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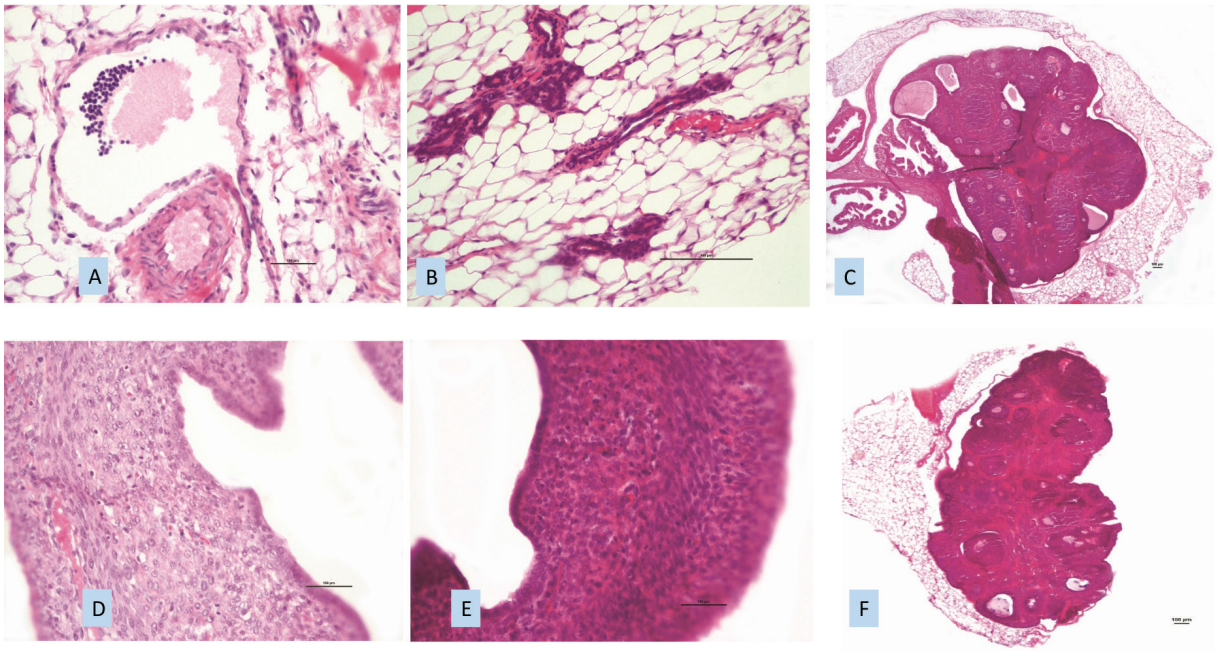

Larger secretory areas (active state) were observed in the adipose tissue of the mammary glands in the propolis group (A) than control group (B). In ovarian tissue, there were more secondary, antral, and corpus luteum follicles in the propolis group (C) than control group (F). The endometrial layer was thicker in the propolis group (E) than control group (D). hematoxylin-eosin pictures. 40x lens, 100 µm scale bar

Effect of Propolis on Precocious Puberty in Female Rats

Polat R et al.

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REVIEW

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Advances in Diagnosis and Management of Childhood Osteoporosis

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Abstract

Childhood osteoporosis leads to increased propensity to fracture, and thus is an important cause of morbidity, pain and healthcare utilisation. Osteoporosis in children may be caused by a primary bone defect or secondary to an underlying medical condition and/or its treatment. Primary osteoporosis is rare, but there is an increasing number of children with risk factors for secondary osteoporosis. Therefore it is imperative that all paediatricians are aware of the diagnostic criteria and baseline investigations for childhood osteoporosis to enable timely referral to a specialist in paediatric bone health. This review will discuss the approach to diagnosis, investigation and management of childhood osteoporosis, with particular consideration to advances in molecular diagnosis of primary bone disorders, and current and emerging therapies for fracture reduction.

Keywords: Childhood, management, osteoporosis

Introduction

Osteoporosis is characterised by low bone mass and microarchitectural deterioration of bone structure, resulting in increased bone fragility and propensity to fracture (1). Importantly, the definition of and treatment options for osteoporosis in children are different to those in adults. Here, we will review the approach to osteoporosis diagnosis and management in children, with particular attention to recent discoveries in the genetic and molecular understanding of bone fragility, natural history of genetic and acquired paediatric bone disorders, recognition of acquired causes of childhood osteoporosis, and development of targeted pharmacotherapy.

Definition and Epidemiology of Childhood Osteoporosis

Fracture in childhood is very common. An estimated one-third of boys and one-fifth of girls will sustain at least one fracture by 18 years old (2). However, osteoporosis in childhood is rare with the exact prevalence unknown. Unlike in adulthood, when osteoporosis and associated fractures have a greater female preponderance due to the post-menopausal decline in bone mass (3), childhood osteoporosis affects both sexes equally.

In adults, osteoporosis is diagnosed solely on bone mineral density (BMD) measured by dual-energy X-ray absorptiometry (DXA) (4). In contrast, the definition of childhood osteoporosis includes both clinical and densitometric criteria. The International Society for Clinical Densitometry defines childhood osteoporosis by the presence of: a) ≥1 vertebral compression fracture in the absence of high-energy trauma or local disease, irrespective of BMD; or b) a clinically significant fracture history accompanied by a DXA BMD Z-score (for age and sex) ≤-2.0 (5). A fracture history is considered clinically significant if there is either ≥2 long bone fractures by 10 years old or ≥3 long bone fractures up to 19 years old (5).

It is, however, recognised that a bone mineral content (BMC) or BMD Z-score of >-2.0 does not rule out the possibility of skeletal fragility and increased fracture risk (5). Furthermore, whilst this strict definition may prevent the overdiagnosis of paediatric osteoporosis given the high rates of fractures during childhood (2), it does not account for the expanding genetic basis of congenital bone fragility and natural history

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of childhood secondary osteoporosis. Clinically relevant bone fragility may still be missed and/or diagnosis delayed whilst waiting until a sufficient number of fractures to fulfil the definition has occurred (6). A more pragmatic diagnostic approach takes into account additional characteristics such as the child's underlying condition, risk factors for fracture, fracture characteristics (site, mechanism and radiographic features), family history and genotype, without overly focusing on a specific BMD Z-score or fracture number (6).

Pathophysiology of Childhood Osteoporosis

Childhood and adolescence is a crucial period to establish a trajectory for lifelong musculoskeletal health. About 95% of skeletal size and bone and muscle mass is achieved by ~18 years of age, with rapid acceleration in bone mineral accrual and muscle mass during the adolescent growth spurt (7). However, peak bone mass is not attained until the mid-late third decade, so approaches to maximising bone mineral accrual should continue into early adulthood.

Bone mass is regulated by modelling (new bone accrual) and remodelling of existing bone, enabled by the co-ordinated action of osteoblasts (which promote new bone tissue formation), osteoclasts (which promote bone tissue resorption) and osteocytes (which regulate activity of osteoclasts and osteoblasts in response to mechanical stimulation, and also promote bone formation) (8). In healthy children, osteoblastic bone deposition dominates osteoclastic bone resorption, resulting in net increase in bone mass. In osteoporosis however, this balance is commonly disrupted resulting in bone mass inadequacy. Signalling between the different cell types involves sophisticated molecular pathways, many of which are dysregulated in genetic causes of bone fragility and represent areas for targeted pharmacotherapy. These key pathways include the:

Receptor Activator of Nuclear Factor Kappa B, RANK Ligand and Osteoprotegerin Pathways

Receptor activator of nuclear factor kappa B (RANK) is expressed on the surface of osteoclast precursors, and RANK ligand (RANKL) is secreted by osteoblasts and osteocytes. The binding of RANKL with RANK stimulates osteoclast differentiation, thereby promoting bone resorption (9). Osteoblasts also express osteoprotegerin (OPG), which acts as a decoy receptor, binding to RANKL and thus blocking RANK-RANKL interaction. The balance of RANKL and OPG therefore determines osteoclast-mediated bone resorption (9). Interleukin (IL)-1, IL-6, tumour necrosis factor and other pro-inflammatory cytokines can trigger this pathway to promote bone resorption and may be implicated in osteoporosis associated with inflammatory conditions (10).

Wingless iNTegration Site Family (Wnt) Signalling Pathway

Wnt proteins are a family of growth factors that bind to membrane receptor complexes, comprising a transmembrane Frizzled G-protein coupled receptor and a low-density lipoprotein receptor (LRP) co-receptor. The Wnt signalling pathway has many roles, including stimulating osteoblast differentiation and inhibiting apoptosis in osteoblast precursor cells. Additionally, Wnt signalling increases the OPG/RANKL ratio to regulate bone resorption. Sclerostin (produced by osteocytes) binds to LRP-5 and LRP-6, inhibiting Wnt signalling (11).

Transforming Growth Factor-β Signalling Pathway

This pathway promotes bone formation by enhancing proliferation and differentiation of mesenchymal precursor cells into osteoblasts. The transforming growth factor-β (TGF-β) superfamily includes members such as TGF-β and bone morphogenic proteins. TGF-β binds to a tetrameric receptor complex at the cell surface, triggering intracellular signalling via the Smad complex or mitogen-activated protein kinase (MAPK) cascade, resulting in cell proliferation, differentiation and migration. Interaction also exists between TGF-β and parathyroid hormone (PTH) and Wnt pathways to promote osteoblast differentiation and bone formation (12).

Primary Osteoporosis

Childhood osteoporosis can be broadly divided into two groups. Primary osteoporosis arises from an intrinsic bone abnormality, usually with an underlying genetic basis or less commonly it is idiopathic. Secondary osteoporosis occurs due to an underlying medical condition and/or its treatment.

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is the commonest cause of primary osteoporosis in children with an incidence of 1:15,000-20,000 births (13). It is regarded as a collagen-related disorder, due to abnormalities not only in collagen structure but also collagen folding, post-translational modification and processing, osteoblast differentiation or bone mineralisation (13,14). Direct defects in type I collagen structure or quantity constitute the majority of OI cases (13). Type I collagen, the major protein in bone, is a triple helix structure comprising two α1-chains and one α2-chain, encoded by the *COL1A1* and *COL1A2* genes respectively. *COL1A1* or *COL1A2* gene mutations account for OI types I-IV.

Clinical manifestations of OI include recurrent fractures, skeletal deformities, short stature, dentinogenesis imperfecta, blue sclerae, ligamentous laxity and hearing loss. Nevertheless, there is wide phenotypic variation depending on the OI type, ranging from mild and almost asymptomatic to very severe and lethal forms (13,14).

The original classification of OI by Sillence in 1979 comprised only four types of OI based on clinical descriptions (15). This has since expanded to include an ever-increasing number of novel subtypes of OI, based on molecular characterisation of defects in genes related to bone metabolism and signalling (13). As of mid-2022, 22 molecular subtypes of OI have been identified according to the Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org/) (Table 1). However, this molecular classification can be confusing in clinical practice, particularly if access to genetic testing is limited, and thus use of the phenotypic descriptions from the original Sillence classification can help to delineate an individual's health needs. This approach is recommended by the Nosology and Classification of Genetic Skeletal Disorders (Table 2) (16).

Other Genetic Causes of Primary Osteoporosis

Genetic mutations in the bone signalling pathways can cause primary osteoporosis. For example, homozygous mutations in the *LRP5* gene, a co-receptor in the Wnt signalling pathway, cause osteoporosis-pseudoglioma syndrome (OPPG), which is characterised by early-onset osteoporosis and vision loss (17). Heterozygous *LRP5* mutations may also cause early-onset osteoporosis (17). Other mediators of the Wnt pathway (e.g. Wnt1, Wnt16, LGR4) are also implicated in osteoporosis (18). Table 1 provides a non-exhaustive list of causes of primary osteoporosis.

Idiopathic Juvenile Osteoporosis

Idiopathic juvenile osteoporosis (IJO) is a diagnosis of exclusion and the underlying pathophysiology is not yet understood, although increasingly children in whom this diagnosis was previously made are being identified to have pathogenic variants in bone signalling pathways (19,20). IJO affects both sexes equally, and manifests insidiously, usually in pre-pubertal children, with back pain, hip and/or lower limb pain, vertebral fractures, long bone fractures and difficulty walking (21). Symptoms may improve during and after puberty, although permanent deformities may occur and long-term outcome is variable (21,22). Low bone turnover may be evident on histomorphometry (22,23).

Secondary Osteoporosis

Secondary osteoporosis develops due to consequences of a disease process and/or its treatment. With advancing medical care and therapies, its prevalence is likely to increase as life expectancy of patients with chronic conditions improves. The commonest causes include chronic systemic inflammatory diseases, malnutrition, conditions related to muscle impairment resulting in immobility, and medications, especially glucocorticoids and some anti-convulsants. For many children with secondary osteoporosis, the cause is multi-factorial.

Chronic Systemic Disease

In children with chronic disease, poor longitudinal bone growth may arise from prolonged inflammation influenced by pro-inflammatory cytokines, and high-dose glucocorticoid therapy (24). The effect on bone mineralisation is complex, including a direct effect of pro-inflammatory cytokines enhancing osteoclast action and inhibiting osteoblast differentiation (25), and the indirect effects of inflammation on downregulation of the growth hormone/insulin-like growth factor-1 (GH/IGF-1) and gonadal axes (24). Delayed puberty, often associated with chronic disease, contributes to diminished bone mineral accrual and higher fracture risk through sex steroid deficiency (26). The impact of anti-inflammatory drugs, including glucocorticoids, and poor nutrition resulting from inflammation-associated anorexia or malabsorptive states (e.g. inflammatory bowel disease) are also important. Children with newly-diagnosed Crohn's disease have low BMD and BMC Z-scores compared with unaffected children (27), highlighting that the underlying disease process and not just glucocorticoids contribute to osteoporosis in this disease model.

Glucocorticoid-induced Osteoporosis

Glucocorticoids remain the mainstay of treatment for numerous inflammatory diseases, such as acute lymphoblastic leukaemia (ALL), nephrotic syndrome, systemic autoimmune conditions and Duchenne muscular dystrophy (DMD). Glucocorticoid-induced osteoporosis (GIO) is the commonest form of secondary osteoporosis in children and adults (28).

The principal effect of glucocorticoid excess on bone is that bone formation is directly impaired through inhibition of osteoblast differentiation and function, and promotion of apoptosis of osteoblasts and osteocytes (28,29). Glucocorticoids also increase RANKL and reduce OPG production, leading to increased bone resorption (30).

Table 1. Causes of primary osteoporosis

Pathology	Condition	Pathogenic mutation	Inheritance
Osteogenesis imperfecta			
Defect in type I collagen structure and processing	OMIM OI types I to IV	*COL1A1, COL1A2*	AD, AR (rare)
Defect in mineralisation	OMIM OI type V	*IFITM5*	AD
	OMIM OI type VI	*SERPINF1*	AR
Defect in collagen modification	OMIM OI type VII	*CRTAP*	AR
	OMIM OI type VIII	*LEPRE1 (P3H1)*	AR
	OMIM OI type IX	*PPIB*	AR
	OMIM OI type XIV	*TMEM38B*	AR
Defect in collagen folding and cross-linking (chaperone defects)	OMIM OI type X	*SERPINH1*	AR
	OMIM OI type XI	*FKBP10*	AR
Impaired osteoblast function and differentiation	OMIM OI type XII	*SP7*	AR
	OMIM OI type XV	*WNT1*	AR
	OMIM OI type XVI	*CREB3L1*	AR
	OMIM OI type XVIII	*TENT5A (FAM46A)*	AR
Defect in regulated intramembrane proteolysis	OMIM OI type XIX	*MBTPS2*	XLR
Defect in collagen processing	OMIM OI type XIII	*BMP1*	AR
	OMIM OI type XVII	*SPARC*	AR
Defect in WNT signalling	OMIM OI type XX	*MESD*	AR
Defect in KDELR2-dependent retrograde Golgi-to-ER transport	OMIM OI type XXI	*KDELR2*	AR
Defect in MAPK signalling	OMIM OI type XXII	*CCDC134*	AR
Others			
Impaired collagen cross-link formation	Bruck syndrome type 1 Bruck syndrome type 2	*FKBP10* *PLOD2*	AR AR
Defect in collagen folding and cross-linking	N/A	*KDELR2*	AR
Defect in WNT signalling	Unnamed/early-onset osteoporosis (non-OI)	*WNT1*	AD
	Osteoporosis pseudoglioma syndrome	*LRP5*	AR
	Unnamed/early-onset osteoporosis (non-OPPG)	*LRP5*	AD
	Unnamed/early-onset osteoporosis	*LRP6*	AD
	Hyper IgE (Job) syndrome	*STAT3*	AD
Defect in TGF-β signalling	Loeys-Dietz syndrome	*TGFBR1, TGFBR2, SMAD3, TGFB2, TGFB3*	AD
Defect in connective tissue	Ehlers-Danlos syndrome	*COL5A1, COL5A2, TNXB, COL3A1*	AD
	Marfan syndrome	*FBN1, TGFBR2*	AD
	Homocystinuria	*CBS*	AR
Defect in osteoclast differentiation	Nasu-Hakola disease	*TYROBP, TREM2*	AR
Bone protein processing disorder	Linkeropathies	*B3GAT3, B4GALT7, B3GALT6*	AR
Impaired catalysis of rearrangement of disulphide bonds	Cole-Carpenter syndrome	*P4HB, CRTAP*	AD
RANK overactivation +/- OPG deficiency	Juvenile Paget's disease	*TNFRS11B*	AR
Impaired bone response to mechanical strain	Bone mineral density quantitative trait locus 18	*PLS*	XLD
Unclear	Cutis laxa with progeroid features	*PYCR1*	AR
Unclear	Geroderma osteodysplasticum	*GORAB*	AR
Unclear	Pseudoachondroplasia	*COMP*	AD
Unclear	RAPADILNO syndrome	*RECQL4*	AR
Unclear	Calvarial doughnut lesions	Unknown	AD
Unclear	Spondylo-ocular syndrome	*XYLT2*	AR
Unclear	Gnathodiaphyseal dysplasia	*ANO5*	AD
Unclear	Mulibrey nanism	*TRIM37*	AR

OMIM: Online Mendelian Inheritance in Man, AD: autosomal dominant, AR: autosomal recessive, XLR: x-linked recessive, XLD: x-linked dominant, OI: osteogenesis imperfecta, OPG: osteoprotegerin, TGF-β: transforming growth factor-β, OPPG: osteoporosis-pseudoglioma syndrome

Sex steroid hormone production may also be inhibited which indirectly impairs bone metabolism (28).

A dose-related increase in fracture incidence and BMD loss occurs with glucocorticoid use in adults (31). A large observational study reported that children who received ≥4 oral corticosteroid courses (average duration of 5 days per course) over a 12 month period for various common childhood illnesses had 1.3 times increased odds of overall fracture risk compared to those who only received non-systemic corticosteroids (32). However the fracture rate was not higher in those who had oral corticosteroids more than 12 months previously, implying long-term recovery of harmful bone effects (32). The use of inhaled corticosteroids in childhood asthma has not been linked to fracture risk (33).

The Canadian STeroid-associated Osteoporosis in the Pediatric Population ("STOPP") Consortium studied glucocorticoid-treated children with different chronic diseases, and found that vertebral fractures were common in GIO, tended to appear early in the treatment course and were often asymptomatic, underscoring the importance of surveillance in this population (34).

Immobility-induced Osteoporosis

Muscle function is important to bone mineral accrual. In children with prolonged immobility [e.g. those with cerebral palsy (CP)], loss of mechanical strain leads to reduced bone tissue strain and consequently reduced bone mass and strength (35). Children with CP have decreased periosteal circumference in their lower extremity bones, giving rise to diminished cortical thickness (36), which increases fracture risk. Thus common fracture sites in this group are the distal femur and proximal tibia (36).

Duchenne Muscle Dystrophy

Children with DMD (and other neuromuscular conditions) have higher risk of osteoporosis. Between 20-60% of boys with DMD have low-trauma extremity fractures, and up to 30% develop symptomatic vertebral fractures (37). Low-trauma vertebral fractures are also common and may be asymptomatic (37). Bone morbidity results from a combination of factors including progressive myopathy leading to immobility with loss of mechanical stimulus on the bone, chronic high-dose glucocorticoid therapy (often about 10 years of exposure by 14 years old), growth failure and pubertal delay (due to steroid-induced hypogonadism) (24,37,38). If left untreated, one vertebral fracture leads to more vertebral fractures (vertebral fracture cascade) (39), causing progressive back pain and spinal deformity. Lower limb fractures may cause earlier loss of ambulation.

Poor Nutrition and Anorexia Nervosa

Conditions associated with malabsorption and poor nutrition, particularly poor absorption of calcium and vitamin D, for example in coeliac disease and inflammatory bowel disease, may result in reduced BMD. In coeliac disease, a gluten-free diet alongside calcium and vitamin D supplementation helps to optimise bone health, although in adults BMD may remain lower than in healthy controls (40). Individuals with coeliac disease may also have co-existing anti-OPG autoantibodies (41), which are expected to increase bone resorption, and thus may further contribute to the aetiology of osteoporosis in this group.

Anorexia nervosa (AN) is characterised by severe undernutrition with associated hypothalamic dysfunction and skeletal disruption (42,43). Functional hypothalamic amenorrhoea is accompanied by low gonadotropin levels and severe oestrogen deficiency. There is an acquired state of high GH with low IGF-1 (i.e. GH resistance), hypercortisolism, and disrupted production of adipokines and appetite-regulating hormones (42,44). Poor bone health in AN is due to body composition alterations (low muscle and bone mass) and these various endocrinopathies. There is diminished bone turnover, bone cortical thickness and volumetric BMD (43). Adolescent girls with AN have higher fracture rates compared to healthy controls (31% versus 19%) (45). The impact of AN on bone health is especially relevant during adolescence, as this is a period of increased bone accrual for attainment of peak bone mass (which predicts future bone health and fracture risk) (44). It is therefore unsurprising that the risk of fracture persists till later life for young women with AN (46).

History and Examination

Assessment of a child with suspected or known osteoporosis should include a targeted medical history and examination (Table 3). Parental recall of their child's fracture history can be inaccurate, so ascertaining radiological confirmation of the fracture is important, where possible (47).

Investigations

Laboratory Tests

The work-up of osteoporosis should be guided by the presenting features and level of suspicion (Table 4). However, osteoporosis may be the presenting feature of coeliac disease, inflammatory conditions and malignancy,

Table 2. Recommended nomenclature of OI syndromes in order of severity (proposed by the Nosology Classification of Genetic Skeletal Disorders)

Name of syndrome	Recommended nomenclature of OI syndrome
Classic non-deforming OI with persistently blue sclerae	OI type 1
Moderate form OI (in adults always normal sclerae)	OI type 4
Progressively deforming OI with normal sclerae	OI type 3
OI with calcification of interosseous membranes and/or hypertrophic callus	OI type 5
Perinatally lethal OI	OI type 2

OI: osteogenesis imperfecta

Table 3. History and examination

History	Examination
- Fracture history (number, location, high/low-impact mechanism, age of occurrence, healing, radiographical confirmation) - Back pain (may indicate vertebral fractures) - Symptoms suggestive of associated disease (e.g inflammatory bowel disease, malabsorption, leukaemia, renal failure) - Growth and puberty (e.g. growth failure, delayed puberty) - Family history (e.g. osteoporosis, fractures, hearing loss, nephrolithiasis) - Diet (e.g. poor nutrition, intake of calcium and protein) - Medications (e.g. steroids) - Physical activity (vigorous, or equally lack of physical activity increases fracture risk)	- Anthropometry including occipitofrontal circumference and body proportions - Sclerae - Teeth - Skin laxity - Joint laxity/ hypermobility - Limb deformities - Scoliosis - Spine tenderness - Pubertal status - Cushingoid features - Thyroid status

Table 4. Laboratory investigations for childhood osteoporosis

Baseline bone metabolism

- Serum calcium
- Phosphate
- PTH
- Magnesium
- Creatinine
- ALP
- GGT
- 25-hydroxyvitamin D
- Full blood count
- Urinary calcium:creatinine ratio (preferably first morning sample)

Assess for cause of secondary osteoporosis

- Erythrocyte sedimentation rate/C-reactive protein (chronic systemic disease)
- Prolactin (hyperprolactinaemia)
- Thyroid function tests (hyperthyroidism)
- Coeliac screen (coeliac disease)
- LH, FSH and testosterone/oestradiol (hypogonadism/pubertal delay)
- IGF-1 (growth hormone deficiency)

Additional investigations to consider

- Store DNA
- Serum homocysteine (homocystinuria)
- 24-hour urinary free cortisol/dexamethasone suppression test (Cushing's syndrome)

ALP: alkaline phosphatase, GGT: gamma glutamyl transferase, PTH: parathyroid hormone, LH: luteinizing hormone, FSH: follicle stimulating hormone, IGF-1: insulin-like growth factor-1

and therefore baseline investigations to assess for these conditions should be considered in all children fulfilling the diagnostic criteria for childhood osteoporosis, if an alternate diagnosis is not evident.

Genetic Investigations

A genetic diagnosis can help to inform management decisions and enable genetic counselling. Currently next generation sequencing (NGS) that includes targeted gene panels, whole-exome sequencing and whole-genome sequencing, are available (19,48,49). As ~90% of all patients with OI possess *COL1A1* or *COL1A2* mutations, some propose screening for these two genes first in children with a suspected genetic aetiology for osteoporosis (50). In one study, NGS panel testing detected pathogenic variants in 35% of children with a clinically significant fracture history, especially in those who had early femoral fracture (48). It should however be recognised that genotype-phenotype correlations can be variable, even within the same family group. Thus, careful consideration should be given to whether cascade screening is appropriate in family members who do not have a clinical history of fracture and for whom, pharmacological management is not presently recommended. If cascade screening is considered, detailed genetic counselling regarding the implications of a pathogenic genetic diagnosis in the absence of clinical symptoms (e.g. the impact on obtaining health insurance) is important. Nevertheless, genetic investigations probably should not be undertaken in asymptomatic children who do not have the capacity to understand these implications, until management options to alter long-term outcomes are available.

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Dual-energy X-ray Absorptiometry

DXA is the preferred technique to measure bone mass, as it is quick to perform, has low radiation exposure and is supported by normative reference data (5). DXA measures BMC (expressed in grams) and the projected area of bone (expressed in cm^2); the areal BMD (aBMD, expressed in grams/cm^2) is then calculated using these values. Raw measurements are converted to age- and sex-specific Z-scores for comparison to the normal population. DXA is usually not performed in children < 5 years old because of movement artefact and lack of age-specific reference data.

The preferred skeletal sites assessed are the anteroposterior lumbar spine and total body less head (TBLH), due to the changing proportional contribution of the skull to whole body bone mass during childhood, and reduced responsivity of the skull to factors that affect BMD at other skeletal sites (5,51). The proximal femur, lateral distal femur and 33% radius may also be used depending on patient-specific circumstances (5), but the requirement for appropriately-trained technicians often limits their usage.

DXA is a two-dimensional measurement (i.e. cannot measure bone depth) resulting in underestimation of BMD in children with short stature, and overestimation in tall children. Various mathematical models are used to account for this, including calculation of bone mineral apparent density (BMAD or volumetric BMD, in g/cm^3) (52) and BMC for height (53). It is important to ensure that the reference database used for these techniques is appropriate to the DXA instrument used. Size adjustment improves the predictive ability of DXA; vertebral fractures are best predicted by lumbar spine BMAD for age and sex, whereas TBLH BMC for lean body mass adjusted for height is superior for long bone fracture prediction (52).

Despite the inclusion of BMD in the definition of childhood osteoporosis, its role remains debatable. A low BMD increases the possibility of osteoporosis, but it is not always diagnostic - BMD can be low for artefactual or non-osteoporotic reasons, may be normal in children with osteoporosis, or even high in sclerosing bone disorders (6). Furthermore, the relationship between BMD and fractures in childhood chronic disease is uncertain (24). Therefore, caution needs to be exercised when interpreting a single low BMD measurement and must be taken in context with the clinical presentation. The trajectory of BMD may be helpful, with a reduction of ≥0.5 SD serving as a threshold to consider further investigations (6,37).

Lateral Spine Radiographs

A lateral thoracolumbar radiograph is currently the gold standard method for detection of vertebral fractures in children (54). It should be employed as an initial screening tool in children at high risk of osteoporosis as vertebral fractures may be asymptomatic, and is also indicated in back pain and in the investigation of suspected osteoporosis in children with multiple long bone fractures.

The Genant semi-quantitative grading system is traditionally used to assess vertebral fractures, although other methods have also been proposed for children (54,55). A ≥20% loss in vertebral height ratio is clinically significant (55).

However, this imaging modality carries high radiation exposure and image quality may be reduced depending on the child's breathing technique and positioning, machine quality, and especially at the T1-T3 vertebral levels where there is visualisation difficulty due to overlying intra-thoracic structures and the patient's shoulders (54).

Vertebral Fracture Assessment

Vertebral fracture assessment (VFA) in DXA software is employed to detect adult vertebral fractures. The newest generation of DXA scanners (e.g. Lunar iDXA scanner) have been shown to be comparable to conventional spine radiography for identifying moderate and severe vertebral fractures in children (56). Advantages of VFA include superior image quality, notably lower radiation dosage than conventional radiography, ability to obtain images simultaneously with bone density measurements, less variability in result interpretation and lower cost. VFA by DXA is thus increasingly favoured as a method to identify vertebral fractures in children and for regular routine screening for asymptomatic vertebral fractures (56,57). Nonetheless, VFA may not possess the spatial resolution of lateral spine radiographs.

Bone Biopsy

Trans-iliac bone biopsy offers detailed qualitative and quantitative information on bone microarchitecture, bone matrix and mineralisation. Dynamic parameters of bone cell function (bone formation and resorption) can also be measured by tetracycline labelling. Bone biopsies allow us to understand histological characteristics and bone metabolic activity, especially when the diagnosis is uncertain or when differentiating types of osteoporosis. For example, low-turnover osteoporosis is demonstrated on bone biopsies performed in patients with *PLS3* and *WNT1* mutations (58). Osteomalacia can be excluded by performing bone biopsies

(59). They should only be performed in highly-specialised centres and research studies, as they are invasive and require general anaesthetic.

Research Techniques

Other bone assessment techniques include quantitative computed tomography (QCT) as low- and high-resolution peripheral QCT and serum/urine bone turnover markers. However, in children they are currently limited to research purposes due to lack of normative data.

Management

Multi-disciplinary Team

A child with osteoporosis should be cared for by a multi-disciplinary team in a specialist centre, comprising a paediatrician with specialist bone expertise, orthopaedic and spinal surgeons, geneticists, physiotherapists, occupational therapists, nurse specialists and psychologists. Other team members include dentists, audiologists, neurosurgeons and a pain management team.

General Measures for Optimisation of Bone Health

Nutrition

Sufficient vitamin D and calcium levels should be maintained through dietary intake and/or supplements, in accordance with current guidelines (60). 25-hydroxyvitamin D levels should be maintained ≥50 nmol/L. Other nutrients (e.g. protein, magnesium, zinc, iron, copper, and vitamins C and K) are also essential in maintaining bone health. Specialist dietetic input may be required for children with poor nutrition or malabsorption.

Exercise and Physical Activity

Exercise tailored to the child's capacity should be encouraged as it promotes anabolic function in the developing skeleton. Children with osteoporosis should be counselled to avoid high-impact repetitive physical activities (e.g. trampolining, gymnastics, horse-riding) that put additional forces on the vertebral column and may cause or exacerbate vertebral fractures, contact sports (e.g. rugby) and sports with high risk of falls (e.g. skiing, ice-skating). Weight-bearing exercises and programmed standing exercises may help to maintain or increase BMD in children with CP (61).

High-frequency, low-amplitude whole body vibration (WBV) may produce anabolic bone effects, either directly through vibrations transmitted to the skeleton, or through indirect neuromuscular effects (62). Many WBV studies report positive bone and muscle outcomes, however results should be interpreted cautiously due to wide variability in study design in many of these studies (63).

Monitoring at-risk Children

In those at risk of osteoporosis, treatment of the underlying medical condition is central to prevention of osteoporosis. A baseline spine radiograph or VFA by DXA should be considered for children with significant osteoporosis risk factors, especially those who will be receiving glucocorticoid therapy for at least three months. Some recommend three months as the threshold given that the earliest incident vertebral fracture observed after starting glucocorticoids is at four months in the paediatric population (64,65). Repeat imaging is then performed at 12 months post-glucocorticoid initiation (the timepoint with the highest rate of vertebral fractures in this cohort) (65). Surveillance with DXA with VFA or lateral spine radiographs should be performed at least every 1-2 years, and if pathological fractures are detected then referral for possible treatment is warranted (24).

In boys with DMD, the UK NorthStar guidance on bone and endocrine monitoring recommends annual BMD screening by DXA, alongside lateral spine imaging or DXA-based VFA (66). Bone protective therapy is considered following a vertebral or a low-trauma long bone fracture. Addressing pubertal delay from long-term glucocorticoid therapy may additionally promote skeletal health (66).

Pharmacological Intervention

A diagnosis of osteoporosis in children does not invariably determine the requirement for immediate pharmacotherapy. A child's skeleton is uniquely programmed to allow spontaneous restoration of bone mass and reshaping of fractured vertebral bodies through bone modelling, especially if insults to bone health are only temporary and there is adequate remaining growth potential (given that bone modelling is a growth-dependent phenomenon) (6).

In the STOPP cohort of children with ALL, over 75% of those with vertebral fractures had spontaneous complete reshaping by six years following ALL diagnosis (67). Gurney et al. (68) reported recovery in BMD Z-scores from adolescence to young adulthood in childhood ALL survivors, again supporting the notion that skeletal recovery can occur following the removal of adverse influences to bone health (i.e. discontinuation of glucocorticoids, increased physical activity, improved nutrition, less cytokine activation, improved linear growth) (24). In contrast, the bone health insults in DMD are so pervasive that vertebral body reshaping

or improvements in BMD without pharmacological intervention have yet to be described (6). The disparity in bone health outcomes between these two conditions illustrates the need to consider the reversibility of osteoporosis risk factors and the remaining growth potential when deciding whether to initiate pharmacological intervention. Earlier treatment may be considered in adolescents compared to young children, as adolescents have more limited potential for natural vertebral body reshaping than younger children. Children with primary osteoporosis are also likelier to benefit from early pharmacological intervention due to long-term persistence of the underlying bone defect. As with all treatments, the decision to initiate treatment should follow a discussion with the child and family, and consideration of benefits and risks of therapy, including the need for frequent intravenous cannulation and hospital visits.

Anti-resorptive Agents

Bisphosphonates

Bisphosphonates are presently the sole recommended medical treatment for childhood primary and secondary osteoporosis, although there is less evidence to advocate their use in secondary osteoporosis due to wide variation in pathology, outcome measures and pharmacological regimes (64). It is postulated that low-bone turnover conditions (e.g. immobility-induced or GIO) are less responsive to osteoclast-targeting bisphosphonates, compared to high bone-turnover conditions (e.g. OI or ALL). For example, in children with OPPG (characterised by impaired bone formation resulting in low bone turnover), although bisphosphonates produced an increase in aBMD, several of these children later suffered fractures even with improvement in DXA Z-scores (69).

Bisphosphonates inactivate osteoclast activity, causing suppression of bone turnover. They also have a positive effect on bone formation despite reduced overall bone remodelling (70). Additionally, bisphosphonates prevent osteoblast and osteocyte apoptosis (71). There is evidence to demonstrate that bisphosphonates improve bone mass acquisition and reduce fracture incidence in some forms of primary and secondary osteoporosis in children (70).

Various bisphosphonate preparations are available, but there is no consensus about the ideal drug, frequency, dose, or duration of therapy. Originally pamidronate was given as 0.5-1 mg/kg/day over three days three-monthly, however regimens with lower and shorter doses have since been developed (72). Zoledronic acid (zoledronate) is now increasingly used. It is effective in the management of OI and other forms of primary osteoporosis and secondary osteoporosis (72,73). Compared to pamidronate, zoledronate is more potent, cheaper, and requires a shorter infusion time and less frequent administration. It is as effective as pamidronate in improving lumbar spine BMD Z-scores and fracture rates in OI (74). In children with GIO, a recent trial demonstrated significant improvement in lumbar spine BMD Z-scores with zoledronate compared to placebo (75).

Although oral bisphosphonates (e.g. risedronate, alendronate) are commonly used for adult osteoporosis, data in children is less clear. Risedronate is the most potent oral bisphosphonate. In children with OI, compared to placebo, risedronate improves lumbar spine BMD, but it is less effective in vertebral body reshaping and its value in reducing fracture risk is less consistent (76,77). Currently, oral bisphosphonates should be reserved for children with less severe forms of OI and no vertebral fractures, or when the intravenous route is unsuitable (78). Oral bisphosphonates may cause significant gastrointestinal side effects.

Side effects of bisphosphonates are well-recognised, and patients and families should be counselled on these. An acute phase reaction typified by flu-like symptoms occurs in most patients within 72 hours of administration of the first dose, and anecdotally is often more severe in those with secondary osteoporosis (79). These symptoms usually respond to paracetamol, non-steroidal anti-inflammatory drugs and anti-emetics. Reducing the initial dose by half may reduce these effects (80). Additional stress-dose steroids should be routinely considered for patients on regular glucocorticoids. Similar reactions occur less frequently in subsequent doses. Transient hypocalcaemia is frequently observed in the first week following bisphosphonate infusion. Ensuring normocalcaemia and adequate vitamin D status prior to the infusion, together with calcium supplementation in the immediate post-infusion period, reduce the risk of symptomatic hypocalcaemia.

The long-term effects of bisphosphonate treatment in children are uncertain. Hypothetically the continuous anti-resorptive action of bisphosphonates arrests bone remodelling, resulting in delayed bone repair and healing following fractures or osteotomies. However, there is evidence demonstrating normal fracture healing time with only slightly delayed osteotomy healing after bisphosphonate treatment (81) which may be improved with advancements in medical and surgical management (82). In bisphosphonate-treated adults, chronic bone turnover suppression may rarely cause osteonecrosis of the jaw (ONJ) and atypical femoral fractures (AFFs). However, no paediatric cases of ONJ have been reported in the literature to date (83). The risk of AFFs is rare in children, and some experts debate that fractures with atypical features mimicking AFFs are simply due to the underlying bone fragility in children with OI (84).

The ideal duration of bisphosphonate therapy in children is also unclear. For high-risk patients with irreversible osteoporosis risk factors, continuation of treatment until final height is attained, with a period of active treatment followed by a lower maintenance dose may be beneficial (64,85). This typically amounts to at least two years, which is the period at which maximal benefit from bisphosphonates has been reported in children with OI (86). In children with transient osteoporosis risk factors, bisphosphonates may be discontinued if there have been an improvement in bone assessment, no new fractures in the preceding 12 months and risk factors eliminated (64). Data suggests that gains in bone mass during bisphosphonate therapy are preserved for at least two years after discontinuation (87). Effects of discontinuation are more marked in growing patients than in those who have attained final height, again supporting the value of continuing therapy as long as linear growth persists (87), at least for high-risk children.

Currently, bisphosphonate use is only recommended after fractures have occurred, despite the recognised high fracture risk in certain medical conditions, such as DMD. Srinivasan et al. (88) showed that prophylactic oral risedronate in 52 boys with glucocorticoid-treated DMD was well-tolerated, stabilised lumbar spine BMD Z-scores and reduced vertebral fracture rate. On the other hand, in childhood ALL, a systematic review reported that the true advantages of bisphosphonates on BMD is inconclusive, and there was inadequate evidence to advocate routine prophylactic use (89). Indeed, the potential risks of long-term bisphosphonate use must be weighed against the benefits, and further understanding of the natural history and fracture prediction in various disease cohorts is required before such an approach can be recommended.

Denosumab

Denosumab is a human monoclonal antibody against RANKL, inhibiting osteoclast activity and hence bone resorption (90). While its effects in adults are recognised, outcomes in children are not well-described. A small number of reports describe its use in paediatric giant cell tumours, fibrous dysplasia, DMD, Paget's disease and OI (90). It offers the advantages of subcutaneous administration, increased potency and quick clearance. Preliminary trial results show that in ten children with OI, denosumab significantly increased lumbar spine aBMD, comparable to bisphosphonate therapy (91). In children with OI type VI, traditionally poorly responsive to bisphosphonates, denosumab reduced bone resorption markers, improved vertebral shape and reduced fracture rate (92). However, a significant rebound increase in bone turnover following denosumab discontinuation has led to severe hypercalcemia in several children requiring hospitalisation (93,94). Bisphosphonates have been proposed as a potential solution for use in conjunction with denosumab to prevent this complication, but more studies are needed to investigate this (95).

Anabolic Agents

Sex Hormone Therapy

Chronic systemic illnesses are commonly associated with delayed puberty, especially in those on long-term glucocorticoids, and pubertal induction should be considered if age-appropriate. Testosterone therapy in boys with delayed puberty may result in BMD increase. In boys with DMD and delayed puberty, testosterone pubertal induction increased lumbar spine BMD and improved muscle function (96). Oxandrolone may be preferred to testosterone to increase BMD, although it is not routinely used for pubertal induction and existing evidence is based on use in children with severe burns (97). As a non-aromatisable testosterone analogue, oxandrolone prevents conversion to oestrogen, which may cause premature epiphyseal closure and affect final adult height.

For girls, in the context of hypothalamic amenorrhoea (e.g. in AN and exercise-related amenorrhoea), the role of oestrogen replacement to improve BMD or reduce fracture risk is less clear. In typical AN, oral oestrogen-progesterone monotherapy has not been shown to improve BMD (98). Conversely, low-dose oestrogen oral contraceptive and dehydroepiandrosterone may improve bone strength and BMD in older adolescents with AN (99). Another study demonstrated that physiological oestrogen replacement increased BMD in girls with AN (100). Ultimately the best way to improve BMD is by regaining weight and restoring menstruation (44,64). In adolescent girl athletes, the "female athlete triad" (inter-relationship of reduced energy availability, menstrual irregularity and low bone density) is a salient concept (101). Again, mixed data exists regarding the efficacy of hormonal interventions in improving bone health (102). Oral contraceptives in amenorrhoeic athletes ≥16 years old may be considered if BMD is falling even after adequate weight gain, as recommended by the American College of Sports Medicine (103).

Parathyroid Hormone (Teriparatide)

Teriparatide is synthetic PTH which promotes bone formation by stimulating osteoblastogenesis and inhibiting osteoblast apoptosis (104). In adults with osteoporosis,

teriparatide improves BMD and reduces fracture risk (105). Teriparatide has not been used previously in children with osteoporosis with open epiphyses, due to concerns of the potential risk of osteosarcoma based on animal studies (106). In late 2020, the US FDA removed both the black box warning of osteosarcoma risk and dosing limitation to 24 months of use, following a conclusion that the osteosarcoma risk was only confined to animal studies. With this update, teriparatide is a promising treatment option and trials in children with osteoporosis are likely to occur soon.

Growth Hormone

GH increases bone cortical thickness and improve muscle mass (107). In children, it acts on the growth plate cartilage, leading to endochondral bone formation and longitudinal growth (104). GH treatment in children with GH deficiency (GHD) increases BMC and bone strength through bone geometry changes rather than BMD (108). GH therapy has been evaluated in non-GHD childhood osteoporosis. It resulted only in modest improvement in bone outcomes in OI type IV but not type III (109). A systematic review of children with juvenile idiopathic arthritis reported largely positive muscle and bone effects of GH therapy (110). Overall evidence of GH as an anabolic therapy for non-GHD childhood osteoporosis remains weak.

Wnt Pathway Inhibitors

Blosozumab and romosozumab are humanised monoclonal antibodies against Sclerostin, an antagonist of Wnt signalling. Romosozumab has been most widely studied. In post-menopausal adults, it resulted in significant BMD improvements and reduction in fracture risk compared to bisphosphonates (111). Similarly in women with post-menopausal osteoporosis, in the only trial of blosozumab, BMD increased compared with placebo, but following treatment discontinuation BMD declined (112,113). A phase 2a trial of another anti-sclerostin antibody (setrusumab) in adults with moderate OI showed improvement in BMD and bone formation, and reduction in bone resorption (114). A phase 1 trial on romosozumab in children with OI commenced in 2021 and is ongoing (NCT04545554).

Dickkopf-1 (Dkk1) blocks Wnt/β-catenin signalling in osteoblasts, inhibiting osteoblast development and activity (115). In animal studies, the anti-Dkk1 monoclonal antibody accelerates bone formation and increases BMD (116), but human studies are awaited.

Anti-TGF-β Therapy

In mouse models of OI, anti-TGF-β antibodies increased bone mass (117). The anti-TGF-β antibody Fresolimumab is presently undergoing clinical trials in children with OI (118).

Losartan, an angiotensin II type 1 receptor blocker may also reduce TGF-β signalling (119). Losartan increased bone mass and accelerated chondrocyte hypertrophy in the growth plate during skeletal development in mice (120). Clinical trials of losartan in children with OI are in development.

Conclusion

Childhood osteoporosis is an important cause of morbidity and healthcare expenditure. Although rare, there is accumulating evidence of groups of children at risk of secondary osteoporosis, in whom a high degree of suspicion needs to be exercised. Early detection with robust monitoring strategies and timely intervention are paramount.

Novel drug therapies born out of advances in genetic and molecular understanding of bone physiology hold promise for the future treatment of childhood osteoporosis. More studies are needed to clarify the role of existing pharmacological therapies, such as bisphosphonates, in the primary prevention of fractures. Studies of therapies for secondary osteoporosis in children remain limited and more are required.

Ethics

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Justin H. Davies, Design: Justin H. Davies, David B.N. Lim, Data Collection or Processing: Justin H. Davies, David B.N. Lim, Analysis or Interpretation: Justin H. Davies, Rebecca J. Moon, David B.N. Lim, Literature Search: Justin H. Davies, Rebecca J. Moon, Writing: Rebecca J. Moon, Justin H. Davies, David B.N. Lim.

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Evaluation of Glucose Metabolism and Cardiovascular Risk Factors in Prepubertal Girls with Premature Pubarche

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What is already known on this topic?

Isolated premature pubarche (PP) is regarded as a warning sign of intrauterine-programmed metabolic syndrome.

What this study adds?

PP is not a risk factor alone for impaired glucose metabolism and insulin resistance in non-obese girls with normal birth weight before puberty.

Abstract

Objective: Premature pubarche (PP) is a risk factor for metabolic syndrome (MS). The aim was to evaluate if glucose-insulin metabolism, cardiovascular risk factors, familial cardiovascular risk factors (FCVRF) created a risk for insulin resistance (IR) and if PP was a risk factor alone for MS in normal weight prepubertal girls with PP.

Methods: Thirty-five prepubertal, non-obese girls with PP with normal birth weight and 35 age-matched control girls were evaluated for FCVRF, anthropometric measurements, blood pressure, lipid profile, fasting blood glucose-insulin, hemoglobin A1c (HbA1c), sex hormone binding globulin (SHBG), leptin, adiponectin, tumor necrosis factor-alpha (TNF-α), androgen levels, and bone age. Oral glucose tolerance test was performed in PP participants. Homeostasis model of assessment of IR (HOMA-IR), fasting glucose/insulin ratio, atherogenic index (AI), and free androgen index (FAI) were calculated. PP participants were further stratified by FCVRF.

Results: HbA1c, lipid profile, testosterone, leptin, adiponectin, TNF-α, HOMA-IR, glucose/insulin ratio, AI, and fasting glucose-insulin levels were similar. In the PP group FAI was significantly higher ($p = 0.001$), whereas SHBG was significantly lower ($p = 0.010$) than the control group. Leptin levels of FCVRF + and FCVRF- subgroups were 15.2 ± 9.1 and 9.7 ± 7.2 ng/mL, respectively and the difference was significant ($p = 0.016$).

Conclusion: As PP does not appear to be a risk factor alone for impaired glucose metabolism and IR in prepubertal non-obese girls with normal birth weight, it is our opinion that it is unnecessary to examine in detail such cases before puberty. Low SHBG levels in the PP group and high leptin levels in FCVRF + subgroup might suggest that these may be predictive for MS in the future.

Keywords: Premature pubarche, prepubertal, leptin, glucose metabolism, adipocytokines, cardiovascular risk

Introduction

Puberty as a result of adrenal gland activity, known as adrenarche, is characterized by an increase in circulating androgen precursors, dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S). Adrenarche is usually accompanied by pubarche; genital and/or axillary hair. If this situation occurs before the age of eight years in girls and before the age of nine years in boys, it is called, "premature pubarche" (PP)' (1). The incidence of PP, depending on societal and racial factors, varies between 5-8.6 % (2). In a study conducted in Turkey, the incidence of PP was reported to be 4.6 % (3). The female/male ratio in PP is approximately

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10/1 (4). Prematurity, intrauterine growth retardation, being overweight or obese predispose to PP (1,2,3,4,5,6).

Even though isolated (normal variant) PP is a benign condition, PP is currently accepted as a warning sign for intrauterine-programmed metabolic syndrome (MS), especially in obese children with a diagnosis of PP. Early diagnosis of MS and its components is important for detection, prevention, and early treatment because patients with MS are at risk of type 2 diabetes mellitus and cardiovascular diseases (5,7,8,9).

It has been shown that isolated PP predisposes to hyperinsulinemia and insulin resistance (IR), not only in obese children, but also in non-obese children. In these cases, low sex hormone binding globulin (SHBG) levels have been shown to be a useful marker for detecting IR (5). It has also been reported that the risk of MS is increased within this group and increased tumor necrosis factor-α (TNF-α) levels may be a predictive marker for MS (10). When compared to healthy controls, SHBG and insulin-like growth factor binding protein-1 have been found to be lower, whereas insulin-like growth factor-1 (IGF-1), TNF-α, adiponectin and insulin levels have been found to be higher (10,11). It is speculated that in these cases, hyperinsulinemia also causes dyslipidemia, and as a result, at an early age, cardiovascular risk begins to increase (5). Family history of type 2 diabetes mellitus and cardiovascular disease has been reported to be more frequent in girls with PP (12). In the light of this evidence, the aim of this study was to evaluate if glucose and insulin metabolism, cardiovascular risk factors, and familial cardiovascular risk factors (FCVRF) are associated with the risk of IR in normal-weight, prepubertal PP girls.

Methods

The patient group consisted of non-obese girls having no breast development and birth weights appropriate for gestational age (AGA), who were in follow-up with the diagnosis of PP from the outpatient clinic of Trakya University Medical Faculty Hospital, Department of Pediatric Endocrinology. Patients with breast development, chronic illnesses, any kind of drug use, congenital adrenal hyperplasia or virilizing tumor were excluded. An equal number of girls, born AGA, and who were non-obese and prepubertal were included as the control group. The patients and the families of the participants were informed about the study and signed informed consent forms were obtained from the mother or father of the participant and the child before they were recruited to the study. The study was conducted after approval was given by the Ethics Committee of Trakya University Faculty of Medicine (TÜTF-TÜBAPK date: 11.06.2013, approval no: 121).

