

The variant was classified as likely pathogenic according to the American College of Medical Genetics and Association for Molecular Pathology 2015 guidelines, based on the following criteria: PVS1, indicating null variant in a gene where loss of

Table 1. Biochemical work-up of the two reported cases to rule out secondary osteoporosis

Laboratory test	Reference value	Patient #1	Patient #2
Ionized calcium (mmol/L)	1.24-1.39	1.216	1.3
Phosphorus (mg/dL)	4.1-5.9	5.68	5.78
25-hydroxyvitamin D (ng/mL)	>30	20.9	23
Parathyroid hormone (pg/mL)	10-65	29.3	-
Alkaline phosphatase (UI/L)	141-460	411	-
Karyotype	-	46,XY	-
Homocysteine (umol/L)	12 -15	6.96	6.26
Thyroid-stimulating hormone (uUI/ml)	0.51-5.5	0.67	
Insulin-like growth factor 1 (ng/mL)	99-655	580.08	-
Cortisol (ug/dL)	5-23	5.79	-
Follicle stimulating hormone (mUI/mL)	1,5-12,4	3.06	-
Luteinizing hormone (mUI/mL)	1,7-8,6	4.02	-
Total testosterone (ng/mL)	0.28-11.1	2.91	-
Sodium (mmol/L)	135-148	141.6	-
Urinary calcium (mg/24 h)	100-300	75.4	-
BUN (mg/dL)	5.0-18.0	9.1	-
Creatinine (mg/dL)	0.67-1.17	0.63	0.47

BUN: Blood urea nitrogen

function is a known disease mechanism; and PM2, reflecting its absence from population databases. *In silico* predictive tools further support a deleterious effect, assigning a pathogenicity score of 1.0 (on a scale from 0 to 1), as calculated by two independent algorithms. Although this variant has not been previously associated with clinical phenotypes in the extant literature, the established pathogenicity criteria, corroborating *in silico* predictions, and the patient's clinical presentation collectively support and confirm the diagnosis.

Initial management included calcium supplementation and optimization of vitamin D levels while awaiting completion of the etiological evaluation. Nutritional counseling and exercise recommendations were initiated to optimize weight control and prevent progression of overweight. The first bone densitometry was performed at age 14 years and showed normal results. Since the patient had not experienced any new fractures, clinical follow-up with serial densitometry was pursued. However, over the next two years, a progressive decline in the Z-score was noted. At 16 years old, a third densitometry revealed a BMD Z-score of -0.5 SD in total body less head and -2.4 SD in the lumbar spine (L1-L4), as shown in Table 2**.** Given these findings bisphosphonate therapy was initiated to improve BMD, reduce fracture risk and enhance quality of life.

Intravenous zoledronic acid therapy (0.05 mg/kg/dose) was initiated, administered every six months. No adverse effects related to the medication were reported. After two doses, equivalent to one year of treatment, follow-up bone densitometry showed marked improvement in the BMD Z-scores (Table 2). During follow-up, the patient remained fracture-free, adhered well to treatment, and achieved a final height of 185.5 cm, weight of 85 kg, and BMI of 24.7 kg/m^2.

Table 2. Evolution of bone mineral density (Z-scores) in the two patients, pre- and post-zoledronic acid therapy

Patient	Age (years)	Total body less head (Z-score)	Lumbar spine L1-L4 (Z-score)	Therapy status*
#1	14	0.0	-1.5	Before
	15	-0.8	-1.4	Before
	16	-0.5	-2.4	Before
	17	-0.3	-1.5	After
#2	11	-2.0	-2.7	Before
	13	-0.5	-0.2	After

*: Therapy status refers to bisphosphonate treatment (zoledronate). Both patients showed substantial improvement in lumbar spine and total body BMD Z-scores after therapy, BMD: bone mineral density

Patient 2

This patient is the younger brother of Patient 1. A 10-year-old male patient was referred to pediatric endocrinology for evaluation of secondary hyperthyroidism due to Graves disease, confirmed by the presence of thyroid-stimulating hormone (TSH) receptor antibodies, and was managed with methimazole. He also had a history of recurrent fractures beginning in early childhood. Aged 3 years, he sustained an elbow fracture, followed by right and left cubitus fractures at ages 8 and 9 years, respectively. When he was 10 years old, he presented with a one-month history of severe lumbar pain. Radiographic evaluation revealed a compression fracture at T12, as well as anterior wedging of 50% at T9 and 20% at L4 (Figure 1). Although the exact mechanisms of injury were not documented, the absence of high-energy trauma suggests a fragility-related cause.

The patient weighed 51 kg and was 155.3 cm tall, corresponding to +1.77 SD; C3REF. His BMI was 21.15 kg/m^2 (+1.68 SD; OMS). Physical examination was unremarkable and showed no dysmorphic features, except for scoliosis. Pubertal assessment revealed Tanner stage G2 for genital development and P2 for pubic hair, with a testicular volume of approximately 6 mL. Biochemical evaluation of phosphocalcic metabolism revealed low serum vitamin D levels, prompting initiation of vitamin D supplementation (Table 1). Nutritional recommendations initially provided to his sibling were extended to Patient 2. However, the initiation of regular exercise was initially constrained by underlying vertebral pathology. Given the known familial mutation, targeted analysis next-generation sequencing identified the same pathogenic *PLS3* c.589_590del variant.

