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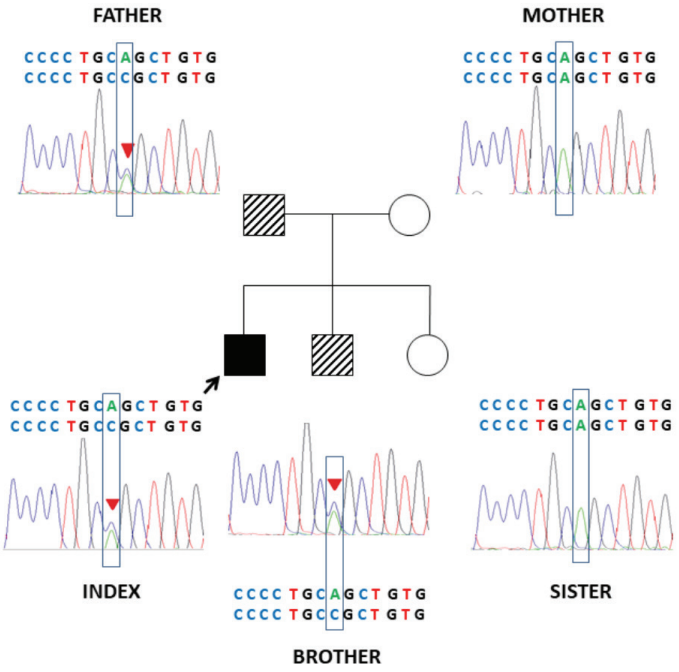

Family pedigree and electropherogram of heterozygous IVS11-2A>C(c.1957-2A>C) mutation in the GLI2 gene. Full-black filled box indicates index case with Culler-Jones syndrome phenotype, shaded boxes indicate father and brother who are also heterozygous for the identical mutation with incomplete phenotype, empty boxes indicate mother and sister with wild type

Ectopic Posterior Pituitary, Polydactyly, Midfacial Hypoplasia and Multiple Pituitary Hormone Deficiency due to a Novel Heterozygous IVS11-2A>C(c.1957-2A>C) Mutation in the *GLI2* Gene

Demiral M et al.

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REVIEW

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Managing Paediatric Growth Disorders: Integrating Technology Into a Personalised Approach

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What is already known on this topic?

There have been few articles specifically linking the human component of growth management, i.e. specialist and nurse interaction with the patient, psychological support and training of healthcare professionals in motivational interviewing together with digital innovations such as electronic monitoring of growth hormone (GH) injections. Both the human and digital components are recognised to contribute to GH adherence, but it is the necessity of their partnership that we emphasize.

What this study adds?

A review on the holistic approach to personalised growth management by multi-disciplinary professionals, but stressing the key importance of the human and technical partnership. Contributions are also provided by a professional coach who is an expert in motivational interviewing and personnel from the UK patient support group, the Child Growth Foundation.

Abstract

Long-term growth management can be challenging for patients, families and healthcare professionals (HCP). Personalised optimal responses to growth hormone (GH) therapy depend on the creation of a good working relationship between the patient and family and the HCPs responsible for care. Current unmet needs in growth management will be discussed, focusing on the likelihood of a poor growth response and its identification and management with emphasis on the importance of good adherence to GH therapy. Digital tools are now available to record injections and communicate accurate adherence data to the HCP and patient. Psychological barriers to good adherence will be covered, with techniques identified to change behaviour and improve outcome. Motivational interviewing is a valuable skill in this respect and should be taught to both medical and nursing HCPs to enhance the quality of the relationship with the patient and family. Key messages are, firstly, the importance of personalised care with the HCP using acquired psychological skills to prevent and manage poor adherence. Secondly, a human-eHealth partnership is necessary to maximise the benefit of new digital tools to aid in successful growth management.