Participants' data, including birth date, gestational age, birth weight, current height and weight measurements, waist-hip circumference measurements, and blood pressure (BP) measurements, and pubertal examination based on Tanner staging, were obtained and recorded. The presence or absence of cardiovascular risk factors, including type 2 diabetes mellitus, polycystic ovary syndrome, MS, dyslipidemia and familial cardiovascular disease (premature atherothrombotic cardiovascular disease, occurring in a first-degree male relative before 55 years of age or in a first-degree female relative before 65 years of age) were recorded (13). Height, weight measurements, and the body mass index (BMI) values and their standard deviation (SD) scores (SDS) were calculated using reference values for Turkish children (14).

Two mL of blood were drawn from all participants at 08.30 in the morning after 10 hours of fasting, and DHEA-S, androstenedione (AS), 17-hydroxyprogesterone (17-OHP), total testosterone (T), lipid profile [high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol, triglyceride (TG)], hemoglobin A1c (HbA1c), SHBG, TNF-α, leptin and adiponectin levels were measured. Free androgen index (FAI), defined as total T/SHBG, and atherogenic index (AI) defined as TG/HDL, were calculated. Oral glucose tolerance test (OGTT) (with 1.75 g/kg glucose, maximum of 75 g glucose) was performed in participants with PP, again at 8.30 in the morning after 10 hours of fasting and blood glucose and insulin values were obtained at baseline (0 minutes) and two hours (120 minutes). From the OGTT results, patients with hyperinsulinemia (those with fasting insulin >15 mIU/mL and/or 120th minute insulin level >75 mIU/mL) and IR [homeostasis model of assessment (HOMA-IR) >2.5] were identified. HOMA-IR was calculated using the standard formula: fasting blood glucose (mmol/L) x 18 x insulin (mIU/L)/22.5 and/or fasting glucose/insulin ratio <7 (15). For the determination of bone age (BA), a radiographic examination of the left wrist was completed for each participant, and BA was assessed by comparison with the Greulich-Pyle radiological atlas. BA SDS were calculated using the programme BoneXpert V3.1 (16). The study group was divided into two subgroups based on the presence (FCVRF+) or absence (FCVRF-) of FCVRF.

Blood glucose, LDL, HDL, TG, and total cholesterol levels were measured by spectrophotometry on an Advia 1800 device; insulin, DHEA-S, and T levels were measured by microparticle chemical immunoassay on an Abbott Architect i2000SR and leptin, adiponectin, and TNF-α levels were measured by immunoassay, also on the Architect i2000SR. SHBG and

AS levels were measured by immunoassay on the Siemens Immulite 2000 (Siemens Healthcare Diagnostics Inc. Flanders, HbA1c level was determined by high performance ion-exchange liquid chromatography using Adams HA-8180V analyzer (Arkray Factory Inc., Shiga, Japan) and 17-OHP levels were measured by radioimmunoassay on the gamma counter DPC Gambyt CR (DPC Diagnostic Products Corporation, Los Angeles, CA, USA) at the department of nuclear medicine. Left hand wrist radiographs were taken with the Philips Optimus Bucky Diagnost TS device.

Statistical Analysis

Normality of the distribution of the parameters was analyzed with Shapiro-Wilk test. Logarithmic or square root transformations were applied to non-normally distributed parameters to obtain normal distribution. Independent samples t-test was used for two group comparisons. All the results except for BA SDS were expressed as mean ± SD also for the data before the transformation was applied for easy understanding clinically. BA SDS data was expressed as median (minimum-maximum) because of normal distribution could not be obtained. Mann-Whitney U test was used for the comparison of BA SDS between two groups. All statistical analysis were performed by SPSS 20 statistical software (IBM Corp., Armonk, NY, USA). The value $p < 0.05$ was accepted as statistically significant.

Results

There were 35 patients in the PP group and 35 healthy girls in the control group. The mean ages of the PP and control groups at the time of the study were 8.3 ± 1.1 and 8.1 ± 1 years, respectively. Birth weights, gestational weeks, presence of FCVRFs, weight SDSs, height SDSs, waist/hip ratios, BA SDSs and BP values of the groups were similar. Although, the mean BMI SDS values were within normal ranges in both groups, they were significantly higher in the PP group than in the control group (Table 1).

There was no difference between the groups in terms of HbA1c level, lipid profiles, HOMA-IR scores, glucose/insulin ratios and AIs. In the PP group, serum DHEA-S, AS, and 17-OHP levels were significantly higher, whereas T levels were similar. Serum leptin, adiponectin, and TNF-α levels were similar in both groups. Fasting and 120th minute glucose and insulin levels of the PP participants and the fasting glucose and insulin levels of the control group participants were within normal limits, and there was no significant difference between the groups in terms of fasting glucose and insulin levels. FAI was significantly higher and SHBG was significantly lower in the PP group. Laboratory findings of the groups are shown in Table 2.

When the PP group was divided by the presence of FCVRF, the clinical and laboratory findings, except for leptin levels, were similar. The FCVRF+ PP group had significantly lower leptin levels than in FCVRP- PP group ($p = 0.016$). Comparisons of the clinical and laboratory findings of the PP sub-groups, stratified by the presence of FCVRF, are shown in Tables 3 and 4.

Discussion

Regardless of age and developmental stage, nutritional status is a physiological regulator of adrenarche. Starting from the age of five, an increase in fat tissue, insulin and IGF-1 levels associated with an increase in BMI enhances the expression of steroidogenic enzymes and the production of androgens in adrenocortical cells (17). Although, weight SDS, height SDS, and BMI SDS were within normal limits in our study, as previously reported (18,19), BMI SDS was found to be significantly higher in the group with PP compared to the control group. In contrast to an earlier study by Ibáñez et al. (20) conducted on participants with similar BMIs, in the present study mean waist/hip ratio was within normal limits and was similar between the groups. However, in the Ibáñez et al. (20) study, in which waist/hip ratio was increased their patients also had and hyperinsulinemia associated with an increase in central adiposity, whereas in our cohort the insulin levels of the PP patients were within normal limits.

The BA of young children normally averages within 33% of chronological age. BA advancement is early evidence of a hyperandrogenic condition, unless it is very mild or of very recent onset. However, BA may be normal early in the course of rapid virilization (21). In our study, BA SDS was similar in both groups, consistent with some literature (12,18) and it was within normal limits for age and pubertal stage. As suggested by Sopher et al. (22), in our study when BMI was within normal limits, PP alone does not appear to be a factor that increased BA, despite hyperandrogenemia.

Systolic and diastolic BP values are components of the MS criteria and are significant in cases with PP. In some studies, in which PP and healthy control participants were compared (10,18,19,23,24), blood pressure values were found to be within normal limits, similar to our study. Based on this evidence, it has been suggested that PP alone does not affect BP, if there is no increase in fat tissue, resulting in obesity or overweight in prepubertal period.

In the PP group, androgen precursors other than for T were significantly higher than in the control group. However, TNF-α, leptin, and adiponectin levels, all adipocytokines, were found to be similar in both groups. When glucose metabolism, another component of the MS, was evaluated

Table 1. Clinical findings of PP and control cases

Clinical findings	PP (n = 35)	Control (n = 35)	p
	Mean ± SD	**Mean ± SD**	
Birth weight (g)	3230 ± 405.9	3290.2 ± 371.7	NS
Gestation week (week)	39.4 ± 0.9	39.4 ± 0.8	NS
Family	**n (%)**	**n (%)**	
FCVRF +	16 (45.7)	14 (40)	NS
FCVRF-	19 (54.3)	21 (60)	NS
At the time of study	**Mean ± SD**	**Mean ± SD**	
Age (year)	8.3 ± 1.1	8.1 ± 1	NS
Weight SDS	0.56 ± 0.97	0.15 ± 0.95	NS
Height SDS	0.92 ± 1.11	0.65 ± 1.23	NS
BMI SDS	0.34 ± 0.83	-0.04 ± 0.78	**0.026**
Waist/Hip ratio	0.86 ± 0.04	0.85 ± 0.04	NS
Systolic BP (mmHg)	93 ± 3.47	93.14 ± 3.44	NS
Diastolic BP (mmHg)	63.29 ± 4.36	63.71 ± 3.5	NS
	Median (minimum-maximum)	**Median (minimum-maximum)**	
BA SDS	-0.40 (-2.5-2.1)	-0.90 (-3.8-2.3)	NS

PP: premature pubarche, SDS: standard deviation score, FCVRF: familial cardiovascular risk factor, BMI: body mass index, BA: bone age, BP: blood pressure, NS: not significant

Table 2. Laboratory findings of PP and control cases

Laboratory findings	PP (n = 35)	Control (n = 35)	p
	Mean ± SD	**Mean ± SD**	
Adipocytokine and adipokines			
TNF-α (pg/mL)	1.95 ± 0.84	1.81 ± 0.93	NS
Leptin (ng/mL)	12.27 ± 8.53	13.33 ± 13.03	NS
Adiponectin (µg/mL)	14.06 ± 5.42	15.81 ± 11.69	NS
Fasting lipid profile (mg/dL)			
Cholesterol	152.3 ± 26.6	155.4 ± 29.4	NS
Triglyceride	65.8 ± 31	67.7 ± 27.6	NS
LDL	90.2 ± 25.8	93 ± 22.8	NS
HDL	58 ± 12.4	57.9 ± 11.3	NS
OGTT			
Glucose (mg/dL)			
0.min	79.7 ± 8.2	80.6 ± 7.6	NS
120.min	97 ± 15	-	-
Insulin (µU/mL)			
0.min	6.6 ± 2.9	7.3 ± 4.1	NS
120.min	31.6 ± 18	-	-
HbA1c (%)	5.1 ± 0.3	5.1 ± 0.2	NS
HOMA-IR	1.2 ± 0.6	1.4 ± 0.8	NS
Fasting glucose/insulin ratio	15.2 ± 8.6	14.7 ± 8.8	NS
SHBG (nmol/L)	52.9 ± 24.2	72.9 ± 38.2	**0.010**
AI	1.2 ± 0.69	1.22 ± 0.62	NS
FAI	1.15 ± 0.62	0.85 ± 0.85	**0.001**

PP: premature pubarche, SDS: standard deviation score, TNF-α: tumor necrotizing factor-α, LDL: low density lipoprotein, HDL: high density lipoprotein, OGTT: oral glucose tolarence test, HbA1c: hemoglobin A1c, HOMA-IR: homeostasis model of assessment-insulin resistance, SHBG: sex homone binding globulin, AI: atherogenic indeks, FAI: free androgen index, NS: not significant

in PP participants, in many studies fasting glucose values (24,25,26,27,28), fasting insulin values, glucose/insulin ratio, HOMA-IR, postprandial glucose-insulin values (12,25,29,30) and HbA1c values (19) were within normal limits, and we obtained similar results. Liimatta et al. (31) evaluated patients diagnosed with PP twice, both at an average age of seven years and at an average age of 18 years. These authors showed that the factor affecting glucose metabolism was not the risk factors present in the prepubertal period, but the adipose tissue present in the pubertal stage. However, in some studies, fasting insulin levels and HOMA-IR were reported to be high and glucose/insulin ratio was reported to

Table 3. Comparison of clinical findings of the PP cases regarding the presence of FCVRF

Clinical findings	FCVRF + (n = 16) Mean ± SD	FCVRF- (n = 19) Mean ± SD	p
Birth weight (g)	3300 ± 445.5	3171 ± 371.1	0.357
Gestation week (week)	39.5 ± 0.8	39.3 ± 1	0.541
Diagnosis age (year)	7.2 ± 0.7	7.1 ± 0.9	0.817
At the time of the study			
Age (year)	8.3 ± 1.1	8.3 ± 1.1	0.960
Weight SDS	0.9 ± 0.81	0.28 ± 1.02	0.069
Height SDS	1.3 ± 1.22	0.59 ± 0.92	0.085
BMI SDS	0.61 ± 0.5	0.12 ± 0.99	0.159
BA SDS	0.15 ± 1.24	-0.52 ± 1.33	0.123
Waist/hip ratio	0.87 ± 0.48	0.86 ± 0.03	0.228
Systolic BP (mmHg)	92.8 ± 3.6	93.1 ± 3.4	0.774
Diastolic BP (mmHg)	62.5 ± 4.4	63.9 ± 4.2	0.336

SDS: standard deviation score, FCVRF: familial cardiovascular risk factor, BMI: body mass index, BA: bone age, BP: blood pressure, PP: premature pubarche

Table 4. Comparison of laboratory findings of the PP cases regarding the presence of FCVRF

Laboratory findings	FCVRF + (n = 16) Mean ± SD	FCVRF- (n = 19) Mean ± SD	p
DHEA-S (µg/dL)	124.9 ± 54.2	109.6 ± 48.4	0.329
17-OHP (ng/mL)	0.81 ± 0.3	0.96 ± 0.28	0.120
AS (ng/mL)	0.75 ± 0.37	0.7 ± 0.41	0.733
T (ng/dL)	16.3 ± 8.5	14.2 ± 3.3	0.341
SHBG (nmol/L)	47.7 ± 16.6	57.4 ± 28.8	0.312
TNF-α (pg/mL)	2.07 ± 0.78	1.84 ± 0.9	0.415
Leptin (ng/mL)	15.2 ± 9.1	9.7 ± 7.2	**0.016**
Adiponectin (µg/mL)	13.4 ± 5	14.5 ± 5.7	0.596
Cholesterol (mg/dL)	154.7 ± 26.8	150.3 ± 26.9	0.635
Triglyceride (mg/dL)	71.8 ± 35	60.6 ± 27.2	0.267
LDL (mg/dL)	97.7 ± 27.1	83.9 ± 23.6	0.117
HDL (mg/dL)	54.8 ± 11.5	60.6 ± 12.9	0.174
Fasting glucose (mg/dL)	79.6 ± 9.5	79.8 ± 7.3	0.765
120. min blood glucose (mg/dL)	96.5 ± 14.4	97.4 ± 15.9	0.778
Fasting insulin (µU/mL)	7.1 ± 3.3	6.1 ± 2.6	0.312
120. min blood insulin (µU/mL)	32.4 ± 14.8	30.9 ± 20.8	0.371
HbA1c (%)	5 ± 0.2	5.1 ± 0.3	0.435
HOMA-IR	1.39 ± 0.72	1.21 ± 0.58	0.508
Fasting glucose/insulin ratio	14.7 ± 9.9	15.7 ± 7.5	0.312
AI	1.38 ± 0.82	1.05 ± 0.53	0.208
FAI	1.34 ± 0.77	0.99 ± 0.41	0.085

SD: standard deviation, FCVRF: familial cardiovascular risk factor, DHEA-S: dehydroepiandrosterone-sulphate, 17-OHP: 17-hydroxyprogesterone, AS: androstenodione, T: total testosterone, SHBG: sex homone binding globulin, TNF-α: tumor necrotizing factor-α, LDL: low density lipoprotein, HDL: high density lipoprotein, HbA1c: hemoglobin A1c, HOMA-IR: homeostasis model of assessment-insulin resistance, AI: atherogenic indeks, FAI: free androgen index, PP: premature pubarche

be low in children with PP (26,32). The reason for impaired glucose metabolism and IR in these studies might possibly be related to the pubertal status of the cases. In our study, it was found that glucose, insulin, and HOMA-IR levels were similar between the PP and the control groups, and there was no impairment in glucose metabolism in the PP group, which could be due to the participants not being overweight or obese, participants being prepubertal and thus not yet experiencing any increase in adiposity. Thus we suggest that isolated PP in the prepubertal period might not pose a risk in terms of MS.

Dyslipidemia is an important biochemical disorder that causes cardiovascular risk to begin at an early age. In PP cases, some studies reported deterioration in lipid profile including an increase in total cholesterol, TG, LDL and decrease in HDL (18,25), whereas other studies reported the absence of dyslipidemia and the lipid profile to be within normal limits (19,29,32). In our study, lipid profile and AI were within normal limits in both groups. In studies in which dyslipidemia was detected, PP subjects were pubertal, and who were also obese and had hyperinsulinemia (18,25). It is possible that the reason lipid profiles were within normal limits, both in the PP group and in the control group in our study, was that the PP subjects were both prepubertal and of normal weight. We therefore speculate that isolated PP in the prepubertal period does not pose a strong risk for the development of dyslipidemia.

In our study, SHBG levels in the PP group were significantly lower than in the control group, and this finding was similar to other published studies (18,19,26,32). FAI was significantly higher in the PP group when compared to the control group. Although, this finding was similar to some studies (18,26,32), some of these studies included pubertal participants (26,32), while some of the others included obese PP participants, who were prepubertal. Ibáñez et al. (18) suggested that elevated FAI in prepubertal participants was a result of hyperinsulinemia. In our study, even though the participants in the PP group were prepubertal, non-obese and had normal birth weight, FAI was significantly elevated. This suggests that some mechanism(s) other than puberty, an increase in adipose tissue and hyperinsulinemia might be responsible for the occurrence of low SHBG and elevated FAI. One of these mechanisms may be intrauterine programming or genetic polymorphisms and other genetic variations that could affect enzyme expression, which plays a role in SHBG production in the liver. In order to test this hypothesis, studies with large numbers of participants, genetic studies and long-term follow-up are needed.

It has been shown that TNF-α, an adipocytokine, is a predictive marker for type 2 diabetes mellitus and coronary heart disease (33). Mathew et al. (10) reported that BP and TNF-α levels were high in both normal weight and overweight PP cases, and that this adipocytokine might be an important factor in the development of hypertension, a component of MS. In another study (32), it was shown that TNF-α levels in PP participants were similar to the control group. In our study, TNF-α level was found to be similar in both the PP group and the control group, and this similarity was primarily attributed to the presence of similar weight SDS and similar insulin levels in both groups. Leptin, also an adipokine, reflects the nutritional status of the body. Teixeira et al. (29) found that serum leptin levels were normal in PP cases with normal BMI and elevated in overweight cases. This finding led them to suggest that an increase in leptin level was not associated with PP but was directly related to increased BMI. Corvalán et al. (34) showed that obesity in 7-year-old children with normal birth weight was directly related to DHEA-S levels, whereas insulin, IGF-1 and leptin levels were only weakly associated with high DHEA-S, but strongly associated with obesity. In our study, serum leptin levels in PP participants were similar to the control group and this finding was attributed to the normal BMI SDS values of PP participants. Once again, we suggest that this is evidence that isolated PP does not lead directly to an elevation in serum leptin levels. It has been shown that adiponectin, another adipokine, increases insulin sensitivity and has a lower level in babies born small for gestational age (SGA) (8,35). Unlike in some studies (28,36), in our study serum adiponectin levels were normal in PP participants. Larqué et al. (28) found that postprandial adiponectin levels were lower in cases with PP when compared to the control group, yet they did not present birth weight data for their cases. Nieuwenhuis et al. (36) reported that adiponectin triggers puberty earlier in obese children. The serum adiponectin levels of the PP participants being similar to the control group in our study might be due to the fact that the study participants had normal birth weight, were non-obese and prepubertal without hyperinsulinemia.

A family history of cardiovascular disease is important for the development of MS, and it has been reported that it is more common in patients with PP than in healthy children (12). There are studies reporting the incidence of FCVRF in PP cases varies greatly, from 31.8% (23) to as high as 92.5% (25). In our study, there was no significant difference in the proportion reporting FCVRF between the PP group (45.7%) and the healthy control group (40%). When the PP group was divided into two subgroups, based on the presence or absence of FCVRF, it was found that mean leptin levels in the FCVRF+ subgroup were significantly higher than in the FCVRF- subgroup. The present study is the first published to divide and compare PP cases according to FCVRF history in

terms of clinical, biochemical, hormonal and adipocytokine statuses. The high serum leptin levels in the FCVRF+ PP group suggested that high serum leptin levels might be a predictive criterion for the development of obesity, MS or cardiovascular disease in the future. Once again, to test this hypothesis, studies with large numbers of participants and long-term follow-up are needed.

This study was conducted only in non-obese and prepubertal girls with normal birth weight, excluding SGA birth, puberty and obesity, which pose risks for the development of MS, independent of PP. The results appear to show that the prepubertal period in these PP patients does not pose a risk in terms of IR and cardiovascular risk.

Study Limitations

This study had several limitations. The sample size was small. OGTT was not performed in the control group and glucose metabolism was evaluated only with fasting glucose and insulin. Low SHBG levels and high leptin levels were found to be predictive factors but to support these findings long term follow up would be needed.

Conclusion

In conclusion, PP alone does not appear to be a risk factor for impaired glucose metabolism and IR in non-obese girls with PP with normal birth weight and before puberty. Additionally, low SHBG levels can be a predictive marker of hyperandrogenism in prepubertal girls with PP and high leptin levels in the FCVR+ subgroup may be a marker of future obesity risk but long term follow up studies are needed to test these hypotheses.

Ethics

Ethics Committee Approval: The ethics committee approval was obtained from Ethics Committee of Trakya University Faculty of Medicine (TÜTF-TÜBAPK date: 11.06.2013, approval no: 121).

Informed Consent: Informed consent was obtained from all parents/guardians and children included in this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Diğdem Bezen, Filiz Tütüncüler Kökenli, Design: Diğdem Bezen, Filiz Tütüncüler Kökenli, Data Collection or Processing: Diğdem Bezen, Analysis or Interpretation: Diğdem Bezen, Filiz Tütüncüler Kökenli, Emine Dilek, Didem Ağ Seleci, Hakan Erbaş, Literature Search: Diğdem Bezen, Filiz Tütüncüler Kökenli, Writing: Diğdem Bezen.

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The Assessment of the Hypothalamic-Pituitary-Adrenal Axis After Oncological Treatment in Pediatric Patients with Acute Lymphoblastic Leukemia

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What is already known on this topic?

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. Treatment of ALL consists of high-dose corticosteroid therapy with the aim of suppressing the hypothalamic-pituitary-adrenal axis (HPAA). Despite successful transient HPAA suppression, survivors of childhood ALL can present with persistent dysregulation of the HPAA in adult life.

What this study adds?

Our data highlights the importance of post-chemo/radiotherapy follow-up assessment of adrenal gland function within five years of therapy cessation. Dehydroepiandrostendione-sulfate seems to be a good marker of adrenal gland function after oncological treatment. The disturbances of the adrenal axis may be associated with early metabolic complications in ALL survivors.

Abstract

Objective: Oncologic treatment can affect the adrenal glands, which in stressful situations may lead to life threatening adrenal crisis. The aim of the study was to assess adrenal function in pediatric acute lymphoblastic leukemia (ALL) survivors and to identify the best markers for this assessment.

Methods: Forty-three ALL survivors, mean age 8.5 ± 3.6 years and 45 age and sex-matched healthy controls were recruited to the study. ALL patients were assessed once within five years following oncological treatment completion. Fasting blood samples were collected from all participants to measure: fasting blood glucose (FBG); cortisol; aldosterone; plasma renin activity (PRA); dehydroepiandrostendione-sulfate (DHEA-S); and adrenocorticotropic hormone (ACTH). Moreover, diurnal profile of cortisol levels and 24-hour urinary free cortisol (UFC) were assessed. ALL survivors underwent a test with 1 ug of synthetic ACTH.

Results: The study revealed lower level of PRA (1.94 ± 0.98 ng/mL/h vs 3.61 ± 4.85 ng/mL/h, p = 0.029) and higher FBG (4.6 ± 0.38 mmol/L vs 4.41 ± 0.39 mmol/L, p = 0.018) in the ALL group compared to controls. UFC correlated with evening cortisol (p = 0.015, r = 0.26), midnight cortisol (p = 0.002, r = 0.33), and DHEA-S (p = 0.004, r = 0.32). UFC also correlated with systolic and diastolic blood pressure (p = 0.033, r = 0.23 and p = 0.005, r = 0.31, respectively). The ACTH test confirmed impaired adrenal function in 4/43 ALL survivors (9%). Two of the patients who needed permanent hydrocortisone replacement had low UFC, midnight cortisol and DHEA-S levels.

Conclusion: These results highlight the importance of reviewing adrenal gland functionality after chemo/radiotherapy in ALL survivors. DHEA-S proved to be a good marker to assess the adrenal glands after oncological therapy. Post-treatment disturbances of the adrenal axis could be associated with metabolic complications.

Keywords: Adrenal insufficiency, acute lymphoblastic leukemia, cortisol, dehydroepiandrostendione-sulfate, adrenocorticotropic hormone test

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Introduction

The incidence of childhood cases of acute lymphoblastic leukemia (ALL) between the ages of 0-14 years old is 3.7 to 4.9 per 100,000 (1). In Poland 250 to 350 young patients are diagnosed with ALL every year (2). Fortunately, the 5-year survival rate of ALL has greatly increased, due advances in medical treatment consisting of multi-agent chemotherapy, radiotherapy and hematopoietic stem-cell transplantation (HSCT). The 5-year survival rate has increased from 10% in the 1960s to 77% in 1985-1994 (3), up to 93.5% nowadays (4).

The first- and second-line treatment for ALL is chemotherapy in addition to radiotherapy in select patients to target the central nervous system (CNS). Destroying cancerous cells by chemotherapy and radiotherapy leads to the consequent damage of healthy cells resulting in endocrine dysfunction. Corticosteroids are the key component of ALL chemotherapy treatment plans and is the first drug to be used to induce remission. It is a cytotoxic agent that arrests growth and induces apoptosis of lymphocytes and thus, like chemotherapy and radiotherapy, can affect the proper functioning of the endocrine system. Research is lacking regarding the effects of treatment on the adrenal axis, which may become life threatening. Adrenal insufficiency (AI) is a chronic and subclinical condition that can occur insidiously in stressful situations and lead to a clinical emergency known as an adrenal crisis.

The aim of this study was to assess the frequency of AI in patients with ALL who had completed oncological treatment, to identify the most useful biochemical and hormonal parameters assessing the adrenal reserve, and to assess adrenal imaging, and antibodies against the adrenal cortex (AAA) in ALL survivors in comparison with healthy controls. Adrenal reserve tests enable early detection of AI and by doing so, minimize the risk of an adrenal crisis developing. Therefore, the secondary aim of the study was to prevent the consequences of adrenal dysfunction in patients after oncological treatment.

The hypothesis of our study was that oncological treatment protocols that include corticosteroids, chemotherapy, and radiotherapy all influence adrenal function and morphology.

Methods

Patients

Forty-three patients treated for ALL and in complete remission, aged 1.17-14.83 years (mean age 8.51 ± 3.55 years) were recruited by the department of pediatric oncology and hematology in Cracow. All consecutive ALL survivors admitted to the oncology clinic between 2019 and 2020 were examined with consent. Any patient with adrenal disease was excluded from the study. A control group of 45 healthy age- and sex-matched children and adolescents were selected among patients, who where diagnosed in the department of pediatric and adolescent endocrinology due to short stature, without other comorbidities including endocrinopathies (aged 3.6-14 years, with the mean age 8.78 ± 3.12 years).

All patients were referred for assessment of adrenal function at the department of pediatric and adolescent endocrinology. All patients in the study group had completed treatment for ALL at a mean age 7.35 ± 2.85 years (range, 1.62-13.8 years). The mean time since the cessation of oncological therapy was 2.4 ± 1.9 years (range, 0.25-7.17 years). In the study group, nine patients (21%) were stratified as high risk (HR) ALL, 27 patients (61%) as intermediate risk (IR) ALL, and seven patients (16%) as standard risk (SR) ALL, in accordance with the ALL stratification protocol. There were 40 patients with B line ALL and three patients with T line ALL. All patients in the research group underwent therapy according to the subsequent prospective randomized trials of the International Berlin-Frankfurt-Münster Study Group (I-BFM-SG) for the management of children and adolescents (up to 18 years of age) with *de novo* diagnosed ALL: ALL-IC BFM 2002 (n = 6 patients) and ALL-IC BFM 2009 (n = 37 patients).

There are five major steps/components of treatment of newly diagnosed ALL. The first step is (1) a remission induction, lasting about five weeks, which is the first block of chemotherapy including steroids, followed by step (2) an early intensification lasting for 4-8 weeks depending on individual stratification by risk group. The third phase of treatment (8-17 weeks) is (3) a consolidation, which aims to eradicate the submicroscopic residual disease that remains after a complete remission is obtained and to maximize synergy and minimize drug resistance, followed by (4) a reinduction therapy (seven weeks). The final part of chemotherapy is (5) a maintenance chemotherapy up to the 104th week of the whole treatment (about two years).

The important component of the treatment of ALL is prophylaxis for patients with subclinical CNS disease or treatment of patients with clinical CNS. It includes direct intrathecal administration of chemotherapy, systemic administration of chemotherapy able to penetrate the blood-brain barrier, and cranial radiation (5).

In these protocols steroids are given two or three times depending on risk group. During the induction treatment/phase, prednisone/prednisolone is administrated at 60 mg/m^2/d, PO/IV, in three single doses per day on days 1-28. This is the first four weeks of the whole therapy. From day 29 tapering to withdrawal of prednisone is used over nine days by halving the dosage every three days in three doses, with the highest dose given in the morning. During the reinduction treatment/phase, dexamethasone 10 mg/m^2/d, PO/IV is given in three single doses for 21 days, on days 1-21. From day 22 taper down stepwise is used to withdrawal over nine days by halving the whole dosage every three days in three doses, giving the highest dose in the morning. This usually occurs at between 18th-30th week of the whole therapy. Additionally, patients who were stratified to HR group received dexamethasone 20 mg/m^2/d, PO/IV, in three divided doses for five days, on days 1-5 in the consolidation phase, at about 10-14 weeks of the whole therapy. The duration of overall therapy in all patients is 104 weeks (24 months). Therefore, all the study patients were at least 74 weeks (about 16 months) after steroid use in the therapy.

In addition to chemotherapy, certain patients with ALL need radiation therapy to prevent or treat their disease. According to the ALL-IC BFM 2009 protocol, prophylactic cranial radiotherapy (CRT) is given to patients without an involvement of CNS or a suspicion/subclinical form of CNS involvement only in patients with T-ALL and white blood cells > 100,000/μL and in patients with ALL stratified as HR non-transplanted (except in B-cell precursor ALL only, due to prednisolone poor responders). It is used only for age ≥1 year (12 Gy), with age attained at the start of irradiation being determinative. In those groups of patients, prophylactic RT is administrated in the first 1.5 weeks after the completion of the reinduction therapy. In previous protocol ALL-IC BFM 2002, prophylactic CRT was used in all SR/IR T-ALL and all non-transplant HR patients without CNS involvement or with only suspicion of CNS involvement, age ≥1 year with the dose of 12 Gy.

All patients with ALL and involvement of CNS received therapeutic CRT at age-adjusted dosage, with age attained at the start of irradiation being determinative, for age ≥1 year. The doses are: for patients aged ≥1 < 2 years 12 Gy; and for patients aged ≥2 years 18 Gy. In those groups of patients therapeutic RT is administrated in the first 2.5 weeks after the completion of the reinduction therapy.

Therefore, all our HR patients (9) were at least one year after prophylactic CRT (12 Gy in eight fractions). None of them needed therapeutic CRT.

According to both the ALL-IC BFM 2002 and ALL-IC BFM 2009 protocols, allogeneic HSCT is recommended for selected subgroups of HR patients on the basis of prognostically unfavorable constellations of disease biology and response quality. One patient in the HR group had to be referred for allogeneic HSCT.

All participants in this study and parents of those under the age of 16 were consented and relevant documentation was signed. The study was then approved by the Jagiellonian University Local Ethical Committee (no. 1072.6120.74.2019, date: 29.04.2019).

All participants had fasting blood samples collected between 7.00 and 8.00 am after waking in order to test for the following: cortisol, aldosterone, plasma renin activity (PRA), dehydroepiandrostendione-sulfate (DHEA-S), adrenocorticotropic hormone (ACTH), AAA, fasting blood glucose (FBG), sodium and potassium. The 24-hour urine was collected to assess free cortisol excretion. Additionally, the study group underwent a low dose (1 μg or 0.5 μg/m^2 BSA) synthetic ACTH test to assess the adrenal reserve. A basal cortisol level was analyzed and then the synthetic ACTH was administered intravenously. After administration, the cortisol levels were measured at 20, 30, and 60 minutes post ACTH injection. Standard biochemical methods were used to test FBG, sodium and potassium levels, while radioimmunological methods in-house that were employed to test for the hormones ACTH (BRAHMS, Germany), cortisol (Beckmann Coulter, Inc., Immunotech, Czech Republic), DHEA-S (Siemens, USA), PRA (Beckmann Coulter, Inc., Immunotech, Czech Republic), aldosterone (Beckmann Coulter, Inc., Immunotech, Czech Republic), and urine free cortisol (Siemens, USA). The AAA concentration was analyzed by enzyme-linked immunosorbent assay with isotope label sets from Brahms (Germany). The morphology of the adrenal gland was investigated using ultrasound and magnetic resonance imaging.

In order to analyze the influence of the duration of remission on the results obtained, the patients were divided into the following groups: 1) up to 2 years remission time [22 patients (51.2%)]; 2) 16 patients (37.2%) in the period 2-5 years in remission; and 3) 5 patients (11.6%) above > 5 years of remission.

Statistical Analysis

Statistical analysis was performed using the Statistica 13.1 64-bit package (StatSoft, Poland, Kraków) using Student's t-test and ANOVA with post-hoc Tukey test and linear and multivariate regression. A value of $p < 0.05$ was assumed to indicate statistical significance.

Results

The study revealed significantly lower level of morning PRA when standing upright (p = 0.029) and significantly higher levels of FBG (p = 0.018) in the ALL study group in comparison to the control group. The other hormonal and biochemical parameters did not differ between groups (Table 1).

In the ALL study group, there were significant positive correlations between urinary free cortisol (UFC) and evening (8.00 pm) cortisol levels (p = 0.016, r = 0.26), midnight cortisol levels (p = 0.002, r = 0.33), and DHEA-S (p = 0.004, r = 0.32). Moreover, UFC also correlated positively with systolic blood pressure (SBP) (p = 0.033, r = 0.23) and diastolic blood pressure (DBP) (p = 0.005, r = 0.31). With age taken into consideration, correlations between UFC and midnight cortisol levels (p = 0.003), and between UFC and DHEA-S (p = 0.008) were significant. The linear positive correlation between UFC and midnight cortisol levels as well as UFC and DHEA-S in ALL survivors are presented in Figure 1 and 2. Additionally, the correlation between UFC and DBP remained statistically significant after age adjustment (p = 0.008). The linear positive correlation between UFC and DBP in ALL survivors is presented in Figure 3. Furthermore, there was also a positive correlation between FBG and SBP (p = 0.035, r = 0.23) in the ALL group. The linear positive correlation between FBG and SBP in ALL survivors is presented in Figure 4. All analyzed parameters did not differ between groups of patients with ALL with regards to the remission time. Moreover, all analyzed parameters did not differ between groups of patients with ALL with regards to groups stratified by intensity of treatment.

In the control group, morning cortisol levels correlated positively with UFC (p = 0.01, r = 0.38). There were also positive correlations between midnight cortisol levels and DHEA-S (p = 0.045, r = 0.33), midnight cortisol levels and SBP (p = 0.006, r = 0.40), and midnight cortisol levels and PRA (p = 0.0002, r = 0.52). Similarly in the ALL study group, there was a statistically significant correlation between UFC and evening cortisol levels (p = 0.0007, r = 0.57), between UFC and midnight cortisol levels (p = 0.046, r = 0.31), between UFC and DHEA-S (p = 0.038, r = 0.35), between UFC and SBP (p = 0.019, r = 0.36), and between DHEA-S and DBP (p = 0.028, r = 0.34). Furthermore, in the control group, a significant negative correlation between UFC

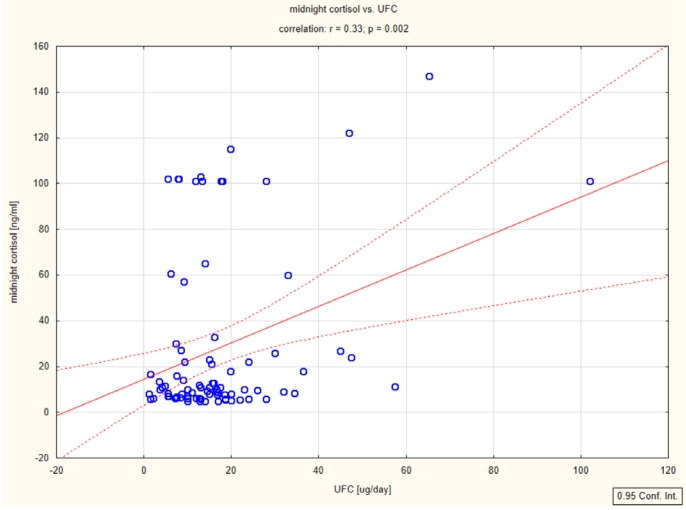

Figure 1. The linear positive correlation between 24-hour UFC and midnight cortisol in childhood acute lymphoblastic leukemia survivors

UFC: urinary free cortisol

Table 1. The results of biochemical and hormonal tests and blood pressure in ALL survivors and in controls

Parameter	ALL survivors (n = 43)	Controls (n = 43)	p
Na (mmol/L)	138.8 ± 1.4	138.9 ± 1.4	NS
K (mmol/L)	4.3 ± 0.2	4.4 ± 0.2	NS
Fasting blood glucose (mmol/L)	4.6 ± 0.4	4.4 ± 0.4	0.02
Systolic blood pressure (mmHg)	109.7 ± 11	105.2 ± 13	NS
Diastolic blood pressure (mmHg)	61.5 ± 8	60.3 ± 8	NS
ACTH (pg/mL)	32.9 ± 18.7	32.4 ± 17.7	NS
Cortisol 8.00 AM (ng/mL)	112.7 ± 41.4	127.5 ± 42	NS
Cortisol 8.00 PM (ng/mL)	19.1 ± 15.7	29.2 ± 37.5	NS
Midnight cortisol (ng/mL)	16.3 ± 25.4	19.5 ± 25.8	NS
UFC (µg/day)	17.9 ± 13.3	14.9 ± 10.6	NS
DHEA-S (µg/mL)	81.2 ± 63.1	65.1 ± 79.4	NS
Aldosterone (pg/mL)	143.3 ± 111	149.8 ± 101.8	NS
Plasma renin activity (ng/mL/h)	1.9 ± 1.0	3.6 ± 4.9	0.03
Maximal cortisol in ACTH test (ng/mL)	246.4 ± 44.3	Not assessed	

DHEA-S: dehydroepiandrostendione-sulfate, ALL: acute lymphoblastic leukemia, ACTH: adrenocorticotropic hormone, UFC: urinary free cortisol

and aldosterone (p = 0.043, r = -0.31) was seen. However, in spite of the correlations between midnight cortisol levels and SBP (p = 0.0098), as well as in midnight cortisol levels and PRA (p = 0.0002), all these correlations became statistically insignificant when patient age was taken into account. Interestingly, there was a positive correlation between DHEA-S and SBP (p = 0.002, r = 0.57), which remained significant (p = 0.0001) after adjusting for subject age. The linear positive correlation between DHEA-S and SBP in healthy patients is presented in Figure 5.

The ACTH test confirmed impaired adrenal function in four children. The first patient with AI was a girl aged 7 years with common ALL with AML/Tel+, stratified into the SR group and treated according to the ALL-IC BFM 2009 protocol. The time in remission prior to adrenal investigations was 2 years and 3 months. She was diagnosed with chronic AI due to our investigations as her maximal cortisol level with the ACTH test was 170.1 ng/mL and therefore treatment with hydrocortisone was initiated. The second patient was an 8-years and 7-months old boy, diagnosed with common ALL, stratified to the IR group, and treated with the ALL-IC BFM 2009 protocol. The time in remission prior to adrenal investigations was 3 years and 10 months. In spite of a lack of signs of AI before our investigations, he was also diagnosed with chronic AI with maximal cortisol level on ACTH test of 176 ng/mL and so hydrocortisone treatment was initiated. Simultaneously with the results of ACTH test confirming AI, both patients had low level of UFC (12.7 and 12.8 µg/day), midnight cortisol levels (6 and 5 ng/mL) and DHEA-S (8 and 28 µg/mL) in comparison to all the other ALL survivors and controls (respectively, mean UFC 17.9 and 14.9 µg/day, mean midnight cortisol levels 16.3 and 19.5 ng/mL, mean DHEA-S 79.4 and 65 µg/mL).

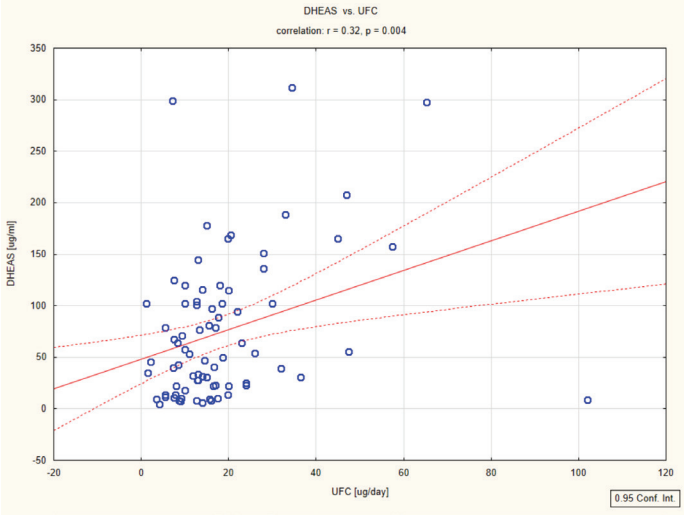

Figure 2. The linear positive correlation between 24-hour UFC and DHEA-S in childhood acute lymphoblastic leukemia survivors

UFC: urinary free cortisol, DHEA-S: dehydroepiandrostendione-sulfate

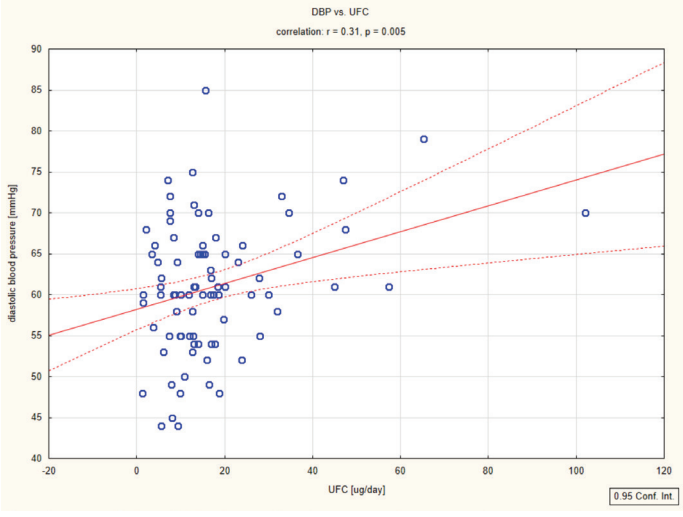

Figure 3. The linear positive correlation between 24-hour UFC and DBP in childhood acute lymphoblastic leukemia survivors

UFC: urinary free cortisol, DBP: diastolic blood pressure

Two additional patients had normal ACTH results but at the lower limit and displayed no AI symptoms. Maximal cortisol levels were respectively 185 ng/mL and 187.6 ng/mL. The first patient was a 10-years and 9-month old boy, with common ALL, in the IR group, treated with the ALL-IC BFM 2009 protocol. The time in remission prior to adrenal investigations was 3 years and 4 months. The second patient, was a 13-years and 7-months old girl with common ALL, in the HR group, treated with the ALL-IC BFM 2009 protocol. Her time in remission prior to adrenal investigations was 1.5 years. Both patients were advised to take hydrocortisone

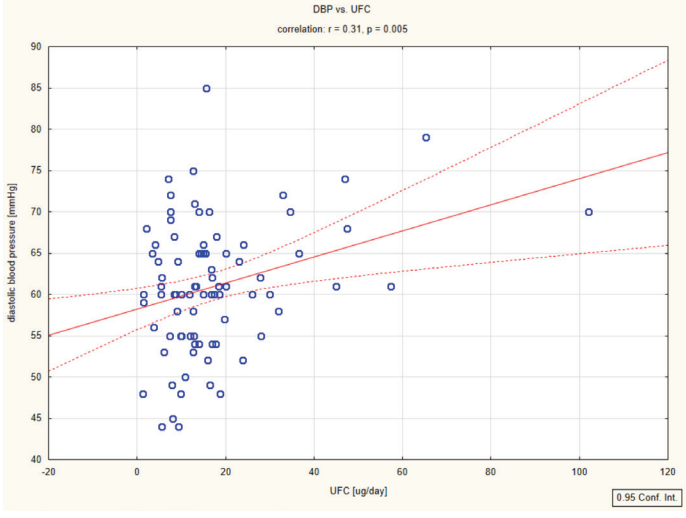

Figure 4. The linear positive correlation between fasting blood glucose and systolic blood pressure in childhood acute lymphoblastic leukemia survivors

UFC: urinary free cortisol, DBP: diastolic blood pressure

supplements in stressful situations. In summary, it appears that the time from completing treatment does not associate with the development of AI.

AAA were not found in any patients. Ultrasound of the abdomen in ALL survivors revealed normal adrenal morphology.

Discussion

The aim of treatment in patients with ALL is for complete disease eradication. First and second line treatment regimens for ALL consist of multi-agent chemotherapy, radiotherapy and, in select subgroups, HSCT. As mentioned previously, cancerous cells are killed and consequently healthy cells are also negatively impacted leading to side effects, such as endocrine dysfunction. Additionally, improper functioning of the adrenal axis can arise as a result of corticosteroid treatment and radiotherapy affecting the CNS.

Glucocorticoids are the key component of ALL chemotherapy protocol treatment regimens and is the first line treatment used to induce remission. The cytotoxic agents of choice are prednisolone/prednisone or dexamethasone which arrest cell growth and induce apoptosis in lymphocytes. Dexamethasone has proven efficacy in CNS penetration and is associated with reduced risk of relapse, but additionally it is associated with increased incidence of toxicities, including avascular necrosis, infection, and linear growth reduction (5,6). Glucocorticoid therapy is highly effective in suppressing the hypothalamic-pituitary-adrenal axis (HPAA) and can cause great effect in as little as five days of treatment (7,8). Therefore, abruptly stopping therapy can lead to secondary AI, which is a life-threatening condition. In a study conducted by Einaudi et al. (9) in 2008, 64 children with ALL underwent low dose ACTH (LD-ACTH) stimulation 24 hours after the last steroid administration. In the event of abnormally low cortisol levels during the LD-ACTH test, the test was repeated every 1-2 weeks until the cortisol values were normal. Adrenal suppression occurred in 52/64 (81.5%) patients and 7-14 days later, the ACTH test result revealed reduced morning cortisol levels in 8/52 (15.4%) patients in addition to an impaired test response in 12/52 (23%) patients. Normal adrenal reserve appeared in all patients within 10 weeks. There were no differences between the patients treated with prednisolone or dexamethasone. Clinical AI appeared in 35% of patients with impaired cortisol values in the first test which differed to results presented in a study by Salem et al. (10). These authors assessed HPAA function at different point in time: before starting therapy, after finishing therapy, and every two weeks after corticosteroid treatment until the adrenal axis recovered. They found that withdrawal syndrome occurred more frequently in patients treated with dexamethasone (75% of patients) than in those treated with prednisolone (50% of patients). The recovery time of the adrenal axis was twice as long with dexamethasone than with prednisolone. Similar results were presented by Mahachoklertwattana et al. (11). In a study by Felner et al. (12), 30% of children who received four weeks of dexamethasone had suppressed adrenal glands for 4-8 weeks. Comparatively, in a study by Petersen et al. (13), adrenal axis suppression was seen lasting 2.5-8 months in 41% of patients who received five weeks prednisolone in the induction phase and three weeks dexamethasone in the re-induction phase. The ALL survivors included in our study were in complete remission and were at least three months after the completion of oncological therapy, therefore at least 1.5 year after steroids use. All these patients were treated with prednisolone and dexamethasone according to the the ALL-IC BFM 2002 or ALL-IC BFM 2009 protocols. Conducted ACTH tests confirmed impaired adrenal function in four children (9%). Two of these children with chronic AI had completed two years of oncological therapy and the other two, who were 1.5 and 3 years post ALL therapy completion, had test results within the normal range but at the lower limit. These children were asymptomatic and were not previously diagnosed with AI prior to our study.

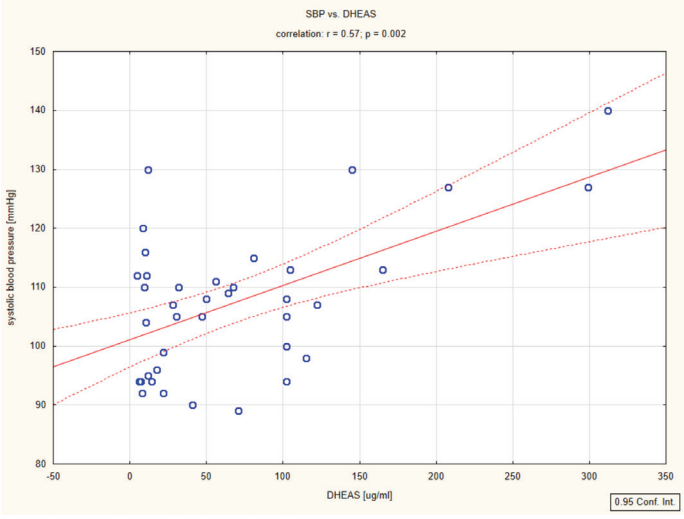

Figure 5. The linear positive correlation between DHEA-S and SBP in healthy ones

SBP: systolic blood pressure, DHEA-S: dehydroepiandrostendione-sulfate

The results of our study suggest that serum level of DHEA-S is a very useful marker in the diagnostic process of adrenal axis recovery in patients previously treated with corticosteroids. According to one pediatric report addressing corticosteroid treatment in ALL, serum levels of DHEA-S returned to normal two weeks before complete

adrenal recovery (10). In comparison with cortisol, the half-life of DHEA-S is longer and lasts 10-20 hours whereas the half-life of cortisol is 2 hours. It also has less fluctuation in concentration ranges than cortisol during the day. DHEA-S seems to be useful as an early indicator of adrenal recovery after the transient suppression of the adrenal axis. DHEA-S could be assessed before initiating steroid therapy from 2 and 4 weeks after the last dose of steroids. DHEA-S level is a reliable and sensitive tool to evaluate adrenal function (14). It is worth highlighting that in our study DHEA S correlated with UFC, indicating DHEA-S to be a very good marker of adrenal gland function. A measurement of UFC is primarily used to evaluate cortisol excess in the context of Cushing syndrome or adrenal cancer, but UFC also represents 24 hour excreted cortisol. We found correlation between UFC and evening and midnight cortisol levels, in addition to UFC with SBP, and DBP with DHEA-S. This could be of great importance diagnostically and clinically in the assessment of adrenal gland function. UFC is used mainly in the diagnostic process of hypercortisolemia and reflects daily production of cortisol and correlates significantly with midnight cortisol. Therefore, midnight cortisol levels are proposed as a diagnostic tool in hypercortisolemia. Our data also confirmed the significance of low midnight cortisol levels being suggestive of AI. Therefore, besides the ACTH test, DHEA-S, UFC, and midnight cortisol levels can be used as screening tests in the assessment of adrenal function in oncological patients. However, the most useful among them was DHEA-S, because it required a single assessment, regardless of the time of day or food intake.