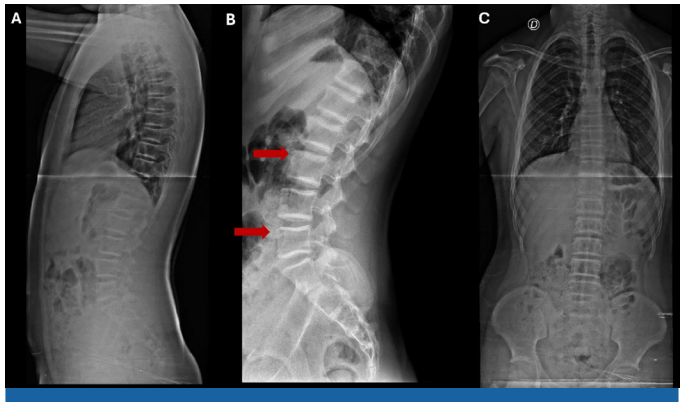

Figure 1. Lateral spine X-ray images of Patient #2 (Panels A and B) prior to treatment show the following findings. (A) Thoracic spine: generalized decrease in bone mineral density. A moderate anterior wedge compression fracture is evident at the level of T9 (arrow). (B) Lumbar spine: diffuse reduction in vertebral radiopacity, also suggestive of decreased bone mineral content. The L4 vertebral body demonstrates a mild reduction in anterior height with a subtle wedge-shaped deformity suggestive of a compression fracture (arrowhead). (C) Anteroposterior radiograph of the thoracolumbar spine showing mild right-convex thoracic scoliosis with compensatory lumbar curvature. Note the subtle anterior height loss and wedge-shaped deformity of the L4 vertebral body (red ellipse)

The first bone densitometry was performed at the age of 11 years and showed abnormal Z-scores for both total body less head and the lumbar spine (L1-L4) (Table 2). The patient was started on intravenous zoledronic acid (0.05 mg/kg/dose). The initial infusion was associated with a mild rash and transient flu-like symptoms, known side-effects of initial zoledronic acid infusion. As expected, subsequent infusions (dose 0.05 mg/kg/dose) were well tolerated without adverse events.

After four doses of zoledronic acid, marked improvement in BMD was observed, with significant increases in Z-scores at both the lumbar spine (L1-L4) and total body less head (Table 2). These improvements were also evident on radiographic follow-up of the vertebral fractures, as described in Figure 2.

Given the trend toward improvement in Z-scores, future doses are scheduled to be reduced to 0.025 mg/kg/dose. Throughout clinical follow-up, the patient has remained fracture-free and has shown continued adherence to and tolerance of bisphosphonate therapy. He was in spontaneous remission of Graves' disease, with negative TSH receptor antibodies and normal thyroid function, having been off methimazole for eight months. At the most recent evaluation, at 13 years of age, his height was 174 cm, with a testicular volume of 15 mL and a bone age of 15 years; his BMI improved to 21.8 kg/m^2 (+0.65 SD, World Health Organization) during follow-up, coinciding with the initiation of regular physical activity.

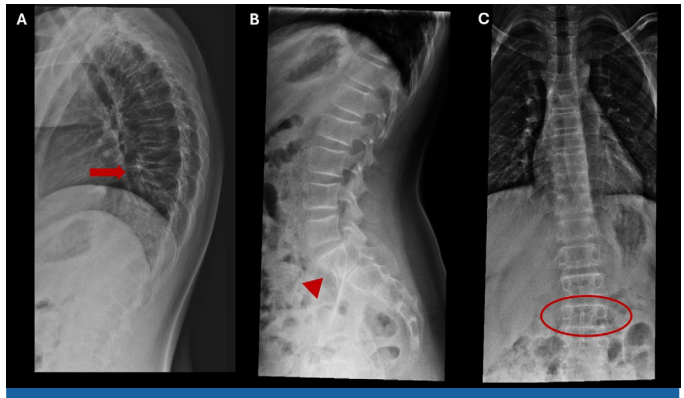

Figure 2. Lateral radiograph (A and B) of the thoracolumbar spine following four doses of zoledronic acid in Patient 2, showing improved bone mineral density and partial restoration of T9 vertebral body height. Diffuse sclerotic lines are observed across multiple vertebral bodies (arrow), consistent with increased bone remodeling activity. Vertebral body contours are well defined, and no new fractures are evident. (C) Anteroposterior radiograph of the spine shows reduction of the previously observed scoliotic curvature

Following confirmation of the diagnosis and in view of the X-linked inheritance pattern, a bone densitometry was performed on the patient`s mother at age 50, showing normal results: lumbar spine (L1-L4) T-score -0.9, BMD 1.073 g/cm^2, and left femoral neck T-score -0.5, BMD 0.962 g/cm^2. Her only relevant history was Graves' disease treated with radioiodine ablation, and she remains euthyroid under levothyroxine replacement. At her current age of 53 years, she has not sustained fractures, spinal radiographs (Figure 3) were normal, and physical examination was unremarkable. Segregation analysis is currently ongoing, and the broader family history is summarized in the pedigree (Figure 3).