Keywords: Growth, growth hormone, adherence, motivational interviewing, eHealth

Introduction

The management of paediatric growth disorders presents a multidisciplinary challenge to healthcare professionals (HCPs) responsible for affected patient care. Several medical HCPs may be involved, including the primary care physician who identifies the initial growth problem, the family general practitioner who refers the child for hospital investigation, the hospital-based paediatrician who

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sees the child at the initial consultation and the specialist paediatric endocrinologist to whom the child is then referred for an expert opinion and further management. In addition, in many hospital paediatric endocrinology units, the developing role of the paediatric endocrinology nurse specialist has directly improved the quality of liaison with the family and contributes to the care of the child through the addition of a skilled HCP to the management team. Pharmacists, biochemists, psychologists, patient support groups and personnel from the pharmaceutical industry also make important contributions to the three key phases of growth management; namely identification of the initial short stature, investigation and diagnosis of the cause, and treatment with hormone therapy, where indicated, all of which implies a long-term commitment to a potentially invasive therapy (1). Early diagnosis and early initiation of growth hormone (GH) therapy is associated with improved long-term height gain (1,2).

The pressures experienced by the patient and family to successfully engage in such a diagnostic and therapeutic journey are also challenging. There are key facts about the nature and implications of the diagnosis to understand and process, including the emotional commitment for therapy to be successful and produce normal growth and adult height. In addition, maintenance of a therapeutic regimen designed to bring long-term improvement, rather than short-term benefit, requires engagement and maturity.

These aspects of short stature management will be discussed in this article. A further component of care, which has emerged in recent years, are the electronic tools to aid therapy and adherence. These tools will also be addressed with emphasis on the importance of the human-eHealth partnership, which is necessary to make patient care optimally beneficial.

We will discuss the challenges encountered by the patient and family through the experience of staff of the UK Child Growth Foundation (CGF), a patient support charity which advises families of patients with short stature. Current unmet medical needs of growth management will also be discussed (1) followed by a description of the psychological basis and management of poor adherence to GH treatment regimens (3). eHealth innovations will be covered, followed by the importance of HCP training in relation to acquisition of motivational skills for improved recognition and intervention in poor adherence situations. Finally, the emerging role of the paediatric endocrinology specialist nurse will be summarised, with conclusions highlighting the rationale for joint human-eHealth collaboration to achieve optimal personalised management of the short child.

Current Unmet Needs in the Management of Short Stature

Early recognition of pathological short stature, as opposed to variants of normal height, remains a challenge, particularly in the UK, where routine height surveillance has been reduced to two measurements at primary school and secondary school entry points (4). The age of diagnosis of disorders of abnormal growth, such as coeliac disease and Turner syndrome, is significantly later than in other countries such as the Netherlands and Finland (5), where investment in primary care identification of growth disorders has resulted in earlier diagnoses (6).

Historically, a high proportion of children, treated with GH therapy for a variety of growth disorders, have not demonstrated a satisfactory degree of catch-up growth during the first year of therapy (7). A number of reasons may underlie this, including incorrect diagnosis, incorrect dose of GH at initiation of therapy and inadequate attention to factors predicting individual growth responses (8). The correct management of poor response to GH remains a priority in such patients (9). However, it is the presence of poor adherence to the GH treatment regimen which has emerged as a key factor, either alone or in combination with other elements that have an impact on growth response (10,11). This issue of non-adherence will be discussed in detail below.

Digital Advances in the Management of Growth Disorders

Digital health, defined as the use of information and communication technologies for health, is becoming a reality in clinical practice and medical education and has made a significant impact in the day-to-day management of diabetes mellitus in children (12). Its application to the treatment of growth disorders is more challenging because therapy is geared to long-term responses and benefits, rather than short-term metabolic control. However, one area where digital technology has been effective is in the electronic monitoring of GH injections (13,14). The use of an electromechanical auto-injector, which records every injection that is given and communicates the data both to the patient and the HCP, is a major advance (15). It is known that self-reporting of adherence tends to be inaccurate and to report artificially high values, compared with digital recording of injections (16). The difference between reported and recorded accuracy, using the electronic device, is significant.

In a large international study of GH therapy using electronic recording, adherence was shown to be good during the first year of treatment, but gradually decreased to approximately 60% after five years (13). These data give two key messages, first that accurately measured adherence decreases over time and secondly that intervention by the HCP is indicated to prevent and correct this trend. The injection device can also demonstrate suboptimal adherence which may not be obvious from auxological measurements.