Hyperglycemia during chemotherapy occurs in approximately 10% to 30% of patients (15). It can happen frequently and transiently. The main chemotherapeutics causing hyperglycemia in the leukemia chemotherapy treatment protocol are glucocorticoids and L-asparaginase. It was used both in the induction and reinduction therapies in all our patients. Complications of glucocorticoid treatment are: increased insulin resistance, diminished insulin secretion and exaggerated hepatic glucose output. L-asparaginase is a cytotoxic chemotherapeutic agent and has a direct toxic effect on pancreatic β-cells, resulting in insulin production and release inhibition. Indirectly, it can also cause pancreatitis, which may lead to impaired β-cell function, even after cessation of chemotherapy (16). L-asparaginase may lead to long-term hyperglycemia more frequently than corticosteroids. Diabetes may occur in up to 15.6% of cancer survivors (15). Diabetes in ALL survivors is of compound etiology due to impaired β-cells function and increase insulin resistance as part of a metabolic syndrome, a well-known marker of cardiovascular morbidity and mortality, and importantly it is a modifiable risk. In the study of Oudin et al. (17), there were 1.025 leukemia survivors. Metabolic syndrome was defined according to the National Cholesterol Education Program's Adult Treatment Panel II criteria and was found in 10.3% of patients. They concluded that in every group (patients after chemotherapy, chemotherapy joined with cranial irradiation, patients transplanted without irradiation and patients transplanted with total body irradiation) there was an increased risk of metabolic syndrome. In a large cohort study (18) of 784 ALL survivors, followed for more than 25 years from diagnosis, metabolic syndrome was identified in 259 survivors (33.6%). Fasting hyperglycemia or treatment for hyperglycemia was prevalent in 246 ALL survivors (31.4%). Hypertension was identified among 364 survivors (46.4%). In our study, in comparison with the control group, ALL survivors had higher blood pressure and FBG levels. Moreover, there was a correlation between FBG levels and SBP. Unique to this study, the abnormalities of SBP and FBG levels appeared as early as five years after the end of ALL treatment. There is data reporting that survivors of childhood ALL can present with persistent dysregulation of the HPAA in adult life. The experience of a stressful life event in the past may cause a long-term dysregulation of the HPAA, as reflected in increased cortisol production and an enhanced negative feedback mechanism (19). This mechanism could be responsible for obesity and metabolic dysregulation often observed in childhood ALL survivors. Glucocorticoid induction of hypertension is complex and tissue dependent. The main pathway is the interconversion of active cortisol to inactive cortisone by hydroxysteroid 11-beta dehydrogenase (11b-HSD). 11b-HSD type 2 (11b-HSD2) is expressed in nonselective mineralocorticoid receptor (MR)-rich tissues, especially the kidney, colon and salivary gland (20,21). MR has a similar affinity for cortisol and aldosterone. Aldosterone occupies the MR only when cortisol is inactivated to cortisone by 11b-HSD2 as this mechanism protects the MR from cortisol excess. 11b-HSD2 is saturated due to increased corticosteroid concentration, resulting in cortisol-induced mineralocorticoid excess. Dexamethasone is poorly metabolized by 11b-HSD2 (22). Corticosteroid-induced hypertension is mediated by excess sodium and water reabsorption by stimulation of the renal MRs (23,24,25).

Besides corticosteroids, other drugs used in the therapy of leukemia increase the risk of metabolic syndrome in ALL survivors. Preclinical evidence has demonstrated endothelial injury and abnormalities in the renin-angiotensin system in animals treated with cyclophosphamide. Therefore, there is biological plausibility for cyclophosphamide-associated hypertension due to vascular injury. However,

cyclophosphamide has not been identified as an independent risk factor for hypertension in cancer survivors (23,26). Another chemotherapeutic, anthracycline, may lead to increased risk of cardiovascular disease in ALL survivors. The Childhood Cancer Survivor Study found that, while both cardiotoxic treatments and hypertension were independently associated with increased risk of coronary artery disease or heart failure, the combination of these factors resulted in a greater increase in risk that yielded an 86-fold increased risk of heart failure in survivors exposed to both anthracyclines and hypertension. This suggests that development of hypertension can exacerbate the damage caused by cardiotoxic cancer treatments (23). Moreover, radiation to the head and neck has been associated with baroreflex failure, which can manifest as labile hypertension or hypertensive crisis (23).

Changes in body salt content are buffered by reciprocal changes in PRA to maintain BP homeostasis (26). The PRA test is useful to define the relative involvement of body sodium-volume and to classify hypertension. Low renin hypertension is a common condition and accounts for 20 % to 30 % of all hypertensive patients (27) and might be associated with high aldosterone levels (Conn syndrome), normal aldosterone levels or low aldosterone levels, as in Liddle syndrome and syndrome of apparent mineralocorticoid excess and glucocorticoid remediable hypertension (28,29). High-dose corticosteroid therapy leads to inappropriate stimulation of the MR, mineralocorticoid excess and low level of PRA, resulting in elevated blood pressure. In our study the PRA was significantly lower than in the control group. ALL survivors also had higher blood pressure. This suggests a reduction in PRA levels compensating for sodium retention due to the stimulation of MR (cortisol-induced mineralocorticoid excess). Our suggestion is also based on our result of the positive significant correlations between UFC, DHEA-S and blood pressure.

Radiotherapy plays an important role in ALL treatment protocols. It stops cancer cells proliferating and often leads to cancer cell apoptosis (30). In parallel, it can lead to HPAA dysregulation, especially during CNS radiotherapy. The risk of AI is significantly reduced when the total radiation dose is less than 30 Gy and fractionated doses are less than 2 Gy (31,32). Prophylactic CRT used in some of our patients with a dose of 12 Gy in eight fractions minimized the risk of pituitary damage and dysfunction of HPAA.

Study Limitations

The ALL survivors included in the study were heterogeneous in regards to their duration of remission after oncological treatment but no precise time was provided how long after treatment the adrenals were assessed. The local ethical committee did not approve the testing of ACTH in the control group and so authors can only assume results of this test are normal in healthy controls.

Conclusion

Our study confirms the effect of ALL treatment protocols on the adrenal glands resulting in transient or occassionally persistent AI. These results highlight the importance of post-chemo/radiotherapy follow-up of adrenal function. The low-dose ACTH test is a reliable and quite sensitive method to exclude chronic, subclinical AI prior to symptoms developing. Our data indicates that DHEA-S, midnight cortisol levels and UFC may be good markers of adrenal function after oncological treatment. However, the most useful among them was DHEA-S, because it requires a single assessment independent of food intake and the time of the day. It is necessary to monitor ALL survivors with importance given to metabolic syndrome surveillance after the cessation of ALL treatment. Regular adrenal and metabolic assessment should be combined to prevent the adverse events caused by chronic, subclinical AI and asymptomatic metabolic disorders, thus promoting the efficacy of anti-cancer therapy and improving quality of life.

Ethics

Ethics Committee Approval: The study was then approved by the Jagiellonian University Local Ethical Committee (no. 1072.6120.74.2019, date: 29.04.2019).

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

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Barbara Hull, Anna Wędrychowicz, Magdalena Ossowska, Aleksandra Furtak, Joanna Badacz, Szymon Skoczeń, Concept: Anna Wędrychowicz, Design: Barbara Hull, Anna Wędrychowicz, Szymon Skoczeń, Data Collection or Processing: Barbara Hull, Anna Wędrychowicz, Magdalena Ossowska, Aleksandra Furtak, Joanna Badacz, Szymon Skoczeń, Analysis or Interpretation: Barbara Hull, Anna Wędrychowicz, Szymon Skoczeń, Jerzy B. Starzyk, Literature Search: Barbara Hull, Anna Wędrychowicz, Writing: Barbara Hull, Anna Wędrychowicz.

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ORIGINAL ARTICLE

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Are Thyroid Functions Affected in Multisystem Inflammatory Syndrome in Children?

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What is already known on this topic?

The health effects of the global Coronavirus disease-2019 (COVID-19) pandemic are still being investigated. In children infected with Severe acute respiratory syndrome-Coronavirus-2, the causative virus of COVID-19, a clinical condition has emerged, called multisystem inflammatory syndrome in children (MIS-C).

What this study adds?

This is the first study to investigate the relationship between MIS-C and thyroid function. Low free triiodothyronine levels were associated with both the diagnosis of MIS-C and severe clinical presentation.

Abstract

Objective: Multisystem inflammatory syndrome in children (MIS-C), associated with Coronavirus disease-2019, is defined as the presence of documented fever, inflammation, and at least two signs of multisystem involvement and lack of an alternative microbial diagnosis in children who have recent or current Severe acute respiratory syndrome-Coronavirus-2 infection or exposure. In this study, we evaluated thyroid function tests in pediatric cases with MIS-C in order to understand how the hypothalamus-pituitary-thyroid axis was affected and to examine the relationship between disease severity and thyroid function.

Methods: This case-control study was conducted between January 2021 and September 2021. The patient group consisted of 36 MIS-C cases, the control group included 72 healthy children. Demographic features, clinical findings, inflammatory markers, thyroid function tests, and thyroid antibody levels in cases of MIS-C were recorded. Thyroid function tests were recorded in the healthy control group.

Results: When MIS-C and healthy control groups were compared, free triiodothyronine (fT3) level was lower in MIS-C cases, while free thyroxine (fT4) level was found to be lower in the healthy group ($p < 0.001$, $p = 0.001$, respectively). Although the fT4 level was significantly lower in controls, no significant difference was found compared with the age-appropriate reference intervals ($p = 0.318$). When MIS-C cases were stratified by intensive care requirement, fT3 levels were also lower in those admitted to intensive care and also in those who received steroid treatment ($p = 0.043$, $p < 0.001$, respectively).

Conclusion: Since the endocrine system critically coordinates and regulates important metabolic and biochemical pathways, investigation of endocrine function in MIS-C may be beneficial. These results show an association between low fT3 levels and both diagnosis of MIS-C and requirement for intensive care. Further studies are needed to predict the prognosis and develop a long-term follow-up management plan.

Keywords: MIS-C, thyroid function, free triiodothyronine, free thyroxine

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Introduction

Multisystem inflammatory syndrome in children (MIS-C), associated with Coronavirus disease-2019 (COVID-19) in children, is defined as the presence of fever, inflammation, and organ dysfunction other than through microbial causes (1). The pathophysiological mechanisms for MIS-C are not yet clear. Severe inflammation, the time interval between Severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) infection and MIS-C, high inflammatory markers, and response to various immunomodulatory treatments suggest an immunological reaction rather than a virus-mediated condition. In addition to the abnormal immune response against the virus, extensive vascular endothelial damage caused by viral infection also contributes to the pathogenesis of MIS-C (2). It remains unclear whether the multi-organ damage observed in MIS-C cases is directly caused by the virus, an abnormal immune response, or both (3).

In the literature, a study reported that non-thyroidal illness syndrome (NTIS) was common in cases of MIS-C. During severe acute illness, changes in thyroid hormones are termed NTIS and are characterized by a rapid decrease in serum triiodothyronine (T3) levels without an increase in thyroid stimulating hormone (TSH) levels (4).

In this study, we aimed to evaluate thyroid function tests in cases diagnosed with MIS-C, understand how the hypothalamus-pituitary-thyroid axis was affected, and investigate the relationship between disease severity and parameters of thyroid function.

Methods

Study Design and Definitions

This case-control study was conducted in the Department of the Pediatric Infectious Diseases, University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital in Turkey between January 2021 and September 2021.

The patient group consisted of MIS-C cases, aged between one month and 18 years, who met the MIS-C case definition according to the Centers for Disease Control and Prevention (CDC) report (5). The control group included healthy children without any known chronic disease.

Demographic characteristics, clinical findings, inflammatory markers, thyroid function tests, thyroid antibody levels, system involvement, treatments, and hospitalizations of MIS-C cases were recorded. Demographic data and thyroid function tests were recorded in the healthy control group. Thyroid function tests were evaluated before treatment in MIS-C cases. For both groups, patients with chronic disease, known thyroid dysfunction, and patients who were treated with steroids before diagnosis were excluded from the study.

The study protocol was approved by the Institutional Ethics Committee of University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/06-33, date: 15.06.2021).

Thyroid Function Test Analysis

Clot activator tubes containing gel barrier (Vacutainer® SST II Advance tube, 5 mL, 13 x 100 mm; Becton Dickinson and Company, NJ, USA) were used for free triiodothyronine (fT3), free thyroxine (fT4), TSH, anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibody assays. Samples were quickly transferred to the laboratory. To separate the serum, SST II tubes were centrifuged at 1500 x g for 10 minutes. Serum fT3, fT4, TSH, anti-TPO, and anti-TG levels were determined using the chemiluminescence immunoassay method (UniCel DxI 800, Beckman Coulter, USA). Thyroid-stimulating hormone level was 0.34-5.76 mIU/L, anti-TPO antibody level was 0-10 IU/mL and anti-TG antibody level was 0-5 IU/mL in all age groups. Other thyroid function tests measurement levels varied according to age ranges. Normal ranges of fT3 were 3.6-7.5 ng/dL in the first year, 4.3-6.8 between one and 12 years, 3.8-6.7 between 12 and 15 years, and 3.5-5.9 ng/dL between 15 and 18 years. The fT4 normal range was 0.5-2.3 between one month and two years and 0.7-1.6 ng/dL between two and 18 years.

NTIS was defined as abnormal thyroid function tests seen in the presence of critical illness and the absence of a pre-existing abnormality in the hypothalamic-pituitary-thyroid axis (4). Thyroid function tests were measured prior to starting any steroid treatment.

Statistical Analysis

Statistical data were analyzed with IBM Statistical Package for the Social Sciences for Windows, version 25.0 (IBM Inc., Chicago, IL, USA). Values for numeric variables are given as median (interquartile range). Categorical variables were presented as numbers and percentages. Continuous variables following normal distribution were compared using a one-way analysis of variance or t-tests. The Mann-Whitney U test was used as a non-parametric test. Categorical variables were compared using the chi-square test. A p value of < 0.05 was considered statistically significant for all predictions.

Results

A total of 108 children were evaluated, 36 of them had MIS-C, meeting the CDC definition criteria, and 72 were

healthy. There was no statistically significant difference in terms of ages and gender ($p > 0.05$).

Clinical and Laboratory Findings in MIS-C Patients

Twenty-three (63.9%) MIS-C cases were male, 13 (36.1%) were female and their age was 98 ± 52 (53-136) months. In terms of MIS-C symptomology, all cases had fever. Other symptoms were: diarrhea in 22 (61.1%); nausea-vomiting in 19 (52.8%); abdominal pain in 13 (36.1%); rash in 11 (30.6%); cough in four (11.1%); headache in three (8.3%); myalgia in three (8.3%); mucositis in three (8.3%); sore throat in two (5.6%); dyspnea in one (2.8%); seizures in one (2.8%); and confusion in one (2.8%). In the medical histories, 26 (72.2%) MIS-C cases reported that they had contact with a SARS-CoV-2 positive case, confirmed by reverse transcription-polymerase chain reaction, in the four weeks preceeding onset of symptoms while five cases (13.9%) reported infection with SARS-CoV-2. In five (13.9%) cases, there was no history of contact or infection. Evidence of inflammation was found in laboratory tests of all MIS-C cases. Laboratory findings of the MIS-C cases, including inflammatory markers, are shown in Table 1. In terms of multi-organ involvement, system involvement of MIS-C cases was analyzed and hematological system involvement was found in all cases. Other systemic manifestations were: 35 (97.2%) gastrointestinal system; 14 (38.9%) cardiovascular system; 11 (30.6%) skin desquamation; four (11.1%) respiratory system; and two (5.6%) central nervous system involvement. Renal involvement was not observed in any of the cases. All patients received hydration and antibiotic therapy. Of the 27 (75%) who received steroid treatment, 25 (69.4%) received low-dose steroid (1-2 mg/kg/day), and two (5.6%) received pulse steroid treatment. When other treatment regimens were examined, intravenous immunoglobulin was administered in 26 (72.2%) cases, antithrombotic agents were administered in 25 (69.4%) cases, inotropic agents in five (13.9%) cases, and antiviral agents (favipiravir) in two (5.6%) cases. Only one (2.8%) case received immunomodulatory agent (anakinra) treatment. Plasmapheresis treatment was used in one of the cases (2.8%). While all patients were hospitalized, six (16.7%) patients were admitted to the intensive care unit (ICU). The median hospital stay was 8 (6-11) days, while the median ICU stay was 5 (3-7) days.

Changes in Thyroid Hormones

The median TSH value in the MIS-C group was 1.919 (1.12-2.577) mIU/L, and in the healthy group was 2.138 (1.571-3.004) mIU/L. The median fT3 level was 2.76 (2.485-3.31) ng/dL in the MIS-C group and 4.45 (4.07-4.79) ng/dL in the healthy group. The median fT4 level was 1.087 (0.976-1.203) ng/dL in the MIS-C group and 0.955 (0.855-1.065) ng/dL in the healthy group. When MIS-C and healthy control groups were compared in terms of thyroid hormones, median fT3 level was lower in MIS-C cases, while the median fT4 level was lower in the healthy group ($p < 0.001$ and $p = 0.001$, respectively).

When thyroid function tests were evaluated by reference intervals according to age, TSH level was within the normal range in all cases. However the fT3 level was low in 35 (97.2%) patients in the MIS-C group but was only low in four (5.6%) patients in the healthy group. The fT4 level was low in one (2.8%) patient in the MIS-C group and was also low in one (1.4%) patient in the healthy group. When both groups were compared, the fT3 level was lower in the MIS-C group ($p < 0.001$). Although the median fT4 level was significantly lower in the healthy group, no significant difference was found compared with the reference intervals according to age ($p = 0.318$).

Anti-TPO was positive in two (5.6%) cases, while anti-TG was positive in one (2.8%) case.

Clinical Comparison Between ICU and non-ICU Admission in MIS-C Patients

A total of six patients were admitted to the ICU. Five (83.3%) of them were male and their median age was 109 (85-168) months. When compared in terms of inflammatory markers, ferritin and D-dimer levels were higher in ICU admission ($p = 0.002$ and $p = 0.007$, respectively). As for thyroid function tests, fT3 level was lower in patients who were admitted to ICU ($p = 0.043$). Hypotension was found in five (83.3%) of the patients who were admitted to the ICU, and the presence of hypotension was found to be a significant finding in ICU admission ($p < 0.001$). ICU admission rates of cases with cardiovascular, skin, and respiratory system involvements were significantly higher ($p = 0.024$, $p = 0.006$ and $p = 0.010$, respectively). Similarly, use of inotropic therapy was also significantly more frequent in MIS-C cases requiring ICU admission ($p < 0.001$). The clinical comparison of MIS-C patients in terms of ICU admission is shown in Table 2.

Clinical Comparison Between Steroid and No Steroid Treatment in MIS-C Patients

In terms of symptoms, patients with vomiting and diarrhea were given steroid treatment more frequently ($p = 0.02$ and $p = 0.048$, respectively). Ferritin, D-dimer, and fibrinogen levels, which are acute phase inflammatory markers, were higher in MIS-C cases treated with steroids ($p < 0.001$, $p = 0.034$, $p = 0.007$, respectively). Conversely, fT3 levels

Table 1. Clinical and laboratory findings in MIS-C patients

	MIS-C group (n = 36)
Gender (male)*	23 (63.9)
Age (months)**	87 (53-136)
Clinical findings*	
Fever	36 (100)
Diarrhea	22 (61.1)
Nausea-vomiting	19 (52.8)
Abdominal pain	13 (36.1)
Rash	11 (30.6)
Cough	4 (11.1)
Headache	3 (8.3)
Myalgia	3 (8.3)
Laboratory findings	
Total WBC (10^3/uL)**	9.8 (7.8-16.4)
ALC (10^3/uL)***	1.1 (0.6-1.9)
C-reactive protein (mg/L)**	124.9 (63.7-189.2)
Procalcitonin (µg/L)***	0.98 (0.35-4.1)
Ferritin (ng/mL)**	145 (87-300)
D-dimer (µg/L)***	2020 (1240-3140)
Fibrinogen (mg/dL)**	481 (369-556)
Troponin I (ng/L)***	2.5 (2.5-17)
TSH (mIU/L)***	1.919 (1.12-2.577)
FT4 (ng/dL)**	1.087 (0.976-1.203)
FT3 (ng/dL)**	2.76 (2.485-3.31)
Anti-TG (IU/mL)***	0.9 (0.9-0.9)
Anti-TPO (IU/mL)***	0.4 (0.3-1.2)
System involvement*	
Hematological system	36 (100)
Gastrointestinal system	35 (97.2)
Cardiovascular system	14 (38.9)
Skin system	11 (30.6)
Respiratory system	4 (11.1)
Central nervous system	2 (5.6)
Treatment*	
Steroid	27 (75)
IVIg	26 (72.2)
Antithrombotic agent	25 (69.4)
Inotropic agent	5 (13.9)
Hospitalization*	36 (100)
Days of hospital stay**	8 (6-11)
ICU admission*	6 (16.7)
Days of ICU stay**	5 (3-7)

*n, % **mean ± SD ***median (IQR).
MIS-C: multisystem inflammatory syndrome in children, WBC: white blood cell, ALC: absolute lymphocyte count, TSH: thyroid stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine, ICU: intensive care unit, IQR: interquartile range, IVIg: intravenous immunoglobulin, SD: standard deviation, TG: thyroglobulin, TPO: thyroid peroxidase

were lower in cases requiring steroid treatment ($p < 0.001$). In terms of organ system involvement, only cardiovascular system involvement was seen more frequently in cases treated with steroids ($p = 0.048$). Duration of hospital stay was longer in patients treated with steroids ($p < 0.001$). The clinical comparison of MIS-C patients in terms of steroid treatment is shown in Table 3.

Discussion

Acute and chronic diseases can cause interactions of some neuroendocrine systems, including the hypothalamic-pituitary-thyroid axis (6). Data in the literature suggest that SARS-CoV-2 infection may have an effect on thyroid tissue and function (7). However, there are scarce data about MIS-C cases associated with SARS-CoV-2.

MIS-C is an immune-mediated phenomenon seen after acute infection. Cases with MIS-C present with single or multiple organ failure, manifested by fever, inflammation, cardiac dysfunction, hypotension, or life-threatening shock (8). Fever was present in all cases with MIS-C in the current study, and the most common organ involvements were the hematological and gastrointestinal systems. There are no data on endocrinological system involvement in MIS-C in current publications. This study was designed to investigate whether thyroid functions are affected in MIS-C cases.

There are some theories regarding hypothalamus-pituitary-thyroid axis abnormalities in COVID-19. The first of these is the appearance of TSH disorders through virus-associated hypophysitis. Another theory is that the thyroid gland is destructively damaged due to virus spread or excessive cytokine production. Finally, NTIS may be associated with severe disease states that are not specific to COVID-19 (9). Similar theories related to excessive cytokine formation and serious disease may be applicable in MIS-C cases. While NTIS was detected in 97.2% of our MIS-C cases, TSH abnormality was not detected in any of them. Since the number of participants was relatively small, it is not possible to exclude hypophysitis in this cohort of MIS-C cases. Thus, larger studies would be required to confirm this finding.

In NTIS, serum T3 level decreases rapidly from the onset of disease and this decrease is proportional to disease severity (10). NTIS typically occurs in critically ill patients and is closely associated with prognosis (11). This is considered a useful adaptation for conserving energy during critical illness (12). Similarly, in our study, fT3 levels were found to be lower in patients who were admitted to the ICU and who were severely unwell. In contrast, serum TSH levels of the participants remained within the normal range, suggesting that thyroidal T3 and T4 production was not greatly reduced.

Table 2. Clinical comparison between ICU and non-ICU admission in MIS-C patients

	ICU admission (n = 6)	Non-ICU admission (n = 30)	p
Gender (male)*	5 (83.3)	18 (60)	0.385
Age (months)**	109 (85-168)	82 (49-132)	0.259
Laboratory findings			
Total WBC (10^3/uL)**	9.8 (5-10.9)	10.5 (7.8-18.6)	0.140
ALC (10^3/uL)***	0.8 (0.4-1.4)	1.2 (0.6-2.5)	0.226
C-reactive protein (mg/L)**	159.1 (107-239.7)	112.7 (60.2-180.4)	0.379
Procalcitonin (µg/L)***	2.78 (0.46-5.94)	0.76 (0.31-4.07)	0.484
Ferritin (ng/mL)**	375 (270-450)	115 (79-223)	**0.002**
D-dimer (µg/L)***	3540 (3070-5400)	1575 (3184-5269)	**0.007**
Fibrinogen (mg/dL)**	519 (388-665)	454 (337-512)	0.340
Troponin I (ng/L)***	36.1 (2.8-82)	2.5 (2.5-3.94)	0.069
TSH (mIU/L)***	1.649 (1.508-2.281)	2.04 (1.085-2.683)	0.610
FT4 (ng/dL)**	1.207 (1.14-1.33)	1.039 (0.973-1.179)	0.273
FT3 (ng/dL)**	2.325 (1.95-2.89)	2.8 (2.58-3.49)	**0.043**
System involvement*			
Gastrointestinal system	6 (100)	29 (96.7)	1.000
Cardiovascular system	5 (83.3)	9 (30)	**0.024**
Skin system	5 (83.3)	6 (20)	**0.006**
Respiratory system	3 (50)	1 (3.3)	**0.010**
Central nervous system	0 (0)	2 (6.7)	1.000
Treatment*			
Steroid	6 (100)	21 (70)	0.303
IVIg	6 (100)	20 (66.7)	0.157
Antithrombotic agent	6 (100)	19 (68.3)	0.148
Inotropic agent	5 (83.3)	0 (0)	**< 0.001**

*n, % **mean ± SD ***median (IQR).
ICU: intensive care unit, MIS-C: multisystem inflammatory syndrome in children, WBC: white blood cell, ALC: absolute lymphocyte count, TSH: thyroid stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine, IQR: interquartile range, IVIg: intravenous immunoglobulin, SD: standard deviation

In a study of patients with sepsis presenting with NTIS, patients with combined low T3 and T4 levels had a worse prognosis than those with low T3 alone (13). In our study, combined low T3 and T4 levels were detected in only one case in the MIS-C group, so it is not possible to reliably comment on this issue.

In the literature, there are studies examining thyroid antibody status, including adult patient populations. In a study conducted in India, anti-TPO seropositivity was found in 13.6% of the participants (14). In a study involving a large number of cases in the European population, 23.6% of participants without known thyroid disease were found to be seropositive for at least one thyroid autoantibody (15). In our study, anti-TPO seropositivity was found in 5.6% of MISC cases. The participants' pre-MIS-C thyroid antibody status was unknown; however, it is thought that thyroid autoimmunity may be triggered in some MIS-C cases. Therefore, it may be important to monitor MIS-C patients for thyroid autoantibody development.

Study Limitations

Our study had some limitations. Due to the relatively small number of participants and the lack of follow-up of patients' thyroid function parameters, more studies are needed to confirm our data.

Conclusion

In conclusion, since the endocrine system critically coordinates and regulates important metabolic and biochemical pathways, investigation of endocrine functions may be beneficial in MIS-C. In our study, low fT3 levels were associated with both the diagnosis of MIS-C and requirement for ICU admission. To the best of our knowledge, there is only one study addressing this issue (4). Further studies are needed to predict the prognosis and develop a long-term follow-up management plan.

Table 3. Clinical comparison between steroid and no steroid treatment in MIS-C patients

	Steroid treatment (n = 27)	No steroid treatment (n = 9)	p
Gender (male)*	16 (59.3)	7 (77.8)	0.317
Age (months)**	85 (49-140)	96 (67-126)	0.881
Laboratory findings			
Total WBC (10^3/uL)**	9.7 (7.4-16.5)	11.3 (7.8-16.2)	0.950
ALC (10^3/uL)***	1.3 (0.6-1.9)	1 (0.6-1.6)	0.522
C-reactive protein (mg/L)**	135 (68.6-208.9)	73 (54.1-137.7)	0.093
Procalcitonin (µg/L)***	1.55 (0.38-4.07)	0.46 (0.12-5.69)	0.622
Ferritin (ng/mL)**	201 (113-400)	81 (49-110)	**< 0.001**
D-dimer (µg/L)***	2180 (1440-3870)	1310 (1110-1850)	**0.034**
Fibrinogen (mg/dL)**	504 (388-621)	337 (243-454)	**0.007**
Troponin I (ng/L)***	2.7 (2.5-20.3)	2.5 (2.5-2.5)	0.113
TSH (mIU/L)***	1.707 (1.085-2.351)	2.397 (2.021-2.683)	0.315
fT4 (ng/dL)**	1.03 (0.947-1.204)	1.105 (1.047-1.172)	0.645
fT3 (ng/dL)**	2.66 (2.31-3.02)	3.55 (3.29-3.98)	**< 0.001**
System involvement*			
Gastrointestinal system	26 (96.3)	9 (100)	1.000
Cardiovascular system	13 (48.1)	1 (11.1)	**0.048**
Skin system	10 (37)	1 (11.1)	0.144
Respiratory system	4 (14.8)	0 (0)	0.553
Central nervous system	1 (3.7)	1 (11.1)	0.443
Days of hospital stay**	9 (7-12)	4 (4-6)	**< 0.001**

*n, % **mean ± SD ***median (IQR).
MIS-C: multisystem inflammatory syndrome in children, WBC: white blood cell, ALC: absolute lymphocyte count, TSH: thyroid stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine, IQR: interquartile range, SD: standard deviation

Ethics

Ethics Committee Approval: The study protocol was approved by the Institutional Ethics Committee of University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital (decision no: 2021/06-33, date: 15.06.2021).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Gönül Çatlı, Concept: Ayşegül Elvan-Tüz, İlkay Ayrancı, Eda Karadağ-Öncel, Bumin Nuri Dündar, Design: Gönül Çatlı, Ahu Kara-Aksay, Eda Karadağ-Öncel, Bumin Nuri Dündar, Data Collection or Processing: İlkay Ayrancı, Analysis or Interpretation: Ahu Kara-Aksay, Dilek Yılmaz, Literature Search: Ayşegül Elvan-Tüz, Yıldız Ekemen-Keleş, İnanç Karakoyun, Writing: Ayşegül Elvan-Tüz.

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Is There a Predictive Factor for an Association with Autoimmune Glandular Disease in Children Diagnosed with Celiac Disease?

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What is already known on this topic?

Celiac disease (CD) can coexist with autoimmune glandular diseases (AGD) such as type 1 diabetes mellitus, Hashimoto's thyroiditis, Graves disease, and other autoimmune diseases. In the literature, there is little information about the clinical or laboratory characteristics of patients with CD and an accompanying AGD.

What this study adds?

In patients with CD there was no predictive value between gender, celiac symptoms, anti-tissue transglutaminase IgA antibody level, human leucocyte antigen type, and histopathological stage and the coexistence of AGD.

Abstract

Objective: A close relationship has been suggested between Celiac disease (CD) and glandular autoimmunity. The aim of this study was to determine the predictive factors for autoimmune glandular disease (AGD) in children with CD.

Methods: The study included 228 pediatric patients, diagnosed with CD between 2010 and 2019. The cases with AGD (Group 1) and those without AGD (Group 2) and the patients with type 1 diabetes mellitus (T1DM) (Group A) and those without T1DM (Group B) were retrospectively reviewed and compared in terms of clinical and laboratory features.

Results: AGD was detected in 8.8% (n = 20) of the patients: T1DM in 13 (65%), T1DM and Hashimoto's thyroiditis (HT) in 3 (15%), HT only in 2 (10%), T1DM and Graves disease (GD) in 1 (5%), and GD only in 1(5%). The mean age at the diagnosis of CD was significantly higher in Group 1 (10.93 ± 4.15 years) compared to Group 2 (8.10 ± 4.19 years) ($p < 0.05$) and also was significantly higher in Group A compared to Group B ($p < 0.05$). Most of the diagnoses of AGD were made before the diagnosis of CD and age was an effective factor. There was no difference between Group 1 and Group 2 and Group A and Group B in terms of gender, typical/atypical CD ratio, tissue transglutaminase IgA (TTGA) level, human leucocyte antigen (HLA)-DQ2 and/or HLA-DQ8 positivity rate, and histopathological stage.

Conclusion: Although patients with a diagnosis of co-existent CD and AGD were significantly older than patients with isolated CD, gender, celiac symptoms, TTGA level, HLA type, and histopathological stage had no predictive value for the coexistence of AGD in patients with CD.

Keywords: Autoimmune glandular disease, Celiac disease, child, diabetes mellitus type 1, Graves disease, Hashimoto's thyroiditis

Introduction

Celiac disease (CD) is a chronic inflammatory enteropathy, characterized by inflammation of the proximal intestine, which is triggered by exposure to gluten, a protein present in dietary wheat, barley, and rye, in genetically susceptible individuals (1).

CD is reported to coexist with autoimmune glandular diseases (AGD) including type 1 diabetes mellitus (T1DM), Hashimoto's thyroiditis (HT), Graves disease (GD), as well as with other autoimmune diseases (2). In various studies, the frequency of T1DM in children with CD has been reported to be 3.2-11.0% (3,4,5,6,7). The human leucocyte antigen

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(HLA) allotypes that are risk factors for CD and T1DM are similar. HLA-DQ2 and HLA-DQ8 genotypes are found to be positive in 40% of the general population, while these are present in approximately 90% of individuals with a diagnosis of T1DM and 100% of individuals with a diagnosis of CD (8).

The prevalence of HT, which is the most common autoimmune thyroid disease (AITD), was found to be 1.2-3% and the prevalence of GD was reported to be 0.02%, in the pediatric age group (9,10). The frequency of AITD is higher in children with CD, and it has been reported to have a frequency of 2.4-41.4% in different populations (11). The coexistence of CD and an AITD is explained by a common genetic predisposition (12). In many studies it has been suggested that this relationship is due to similar HLA haplotypes or the defects of genes encoding the autoimmune-predisposing cytotoxic T-lymphocyte-associated antigen-4 (13,14,15).

The aim of this study was to determine the predictive factors for AGD in children with a pre-existing diagnosis of CD.

Methods

In this retrospective study, the files of 228 patients aged between 0-18 years who were diagnosed with CD between 2010 and 2019 in the Pediatric Gastroenterology Clinic of İnönü University Medical Faculty, were reviewed. Age at diagnosis, gender, symptoms at the time of diagnosis (typical/atypical), anthropometric findings [body weight, height, body mass index (BMI) and their respective standard deviation (SD) scores (SDS)], tissue transglutaminase IgA antibody (TTGA) levels, the presence of HLA DQ2 and HLA DQ8 genotypes, histopathological stage by endoscopic biopsy, and accompanying AGD's were recorded.

The diagnosis of CD was made according to the revised criteria of the European Committee of Pediatric Gastroenterology, Hepatology and Nutrition. The patients were considered positive if titration of TTGA increased 3 times the upper limit of normal values (18 Ru/mL). Histopathological staging was performed using the Modified Marsh-Oberhuber Classification, and patients with stage 2 and above were considered to have CD. The patients were divided as typical and atypical, according to the complaints at the time of diagnosis (16). The diagnosis of T1DM was made with a fasting blood sugar of 126 mg/dL and above, a postprandial blood sugar of 200 mg/dL and above, and a HbA1c value above 6.5% (17). The diagnosis of HT was made with the positivity of thyroid autoantibodies (anti-thyroglobulin Ab and/or anti-thyroid peroxidase antibody) in the patient (18). The diagnosis of GD was made with high free T3 and free T4 levels, low TSH level and positive anti-TSH receptor antibody (19).

Clinical and laboratory findings of the patients with AGD (Group 1) and those without AGD (Group 2) and the patients with T1DM (Group A) and those without T1DM (Group B) were compared.

Ethical approval (no: 2020/1351, date: 01.06.2021) for the study was obtained from the Scientific Research Ethics Committee of İnönü University and the study was carried out in accordance with the principles of the Helsinki Declaration.

Statistical Analysis

Statistical analyses of the data were performed using Statistical Package for the Social Sciences, version 20.0 (IBM Inc., Armonk, NY, USA). Normality of distribution of the data were examined using visual (histogram and probability charts) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive analyzes were expressed as percentage, mean ± SD (for normally distributed data), and median (minimum-maximum) (for non-normally distributed data). Normally distributed numerical data were compared by using independent samples t-test and non-normally distributed numerical data were compared using the Mann-Whitney U test. Pearson's chi-square and Fisher's Exact tests were used to compare the frequency rates of categorical variables. A value of $p < 0.05$ was considered statistically significant.

Results

The mean age at diagnosis of CD of the 228 patients included in the study was 8.35 ± 4.25 years and 69.3% (n = 158) of the patients were female. Most of the patients (n = 174, 76.3%) presented with atypical findings (short stature and anemia). AGD was detected in 8.8% (n = 20) of the patients, including T1DM in 13 (65%), T1DM and HT in 3 (15%), HT only in 2 (10%), T1DM and GD in 1 (5%), and GD only in 1 (5%). The mean age of the CD patients at the time of diagnosis of T1DM was 9.24 ± 4.53 years, and similarly the mean age of diagnosis of HT was 10.71 ± 2.57 years. GD was diagnosed when both patients were over the age of 15 years. T1DM was diagnosed before CD in ten patients, concurrently with CD in six patients, and after CD in only one patient. The diagnosis of HT was made before the diagnosis of CD in three patients, and after the diagnosis of CD in two patients. The diagnoses of GD were made simultaneously with CD.

The average age of diagnosis of CD was 10.93 ± 4.15 years

in cases with AGD (Group 1) but was significantly younger in patients without AGD (Group 2) at 8.10 ± 4.19 years ($p < 0.05$). There was no difference between Group 1 and Group 2 in terms of gender, typical/atypical CD ratio, serum TTGA level, HLA-DQ2 or HLA-DQ8 positivity rate, and histopathological stage. The mean weight SDS, height SDS, and BMI SDS were significantly higher in Group 1 compared to Group 2 ($p < 0.001$, $p = 0.003$ and $p = 0.01$, respectively) (Table 1).

The mean age at diagnosis of CD was 10.62 ± 4.09 years in patients with T1DM (Group A), and 8.17 ± 4.22 years in those without T1DM (Group B). The mean age at diagnosis of CD was significantly higher in Group A ($p < 0.05$). There was no difference between Group A and Group B in terms of gender, frequency of the presece of typical or atypical CD, serum TTGA level, HLA-DQ2 or HLA-DQ8 positivity rate, and histopathological stage. However, the mean weight SDS, height SDS, and BMI SDS were significantly higher in Group A compared to Group B ($p < 0.001$, $p = 0.006$ and $p = 0.001$, respectively) (Table 2).

The positivity of both HLA-DQ2 and HLA-DQ8 genotypes was approximately twice as frequent in Group 1 (22.2%) compared to Group 2 (11.2%), butthis was not statistically significant. Similarly, the positivity of both HLA-DQ2 and HLA-DQ8 genotypes was 26.7% in Group A, and was more than twice as frequent as in Group B (11%), but again the difference was not statistically significant (Tables 1, 2).

Discussion

There are studies reporting that the prevalence of autoimmune diseases are higher in children with CD compared to the normal population. Ventura et al. (20), found the prevalence of autoimmune disease was 14% in 909 Italian patients between the ages of 10 and 25 with a diagnosis of CD and 2.8% in controls ($p < 0.001$). In the same study, the frequency of AGD was 6.3%, and the most common autoimmune disease was T1DM (3.9%) (17). In a study conducted in Iran, it was reported that 15.4% of 130 pediatric patients diagnosed with CD had T1DM and 7.7% had hypothyroidism (5). In a study conducted in India, on 363 patients with CD aged between 2 and 50 years (mean 19 years), it was found that T1DM was present in 3.5%, hypothyroidism in 3%, and GD in 0.2% (21). In a study conducted in Turkey, it was reported that AGD was present in 8.7% of 148 pediatric CD patients (4% T1DM, 4.7% HT) (4). Another study conducted in Turkey reported that anti-thyroid antibodies were negative in all of the pediatric patients with CD, but after 2-3 years, 16.4% (11/67) of the patients became positive. It has been reported that only 3/11 (27.2%) CD patients with positive anti-thyroid antibodies have clinical hypothyroidism (22). In our study, the prevalence of AGD in pediatric patients with CD was 8.8%, and, as previously reported, the most common accompanying diseases were T1DM (7.5%) and HT (2.2%). In our study, the prevalence of T1DM detected in children with CD was relatively high compared to the prevalence in the general pediatric population in Turkey (0.075%) (23). Again in our study, the prevalence of GD in children with CD was 0.9% and this rate was found to be significantly higher compared to the general pediatric population (0.02%), while the prevalence of HT was similar to the general pediatric population. Although the rates vary according to

Table 1. Comparison of CD patients with (Group 1) or without (Group 2) an accompanying autoimmune disease

	Group 1 (n = 20)	Group 2 (n = 208)	p
Age	10.93 ± 4.15	8.10 ± 4.19	0.004
Gender	80% female 20% male	68.3% female 31.7% male	0.277
Clinical findings	90% atypical 10% typical	75.4% atypical 24.6% typical	0.174
Weight SD	-0.77 (-2.11-1.74)	-1.6 (-5.15-7.8)	0.001
Height SD	-0.77 (-3.2-1.08)	-1.73 (-2.5-1.06)	0.003
BMI SD	-0.19 (-3-1.4)	-0.82 (-8.17-2.26)	0.01
TTGA level	100 (54.9-300)	100 (54-300)	0.831
Positive HLA DQ2	88.9%	86.7%	1.000
Positive HLA DQ8	27.8%	23.0%	0.771
Positive HLA DQ2&DQ8	22.2% (4/18)	11.2% (21/188)	0.245
Hystopathological examination (Marsh-Oberhuber staging distribution)	10.0% type 2 30.0% type 3A 40.0% type 3B 20.0% type 3C	5.3% type 2 31.7% type 3A 43.3% type 3B 19.7% type 3C	0.856

SD: standard deviation, BMI: body mass index, HLA: human leucocyte antigen, CD: Celiac disease, TTGA: tissue transglutaminase IgA

populations, it has been reported that the prevalence of AGD is higher in children with CD, and T1DM or AITD are the most common AGDs. Moreover, the prevalence of CD in children with T1DM was higher (0.6-16.4%) than the general population (3). In these patients, CD is often asymptomatic or presents with atypical symptoms. As delayed diagnosis increases morbidity, it is recommended to screen for CD in children with T1DM (16).

In the literature, it was not specified which disease was diagnosed first in cases with concomitant CD and AGD. In our study, all of the cases of accompanying CD were diagnosed simultaneously with T1DM or as a result of screening performed following the diagnosis of T1DM. It was thought that, this situation caused the frequency of T1DM to be found misleadingly high in CD. In contrast, Nijhawan et al. (21) reported that in 10 of 13 (76.9%) patients with accompanying T1DM and CD, CD was diagnosed before T1DM and T1DM was detected later during screening. In order to clarify this issue, there is a need for prospective studies examining the frequency of AGD in patients diagnosed with CD.

More than one autoimmune disease can be present in CD patients. Ventura et al. (20), found that multiple autoimmune diseases (coexistence of AGD and other autoimmune diseases like dermatitis herpetiformis, alopecia areata, psoriasis etc.) were present in 1.7% (16/909) of the patients with CD, and multiple AGD were present in only three patients. In our study, T1DM and HT were found to accompany CD in three patients and T1DM and GD were found concurrently in one patient with CD.

In the literature, although the frequency of AGD in patients with CD has been reported in various studies, there is little information about the clinical or laboratory characteristics of patients with an accompanying AGD. Ventura at al. (20), reported that, the frequency of accompanying autoimmune diseases (T1DM and AITD) increased as the age of diagnosis increased in patients with CD. They reported that, this rate was four times higher in children diagnosed with CD after 10 years of age compared to those who were diagnosed at the age of two years. They reported that the age at the time of diagnosis is the only significant predictor of the development of an autoimmune disease ($r = 0.3$; $p < 0.001$) (17). In a study conducted by Rasheed et al. (24), it was reported that the mean age of the children with an accompanying AITD at the time of the diagnosis of CD was higher compared to those without AITD. However, the timing of diagnosis was not specified in either of the studies, so whether CD preceeded AGD was not clear. In our study, consistent with the above mentioned studies, the mean age at the time of diagnosis of CD was found to be higher in cases with an AGD. However, as most of the patients in our cohort were diagnosed with CD simultaneously with AGD or after the diagnosis of in asymptomatic cases, there may be some bias in the age of diagnosis which may be misleadingly high. In a prospective study conducted by Kalyoncu and Urganci (22), it was reported that CD patients with positive antithyroid antibodies were significantly younger compared to patients with negative antithyroid antibodies.

In our study, no difference was found between the CD patients with an AGD or T1DM and those without in terms of gender, symptoms on admission, serum TTGA levels, HLA allotypes, and histopathological CD stage. In two studies conducted previously, it was found that gender had no effect on the frequency of an accompanying autoimmune

Table 2. Comparison of CD patients with (Group A) or without (Group B) an accompanying T1DM

	Group A (n = 17)	Group B (n = 211)	p
Age	10.62 ± 4.09	8.17 ± 4.22	0.022
Gender	76.5% female 23.5% male	68.7% female 31.3% male	0.505
Clinical findings	88.2% atypical 11.8% typical	75.7% atypical 24.3% typical	0.372
Weight SD	-0.56 (-2 to 1.74)	-1.6 (-5.15 to 7.8)	< 0.001
Height SD	-0.77 (-3.2 to 1.08)	-1.73 (-2.5 to 1.06)	0.006
BMI SD	-.08 (-1.22 to 1.4)	-0.84 (-8.17 to 2.26)	0.001
TTGA level	100 (54.9-300)	100 (54-300)	0.799
Positive HLA DQ2	93.3%	86.4%	0.698
Positive HLA DQ8	33.3%	22.6%	0.350
Positive HLA DQ2&DQ8	26.7% (4/15)	11.0% (21/191)	0.091
Hystopathological examination (Marsh-Oberhuber staging distribution)	11.8% type 2 29.4% type 3A 35.3% type 3B 23.5% type 3C	5.2% type 2 31.8% type 3A 43.6% type 3B 19.4% type 3C	0.66

T1DM: type 1 diabetes mellitus, SD: standard deviation, BMI: body mass index, HLA: human leucocyte antigen, CD: Celiac disease, TTGA: tissue transglutaminase IgA

disease (20,22,24). To the best of our knowledge, there are no studies evaluating other parameters. In our study, anthropometric measurements of the patients were also evaluated. The higher mean values of weight, height and BMI in CD patients with an accompanying AGD or T1DM can be explained by the fact that the mean age at the time of diagnosis was higher in these groups. The higher mean weight SDS, height SDS, and BMI SDS in groups with accompanying AGD or T1DM compared to those without may be due to the higher but non-significant rate of patients presenting with atypical clinical presentation in these groups. Especially in patients with T1DM, the diagnosis of CD was mostly made by screening during or after the diagnosis of T1DM. Therefore, we hypothesize that anthropometric findings were less affected in these groups.

It is known that autoimmune diseases are associated with alleles in genes in the major histocompatibility complex, especially DQ2 (DQA1*05/ DQB1*02) and DQ8 (DQA1*0301/ DQB*302) (25). It has been reported that the HLA DQ2 and/or DQ8 locus is the most important predictor of susceptibility to T1DM (8). In the literature, the presence of HLA-DQ2/ HLA-DQ8 alleles or HLA-DR3/HLA-DR4 alleles has been reported as a risk factor for accompanying T1DM in CD patients (26). In our study, DQ2 was found to be positive in 93.3% of the patients with T1DM. HLA-DQ2 and HLA-DQ8 genotypes were found to be present together, twice as often in the group with T1DM compared to the group without T1DM and in the group with AGD compared to the group without AGD, but the frequency difference was not statistically significant. The lack of a significant difference is likely due to the small number of patients.

Understanding of the likelihood of AGDs that may accompany CD and screening for CD in these patients will facilitate early diagnosis and treatment. It has been reported that a gluten-free diet improves the metabolic control of diabetes, has a protective effect on the development of vascular complications, and prevents growth retardation in patients with T1DM and CD (27). In addition, increasing awareness of other autoimmune diseases that may accompany CD in children is important for their early diagnosis and treatment.

Study Limitations

The retrospective nature of our study and small sample size are limitations, and prospective studies with a larger number of patients are needed.

Conclusion

In our study, although patients with a diagnosis of co-existent CD and AGD were significantly older than patients with isolated CD, gender, celiac symptoms, TTGA level, HLA type, and histopathological stage were not found to have a predictive role in predicting the presence of AGD in CD patients. There is a need for prospective studies in larger pediatric patient populations.

Ethics

Ethics Committee Approval: Ethical approval (no: 2020/1351, date: 01.06.2021) for the study was obtained from the Scientific Research Ethics Committee of İnönü University and the study was carried out in accordance with the principles of the Helsinki Declaration.

Informed Consent: Retrospective study.

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Authorship Contributions

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

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Effect of Propolis on Precocious Puberty in Female Rats

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What is already known on this topic?

Nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset.

What this study adds?

This study investigated the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats. Although propolis has estrogenic effects, to the best of our knowledge, no previous study has evaluated the relationship between propolis and puberty onset. This study provides comprehensive information about the stimulant effect of propolis on puberty.

Abstract

Objective: Nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset. The aim of this study was to identify the effects of propolis, which has been reported to have oestrogenic effects, on precocious puberty and the reproductive system in prepubertal female rats (ovary, endometrium, breast).

Methods: Thirty-four 25-day-old, prepubertal, female Sprague-Dawley rats were included. Rats were randomly divided into the propolis (n = 17) and control groups (n = 17). The primary endpoint was the number of rats that developed vaginal opening, a sign of puberty, at 12-day follow-up. In addition, the effect of propolis on ovary, uterus and breast tissue was evaluated histologically.

Results: Vaginal patency occurred earlier (about 7.5 days sooner) in the propolis group and all animals in the propolis group had vaginal patency by day 12. The number of ovarian follicles (in all follicles), endometrial thickness, and mammary gland secretory gland area were significantly higher in the propolis group than in the control group (all $p < 0.001$). In addition, Ki-67 activity in the endometrium, breast tissue and ovary was more intense in the propolis group compared to the control group (all $p < 0.001$).