Discussion

Various *PLS3* gene variants have been described. Here, we report the cases of two brothers presenting with osteoporosis in hemizygous male affected, manifested by long bone and vertebral compression fractures. A novel hemizygous frameshift variant, NM_005032.5:c.589_590delTT (p.Leu197GlufsTer39), was identified. This variant introduces a premature stop codon 39 amino acids downstream from the leucine at position 197, leading to a truncated protein or non-sense-mediated mRNA decay, and has not been previously reported in the literature.

Consistent with previously reported cohorts, both patients exhibited normal growth and neurodevelopment, and lacked classical extra-skeletal features of osteogenesis imperfecta, such as blue sclerae, dentinogenesis imperfecta, or hearing loss (4). The only notable clinical finding in these siblings was scoliosis, which has also been reported in other patients with *PLS3* mutations. In our experience, scoliosis improved with bisphosphonate therapy, possibly due to vertebral stabilization and reshaping linked to increased BMD (3).

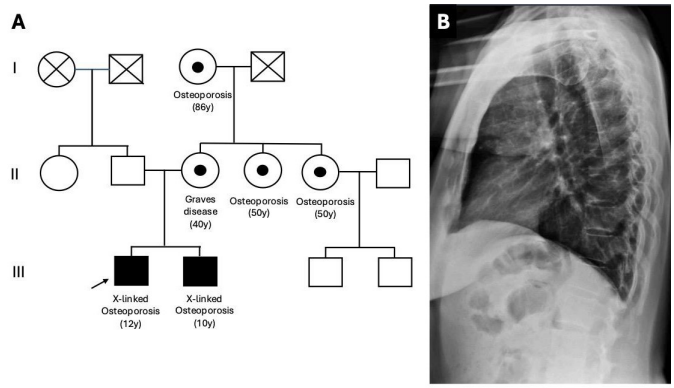

Figure 3. Clinical and radiographic findings in a family with *PLS3*-associated X-linked osteoporosis. (A) Pedigree showing the segregation of the pathogenic *PLS3* variant consistent with X-linked inheritance. (B) Lateral spine radiograph of the mother at age 53 years, showing no evidence of vertebral fractures

Although most patients with PLS3-related osteoporosis do not exhibit extra-skeletal findings, some reports have described variable features, such as pectus excavatum, flat feet, broad and short thumbs, bilateral syndactyly of digits 4-5, and gait disturbances including waddling or clumsiness (7). The reason for this variability remains unclear. It is possible that PLS3 may have a broader role in collagen metabolism or extracellular matrix composition, as similar features have been reported in unrelated individuals with different *PLS3* variants (3,5,7,8).

Heterozygous females carrying *PLS3* variants are generally reported to present with a mild and variable phenotype, most often characterized by normal BMD and absence of fractures, although some cases show reduced bone mass at peripheral sites or skeletal fragility (3,4). No consistent genotype-phenotype correlation has been established, and progression appears to be more strongly associated with age and the degree of BMD reduction than with the specific mutation (3). In the family in our report, the mother, a heterozygous carrier, exhibited normal BMD, no fractures, and no significant clinical findings at 53 years of age, which contrasts with maternal aunts who were diagnosed with osteoporosis around the age of 50 years. This intrafamilial variability illustrates the wide spectrum of phenotypes in heterozygous females, ranging from completely asymptomatic to clinically significant osteoporosis. Taken together, these findings reinforce the suggestion that additional modifiers, including age, hormonal and metabolic factors, and lifestyle, play an important role in shaping bone health in female carriers and highlight the need for individualized longitudinal follow-up, even in apparently unaffected women (3,4).

Optimizing bone health requires a multifaceted approach, including sufficient nutritional intake, regular weight-bearing exercise, and targeted treatment of the underlying condition contributing to skeletal fragility (8). Both of the siblings in this report presented with BMI values above the 95th percentile, highlighting the need to critically evaluate the potential contribution of overweight/obesity to their skeletal phenotype. Although obesity in childhood is often associated with higher absolute BMD values, this increase is not proportional to the greater body mass, resulting in insufficient mineralization to support the excess load and a paradoxical increase in fracture risk (6,7). In Patient 1, insulin-like growth factor-1 and testosterone levels were within the normal range, indicating that endocrine alterations commonly described in obese boys were unlikely to explain the skeletal fragility (6,7). These findings reinforce the suggestion that obesity is not uniformly protective for bone health and, in fact, may act as a confounding factor in the interpretation of densitometry results, particularly in patients with an underlying genetic predisposition, such as the *PLS3* variant.

Bisphosphonates, synthetic analogs of pyrophosphate, are the most extensively studied pharmacologic agents for pediatric osteoporosis. Due to the rarity of this condition, however, clinical trials in pediatric populations remain limited (8). Zoledronic acid, a nitrogen-containing bisphosphonate, inhibits osteoclastic activity by binding to bone hydroxyapatite, thereby reducing bone turnover and preserving skeletal integrity. Clinical trials have shown significant improvements in vertebral body height in patients treated with intravenous bisphosphonates compared to oral formulations (8).

Several studies on patients with *PLS3* mutations have confirmed the effectiveness of bisphosphonate therapy, indicating that younger children may derive greater benefit, likely due to the accelerated accrual of bone mass during these critical developmental stages (4). In Patient 1, although BMD initially improved during adolescence but then declined rapidly, prompting the need for bisphosphonate treatment. This illustrates the importance of early bone densitometry screening and timely intervention to maximize peak bone mass in affected individuals. Both patients exhibited marked improvements in BMD Z-scores after one year of zoledronic acid therapy, reinforcing the value of early diagnosis and initiation of treatment (9).