Psychological Factors Predisposing to Non-adherence: The Human-digital Model of Collaborative Care

Adherence or compliance can be defined as the extent to which the patient follows a prescribed therapeutic regimen, and in the case of GH, the extent to which daily GH treatment is taken. There are three phases in understanding the way adherence develops. First, there is the uptake stage, which describes the way in which the patient begins to accept the treatment and indeed whether they actually start to take the treatment. It is known that 10% to 15% of patients never start taking the treatments they are prescribed (3). This is known as primary non-adherence. The second phase, which is really critical for long-term progress, is the way in which the patient, or the family, incorporates the treatment into the habitual pattern of daily life. The last phase describes how long the patient stays with the treatment.

It is known that patients may give up after months or years of treatment and there is evidence for a wide range of adherence to GH therapy (3,11). Overall, there are figures of up to 50%, 60%, or even 70% of patients not taking GH treatment in a regular and useful way, with a clear relationship demonstrated between non-adherence and not achieving linear growth targets (10).

Given that GH therapy is evidence-based, the question is why are patients not adherent? Older explanations were essentially based around the idea that people did not follow treatment because they did not understand or remember what they had to do (17). This was often taken to be a symptom of poor communication in healthcare, so interventions were designed to improve communication and patient understanding and the ability to remember and plan treatment. This, unfortunately, is only a small part of the answer.

Intentional and Non-intentional Non-adherence

It is now clear that there are different categories and certainly different causes of non-adherence. Two distinct types are recognised, known as intentional and unintentional non-adherence, which have very different drivers, or different origins. The reasons for the two different categories can be summarised in terms of what is known as the COM-B model (Figure 1) (18). In the COM-B acronym, C stands for capability, O for opportunity and M for motivation. In intentional non-adherence many patients know what they have got to do, ie it is not a question of misunderstanding or not remembering, but they are reluctant to adhere, because either the treatment does not make sense to them, or they have worries or concerns about it. In unintentional non-adherence some of the older factors can be responsible such as poor communication, poor experience or satisfaction with the organisational challenges of doing something regularly on a daily basis. There may also be other barriers outside the individual, such as financial or practical constraints.

The COM-B Model

If this is mapped onto the COM-B model (Figure 1) we can see that under **Capability**, there is a range of factors, such as psychological difficulties; eg, people not remembering or not being able to plan. There are also some physical capability issues, eg, not being able to administer the treatment in a way that is effective. Under **Opportunity**, there are physical factors such as getting access or having barriers to treatment, which lie outside the patient, together with psychological barriers, such as poor support and communication from people close to the patient. However, the really important factors for many patients, particularly related to intentional non-adherence, are the **Motivational** influences, such as negative or mistaken beliefs about their condition and their treatment.

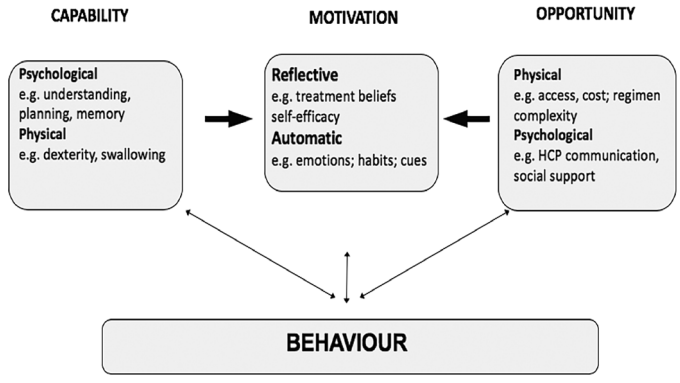

Figure 1. The COM-B model: A new approach to classifying adherence to diseases (adapted from reference: 18)

HCP: healthcare professionals

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Human and Digital Interventions

Accepting this variety of factors, it is not surprising there is a range of ways that we have of working with families and patients, to improve their adherence. These can involve both human and digital interventions. Two available strategies are equally important. It is fundamental to use the direct experience in the healthcare situation, ie the consultation, to understand the patient's issues and perspectives and to anticipate factors around non-adherence which can be managed. Going beyond that, there is a range of digital and personalised interventions available; for example, an initial brief screening questionnaire to identify the particular problems each patient and family may be experiencing. Then, following that, interventions can be developed which are tailored to each patient.