Conclusion: Propolis triggers precocious puberty in female rats, possibly by interacting with the oestrogen receptor. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

Keywords: Phytoestrogens, propolis, precocious puberty, rat

Introduction

Adolescence is the transition period from childhood to adulthood and involves the development of secondary sexual characteristics and reproductive ability, and sexual maturation (1). Even under similar living conditions, the timing of puberty varies significantly among individuals, suggesting that many factors affect the onset of puberty, such as genetic and environmental factors, socioeconomic status, stress, metabolic rate, bone maturation, and body fat ratio (2,3,4). In addition, nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens, which have oestrogen-like effects, are environmental factors that affect puberty onset (2,5,6,7,8). Propolis is a product from *Apis mellifera* (honey bee) hives, containing plant resins, beeswax, and minor

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constituents, including pollen and minerals (9). Propolis is very heterogeneous and the composition is dependent upon plant sources and/or types of bees (10). Although propolis has estrogenic effects (11,12,13), to the best of our knowledge, no previous study has evaluated the relationship between propolis and pubertal onset. The aim of this study was to investigate the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats.

Methods

This study was approved by the Animal Experiments Local Ethics Committee, Sakarya University, Turkey (date: 01.07.2020, decision no: 34). Thirty-four 25-day-old, prepubertal, female, Sprague-Dawley rats were included. The number of rats was determined using G Power analysis (95% confidence interval, 80% power). Rats were randomly divided into the propolis (n = 17) and control groups (n = 17). The weight of the rats was recorded before the experiments. The rats were sedated using anaesthetic doses of ketamine and xylazine; blood was obtained from the rats to measure the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), oestradiol, and testosterone. Water-soluble propolis (1 cc at a dose of 200 mg/kg; based on other similar studies) (11,14) was administered to the propolis group by gavage for 12 days (equivalent to approximately 1 human year, comparing relative expected life-spans). Water-soluble propolis contained 10% pure propolis and was prepared using water and glycol solution for gavage. The content of the propolis used is presented in Supplementary Table 1. The control group was administered 1 cc of water by gavage. The animals were provided food and water *ad libitum*. To determine the time of puberty onset, vaginal openness was measured, and estrous cycle status was observed with vaginal smear at baseline and then daily. Vaginal openness was measured at baseline and then daily to determine the time of puberty onset. The number of rats attaining puberty after 12 days of treatment was recorded. After 12 days of treatment, the rats were weighed. Then the rats were sedated with the appropriate anaesthetic dose, blood was obtained to measure hormone levels, and the rats were sacrificed. Uterine, ovarian, and breast tissues were obtained for histopathological and immunohistochemical evaluation. This study was not designed as part of a translational medicine study.

Histopathological and Immunohistochemical Evaluation

The tissue samples were fixed with 10% formalin solution for 48 hours and dehydrated with 60%, 70%, 80%, 96%, and 100% alcohol. Then the samples were passed through a xylol series to render the tissues transparent. The tissues were embedded in paraffin and cut using a microtome. The sections were stained with hematoxylin-eosin to observe the histological changes in the ovary, endometrium, and mammary gland tissues. Photographs were acquired under a light microscope (Olympus CX31-Japan). Ten sections (10 µm each) were obtained from each ovary to determine the effects of propolis on the number of follicles. Only follicles with oocyte nuclei were counted to determine the follicle count. Follicles were classified into five stages: primordial, primary, secondary, antral, and atretic follicles (15). The automated image analysis software, Image J®, was used to measure endometrial thickness (in µm). All slides were examined under the microscope at 100× magnification (14). Mammary gland tissues were examined using a Nikon eclipse inverted microscope (Nikon Corp., Tokyo, Japan), and the area (μm^2) of secretory epithelium and fat cells and the area of stroma were calculated using the NIS-element imaging system from the same manufacturer. The ratio of

Table 1. Comparison of the laboratory data of rats in the propolis and control groups

	Control group (n = 17) Median values (min-max)	Propolis group (n = 17) Median values (min-max)	p
Starting weight (g)	50 (30.1-61.9)	46.6 (38.2-52.3)	0.540
Final weight (g)	92 (77.8-104.0)	93.4 (76.6-104.8)	0.812
Starting LH	1.12 (0.57-2.13)	1.40 (0.70-2.97)	0.345
Final LH	1.39 (0.61-2.88)	1.52 (0.77-3.83)	0.563
Starting FSH	2.35 (1.88-3.13)	2.70 (1.43-4.17)	0.160
Final FSH	2.48 (1.61-3.55)	2.63 (2.35-3.52)	0.170
Starting oestradiol	86.48 (63.2-114.3)	86.48 (54.9-119.1)	0.683
Final oestradiol	62.83 (48.8-83.0)	77.78 (60.3-108.9)	0.020*
Starting testosterone	209.30 (160.3-272.2)	200.30 (98.6-302.3)	0.540
Final testosterone	146.10 (93.1-278.4)	276.50 (200.7-338.5)	< 0.001*

Mann-Whitney used for continuous variables. *Statistical significance ($p < 0.05$).
FSH: follicle stimulating hormone, LH: luteinizing hormone, min-max: minimum-maximum

the area of the secretory epithelium and fat cells to the area of the stroma was then calculated (16). Ki-67 staining was used to demonstrate tissue stimulation and proliferation in the endometrium, mammary glands, and ovaries. Four micron thick tissue samples were cut from paraffin-embedded blocks and deparaffinized using a decreasing alcohol series. Citrate buffer was heated in the microwave for 20 minutes. Endogenous peroxidase activity was blocked with 3% H_2O_2. The primary antibody used was anti-Ki-67 (1/400 dilution, GeneTex; Cat. No: GTX16667; USA). The secondary antibody [Ultra Vision Large Volume Detection System Anti-rabbit by LabVision, conjugated with horse radish peroxidase (HRP)] was used in accordance with the manufacturer's instructions. DAB (3,3'-diaminobenzidine) was used for immunohistochemical staining of HRP-conjugated secondary antibody-labeled proteins in tissues. Mayer's hematoxylin was used as the counterstain. The prepared slides were covered in mounting medium (Aqueous Mounting Medium by ScyTek). Proliferative activity, as assessed by Ki-67 staining, was semi-quantitatively analysed (h-score) by selecting 10 random fields, and 100 epithelial cells were photographed in each area. The Ki-67 index was calculated as the percentage of positively stained cells among the total cells assessed (17,18). In the immunohistochemical analysis, Ki-67 staining and cell division rates in the mammary glands, ovary, and endometrium were compared between the control and propolis groups.

Hormonal Assessment

The rats were sacrificed and blood samples were collected. When the specimens had completely clotted, they were centrifuged at 1500 *g* for 10 minutes. Serum fractions were collected and frozen at -40 °C until further use. LH, FSH, testosterone, and oestradiol levels were determined using a double antibody enzyme-linked immunosorbent assay [YLBiont brand Sandwich enzyme linked immunosorbent assay (ELISA); Shanghai YL Biotech Co., Ltd., Shanghai, China] containing hormone-specific monoclonal antibody coated wells. 40 µL of rat serum and 10 µL of antibody were added to the wells. 50 µL of streptavidin HRP conjugate was added to all wells except the blank well (standard and sample well) and the wells were incubated at 37 °C for 60 min. After incubation, the wells were washed to remove unbound antibody. The specimens were incubated with chromogen at 37 °C for 10 min to develop a blue colour. Stop solution was added to terminate the reaction, reflected by a change in the colour of the solution from blue to yellow. The intensity of the yellow colour was directly proportional to the analyte concentration. The colorimetric readings were performed using the inappropriate wavelength for the micro ELISA reader. A standard curve was generated to calculate the sample concentrations. The results and the measurement range were specified as rat LH 0.1-38 mIU/mL, rat FSH 0.2-60 mIU/mL, rat testosterone 10-3000 ng/L, rat oestradiol 3-900 ng/L respectively. The within-run and between-run CV% of the assays were given as $<10\%$.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 20.0 software (IBM Inc., Chicago, IL, USA). Numerical variables were summarized using median values (minimum-maximum) as appropriate. Normality of the numerical variables was assessed with the Kolmogorov-Smirnov test. To compare independent groups, the number of rats in the groups was low and therefore nonparametric tests were used, including the Mann-Whitney U test. A p-value less than 0.05 was considered statistically significant.

Results

Laboratory, histopathological, and immunohistochemical data are presented in Tables 1 and 2. Histopathological and immunohistochemical images are shown in Figures 1 and 2, respectively. The control and propolis groups had similar initial ($p=0.535$) and final weights ($p=0.809$) and baseline levels of LH ($p=0.241$), FSH ($p=0.158$), testosterone ($p=0.524$), and oestradiol ($p=0.667$). On day 12, the oestradiol and testosterone levels were higher in the propolis than control group ($p=0.021$ and $p<0.001$, respectively). The testosterone level decreased from baseline to day 12 in the control group, whereas it increased in the propolis group. Although the oestradiol level decreased in both groups, the decrease was smaller in the propolis compared with the control group. Vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up in the control group, whereas it was observed in all rats in the propolis group. Furthermore, in the propolis group nine (52.9%) rats exhibited vaginal openness on day 4 and eight (47.1%) rats on day 5. The number of ovarian follicles, endometrial thickness, and mammary gland secretory area were significantly higher in the propolis than control group ($p<0.001$, $p<0.001$, and $p<0.001$, respectively). In addition, Ki-67 activity in the endometrium, breast, and ovarian tissues was greater in the propolis than control group ($p<0.001$, $p<0.001$, and $p<0.001$, respectively).

Discussion

Many factors affect the age of puberty onset, including nutrition and exposure to environmental pollution, insecticides, and plant phytoestrogens, which have

oestrogen-like effects (2, 5-8). Propolis consists of many chemicals that vary depending on the type of plant the bees have collected the pollen and nectar from. Several studies have reported that some of these chemicals, such as flavonoids, coumaric acids, and caffeic acids, have oestrogen-like activity (11,12). Okamoto et al. (11) showed that propolis increased the uterine wet weight and endometrial thickness in ovariectomized rats and stimulated ductal cell proliferation in the mammary glands via the oestrogen receptor. In the present study, propolis increased the endometrial thickness and secretory area of adipose tissue in the mammary glands. Additionally, it increased the number of follicles in the ovaries and Ki-67 staining in the ovary, uterus, and breast tissues, suggesting increased cell proliferation.

In female rats, vaginal opening, the first external sign of ovarian activity, is considered a sign of puberty and occurs at approximately postnatal 35-37 days. The estrous cycle may start immediately after vaginal opening or within a week (19,20). Our study showed that the vaginal opening developed significantly earlier in the propolis compared with the control group. In the control group vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up, whereas it was observed in all rats in the propolis group. Furthermore, in the propolis group nine rats exhibited vaginal openness on day 4 and eight rats on day 5. In addition, while none of the rats in the control group could enter the estrous cycle (in vaginal smear), all the rats in the propolis group were in the estrous cycle.

Although genistein is predominantly found in soy, it is also one of the main components of propolis and estrogenic effect has been shown in previous studies (21,22,23,24). Chrysin, the flavone group found in propolis, was found to inhibit the aromatase enzyme in most *in vitro* studies, leading to reduced oestrogen production (25). A human study found no increase in urine testosterone level after chrysin administration, suggesting that aromatase was not inhibited (26). In the present study, the testosterone level was higher in the propolis than control group, suggesting that the propolis used in this experiment inhibits aromatase. However, the oestradiol level was also significantly higher in the propolis group at the day 12 timepoint.

This study also demonstrated a significant stimulating effect of propolis on the ovary, endometrium, and breast. However, the unexpected low estrogen level detected at the end of the study was interpreted as the increase in the cumulative estrogenic effect due to the estrogen-like effect of propolis rather than a direct estrogen increase. These changes may be due to a large intra-cycle oestradiol change due to the high number of rats in the estrous cycle in the propolis group. For this reason, estradiol values can be very different according to the period of the rats in the propolis group (especially in the proestrus period). In fact, it is inaccurate to compare estradiol between groups because we do not know exactly what stage of the estrous cycle the rats in the propolis group were in at sacrifice. At the end of the study, no difference was found in the gonadotropin level between the propolis and control groups. Presumably, propolis triggers precocious puberty by interacting with the oestrogen receptor and oestradiol/testosterone ratio, rather than increasing the gonadotropin or oestradiol level. In addition, the steroid/oestrogen-like side chain rings of some flavonoids/phenolics, which are abundant in propolis, may induce changes in the steroid pathway and trigger precocious puberty due to interaction with the oestrogen receptor. This condition can only be determined by conducting studies at the estrogen receptor level. Contrary to our study, some

Table 2. Comparison of the immunohistochemical findings in the propolis and control groups

	Control group (n = 17) Median values (min-max)	Propolis group (n = 17) Median values (min-max)	p values
Number of primordial follicles	52 (37-59)	61 (54-65)	**< 0.001***
Number of primary follicles	24 (20-29)	32 (25-36)	**< 0.001***
Number of secondary follicles	4 (2-6)	12 (9-15)	**< 0.001***
Number of antral follicles	3 (1-4)	5 (4-6)	**< 0.001***
Number of atretic follicles	2 (0-2)	4 (3-5)	**< 0.001***
Endometrial thickness (μm)	178 (156-189)	193 (185-198)	**< 0.001***
Mammary gland entire area (μm^2)	44.18 (39.1-49.3)	77.84 (69.5-95.7)	**< 0.001***
Mammary gland secretory area (μm^2)	6.64 (4.1-45.1)	36.08 (32.1-39.5)	**< 0.001***
Ovarian Ki-67 (%)	15 (9-22)	33 (25-44)	**< 0.001***
Endometrium Ki-67 (%)	13 (7-16)	40 (35-46)	**< 0.001***
Mammary gland Ki-67 (%)	17 (10-21)	43 (39-46)	**< 0.001***

Mann-Whitney used for continuous variables. *Statistical significance ($p < 0.05$).
Min-max: minimum-maximum

studies have shown that polyphenols in green tea prevent prepubertal puberty (27,28). This opposite effect of green tea compared to propolis may be due to the different ratios of polyphenols in the food used as the proportion of catechin was high in green tea, while the proportion of chrysin and caffeic acid phenethyl ester was higher in the propolis used in the present study.

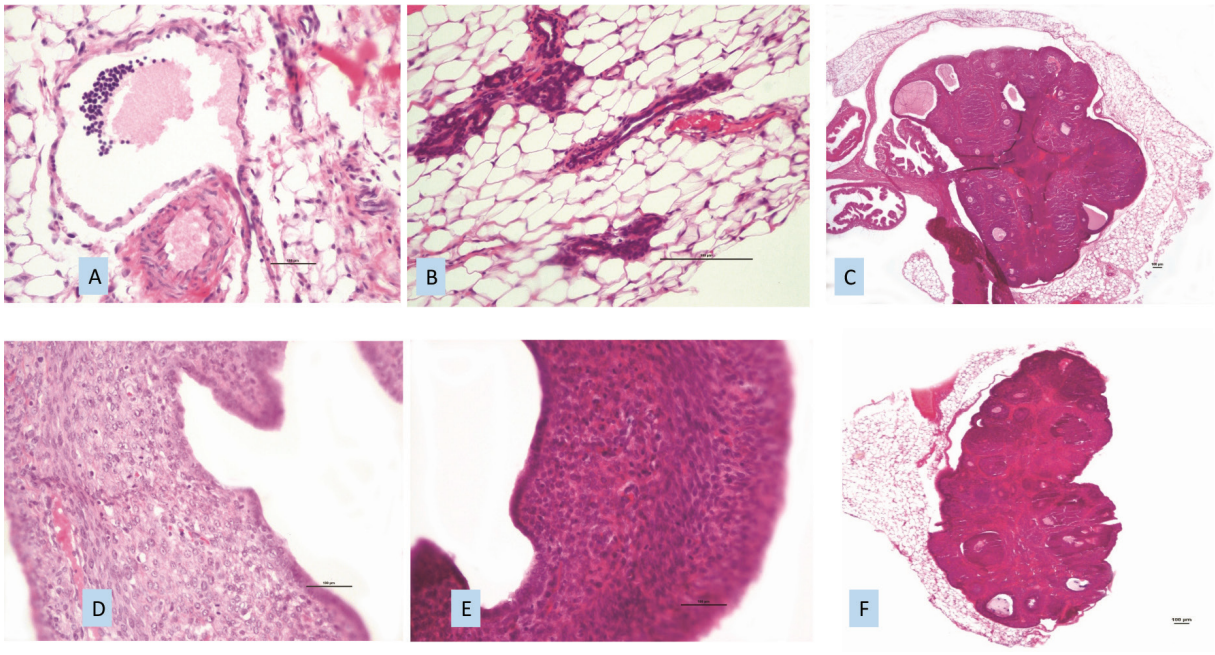

Figure 1. Larger secretory areas (active state) were observed in the adipose tissue of the mammary glands in the propolis group (A) than control group (B). In ovarian tissue, there were more secondary, antral, and corpus luteum follicles in the propolis group (C) than control group (F). The endometrial layer was thicker in the propolis group (E) than control group (D). hematoxylin-eosin pictures. 40x lens, 100 µm scale bar

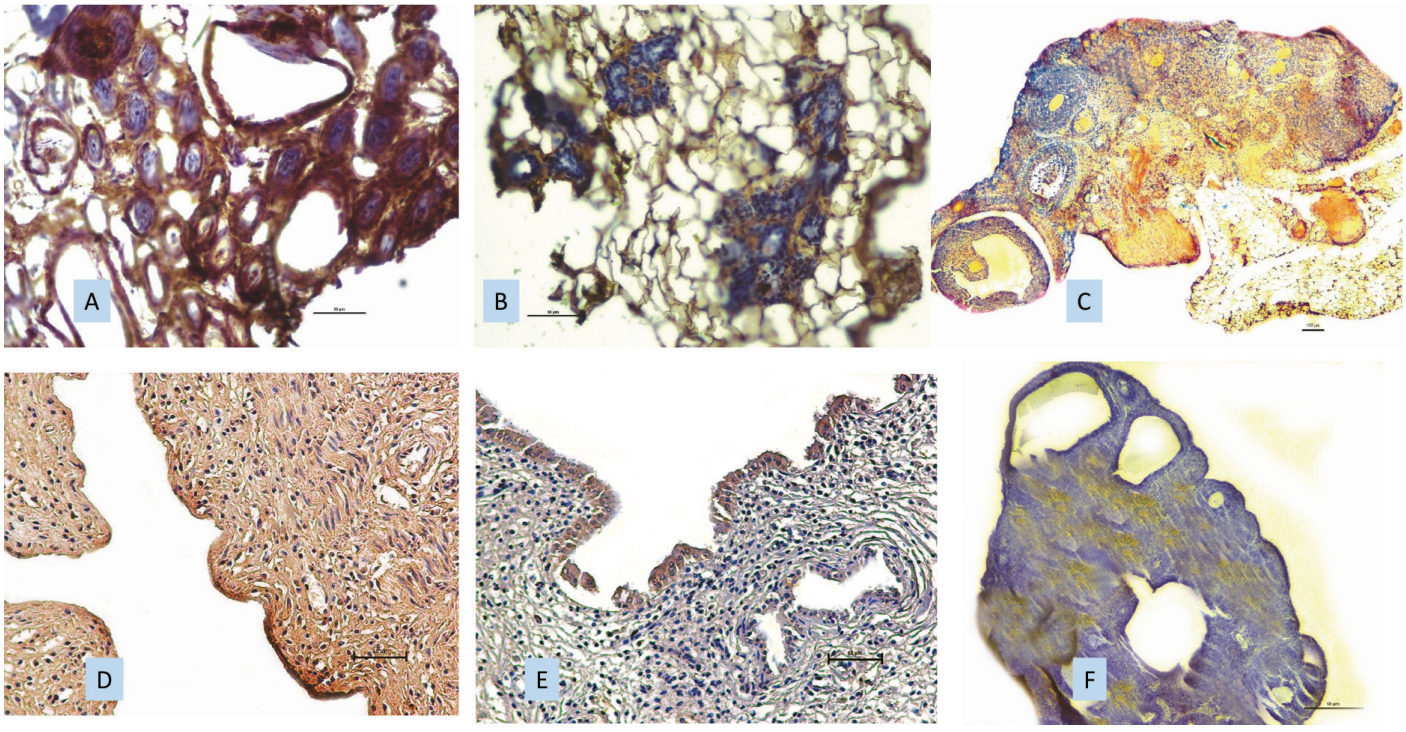

Figure 2. Due to cell development and proliferation, Ki-67 staining intensity was greater in the mammary glands, ovary, and endometrium (due to thickening) in the propolis group (A, C, and D, respectively) compared with the control group (B, E, and F, respectively). Ki-67 immunoreactivity preparations. 200x lens, 100 µm scale bar

Study Limitations

The small sample size limits our results. To account for this, conservative statistical methods, including nonparametric tests, were employed to mitigate the risk of type 1 error. Clinical findings and histological findings were supported by immunohistochemical staining.

Conclusion

This was the first study to evaluate the relationship between propolis and puberty, to the best of our knowledge. Propolis triggered precocious puberty in female rats, possibly by interacting with the oestrogen receptor. This is an interesting finding which should be investigated further. However, this finding does not yet have a direct impact on clinical practice. Composition and effective dose of propolis varies from product to product. Moreover, duration of use, differences in individual receptor sensitivity, and exposure to other chemicals with anti-estrogenic/androgenic effects (cumulative effect) might differ for each individual. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

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The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/yFiRRO

Ethics

Ethics Committee Approval: This study was approved by the Animal Experiments Local Ethics Committee. Sakarya University, Turkey (date: 01.07.2020, decision no: 34).

Informed Consent: Animal experiments.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Recep Polat, Fatıma Betül Tuncer, Design: Recep Polat, Erdem Çokluk, Özcan Budak, Data Collection or Processing: Recep Polat, Erdem Çokluk, Özcan Budak, Fatıma Betül Tuncer, Analysis or Interpretation: Recep Polat, Erdem Çokluk, Özcan Budak, Fatıma Betül Tuncer, Literature Search: Recep Polat, Erdem Çokluk, Fatıma Betül Tuncer, Writing: Recep Polat, Erdem Çokluk, Özcan Budak.

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Click for Supplementary Table 1 access link: http://glns.co/fkyd7

ORIGINAL ARTICLE

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The Effect of Growth Hormone Therapy on Cardiac Outcomes in Noonan Syndrome: Long Term Follow-up Results

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What is already known on this topic?

Noonan syndrome (NS) is a multisystem disorder, with short stature and cardiac anomalies being the most common systemic effects. Theoretically, there is a risk of increased ventricular wall thickness and cardiac side effects associated with the use of recombinant human growth hormone (rGH).

What this study adds?

This study aimed to investigate the effect of rGH use in patients with NS on ventricular wall thickness and a possible increased risk of cardiac side effects. Patients were divided into two groups based on whether or not they received rGH. There was no difference in this cohort between the rGH and non-rGH groups in terms of echocardiographic parameters, pre-and post-treatment.

Abstract

Objective: Cardiac involvement is common in Noonan syndrome (NS). Concerns have been raised regarding the effect of recombinant growth hormone (rGH) use on ventricular wall thickness and a possible increased risk of cardiac side effects. This study aimed to investigate the effect of rGH on the development of hypertrophic cardiomyopathy and other cardiac findings in NS.

Methods: Patients under the age of 18 years and diagnosed with NS according to the Van der Burgt criteria, were included. Patients were divided into two groups according to those receiving rGH or not at the time of obtaining cardiac measurements. Before and after the treatment, electrocardiographic and echocardiographic (ECHO) assessments were made, including interventricular septal thickness, left ventricular internal diameter, and left ventricular posterior thickness. Results were expressed as Z scores.

Results: Twenty-four NS subjects (16 boys, eight girls) were included. At the beginning of the follow up, the overall height standard deviation score was -2.56 ± 0.94. Sixteen were on rGH. The mean rGH treatment duration was 8.3 ± 3.8 years, and the mean dose was 0.22 ± 0.04 mg/kg/week. The final height was 169 ± 8.2 cm, and 10 of 11 patients who reached the final height received rGH. There was no difference between the rGH and non-rGH groups in terms of ECHO parameters pre-and post-treatment.

Conclusion: In this cohort, there was no change in ECHO parameters on rGH and during follow-up. These results suggest that rGH is safe in NS patients with cardiac pathology under close follow-up.

Keywords: Recombinant growth hormone therapy, Noonan syndrome, hypertrophic cardiomyopathy, left ventricular dimension

Introduction

Noonan syndrome (NS; OMIM 163950) is characterized by typical facial findings (hypertelorism, ptosis, downward-sloping palpebral fissures, epicanthus, depressed nasal bridge, low-set posterior-turned ears, and micrognathia), developmental delay, learning disability (usually mild), short stature, congenital heart disease, kidney anomalies, lymphatic malformations, and bleeding disorders (1,2,3). Mutations in the RAS-mitogen-activated protein kinase (RAS-MAPK) pathway cause NS by changing protein-coding genes (4). To date, 20 genes associated with NS (*PTPN11, SOS1, SOS2, KRAS, NRAS, RIT1, RRAS, RASA1, RASA2, MRAS, RAF1, BRAF, MAP2K1, MAP3K8, SHOC2, PPP1CB, SPRY1,*

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LZTR1, MYST4, A2ML1, CBL) have been identified. Over the years, the identification of genes associated with NS has increased (1). Candidate genes may be associated with clinically diagnosed cases without molecular confirmation.

Congenital heart disease is frequently described in patients with NS. The syndrome is inherited mostly in an autosomal dominant pattern. It has an estimated incidence of 1:1000 to 1:2500 live births (2,3,4,5). More than 80% of the cases have cardiovascular system anomalies. The most common congenital heart defect is pulmonary stenosis (PS), with a rate of about 40%. Twenty percent of patients have hypertrophic cardiomyopathy (HCMP) with asymmetric septal hypertrophy. Other common cardiac anomalies in NS include atrial septal defect (ASD) in 6-10%, mitral stenosis (MS) in 6%, aortic stenosis (AS) and aortic coarctation in 9%, ventricular septal defect (VSD) in 5%, and patent ductus arteriosus in 3%. HCMP can be mild or severe and present from prenatal ages to late childhood. In some infants, HCMP resolves, while in others, it becomes rapidly progressive and can be fatal. Left obstructive lesions can also develop in adulthood (1,2,3,4).

Recombinant growth hormone (GH) therapy (rGH) has been given to patients with NS because of short stature, typical of NS. It has been reported that the use of rGH in NS can result in reaching the average height of normal healthy adult individuals (4). However, persistent high insulin-like growth factor-1 (IGF-1) levels may cause pathological cardiac hypertrophy and heart failure (2). Thus there are concerns about the use of rGH in NS because of the already existing cardiac involvement in the pathophysiology of the disease and the high frequency of cardiac hypertrophy. Theoretically, the risk of increased ventricular wall thickness and cardiac side effects associated with the use of rGH is predicted, but studies providing empirical evidence in this area are very limited (Table 1) (6-16).

The aim of this study was to describe the presence of accompanying cardiac anomalies and the effects of rGH use in NS patients. Other objectives were to investigate the cardiac effects of rGH treatment on ventricular wall thickness and other possible cardiac anomalies during follow-up in patients with NS who did not receive rGH, and to increase awareness of the importance of cardiac monitoring in pediatric NS.

Methods

The study was approved by the Ankara University Faculty of Medicine Local Ethics Committee (decision number:

Table 1. The studies, which were published earlier about the cardiac involvement in Noonan syndrome

Study	n	Mean rGH dose, mg/kg/week	Mean duration of therapy, years	Result	Cardiac safety of rGH
Brown et al. (6)	23	0.33	3	HCMP did not develop in the follow-up.	√
Noordam et al. (7)	27	0.35	3	Long-term use of high-dose rhGH has no effect on left ventricular thickness.	√
Ozono et al. (8)	51	0.23	2	No change in ventricular wall thickness with rGH	√
Noordam BJOK (9) KIGS study	85	0.25	3	No relationship between these cardiac events and rGH	√
Romano et al. (10) NCGS	65	0.28-0.38	5.6 ± 2.6*	No cardiac side effects	√
Osio et al. (11)	25	0.33 (n = 10), 0.66 (n = 15)	2 (1-9)	No cardiac side effects	√
Lampit et al. (12)	22	0.9**	2	No effect of rGH treatment on left ventricular wall thickness	√
MacFarlane et al. (13)	23	0.33	3	One mild left ventricular hypertrophy at initial evaluation had no change. The other two subjects developed an increase in left ventricular wall thickness, close to the upper limit, without other features of HCMP.	√
Apperley et al. (14)	12	0.25	3	No progression in findings or cardiac side effects	√
Cotterill et al. (15)	27	0.33	1	No change in the cardiac mass in the treated group	√
Romano et al. (16)	412	0.33	3	One abdominal aortic aneurysm, one PS, three unspecified cardiovascular event	√
Our study	24	0.22	4.5	No worsening of cardiac findings, and no change in ECHO parameters in either the rGH group or the non-rGH group.	√

*Mean ± SD. **mg/m^2/day.
rGH: recombinant growth hormone therapy, HCMP: hypertrophic cardiomyopathy, PS: pulmonary stenosis, NCGS: National Cooperative Growth Study, KIGS: The International Growth Database, ECHO: echocardiography, rhGH: recombinant human growth hormone

I2-93-21, date: 11.02.2021). Children and adolescents diagnosed with NS, either clinically according to the Van der Burgt (4) criteria, or genetically and were followed up in our clinic between January 1st, 2000, and January 1st, 2021, were eligible for inclusion. Exclusion criteria comprised pre-existing HCMP before starting rGH, and less than six months rGH treatment prior to detailed cardiac assessment.

Patient data were retrieved from the archive records and included anthropometry and physical examination findings at diagnosis and during follow-up, laboratory and genetic evaluations, systemic disease screening results, and treatment response. Clinical features, such as birth weight and length, weight standard deviation score (SDS), height SDS, body mass index, bone age at diagnosis, and target height were also recorded. Systemic problems at presentation and during follow-up were recorded. The karyotype of all female cases was 46, XX. The definitive diagnosis of NS was made according to the Van der Burgt (4) criteria as follows: 1) Typical facial appearance + 1 major or two minor clinical characteristic findings, or 2) Facial findings suggestive of NS + 2 major or three minor clinical characteristics verified for each case.

At presentation, all cases were evaluated in detail in terms of short stature. In addition, all of them had normal hemogram, liver, and kidney function tests, blood glucose, thyroid function test, total immunoglobulin A (IgA) level, and tissue transglutaminase IgA antibody were negative. Complete urine analysis was performed on all patients. Data retrieved from laboratory records included IGF-1 levels, IGF binding protein 3 (IGFBP3) levels, GH stimulation test results and bone age. Serum GH (ng/mL) level was measured by a chemiluminescence method. Serum IGF-1 and IGFBP3 were tested by immunochemiluminescence (MyBioSource, Inc. P.O. Box 153308 San Diego, CA 92195-3308, USA). An adequate response to GH stimulation tests was considered to be > 10 ng/mL. Annual growth velocity, change in height SDS (Δheight SDS), and growth velocity SDS values of each case were calculated. All of the cases were evaluated with electrocardiography (ECG) and echocardiography (ECHO) at presentation and during follow-up by the pediatric cardiology department.

Each case was recalled for physical examination and cardiac evaluation. Anthropometric measurements such as body weight (kg), height (cm), and head circumference (cm) were measured during the examinations. The weight was measured with a scale tool approved by the Turkish Standards Institute (TSE) with 0.1 kg intervals, and height measurements were made with a TSE-approved measuring device with 0.1 cm intervals. Weight and height SDS values were calculated according to the norms of Turkish children, based on data by Neyzi et al. (17) and according to the standard curves from data by Ranke et al. (2) for cases with NS. The growth velocity was calculated. The growth velocity SDS calculation was evaluated in accordance with Baumgartner references (3). Puberty staging was assessed according to Tanner Staging (18). Left wrist radiographs of the patients were evaluated using the Greulich-Pyle Atlas, and the bone age was assessed (19). Interventricular septal thickness in diastole (IVSed), interventricular septal thickness in systole (IVSes), left ventricular internal end-diastolic diameter (LVIDed), left ventricular internal end-systolic diameter (LVIDes), left ventricular posterior wall thickness in end-diastole (LVPWed), and left ventricular posterior wall thickness in end-systole (LVPWes) were measured with ECHO through a parasternal long-axis view using M-Mode Doppler. All measurements were made in triplicate, and the mean was used for statistical analysis. Z scores of IVSed, LVPWed, LVIDed, and LVIDes were calculated (20). This detailed evaluation was carried out at the beginning of the follow-up, on follow-up, and at last control. Previous ECHO reports of the cases were classified according to the evaluated periods before, during, and after GH. In addition, the Z-score of pre- and post-treatment ECHO parameters (IVSed, LVPWed, LVIDed, LVIDes) were evaluated with regard to being > + 2, and if the finding was above + 2 this was considered significant in terms of hypertrophy.

All cases were divided into two groups: those who were on rGH and those who were not on rGH treatment. The study was designed to show the change in ECHO parameters of the rGH group at the beginning and end of treatment.

Statistical Analysis

Data Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences for Windows, version 22.0 (IBM Inc., Armonk, NY, USA). The conformity of the variables to a normal distribution was examined using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Differences between independent groups were analyzed with the Mann-Whitney U test. The study was longitudinal and included multiple, repeated measurements of parameters measured in each patient, so Repeated Measurements ANOVA test statistics were used to assess differences in outcomes of Z scores of ECHO parameters (IVSed, LVPWed, LVIDed, LVIDes) before and after treatment over time. Statistically, $p < 0.05$ was considered significant.

Results

Features at the Presentation of the Participants

Twenty-four patients (16 boys, eight girls) were included, of whom 16 were treated with rGH and eight were not. Their details are shown in Table 2. Congenital heart lesions were present in 11/16 of the rGH group and in 6/8 of the non-rGH group. The most common finding was short stature (n = 22, 91.6%). For the whole cohort, the mean age at presentation was 8.02 ± 4.30 years. Patients in the rGH group were significantly older than the non-rGH group (p = 0.02). At the beginning of the follow up, the overall height SDS was -2.56 ± 0.94 [data by Neyzi et al. (6)], and + 0.25 ± 1.07 [Noonan specific data by Ranke et al. (2)].

Protein tyrosine phosphatase, nonreceptor type 11 gene (*PTPN11*) sequence analysis was performed in 10 cases, and mutation was present in five cases. One patient had normal *PTPN11* sequence analysis, and *MAP3K7* (NM_145331.3) heterozygous p.P22H (c.65c > A) missense mutation was detected in the WES analysis. In other patients, the diagnosis of NS was made according to the Van der Burgt criteria.

Laboratory Features

There was an insufficient response to the GH stimulation test in the group receiving rGH, and mean serum IGF-1 was -0.92 ± 1.31 SDS (n = 15) and IGFBP-3 was -0.70 ± 1.70 SDS (n = 15).

Growth Hormone Treatment and Long-term Follow-up Findings

Sixteen of the patients were on rGH. The follow-up period of the rGH group was 8.3 ± 3.8 years, and the age of rGH initiation was 9.7 ± 3.2 years. The mean rGH dose was 0.22 ± 0.04 mg/kg/week, and the mean duration of treatment was 4.5 ± 2.1. The mean first-year growth velocity was 1.13 ± 0.83 SDS (n = 14). First-year Δheight SDS gain was + 0.50 ± 0.32, second-year Δheight SDS gain was + 0.39 ± 0.48, and third-year Δheight SDS gain was + 0.16 ± 0.60 (Figure 1).

In the follow-up, 10 of the 11 patients who reached final height had received rGH.

Cardiac Features

In this cohort, cardiac pathology was present in 17 (70.8%) cases, and 11/17 (64.7%) were in the rGH group. PS (n = 6, 25%) and ASD (n = 6, 25%) were the most common findings at the beginning of the follow-up. Other findings in order of frequency were: VSD (n = 4, 16.7%), mitral regurgitation (n = 2, 8.3%), and one each (n = 1, 4.2%) with aortic regurgitation, coarctation of the aorta, bicuspid aortic valve, HCMP, interventricular septal hypertrophy, interatrial septal aneurysm, isolated left ventricular noncompaction cardiomyopathy (noncompaction CMP), patent foramen ovale, and right ventricular hypertrophy (Figure 2). Two patients with PS underwent pulmonary valvuloplasty, and one subject with VSD and coarctation of the aorta required surgical correction.

Pre-treatment ECHO evaluation was performed in 12 (66.7%) patients in the rGH group and in 5 (62.5%) patients in the non-rGH group. In terms of ECHO parameters, the Z scores of the rGH group at baseline were significantly higher compared to the non-rGH group: IVSed Z_{score} p = 0.02; IVSes Z_{score} p = 0.008; LVIDed Z_{score} p = 0.05; LVIDes Z_{score} p = 0.02; LPWed Z_{score} p = 0.04; and LPWS Z_{score} p = 0.01.

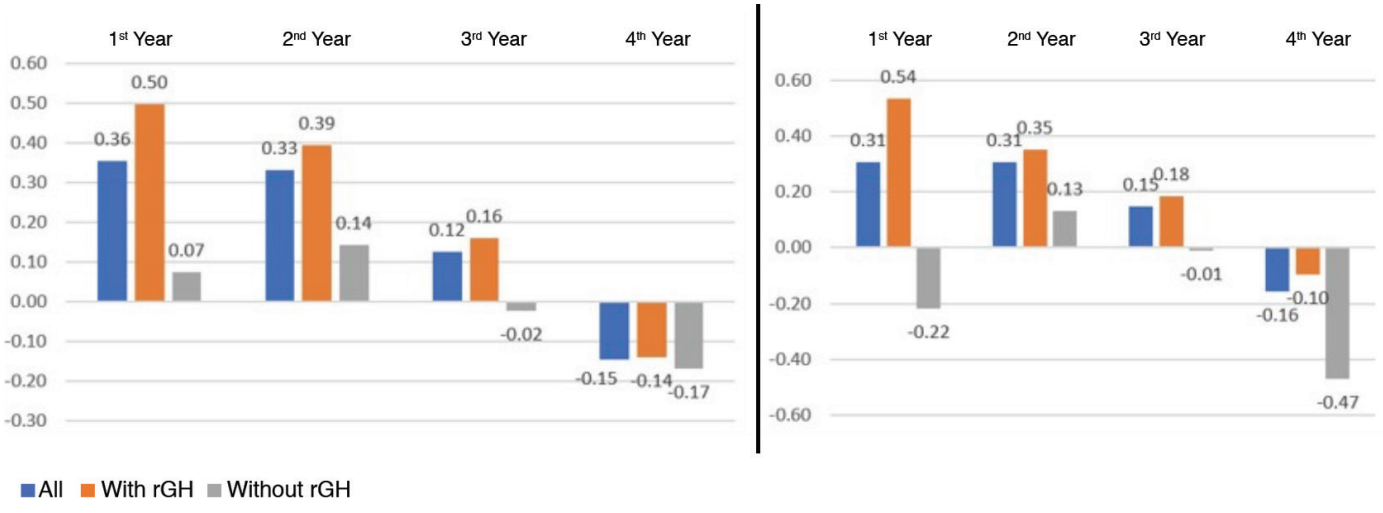

Figure 1. ΔHeight SDS gain in all, the rGH group, and the non-rGH group according to data by Neyzi et al. (17) (left graph) and data by Ranke et al. (2) (right graph)

SDS: standard deviation score, BMI: body mass index, rGH: recombinant growth hormone therapy

When the post rGH therapy ECHO parameter Z_{score} of the rGH group (n = 16) was compared to the non-rGH group (n = 8), no significant differences were observed between the two groups: IVSed Z_{score} p = 0.5; IVSes Z_{score} p = 0.3; LVIDed Z_{score} p = 0.08; LVIDes Z_{score} p = 0.2; LPWed Z_{score} p = 0.27; and LPWS Z_{score} p = 0.41.

When the ECHO parameters of rGH group at the beginning and the end of the treatment were compared, there was no

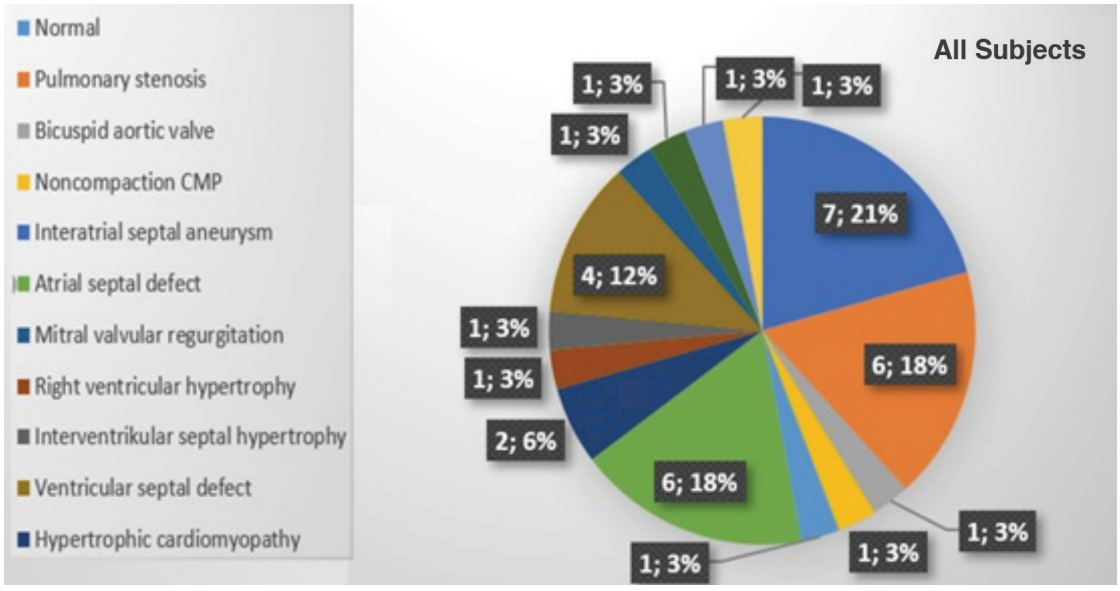

Figure 2. Distribution of cardiac pathologies

CMP: cardiomyopathy

Table 2. Clinical characteristics of patients with Noonan syndrome and the effect of rGH

	All (n = 24)	With rGH (n = 16)	Without rGH (n = 8)	p
At the beginning of the follow-up	8.02 ± 4.30	9.72 ± 3.23	4.60 ± 4.15	**0.02**
Gender (M:F)	16:8	10:6	6:2	0.81
%BMI	91.19 ± 12.82	90.85 ± 13.64	91.86 ± 11.87	0.85
Height SDS at admission	-2.56 ± 0.94	-2.78 ± 0.89	-2.12 ± 0.89	0.11
Puberty Stage (prepubertal/pubertal)	21/3	14/2	7/1	1
Bone age	6.73 ± 4.00 (n = 21)	7.12 ± 3.65 (n = 16)	5.50 ± 4.75 (n = 5)	**0.01**
Target height (cm)	166.52 ± 8.31	166.06 ± 8.3	167.44 ± 8.82	0.6
Target height SDS	-0.59 ± 0.78	-0.52 ± 0.79	-0.74 ± 0.76	0.39
Target height SDS-Height SDS	-2.12 ± 1.08	-2.49 ± 1.07	-1.38 ± 0.61	**0.02**
IGF-1 SDS	-1.00 ± 1.24 (n = 20)	-0.92 ± 1.31 (n = 15)	-1.25 ± 0.97 (n = 5)	0.32
IGFBP3 SDS	-0.87 ± 2.0 (n = 19)	-0.70 ± 1.70 (n = 15)	-1.53 ± 2.75 (n = 4)	0.19
Cardiac pathology (n, %)	17 (70.8%)	11 (64.7%)	6 (35.3%)	-
rGH dose (mg/kg/week)	x	0.22 ± 0.04	x	-
rGH duration (year)	x	4.5 ± 2.1	x	-
ΔHeight SDS gain in the 1st year	+ 0.36 ± 0.42 (n = 24)	+ 0.50 ± 0.32 (n = 16)	+ 0.07 ± 0.46 (n = 8)	**0.04**
ΔHeight SDS gain in the 2nd year	+ 0.33 ± 0.46 (n = 20)	+ 0.39 ± 0.48 (n = 15)	+ 0.14 ± 0.32 (n = 5)	0.16
ΔHeight SDS gain in the 3rd year	+ 0.12 ± 0.55 (n = 16)	+ 0.16 ± 0.60 (n = 13)	-0.02 ± 0.12 (n = 3)	0.09
Subjects reached final height				
Subjects reached final height (n)	11	10		
Final height (SDS)	-0.94 ± 1.31	-0.90 ± 1.37		
Target height SDS-Final height SDS	-0.40 ± 1.33	-0.41 ± 1.39		

*p < 0.05: Level of significance *Mann-Whitney U test.
SDS: standard deviation score, BMI: body mass index, rGH: recombinant growth hormone therapy, min-max: minimum-maximum, M: male, F: female, IGF-1: insulin-like growth factor-1, IGFBP3: IGF binding protein 3

significant increase in Z score of the IVSed, IVSes, LVIDed, LVIDes, LVPWed, LVPWes (Figure 3).

ECHO parameters of the rGH group and the non-rGH group (IVSed Z_{score} p = 0.32, IVSes Z_{score} p = 0.1, LVIDed Z_{score} p = 0.23, LVIDes Z_{score} p = 0.15, LPWed Z_{score} p = 0.97, LPWS Z_{score} p = 0.26) before and after treatment were not different (Table 3).

When each patient was evaluated individually, two cases in the rGH group had an IVSed Z score of > +2 at the last follow-up visit, and an increase in the Z score of IVSed from +0.59 to +2.26 at the last follow-up was noticed in one of these. The IVSed Z scores of 2 of 3 patients with an IVSed Z score of > +2 at presentation fell below < +2 at the last follow-up. Although one of these patients had a Z score > +2 at the last follow-up, the Z score of this patient also decreased from +2.65 to +2.09. There was only one case with a LVIDed Z_{score} > +2 at presentation. Afterward, the Z_{score} dropped below < +2. In the non-rGH group, there were no patients with LVIDed and LVIDes Z_{score} > +2 SDS at presentation or at final follow-up. LVPWed Z_{score} of the only case with a Z score > +2 at presentation decreased from +2.81 to -0.35 during follow-up. In contrast to this, during follow-up, the Z scores of the two cases increased from 0.19 and 1.88 to 2.25 and 2.65, respectively, whereas both patients had a Z score <2 at the last visit. When each group was analyzed on its own, in both groups, there was no significant difference in the Z scores of IVSed, IVSes, LVIDed, LVIDes, LVPWed, LVPWes Z_{score} between presentations (Table 3). At the final follow-up, none of the patients had a hemodynamically significant problem.

Discussion

Severe short stature is the major finding of NS. It is known that patients benefit from rGH treatment. *PTPN11,* the gene most associated with the syndrome, encodes the intracellular protein tyrosine phosphatase SHP-2. SHP-2 is a negative regulator of GH activity. Although the cause of GH deficiency is heterogeneous, in NS cases, IGF-1 is generally low, and the response to rGH is good (1,21,22,23). In the present study, IGF-1 SDS was low in the rGH group, and all of them had an insufficient response to the GH stimulation test. No resistance to rGH was observed in *PTPN11* positive cases.

Şıklar et al. (10) conducted the first national multicenter study in Turkey, and 124 cases with NS were evaluated retrospectively. Forty-seven of those had received rGH treatment and, comparable to our results, height gain in the first and second year of the treatment was 0.40 ± 0.44 and 0.75 ± 0.55 SD, respectively; however, a difference occurred in the third year. While an increase of 0.76 ± 0.41 SDS was observed in the third year in the Şıklar et al. (10) cohort, the third year Δheight SDS gain decreased to +0.16 ± 0.60 SDS in the present study (21). This may be

Table 3. Z score of ECHO parameters comparision in all groups

ECHO parameters	Group with rGH		Group without rGH		p
	$\overline{X}$ ± SDS	$\overline{X}$ [min-max]	$\overline{X}$ ± SDS	$\overline{X}$ [min-max]	
IVSed	0.45 ± 1.13	0.19 [-1.5-2.23]	0.71 ± 0.85	0.48 [-0.44-2.46]	group: F = 0.032 p = 0.859 time: F = 3.403 p = 0.079 group*time: F = 1.033 p = 0.321
IVSed	1.01 ± 0.65	1.16 [-0.34-1.59]	0.87 ± 0.92	0.67 [-0.75-2.53]	
IVSes	0.73 ± 1.29	0.2 [-1.12-2.43]	1.05 ± 0.93	0.78 [0-2.65]	group: F = 0.238 p = 0.631 time: F = 0.017 p = 0.896 group*time: F = 2.880 p = 0.104
IVSes	1.21 ± 0.66	1.28 [0.12-2.28]	0.48 ± 1.57	0.99 [-2.9-2.5]	
LVIDed	-0.72 ± 0.85	-0.44 [-2.31-0]	-0.67 ± 1.37	-0.08 [-3.03-2.08]	group: F = 1.669 p = 0.210 time: F = 6.224 p = 0.021 group*time: F = 1.561 p = 0.225
LVIDed	-1.98 ± 1.24	-2.27 [-3.76-0.22]	-1.09 ± 0.95	-1.06 [-2.75-0.39]	
LVIDes	-0.63 ± 0.89	-0.04 [-2.21-0.03]	-0.73 ± 1.25	-0.41 [-3.34-1.3]	group: WTS = 0.297 p = 0.586 time: WTS = 6.181 p = 0.013 group*time: WTS = 2.110 p = 0.146
LVIDes	-1.7 ± 1.24	-1.52 [-3.27-0.3]	-1.02 ± 1.05	-0.92 [-3.11-0.31]	
LVPWed	0.97 ± 1.09	0.65 [0-2.81]	0.62 ± 1.23	0.19 [-1.47-3.18]	group: F = 1.277 p = 0.271 time: F = 0.197 p = 0.661 group*time: F = 0.001 p = 0.971
LVPWed	1.13 ± 1.28	1.47 [-1.06-2.65]	0.76 ± 0.8	0.66 [-0.6-2.35]	
LVPWes	0.64 ± 1.23	0.13 [-1.19-2.24]	0.19 ± 1.02	0.01 [-1.87-1.98]	group: F = 0.042 p = 0.840 time: F = 0.039 p = 0.846 group*time: F = 1.354 p = 0.257

IVSed: interventricular septal thickness in diastole, IVSes: interventricular septal thickness in systole, LVIDed: left ventricular internal end-diastolic diameter, LVIDes: left ventricular internal end-systolic diameter, LVPWes: left ventricular posterior wall thickness in end-systole, SDS: standard deviation score, ECHO: echocardiography, rGH: recombinant growth hormone therapy, min-max: minimum-maximum

related to the fact that 70% of our patients had cardiac findings, so we could not increase the rGH dosage. No safe dose range in terms of cardiac exposure has been reported in studies conducted so far. Due to the lack of evidence, we believe that the results reported herein, under standard-dose treatment, are a valuable addition to the limited published data in this field.