Advances in molecular diagnostics have significantly improved our ability to identify osteoporosis of genetic origin, particularly in patients where secondary causes and more common primary etiologies, such as osteogenesis imperfecta, have been excluded. Early recognition of clinical signs, such as recurrent low-impact fractures and vertebral deformities, should prompt timely genetic evaluation. Early genetic diagnosis not only facilitates appropriate clinical management but also enables targeted family screening, which is important in X-linked conditions, such as *PLS3*-related osteoporosis. Patients should be closely monitored for early vertebral collapse, and any underlying mineral or hormonal deficiencies should be addressed. Encouraging physical activity and initiating bisphosphonate therapy, when indicated, can positively impact bone health and reduce fracture risk. Continued research into pharmacologic strategies for managing genetically driven osteoporosis remains key to optimizing long-term outcomes.

Ethics

Informed Consent: Informed consent was obtained for the use of patients' medical history and images.

Footnotes

Authorship Contributions

Surgical and Medical Practices: María Camila Velandia-Avendaño, María Paula Sarmiento-Ramón, Concept: María Camila Velandia-Avendaño, María Paula Sarmiento-Ramón, Design: María Camila Velandia-Avendaño, Data Collection or Processing: María Paula Sarmiento-Ramón, Analysis or Interpretation: María Camila Velandia-Avendaño, María Paula Sarmiento-Ramón, Literature Search: María Camila Velandia-Avendaño, María Paula Sarmiento-Ramón, Writing: María Camila Velandia-Avendaño.

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CASE REPORT

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Hereditary Hypophosphatemic Rickets with Hypercalciuria-Importance of Further Evaluation when Clinical Suspicion is Strong

Chathupani Anuradha Wettasinghe, Ishara Minuri Kumarasiri, Mahendralingam Vidushajini, Thabitha Jebaseeli Hoole, Manimel Wadu Akila Nimanthi, Imalka Jayasundara, Reha Balasubramaniam, Navoda Atapattu

Lady Ridgeway Hospital, Clinic of Pediatric Endocrinology, Colombo, Sri Lanka

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ABSTRACT

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is a rare genetic condition with autosomal recessive inheritance and a prevalence of 1 in 250000. It is due to mutation of the *SLC4A3* gene. Correct diagnosis of this condition is important as treatment with active vitamin D metabolites is contraindicated. Evolution of the disease despite initial completely normal biochemistry has been observed, potentially creating diagnostic confusion. The first child presented at the age of 5.5 years with features of rickets. He had an abnormal bone profile with normal vitamin D levels. Urinary phosphate studies were compatible with HHRH. He was treated with phosphate supplementation and potassium citrate. He has well responded well to treatment. The second child initially presented at 1.5 years of age with leg bowing and a family history of hypercalciuria. All investigation findings, including urinary phosphate studies, were within normal limits. At the age of 2.5 years, he again presented with worsening of bowing. Biochemical and urinary investigations were repeated and laboratory findings were now compatible with HHRH. These cases highlight the importance of repeated investigations despite initial normal parameters, if the initial clinical suspicion is strong. Clinical- and investigation-based diagnosis of this rare genetic disease, HHRH, is feasible in resource limited setting.

Keywords: Hereditary hypophosphatemic rickets with hypercalciuria, evolution of investigation findings, clinical diagnosis of the disease

What is already known on this topic?

Hereditary hypophosphatemic rickets with hypercalciuria is a rare genetic disease. Correct diagnosis is essential as vitamin D metabolites which are commonly used in the treatment of rickets is contraindicated in this unique disease.

Corresponding Author: Navoda Atapattu, Lady Ridgeway Hospital, Colombo, Clinic of Pediatric Endocrinology, Colombo, Sri Lanka
E-mail: navodaa@gmail.com **ORCID:** orcid.org/0000-0002-3398-2536
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What this study adds?

This study shows the possibility of evolution of biochemical findings over time despite completely normal initial reports. This report also highlights the role of clinical- and laboratory-based diagnosis in resource limited settings, where genetic diagnosis is not feasible.

Introduction

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH-OMIM: 241530) is a genetic condition with autosomal recessive inheritance (1). It is a rare condition with a prevalence of 1 in 250000 (1). HHRH occurs due to a loss of function mutation in *solute carrier family 34 member 3* (*SLC4A3*) gene located on chromosome 9q34, which encodes a sodium dependent phosphate transporter (NaPi-IIc) that is highly expressed in the proximal tubule of the nephron (2,3). Dysfunction of the transporter results in phosphate wasting. It is a distinct form of hypophosphatemic rickets compared to other types as affected individual present with hypercalciuria (2).

HHRH was first described by Tieder et al. (4) in 1985. Investigators identified consanguineous Bedouin kindreds consisting of six members with hypophosphatemic rickets and hypercalciuria. The condition began in early childhood with short stature, clinical features of rickets and increased renal clearance of phosphate and hypercalciuria. Apart from that these patients had low or low-normal parathyroid hormone (PTH) levels. Supplementation with phosphate resulted in complete resolution of symptoms and biochemical findings except for the maximal tubular reabsorption of phosphate (TRP) to glomerular filtration rate ratio (TmP/GFR).