In terms of the consultation, a structure is recommended for each family to analyse their understanding of the primary short stature condition and the treatment regimen they are being asked to follow. It is important to make sure that they have a clear rationale for the need for treatment and for daily injections. A recent study in adults with GH deficiency showed that non-adherence was related to lack of understanding of the primary disorder, which can be improved through focused education (19). A practical plan needs to be agreed for how, where and when the GH injections are given to ensure that treatment is administered more regularly.

More generally, factors which cause adherence problems for each individual need to be identified. At the beginning and during treatment, brief screening questionnaires can be used to identify relevant personal issues. Information from the screening questionnaires can be used to start a personalised conversation to understand what is going wrong. From there, basic behaviour change approaches, such as motivational interviewing by HCPs, can attempt to target individual factors.

Beyond the consultation, many other digital approaches are available which patients and parents can access on a daily basis. These could be personalised web-based tools, mobile phone applications, daily text messaging or interactive programmes which address particular issues.

Training of Medical and Nursing HCPs in Motivational Interviewing to Address Family Interactions Related to Growth Management

The main role of a professional coach in the healthcare environment is to support HCPs in learning how to help patients to make healthy choices and decisions in their lives. This can be challenging because patients can struggle to make such choices, particularly when emotional barriers block the logical courses of action. A number of questions can be asked. How can HCPs really influence the behaviour of patients and families, particularly when they have decided they do not want to change? Why can some patients move forward when others are resistant to making progress?

These questions and observations have led to the exploration of motivational interviewing practised by HCPs which can be applied in the clinical scenario of outpatient consultations to help patients with adherence to GH therapy. It is proposed that motivational interviewing skills can motivate patients and families to overcome the practical and emotional barriers related to therapy.

Motivational Interviewing

Motivational Interviewing, which is based on the work of Miller and Rollnick (20), is a collaborative conversation style which aims to strengthen a person's motivation and commitment to change. It is a structured, person-centred approach which helps patients and families to resource their own inner motivation to be translated into improving adherence to GH therapy. Motivational interviewing is a skill which needs to be taught and thus learnt by both medical and nursing HCPs.

Examples of the benefits of motivational interviewing can be taken from experience in making healthy life choices, such as giving up smoking, reducing alcohol intake or eating in a healthier way. When considering these choices, reaction to the individual can be unhelpful, such as not listening or negatively encouraging regressive behaviour. By contrast, a helpful response to the same life choices would consist of positive reactions such as genuine empathetic listening and exploration of the individual's feelings without judgement. This behaviour typifies the spirit of motivational interviewing.

The principles of motivational interviewing are collaboration, acceptance and compassion. Collaboration is very important because partnership on an equal level with the patient is a key aim. Acceptance leads to better understanding of the decisions and choices that patients and families are making without judgement. These choices are accepted and the HCP responds with guidance. Compassion is a further component that is combined with evocation, which means drawing out a patient's inner motivation and commitment, and building on this to effect change.

The OARS Model

Core skills in motivational interviewing can be discussed under the acronym OARS, which stands for Open questions, Affirmations, Reflective listening and Summarising. The conversation can be structured by following these headings. Open questions such as what, how and why will open conversations and evoke dialogue. Other examples would be 'what are your hopes for your consultation today?' and 'I am curious to learn how you have been getting on with your injections?' These questions can be prefaced by saying 'help me understand …' and the conversation can develop by inviting the patient or family to talk about what is on their mind, what are their needs and their priorities. Affirmations are about helping patients to recognise their own strengths and positive beliefs that are going to help them to adhere to GH therapy. Examples could be to say to a patient 'I can see it took courage for you to try this out today' or to a parent 'your creative ideas around this are very helpful'. Reflective listening consists of not only listening and reflecting back what is said, it also helps in verbalising the thinking and feelings that lie underneath, showing a depth of empathy that leads to further conversations. The last skill here is summarising, which serves the useful purpose of wrapping up conversations and can be started by saying 'let me see if I have got this right, you are feeling this on one hand and perhaps feeling this on the other?'