Due to the common finding of cardiac involvement in NS and the frequency of cardiac hypertrophy, there are concerns about the risk of increased ventricular wall thickness and the frequency of cardiac side effects when giving rGH therapy. Studies investigating the cardiac effects of rGH consist of retrospective or short-term prospective studies. To the best of our knowledge, there is no other study investigating the changes in ECHO parameters in NS patients not on rGH and comparing these with the group who had undergone rGH treatment.

There are questions concerning the use of rGH in NS, which include "Would cardiac pathology occur with standard-dose rGH by close monitoring of IGF-1?" and "Is concomitant cardiac pathology an expected finding due to the nature of the syndrome?". Assessment of published studies and the present study shows that there is no clear consensus on the effect of rGH use in NS on cardiac findings. Again, since HCMP cases were excluded in some studies, there is no clear consensus in the literature regarding the follow-up process of HCMP cases. One of the patients in the non-rGH group had HCMP, and thus rGH was not initiated due to hemodynamic instability.

There are several studies investigating the effect of rGH in NS cases (Table 1). Brown et al. (13) showed that HCMP did not develop in the follow-up. The effect of rGH could not be clarified in cases with HCMP, since they were excluded from the study (6). Seo and Yoo (14) showed that rGH was not a risk for HCMP progression and tumor development (24). Of the six articles evaluating adult height in NS patients receiving rGH (n = 889), cardiac adverse events were described in only five patients, including two mild PS progression, one HCMP, one increased biventricular hypertrophy, and one cardiac decompensation (25). Noordam et al. (16) evaluated left ventricular thickness in 27 patients with NS. Mild, non-progressive HCMP was detected in one. Patients were divided into group A (rhGH was started immediately and stopped for two years) and

Figure 3. Z score of echocardiography parameters in all groups during follow-up

IVSed: interventricular septal thickness in diastole LVIDed: left ventricular internal end-diastolic diameter, LVIDes: left ventricular internal end-systolic diameter, LVPWed: left ventricular posterior wall thickness in end-diastole, GH: growth hormone

group B (one-year treatment was started and two years of treatment was given), and patients were compared over three years of treatment. Although initially, the left ventricular internal diameter was smaller and the posterior wall thickness was thicker than normal, there was no difference between the pre-treatment and at the fourth year of treatment (7). Ozono et al. (17) reported serious cardiac adverse events in four of 51 (7.8%) patients on rGH (8). These authors concluded that the treatment had no cardiac side effects, and no progression or new HCMP cases were reported. In a review, it was reported that there was no change in ventricular wall thickness with rGH (26). In most studies, it was shown that the left ventricular wall thickness was normal in prospective evaluations of NS patients on rGH. Studies have investigated the effect of rGH on left ventricular thickness, but there are very few studies examining left ventricular thickness progression, as in our study. Since rGH may theoretically worsen HCMP, no study has examined the effect of rGH on HCMP. HCMP was detected in one patient in our cohort in the non-rGH sub-group, and improvement in HCMP was observed in the seven-year follow-up. However, since this improvement is seen on a case-by-case basis, studies of patients with NS with HCMP are needed to provide more robust data.

The International Growth Database (KIGS) study is the largest NS case cohort in the literature. According to the twenty-year KIGS database, cardiac side effects were found in seven of 429 children with NS who received rGH, and it was reported that there was no relationship between these cardiac events and rGH (9). At the end of 25 years (1987-2012) of the same KIGS database, pacemaker implantation was required in one of the cases due to arrhythmia. The most serious adverse events reported to date have been left ventricular hypertrophy (after 2.0 years) and CMP requiring cardiac transplantation (after 10.7 years). Cardiac side effects were reported in only four of the NS patients receiving rGH treatment (27). The National Cooperative Growth Study group also reported no cardiac side effects in 150 children (97 males) treated with rGH (10).

There are studies of treatment with a higher rGH dose than was used in our study. Osio et al. (22) investigated the effect of rGH in 25 prepubertal NS patients without major cardiac anomalies. No cardiac side effects were detected and the authors suggested that there was no risk for HCMP progression or tumor development (11). Lampit et al. (23) performed ECHO follow-up for two years in 22 cases (12 in the control group) and did not detect any effect of rGH treatment on left ventricular wall thickness (12). MacFarlane et al. (24) evaluated 23 patients with NS using ECHO before rGH treatment. Cardiac anomalies were detected in 18 and moderate cardiac hypertrophy in three. There was no change in the findings after the twelfth month of treatment. The findings were re-evaluated three years later. At initial evaluation, no change was found in the wall thickness measurement in the patient with mild left ventricular hypertrophy. The other two subjects developed an increase in left ventricular wall thickness, close to the upper limit of normal for age and body surface area, without other features of HCMP. No cardiac side effects were detected in other cases (13). Apperley et al. (25) investigated patients with NS who received a mean of 0.037 mg/kg/day rGH and detected cardiac anomalies in 8 (88%) of 12 patients, including PS, two with ASD, and one with HCMP. There was no progression in findings or cardiac side effects over an average of three years of rGH treatment (14). Cotterill et al. (26) excluded cases with an average left ventricular wall thickness of more than 10 mm in their multidisciplinary study. At the end of the first year of treatment, no change was found in the cardiac mass in the treated group (15).

Recently, Romano et al. (27) assessed the cardiovascular safety of GH in patients with NS (n = 412). Of the 18 patients with PS and three with HCMP at baseline, they had no worsening during treatment. After the beginning of rGH, one ruptured abdominal aortic aneurysm, one PS, and three unspecified cardiovascular events were observed. Given the low prevalence of cardiovascular comorbidities, they reported a safe profile of rGH treatment in patients with NS (16).

Since NS individuals with HCMP were excluded from treatment, the effect of rGH on NS cases with HCMP could not be evaluated. Except for the KIGS study, the follow-up times of all reported studies are very limited. The present study includes one of the longer follow up periods, outside of KIGS, at 8.3±3.8 years of follow-up in patients who received rGH. After long-term follow-up, we did not observe any worsening of cardiac findings in either the rGH group or the non-rGH group. Although we used the standard dose of rGH, the difference between the target and final height improved.

The genotype-phenotype correlation in cardiovascular disease in NS is well established (28). *PTPN11* is the most commonly implicated NS gene (~50% of cases) and is found in 59% of familial cases and 37% of sporadic cases. SHP-2 plays an important role in valvular morphogenesis and the development of heart defects (4,28,29). While *PTPN11* mutations are found in 80% of NS patients with pulmonary valvular stenosis and ASD, there is an inverse relationship with HCMP. However, in *RAF1* variants, HCMP

is inversely related to PS (7,28,29). In our study, genetic evaluation was performed in a very limited number of cases. Therefore, the statistical correlation could not be investigated. Although the genotype-phenotype correlation has been established, it should not be assumed that *PTPN11* mutation will never be associated with HCMP. Thus, in our cases with *PTPN11* mutation, there was a case with HCMP. Athota et al. (30) also reported that 84% of 117 NS individuals with *PTPN11* mutation had cardiac defects, and 8.5% of them had HCMP. This confirms the wide genetic variability observed in NS patients. A compound heterozygous mutation of *MYBPC3* and *PTPN11* was reported in a patient with NS and HCMP (31). Although a relationship between genotype and phenotype has been established, no study has revealed a definite mechanism (32,33,34,35). However, mutation positivity in the relevant gene for the diagnosis is a confirmatory diagnostic criterion for NS, a normal result does not exclude the syndrome. Therefore, in 1994 Van der Burgt et al. (35) developed a clinical diagnostic system, which remains current today (4).

A cardiologist should evaluate all individuals with NS with ECG and ECHO at diagnosis. Those who are found to have cardiac defects should be followed up regularly. It is recommended that cardiac reassessments should be performed every five years in individuals without any evidence of heart disease at their initial assessment. If congenital heart disease or HCMP is detected in early infancy, close ECHO monitoring is recommended. Periodic cardiac evaluations should be continued in adults, even if evaluations are normal during childhood or adolescence. It should not be forgotten that unexpected cardiac findings may develop over time (25,28,29). Recently, Rohrer et al. (36) also showed no higher prevalence of cardiac comorbidities in patients with NS who had been treated with rGH but close follow-up was recommended.

Study Limitations

The limitations of our study include the low number of participants despite all patients diagnosed in a single center over a 21-year period being eligible. The number of participants is as many as one-fifth of the participants in the Turkish national study (21). Only five participants were included the national study. The number of participants in the non-rGH group was lower, and the follow-up period was shorter than the rGH group. Since rGH was given at a standard dose, the relationship between high-dose rGH and cardiac parameters could not be evaluated.

Conclusion

In this study, rGH was effective in achieving good final height in patients with NS. The difference between final height SDS and target height SDS was small even when using standard dose rGH. Although cardiac pathology was observed in 70% of cases at presentation, there was no change in ECHO parameters from the point of left ventricular hypertropia or dilatation on rGH therapy and during follow-up. We, therefore, conclude that the use of rGH was safe in this small cohort of NS patients, most with cardiac pathology, under close follow-up.

Ethics

Ethics Committee Approval: The study was approved by the Ankara University Faculty of Medicine Local Ethics Committee (decision number: I2-93-21, date: 11.02.2021).

Informed Consent: Published written consent was obtained from parents and in children also their assent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sirmen Kızılcan Çetin, Mehmet Gökhan Ramoğlu, Zeynep Şıklar, Elif Özsu, Zehra Aycan, Hasan Ercan Tutar, Merih Berberoğlu, Concept: Sirmen Kızılcan Çetin, Zeynep Şıklar, Hasan Ercan Tutar, Merih Berberoğlu, Design: Sirmen Kızılcan Çetin, Zeynep Şıklar, Merih Berberoğlu, Data Collection or Processing: Sirmen Kızılcan Çetin, Mehmet Gökhan Ramoğlu, Elif Özsu, Zehra Aycan, Hasan Ercan Tutar, Analysis or Interpretation: Sirmen Kızılcan Çetin, Mehmet Gökhan Ramoğlu, Merih Berberoğlu, Literature Search: Sirmen Kızılcan Çetin, Mehmet Gökhan Ramoğlu, Elif Özsu, Zehra Aycan, Hasan Ercan Tutar, Merih Berberoğlu, Writing: Sirmen Kızılcan Çetin, Mehmet Gökhan Ramoğlu, Zeynep Şıklar, Merih Berberoğlu.

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Fibroblast Growth Factor 21 Levels and Bone Mineral Density in Metabolically Healthy and Metabolically Unhealthy Obese Children

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What is already known on this topic?

Fibroblast growth factor 21 (FGF21) is produced by the liver and plays a unique role in the regulation of carbohydrate and lipid metabolism. FGF21 increases glucose uptake into fat cells, thermogenesis, energy expenditure, fat use, and insulin sensitivity. There are limited studies evaluating the role of FGF21 in obese children and its effects on bone metabolism, and the results of these studies are contradictory.

What this study adds?

Although FGF21 levels were higher in obese children compared to non-obese children, this difference was not statistically significant. No correlation was found between FGF21 levels and bone mineral density.

Abstract

Objective: The harmful or beneficial effect of obesity on bone mineral density (BMD) remains controversial in children and adolescents. Fibroblast growth factor 21 (FGF21) is a metabolic factor that plays a specific role in the regulation of carbohydrate and lipid metabolism. However, the role of FGF21 in bone metabolism appears paradoxical and is complex. To determine whether serum FGF21 level was associated with BMD in obese children and adolescents.

Methods: The study was conducted with the participation of children and adolescents aged 8-18 years. Ninety-eight obese children were included in the study group and 44 children were included in the control group. BMD, in addition to the routine obesity workup, which includes fasting blood glucose, fasting insulin levels, lipid profile, and liver enzymes; serum FGF21 levels have been analyzed.

Results: The mean age of the obese group (n = 98) was 13.34 ± 2.24 years and the mean age of controls (n = 44) was 13.48 ± 2.87 years. Based on International Diabetes Federation criteria, 15 of 98 (15.3%) patients were metabolically unhealthy. FGF21 levels were 193.54 ± 139.62 mg/dL in the obese group and 158.69 ± 151.81 mg/dL in the control group ($p = 0.06$). There was no difference between the FGF21 and BMD z-score values of girls and boys in the obese and control groups ($p > 0.05$).

Conclusion: BMD-z-score was increased in obese children compared to healthy control. Moreover, BMD-z-score tended to be higher when more metabolic risk factors were present. However, there was no significant relationship between FGF21 levels and BMD z-score values in obese children.

Keywords: Obesity, children, bone mineral density, FGF21

Introductıon

Since obesity is an important multifactorial problem caused by the interaction of eating behavior, physical activity, environmental conditions and genetic characteristics, it has proven challenging both to treat existing obesity and to design effective strategies for prevention (1). Obesity causes metabolic problems, such as insulin resistance, type 2 diabetes, atherosclerotic heart disease, non-alcoholic fatty liver disease, hypertension and hyperlipidemia (2).

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The association between obesity and healthy bone tissues has recently been highlighted as an area of concern (3). However, it is difficult to determine whether excess adipose tissue in the body is beneficial or harmful to growing bone tissue. While some studies reported increased bone mass in overweight children and adolescents compared to peers of normal weight (4,5,6), others concluded that obesity was associated with less bone mass (7,8,9). It has been suggested that bone mass gain may be reduced in adolescents with obesity-related metabolic disorders, and suboptimal peak bone mass gain in this period will bring the risk of osteoporosis in later life. It has been reported that bone mineral density (BMD) was decreased in obese adolescents, especially as the degree of hyperinsulinism increased and cardiometabolic risk factors accumulated (3).

Fibroblast growth factor (FGF) 21 (FGF21), a member of the family of FGF, is produced by the liver, and consists of 209 amino acids with a molecular weight of 23 kilodaltons. FGF21 plays a unique role in the regulation of carbohydrate and lipid metabolism (10). FGF21 increases glucose uptake into fat cells, thermogenesis, energy expenditure, fat use and insulin sensitivity. It has been suggested that FGF21 may be beneficialfor metabolic health by reducing blood glucose and lipid levels (11). The role of FGF21 in bone metabolism appears paradoxical because it can both improve metabolic health but also reduce bone formation. The relationship between various metabolic health parameters, including FGF21, insulin sensitivity and body composition in obese adolescents is not fully understood (12). Therefore, the aim of this study was to investigate differences in BMD between metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO) children and the healthy controls and the association with metabolic parameters including serum FGF21 levels.

Methods

The study was conducted with the participation of children and adolescents who attended a single Pediatric Endocrinology outpatient clinic. Informed consent was obtained from the patients and their families. Ethical approval was obtained from Ankara University Faculty of Medicine Clinical Research Ethics Committee with the number 08-418-17 and made in accordance with the Helsinki Declaration (date: 24.04.2017). The study group included obese children, defined as body mass index (BMI) $>95^{th}$ percentile, aged between >8 and <18 years of age, who had no chronic systemic disease and did not use any medication. Patients with an active infection at the time of blood collection or who refused to give consent were excluded.

The weight of the participants was measured wearing light clothing. The height measurements were made with 1 mm spaced fixed meter with heels, hips and head against the wall without shoes, and measurements were evaluated according to the norms of Turkish children with age and are sex taken into consideration (13). Height standard deviation (SD) score (SDS), BMI, BMI %, BMI z-score, waist circumference, hip circumference, and waist/hip circumference ratios were determined. BMI was calculated with the formula of body weight (kg)/square of height (m^2) and children over the 95^{th} percentile were accepted as obese. Waist circumference was measured from the 10^{th} costa and iliac crest where the waist was the thinnest, while the children were standing upright while the abdomen was relaxed. Hip circumference was measured around the large trochanter while children were standing upright. Measurements were evaluated in accordance with previously published hip circumference reference values (14).

Blood pressure measurements were performed with a blood pressure measuring device having a suitable sleeve to cover the upper 2/3 of the arm using the upper right arm. Measurements were made in the morning before breakfast, after at least 30 minutes rest and in a sitting position. The patients were evaluated according to age, sex and height using the appropriate percentile curve. Patients with hypertension were identified. Physical examination was performed to identify acanthosis nigricans and puberty staging was undertaken according to the Tanner-Marshall classification (15,16).

Blood samples were taken between 8 am and 9 am after fasting for 12 hours at night. Fasting blood glucose, fasting insulin, lipid profile, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were measured. In addition, after the blood sample was taken for FGF21 level, serum was separated and stored within an hour of collection at -80 °C. Biochemical evaluations were performed in Central Biochemistry and Endocrinology Laboratories.

Methodologies for testing were as follows.

Serum FGF21 levels were measured using a commercially available enzyme-linked immunosorbent assay (Biovendor Research and Diagnostic Products, Czech Republic). Absorbance measurements at 450 nm were performed on a microplate reading device ELx800 (Bio-Tek Instruments, Inc., USA). Serum samples were diluted 1:2 with buffer dilution prior to analysis to measure FGF21 levels according to manufacturer instruction. The standard curve range for analysis was 30-1920 pg/mL and sensitivity is reported as 7 pg/mL. Intra-assay and inter-assay coefficient of variation were 3.0-4.1% and 3.6-3.9%, respectively.

Glucose was tested by the glucose hexokinase method, total cholesterolwas determined enzymatically by an oxidase method while high-density lipoprotein-cholesterol (HDL-C) was measured directly by a non-immunological method. Triglyceride (TG) at concentrations of < 400 mg/dL was derived from the Friedewald formula but TG > 400 mg/dL was measured by homogeneous enzymatic method on a Roche Modular automatic biochemistry analyzer (Roche Diagnostics, Germany). Creatinine was determined by the Jaffe rate blanked method and fasting insulin levels were measured by radioimmunoassay.

Metabolic health status was assessed by both the presence of metabolic syndrome (MS) and assessment of cardiometabolic risk factors clustering (CMRFC).

The presence of MS in the study group was investigated according to the criteria by International Diabetes Federation (IDF) for children aged 10-18 years (17). MHO and MUO groups were determined according to IDF criteria. The IDF criteria for MS in obese children and adolescents are: waist circumference percentile ≥90th with at least two of the following:

1. Dyslipidemia: TG ≥150 mg/dL or HDL-C ≤40 mg/dL

2. Blood pressure: Systolic ≥130 mmHg/diastolic ≥85 mmHg systolic ≥130 mmHg or diastolic ≥85 mmHg-

3. Fasting blood glucose > 100 mg/dL or known type 2 diabetes history.

The presence of CMRFC was accepted if at least two of the following criteria were present (18).

1. Waist circumference percentile ≥90

2. TG ≥150 mg/dL

3. HDL-C ≤40 mg/dL

4. Blood pressure: Systolic and diastolic ≥90-95th

5. Fasting blood sugar > 100 mg/dL.

BMD measurements were performed by the DEXA Norland method on a Hologic Discovery Discovery DXA (Hologic) device. The children were supine with knees slightly bent to correct physiological lumbar lordosis. Hips were positioned in the supine decubitis position. The antero-posterior vertebral L2-4 was measured in three minutes, and evaluated for BMD, bone mineral content (BMC) and length (cm). Deviations in BMD were evaluated with z-score according to the reference values for each age and sex of the childhood age group. The z-scores of the subjects were calculated according to the following formula (19): BMD z-score = (measured BMD - age and gender matched control BMD)/ age and gender matched control standard deviation). If the z-score calculated according to this formula is lower than "-2 SD", the patient's BMD was considered low (the z-score calculated according to this formula that was considered to be less than "-2 SD" BMD).

Statistical Analysis

Statistical analysis of the data was performed using Statistical Package for the Social Sciences for Windows, version 20.0 (IBM Inc., Armonk, NY, USA). Descriptive statistics are presented as both mean ± SD and median (minimum-maximum) for continuous variables. Nominal changes were shown as number and frequency. Chi-square or Fisher's exact test were used to compare percentages between groups. In order to compare the continuous variables in two groups, the fit of the data to the normal distribution was tested (with chi-square), then the Mann-Whitney U test or t-test while for comparison of more than two groups ANOVA or Kruskal-Wallis variance analysis was used. Tukey test and Kruskal-Wallis multiple comparison tests were used to investigate which group or groups were the cause of any significant differences. Spearman correlation coefficient was used to investigate the correlation between continuous data. Statistical significance was assumed when $p < 0.05$.

Results

A total of 142 subjects, of whom 98 (69%) were obese and 44 (31%) were healthy controls, were included. The mean age of the obese group was 13.34 ± 2.24 years and the mean age of the control group was 13.48 ± 2.87 years. The sex dstribution in the two groups were 56.1% girls and 43.9% were boys in the obese group and 72.2% girls and 27.3% boys in the control group. There was no significant difference between the obese and control groups in terms of age and gender ($p > 0.05$).

There was no significant difference between obese and control groups in terms of diastolic and systolic blood pressure, height, and height SDS values. As expected bodyweight (BW), BW SDS, BMI, BMI%, BMI percentile, BMI SDS, waist circumference, hip circumference and waist/hip ratio values were significantly higher in the obese group compared to the control group ($p < 0.001$) (Table 1).

The mean values of fasting insulin, total cholesterol, TG, very low-density lipoprotein-cholesterol (VLDL-C), low-density lipoprotein-cholesterol (LDL-C), and ALT were significantly higher in the obese group compared to the control group ($p < 0.001$) (Table 2).

Based on IDF criteria, 15 of 98 (15.3%) patients in the obese group met the MS criteria, occurring at a rate of 16.3% in obese girls and 14% in obese boys. Thus the group was further stratified into 15 (15.3%) MUO and the remaining

Table 1. Clinical characteristics of all cases

	Obese (n = 98)	Control (n = 44)	p value
Female/male (n %)	55 (56.1 %)/43 (43.9 %)	32 (72.7 %)/712 (27.3 %)	0.060
Prepubertal/pubertal (n %)	9 (9.2 %)/89 (90.8 %)	3 (6.8 %)/741 (93.2 %)	0.754
Age (years)	13.34 ± 2.24 13.13 (9.06-17.7)	13.48 ± 2.87 14.08 (8.17-17.74)	0.771
Diastolic blood pressure (mmHg)	65.89 ± 10.18 60 (50-112)	63.84 ± 6.89 60 (60-85)	0.471
Systolic blood pressure	101.59 ± 15.81 100 (80-162)	103.25 ± 14.70 100 (80-135)	0.467
Height SDS	0.78 ± 1.18 0.70 (-2.11-3.95)	0.59 ± 1.23 0.61 (-1.69-3.88)	0.404
Weight SDS	2.17 ± 0.97 2.09 (0.39-5.32)	-0.20 ± 1.26 -0.41 (-2.55-3.12)	< 0.001
% BMI	139.94 ± 17.17 136.5 (107.4-200.36)	95.33 ± 1786 90.32 (67.74-152.69)	< 0.001
BMI (kg/m^2)	27.96 ± 3.79 27.78 (20.9-38.27)	18.82 ± 3.54 18.55 (12.16-29.05)	< 0.001
BMI percentile (%)	96.02 ± 5.12 97.35 (64.43-99.98)	36.16 ± 33.89 19.22 (0.18-99.06)	< 0.001
BMI SDS	2.08 ± 0.63 1.94 (0.37-3.78)	-0.55 ± 1.28 -0.87 (-2.91-2.35)	< 0.001
Waist circumference (cm)	92.47 ± 10.23 93 (70-115)	72.79 ± 11.54 75 (48-96)	< 0.001
Hip circumference (cm)	104.59 ± 10.49 105 (80-130)	86.14 ± 10.89 87.5 (60-104)	< 0.001
Waist/hip ratio	1.13 ± 0.14 1.13 (0.10-1.36)	1.17 ± 0.14 1.18 (0.58-1.47)	0.038

*Data are given as mean ± standard deviation and median (min-max) except for sex and puberty which are shown as frequency, given as n (%).
BMI SDS: body mass index standard deviation score, min-max: minimum-maximum

Table 2. Laboratory parameters of all cases

	Obese (98)	Control (44)	p
	Mean ± SD* Median (min-max)**	Mean ± SD Median (min-max)	
FBG (mg/dL)	87.78 ± 7.32 88 (60-104)	83.55 ± 5.71 83 (68-95)	0.001
Fasting insulin (mIU/mL)	25.19 ± 13.22 21.85 (10.1-112-4)	10.09 ± 3.01 10.20 (4-16)	< 0.001
Total cholesterol (mg/dL)	169.50 ± 30.97 171.5 (110-246)	152.43 ± 25.82 146 (102-204)	0.002
Triglyceride (mg/dL)	108.69 ± 45.02 102.5 (42-289)	74.52 ± 1.77 70.5 (36-136)	< 0.001
HDL-C (mg/dL)	45.57 ± 12.99 44 (30-129)	49.64 ± 10.13 48.5 (31-75)	0.045
LDL-C (mg/dL)	101.50 ± 24.71 98.5 (46-165)	89.66 ± 24.45 84 (55-154)	0.006
VLDL-C (mg/dL)	21.97 ± 8.98 21 (8-58)	14.82 ± 3.80 14 (7-27)	< 0.001
ALT (U/L)	18.39 ± 8.84 17 (4-69)	12.93 ± 4.55 12 (7-28)	< 0.001
AST (U/L)	21.26 ± 7.86 20 (11-75)	20.48 ± 5.19 19.5 (11-32)	0.815

*Mean ± SD. **Median (minimum-maximum).
HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol, VLDL-C: very low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase, FBG: fasting blood glucose, SD: standard deviation

83 (84.7%) were considered MHO. All patients with MS were over 10 years old. No patient met the MS criteria in the control group. CMRFC, another metabolic health parameter, was found in 57.1% of the obese group and 13.6% in the control group. As expected, the presence of MS and CMRFC were significantly higher in the obese group compared to the control group ($p < 0.06$ and $p < 0.001$, respectively). Fasting blood glucose, fasting insulin, total cholesterol, TG, LDL-C and ALT levels were significantly higher in the MUO group than in the control group (Table 3).

BMD z-score were 1.19 ± 1.48 (g/cm^2) in the obese group and 0.48 ± 1.75 g/cm^2 in the control group. BMD z-score values were significantly higher in the obese group compared to the control group ($p < 0.013$) (Table 4). The BMD z-score in the obese group was 1.28 ± 1.42 in girls [median 0.95 (-1.3-6.17)] and 1.09 ± 1.58 [median 0.89 (-1.44-5.6)] in boys. There was no difference between the BMD z-score values of girls and boys in the obese group ($p > 0.05$).

BMD z-score values were different between MUO and MHO and control groups. BMD z-score values were significantly higher in the MHO compared to the control group ($p < 0.044$) (Figure 1a). BMD z-score values were found to be significantly different between obese without CMRFC, obese with CMRFC, and control groups ($p < 0.016$). BMD z-score values were significantly higher in the obese patients without CMRFC group compared to the control group (Figure 1b). There was no significant correlation between BMD z-score and total cholesterol, TG, LDL, VLDL, HDL and ALT ($r = 0.09$ $p = 0.36$, $r = 0.09$ $p = 0.4$, $r = 0.09$ $p = 0.4$, $r = 0.1$ $p = 0.3$, $r = 0.02$ $p = 0.88$, and $r = 0.09$ $p = 0.37$, respectively).

No relation was found between FGF21 level and age, body weight, BW SDS, BMI, BMI%, BMI percentile, BMI SDS, waist circumference, hip circumference, fasting blood sugar, fasting insulin, total cholesterol, HDL-C, LDL-C, ALT, AST values and BMD z-score in obese and control groups ($p > 0.05$). There was a negative correlation between FGF21 and height SDS, and a positive correlation with TG and VLDL-C ($p < 0.045$, $p < 0.049$, $p < 0.025$, respectively) (Table 5). No significant correlation was found between HOMA-IR, BMD z-score and FGF21 ($r = -0.01$, $p = 0.90$ and $r = 0.04$, $p = 0.70$, respectively).

Table 3. Laboratory parameters and BMD z-scores of MHO, MUO and control groups (FGF21 values were not different between MHO, MUH and control groups. Fasting blood glucose, fasting insulin, total cholesterol, triglyceride, LDL-C and ALT levels were significantly higher in the MUO group than in the control group. BMD z-score values are significantly higher in the MHO group than in the control group)

	MHO (15)	MUO (83)	Control (44)	p value
	Mean ± SD* Median (min-max)**	Mean ± SD Median (min-max)	Mean ± SD Median (min-max)	
FGF21 (mg/dL)	196.69 ± 140.09 149.57 (32.48-735.79)	176.08 ± 140.47 141.92 (22.53-451.87)	158.69 ± 151.81 102.31 (11.82-853.65)	0.135
Fasting blood glucose (mg/dL)	87.18 ± 6.93 88 (60-104)	91.07 ± 8.74 89 (71-103)	83.55 ± 5.71 83 (68-95)	0.001
Fasting insulin (mIU/mL)	24.58 ± 13.20 20 (10.1-112.4)	28.55 ± 13.29 22.7 (14.9-63.8)	10.09 ± 3.01 10.20 (4-16)	< 0.001
Total cholesterol (mg/dL)	170.84 ± 31.55 175 (110-246)	162.07 ± 27.33 164 (114-214)	152.43 ± 25.82 146 (102-204)	0.004
Triglyceride (mg/dL)	104.65 ± 43.61 99 (42-289)	131.07 ± 47.64 127 (60-218)	74.52 ± 1.77 70.5 (36-136)	< 0.001
HDL-C (mg/dL)	47.96 ± 13.54 45 (30-129)	40.32 ± 6.62 38.8 (33-59)	49.64 ± 10.13 48.5 (31-75)	0.004
LDL-C (mg/dL)	102.59 ± 25.45 101 (46-165)	95.47 ± 19.68 94 (63-134)	89.66 ± 24.45 84 (55-154)	0.016
VLDL-C (mg/dL)	21.19 ± 8.70 20 (8-58)	26.27 ± 9.57 25 (12-44)	14.82 ± 3.80 14 (7-27)	< 0.001
ALT (U/L)	17.98 ± 7.54 17 (4-46)	20.67 ± 14.22 18 (8-69)	12.93 ± 4.55 12 (7-28)	< 0.001
AST (U/L)	21.34 ± 8.20 20 (11-75)	20.80 ± 5.85 20 (12-34)	20.48 ± 5.19 19.5 (11-32)	0.969
BMD z-scores	1.22 ± 1.53 0.97 (-1.44-6.170)	1.09 ± 1.25 0.56 (-0.13-3.31)	0.48 ± 1.75 0.13 (-2.67-5.76)	0.044

*Mean ± SD. **Median (min-max).
FGF21: fibroblast growth factor 21, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol, VLDL-C: very low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMD: bone mineral density, MHO: metabolically healthy obese, MUO: metabolically unhealthy obese, SD: standard deviation, min-max: minimum-maximum

It was demonstrated that FGF21 values were not different between obese without MS, obese with MS and control groups ($p > 0.05$). Similarly, FGF21 values were not found to be significantly correlated for obese without CMRFC, obese with CMRFC, and control groups (Figure 2a, 2b) ($p > 0.05$).

FGF21 levels were 193.54 ± 139.62 pg/mL in the obese group and 158.69 ± 151.81 pg/mL in the control group. The difference was not significant ($p > 0.06$) (Table 4).

Discussion

The prevalence of obesity in childhood can lead to psychological and social problems, as well as increasing the burden of chronic diseases, creating an important public health problem (20). The presence of MS in obese populations has been described for many years, identified by reference to a range of metabolic health parameters (21). MS refers to a combination of risk factors for cardiovascular diseases, including abdominal obesity, dyslipidemia, glucose intolerance and hypertension. The presence of these risk factors in childhood increases the likelihood of developing MS, type 2 diabetes and cardiovascular disease in adulthood. In addition to MS, CMRFC has been defined in recent years and accepted as another metabolic health parameter (18). In the present study both MS and CMRFC were compared and used for metabolic health evaluation. Since CMRFC has at least two parameters, its rate would be expected higher than MS, as in our cases. As expected, the presence of MS and CMRFC were significantly higher in the obese group compared to the control group ($p < 0.05$).

Different results have been obtained in different studies researching BMD values in adolescent obesity. In our study, BMD z-score levels were significantly higher in the

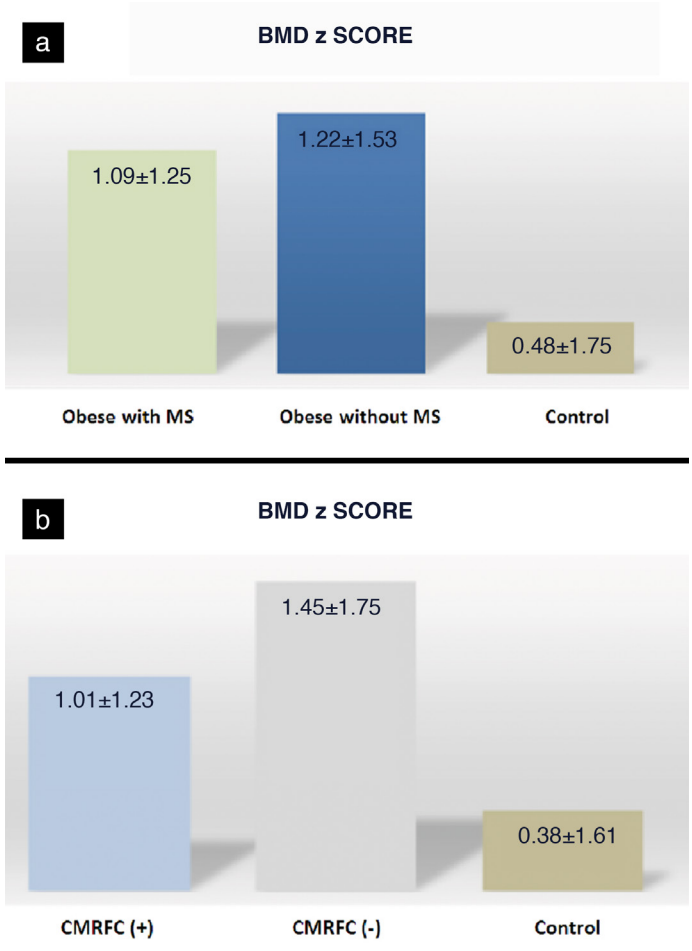

Figure 1. a) BMD z-score (g/cm^2) in obese with MS, obese without MS and control groups [BMD z-score values are significantly higher in the obese without MS group than in the control group ($p < 0.044$)]. **b)** BMD z-score (g/cm^2) in CMRFC (+) obese, CMRFC (-) obese and control groups [BMD z-score values were significantly higher in the CMRFC (-) obese group than in the control group ($p < 0.016$)]

BMD: bone mineral density, MS: metabolic syndrome, CMRFC: cardiometabolic risk factors clustering

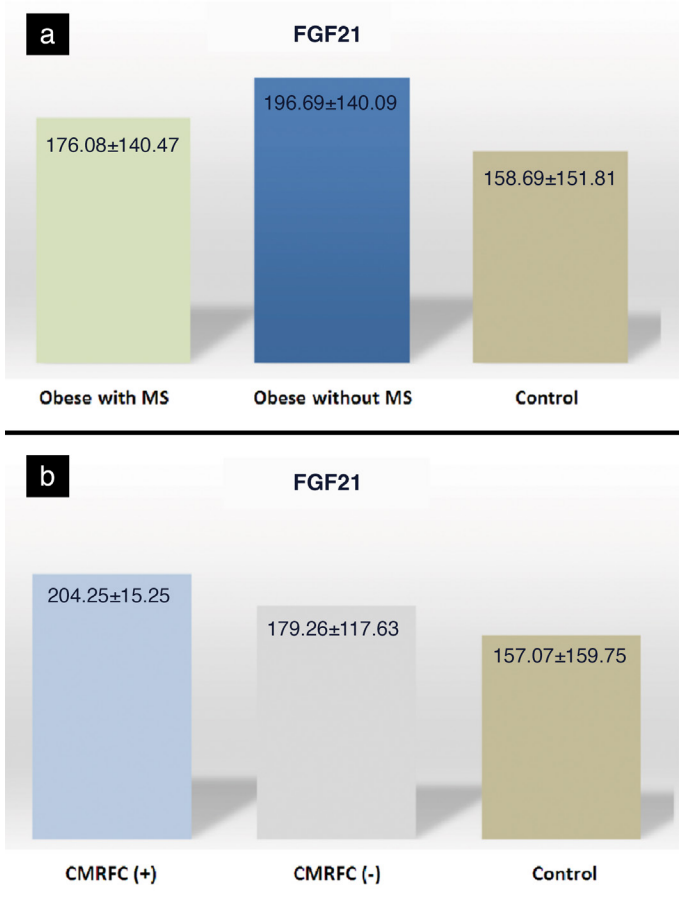

Figure 2. a) FGF21 (pg/mL) in obese with MS, obese without MS and control groups ($p > 0.05$). **b)** FGF21 (pg/mL) in CMRFC (+) obese, CMRFC (-) obese and control groups ($p > 0.05$)

MS: metabolic syndrome, CMRFC: cardiometabolic risk factors clustering, FGF21: fibroblast growth factor 21

Table 4. Comparison of obese and control group FGF21 (pg/mL) and BMD z-score (g/cm²) values

	Obese (98)	Control (44)	p value
	Mean ± SD Median (min-max)	Mean ± SD Median (min-max)	
FGF21	193.54 ± 139.62 147.66 (22.53-735.79)	158.69 ± 151.81 102.31 (11.82-853.65)	0.064
BMD z-score	1.19 ± 1.48 0.93 (-1.44-6.17)	0.48 ± 1.75 0.13 (-2.67-5.76)	0.013

FGF21: fibroblast growth factor 21, SD: standard deviation, min-max: minimum-maximum, BMD: bone mineral density

Table 5. Correlation between variables with FGF21 values in the obese and control groups

		FGF21	
		Obese	Control
Age (years)	r	0.094	-0.033
	p	0.358	0.833
Weight	r	0.095	-0.031
	p	0.353	0.841
Height	r	0.005	-0.159
	p	0.963	0.304
Height SDS	r	-0.051	-0.304
	p	0.616	0.045(*)
BMI (kg/m²)	r	0.132	0.017
	p	0.196	0.915
BMI percentile (%)	r	0.070	0.038
	p	0.493	0.807
BMI SDS	r	0.074	0.038
	p	0.473	0.807
Waist circumference (cm)	r	0.197	-0.075
	p	0.051	0.626
Hip circumference (cm)	r	0.019	-0.105
	p	0.856	0.499
Fasting blood glucose (mg/L)	r	-0.803	0.069
	p	0.416	0.657
Fasting insulin (mIU/mL)	r	0.056	0.053
	p	0.581	0.731
Total cholesterol (mg/dL)	r	-0.096	0.066
	p	0.349	0.669
Triglyceride (mg/dL)	r	-0.059	0.298
	p	0.562	0.049(*)
HDL-C (mg/dL)	r	0.103	-0.024
	p	0.314	0.875
LDL-C (mg/dL)	r	-0.109	0.036
	p	0.285	0.817
VLDL-C (mg/dL)	r	-0.078	0.338
	p	0.446	0.025(*)
ALT (U/L)	r	0.115	-0.197
	p	0.260	0.200
BMD z-scores (g/cm²)	r	-0.151	0.003
	p	0.137	0.985
BMD	r	-0.110	-0.010
	p	0.279	0.949

(*): $p < 0.05$.
HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol, VLDL-C: very low-density lipoprotein-cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMD: bone mineral density, FGF21: fibroblast growth factor 21, BMI SDS: body mass index standard deviation score, min-max: minimum-maximum

obese group compared to the control group. Adolescence is a critical period for bone development and about 40% of adult skeletal calcium accumulates during this period (22). Therefore, maximizing BMD during this period may result in less osteoporosis and better protection against fracture in adulthood. Irreversible factors, such as gender, race, ethnicity, and genetics contribute to 60-80% of bone mass and environmental and lifestyle factors contribute to the remaining 20-40%. Diet and physical activity are the most widely studied factors to maximize BMD (23).

Bone regeneration and consequently skeletal homeostasis is governed by endocrine and/or humoral factors. Among anthropometric and metabolic factors, body weight is the main determinant of bone density (24). Many studies on the effect of excess adipose tissue on growing bone tissue have yielded variable results. Some studies reported more bone mass in overweight children and adolescents than in normal-weight peers, while other studies concluded that it was associated with less bone mass (4,25). Differences in these studies can be attributed to methodological limitations (3). The greater bone mass in obesity may be due to greater mechanical load on the bone. In addition, regional fat distribution may affect bone mass, independent of obesity (26). In our study, BMD z-score levels were significantly higher in the obese group compared to the control group, suggesting a positive association between obesity and increased BMD.

While a minimum level of fat is required for bone tissue to mature, excess adiposity is associated with increased bone size. However, this can have a negative effect on bone quality. During adolescence, body fat has been associated with larger bones in boys and larger and denser bones in girls (26). Baxter-Jones et al. (27), in a study of children aged 8-19 years, reported that total body and femoral neck BMD were higher in boys than in girls, but no gender differences were observed in vertebral BMD. In a study by Singhal et al. (28), which included 153 adolescent girls, parameters related to regional bone density and degree of obesity were higher in normal-weight adolescent girls compared to obese adolescent girls. In a study by Singhal et al. (28) involving 153 adolescent girls, bone density including areal BMD

z-scores from all regions was found to be higher in obese adolescent girls than in normal-weight adolescent girls. Other studies have shown that there is no difference in total BMD between boys and girls in the 9-11 age range (29,30). In the present study, there was no difference between BMD z-score values in obese girls and boys.

The metabolic effects of obesity may have an impact on bone development. Although some studies (31) have reported BMD increases in adults with MS, other studies have reported the opposite (32). These contradictory reports may partly depend on the heterogeneous samples examined but the findings suggest that the increase in BMD does not persist as metabolic health begins to deteriorate. The idea that insulin resistance due to obesity in children with MS may adversely affect BMD was first proposed by Afghani et al. (33). These authors found that BMD decreased in overweight children with insulin resistance. It has been suggested that the most important metabolic factor that may cause a negative assocaition between insulin levels and BMD is insulin resistance. Impaired glucose homeostasis has been shown to have a negative effect on the growing skeleton (34). Kindler et al. (35), conducted a study on children between the ages of 7-15 where body fat mass, waist circumference and insulin resistance were found to be negatively correlated with total body and vertebral BMD. In another study by Kindler et al. (36), insulin resistance was determined to be a potential inhibitor of IGF-I-dependent cortical bone development. Body fat mass and insulin resistance were found to be inversely associated with bone mass. Improvement in insulin resistance was seen to increase BMD in obesity (37). In addition, as the number of cardiometabolic risk factors increased, BMD decreased (3). In the present study the mean BMD z-score values were higher in the MHO obese group. In addition, no statistically significant relationship was found between insulin and HOMA-IR levels and BMD. However, there appears to be a decrease in BMD in the presence of MS or CMRFC. These results suggest a significant increase in BMD in simple obesity but only when MS or CMRFC has not yet occurred. However, a decrease in BMD was evident in the presence of MS or CMRFC. This suggests that obesity-related metabolic problems have negative effects on BMD and that a metabolic unhealthy state in obesity negatively affects BMD.

Adipokines secreted from adipose tissue and cytokines secreted from other tissues have a regulatory role in metabolism in obesity. One of these regulators is FGF21. It is produced in metabolically active tissues such as the pancreas, skeletal muscle, adipose tissue and placenta, but mainly in the liver. Experimental and clinical data revealed that FGF21 is a potent endocrine regulator with physiological effects on weight loss, insulin sensitivity, glucose and lipid metabolism (38). FGF21, which contributes to the regulation of insulin synthesis, inhibits β cell proliferation in pancreatic islet cells (11). FGF21 increases the effect of insulin as an insulin sensitizer and decreases glucose production during long post-starvation feeding or overeating (39). FGF21is also an important energy metabolism regulator with its beneficial effects on glucose and lipid metabolism (40). In animal studies, FGF21 has been shown to lower blood sugar levels and inhibit glucagon secretion (11). Animal studies have also shown that FGF21 improves hyperglycemia, hyperlipidemia and insulin resistance and thus it is suggested that FGF21 may ameliorate the development of type 2 diabetes (41). We did not find any relationship between FGF21 levels and anthropometric parameters, fasting blood sugar, fasting insulin, blood lipid profile, and ALT in both the obese group and the control group. These results suggest that not only FGF21 but also other factors may affect the mentioned parameters.

Although FGF21 acts as a protective molecule, most studies in adults reported that increased FGF21 levels are associated with an increased risk of obesity, MS and type 2 diabetes mellitus. This increases the likelihood of FGF21 resistance playing a role in the pathogenesis of some human metabolic disorders. However, to date, data specific to children is limited and remains controversial (42). For example, there are some studies in the literature that correlate circulating FGF21 levels with the amount of adipose tissue in the body (43) and there are other studies that do not detect this relationship (12). A similar situation exists for insulin resistance (42).

In a study by Reinehr et al. (43), comparing normal-weight children with obese children, FGF21 levels were significantly increased in obese children but this significant increase was not observed in the MS group. In a study by Korwutthikulrangsri et al. (44), serum FGF21 levels were high in obese children with insulin resistance or abnormal glucose tolerance. In another study, 210 children over nine years of age were evaluated, and no relationship was found between MS and FGF21 levels (45). The reason for these differences between studies may be due in part to the possibility of pubertal effects on small sample sizes and FGF21 levels (46). In addition, two studies showed no significant relationship between FGF21 levels and MS (43,45). FGF21 levels in obese children and adults were found to be significantly higher than normal-weight children and adults (43,47). In this study, we investigated whether FGF21 levels were different in obese children and their potential relationship with BMD in terms of metabolic health. Although FGF21 levels were higher in the obese

group compared to the control group, this difference was not significant (p = 0.06). Obese subjects did not differ from control groups according to their metabolic health status, when defined by either MS or CMRFC criteria. These results were similar to some alreay reported. Actually had similar results with the studies in the literature. We cannot rule out an effect of small sample size in our study as there were only 15 obese children who met the IDF criteria for being MUO. A further reason for the lack of significant differences may be the shorter duration of obesity compared to adult obesity. Finally, FGF21 might exert less effect on metabolic health for this age group.

FGF21 is a peptide that has also been reported to have a direct effect on bone as well as its metabolic effects. It has been suggested that FGF21 reduces osteoblast production from mesenchymal stem cells. However, it also increases fat cell production and ultimately reduces bone formation. Experimental animals treated with FGF21 were found to have bone loss, especially in the trabecular cortex of the bone. FGF21 is thought to directly disrupt bone formation (46). Data on FGF21 levels in obese children and adolescents are very limited. In our study, no statistically significant relationship was found that could relate changes in BMD and insulin levels with FGF21 levels. A recent study reported an inversely proportional relationship between FGF21 and lean muscle mass in girls aged 7-12 years. In addition, an inverse relationship was found between BMC and FGF21 in all cases (12). In another study of obesity accompanied by insulin resistance and hyperinsulinism, increased levels of FGF21 were reported. These authors reported that FGF21 receptor expression was decreased and FGF21 resistance, manifested as increased serum levels, was found (48).

Study Limitations

Limitations of our study should be noted. These include the number of patients in the control group being around 45% of the number in the obese group. Furthermore, the number of patients in the obese group who met the IDF criteria for MS was only 15 (15.3%) of the patients although none of the healthy controls met these criteria. The proportion of the obese group meeting the CMRFC criteria was higher at 57% but nearly 14% of controls also met these criteria.

Conclusion

Metabolic health parameters, such as hyperinsulinism and dyslipidemia were higher, and the frequency of MS and CMRFC was higher in the obese group, as expected. FGF21 levels did not differ between obese and healthy groups. In the control group, FGF21 level was negatively correlated with height SDS and positively correlated with TG and VLDL. Although BMD was significantly higher in the obese group than in the control group, it began to decrease with deterioration of metabolic health status. Changes in BMD in MUO obese children was shown to become more evident. This study suggests that metabolic changes should be considered together, without expecting isolated changes in insulin level or FGF21 level to be significant.

Ethics

Ethics Committee Approval: Ethical approval was obtained from Ankara University Faculty of Medicine Clinical Research Ethics Committee with the number 08-418-17 and made in accordance with the Helsinki Declaration (date: 24.04.2017).

Informed Consent: Informed consent was obtained from the patients and their families.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Filiz Akduman, Concept: Zeynep Şıklar, Merih Berberoğlu, Design: Zeynep Şıklar, Merih Berberoğlu, Data Collection or Processing: Filiz Akduman, Elif Özsu, Özlem Doğan, Metin Kemal Kır, Analysis or Interpretation: Zeynep Şıklar, Özlem Doğan, Metin Kemal Kır, Merih Berberoğlu, Literature Search: Filiz Akduman, Zeynep Şıklar, Elif Özsu, Writing: Filiz Akduman, Zeynep Şıklar, Merih Berberoğlu.

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Cord Blood Levels of Spexin, Leptin, and Visfatin in Term Infants Born Small, Appropriate, and Large for Gestational Age and Their Association with Newborn Anthropometric Measurements

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What is already known on this topic?

Birthweight is associated with an increased risk of obesity and cardiovascular disease later in life. Umbilical cord blood adipokines serving as a measure of adipose tissue activity are associated with birth outcomes.

What this study adds?

Cord blood spexin (SPX) levels were found to be associated with neonatal anthropometric measurements. The lowest SPX levels were found in SGA babies.

Abstract

Objective: Children born small for gestational age (SGA) are at risk of future obesity and associated comorbidities. Therefore the identification of risk factors and novel biomarkers which are associated with this risk are needed for early detection and to improve preventive strategies. Spexin (SPX), a novel neuropeptide that is involved in the regulation of obesity and fat metabolism, is a candidate biomarker for predicting obesity and related comorbidities at an early age. The aim of this study was to investigate serum levels of SPX in term infants born small, appropriate, and large for gestational age (LGA) and its association with newborn anthropometric measurements.

Methods: One hundred and twenty term newborn babies classified as SGA, appropriate for gestational age (AGA), or LGA and their mothers were included. SPX, leptin and visfatin were measured in cord blood and maternal serum by enzyme-linked immunosorbent assay.

Results: Fifty-six (46.7%) neonates were girls and 64 (53.3%) were boys. The mean birth weight was 3170.70 ± 663 g, birth length was 48.9 ± 2.79 cm, and head circumference was 34.5 ± 1.67 cm. Birth weights, lengths, and head circumferences of the neonates in the SGA, AGA, and LGA groups were significantly different. Cord blood SPX and leptin levels in the SGA groups were significantly lower than those of both the LGA and AGA groups. Cord blood visfatin levels were significantly lower in the AGA group than the LGA and SGA groups. Maternal SPX levels of SGA babies were significantly lower than those of the mothers in both the LGA and AGA groups, but no significant difference was observed between the SGA and LGA groups. Maternal visfatin levels of the AGA babies were significantly higher than the maternal levels of SGA and LGA groups. There was no difference in terms of maternal leptin levels. Cord blood SPX and leptin levels were positively correlated with birth weight, length and head circumference. Birth weight increased significantly in line with maternal pregestational body mass index.