Twenty-one years after the initial diagnosis of HHRH the genetic abnormality causing the disease was identified by Bergwitz and Miyamoto (2) in 2006. They identified a homozygous single nucleotide deletion (c.228delC) in all individuals affected with HHRH by nucleotide sequencing analysis.

Hypophosphatemia in patients with HHRH causes an appropriate stimulation of renal 1α-hydroxylase leading to increased synthesis of the biologically active vitamin D metabolite 1,25 dihydroxyvitamin D [1,25(OH)2D] (5). Active vitamin D metabolites cause increased intestinal absorption of calcium and reduced PTH-dependent calcium absorption in distal renal tubules resulting in increased urinary calcium excretion.

HHRH usually presents in early childhood with features of rickets, bone pain, skeletal deformities, muscle weakness and short stature (6). It has been reported that HHRH is strongly associated with renal cysts (7).

Diagnosis of the condition depends on several biochemical investigations combined with imaging studies and genetic tests. Biochemical tests may include a fasting bone profile demonstrating low serum phosphate with normal corrected calcium, elevated 1,25 OH vitamin D levels and low or low normal PTH level. Urinary studies will show hypercalciuria with phosphate wasting (inappropriately low Tmp/GFR) in the absence of features of Fanconi syndrome. Imaging studies are likely to provide evidence of rickets with ultrasound (US) scan evidence of nephrocalcinosis or renal stones (8,9).

As there is a marked heterogenicity in clinical spectrum of HHRH and investigation findings may evolve with time, periodic close follow-up is mandatory in this unique disease. Here in we describe patients from two different kindreds reflecting the diversity of clinical presentation in HHRH.

Clinical Presentation

Patient 1

A five years eight months old boy presented with leg bowing. He was the first born to non-consanguineous, healthy parents with two healthy younger siblings. His past history was unremarkable. He had no clinical features of malabsorption. On examination, there was widening of wrists and bilateral genu valgum deformity, suggestive of a clinical diagnosis of rickets.

Imaging studies also revealed evidence of rickets. Initial biochemical results are depicted in Table 1 which shows normal biochemical findings with the exception of elevated alkaline phosphatase (ALP).

During follow-up investigations he had both normal and low serum phosphate values. Due to normal vitamin D level, persistently elevated ALP level and intermittent subnormal serum phosphate levels, he underwent urinary phosphate and calcium studies. Results are depicted in Table 2. These investigations showed hypophosphatemia, renal phosphate wasting and hypercalciuria.

US of kidney, ureter and bladder revealed bilateral grade II-III medullary nephrocalcinosis, mild hydronephrosis and proximal hydroureter on the left side, a 3.2 mm size calculus within the left sided pelvicalyceal system and bilateral simple renal cysts. He was started on phosphate buffer due to unavailability of phosphate tablets at that time. Hydrochlorothiazide and potassium citrate were also added. With treatment his ALP and serum phosphate levels became normal, rickettsial changes resolved on X-rays and bowing of legs completely resolved.

Table 1. Initial biochemical findings of Patient 1

Test	Value	Reference range
Corrected Calcium (mmol/L)	2.22	2.2-2.7
ALP (U/L)	650	60-425
Serum phosphate (mmol/L)	1.27	1.25-2.1
Serum creatinine (umol/L)	51	26-62
Vitamin D (nmol/L)	63	>50 sufficient
PTH (pmol/L)	2.42	1.54-7.2

ALP: alkaline phosphatase; PTH: parathyroid hormone

Table 2. Follow-up results of Patient 1

Test	Value	Reference range
Serum phosphate (mmol/L)	0.86	1.25-2.1
Urine phosphate creatinine ratio (mmol/mmol)	7.54	1.2-12
TRPO4 (%)	68%	>90%
TmPGFR (mmol/L)	0.8	1.15-2.6
Urine calcium creatinine ratio (mmol/mmol)	1.92	0.07-1.5

Currently, he is 10 years and 1 month old and the bowing of legs has completely disappeared with no progression of medullary nephrocalcinosis or calculi. The renal functions are within normal limits. He is on phosphate supplements and potassium citrate with good compliance. He is studying in grade 5 with good scholastic skills.

Patient 2

The second case presented was a 2 year 6-month-old boy who presented with bowing (mild genu valgum deformity) who had completely normal investigation findings one year earlier. However, due to the worsening clinical features and a high degree of clinical suspicion, he was re-evaluated and found to have abnormal investigation findings one year after the initial presentation. This underlines the importance of a combination of high degree of clinical suspicion triggering periodic re-evaluation, despite completely normal initial biochemical findings.

He initially presented to our unit at the age of one year six months with bilateral bowing of the legs. He was the first of dizygotic twins, born at term with a birth weight of 2.37 kg to second degree consanguineous parents with a healthy elder sister and a twin sister. Antenatal and post-natal periods were unremarkable. He was on formula and breast milk from the second week of life. Weaning started from 5 months of age and his diet was rich in calcium and vitamin D-containing food. There was no history of convulsions or tetany. He exhibited no features to suggest malabsorption. There was a family history of hypophosphatemia with hypercalciuria in two of paternal cousins, a boy and a girl, neither of whom were evaluated. The pedigree chart is shown in Figure 1.

This boy was extensively investigated during the admission, including urinary phosphate studies, urinary calcium excretion, intact PTH levels and all were within normal limits.