Conclusion: The lowest SPX levels were found in the SGA babies and cord SPX level was significantly correlated with newborn length, weight, and head circumference.

Keywords: Adipokine, speksin, antropometry, newborn, umblical cord

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Introduction

Intrauterine growth is under control of genetic, growth and nutritional factors related to the fetus, mother, and placenta (1). Birth weight, length, and head circumference values according to gestational age and gender in neonates are evaluated on the basis of growth curves, and newborns are classified as small for gestational age (SGA), appropriate for gestational age (AGA), or large for gestational age (LGA) (2).

Infant birth weight is one of the determinants of perinatal mortality and morbidity. Placental insufficiency and adverse intrauterine conditions are also associated with numerous long- and short-term sequelae in postnatal life, with an increase in perinatal morbidity and mortality, by affecting the development of the fetus. Obesity, insulin resistance, metabolic syndrome, chronic kidney diseases, and cardiovascular diseases are thought to be associated with epigenetic changes occurring in fetal metabolic programming (Barker's hypothesis) affected by adverse conditions during the intrauterine period (3). Studies of control mechanisms in natal and postnatal growth have shown that adipose tissue behaves like an active endocrine organ (4) and regulates numerous physiological functions in the body, such as insulin sensitivity, inflammation, growth, puberty, and cardiovascular functions by secreting messenger molecules known as 'adipokines' (5). Adipokines are circulating factors that mediate the cross-talk between metabolic systems and different organs (5).

The most frequently studied adipokine is leptin. Leptin and its receptors are widely produced and expressed in fetal and placental tissues (6). It is predominantly released by white adipose tissue and affects appetite and food intake, gonadotropin release, immune modulation, and lipogenesis (7). Low serum and placental leptin levels have been demonstrated in newborns with intrauterine growth retardation (IUGR), and high concentrations in the macrosomic babies of diabetic mothers (6). Visfatin is specific to visceral adipose tissue and is a marker of insulin resistance-associated fat deposition (8). Studies have suggested that high visfatin levels may be a prognostic marker of the probable future development of metabolic syndrome (9,10,11). Spexin (SPX), also known as neuropeptide Q, is a novel, 14 amino acid peptide first described by Mirabeau et al. (12) in 2007. SPX binds to and activates galanin receptor 2/3 (GALR2/3) (13,14). SPX mRNA is expressed in different parts of the human body, particularly in white adipose tissue, but also in the brain, heart, thyroid, gonads, gastrointestinal system, and pancreas. Studies of SPX have shown that it is involved in energy homeostasis, glucose and lipid metabolism, obesity, gastrointestinal functions, pain, regeneration and neuron renewal, Alzheimer's disease, cerebral ischemia and stroke, epilepsy, anxiety disorders, and cardiovascular and renal functions (15). The few studies of SPX in the pediatric age group have investigated its association with obesity and metabolic diseases (16). Two of these studies were performed in the neonatal period and only one of them investigated the levels of SPX in umbilical cord blood from term LGA, SGA, and AGA infants and the association with anthropometric measurements. This study reported no significant difference in terms of SPX levels (17,18).

Anthropometric measurements in newborns have long been known as a risk factor for conditions such as obesity, cardiovascular disease and type 2 diabetes in adulthood (19). Changes in adaptation to life in the early period, known as "early life programming", may become maladaptive with advancing age and increase the risk of obesity associated cardiometabolic diseases. It is well established that children born SGA are at risk of future obesity and associated comorbidities. Therefore the identification of risk factors and novel biomarkers which are associated with this risk are needed for early detection and to improve preventive strategies. SPX is a novel neuropeptide that has an effect in many systems and has been shown to be involved in the regulation of obesity, and fat metabolism. It is a candidate biomarker for predicting obesity and related comorbidities at an early age. The aim of the present study was to investigate serum levels of SPX in term SGA, AGA and LGA infants and its association with newborn anthropometric measurements. SPX levels, together with those of the well-known and frequently studied leptin and visfatin, measured in umbilical cord serum specimens obtained from SGA, AGA and LGA infants were investigated. The relationship between the results and neonate anthropometric measurements was then examined. Potential relationships between mother-infant adipokine levels and anthropometric measurements were evaluated by analyzing the serum leptin, visfatin, and SPX levels of the mothers in blood samples collected during labor simultaneously with those of the babies.

Methods

SGA, AGA and LGA neonates born in the Pamukkale Medical Faculty Hospital, Turkey, between September 2019 and April 2020, and their mothers, were eligible for the study. Written informed consent was obtained from all parents and mothers prior to their participation in the study. The research was approved by the Pamukkale University Ethics Committee (approval number: 10.09.2019/21, date: 10.12.2019) and was performed in accordance with the Declaration of Helsinki.

Infants' gestational ages were determined from the date of the last menstrual cycle based on the New Ballard scoring system. Term neonates born between 37 and 42 weeks were divided into three groups using Lubchenko's intrauterine development curves - SGA, consisting of babies under the 10th percentile, AGA, babies with birth weights for gestational age between the 10th and 90th percentiles, and LGA, babies above the 90th percentile (2). One hundred and twenty newborn babies, 40 in each group, and their mothers were included in the study. Babies with congenital malformations, syndromic babies, and those with chromosomal disease, severe infection, hypothyroidism, and similar disorders capable of causing growth and development retardation, and the babies of mothers with histories of maternal hypothyroidism, early membrane rupture, pre-eclampsia, and gestational/pregestational diabetes were excluded. The blood specimens required for the study were collected from the umbilical cord blood supply together with blood samples (blood gas, blood group) collected routinely in order to evaluate the baby's condition in the delivery room, and from the mothers of the babies before they left the delivery room.

Blood samples for SPX, leptin, and visfatin measurement were collected from the umbilical vein on the placental side of the umbilical cord, and venous blood samples collected from the antecubital veins of the mothers were placed into pre-prepared biochemistry tubes containing a gel separator. Following a maximum waiting period of 30 minutes, the sera were separated by centrifugation at 3000xg for 10 minutes. These were then placed into Eppendorf tubes and stored at -80 °C until analysis. The birth weights, lengths, and head circumferences of all the babies in the study were measured by the same individual. Body weight was measured using electronic scales sensitive to 5 g, with the baby wearing no clothing or nappy. Length was measured with the baby lying in a supine position, and head circumference using a non-elastic tape measure, and passing this over the most protruberant point on the back of the head, the parietal region on the side, and the glabella on the front. Serum SPX levels were measured using an Enzyme Amplified Sensitivity Immunoassay (EASIA) method on an Lbiont Human SPX (C12orf39) Enzyme-linked Immunosorbent Assay (ELISA) (Catalog no. YLA1034HU) test kit. The sensitivity was 4.95 pg/mL and detection range was 10 pg/mL-4000 pg/mL. Serum leptin levels were measured using EASIA on a Boster Picokine Human Lep pre-coated ELISA (catalog no. EK0437) test kit. The sensitivity was 4.95 pg/mL and detection range was 10 pg/mL-4000 pg/mL respectively. Serum visfatin levels were calculated using the ELISA method on a CUSABIO Human Visfatin ELISA (Catalog no. CSB-E08940h) test kit. The sensitivity and detection ranges were 0.156 ng/mL and 0.625 ng/mL-40 ng/mL, respectively. No data out of read limits

Statistical Analysis

Statistical analysis of the study findings was performed on Statistical Package for Social Sciences for Windows, version 22.0 (IBM INC., Armonk, NY, USA). In addition to descriptive methods, the Kruskal-Wallis and Mann-Whitney U tests were used in the comparison of numerical data since the parameters were all non-parametric, and Bonferroni correction was applied. The chi-square test was employed for the comparison of qualitative data. Since the parameters were not normally distributed, Spearman's correlation test was applied to examine relationships between parameters, and the partial correlation test was performed in case of the presence of potentially confounding factors. Robust regression analysis was performed, again due to non-normal distribution. A multiple regression model was employed in order to examine the effect of each adipokine on newborn anthropometric measurements. Cord blood SPX, leptin, and visfatin were used as dependent variables in this model, and length, weight, and head circumference as independent variables. A $p < 0.05$ was regarded as statistically significant.

Results

One hundred and twenty neonates and their mothers who meet the study criteria were included in the research. Fifty-six (46.7%) neonates were girls and 64 (53.3%) were boys. Across the whole cohort the mean birth weight was 3170.70 ± 663 g, mean birth length was 48.90 ± 2.79 cm and mean head circumference was 34.50 ± 1.67 cm. The mean age of the mothers was 30.38 ± 5.83 years, and the mean gestational week was 38.05 ± 0.77. The mean weight of the mothers prior to delivery was 62.46 ± 6.32 kg, their mean height was 159.10 ± 4.17 cm, and their mean body mass index (BMI) was 24.7 ± 2.8. Mean weight gain during pregnancy was 11.60 ± 1.43 kg (range 9 to 15 kg). Based on their pre-pregnancy BMI values, 40.83% of mothers were overweight or obese. Birth weights (2407.7 ± 296.9 vs. 3219.2 ± 245.9 vs. 3885.1 ± 264.6), lengths (46.2 ± 2.6 vs. 49.2 ± 1.3 vs. 51.1 ± 1.6), and head circumferences of the neonates (32.8 ± 1.3 vs. 34.6 ± 0.7 vs. 36 ± 1.1) in the SGA, AGA, and LGA groups, exhibited statistically significant differences, as expected. The mean age of the mothers in the SGA group was significantly higher than that of the mothers in the AGA group ($p < 0.05$).

Across the whole study cohort neonatal median cord blood SPX concentration was 304.88 pg/mL (12.564-1739.22), median leptin concentration was 6.50 ng/mL (0.58-41.60), and median visfatin concentration was 3.28 ng/mL (0.39-

8.50). Similarly, for the mothers, median plasma SPX concentration was 336.09 pg/mL (15.62-1216.45), median leptin concentration was 19.59 ng/mL (1.73-53.94), and median visfatin concentration was 3.28 ng/mL (0.39-8.56).

SPX, leptin, and visfatin concentrations of the neonates in the SGA, AGA, and LGA group were measured and compared togethere with those values obtsined from their mothers (Table 1). Cord blood SPX concentrations of SGA neonates were significantly lower than those of both the LGA and AGA groups. The venous blood SPX concentrations of mothers of SGA babies were significantly lower than those of the mothers in both the LGA and AGA groups. The SPX concentration of mothers in the AGA and LGA groups did not differ.

Comparison of leptin concentrations in cord blood samples showed that neonates from the SGA group had significantly lower median leptin concentrations than those found in either the LGA or AGA groups. Furthermore, median cord blood leptin levels were also significantly lower in AGA group neonates than in LGA group neonates (Table 1). No statistically significant difference in maternal venous blood leptin concentrations was observed between the groups.

Cord blood visfatin concentrations were significantly lower in the neonates from the AGA group compared to those from both the LGA and SGA groups. However, median concentration values of visfatin obtained from babies in the SGA and LGA groups did not differ (Table 1). Similarly, maternal visfatin levels were significantly higher in the mothers of neonates from the AGA group compared to those of neonates from both the SGA and LGA groups (Table 1).

In correlation analysis, cord blood SPX and leptin levels were positively correlated with birth weight, length and head circumference (Table 2). Birth weight increased significantly in line with maternal pregestational BMI ($r = 0.505$, $p < 0.001$). Correlation analysis of the adipokine concentration of all the neonates and their mothers showed strong correlation between infant and maternal SPX levels ($r = 0.818$, $p < 0.001$) but a weak correlation between infant and maternal leptin levels ($r = 0.214$, $p = 0.019$) (Table 2).

Regression analysis showed cord blood leptin concentrtion had a significant positive association with birth weight and head circumference [Odds ratio (OR): 0.584, 95% confidence interval (CI): 0.003-0.011, $p = 0.002$ and OR: -0.334, 95% CI: -3.00-0.142, $p = 0.032$], but that SPX and visfatin levels exerted no significant effects on birth indices.

Discussion

This study investigated the relationships between anthropometric measurements and levels of the novel adipocytokine SPX and of leptin and visfatin in the cord blood of term SGA, LGA, and AGA babies and in plasma specimens from their respective mothers. Cord blood SPX and leptin levels were lowest in the SGA neonates, while visfatin levels were similar between babies from the SGA and LGA groups but higher than those in babies from the AGA group. Significant correlation was found between neonatal cord blood SPX and leptin levels and newborn length, weight, and head circumference.

The birth weight of the newborn baby is the most important anthropometric measurement used in the evaluation of pregnancy, and also one of the most important predictors of perinatal mortality and morbidity (20). The understanding that numerous common chronic diseases are caused by adaptations developed to combat adverse intrauterine conditions in the early period of life has significantly

Table 1. Serum and cord blood levels of spexin, leptin, and visfatin in SGA, AGA, LGA newborns and mothers

	SGA (1) (n = 40)	AGA (2) (n = 40)	LGA (3) (n = 40)	p
Cord blood spexin (pg/mL)	191.2 (12.6-1739.2)	6.34 (1.8-25.1)	9.75 (1.2-35.4)	**0.0001 (1-2)[§] (1-3)** **0.413 (2-3)**
Cord blood leptin (ng/mL)	3.54 (0.4-34.7)	6.34 (1.8-25.1)	9.75 (1.2-35.4)	**0.046 (1-2)** **0.0001 (1-3)[®]** **0.035 (2-3)**
Cord blood visfatin (ng/mL)	5.84 (0.4-34.7)	3.20 (0.3-8.6)	5.69 (2.8-17.8)	**0.0001 (1-2/2-3)** 1.000 (1-3)
Maternal spexin (pg/mL)	196.28 (15.6-1216.4)	366.047 (129.6-837.3)	392.34 (24.56-1148.96)	**0.0001 (1-2/1-3)**
Maternal leptin (ng/mL)	19.18 ± 8.39	16.73 ± 9.78	22.03 ± 11.94	0.069*
Maternal visfatin (ng/mL)	1.55 (0.39-6.85)	4.43 (0.42-7.38)	1.66 (0.41-8.56)	**0.0001 (1-2) (2-3)[¶]** 1.000 (1-3)

Values are expressed as mean ± standard deviation, median (minimum-maximum). Comparison between groups: Kruskal-Wallis test, *Anova.
SGA: small for gestational age, AGA: appropriate for gestational age, LGA: large for gestational age
[§](1-2) Comparison between SGA and AGA
[®](1-3) Comparison between SGA and LGA
[¶](2-3) Comparison between AGA and LGA

increased interest in endocrine programming. Following the initial observation of an association between the risk of mortality due to ischemic heart disease and the individual's birth weight, similar relationships were found in other diseases, such as stroke, type 2 diabetes mellitus, and dyslipidemia (21).

Obesity is a global health problem and a risk factor for chronic diseases (22). There is a known link between maternal obesity and health problems in newborn babies, and the increase in obesity rates among women of reproductive age is worrying. The effect on the fetus of adverse intrauterine conditions is known to affect the risk of development of disease in subsequent periods of life, and the process, known as "early life programming", in which the fetus adapts to the adverse intrauterine environment, increases the likelihood of its survival (23). An adverse intrauterine environment can affect the amount and function of adipose tissue, extending into adulthood (24,25). Changes due to early life programming may become maladaptive with advancing age and exacerbate the risk of several chronic diseases, such as obesity, diabetes, and hypertension (26).

The placenta, and the hormones and adipokines produced by it, are of considerable importance in providing the nutritional resources essential for growth and development of the fetus and in maternal metabolic adaptation to pregnancy (27). The relationship between neonatal cord blood adipokine levels and anthropometric measurements has been the subject of frequent investigation, and efforts have been made to predict the risk of obesity, metabolic syndrome, and cardiovascular disease development in at-risk newborn babies (25,28,29). In this respect, one of the most studied adipokines in the neonatal period is leptin. Placental leptin production is the main maternal source of leptin. High molecular weight leptin of maternal origin is unable to cross the placenta, and measurement of cord blood leptin levels therefore show fetal adipose tissue and placental production. Umbilical cord blood leptin levels have been found to be associated with placental weight, and the infantile body weight, length, head circumference, ponderal index, adiposity, and bone mineral density. These studies have shown higher cord blood leptin values in LGA neonates compared to those born AGA and SGA (30), and lower cord blood leptin levels and decreased leptin gene expression in those with IUGR (30,31,32). In their meta-analysis examining the relationship between cord blood leptin levels and anthropometric measurements, Karakosta et al. (33) reported a correlation between leptin levels and birth weight (r = 0.46). A significant difference was also determined in cord blood leptin levels between the groups in the present study. As expected, the lowest leptin level was observed in the SGA group, and the highest in the LGA group. Leptin level elevation at birth may result in adverse programing of the hypothalamus (via an impaired leptin surge), the effects of which usually manifest after the third year of life. The initial negative correlation between cord leptin and adiposity may be due to the anorexigenic effect of leptin, with subsequent leptin resistance, in turn leading to hyperphagia and increased adiposity (25,34,35,36). Studies investigating the relationship between maternal leptin levels and neonatal anthropometric measurements have reported positive, negative, and even no correlation (37,38,39). There are also studies showing a direct association between maternal obesity markers and neonatal leptin levels (40,41). These inconsistent observations in pregnant women suggest that most of the leptin levels measured in mothers originate from the placenta, and that other factors or complications of pregnancy may affect leptin levels (42). In the present study, individual evaluation of the groups revealed weak correlation between maternal leptin and cord blood leptin levels, but only between the babies and their mothers in the AGA group. Positive correlation was observed between cord

Table 2. Correlation analysis of birth weight, length, head circumference, maternal BMI, spexin, leptin, and visfatin levels

	Cord spexin (n = 120)		Cord leptin (n = 120)		Cord visfatin (n = 120)	
	r	p	r	p	r	p
Birth weight	**0.417**	**0.0001**	0.404	**0.0001**	0.045	0.628
Birth length	**0.363**	**0.0001**	0.293	**0.001**	0.074	0.421
Birth head circumference	**0.375**	**0.0001**	0.314	**0.0001**	-0.124	0.178
Maternal BMI	**0.21**	**0.028**	0.207	**0.023**	0.047	0.613
Maternal spexin	0.818	**0.000**	0.05	0.958	0.44	0.630
Maternal leptin	-0.076	0.409	0.214	**0.019**	-0.90	0.326
Maternal visfatin	0.20	0.831	-0.107	0.247	0.09	0.329
Cord spexin			0.155	0.091	-0.43	0.637
Cord leptin	0.155	0.091			-0.175	0.055
Cord visfatin	-0.043	0.637	-0.175	0.055		

BMI: body mass index

blood leptin levels and maternal BMI, but no correlation was found between maternal BMI or weight gain in pregnancy and maternal leptin levels.

Visfatin is an adipocytokine mostly produced in visceral adipose tissue, and is also secreted by the placenta and amniotic membranes, and that results in increased synthesis of proinflammatory cytokines (43). The pathophysiological role of visfatin has frequently been investigated in conditions such as chronic inflammatory and rheumatological diseases, cardiovascular diseases, diabetes mellitus, and obesity in which glucose metabolism is affected (43). Insulin resistance increases the expression of visfatin. Levels of visfatin also rise in normal pregnancies in which physiological insulin resistance is observed, peaking between the nineteenth and twentyfifth weeks (44). This increase becomes more pronounced when accompanied by obesity and related diseases. Changes in visfatin levels have been linked to pre-eclampsia, IUGR, premature birth, and gestational diabetes (GDM) (45). It is not always easy to ascertain whether these differences derive from the mother, placenta, or fetal metabolism alone, or from a collective interaction. In research on this subject, that may be regarded as pioneering, Malamitsi-Puchner et al. (11) compared two newborn groups, AGA and IUGR, and reported no significant difference in umbilical cord visfatin levels, but observed significantly higher serum visfatin levels in mothers who gave birth to babies with IUGR than those giving birth to AGA babies. Similarly, Estrada-Zúñiga et al. (46) investigated cord blood visfatin levels in 128 newborns divided into groups according to birth weight and reported no difference in cord blood levels, while Mazaki-Tovi et al. (47) found no difference in cord blood visfatin concentrations in normotensive mothers of babies born AGA and SGA. In addition, in a study of mothers and children with no morbidity, Meral et al. (10) reported higher visfatin concentrations in LGA neonates compared to SGA neonates, and in SGA neonates compared to AGA babies. Shang et al. (48) reported higher visfatin levels in neonates with IUGR compared to a macrosomic neonate control group, while Cekmez et al. (49) reported slightly higher cord visfatin levels in neonates in an SGA group compared to an AGA group. Similarly, in the present study, cord blood visfatin levels were significantly higher in the LGA and SGA group neonates compared to the AGA group. Visfatin is produced in visceral adipose tissue, and we hypothesize that increased visceral adipose tissue in LGA and SGA neonates may have resulted in this elevation. Visfatin may be an early marker of the development of insulin resistance capable of emerging later in life, and it has also been suggested that it possesses prognostic value in terms of the future development of metabolic syndrome in neonates with IUGR (10).

Information concerning the physiological functions of SPX has increased as studies have revealed the presence of SPX in a wide range of tissues and organ systems including the central nervous system (hypothalamus, pons, cerebral cortex, and anterior pituitary gland), white adipose tissue, kidneys, ovaries, thyroid, stomach, adrenal glands, pancreas and many other human tissues (50). These studies have shown that SPX is associated with feeding behavior, body weight, obesity, diabetes mellitus, gastrointestinal motility, mental illness, and reproductive and cardiovascular functions (51). Studies of adult and pediatric obese patients have shown lower SPX levels than those of normal-weight controls, with SPX levels being reported to exhibit inverse correlation with such metabolic parameters as BMI, waist circumference, low-density lipoprotein, triglycerides, leptin, ghrelin, insulin, and HOMA-IR (52,53,54,55,56). However, there are also studies reporting no difference in SPX levels between adolescent obese, normal, and type 2 DM groups (57). While some studies have reported decreased serum SPX levels in patients with type 1 and type 2 DM compared to a control group and that serum SPX levels are not associated with glycemic parameters, lipids, or BMI, and that the decreased SPX levels in patients with Type 2 DM are related to fasting glucose and HbA1c, others have concluded that SPX is positively associated with blood lipid levels and that SPX levels increase in patients whose blood sugars improve following intervention (58).

Studies have sought to identify the facilitating or preventive role of different adipokines in the emergence of GDM, which can develop as a result of insulin resistance during the natural course of pregnancy and that have adverse effects on maternal and infant health (59). There has been an increase in the numbers of studies investigating the physiological role of SPX during pregnancy. Akbas et al. (60) compared pregnant women with and without GDM in the third trimester of pregnancy and observed higher serum SPX levels in women with GDM compared to controls with no GDM. In that study, serum SPX levels were directly correlated only with HOMA-IR ($r = 0.234$, $p = 0.04$), while no correlation was observed with patient age, glucose, insulin, BMI, or fetal weight. Al-Daghri et al. (61) followed-up 102 pregnant women, 63 non-GDM and 39 with GDM and reported that serum SPX levels in pregnant women with GDM increased significantly in line with glucose levels, while serum SPX levels decreased in the non-GDM patients, although this decrease was not associated with glucose levels. Yavuzkir et al. (62) investigated subfatin and SPX levels in the serum of pregnant women with and without GDM, before and during an oral glucose tolerenace test performed between weeks 24 and 28 weeks of pregnancy, and in baby cord blood at birth. These authors reported significantly higher SPX and

subfatin levels in women with GDM during pregnancy and during delivery, while cord blood SPX and subfatin levels in babies born to mothers with GDM were significantly higher than those of babies born to mothers without GDM. Serum levels of these adipokines were positively correlated with the lipid, glucose, and HOMA-IR levels of mothers with GDM ($p < 0.05$). The common conclusion of all these studies is that the SPX levels of pregnant women with GDM were significantly higher than in the controls, and this has led to SPX being proposed as a potential marker in the diagnosis and follow-up of GDM. SPX levels in mothers with and without GDM and in babies' umbilical cord blood were examined in only one of these studies to date. Sanli et al. (17) investigated the effect of SPX, leptin, ghrelin, free 25(OH) vitamin D3, glucose, and insulin levels in umbilical cord blood from term LGA, SGA, and AGA infants on anthropometric measurements. They observed that only leptin levels were higher in the LGA group than in the SGA and AGA groups, while no significant difference was observed in terms of SPX, ghrelin, free 25(OH) vitamin D3, or insulin levels. To the best of our knowledge, the present study is the first to investigate SPX levels measured in healthy pregnant women and cord blood from newborns classified as LGA, SGA, and AGA, with the lowest SPX levels being found in the SGA babies and mothers. No statistically significant difference was observed between AGA and LGA babies and mothers' SPX levels. Cord blood SPX levels were positively correlated with neonatal birth weight, birth length, and head circumference, and maternal BMI, and maternal SPX levels. We speculate that the presence of this correlation suggests that adverse conditions affecting fetal development in that period cause a decrease in SPX synthesis or SPX levels exert a positive effect on growth in the intrauterine period. Compared with the research of Sanli et al. (17) which is the only other study to report results at least partly similar to our own findings, cord blood leptin levels exhibited a positive effect on anthropometric measurements, but in contrast to their findings, we found that cord blood SPX levels were associated with neonatal anthropometric measurements. These differences in results may be due to the difference between the two study populations. Sanli et al. (17) conducted their study in Istanbul, which is home to different ethnic groups and that has received considerable immigration in recent years, and is the world's 14th largest metropolis. In contrast, our study was conducted in a small city with a relatively homogenous population. The variation in SPX levels may be attributable to the difference in the numbers of patients, and to that study being conducted in a city such as Istanbul. Being born SGA is now well established as a risk factor for type 2 DM, obesity, hypertension, and cardiovascular diseases in which insulin resistance is a common feature (18). The most interesting finding of the present study was that SPX levels, which has been shown to be negatively correlated with insulin resistance parameters in insulin resistance and associated metabolic disorders, were also low in SGA neonates (18). Although maternal SPX levels correlated with maternal BMI and cord blood SPX levels, no difference was observed between the mothers of SGA and AGA babies in terms of BMI values. We speculate that placental production of SPX might contribute to the levels of both fetal and maternal SPX levels. We think that this hypothesis can be tested by studying the placental weights and SPX expressions of pregnant women with GDM in order to explain the high SPX levels in pregnant women with GDM, in contrast to the low SPX levels in type 2 DM in particular.

Study Limitations

There are a number of limitations to this study. First, neonatal and maternal insulin resistance parameters were not investigated. Second, placental weights were not measured and SPX expression in placental tissue was not evaluated. Third, the neonates were not followed-up long term. Thus, more extensive studies including the measurement of cord levels at birth and long-term follow-up of these patients will clarify whether SPX may be a clinially useful predictive marker for metabolic disorders and cardiovascular diseases that may not present until adolescence or adulthood.

Conclusion

SPX level, which has previously been shown to be negatively correlated with insulin resistance parameters in insulin resistance and associated metabolic disorders, was also found to be low in SGA neonates in this study. Furthermore, cord SPX concentration significantly correlated with newborn length, weight, and head circumference. Given the clinical association between SGA infants and the later risk of diseases associated with insulin resistance, we believe that this association warrants further extensive research.

Ethics

Ethics Committee Approval: The research was approved by the Pamukkale University Ethics Committee (approval number: 10.09.2019/21, date: 10.12.2019) and was performed in accordance with the Declaration of Helsinki.

Informed Consent: Written informed consent was obtained from all parents and mothers prior to their participation in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Yücel Pekal, Bayram Özhan, Design: Yücel Pekal, Bayram Özhan, Yaşar Enli, Özmert M.A. Özdemir, Hacer Ergin, Data Collection or Processing: Yücel Pekal, Yaşar Enli, Analysis or Interpretation: Yücel Pekal, Bayram Özhan, Yaşar Enli, Literature Search: Yücel Pekal, Bayram Özhan, Writing: Yücel Pekal, Bayram Özhan.

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Thyroid Function in 509 Premature Newborns Below 31 Weeks of Gestational Age: Evaluation and Follow-up

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What is already known on this topic?

Preterm and low birth weight (LBW) newborns are at risk of thyroid dysfunction during a critical period for neurodevelopment and this dysfunction can be missed in the congenital hypothyroidism screening program performed in whole-blood. The utility of a second screening, its optimal timing and the need of levothyroxine (LT4) still remain subjects for debate.

What this study adds?

This study included a large number of preterms and their follow up. This protocol was able to detect thyroid dysfunction in neonates who were not identified by the current program based on thyroid stimulating hormone determination in whole-blood. Most cases of thyroid dysfunction resolve spontaneously in a few months, but in some cases LT4 could be needed.

Abstract

Objective: Preterm and low birth weight (LBW) neonates may present with thyroid dysfunction during a critical period for neurodevelopment. These alterations can be missed on routine congenital hypothyroidism (CH) screening which only measures thyroid stimulating hormone (TSH). The objective of this study was to evaluate a protocol for thyroid function screening (TFS) six years after national implementation.

Methods: Serum TSH and free thyroxine (fT4) were measured during the second week of life in neonates below 31 weeks. Patients with abnormal TFS (fT4 < 0.8 ng/dL and/or TSH > 5 mU/L) were followed up with repeated tests until normal levels were reported. Patients who were still on levothyroxine (LT4) at three years of age were re-evaluated.

Results: Five-hundred and nine neonates were included. Thyroid dysfunction was detected in 170 neonates (33%); CH n = 20 (3.9%) including typical CH n = 1; delayed TSH elevation CH n = 19; hypothyroxinemia of prematurity (HOP) n = 15 (2.9%); and transient hyperthyrotropinemia n = 135 (26.5%). Twenty-one neonates (4.1%) were treated (20 for CH and 1 for HOP). At 3-year follow-up only three patients were diagnosed with permanent CH and still need treatment. LBW infants tended to have TSH levels higher than those with adequate weight.

Conclusion: This protocol was able to detect thyroid dysfunction in preterm neonates who were not identified by the current program based on TSH determination in whole-blood. This thyroid dysfunction seems to resolve spontaneously in a few months in the great majority of neonates, but in some cases LT4 could be needed. There is a critical need for specific guidelines regarding the follow-up and re-evaluation of transient CH in preterm neonates.

Keywords: Preterm newborn, low birth weight, congenital hypothyroidism, hypothyroxinemia of prematurity, delayed TSH rise

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Introduction

Thyroid hormones are essential for the growth and development of the central nervous system, as well as for bone, pulmonary and cardiac maturation throughout foetal and neonatal life (1,2,3). Preterm neonates usually exhibit lower thyroid hormones levels compared to term neonates, in proportion to their degree of prematurity (4). The immaturity of the hypothalamic-pituitary-thyroid axis and the influence of pathologies, such as respiratory distress syndrome, sepsis, and intraventricular hemorrhage, and the therapeutic measures (dopamine, corticosteroids) make these infants prone to thyroid dysfunction (5,6).

Hypothyroxinemia of prematurity (HOP) refers to low levels of free thyroxine (fT4) generally without elevation of thyroid stimulating hormone (TSH) (4,7,8). This condition is difficult to differentiate from central hypothyroidism and from non-thyroidal illness syndrome. Although in preterms with HOP low thyroid hormone levels have been related to worse neurodevelopmental outcome, a causal relationship has not been clearly established as it is difficult to adjust for other co-morbidities present in this population (9,10,11,12). However, some preterm neonates, especially those with low birth weight (LBW), have congenital hypothyroidism (CH) with delayed elevation in serum TSH levels (5,13,14). The immaturity of the hypothalamic-pituitary axis, iodine overload due to any procedure involving iodine-contining antiseptics, drugs, and acute nonthyroidal illness can contribute to the elevation of TSH and its later occurrence in time (5,15,16). This CH will be transient in the majority of infants but it could be permanent in some cases. Moreover, it is unclear whether treatment with levothyroxine (LT4) is necessary for milder elevations of TSH (13).

Although the incidences of permanent CH and central hypothyroidism are similar in preterm and term newborns, these disorders can be missed in CH screening performed using only TSH determination in a dried blood spot test taken at 48-72 hours of life. Accordingly, guidelines of the European Society for Paediatric Endocrinology and the European Society for Endocrinology strongly recommend a second screening for preterm neonates, low or very LBW neonates, and sick neonates admitted to the neonatal intensive care unit (NICU) (17). The utility of the second screening, its optimal timing, whether it measures TSH alone in dried blood spot or TSH and fT4 in a serum sample, the TSH cutoffs to be used, and the need to start replacement therapy still remain subjects of active debate (18).

The Neonatal Screening Program of Catalonia centralizes all the birth centers throughout Catalonia and only mandates a TSH determination in dried blood spot and does not currently require a routine second sample on preterm neonates or LBW neonates. Therefore, thyroid function screening (TFS) based on measurement of serum TSH and fT4 has been implemented in our tertiary hospital NICU for preterm neonates born below 31 weeks of gestational age (GA). This protocol has been conducted in addition to routine CH screening in dried blood spot samples.

The aims of the present study were: first, to determine the incidence of thyroid dysfunction detected by the application of this protocol in preterm neonates below 31 weeks of GA; and second, to describe the follow-up of the treated patients at reassessment. In addition, thyroid function of preterm neonates with LBW for GA was evaluated separately.

Methods

This was a prospective, observational and descriptive study. TFS based on measurement of serum TSH and fT4 during the second week of life was performed on all preterm neonates below 31 weeks GA admitted in a tertiary hospital NICU from January 2011 to March 2017. Patients who died before 14 days of age, those who were transferred from other centers after 14 days, those born to mothers with thyroid disorders and those who had no TFS performed for any other reason were excluded.

TFS was conducted at the same time as routine blood tests scheduled during the second week after birth for preterm neonates and did not involve an extra blood test or a larger volume of blood to be drawn. TSH and fT4 were measured by immunochemoluminescence in an automated analyzer (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA) using commercially-available kits.

HOP was defined by low fT4 (lower than 0.8 ng/dL) and normal TSH (lower than 5 mU/L) according to the literature reviewed and a previous study published by our group (4,7,19). In those preterm neonates with increased TSH levels, the differential diagnosis between CH and hyperthyrotropinemia was based on if they were treated or not (13). A TSH serum cut-off value of 12 mU/L to start LT4 was used, based on consensus guideline recommendations to treat patients if TSH was between 10-20 mU/L and on the experience of previous cases, and with the intention of evaluating whether this cut-off point could be raised (17,20). CH was diagnosed in neonates with increased serum TSH (>12 mU/L) and normal or low fT4 who were treated with LT4. It was categorized as transient or permanent CH, depending on whether treatment could be withdrawn before or at 3 years of age. CH was classified as typical when it was detected by routine CH screening on whole-blood or with delayed TSH rise (TSH levels increased from the first

or more TFS). Hyperthyrotropinemia was defined as the presence of increased TSH (but always below 20 mU/L) with fT4 in the normal range in neonates in whom LT4 was not started either because this TSH elevation was transient or lower than 12 mU/L.

LBW for GA was defined as birth weight below -2 standard deviation (SD) score according to the Spanish newborn reference population (21,22).

Maternal and neonatal data were collected from the NICU database including maternal thyroid dysfunction, type of pregnancy (single or twin), sex, cause of preterm birth, administration of prenatal corticosteroids and magnesium sulfate, GA, anthropometry at birth, Apgar test, type of resuscitation, endotracheal surfactant administration, neonatal evolution variables (respiratory and hemodynamic support, intraventricular hemorrhage, early and late sepsis, death), genetic diseases of the neonate, and iodine overload procedures.

The TFS flow-chart is shown in Figure 1. Patients with abnormal TFS (fT4 < 0.8 ng/dL and/or TSH > 5 mU/L) were followed up with repeated thyroid function test until normal levels of thyroid hormones were reported. Patients with TSH level > 12 mU/L, persistently elevated TSH 5-12 mU/L and/or persistently low fT4 below 0.8 ng/dL were started on LT4 after confirmation and taking into account the patient's clinical condition and the preceding TFS. Oral LT4 replacement was administered at 4-6 mcg/kg/day for HOP and 10-15 mcg/kg/day for CH (7,17,20). In those preterm infants with normal TFS at first sample, TFS was repeated if iodine overload occurred. A thyroid ultrasound was performed on all treated patients. Those who were still on replacement therapy at discharge from the NICU were followed up in the pediatric endocrinology outpatient clinic. All patients who were still on replacement therapy at three years of age were re-evaluated, and those who still showed thyroid dysfunction after three weeks without LT4 were assessed by genetic screening for thyroid dyshormonogenesis with Next Generation Sequencing. This consisted of PCR amplification of coding exonic sequences and flanking intronic regions of *DUOX2, DUOXA2, IYD, TPO, SLC26A4, SLC5A5, TG, TSHR,* and *PAX8* genes using the GeneRead (Quiagen) methodology and sequencing using the Illumina MiSeqd sequencer. Data analysis was performed with the following platforms MiSeq Control Software (MCS), MiSeq Reporter (Illumina Inc, San Diego CA, USA) and GeneRead SeqVariant Analysis software (Quiagen).

All neonates were tested for CH within the Catalonia Neonatal Screening Program. This program uses a primary TSH test strategy from whole-blood sample on filter paper following a heel-prick at 48-72 h of life. Whole-blood TSH concentration is measured by fluorescence immunoassay in an autoanalyzer (AutoDELFIA Neonatal hTSH kit, Perkin Elmer, Turku, Finland), using commercially-available kits. If the TSH level is higher than 20 mU/L, the patient is referred to our endocrinology unit for confirmation and treatment. If TSH is between 10-20 mU/L a second dried blood spot sample is requested, and if TSH persists above 10 mU/L the patient is also referred to our endocrinology unit.

Serial thyroid hormone determinations (date of analysis, TSH and fT4 levels), as well as the indication for treatment with LT4, its start date and, if it occurred, end date, were

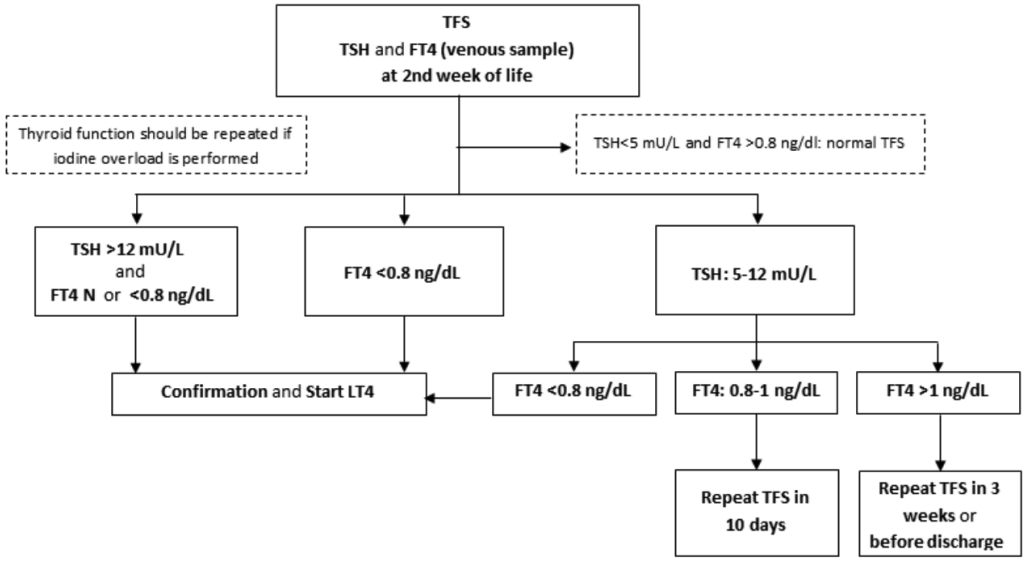

Figure 1. Thyroid function screening protocol

TFS: thyroid function screening, fT4: free thyroxine, TSH: thyroid stimulating hormone, LT4: levothyroxine

collected from the NICU database. TFS data from routine CH screening were provided from the Neonatal Screening Program of Catalonia. Treated patients follow up data were collected from the Pediatric Endocrinology Unit outpatient clinic medical reports.

The study was conducted in compliance with the terms of the Helsinki II Declaration and was approved by the Drug Research Committee and the Research Project Committee of Vall d'Hebron University Hospital [PR (AMI)271/2018]. Informed consent was obtained from all patients.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences, version 20.0 (IBM Inc., Armonk, NY, USA). Normal distribution was assessed by the Kolmogorov-Smirnov test. Comparisons between groups were performed using Student's t-test or the Mann-Whitney U test, as appropriate. The Pearson test was used in the correlation analysis in normally distributed data. The x^2 test was used to test for differences in patient group distribution. Data are expressed as frequency, mean ± SD and median and interquartile range (IQR), whenever appropriate. In some variables, range (minimum-maximum) was also shown. Statistical significance was assumed when $p < 0.05$.

Results

A total of 550 preterm neonates below 31 weeks GA were born in the observation period. Forty-one neonates were excluded: 30 died within two weeks of birth, nine did not have TFS performed and two had mothers with thyroid pathology. Thus, 509 preterm neonates were enrolled. Median (IQR) of GA at birth was 28 weeks (26.4,29.4) and the birth weight was 1000 g (800,1230). The distribution by weeks of GA was: 23 (n = 3), 24 (n = 30), 25 (n = 54), 26 (n = 59), 27 (n = 94), 28 (n = 91), 29 (n = 91) and 30 (n = 87). Fifty-six neonates (11 %) were LBW. Nineteen neonates died after TFS was performed at a median of 30 days. A total of 687 TFS were performed (Table 1). The total number of repeat analyzes were 104, for abnormal values (n = 58) or insufficient sample (n = 46).

Thyroid dysfunction was detected in 170 patients (33.3 %); CH was diagnosed in 20 (3.9 %); and hyperthyrotropinemia in 135 neonates (26 %) [TSH between 13-20 mU/L n = 9; TSH between 5-12 mU/L n = 126 patients]; HOP was diagnosed in 15 neonates (2.9 %). Results are shown in Figure 2. Twenty-one neonates (4.1 %) were treated with LT4 (20 for CH and one for HOP). Characteristics of neonates treated are shown in Table 2.

Congenital Hypothyroidism

Twenty preterm neonates were diagnosed with CH of whom one was typical (patient 16) and 19 with delayed TSH rise) with median (IQR) TSH levels of 25.9 (16.8,42) mU/L and mean ± SD fT4 levels of 1.0 ± 0.3 ng/dL. Remarkably, 11 (55 %) of the 20 patients received an iodine overload due to routine procedures (intestinal surgery, lumbar puncture and surgical closure of ductus arteriosus). Seven patients were LBW. To highlight, all patients with delayed TSH rise presented with TSH levels at the first TFS above 5 mU/L (14 patients between 13-20 mU/L and five patients between 5-12 mU/L).

In relation to the Neonatal Screening Program, all neonates were tested at a median (IQR) of 4 (3,13) days of life. Three patients had a TSH above 10 mU/L in the first dried spot sample. Patient 16 was diagnosed with typical CH because he had a TSH level of 18.6 mU/L in dried spot sample and TSH levels of 66 mU/L in the serum sample at fourteenth day of life. Patients 15 and 17 had TSH levels in the first sample of 13.6 and 13.2 mU/L, but showed normal TSH levels in the second blood spot sample.

LT4 was started at a median (IQR) of 27.9 (18,33) days of life and stopped at median (IQR) of 12.5 (2,36) months. Indication for treatment was done according to the protocol

Table 1. Number of TFS performed

Number of TFS	Number of patients	Days of life (min-max)	Number of patients with TSH > 12 mU/L (TSH min-max)
1st TFS	509	15 (3-61)	21 (12.4-66)
2nd TFS	104	28 (5-106)	10 (12.5-58.3)
3rd TFS	46	38 (13-278)	1 (17.9)
4th TFS	11	42 (23-76)	1 (19.6)
5th TFS	11	51 (34-64)	1 (13)
6th TFS	4	60.5 (42-80)	0
7th TFS	1	98	0
8th TFS	1	118	0
	687		

TFS: thyroid function screening, min-max: minimum-maximum, TSH: thyroid stimulating hormone

shown in Figure 1, except for patient 9 who was treated due to persistent elevated TSH levels (9.37 mU/L) at 66 days of life on the third TFS. All the patients had normal thyroid ultrasound. At re-evaluation after more than three years of follow up, three patients were finally diagnosed with permanent CH. One patient was diagnosed with Williams syndrome, another one with Down syndrome at follow up and the last one was diagnosed with probable thyroid dyshormonogenesis. A heterozygous variant of unknown significance (VUS), c.2654G>A, (p.Arg885Gln), was found in exon 20 of the *DUOX2* gene. This variant has been previously described in the bibliography and in the HGMD database as a variant associated with transient hypothyroidism resulting from a single compromised allele, but at re-evaluation TSH levels had increased up to 8.6 mU/L (after three weeks without LT4) (23). This patient is currently 9 years old and is still receiving 2 mcg/kg of LT4. In contrast, 15 patients were diagnosed with transient CH because LT4 replacement was withdrawn before 6 years of age (before 3 years of age in 13 patients, and between 4 and 6 years of age in another two). Of the remaining two patients, one is currently 2 years old and has not been re-evaluated and the other one died 15 days after birth. Six patients (28.6%) were lost to follow-up because they returned to their country or region of origin.

Low Birth Weight for Gestational Age Neonates

Fifty-six patients (11%) were LBW. These neonates were around two weeks of GA older than those preterm neonates born with adequate birth weight for GA (ABW) ($p<0.001$). LBW neonates presented with TSH levels at first and second TFS higher than those of ABW neonates. However, no statistically significant differences were found for fT4 levels between those groups. Results are shown in Table 3. Remarkably, there was a statistically significant relationship between being LBW and having a TSH >12 mU/L at first TFS ($p<0.0001$) and receiving treatment with LT4 ($p=0.006$). Of the LBW neonates, only one was finally diagnosed with permanent CH corresponding to the newborn with Williams syndrome.

Hypothyroxinemia of Prematurity

Fifteen neonates presented with HOP during the second week of life [median (IQR) 15 (9,17) days] with TSH

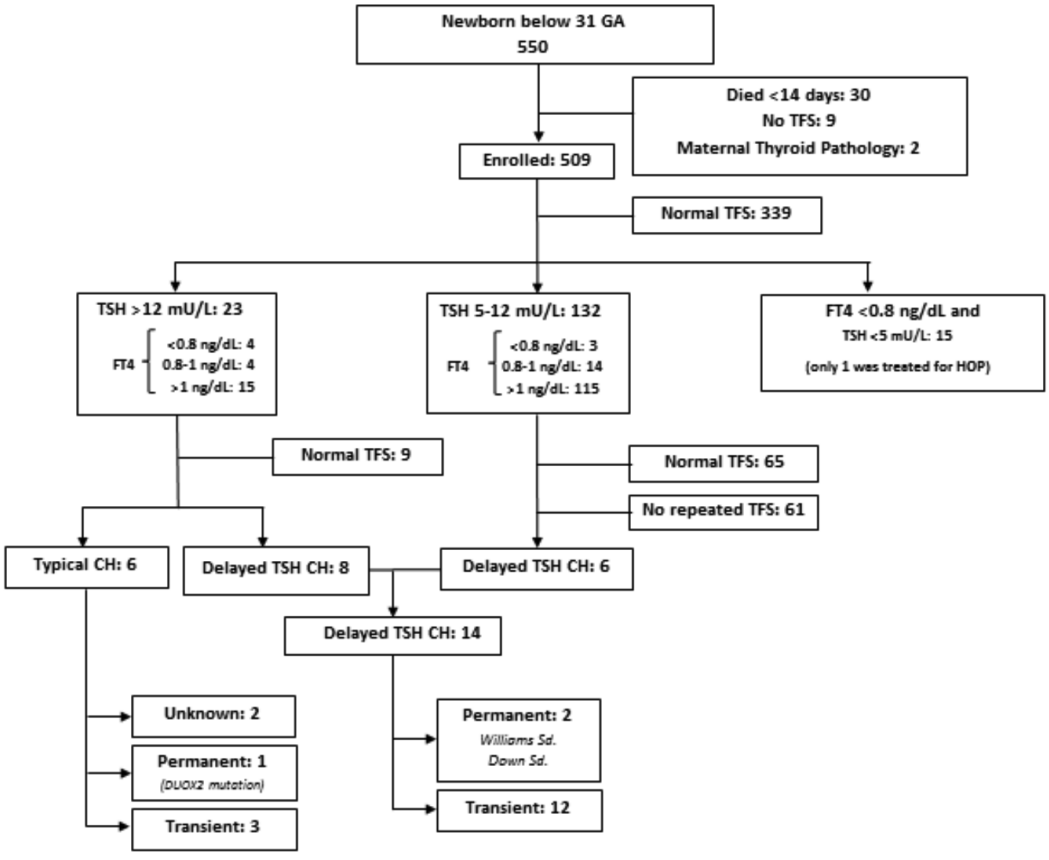

Figure 2. Flowchart of thyroid function screening results of premature neonates

GA: gestational age, TSH: thyroid stimulating hormone, fT4: free thyroxine, TFS: thyroid function screening, TSH: thyroid stimulating hormone, CH: congenital hypothyroidism

Table 2. Characteristics of premature newborns treated with levothyroxine

	Weeks of GA/ weight (g)/ LBW or ABW	TSH (mU/L)/ fT4 (ng/dL) when levothyroxine was started	Days of life / TFS number when levothyroxine was started	TSH (mU/L) at CH screening (whole-blood)/ days of life (2nd sample)	Surgical or medical procedure with iodine overload/days of life	Levothyroxine therapy duration (months)	Thyroid dysfunction	Transient/ permanent CH	Follow up: Current age (years)/ dyshormonogenesis genetic test
1	28/700/ ABW	19.6/1.02	33/4	1.4/20	Intestinal surgery/4	48	Delayed TSH rise	Transient, although therapy until 4 years old	4y Dyshormonogenesis test negative
2	24/750/ ABW	25/0.76	19/2	4.8/30	PDA surgery/12	30	Delayed TSH rise	Transient	3y
3	28/820/ ABW	26.8/1.05	21/2	7.5/3	LP/10	72	Delayed TSH rise	Transient, although therapy until 6 years old	6y Dyshormonogenesis test negative
4	25/630/ ABW	15.6/1.18	25/2	3.2/3	PDA surgery/20	1	Delayed TSH rise	Transient	3y LOFU
5	29/900/ LBW	94/0.65	18/2	1.7/3	-	Still on LT4	Delayed TSH rise	Permanent	3y Williams Sd
6	24/495/ ABW	13/1.2	60/5	4.63/40	PDA surgery/8	4	Delayed TSH rise	Tansient	4y
7	28/1220/ ABW	58/0.55	33/2	1.12/8	-	12	Delayed TSH rise	Transient	5y LOFU
8	30/685/ LBW	12.5/1.62	24/2	1.7/4	-	6	Delayed TSH rise	Transient	3y
9	30/645/ LBW	9.37/1.19	66/3	3.61/19	-	36	Delayed TSH rise	Transient	3y
10	27/600/ LBW	32.4/1.23	30/2	1.9/3	-	6	Delayed TSH rise	Transient	5y
11	27/730/ ABW	18/1.6	36/2	4/5	Inguinal hernia surgery/33	42	Delayed TSH rise	Transient, although therapy until 3.5 years old	5y Dyshormonogenesis test negative
12	29/1600/ ABW	20.8/1.2	18/2	4.2/5	-	Still on LT4	Delayed TSH rise	Permanent	2y Down Sd
13	29/750/ LBW	39.8/0.94	20/2	3/4	Intestinal surgery/7	36	Delayed TSH rise	Transient	3y LOFU
14	28/1070/ LBW	15/1.05	51/2	3.02/4	Intestinal surgery/60	13	Delayed TSH rise	Transient	2y
15	30/1450/ LBW	41/0.48	25/1	13.6/13 0.6 (2nd sample)	-	2	Delayed TSH rise	Transient	6y LOFU
16	26/790/ ABW	66/0.6	14/1	18.6/4	Intestinal surgery/7	1.5	Typical CH	Transient	2y LOFU
17	28/815/ ABW	44.4/0.85	17/1	13.2/4 1 (2nd sample)	Intestinal surgery/2	-	Delayed TSH rise	?	Died at 15 days of life
18	30/1410/ ABW	46/0.79	23/1	3.5/13	-	Still on LT4	Delayed TSH rise	Permanent	6y Dyshormonogenesis test: Mut in *DUOX2*
19	30/825/ LBW	37/0.94	11/1	5.3/4	-	Still on LT4	Delayed TSH rise	?	2y
20	29/600/ LBW	22.9/1.29	14/1	10/13	LP/1	13	Delayed TSH rise	Transient	2y
21	24/720/ ABW	1.27/0.41	15/1	0.24/60	Hemodynamic instability, dopamine/2	0.5	HPO	Transient	6y LOFU

ABW: adequate bierth weight for gestational age, LBW: low birth weight for gestational age, NA: not available, PDA: surgical closure of patent ductus arteriosus, LP: lumbar puncture, TG: tyroglobulin: LOFU: lost of follow up: US: ultrasound, HPO: hypothyroxinemia of prematurity, CH: congenital hypothyroidism, LT4: levothyroxine, Sd: syndrome

median (IQR) levels of 2 (1.3-3.6) mU/L and fT4 mean levels of 0.68 ± 0.1 ng/dL. Two neonates with HOP were LBW. Only one patient (26 weeks of GA, TSH 2.1 mU/L, fT4 0.41 ng/dL) was treated with LT4 at 15 days of life while suffering a septic shock; his thyroid ultrasound was normal and LT4 and hydrocortisone were stopped after two weeks. On follow up, 10 patients had TFS with normal levels, two patients had died and in two patients TFS was not repeated.

Correlation and Association Analysis

FT4 levels at first TFS correlated positively with birth weight ($r = 0.193$, $p < 0.001$) and GA ($r = 0.343$, $p < 0.001$), although no association was found with the other variables (LBW, prenatal corticoid administration, ventilatory support, oxygen therapy, inotropic support, intraventricular hemorrhage and neonatal sepsis).

TSH levels at first TFS correlated negatively with birth weight ($r = -0.146$, $p = 0.002$), although no correlation was found with GA. In association analysis, neonates who received dopamine had TSH levels [3.98 mU/L (0.6,22.9)] slightly higher than neonates who had not received dopamine [3.16 mU/L (0.2,37); $p = 0.019$], but no association was found with the other variables.

Discussion

Our study focuses on the incidence of thyroid dysfunction in a cohort of 509 preterm neonates below 31 weeks of GA evaluated with a protocol of TFS in a venous sample during the second week of life, in addition to routine CH screening in a dried blood spot (13,16). Thyroid dysfunction was identified in 170 neonates (33%), although only 21 of them (4.1%) were finally treated, 20 for CH and one for HOP.

The diagnosis of CH in the preterm population is challenging, especially in the more immature infants because TSH may not be elevated in initial samples. Remarkably, in this study only one patient was diagnosed by the routine CH screening program in a dried blood spot sample, highlighting the need for specific protocols for this population. Whether repeat screenings should be done at the state level or the individual NICU level remains a matter of debate (14). Some authors have emphasized the need for a standard repeat of whole-blood TSH samples in very preterm infants as taking only a second sample could miss a significant proportion of neonates with delayed TSH rise (48% in the study of McGrath) (14,18). Catalonia's CH screening program does not currently require a routine second sample on preterm or LBW neonates, and, given this, it seems more efficient and practical that each NICU undertakes the TFS (in venous sample and with TSH and fT4 determination) in a consistent way integrated with any other screening of co-morbidities related to prematurity (intraventricular haemorrhage, anaemia or retinopathy). Furthermore, the results can be obtained faster and hypothyroxinemic states with low TSH values can be treated earlier.

In the present NICU-based protocol a single serum determination of TSH and fT4 is performed, unlike those described in other studies in which serial determinations of whole-blood TSH were required. However, through the requisition of repeating all serum TSH above 5 mU/L, we consider that cases of CH will not be missed, and are not aware of any infant who was diagnosed late with CH, to date. The second week of life appears to be an optimal moment to detect affected neonates in order to start treatment as soon as possible and preferably no later than the second week after birth, as European Guidelines recommend (17).

Table 3. Comparison of TSH and fT4 levels at first and second TFS between LBW for gestational age and ABW for gestational age

		LBW	ABW	p value
1st TFS	N	56	393	
	Gestational age median (IQR)	29.1 (28,30)	27.6 (26.2,29.2)	0.001
	Birth weight median (IQR)	735 (630,735)	1045 (830,1251.2)	< 0.001
	Days of life when TFS was performed median (IQR)	14 (13,16)	15 (13,17)	NS
	fT4 (ng/dL) mean ± SD and range	1.2 ± 0.2 0.84-1.76	1.18 ± 0.2 0.8-2.05	NS
	TSH (mU/L) median (IQR)	5.3 (3.6,9)	3.3 (2.1,5.2)	< 0.001
2nd TFS	N	21	94	
	Days of life when TFS was performed median (IQR)	28 (22,32.5)	28 (21,34)	NS
	fT4 (ng/dL) mean ± SD and range	1.31 ± 0.2 0.96-1.84	1.22 ± 0.2 0.83-1.76	NS
	TSH (mU/L) median (IQR)	6.9 (5.1,9.4)	3.8 (2.6,6)	< 0.001

TFS: thyroid function screening, LBW: low birth weight for gestational age, ABW: adequate birth weight for gestational age, NS: non-significant, SD: standard deviation, IQR: interquartile range, fT4: free thyroxine, TSH: thyroid stimulating hormone

Several longitudinal studies have shown that some preterm neonates have a characteristic fluctuation in thyroid hormone levels during the first few weeks of postnatal life, consisting of transient mild lower levels of fT4, followed by a mild and transient elevation in serum TSH levels. Some preterm neonates present with delayed TSH elevations greater than this mild compensatory rise and the etiology of this greater delayed TSH elevation remains unclear (4). It may reflect true primary hypothyroidism or the recovery of illness-induced suppression of the hypothalamic-pituitary-thyroid axis. In addition, iodine deficiency or excess iodine levels could also be associated with the development of delayed TSH elevation in this population (13,24). Although TSH elevation usually resolves spontaneously in a few months, some of these neonates have low fT4 concentrations at diagnosis and in a small but significant proportion of neonates, thyroid dysfunction is permanent (13). Infants with extremely LBW, have greater and more persistent increase in TSH levels (4,6). In general, the strategy is to start replacement treatment with LT4 (see protocol) and attempt to withdraw it at 3 years of age when neurodevelopment is completed. In our study some neonates with mild and delayed TSH elevations were re-evaluated earlier during these 36 months post-birth because of the low doses of LT4 related to their weight. New, recently published guidelines recommend re-evaluating a child with no permanent CH diagnosis and a gland *in situ* if thyroxine dose required is less than 3 mcg/kg per day at the age of 6 months (17,25). Another important factor to be considered is the susceptibility of preterm neonates to iodine overload, despite the very restrictive protocols for iodine use in NICU. In our sample, 55% of neonates diagnosed with CH received an iodine overload and in 60% of these cases replacement therapy was withdrawn before 3 years of age. Our study highlights the importance of repeating TFS and close monitoring when iodine overload occurs, as was also reported by Mcgrath et al. (18) who found an incidence of 25% of iodine overload in infants with delayed TSH rise in their cohort.

After 6 years of follow up only three of the treated neonates were diagnosed with permanent CH, one with an heterozygous *DUOX2* mutation of unknown significance and the other two were syndromic, Down and Williams syndrome, respectively. To highlight, none of them were diagnosed using routine dried blood spot TSH-based screening. The global incidence of primary CH in our community is 1:2305 and in our study the incidence of CH is 1:509 (patients with Down and Williams syndrome were excluded because these syndromes already predispose to thyroid disorders) in preterm newborn < 31 weeks of GA. In addition to the GA effect, this may be due to the fact that our NICU has a higher number of complex pregnancies (fetal pathologies such as genetic and chromosomal syndromes) and also to the smaller size of the study cohort compared to the general population. Our data are similar to other studies with extremely preterm neonates: Kaluarachchi et al. (5) reported an incidence of 1:143 and 1:64 in < 32 weeks of GA neonates and Woo et al. (26) reported 14-fold higher incidence of CH in very LBW infants.

The TSH cut-off point for starting treatment is unclear, but given the evolution of our patients, perhaps an increase in this cut-off from 12 to 15 mU/L could be considered, as long as fT4 levels remain within normal range. A recent study by Kaluarachchi et al. (27) suggested using age-related TSH cut-offs from birth to term equivalent gestation to avoid missed diagnoses.

In preterm neonates with low levels of fT4 and normal levels of TSH it is difficult to distinguish between HOP, central hypothyroidism and non-thyroidal illness syndrome. Central hypothyroidism is an extremely rare condition and other pituitary hormone deficiencies and midline brain defects could suggest this diagnosis. The incidence of HOP is difficult to compare in the studies published because of different definitions (free T4 or total T4) and different cut-off levels. It is reported from 7 to 50% of preterm neonates below 28 weeks of GA (7,28,29). In our study the incidence of HOP was lower (3%), but is comparable with that reported in a previous study (7). In the present study fT4 levels correlated positively with GA and levels rise throughout the first postnatal weeks (7,28,29). Only one newborn received LT4 for HOP while suffering a septic shock. This data confirms that not all preterm infants are hypothyroxinemic and there is no reason for indiscriminate treatment with LT4 in this population (4).

Study Limitations

Limitations of our study are that some neonates might have been acutely ill at the time of TFS. However, this limitation may have been mitigated with repeated abnormal findings. Another issue is that starting LT4 treatment was decided on an individual basis and not from a randomized control trial, denoting the lack of consensus on the start of treatment in this group of neonates. It should also be added that the choice of the serum TSH cut-off point of 12 mU/L to start treatment with LT4 was used based on consensus guidelines recommendation to treat patients if TSH is between 10-20 mU/L and on the experience of previous cases (17,20).

Conclusion

In conclusion, this protocol was able to detect thyroid dysfunction in preterm neonates that were not detected by the current routine program base on TSH determination in whole-blood. Preterm neonates, especially of lower GA, LBW or those having had an iodine overload show a risk of thyroid dysfunction during a critical period of brain development, and therefore a TFS with serum TSH and fT4 is proposed. The second week of life seems to be an appropriate time and this TFS does not involve an extra blood test as it is performed at the same time as a routine blood test. Thyroid dysfunction seems to resolve spontaneously in a few months in the great majority of preterm neonates, but in some cases replacement treatment could be needed. There is a critical need for specific guidelines regarding the follow-up and re-evaluation of transient thyroid dysfunction, especially in preterm neonates.

Ethics

Ethics Committee Approval: The study were approved by the Drug Research Committee and the Research Project Committee of Vall d'Hebron University Hospital [PR (AMI)271/2018].

Informed Consent: Informed consent was obtained from all patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ariadna Campos-Martorell, Karla Narváez Barros, Alicia Montaner Ramon, María Clemente León, Diego Yeste Fernández, Concept: Ariadna Campos-Martorell, Karla Narváez Barros, Alicia Montaner Ramon, María Clemente León, Design: Ariadna Campos-Martorell, Karla Narváez Barros, Alicia Montaner Ramon, María Clemente León, Data Collection or Processing: Ariadna Campos-Martorell, Karla Narváez Barros, Alicia Montaner Ramon, María Clemente León, Jose Luis Marin Soria, Rosa Maria López Galera, Analysis or Interpretation: Ariadna Campos-Martorell, Alicia Montaner Ramon, María Clemente León, Jose Luis Marin Soria, Rosa Maria López Galera, Diego Yeste Fernández, Literature Search: Ariadna Campos-Martorell, Alicia Montaner Ramon, María Clemente León, Writing: Ariadna Campos-Martorell, Alicia Montaner Ramon, María Clemente León, Jose Luis Marin Soria, Rosa Maria López Galera, Diego Yeste Fernández.

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CASE REPORT

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Menstrual Suppression in Gender Minority Youth

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What is already known on this topic?

Menstrual distress is frequently reported in gender minority youth (GMY) in sex assigned-at-birth female adolescents identifying as male or gender non-conforming. Menstrual suppression refers to the practice of using hormonal management to reduce menstrual bleeding which may be an option to treat menstrual dysphoria in GMY.

What this study adds?

Menstrual suppression should be offered to GMY when pubertal suppression is not an option. Each treatment plan should be individualized.

Abstract

The purpose of this case series was to evaluate menstrual suppression in sex assigned at birth female adolescents identifying as male or gender non-conforming. A retrospective chart review of four gender minority youth (GMY), age 14-17, was performed for gender identity history, type and success of menstrual suppression, method satisfaction, side effects and improvement in menstrual distress. Menstrual suppression was successful in three patients, one patient discontinued use due to side effects that caused an increase in gender dysphoria. Menstrual distress and bleeding pattern improved in the majority of GMY in this series but side effects, as well as contraindications, may limit their use. In conclusion, menstrual dysphoria can be life-threatening for GMY and it is important that clinicians consider menstrual suppression in GMY with menstrual dysphoria. This series emphasizes the importance of individualized treatment plans.

Keywords: Gender minority youth, menstrual suppression, menstural dysphoria

Introduction

At our pediatric hospital in Ankara, Turkey, visibility of trans gender youth is growing and we are receiving more applications and referrals of young people whose gender identity does not align with their sex assigned at birth (SAB) more frequently than ever before (1). Gender minority youth (GMY) is an umbrella term that is used to describe this community (2) and gender dysphoria is the term used to describe the stress experienced by these young people (3).

For SAB female adolescents who identify as male or gender non-conforming, puberty and the development of unwanted sex characteristics can be associated with significant psychological stress and may be the trigger for gender dysphoria (4). Menarche can be particularly distressing, as the initiation of menstruation represents a social category of gender to which the person does not belong, and which may lead to stress, anxiety, and dysphoria (5,6).

Menstrual dysphoria is defined as a sense of distress or anxiety associated with menses (7). Menstruation is a monthly reminder to these teens that their SAB does not align with their gender identity. Studies have reported increased rates of depression, thoughts of self-harm and suicidal ideation in GMY while menstruating (8).

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The first approach to an adolescent with gender dysphoria is confirmation of the diagnosis and continuous follow-up by a mental health professional. The social transition process then continues with the adoption of a new name, and style of dressing congruent with their gender identity, while therapy may include the reversible phase of treatment such as the suppression of puberty with gonadotropin-releasing hormone (GnRH) analogues (4,9). Pubertal suppression has been shown to have a positive effect on psychological function, improve well-being, and alleviate menstrual dysphoria since it provides therapeutic amenorrhea (10,11).

Pubertal suppression should be offered to adolescents experiencing gender dysphoria, but many GMY still struggle to access this treatment (4). Gender affirmative care, especially in the adolescent population, is still developing in Turkey which means the main barrier to treatment is lack of experienced medical providers (1). Furthermore, failure to secure parental consent is another major obstacle to treatment. Even when the patient finds an experienced provider and has parental consent, obtaining the medication may take some time due to the gender informed mental health assessment not yet being completed or the formalities associated with prescribing this treatment.

Provisional therapy to suppress menstruation in GMY with menstrual dysphoria may be used for a number of reasons, including those who do not have the option of pubertal blockers or as an interim approach pending completion of evaluation or until official approval for use of pubertal blockers (7). Menstrual suppression refers to the practice of using hormonal management to reduce menstrual bleeding with the goal of achieving therapeutic amenorrhea (12). The hormonal methods used to manipulate menstruation are totally reversible and studies have shown they may increase quality of life and relieve the menstrual dysphoria experienced by GMY (8).

The objective of the current case series was to describe the experiences of GMY after using a hormonal menstrual suppression method of their choice for menstrual dysphoria.

Case Report

A retrospective chart review was performed of all GMY evaluated for the first time during September 2018 and May 2020. The diagnosis of gender dysphoria was made according to DSM-5 criteria (13).

Eligibility for the study required that patients had been seen in the adolescent medicine clinic for at least two visits. A total of nine patients were evaluated for gender dysphoria during this time period, and of these two were SAB males who identified as trans female. Menstrual suppression was offered to the remaining seven SAB female patients. Parental consent for menstrual suppression was not obtained in two and one patient was started on a GnRH analog soon after the first evaluation. Thus, a total of four patients between the ages of 14-17 received this treatment for menstrual dysphoria. All patients had regular menstrual patterns before treatment.

Management of Menstrual Distress

At our institution, menstrual distress related to gender identity is explored with patients at their initial visits. Menstrual suppression is offered to SAB female GMY who request it, and patients are counseled concerning the following three methods:

i. Combined hormonal contraceptives given continuously

ii. Progestin only contraceptive pill

iii. Intramuscular (IM) depot medroxy progesterone acetate (DMPA)

Method of application (oral vs. IM), possible side effects, contraindications and success rates are discussed. All patients receive counselling concerning the efficacy of treatment and it is explained that suppression of menstrual bleeding is not immediately 100% effective, and that with many options the possibility of "breakthrough bleeding" still exists although it becomes less frequent with time.

Long acting reversible contraceptives such as hormonal implants and intrauterine devices are not offered to the patients due to the lack of provider experience.

Descriptive Data of the Case-series

Data concerning gender history including identified gender, current age and age of onset of gender dysphoria, age of menarche, baseline hormonal evaluation, psychiatric diagnosis, disclosure of gender identity to family/friends and social transitioning is shown in Table 1.

Information concerning menstrual suppression, such as reason GnRH was not initiated, method chosen, reason for choice of method, side effects and efficacy of the method, patient satisfaction and treatment duration is shown in Table 2.

All patients and parents gave verbal informed consent for the publication of this case-series.

Discussion

The majority of research concerning GMY health is related to mental health concerns and the use of gender affirming

hormonal treatments, whereas little data is available related to menstrual health and dysphoria (14). Menstrual distress related to gender identity is frequently reported in GMY and often has a negative impact on mental health (7). This case series aimed to evaluate menstrual suppression in a group of GMY and, to the best of our knowledge, this is the first report from an adolescent population from Turkey. This case series shows the importance of individualized treatment since although all patients were given the same counseling each individual chose a different path.

Historically it was believed that cyclic menstruation was necessary for health but the concept that it did not have beneficial effects, and that menstruation could be controlled was first raised in the 1960s and many studies and systemic reviews have shown both its efficacy and safety (15,16). Since then, menstrual suppression has been used to manage a number of medical conditions (17). Primarily used to control gynecological problems, such as dysmenorrhea, abnormal uterine bleeding, premenstrual syndrome and endometriosis, it can also be used for patients with medical conditions associated with excessive blood loss which includes patients with bleeding disorders or those receiving chemotherapy or conditions where menstruation causes an exacerbation in symptoms, such as catamenial seizures or migraines. It can also be used for menstrual hygiene related concerns in patients with intellectual or developmental delays (12).

The most commonly used method for menstrual suppression used for other indications are combined hormonal contraceptives which contain both estrogen and progesterone (12). For contraceptive purposes these methods have traditionally been used in a cyclic fashion, with 21 days of hormones, followed by a 7-day hormone-free interval during which withdrawal bleeding occurs. With extended or continuous use, the hormone-free week is taken out, which either reduces or totally eliminates the withdrawal bleeding (18). When giving counseling concerning this method it is important to discuss with the patient that although breakthrough bleeding is common in the initial months the rate of amenorrhea increases with continuous use and has been shown to be about 50% after the first year of use (19).

Although the combined hormonal formulations continue to be the most popular method for suppressing menstruation for other indications, GMY may want to avoid this method due to the perceived feminizing effects of these hormones

Table 1. Gender history of the participants

	Gender identity of the patient	Current age (years)	Age of onset of gender dysphoria (years)	Age of menarche (years)	Baseline hormonal evaluation	Psychiatric diagnosis	Disclosure of gender identity to family/friends	Social transitioning process
Case 1	Transmale	16	12	11.9	FSH (mU/mL): 4.36 LH (mU/mL): 1.95 Estradiol (pg/mL): 37 Testosterone (ng/dL) :12 $DHEASO_4$ (mcg/dL): 174 Androstenedion (ng/dL): 172 SHBG (mg/L): 30.6	Gender dysphoria Major depression	Only with family	Adopted a new name Gender congruent hair style and clothing
Case 2	Transmale	14	13	12.2	FSH (mU/mL): 2.36 LH (mU/mL): 4.95 Estradiol (pg/mL): 29 Testosterone (ng/dL) 24.7 $DHEASO_4$ (mcg/dL): 257 Androstenedion (ng/dL): 76.6 SHBG (mg/L): 45.5	Gender dysphoria Anorexia nervosa	Family and very few closest friends	Gender congruent clothing
Case 3	Gender non-conforming	14.6	11	11.8	FSH (mU/mL): 6.2 LH (mU/mL): 3.2 Estradiol (pg/mL): 62 Testosterone (ng/dL): 22 $DHEASO_4$ (mcg/dL): 212 Androstenedion (ng/dL): 66.6 SHBG (mg/L): 37	Gender dysphoria Generalized anxiety disorder	Parents and only those closest to them	Gender congruent hair style and clothing Used birth name
Case 4	Transmale	17	12	12.4	FSH (mU/mL): 6.2 LH (mU/mL): 4.11 Estradiol (pg/mL): 17 Testosterone (ng/dL): 12 $DHEASO_4$ (mcg/dL): 220 SHBG (mg/L): 49	Gender dysphoria	Only with family	Adopted a new name Gender congruent hair style and clothing

FSH: follicle stimulating hormone, LH: luteinizing hormone, $DHEASO_4$: dehydroepiandrosterone sulfate, SHBG: sex hormone binding globulin

and association with an incongruent gender (20), which was the situation for Case 1. It is also important to discuss the possible side effects of the medication, such as its effect on breast tissue. Changes in breast size and/or breast tenderness are known effects of this treatment and these symptoms are made increasingly complicated for those who bind their chests (7). Case 2 discontinued this treatment as the side effects in breast tissue increased his gender dysphoria.

Other methods of choice offered to patients were the progestin only medications, including progestin only pills and the IM DMPA, which are advantageous for GMY as they do not contain estrogen. DMPA is usually administered IM every 12-13 weeks, at a convenient dose schedule of four times per year, which makes it appealing especially to adolescents and the most common bleeding pattern in this method is break through bleeding close to the next injection (21). A modification clinicians can offer to GMY is administering the injection every 10 weeks, which could decrease the break through bleeding when given in shorter cycles. Again, it is important to discuss realistic expectations, as therapeutic amenorrhea may not be achieved straight away and is more likely with continuous use. DMPA is typically associated with amenorrhea in about 50-60% of users at the end of one year and 70% by the end of the second year (22). A study by Kanj et al. (6) evaluated menstrual suppression choices among transmale individuals and showed DMPA to be the most common method selected. It is also important to discuss side effects and possible contraindications. The use of DMPA is associated with loss of bone mineral density (BMD) (23). As Case 2 had a history of low BMD, secondary to an eating disorder, use of DMPA was not recommended for this patient. Oral progestin pills were the second choice in Case 3 who continued to have cyclic periods with oral contraceptives. It is important to remember that although progestin only pills may induce amenorrhea they should not be used for patients that additionally require birth control. This patient was not sexually active.

The most common reason for inability to prescribe a GnRH analog in this case series was the time taken to complete mental health evaluation. Our clinic is in a tertiary

Table 2. Information concerning menstrual suppression

	Reason GnRH was not initiated	Method chosen	Reason for choosing this method	Observed side effects	Efficacy of the method	Patient satisfaction	Treatment duration
Case 1	Needed time to complete mental health evaluation	DMPA	1. Did not want to use a method containing estrogen 2. Did not want to take daily medication	None	DMPA was initiated 3 times every 10 weeks. Bleeding occurred a few days before each injection	High	DMPA was stopped after 1 year as a GnRH analog was started
Case 2	Needed time to complete mental health evaluation	COC given continuously	DMPA was contraindicated in this patient due to very low Bone mineral density	Breast enlargement and increased breast sensitivity	Only used the treatment for a month	Low Due to the breast changes the patient decided to discontinue treatment.	Was started on a GnRH analog
Case 3	Lack of parental consent	1. COC given continuously 2. POC	Was scared of receiving an injection	Patient continued to have cyclic bleeding on COC and was switched to a POC	Low with COC, achieved therapeutic amenorrhea with POC. Had one break through bleed on POC.	Low with COC, high with POC	Continues to use POC
Case 4	Lack of parental consent	1. COC 2. DMPA	Was started on COC as they were scared of daily injection but they constantly forgot to take it, which lead to break through bleeding and was switched to DMPA	None	COC was not effective as patient was non-compliant DMPA bleed a few days every 3 months.	Low on COC High on DMPA	DMPA was stopped after 1 year as a GnRH analog was started

DMPA: depo-medroxy progesterone acetate, COC: combined oral contraceptive, POC: progestin only contraceptive, GnRH: gonadotropin-releasing hormone

pediatric hospital and one of the very few to provide gender affirmative care in the country. Due to lack of training, health care providers may not have the means to provide pubertal suppression, but menstrual suppression can be prescribed by both pediatricians and pediatric endocrinologists and, in our opinion, should be until the youth is sent to a center where they can receive gender affirmative care. Another common reason for failure to prescribe pubertal suppression is lack of parental consent, as seen in Case 4, but after a year of DMPA and counseling of both the GMY and his parents, they decided to initiate GnRH therapy. Menstrual suppression can act as a bridge to gender affirmative treatment by giving the family time to adjust to this situation.

Conclusion

In conclusion, the methods utilized for menstrual suppression in this small group of GMY were well tolerated and beneficial. Menstrual dysphoria can be life threatening for GMY and it is important that menstrual suppression should be considered by health care providers caring for these youths. After detailed counseling, concerning different method types and their possible side effects an individualized treatment plan should be made.

Ethics

Informed Consent: All patients and parents gave verbal informed consent for the publication of this case-series.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sinem Akgül, Zeynep Tüzün, Melis Pehlivantürk Kızılkan, Zeynep Alev Özön, Concept: Sinem Akgül, Design: Sinem Akgül, Data Collection or Processing: Sinem Akgül, Zeynep Tüzün, Melis Pehlivantürk Kızılkan, Zeynep Alev Özön, Analysis or Interpretation: Sinem Akgül, Zeynep Tüzün, Melis Pehlivantürk Kızılkan, Zeynep Alev Özön, Literature Search: Sinem Akgül, Writing: Sinem Akgül, Zeynep Tüzün, Melis Pehlivantürk Kızılkan, Zeynep Alev Özön.

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CASE REPORT

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GATA-4 Variants in Two Unrelated Cases with 46, XY Disorder of Sex Development and Review of the Literature

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What is already known on this topic?

The genetic cause of 46, XY disorders of sex development (DSD) still cannot be determined in about half of the cases. *GATA-4* haploinsufficiency is one of the rare causes of DSD in genetic males (46, XY).

What this study adds?

Twenty-two cases with 46, XY DSD due to *GATA-4* haploinsufficiency (nine missense variant, two copy number variation) have been reported in the literature. Phenotype varied from a mild insufficient virilization to complete female appearance. There is remarkable phenotype-genotype variation in the *GATA-4*-related conditions, associated with incomplete penetrance or variable expressivity.

Abstract

The genetic cause of 46, XY disorder of sex development (DSD) still cannot be determined in about half of the cases. *GATA-4* haploinsufficiency is one of the rare causes of DSD in genetic males (46, XY). Twenty-two cases with 46, XY DSD due to *GATA-4* haploinsufficiency (nine missense variant, two copy number variation) have been previously reported. In these cases, the phenotype may range from a mild undervirilization to complete female external genitalia. The haploinsufficiency may be caused by a sequence variant or copy number variation (8p23 deletion). The aim of this study was to present two unrelated patients with DSD due to *GATA-4*variants and to review the phenotypic and genotypic characteristics of DSD cases related to *GATA-4* deficiency.
Keywords: Disorder of sex development, *GATA-4*, Gonad, heart

Introduction

Disorder of sex development (DSD) is defined as atypical development of gonadal, chromosomal, or anatomical sex (1). It may be related to aneuploidy, copy number variations, or single nucleotide variants causing defects of sex hormone biosynthesis/action, and/or gonadal differentiation/development (2,3). The genetic cause of 46, XY DSD still cannot be determined in about half of the cases. *GATA-4* haploinsufficiency is one of the rare causes of DSD in genetic males (46, XY).

The *GATA-4* gene, located on chromosome 8p23.1, encodes GATA-binding protein 4 (*GATA-4*), a transcription factor that is essential for cardiac and gonadal development (4,5,6). By interacting with NR5A1, FOG-2, and WT1, the *GATA-4* protein regulates the expression of sex-determining genes, *SRY, SOX-9*, and anti-Müllerian hormone (AMH) (7). It has also been shown that the protein modulates a couple of steroidogenic genes that are essential for sexual differentiation (7,8).

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GATA-4 haploinsufficiency as a cause of congenital heart disease (CHD) is a well-known association and nearly 200 variants have been reported to date. However, to the best of our knowledge, there are only twenty-two cases of *GATA-4* related DSD in the literature (7,9,10,11,12,13,14). In these cases, the phenotype may range from a mild undervirilization to complete female external genitalia. The haploinsufficiency may be caused by a sequence variant or copy number variation (8p23 deletion). Based on a large international cohort study, only 1-2% of 46, XY DSD cases may be related to *GATA-4* gene (12,15).

The aim of this study was to present two unrelated patients with DSD due to *GATA-4* variants and to review the phenotypic and genotypic characteristics of DSD cases related to *GATA-4* deficiency.

Case Reports

DNA was extracted from peripheral blood sample by using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to manufacturers instructions. A targeted gene panel for 46, XY DSD was used and samples were analyzed by a next-generation sequencing technique using a custom panel kit (Twist Bioscience, San Francisco, CA, USA). The gene panel included *AMH*, *AMHR2*, *AKR1C2*, *AR*, *ARX*, *ATRX*, *B3GALTL*, *CYB5A*, *CYP11A1*, *CYP17A1*, *DHCR7*, *DHH*, *GATA4*, *HCCS*, *HSD17B3*, *LHCGR*, *MAMLD1*, *MAP3K1*, *NR5A1*, *OPHN1*, *SOX9*, *SRD5A2*, *SRY*, *WT1*, *ZFPM2*. The Genemaster (www.egenemaster.com) program was used for the analysis of the obtained data. Detected changes were analyzed using genomAD (https://gnomad.broadinstitute.org), dbSNP (16), VarSome (17), and Clinvar (18) databases and interpreted according to The American College of Medical Genetics and Genomics (ACGM) criteria (19). Written consent was obtained from parents of the probands.

Follicle stimulating hormone, luteinizing hormone, estradiol, total testosterone, AMH, adrenocorticotrophic hormone, cortisol and dehydroepiandrosterone sulfate (DHEA-SO4) levels were measured by an automated electrochemiluminescence immunoassay (Roche Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany) using the standard reagent kits supplied by the instrument manufacturer. Dihydrotestosterone and 17-OH progesterone levels were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Case 1

A neonate was hospitalized to the neonatal intensive care unit due to prematurity, and respiratory distress. The baby was delivered from a 20-year-old mother at 32 weeks 3 days, with a birth weight of 1960 g. The parents were consanguineous and the baby was a first child. Delivery was by cesarean section, due to loss of Doppler activity and polyhydramnios. Physical examination revealed dysmorphic ears, epicanthus, hypertelorism, umbilical hernia, standing trigger finger, bilateral simian line, central hypotonicity, micropenis (1.5x1 cm), scrotal hypoplasia, and bilateral undescended testis. Atrial septal defect (ASD), patent ductus arteriosus, and pulmonary stenosis were diagnosed with echocardiographic assessment at the fourth month of age. Adrenal gland hormones were within normal limits according to age and gender. Pituitary-gonadal functions were in the normal range, consistent with mini puberty. Gonad and adrenal function tests are presented in Table 1.

The chromosomal analysis revealed a 46, XY karyotype. Targeted gene panel sequencing for 46, XY DSD identified a heterozygous, novel variant in Exon 2 of the *GATA-4* gene, c.337A>C (p.Thr113Pro). This variant had not been previously reported. VarSome classified this substitution as "Variant of Uncertain Significance". In silico analysis revealed

Table 1. 15th day basal gonadal and adrenal functions of the cases

	Case 1	Case 2	References
FSH, IU/L	4	0.5	0.16-4.1
LH, IU/L	4	3.79	0.02-7
Estradiol, pmol/L	<12	<12	0.3-1
Total testosterone, nmol/L	15.09	5.24	2.6-13.86
Dihydtestosterone, nmol/L	1.64	1.88	0.4-2.92
AMH*, pmol/L	153.07	772.86	100-3328
ACTH, pmol/L	4.22	3.47	1.32-10.47
Cortisol, nmol/L	105.94	121.39	55-303
DHEA-SO_4, µmol/L	8.35	14.32	0.84-11.68
17-OH-pProgesterone, nmol/L	14.57	2.88	0.1-6.06

ACTH: adrenocorticotrophin, AMH: anti-Müllerian hormone, DHEA-SO4: dehydroepiandrosterone sulfate, FSH: follicle stimulating hormone, LH: luteinizing hormone

eight pathogenic predictions (BayesDel_addAF, DEOGEN2, FATHMM-MKL, M-CAP, MutationTaster, PrimateAI and SIFT) and four benign predictions (DANN, EIGEN, MVP and MutationAssessor). Segregation analysis showed that the variant was *de novo* (Figure 1). We believe this new variant is compatible in terms of genotype and phenotype correlation.

Case 2

A three day-old patient was referred to the endocrinology clinic due to ambiguous genitalia. He was born to non-consanguineous parents at 38 gestational weeks, with a birth weight of 3185 g. Microphallus, bifid scrotum, perineo-scrotal hypospadias were evident on physical examination. Bilateral gonads were palpable in the scrotum. System examination was normal, except for ptosis in the left eye. CHD was not detected by echocardiography. Adrenal gland hormones were within normal limits according to age and gender. Pituitary-gonadal functions were in the normal range, consistent with mini puberty. Gonad and adrenal function tests are presented in Table 1. Chromosome analysis revealed a 46, XY karyotype. Targeted gene panel sequencing for 46, XY DSD identified a heterozygous, likely pathogenic variant in Exon 2 of the *GATA-4* gene, c.487C > T (p. Pro163Ser). In the segregation analysis, the mother did not carry this variant, The analysis could not be done for the father (Figure 1).

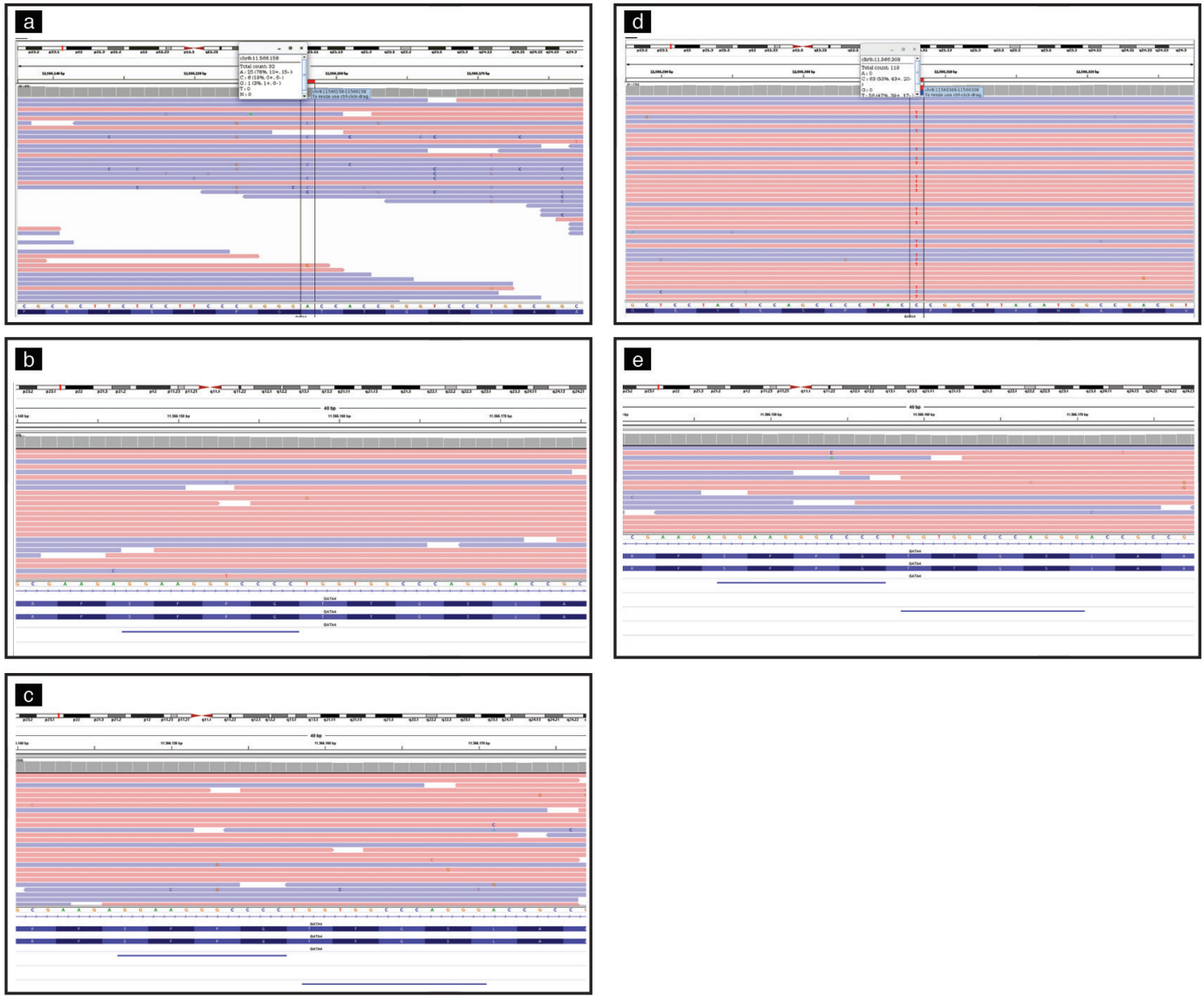

Figure 1. Integrative Genomics Viewer (IGV) images of NGS results of *GATA-4* gene located at chromosome 8. **a)** Shows the heterozygous c.337 A > C variant (p.Thr113Pro) in the *GATA-4* gene in Case 1. **b, c)** The relevant gene variant was not detected in parents of Case 1. **d)** Shows the c.487 C > T variant (p.Pro487Ser) in the *GATA-4* gene in Case 2. **e)** The relevant gene variant was not detected in mother of the Case 2 (the analysis could not be done for the father)

Discussion

Twenty-two cases with 46, XY DSD due to *GATA-4* haploinsufficiency (nine missense variant, two copy number variation) have been previously reported (Table 2). Eighteen of these cases (82%) were raised as a male. Only two (9%) cases were accompanied by CHD, specifically ASD and ventricular septal defect. Phenotype varied from mild insufficient virilization to a complete female appearance.

The first report by Lourenço et al. (9) reported three DSD cases having the same missense variant in the *GATA-4* gene. This variant was located in the zinc finger domain, which is responsible for DNA binding and protein interaction of the *GATA-4* protein. Moreover, they showed a 50% reduction in AMH activity with expression analysis of this variant. While the index case had only DSD, the brother and one cousin with the same variant as the index case both had DSD and CHD. Furthermore, the mother and aunt of the index case, who carried the same variant, had neither DSD nor CHD.

In another study evaluating 278 cases with 46 XY DSD, four different *GATA-4* variants were detected in seven cases (12). However, the authors reported that only one of the four variants was pathogenic, and the others were benign, in their later work (15). In particular, they suggested that variants in the *GATA-4* gene located outside the N-terminal region of the zinc finger domain should be approached with suspicion that there will be a causal relationship with DSD. Although van den Bergen et al. (15) mentioned that the p.P407Q variant in the *GATA-4* gene, the most commonly reported *GATA-4* variant in 46 XY DSD, was benign, it has been shown in experimental studies that the variant causes reduced expression of both *AMH* and *SRY* genes (13,14).

Table 2. Summary of *GATA-4* related cases with disorder of sex development

Case	Sex of rearing	Additional findings	CHD	Phenotype	Genotype	References
1	M			Fused hypoplastic labioscrotal fold, perineal hypospadias, hypoplasia of corpus cavernosum, bilateral cryptorchidism (inguinal)	p.G221R (n = 3)	9
2	M		ASD	Microphallus, bilateral cryptorchidism (inguinal)		
3	M			Fused labioscrotal folds, hypospadias, bilateral cryptorchidism (inguinal)		
4	M	Congenital adrenal hypoplasia		Complete gonadal dysgenesis, female external genitalia	8p23 deletion	10
5	M			Perineal hypospadias, bifid scrotum, bilateral cryptorchidism, Mullerian structures absent	8p23 deletion	11
6	M			Micropenis, cryptorchidism	p.W228C	12
7	M			Perineal hypospadias, chordee, and penoscrotal transposition, cryptorchidism	p.A346V	
8	M			Perineal hypospadias, (gonad position unknown)	p.P394T	
9	F			Female (no virilization), inguinal bilateral testes, no uterus		
10	M	Imperforate anus		Penile hypospadias, cryptorchidism	p.P407Q	
11	M			Scrotal hypospadias, testes palpable, hypoplastic uterus		
12	M			Perineal hypospadias cryptorchidism		
13	M			Male type genitalia, cryptorchidism with or without micropenis	p.R265C n = 1	13
14-17	M				p.P407Q n = 4	
18	F	Autism	VSD	Clitoral hypertrophy, fused labia with posterior raphe, gonads palpable in inguinal canal, rudimentary uterus	p.C238R	7
19	M			Micropenis, hypospadias, bilateral cryptorchidism	p.W228C	
20	M	Severe obesity		Micropenis, bilateral cryptorchidism (inguinal)	p.P226L	
21	M			Micropenis, perineal hypospadias, bilateral cryptorchidism	p.R215G	14
22	F			Complete female genitalia	p.P407Q	
23	M	Dysmorphic ear, epicanthus hypertelorism umbilical hernia	ASD, VSD, PS	Microphallus, scrotal hypoplasia, bilateral cryptorchidism (inguinal)	p.T113P	
24	M	Ptosis		Perineoscrotal hypospadias, microphilus, bifid scrotum	p.p163S	

ASD: atrial septal defect, CHD: congenital heart disease, F: female, M: male, PS: pulmonary stenosis, VSD: ventricular septal defect

Furthermore, this variant was found to be associated with CHD in previous studies (15).

Our unrelated patients had two different variants in the *GATA-4* gene. Undoubtedly, expression analysis is needed to establish a causal relationship between these variants and DSD, which is the most important limitation of the study. However, although these two variants were not located in the zinc finger domain, they were close to the N-terminal part of the domain. On the other hand, the first case (Case 1) with a novel variant of uncertain significance also had CHD, which may be explained by *GATA-4* deficiency. We also performed the microarray analysis due to other accompanying syndromic findings in the first case, and this was evaluated as normal. Further genetic studies are needed in this case. The *GATA-4* variant in the second case (Case 2), which was reported in cases with previous patients with CHD (20,21,22), was classified as likely pathogenic according to ACMG criteria. To the best of our knowledge, the latter variant has not been previously associated with DSD. This may be a striking example of the phenotype-genotype mismatch associated with the *GATA-4* gene.

The phenotype-genotype variation in *GATA-4* related conditions may be associated with incomplete penetrance or variable expressivity. However, it is unclear why *GATA-4* variant related CHD is encountered more often than DSD. The answer of this question will perhaps enable us to better understand the phenotype-genotype relations.

Conclusion

Variants of the gene encoding the *GATA-4* protein may be responsible for the etiology in 46, XY DSD. The phenotype may range from a mild undervirilization to complete female external genitalia. The CHD or DSD can be isolated or combined; *GATA-4* gene defects should be considered in cases with both CHD and DSD.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Nurullah Çelik, Hande Küçük Kurtulgan, Fatih Kılıçbay, Gaffari Tunç, Ayça Kömürlüoğlu, Data Collection or Processing: Nurullah Çelik, Hande Küçük Kurtulgan, Onur Taşçı, Cemile Ece Çağlar Şimşek, Taha Çınar, Analysis or Interpretation: Nurullah Çelik, Hande Küçük Kurtulgan, Fatih Kılıçbay, Gaffari Tunç, Literature Search: Nurullah Çelik, Hande Küçük Kurtulgan, Yeşim Sıdar Duman, Writing: Nurullah Çelik.

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CASE REPORT

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A Novel Mutation in the *TRIP11* Gene: Diagnostic Approach from Relatively Common Skeletal Dysplasias to an Extremely Rare Odontochondrodysplasia

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What is already known on this topic?

Most patients with odontochondrodysplasia (ODCD) have a compound heterozygous mutation. ODCD is a rare skeletal dysplasia that is associated with dentinogenesis imperfecta.

What this study adds?

The c.3296_3298delinsTG is a novel pathogenic variant in the *TRIP11* gene, inherited in a compound heterozygous fashion, that led to ODCD. Joint limitation and craniocervical stenosis have not been observed in patients with ODCD to date. In this respect, our patient is the first such case in the literature.

Abstract

Odontochondrodysplasia (ODCD, OMIM #184260) is a rare, non-lethal skeletal dysplasia characterized by involvement of the spine and metaphyseal regions of the long bones, pulmonary hypoplasia, short stature, joint hypermobility, and dentinogenesis imperfecta. ODCD is inherited in an autosomal recessive fashion with an unknown frequency caused by mutations of the thyroid hormone receptor interactor 11 gene (*TRIP11*; OMIM *604505). The *TRIP11* gene encodes the Golgi microtubule-associated protein 210 (GMAP-210), which is an indispensable protein for the function of the Golgi apparatus. Mutations in *TRIP11* also cause achondrogenesis type 1A (ACG1A). Null mutations of *TRIP11* lead to ACG1A, also known as a lethal skeletal dysplasia, while hypomorphic mutations cause ODCD. Here we report a male child diagnosed as ODCD with a novel compound heterozygous mutation who presented with skeletal changes, short stature, dentinogenesis imperfecta, and facial dysmorphism resembling achondroplasia and hypochondroplasia.

Keywords: Odontochondrodysplasia, *TRIP11*, skeletal dysplasia, dentinogenesis imperfecta, rare disease

Introduction

Odontochondrodysplasia (ODCD, OMIM #184260) is a rare, non-lethal skeletal dysplasia characterized by involvement of the spine and metaphyseal regions of the long bones, pulmonary hypoplasia, short stature, joint hypermobility, and dentinogenesis imperfecta (1). Spondylo-metaphyseal dysplasias (SMD) are a group of skeletal dysplasia that includes miscellaneous disorders with vertebral and metaphyseal defects. ODCD is inherited in an autosomal recessive fashion with an unknown frequency and is caused by mutations of the thyroid hormone receptor interactor 11 gene (*TRIP11*; OMIM *604505). Initially, homozygous mutations in *TRIP11* were associated with a lethal skeletal dysplasia, achondrogenesis type 1A (ACG1A, OMIM #200600) associated with severe thorax hypoplasia, hypomineralization of several bones, and short extremities (2). ODCD is towards the milder end of the spectrum of *TRIP11* gene mutations compared to ACG1A which is at the severe end. Maroteaux et al. (3) reported two cases with short limbs, and metaphyseal irregularities and dentinogenesis imperfecta; they used the term ODCD for

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this condition. ODCD is also called Goldblatt syndrome or SMD with dentinogenesis imperfecta. Short stature is one of the most common complaints referred to genetics clinics. Here we report a male child diagnosed as ODCD with a novel compound heterozygote mutation who presented with skeletal changes, short stature, dentinogenesis imperfecta, and facial dysmorphism resembling achondroplasia (ACH) and hypochondroplasia (HCH).

Case Report

The patient was the second child of a nonconsanguineous couple of Turkish origin. Maternal history revealed short limbs on second-trimester ultrasonography (US) that ended with a full-term male baby born through cesarean section due to breech presentation. Fetal mobility and amniotic fluid were normal. Birth weight was 4035 g (90th percentile), the length was 47 cm (3-10th percentile) and head circumference was 38 cm (≥97th percentile). After birth, he was admitted to the neonatal intensive care unit and treated for respiratory distress for 17 days. The mother and father were 40 and 35 years old, respectively, at the time of delivery. The patient has an older healthy sister. The family history was unremarkable. The patient was referred at the age 2.5 months for evaluation for possible skeletal dysplasia. On physical examination, the patient's body weight was 4.9 kg (10th percentile), height was 55 cm (10th percentile) head circumference was 40.5 cm (50-75th percentile). Anterior fontanelle was 5x4 cm. Relative macrocephaly, midfacial hypoplasia, frontal bossing, downslanting palpebral fissures, depressed nasal root, short nose, anteverted nares, short neck, and redundant nuchal skin were noted as dysmorphic features. Narrow thorax, disproportionately short extremities, redundant skin folds on upper and lower limbs with skin dimpling over the knees, inability to extend elbow/knee fully and brachydactyly were present. No head control was observed on neurologic examination. Examination at 15 months of age revealed his body weight to be 7.2 kg [-3.2 standard deviation score (SDS)], height 67 cm (-3.9 SDS) head circumference 46 cm (25-50th percentile). At this time anterior fontanelle was 3x3 cm. and at 12 months of age, the proband had attained neck control. He could stand with support by 14 months.

The first tooth erupted at age 9 months and it was blue-gray and translucent, which was confirmed as dentinogenesis imperfecta by a pedodontist. It was learned that he was suffering from insomnia. Intellectual development was normal based on the Denver developmental screening test. Echocardiogram and abdomen US were normal. Extra cerebrospinal fluid space was at the upper limit of normal on cranial US.