Results are depicted in Table 3. X-ray examinations did not show any evidence of rickets and US of kidney, ureter, and bladder was normal. He was followed up at a local hospital with a healthy diet rich in calcium and vitamin D. One year later he was referred to our unit due to worsening of bowing and cloudy urine. Due to the high degree of clinical suspicion, he was re-evaluated. These results are also depicted in Table 4. Repeat investigations revealed hypercalciuria, hypophosphatemia, renal phosphate wasting with a TmpGFR of 0.92, compatible with a diagnosis of HHRH. He was started on oral phosphate and potassium citrate and is being followed up at our clinic routinely.

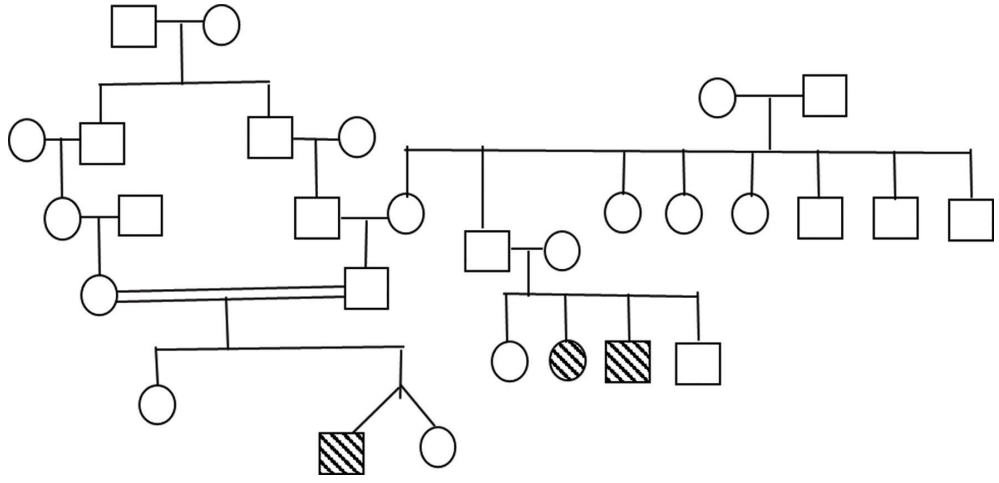

Figure 1. Pedigree chart of case 2 patients

Discussion

Correct diagnosis of HHRH is important due to the unique nature of treatment modalities, but may be challenging because of initial phenotypic variability. This heterogenicity may vary from full-blown HHRH at the most severe to asymptomatic idiopathic hypercalciuria in the other. According to Tieder et al. (4), the magnitude of the hypophosphatemia which regulates active vitamin D levels appeared to determine which subjects would have hypercalciuria combined with bone disease.

Table 3. Initial evaluation of Patient 2

Test	Value	Reference range
Corrected calcium (mmol/L)	2.52	2.2-2.45
Serum phosphate (mmol/L)	1.46	1.25-2.1
ALP (U/L)	416	60-425
Serum creatinine (umol/L)	21	26-62
Serum sodium (mmol/L)	137	135-145
Serum potassium (mmol/L)	5.1	3 5-5.5
Urine phosphate: creatinine ratio (mmol/mmol)	5.5	1.2-12
TRPO4 (%)	92.04%	>90%
TmpGFR (mmol/L)	1.53	1.15-2.6
Urine calcium: creatinine ratio (mmol/mmol)	0.85	0.07-1.5
PTH (pmol/L)	1.0	1.54-7.2
Vitamin D (nmol/L)	60	>50 Sufficient

ALP: alkaline phosphatase; PTH: parathyroid hormone

Table 4. Follow-up results in Patient 2

Test	Value	Reference range
Corrected calcium (mmol/L)	2.5	2.2-2.7
Serum phosphate (mmol/L)	1.08	1.25-2.1
ALP (U/L)	446	60-425
Serum creatinine (umol/L)	28	26-62
Serum sodium (mmol/L)	137	135-145
Serum potassium (mmol/L)	4.5	3.5-5.5
Urine phosphate: creatinine ratio (mmol/mmol)	5.68	1.2-12
TRPO4 (%)	85%	>90%
TmpGFR (mmol/L)	0.92	1.15-2.6
Urine calcium: creatinine ratio (mmol/mmol)	1.8	0.07-1.5
Urine magnesium: creatinine ratio (mmol/mmol)	0.59	0.3-1.6
PTH (pmol/L)	0.77	1.54-7.2
Vitamin D (nmol/L)	55.5	>50 Sufficient

ALP: alkaline phosphatase; PTH: parathyroid hormone

Zhu et al. (3) reported that compound heterozygous/homozygous carriers showed >90% penetrance for kidney and bone phenotypes. The biochemical phenotype for heterozygous carriers was intermediate, with decreased serum phosphate, TRP%, fibroblast growth factor 23 and intact PTH, but increased serum 1,25-dihydroxyvitamin D and urine calcium excretion, causing idiopathic hypercalciuria in 38%, with bone phenotypes observed in 23% of patients. Some heterozygous NaPi-IIc mutations have been associated with isolated hypercalciuria which may increase the risk of nephrocalcinosis (3).

Patients may initially present with normal laboratory investigations with a mild degree of rickets symptoms, as in Patient 2. These patients, when undergoing periodic evaluation, may develop biochemical evidence of HHRH. A rare case of initially normal biochemical findings, later manifesting as abnormal laboratory values compatible with HHRH was reported by Karakilic-Ozturan et al. (10). These authors described two siblings with a similar condition. The first sibling was a sixteen year old boy with severe deformity of lower limbs and recurrent fractures. His lower limb X-rays did not suggest rickets but his biochemical profile revealed low phosphate, high ALP, and hypercalciuria with low TRP. Renal US suggested bilateral renal calculi. He has underwent genetic analysis, revealing *SLC34A3* homozygous mutation. As his younger sibling had mild genu valgum deformity with bone pain, she underwent biochemical investigation and genetic analysis. In her case, biochemistry revealed phosphate in the lower region of normal with elevated ALP but without hypercalciuria. TRP was normal at that time. There were no X-ray changes suggestive of rickets in her lower limbs. However, her genetic analysis also showed she harbored a homozygous *SLC34A3* mutation.

Due to the expected risk of developing the full clinical spectrum of HHRH she was periodically followed up in clinic. Two and half years after initial presentation, she developed hypophosphatemia and reduced TRP and was started on phosphate supplementation. This was the first case history reflecting the evolution of clinical and biochemical changes over time, despite initial normal biochemical parameters. These authors proposed that the phenotypic variation in HHRH may be due to genetic and epigenetic factors. The clinical sequence and biochemical progression of the younger sibling is very similar to the progression seen in Patient 2 in the present report.

Unfortunately, as a developing country with economic constraints, genetic testing is not available for our patients. Close clinical follow-up and timely re evaluation enabled us to diagnose these two patients with HHRH.

Treatment of HHRH includes phosphate supplementation which is the main stay of treatment. Clinical features of rickets improve rapidly with phosphate supplementation and hypercalciuria resolves as FGF 23 levels increase. Activated vitamin D analogues are contraindicated in HHRH because they compound the effect of excess endogenous 1,25 vitamin D in hypercalciuria. There is a lack of long-term data to determine whether oral phosphate supplementation alone is sufficient to prevent renal calcification and bone loss. The monitoring frequency and biochemical parameters are also not well established (1).

Conclusion

HHRH is due to genetic mutation in *SLC34A3* gene mutation causing variable clinical presentations ranging from completely asymptomatic, isolated hypercalciuria with or without nephrocalcinosis or nephrolithiasis and HHRH causing rickets and related sequelae HHRH is diagnosed by clinical history, examination, biochemical and imaging findings, while specific genetic mutational analyses, where available, are used to confirm the diagnosis. However, HHRH may present a diagnostic challenge due to the phenotypic variability and completely normal initial investigations, as in Patient 2, although findings later progressed to those suggestive of HHRH. This case report highlights the importance of periodic monitoring of biochemical parameters despite completely normal initial biochemistry, if the clinical suspicion is high. These two cases also provide a description of the diagnosis of HHRH using clinical presentation and laboratory parameters in a resource limited setting.

Ethics

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

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Footnotes

Authorship Contributions: Concept: Chathupani Anuradha Wettasinghe, Ishara Minuri Kumarasiri, Navoda Atapattu, Design: Chathupani Anuradha Wettasinghe, Reha Balasubramaniam, Manimel Wadu Akila Nimanthi, Navoda Atapattu, Data Collection or Processing: Chathupani Anuradha Wettasinghe, Mahendralingam Vidushajini, Thabitha Jebaseeli Hoole, Imalka Jayasundara, Navoda Atapattu, Analysis or Interpretation: Chathupani Anuradha Wettasinghe, Ishara Minuri Kumarasiri, Navoda Atapattu, Literature Search: Chathupani Anuradha Wettasinghe, Ishara Minuri Kumarasiri, Mahendralingam Vidushajini, Thabitha Jebaseeli Hoole, Manimel Wadu Akila Nimanthi, Imalka Jayasundara, Reha Balasubramaniam, Navoda Atapattu, Writing: Chathupani Anuradha Wettasinghe, Navoda Atapattu.

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LETTER TO THE EDITOR

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Anorexia Nervosa Emerging after Swyer Syndrome Diagnosis

Demet Aygün Arı[1], Semra Çetinkaya[2]

[1]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, Ankara, Türkiye
[2]University of Health Sciences Türkiye, Ankara Etlik City Hospital, Clinic of Pediatrics, Division of Pediatric Endocrinology, Ankara, Türkiye

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Dear Editor,

Swyer syndrome (46,XY complete gonadal dysgenesis) is a rare disorder of sex development (DSD) characterized by female external genitalia, hypergonadotropic hypogonadism, primary amenorrhea, and increased risk of gonadal malignancy (1,2). Beyond endocrine and oncologic complications, psychosocial difficulties are increasingly recognized in individuals with DSD. We report an adolescent with Swyer syndrome who developed anorexia nervosa during follow-up after diagnosis and treatment.