Skeletal survey at the age of 2.5 months showed short long bones with irregular flaring of metaphyses, small thoracic cage, small sacroiliac notch, flat acetabular roof, enlargement of iliac wings, and short tibia in relation to fibula. Additionally, when he was 15 months old, platyspondyly, coronal clefts in the lower thoracal vertebrae, broad-cupped metacarpals, short phalanges, cone-shaped epiphyses, and mildly delayed carpal ossification were detected on roentgenogram. His radiologic examination was otherwise unchanged from the previous visit. Photographs and X-rays of the patient, who was 2.5 and 15 months old, are shown in Figures 1 and 2, respectively. Although there was no neurological abnormality, the patient was referred to neurosurgery in terms of craniocervical involvement when he was about 2 years old. His magnetic resonance imaging showed craniocervical stenosis and he was operated by neurosurgery. Metabolic screening (calcium, phosphorus, alkaline phosphatase, parathyroid hormone, 25-hydroxy vitamin D, and thyroid hormone) was normal. After obtaining written informed consent, genetic analysis was performed on blood samples from the proband and his parents. Karyotyping was normal 46, XY in standard resolution in the proband. No mutation was detected in the FGFR3 gene in the patient. Afterward, whole-exome sequencing was performed. Two heterozygous variants, c.1225G>T (p.Asp409Tyr) and c.3296_3298delinsTG (p.Lys1099Metfs*6), were identified in the *TRIP11* (NM_001321851.1) gene. The c.1225G>T variant is located in the first base of exon 9 and has previously been reported to cause missplice, resulting in ODCD (4). It was reported in the ClinVar database and submitted as a likely pathogenic variant. DANN, EIGEN, FATHMM-MKL, M-CAP, MutationAssessor, MutationTaster, and SIFT computational algorithms predicted the variant as deleterious. The novel c.3296_3298delinsTG variant causes frameshift and leads to a premature stop codon after six residues. Neither variant was found in the gnomAD exomes and genomes. Finally, both variants were considered pathogenic according to American College of Medical Genetics guideline (5). Finally, the c.1225G>T variant in the mother and the c.3296_3298delinsTG variant in the father were present in a heterozygous state, thus confirming the compound heterozygosity in the patient. Images of variants in the Integrative Genomics Viewer are shown in Figure 3. His sister had none of the variants in *TRIP11* present in her parents or sibling. As a result, our patient was diagnosed with ODCD given the evidence of the clinical, radiological, and molecular findings.

Discussion

ODCD was first described by Goldblatt et al. (6) in a 3.5-year-old male patient characterized by SMD, joint hypermobility,

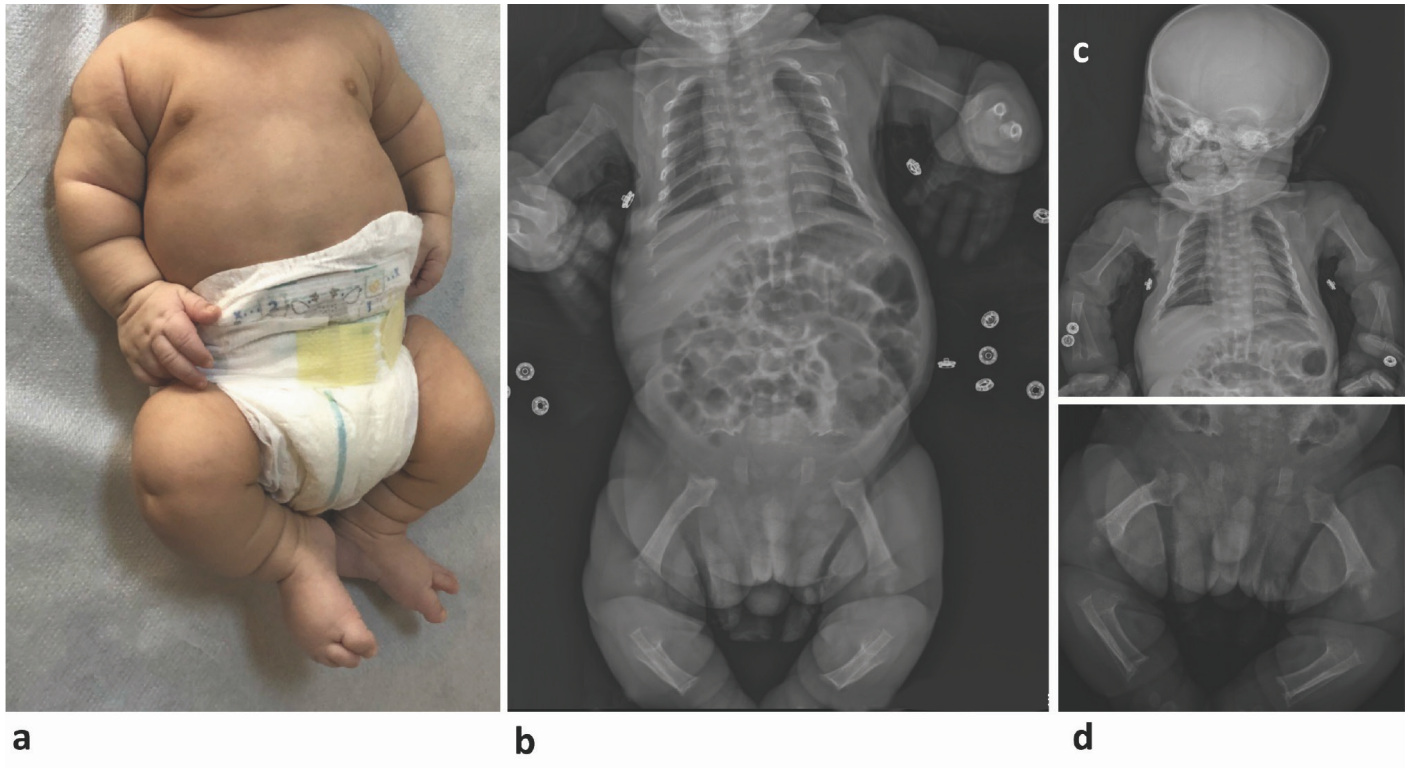

Figure 1. Clinical and radiologic findings of the proband at the age of 2.5 months. **a)** Narrow thorax, short extremities, and redundant skin folds on upper and lower limbs with skin dimpling over knees. **b, c, d)** Short long bones with irregular flaring of metaphyses, small thoracic cage, small sacroiliac notch, flat acetabular roof, enlargement of iliac wings, and short tibia in relation to the fibula

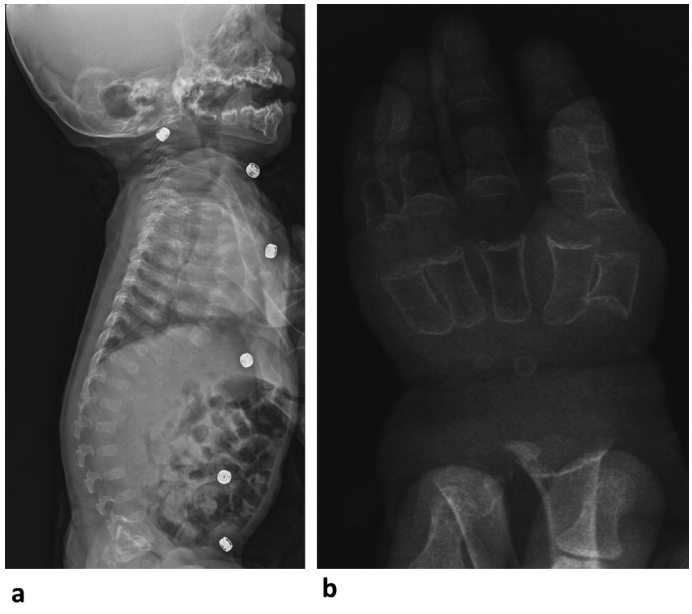

Figure 2. Radiologic findings of the proband at the age of 15 months. **a)** Platyspondyly and coronal clefts at lower thoracal vertebrae. **b)** Cupping of the metaphyses of the radius and ulna, broad-cupped metacarpals, short phalanges, cone-shaped epiphyses, and mildly delayed carpal ossification

and dentinogenesis imperfecta in 1991. Until Wehrle et al. (4) shoed *TRIP11* gene mutations leading to ODCD in 2019, the diagnosis of ODCD was based on clinical and radiologic features. The *TRIP11* gene is located at 14q32.12 and contains 21 exons. *TRIP11* encodes the Golgi microtubule-associated protein 210 (GMAP-210), which is an indispensable protein for the function of the Golgi apparatus (7). In 2010, Smits et al. (8) investigated lethal skeletal dysplasia and showed that the GMAP-210 protein was essential for glycosylation and cellular transport of proteins. In the absence of GMAP-210 protein, endochondral and intramembranous ossification is dramatically decreased (9). *TRIP11* is the only known gene associated with ODCD. Mutations of *TRIP11* also causes ACG1A. Null mutations of *TRIP11* lead to ACG1A, a lethal skeletal dysplasia, while hypomorphic mutations cause ODCD (4). Loss of function mutations in the *TRIP11* gene, leading to ACG1A, are characterized by short limbs, small thorax, domed skulls, absence of several bone ossifications, and decreased alveolar formation in the lungs in mice and humans (8). ODCD is classified in group 12 of the SMD because of involvement of vertebrae and affecting metaphyses of all tubular long bones at Nosology and classification of genetic skeletal disorders in the last revision published in 2019 (10).

Figure 3. The Integrative Genomics Viewer visualization of the c.1225G > T and c.3296_3298delinsTG variants

Only one patient in the literature, the product of a consanguineous marriage, had a homozygous mutation as expected (11). In 2019, Wehrle et al. (4) suggested an autosomal recessive pattern in this disorder. Almost all patients diagnosed with ODCD have had compound heterozygous mutations. The compound heterozygote appearance of this mutation in our patient is in agreement with the autosomal recessive trait of ODCD. The mutation mechanisms found in ODCD can be listed in order of frequency as missense, small deletion, and splice-site mutations but for that, there were no hotspot regions in *TRIP11* gene related to ODCD (4). Unger et al. (1) published a case series of six patients that were diagnosed with clinical and radiographic findings in 2008. These authors reported mesomelic limb shortening (6/6 cases), narrow chest (5/6 cases), dentinogenesis imperfecta (5/6 cases) (1 patient could not be evaluated because he died at the age of 4 months), and scoliosis (2/6 cases). Wehrle et al. (4) confirmed this skeletal dysplasia by studying the molecular diagnosis of these patients. The comparison between common abnormalities of the present case and the patients reported by Unger et al. (1) and Medina et al. (11) are summarized in Table 1.

Cystic renal disease, pulmonary dysplasia, and non-obstructive hydrocephaly were seen in a few cases with ODCD (4). While generalized joint hypermobility is observed in ODCD, our patient had limitation of extension in some joints. These joint limitations are not a typical finding observed in ODCD patients, and thus this is the first report of such an association. There was no joint hypermobility or limitation in one of the six patients in the publication of Unger et al. (1). Short limbs on the prenatal US, large head with protruding forehead, midface hypoplasia, brachydactyly, and short tubular bones with metaphyseal flare are also observed in HCH and ACH, as in ODCD. In this respect, ODCD can be confused with these genetic skeletal disorders. Nevertheless, lumbar lordosis,

Table 1. Clinical and genetic outcomes of odontochondrodysplasia

	Case 1 (Sibling 1) Unger et. al. (1)	Case 2 (Sibling 2) Unger et. al. (1)	Case 3 Unger et. al. (1)	Case 4 Unger et. al. (1)	Case 5 Unger et. al. (1)	Case 6 Unger et. al. (1)	Case 7 Medina et. al. (1)	Case 8 Our patient
Sex	Male	Female	Female	Female	Female	Male	Female	Male
Relationship	-	-	-	-	-	-	+	-
Relative macrocephaly	-	-	-	-	-	-	+	+
Short extremities	+	+	+	+	+	+	+	+
Joint laxity	?	+	+	+	-	+	+	-
Restriction of joints	-	-	-	-	-	-	-	+
Redundant skin folds	-	-	+	-	-	+	-	+
Skin dimpling over limbs	-	-	-	-	-	-	-	+
Dentinogenesis imperfecta	?	+	+	+	+	+	+	+
Neuromotor development delay	?	+	?	+	?	+	-	+
Molecular analysis	c.(1314+5G>A); (chr14:g.(?_92.474.069)_(92.597.431_?)del	c.(1314+5G>A); (chr14:g.(?_92.474.069)_(92.597.431_?)del	c.(1228G>T); (4815_4818delAGAG)	c.(586C>T); (4534C>T)	c.(1228G>T); (2128_2129delAT)	c.(1622delA); (5416A>G)	c.1314+5G>A	c.1225G>T; c.3296_3298delinsTG

progressive narrowing, or unchanged interpedicular distance in lumbar vertebrae, short iliac bones, short femoral neck, and bowing of legs are expected in HCH and ACH (12). However, the skeletal anomalies of HCH are milder and can be detected in late childhood. Characteristic facial features are more pronounced in ACH compared with ODCD and HCH. However, dentinogenesis imperfecta is only seen in ODCD. Even though his phenotype overlapped significantly with ACH and HCH, as we expected, the known causal gene for these was not found in the presented patient. Our patient had suffered from respiratory distress in the newborn period due to pulmonary hypoplasia. Thoracic hypoplasia is observed in skeletal ciliopathies, notably in Jeune asphyxiating thoracic dysplasia (JATD). A small thoracic cage is more severe in JATD and that is accompanied by short extremities with brachydactyly, as in ODCD. Whereas short stature is less conspicuous than ODCD, the skull and spine are unaffected in JATD. Polysyndactyly is another distinctive feature seen in JATD and the other ciliopathies. Renal involvement, which is a typical feature of ciliopathies, is rarely seen in ODCD. In this respect, it has a common feature with ciliopathies besides lung hypoplasia.

Dentinogenesis imperfecta is one of the most characteristic features of ODCD. Nearly all patients with ODCD have dentinogenesis imperfecta although dentinogenesis imperfecta is extremely rare in other genetic skeletal diseases, with the exception of osteogenesis imperfecta. Dentinogenesis imperfecta is a genetic disorder caused by impaired dentine development that results in discolored and fragile teeth. Dentine is formed by odontoblasts that secrets an extracellular matrix after mineralization and this matrix is comprised of 90% of type 1 collagen and 10% of non-collagenous proteins and lipids (13). This entity influences both deciduous and permanent teeth. The incidence is estimated to be 1 in 7000 in the USA, according to the last published study in 1975 (14). The coexistence of dentinogenesis imperfecta and skeletal dysplasia is rare except in osteogenesis imperfecta. However, osteogenesis imperfecta has rather different clinical and radiological features compared with ODCD, including blue sclera, hearing loss, and increased frequency of fracture.

The most characteristic radiologic features of ODCD include small thorax, platyspondyly with coronal clefts, broadened iliac wings with horizontal acetabulum, cupping of metaphyses and shortening of all long bones (1). All of these radiologic findings were found in

our patient. With advancing ages, metaphyseal alterations in metacarpals deteriorate, which mimics enchondroma, and mesomelic shortening becomes more apparent (1). The most characteristic clinical features are macrocephaly, short stature, pulmonary hypoplasia, and dentinogenesis imperfecta. The presented patient had all of these findings. There is no intellectual disability, hearing loss, and ophthalmologic involvement in this skeletal dysplasia (1).

The diagnosis of ODCD is based on clinical and radiologic keystones of prenatal onset of short stature, pulmonary hypoplasia with a narrow thorax, short long bones, and dentinogenesis imperfecta and confirmed by molecular analysis. Wehrle et al. (4) reported significant clinical variability within affected families with this skeletal dysplasia.

Conclusion

In summary, ODCD is a very rare skeletal dysplasia. We describe a male affected with ODCD who had facial dysmorphism, dentinogenesis imperfecta, short stature, and joint hypermobility, due to a novel compound heterozygote mutation in the *TRIP11* gene. It is predicted that this mutation has a pathogenic and damaging influence on the protein product of *TRIP11*. More than 20 parients have been reported to date but to the best of our knowledge, this is only the third published Turkish case with a clinical, radiographic, and molecular diagnosis of ODCD. Furthermore, reporting of novel mutations, as found in this case, are crucial to extend the molecular spectrum of ODCD and to clearly understand the genotype-phenotype correlation in a larger number of patients. In skeletal dysplasias accompanied by dentinogenesis imperfecta, ODCD should be included in the differential diagnosis, unless the diagnosis is clearly osteogenesis imperfecta. We suggest that the presence of dentinogenesis imperfecta may make the diagnosis easier.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Burcu Yeter, Ayça Dilruba Aslanger, Nursel H. Elçioğlu, Concept: Gözde Yeşil, Design: Gözde Yeşil, Data Collection or Processing: Burcu Yeter, Ayça Dilruba Aslanger, Nursel H. Elçioğlu, Analysis or Interpretation: Burcu Yeter, Ayça Dilruba Aslanger, Literature Search: Burcu Yeter, Gözde Yeşil, Nursel H. Elçioğlu, Writing: Burcu Yeter, Nursel H. Elçioğlu.

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CASE REPORT

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A Novel Heterozygous *ACAN* Variant in a Short Patient Born Small for Gestational Age with Recurrent Patellar Dislocation: A Case Report

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What is already known on this topic?

ACAN is located on chromosome 15q26.1 and variants in this gene impair chondrogenesis at the growth plate, resulting in defects in longitudinal bone growth and skeletal development. Heterozygous *ACAN* variants can induce several clinical characteristics in patients, including variable degrees of short stature and mild skeletal dysplasia, such as midface hypoplasia, joint problems, brachydactyly, broad great toes, and lumbar lordosis.

What this study adds?

We report a Korean patient with a novel nonsense variant, c.1968C > G, p.(Tyr656*), in *ACAN*. The patient was born small for gestational age (SGA) and presented with short stature and displayed several dysmorphic features including genu valgum, cubitus valgus, and recurrent patellar dislocations. Patellar dislocation is a rare clinical feature of patients with *ACAN* defects. *ACAN* variants should be considered in short stature patients born SGA with joint problems, particularly those with recurrent patellar dislocation and genu valgum.

Abstract

ACAN variants can manifest as various clinical features, including short stature, advanced bone age (BA), and skeletal defects. Here, we report rare clinical manifestations of *ACAN* defects in a 9 year, 5 month-old girl born small for gestational age (SGA), who presented with short stature, and was initially diagnosed with idiopathic growth hormone deficiency. She displayed several dysmorphic features, including genu valgum, cubitus valgus, and recurrent patellar dislocations. She presented with progressive advancement of BA compared with chronological age. Whole exome sequencing confirmed the presence of a novel heterozygous nonsense variant, c.1968C > G, p.(Tyr656*), in *ACAN*. *ACAN* variants should be considered in short stature patients born SGA with joint problems, particularly those with recurrent patellar dislocation and genu valgum.

Keywords: *ACAN*, short stature, patellar dislocation, small for gestational age

Introduction

Aggrecan is a component of the extracellular matrix around the cartilage growth plate and provides a hydrated gel structure for the load-bearing properties of joints. The aggrecan gene, *ACAN*, is located on chromosome 15q26.1 and variants of this gene impair chondrogenesis at the growth plate, resulting in longitudinal bone growth and skeletal development defects (1). Heterozygous *ACAN* variants can induce several clinical characteristics in patients, including variable degrees of short stature and mild skeletal dysplasia, such as midface hypoplasia, joint problems, brachydactyly, broad great toes, and lumbar lordosis (2,3,4). Short patients with *ACAN* variants often present with advanced bone age (BA), which is different from the short stature associated with endocrine diseases, such as idiopathic growth hormone deficiency (IGHD) and hypothyroidism, which are related to delayed BA. Therefore, advanced BA may lead clinicians to test specifically for *ACAN* variants in patients with short stature and other clinical features, such as skeletal defects (5,6). However, as *ACAN* variant patients without advanced BA have also been reported, advanced BA may suggest

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the presence of *ACAN* variant but cannot be referred to as a specific characteristic of *ACAN* variants (7). In clinical practice, *ACAN* variants are difficult to diagnose because their phenotype-genotype relationships are inconsistent.

Here, we report a case of a patient with a novel *ACAN* variant presenting with patellar dislocation and suggest that this rare clinical feature of *ACAN* variant may be a definitive clue for diagnosis.

Case Report

A girl aged 9 years and 5 months was referred to a pediatric endocrinology clinic for evaluation when she presented with abnormally reduced stature. Her height was 115 cm [-3.93 Korean standard deviation score (SDS)] and weight 35.2 kg (0.60 SDS) (Figure 1A) (8). According to the Tanner stage for puberty, her breast development was at stage 3 and pubic hair development was at stage 1. She was born at 40 weeks through normal vaginal delivery with birth weight 2800 g (-1.53 SDS; ≤10th percentile); she was diagnosed as small for gestational age (SGA). Her mother's height was 161 cm (-0.01 SDS) and the father's height was 175 cm (0.10 SDS), yielding a predicted adult height - calculated using mid-parental height - of 161.5 cm (0.09 SDS) (Figure 1A). Family history also included short stature, with a recorded height of 150 cm (-2.36 SDS) in her paternal grandmother (Figure 1B). The subject exhibited various skeletal defects, including cubitus valgus, genu valgum, and recurrent patellar dislocation. BA was 10 years (1.37 SDS) and within the normal range when compared with her chronological age (CA). No abnormalities were identified in either chemistry or thyroid function and the karyotype of the subject was normal (46, XX). The levels of basal luteinizing hormone, follicle-stimulating hormone, and estradiol were 2.02 mIU/mL, 6.16 mIU/mL, and 25 pg/mL, respectively, confirming that the pubertal stage. We performed growth hormone provocation tests using arginine and L-dopamine without sex steroid priming, and the peak levels were 0.29 ng/mL and 2.3 ng/mL, respectively. No abnormal findings were identified using sellar magnetic resonance imaging and the patient was treated with 0.23 mg/kg/day of recombinant human growth hormone (rhGH) to combat IGHD. One year after initiation of treatment, the age of the subject was 10 years and 6 months, and her height had increased to 124.1 cm (-2.97 SDS). Serum insulin-like growth factor-1 levels were in the upper half of the reference range (0.69 SDS) after rhGH treatment. BA was shown to be advanced by 1 year and 6 months (2.31 SDS). Gonadotropin-releasing hormone agonist (GnRHa) treatment was added to the rhGH treatment to suppress bone maturation. She developed knee deformity over the previous four years, with recurrent intermittent pain during walking. She showed no improvement in her knee deformity and pain with conservative treatment. At the age of 10 years and 10 months, she underwent knee realignment surgery to correct a progressive bilateral genu valgum deformity and address her recurrent patellar dislocations.

DNA was extracted from EDTA blood samples and the entire exome was captured using SureSelectXT Human A11 Exon v5 (Agilent Technologies Inc., Santa Clara, CA, USA), followed by sequencing using NextSeq (Illumina Inc., San Diego, CA, USA). The reported variant was confirmed using Sanger

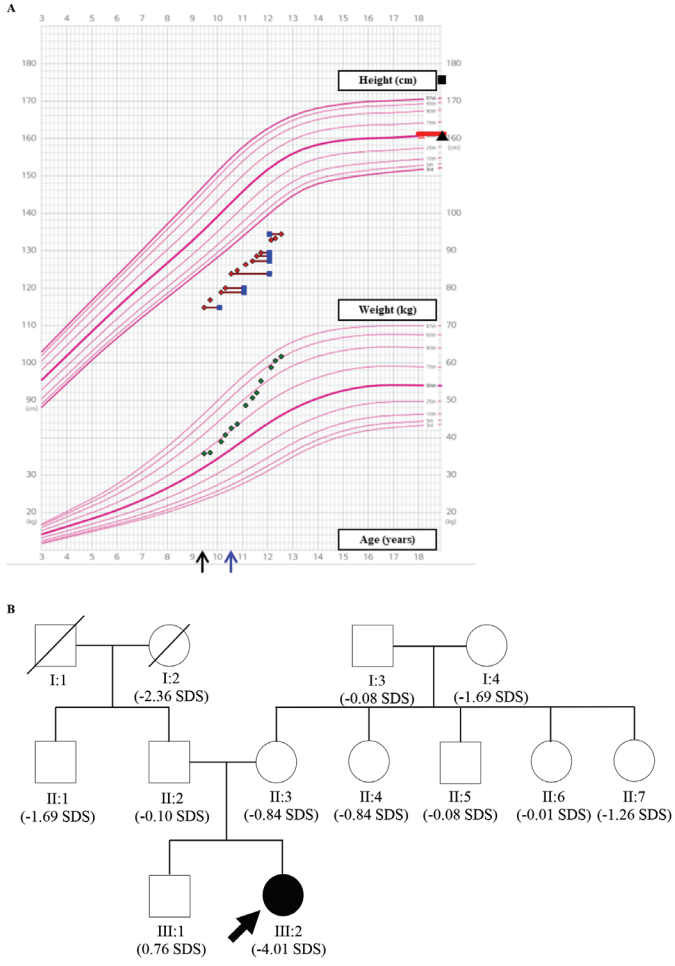

Figure 1. (A) Reference growth chart for Korean females (3-18 years). Heights and weights are shown as red and green dots, respectively. bone age is represented as blue dots. Black squares and triangles indicate the height of the father and the mother, respectively. Mid-parental height is shown as the red line. The time point at which the patient started treatment with recombinant human growth hormone is indicated by a black arrow, and the time point at which gonadotropin-releasing hormone agonist treatment was started is indicated by a blue arrow. (B) Pedigree of the patient. The patient is indicated by a black arrow; the height SDS corresponding to the age of each family member is also indicated. SDS: standard deviation score

SDS: standard deviation score

sequencing. WES revealed a novel heterozygous variant, NM_013227.3:c.1968C > G, p.(Tyr656*), in exon 10 of *ACAN* (Figure 2). The pathogenicity of the variant was evaluated in accordance with the American College of Genetics and Genomics (9). This variant was considered pathogenic in accordance with the PSV1, PM2, and PP3 criteria, and was not found in the Genome Aggregation Database (gnomAD) or the Korean Reference Genome Database (KRGDB). Thus, we concluded that it represents a novel pathogenic variant that results in a nonsense variant of the tyrosine residue to a stop codon.

After 3 years and 6 months of rhGH treatment, the patient was receiving rhGH (0.3 mg/kg/day) and her height was 137.9 cm (-2.91 SDS). She has since been rehabilitated and her walking has stabilized.

Discussion

In this report, we confirmed a novel heterozygous *ACAN* variant using WES. The subject presented with short stature, SGA, IGHD, progressive advanced BA, and various skeletal defects including cubitus valgus, genu valgum, and recurrent patellar dislocations.

The increase in genetic testing to determine the underlying cause of idiopathic short stature (ISS) has highlighted the importance of *ACAN* variants. Recently, the *ACAN* variant has been shown to be one of the most common causes of ISS with a prevalence of 1-6% (10,11). In addition, *ACAN* presents with autosomal dominant (AD) inheritance, i.e., *ACAN* variants are also a common cause of familial ISS (12).

Aggrecan is a major proteoglycan component of the extracellular matrix in joints and intervertebral disc cartilage and provides the hydrated gel structure necessary to facilitate the load-bearing properties of skeletal joints (1). Therefore, aggrecanopathy resulting from *ACAN* variant has been linked to several degrees of skeletal defects and presents with some consistent clinical characteristics, such as advanced BA and early growth cessation (13). However, *ACAN* variants with genotype–phenotype correlation are not always consistent, resulting in a high degree of clinical heterogeneity.

Advanced BA is considered a hallmark of *ACAN* variant but some patients develop delayed BA. *ACAN* variant with short stature and delayed BA was first reported in 2017 (7). A study showed that 79 out of 110 affected patients (72%) experienced advanced BA, suggesting that accelerated skeletal maturation may be a useful indicator of *ACAN* variant, but is insufficient as a clear clinical feature (2,4,10). The initial BA of the subject was within the normal range compared to that for her CA, but her BA progressed to more than 12 months in relation to CA after one year of rhGH treatment. It can be assumed that IGHD influences the progression of BA. Previous studies have reported that GnRHa treatment was successfully applied to prevent bone aging (2,4), as was also evident in this case.

It has been suggested that the possibility of *ACAN* variant in short-statured patients born SGA should be considered (4). A flowchart to determine the possibility of having an *ACAN* variant was proposed by these authors. The clinical features of midface hypoplasia, joint problems, and broad great toes among short-statured patients born SGA were considered to be linked to the possibility of having *ACAN* variant. In this earlier study, since only 13.8% of patients with *ACAN* variant with advanced BA were found, the researchers reported that advanced BA was not sufficient as an indicator of *ACAN* variant. Among the major clinical symptoms presented in the literature, that is midface hypoplasia, joint problems, and broad great toes, the subject of this case had only one characteristic feature, namely joint problems. According to

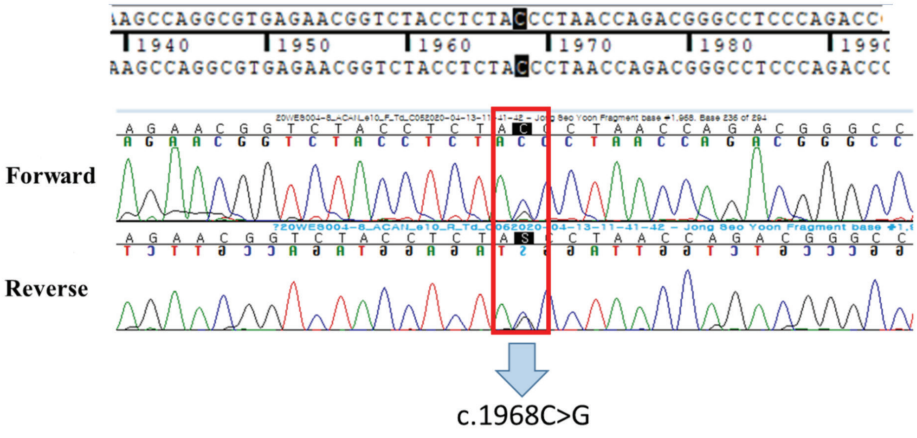

Figure 2. Results of *ACAN* sanger sequencing in the patient NM_013227.3:c.1968C > G, p.(Tyr656*), heterozygote, nonsense

the proposed flowchart, the probability of *ACAN* variant was rare in this subject.

Skeletal defects from *ACAN* variants diagnosed in ISS do not present with serious defects. Osteoarthritis is a representative feature of the joint problems associated with the *ACAN* variant; however, the subject in this case did not experience any osteoarthritis, but developed genu valgum and recurrent knee dislocations at a young age. In a previous study, knee dislocation was found to be a relatively rare clinical feature reported in only a few affected patients (3). Knee dislocation is a very rare symptom and considered a hallmark of suspected *ACAN* variant when accompanied by short stature.

ACAN variant is inherited in an AD fashion, but the parents of the subject were not short in stature. The grandmother of the subject was short in stature, but she grew up in a period of poor nutrition and hence environmental factors cannot be excluded. The parents of the subject did not want to undergo genetic testing, so we could not test them for genetic abnormalities. Owing to these limitations, we have assumed that the patient had a de novo heterozygous *ACAN* variant.

Conclusion

We report a novel heterozygous variant of *ACAN*. If short statured children born SGA without catch-up growth exhibit joint problems, particularly recurrent patellar dislocation and genu valgum, we would suggest that these patients be immediately referred for genetic testing as there is a possibility that they may harbor an *ACAN* variant.

Ethics

Informed Consent: A written informed consent was obtained from her parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Il Tae Hwang, Concept: Su Ji Kim, Jong Seo Yoon, Il Tae Hwang, Design: Jong Seo Yoon, Il Tae Hwang, Data Collection or Processing: Su Ji Kim, Analysis or Interpretation: Su Ji Kim, Jong Seo Yoon, Il Tae Hwang, Literature Search: Su Ji Kim, Jong Seo Yoon, Il Tae Hwang, Writing: Su Ji Kim, Jong Seo Yoon, Il Tae Hwang.

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CASE REPORT

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Central Precocious Puberty in a Boy with Pseudohypoparathyroidism Type 1A due to a Novel *GNAS* Variant, with Congenital Hypothyroidism as the First Manifestation

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What is already known on this topic?

Resistance to multiple hormones as evidenced by pseudohypoparathyroidism, hypothyroidism and hypogonadism is characteristics of pseudohypoparathyroidism type 1A (PHP1A). Mild congenital hypothyroidism may manifest as the first and only clinical presentation of patients with PHP1A.

What this study adds?

Boys with PHP1A might develop central precocious puberty, despite having multiple hormone resistance.

Abstract

Pseudohypoparathyroidism (PHP) type 1A (PHP1A) is a disorder of multiple hormone resistance, mainly parathyroid hormone. It is associated with Albright hereditary osteodystrophy phenotypes. Patients with PHP1A may initially present with hypothyroidism during infancy and later develop typical PHP1A characteristics during their childhood. Central precocious puberty (CPP) is extremely rare among PHP1A patients in whom gonadotropin resistance is more usual. This is a case report of a 9.5-year-old boy with congenital hypothyroidism who developed hypocalcemia secondary to PHP. He had relatively short stature with height standard deviation score of -0.9. Obesity had been noted since the age of two years. At the presentation of PHP, pubertal-sized testes of 10 mL were observed, and CPP was documented with serum testosterone concentration of 298 ng/dL (normal for Tanner stage III, 100-320), luteinizing hormone of 3.9 IU/L (normal, 0.2-5.0), and follicle stimulating hormone of 4.8 IU/L (normal, 1.2-5.8). Pituitary magnetic resonance imaging was unremarkable. Genetic analysis confirmed the diagnosis of PHP1A with a novel heterozygous missense variant of *GNAS* gene in exon 13, c.1103A > G (p.Asp368Gly). Awareness of PHP1A diagnosis in patients with congenital hypothyroidism and early childhood-onset obesity is important for early diagnosis. Apart from multiple hormone resistance, CPP may manifest in patients with PHP1A.

Keywords: Pseudohypoparathyroidism, precocious puberty, hypothyroidism

Introduction

Pseudohypoparathyroidism (PHP) type 1A (PHP1A) is a disorder of multiple hormone resistance in which a decreased responsiveness to parathyroid hormone (PTH), leading to hypocalcemia and hyperphosphatemia, is a main defect (1). It is caused by heterozygous loss-of-function variants in the coding sequence of GNAS gene that cause defects in the α-subunit of the stimulatory G protein (Gsα) (2). Resistance to other hormones, which includes thyroid stimulating hormone (TSH), gonadotropins and growth hormone (GH)-releasing hormone, causes hypothyroidism, hypogonadism and GH deficiency, respectively (2). In addition, PHP1A is associated with Albright hereditary osteodystrophy (AHO) phenotypes, including short stature, brachydactyly, obesity, round face, and ectopic ossifications (1,2). TSH resistance has frequently been described as the presenting feature of PHP1A with diverse severity, from isolated hyperthyrotropinemia to overt hypothyroidism,

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and might present at birth as congenital hypothyroidism or later during infancy and childhood (2,3). The diagnosis of PHP1A was reported to often be delayed in patients presenting with isolated TSH resistance, until other manifestations became apparent (4,5,6,7).

Some PHP1A patients also demonstrate elevated gonadotropin levels which represents gonadotropin resistance (8,9,10,11,12). This finding was mainly reported in female PHP1A patients who presented with either amenorrhea or oligomenorrhea (9,10,11). In contrast, male PHP1A patients were rarely reported to have gonadotropin resistance (10,11,12).

Central precocious puberty (CPP), a condition which is the biological opposite of gonadotropin resistance, is unlikely to be present in PHP1A patients. However, there are at least two reports of CPP in two PHP boys who had PTH as the only hormone resistance (13,14). Herein, we report another boy with PHP1A who was diagnosed with isolated hyperthyrotropinemia during infancy, and developed symptomatic hypocalcemia secondary to PHP1A later in his childhood, at which time CPP was also diagnosed.

Case Report

A 9.5-year-old boy who had been diagnosed as having mild congenital hypothyroidism since the age of 11 months presented to our hospital for the first time with viral infection and tetany. Physical examination revealed body temperature of 38.8 °C, heart rate of 82 beats per minute, blood pressure of 97/61 mmHg, height of 128 cm [-0.9 standard deviation score (SDS)], weight of 38 kg (+1.8 SDS) and body mass index of 23.2 kg/m^2 (+2.6 SDS). Carpopedal spasm and positive Chvostek's sign were noted. He had a round face and short fourth and fifth metacarpal bones. No subcutaneous calcification was observed. Chest, abdominal and neurological examinations were unremarkable. Investigations for his tetany revealed a finding which was consistent with PTH resistance or PHP, including hypocalcemia, hyperphosphatemia and elevated intact PTH level concomitant with hypocalciuria and high tubular reabsorption of phosphate (Table 1). His clinical presentations including AHO phenotype (obesity, round face, short metacarpal bones and relatively short stature) and PHP led to the provisional diagnosis of PHP1A. Supportive treatment for viral infection was administered. Hypocalcemia secondary to PHP was initially treated with

Table 1. Clinical characteristics of the reported patient and previous reports of boys with pseudohypoparathyroidism (PHP) who had central precocious puberty at the diagnosis of PHP

Characteristics	This report	Kagami et al. (13)	Rossodivita et al. (14)
Age at diagnosis, years	9.5	10.0	11.5
Height, cm (SDS)	128 (-0.9)	138 (+0.2)	157 (+1.9)
Predicted adult height, cm (SDS)	151 (-3.5)	162 (-2.0)	179 (+0.3)
Weight, kg (SDS)	38 (+1.8)	39 (+0.7)	46 (+1.2)
Body mass index, kg/m^2 (SDS)	23.2 (+2.6)	20.2 (+1.6)	18.9 (+0.7)
Bone age, years	13.0	13.0	13.5
Testicular volume, mL	10	6-8	12-15
Serum calcium, mg/dL	6.0	5.3	4.2
Serum phosphorus, mg/dL	8.3	11.1	10.3
Serum magnesium, mg/dL	1.7	NA	1.5
Serum intact PTH, pg/mL	137	363	191
Serum 25-hydroxyvitamin D, ng/mL	35	16	20
Serum testosterone, ng/dL	298	250	384
Serum LH, IU/L	3.9 (random)	18.0 (GnRH-stimulated)	2.8 (random), 29.9 (GnRH-stimulated)
Serum FSH, IU/L	4.8 (random)	7.0 (GnRH-stimulated)	6.9 (random), 13.3 (GnRH-stimulated)
Urine calcium	0.01 mg/mg of creatinine (N, 0.03-0.26)	NA	4.9 mg/kg/day (N, 0.8-2.8)
Tubular reabsorption of phosphate, %	97.6	NA	95.0
GNAS variant	c.1103A>G; p.Asp368Gly	c.568dupT; p.Tyr190Leufs*20	NA

Normal range: serum calcium 8.7-10.7 mg/dL, phosphorus 3.3-5.4 mg/dL, magnesium 1.6-2.4 mg/dL, intact PTH 10-65 pg/mL, 25-hydroxyvitamin D 30-100 ng/mL, testosterone (Tanner stage III) 100-320 ng/dL, tubular reabsorption of phosphate 90-95%.
FSH: follicle-stimulating hormone, GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone, N: normal, NA: not available, PTH: parathyroid hormone, SDS: standard deviation score

intravenous calcium gluconate concomitant with oral calcium carbonate and calcitriol. Tetany resolved following intravenous calcium gluconate treatment and normalization of serum calcium and phosphorus concentrations was gradually achieved with oral calcium carbonate and calcitriol.

Regarding his past medical and congenital hypothyroidism history, he was born at 35 weeks of gestation with a birth weight of 2.6 kg (+0.1 SDS) and length of 47 cm (+0.4 SDS). His developmental milestones were normal. He had a positive serum TSH screening level of 29 mU/L (normal, <25). Subsequent thyroid functions at ages 11 days to 7 months showed serum TSH levels of 5.6-12.1 mU/L (normal, 0.7-4.2) and free thyroxine (T4) levels of 1.1-1.2 ng/dL (normal, 0.9-1.7), indicating isolated hyperthyrotropinemia. Levothyroxine was not started until the age of 11 months when his TSH level rose to 16.2 mU/L with normal free T4 of 0.9 ng/dL. Free T4 and TSH normalized within four weeks following levothyroxine treatment. Taken together, congenital hypothyroidism or TSH resistance, one of the PHP1A phenotypes, was the first manifestation of PHP in this patient.

His growth trajectory had been tracking along the 75th percentile for weight and the 3rd percentile for height since he was 2 years of age. His mid-parental height was 159 cm (-2.0 SDS). His height velocity had strikingly increased by 9.5 cm during the past year. Pubertal assessment demonstrated testicular size of 10 mL, penile length of 7 cm and Tanner stage III pubic hair. Based on his height velocity and secondary sex characteristics, pubertal onset had presumably begun before 9 years of age. Therefore, the findings were consistent with gonadotropin-dependent precocious puberty or CPP. The diagnosis was confirmed by the findings of pubertal levels of serum testosterone, luteinizing hormone and follicle-stimulating hormone (FSH), and advanced bone age (Table 1). Pituitary magnetic resonance imaging was normal. He was commenced on depot gonadotropin-releasing hormone analog to preserve final adult height.

A novel heterozygous missense variant of the *GNAS* gene (NM_000516.5) in exon 13, c.1103A>G (p.Asp368Gly) was identified in the patient. The variant was classified as likely pathogenic based on the American College of Medical Genetics criteria (15). The variant has not been reported in individuals with PHP1A or in the gnomAD, ExAC and in-house Thai Exome databases. Multiple computer prediction algorithms including SIFT, DANN, PrimateAI REVEL, MutationTaster, MVP, Polyphen2HVAR, BayesDel_addAF, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, and MutationAssessor classified the variant as damaging or deleterious. The sequence data has been submitted to the GenBank database under accession number MW503931.

Maternally-inherited heterozygous inactivating GNAS mutation is usually the cause of PHP1A. His mother's height was 140 cm (-3.6 SDS) and she had short fourth and fifth metacarpal bones. Her serum calcium, phosphorus and intact PTH levels were normal at 9.2 mg/dL (normal, 8.5-10.5), 3.2 mg/dL (normal, 2.4-4.4) and 54 pg/mL (normal, 10-65), respectively. Since AHO phenotype was observed in the absence of PTH resistance, the diagnosis of pseudopseudohypoparathyroidism was likely in his mother. Genetic testing revealed the same variant in GNAS gene as found in the patient.

The report was approved by the Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (date: 23.02.2021, MURA 2021/161) and conformed to the Declaration of Helsinki.

Informed assent and consent were obtained from the patient and his parents, respectively.

Discussion

The patient in this report had mild congenital hypothyroidism without documented AHO phenotypes (such as finger abnormalities) during the early life, as the first manifestation of PHP1A. In fact, hyperthyrotropinemia representing TSH resistance, is a common finding and could be the earliest hormonal dysfunction in PHP1A patients, because elevated TSH concentration might be detected at the time of neonatal screening (2,3,4,5). Unlike resistance to TSH, PTH resistance, the hallmark of PHP1A, usually manifests after the first few years of life due to gradual silencing of paternal Gsα in the renal proximal tubule (4). As a result, the diagnosis of PHP1A is often delayed, especially in patients with non-specific features such as obesity or short stature (2,4,5,6,7). Childhood obesity was proposed as an early clinical sign of PHP1A as it might develop in very early life and could even be recognized before any other endocrine disturbances (2,5). Our patient developed obesity from the age of 2 years and later developed symptoms of PTH resistance at the age of 9.5 years. Therefore, PHP1A diagnosis should be suspected in children who present with isolated hyperthyrotropinemia or mild congenital primary hypothyroidism and obesity early in life to avoid delayed diagnosis.

Gonadotropin resistance has been described in patients with PHP1A (2,3,7). Delayed or incomplete puberty with elevated gonadotropin levels could thus be one of the

PHP1A phenotypes. Symptomatic gonadotropin resistance has been detected more commonly in female PHP1A patients while elevation of gonadotropin levels without symptoms has rarely been reported in male PHP1A patients (2,3,8,9,10,11,12). Interestingly, our patient developed CPP instead, which is exceptionally rare among patients with PHP1A. To the best of our knowledge, there have only been two male PHP patients with CPP reported in the English literature (Table 1) (13,14). Both of them and our patient had CPP at the presentation of PHP. One patient had clinical features of PHP1A and the diagnosis was confirmed by demonstrating a heterozygous frameshift variant in exon 7 of the *GNAS* gene (13). The other patient manifested PTH resistance in the absence of AHO phenotypes; PHP type 1B, the disease associated with epigenetic alterations at the GNAS locus, was likely the diagnosis (14). Hence, CPP could manifest regardless of the type of genetic defect underlying PHP. The mechanism of CPP remains unclear. In parallel with PHP patients who developed CPP, CPP was previously described in girls with Turner syndrome who commonly have ovarian failure (16,17). Functioning ovarian tissue, which is infrequently present in girls with Turner syndrome and could be responsive to FSH surge preceding the ovarian failure, may be the cause of CPP found in Turner syndrome patients (16). Similarly, CPP in boys with PHP1A might be mediated by testicular androgen production in response to elevated gonadotropin levels during childhood before developing partial gonadotropin resistance later in life. Nevertheless, coincidental idiopathic CPP in our patient cannot be excluded. Idiopathic CPP in girls is indeed much more common than in boys (18). Interestingly, to the best of our knowledge, CPP in girls with PHP1A has not been reported. This might be due to a gender discordance of resistance to gonadotropins in patients with PHP1A. Inactivating mutation of Gsα-coupled receptor along hypothalamic-pituitary-gonadal axis that causes CPP has not been identified. Indeed, Gsα-coupled receptor has not been shown to be a part of the neuroendocrine regulators of male puberty (19).

Conclusion

In conclusion, isolated hyperthyrotropinemia presenting with congenital hypothyroidism may be the first manifestation of PHP1A. Apart from the typical gonadotropin resistance, CPP may also be found in male PHP1A patients.

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Ethics

Informed Consent: Informed assent and consent were obtained from the patient and his parents, respectively.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Somboon Wankanit, Pat Mahachoklertwattana, Preamrudee Poomthavorn, Concept: Somboon Wankanit, Pat Mahachoklertwattana, Preamrudee Poomthavorn, Design: Somboon Wankanit, Pat Mahachoklertwattana, Preamrudee Poomthavorn, Data Collection or Processing: Somboon Wankanit, Pat Mahachoklertwattana, Preamrudee Poomthavorn, Analysis or Interpretation: Somboon Wankanit, Pat Mahachoklertwattana, Thipwimol Tim-Aroon, Kinnaree Sorapipatcharoen, Preamrudee Poomthavorn, Literature Search: Somboon Wankanit, Pat Mahachoklertwattana, Thipwimol Tim-Aroon, Kinnaree Sorapipatcharoen, Preamrudee Poomthavorn, Writing: Somboon Wankanit, Pat Mahachoklertwattana, Thipwimol Tim-Aroon, Kinnaree Sorapipatcharoen, Preamrudee Poomthavorn.

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Beta-blocker Rebound Phenomenon in an Adolescent with Graves' Disease

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Dear Editor,

Graves' disease is the most common cause of hyperthyroidism in children (1). The most common complaints at admission are palpitation, sweating, tremor, and irritability (2). Beta-blockers such as propranolol (0.5-2 mg/kg/day) are mostly used for adrenergic symptoms of children with Graves' disease until euthyroidism is achieved (3).

Sudden withdrawal of beta-blocker drugs in adults can lead to unwanted effects such as tachycardia, arterial hypertension, angina pectoris, and an increase in heart failure symptoms. These symptoms and signs following sudden cessation of beta-blockers are called the "beta-blocker rebound phenomenon", also known as beta-blocker withdrawal syndrome (4). Adult patients may exhibit hypertension, headache, palpitation, sweating, and chest pain. These symptoms occur within the first few days after discontinuation of the drug (4). In adults with underlying overt or occult ischemic heart disease, this phenomenon may cause major complications. Moreover, an exacerbation of migraine headache attack may develop in patients withdrawn from beta-blocker therapy (5). Although the optimal prevention methods for the beta-blocker rebound phenomenon are not known, sudden discontinuation of long-term propranolol therapy (longer than 3 months) should be avoided to prevent rebound effects in the adult population (4,6). The daily dose of propranolol in adults with propranolol withdrawal syndrome is 160-320 mg and a lower propranolol dose is less likely to lead to this phenomenon (4,5,6,7).

A 17-year-old female patient presented with complaints of palpitation, irritability, and weight loss. Physical examination revealed a weight of 0.2 standard deviation score (SDS), height of -1.0 SDS, body mass index of 1.0 SDS, heart rate 164/min, arterial blood pressure 120/80 mmHg, goiter, and fine tremor. Laboratory test results were as follows: free thyroxine (fT4) 4.9 ng/dL (N: 0.7-1.48), free triiodothyronine (fT3) 16.5 pmol/L (N: 3.0-9.0), thyroid-stimulating hormone (TSH) 0.01 uIU/mL (N: 0.35-4.94) and anti-thyroglobulin 5.1 IU/mL (N: <4.1). Thyroid ultrasonography showed an enlarged thyroid gland (volume 16.9 mL, 4.8 SDS), hypoechoic heterogeneous thyroid echotexture, and hypervascularity. The patient was diagnosed with Graves' disease and methimazole 20 mg/day (0.33 mg/kg/day), propranolol 80 mg/day (1.3 mg/kg/day) treatments were started. The dose of methimazole was reduced and propranolol was discontinued on the 16th day. One day after the discontinuation of propranolol, the patient developed severe headache, palpitation, and malaise. Physical examination at that time revealed systemic hypertension (arterial blood pressure 140/90 mmHg) with normal systemic findings. Serum levels of thyroid hormones were as follows: fT3 4.68 pg/mL (N: 1.71-3.71), fT4 1.27 ng/dL (N: 0.7-1.48), TSH 0.001 uIU/mL (N: 0.35-4.94). All the complaints spontaneously disappeared within 24 hours. The patient was normotensive and physical examination findings were normal after 24 hours. Typical findings and spontaneous regression of symptoms suggested that this attack may be due to beta-blocker withdrawal but there are no data regarding the incidence and characteristics of withdrawal reactions in children receiving relatively low dose propranolol for a short period.

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In conclusion, the beta-blocker rebound phenomenon may occur in adolescents with Graves' disease who are treated with short-term and relatively low-dose propranolol and whose treatment is rapidly discontinued. Although the clinical significance of this situation in adolescents without underlying ischemic heart disease or migraine is not known, it may lead to discomfort and a decrease in the quality of life of the patient. It is not known whether tapering, rather than rapid, discontinuation of propranolol in adolescents with Graves' disease will prevent this condition. However, pediatric endocrinologists must be aware of beta-blocker rebound to inform their patients about it.

Ethics

Peer-review: Internally peer-reviewed.

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