A 15.5-year-old phenotypic female presented with primary amenorrhea despite spontaneous breast and pubic hair development since age 11 years. Physical examination showed Tanner stage IV breast and pubic hair development with normal female external genitalia. Laboratory evaluation demonstrated hypergonadotropic hypogonadism (follicle-stimulating hormone 138.07 mIU/mL, luteinizing hormone 48.35 mIU/mL, estradiol <12.1 pg/mL). Pelvic imaging revealed absent ovarian tissue and a left adnexal nodular lesion. Karyotype analysis showed 46,XY with positive SRY expression. During the diagnostic process, tumor markers increased (alpha-fetoprotein 5.6 ng/mL, β-hCG 6.6 mIU/mL), raising suspicion for gonadal malignancy. Laparoscopic bilateral gonadectomy was performed, and histopathological examination demonstrated gonadoblastoma in both gonads with coexisting dysgerminoma in the left gonad. Postoperatively, estrogen replacement therapy was initiated and later transitioned to cyclic estrogen-progesterone therapy.

At initial psychosocial assessment, no apparent psychiatric risk factors were identified. However, six months after diagnosis, she had intentionally lost 16 kg over three months through severe caloric restriction. At that time, her weight was 45 kg [-1.94 standard deviation score (SDS); 2.6th percentile], height was 164 cm (0.26 SDS; 60.3rd percentile), and body mass index (BMI) was 16.7 kg/m^2 (-2.63 SDS; 0.4th percentile), corresponding to 78.8% of median BMI. Further psychosocial assessment revealed increasing distress related to her diagnosis, concerns regarding gender identity, and dissatisfaction with physical appearance. She demonstrated distorted body image and intense fear of gaining weight and was diagnosed with anorexia nervosa according to the fifth revision of the Diagnostic and Statistical Manual of Mental Disorders criteria. As she remained medically stable, outpatient management was initiated. Her caloric intake was gradually increased, and she was closely monitored during

Corresponding Author: Demet Aygün Arı, MD, PhD, Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Adolescent Medicine, Ankara, Türkiye
E-mail: demetaygunari@gmail.com **ORCID:** orcid.org/0000-0002-8701-6085
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follow-up. No signs of refeeding syndrome or other medical complications were observed during nutritional rehabilitation. She was also evaluated regularly by psychiatry and dietetics teams as part of multidisciplinary care. During follow-up, she regained a healthy weight and maintained positive body image.

Patients with DSD are known to experience increased rates of psychological distress, including anxiety, depression, stigma, impaired self-esteem, and body dissatisfaction (3,4,5). Current consensus guidelines emphasize psychosocial support as part of multidisciplinary care (4). A large European study reported eating disorders in 11.1% of adults with DSD, particularly among individuals with an XY karyotype raised as females (3). In contrast, studies in adolescents with DSD have focused mainly on anxiety, depressive symptoms, and attention-deficit/hyperactivity disorder, while eating disorders have rarely been discussed (5). To the best of our knowledge, reports of anorexia nervosa in adolescents with Swyer syndrome are scarce (6). Our case highlights that eating disorders may emerge shortly after diagnosis and treatment, even in adolescents without previous psychosocial risk factors.

This report highlights the importance of multidisciplinary management in Swyer syndrome, including routine psychosocial assessment during follow-up. Clinicians caring for adolescents with DSD should remain aware of the potential for eating disorders and body image concerns during the diagnostic and treatment process.

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In the article "*Haliloğlu B, Seven Menevşe T, Güleç Yılmaz S, Akdeniz T, Gürpınar Tosun B, Demircioğlu Turan S, Güran T, Gökdemir Y, Erdem E, Karadağ B, İşbir T, Bereket A. Isolated hypoglycemia in children with cystic fibrosis: role of pancreatic insufficiency and glucagon response. J Clin Res Pediatr Endocrinol. 2026;18(2):233-242*", the previous institutional information of the author named Tuba Akdeniz was added, but the new institutional information was inadvertently omitted by the author.

The corrections made on page 233 are as follows;

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Belma Haliloğlu[1,2], Tuba Seven Menevşe[1], Seda Güleç Yılmaz[2], Tuba Akdeniz[2], Büşra Gürpınar Tosun[1], Serap Demircioğlu Turan[1], Tülay Güran[1], Yasemin Gökdemir[3], Ela Erdem[3], Bülent Karadağ[3], Turgay İşbir[2], Abdullah Bereket[1]

[1]Marmara University Faculty of Medicine, Department of Child Health and Disease, Division of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye

[2]Yeditepe University Faculty of Medicine, Department of Medical Biology, İstanbul, Türkiye

[3]Marmara University Faculty of Medicine, Department of Child Health and Disease, Division of Pediatric Pulmonology, İstanbul, Türkiye

Corrected on page 233

(The corrected sections are indicated in bold)

Belma Haliloğlu[1,2], Tuba Seven Menevşe1, Seda Güleç Yılmaz[2], Tuba Akdeniz[**2,3**], Büşra Gürpınar Tosun[1], Serap Demircioğlu Turan[1], Tülay Güran1, Yasemin Gökdemir[**4**], Ela Erdem[**4**], Bülent Karadağ[**4**], Turgay İşbir[2], Abdullah Bereket[1]

[1]Marmara University Faculty of Medicine, Department of Child Health and Disease, Division of Pediatric Endocrinology and Diabetes, İstanbul, Türkiye

[2]Yeditepe University Faculty of Medicine, Department of Medical Biology, İstanbul, Türkiye

[**3**]**İstanbul Okan University Faculty of Engineering and Natural Sciences, Department of Genetics and Bioengineering, İstanbul,** Türkiye

[**4**]Marmara University Faculty of Medicine, Department of Child Health and Disease, Division of Pediatric Pulmonology, İstanbul, Türkiye