Challenges with Adherence from the Patient and Family Perspectives

When patients and families are asked about the difficulties they face related to management of short stature, a wide range of opinions and comments are given. The UK CGF (https: www.childgrowthfoundation.org) is a non-profit patient support group, which was originally founded as a charity in 1977 (UK Registered Charity number 1172807). The CGF receives many requests for information and support and delivers management advice on a wide range of growth disorders.

In relation to adherence to GH therapy, the CGF reports that in the consultation setting some HCPs do not have sufficient time or experience of GH treatment which results in them giving conflicting advice to families. Insufficient knowledge of the primary growth disorder results in communication of inadequate or incorrect information. In particular, the patient may not realise how effective and worthwhile long-term therapy with GH can be. Insufficient education of the patient by the HCP can result in the family seeking alternative advice on the internet and thus receiving more confusing, incorrect and worrying messages. More accurate information needs to be available regarding the benefits of GH therapy with advantages outside growth being emphasised, such as improved general health and self-esteem (19). Accurate information regarding GH injection devices needs to be given with the choice of the most suitable injection device made by the family before the initiation of therapy. Size, comfort and storage requirements should also be considered, together with family dynamics and travel.

Patient Choice of GH Brand and Injection Device

The concept of patient choice is an organisational decision which is not universally adopted in the framework of growth consultations. Ideally however, the patient and family should be offered the choice of GH brand and injection device and this has been demonstrated to increase the likelihood of good adherence (21,22). In 2019 the CGF conducted an online survey amongst its members about initiation of GH therapy (Figure 2). One hundred and eleven responses were received, mostly from patients with GH deficiency, multiple pituitary hormone deficiencies, Silver Russell syndrome, small for gestational age and intrauterine growth retardation. The two most relevant questions were, 'Were you offered a choice of GH brand and device?' and 'How often does your child miss a GH dose?' Out of 111 responses, 31% of patients were not offered a choice of GH brand or injection device, demonstrating that within the UK, patient choice remains very inconsistent. Guidelines for England and Wales, regarding GH treatment, https: www.nice.org.uk/guidance/ta188/chapter/1-Guidance are not being followed. The survey indicated that 58% of patients never missed a GH dose, with lower values of 30% of non-GH deficient cases compared with 78% in multiple pituitary hormone deficiency cases.

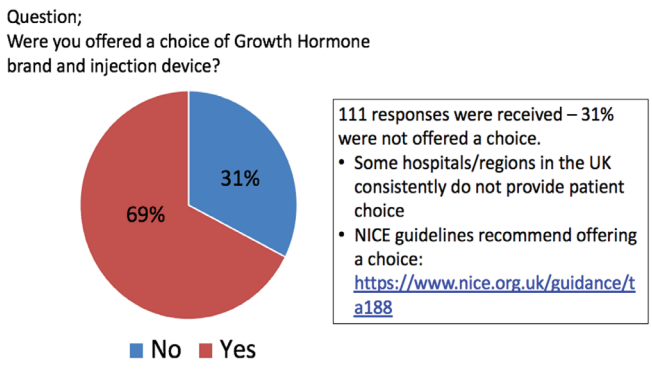

Figure 2. Results of a patient-directed survey on choice of growth hormone brand and injection device

Family Logistical Barriers to Good Adherence

From many years' experience of handling requests for information and from managing the CGF Facebook page, the CGF reports topics, which are frequently repeated, related to barriers to good GH adherence. The first of these is logistical barriers. A daily subcutaneous injection should become part of the family's routine, provided the routine is not disturbed. However when changes do occur, such as a play-date, a school trip, a sleep-over, a camping trip with refrigeration necessary or particularly when the child's care is shared between parents in different locations or with grandparents, the first casualty is the GH injection. As the effect of missing one or several GH injections is not immediately apparent, the long-term objective of regular therapy tends to be forgotten leading to chronic poor adherence. Another practical aspect is the maintenance of regular GH supplies, which may not occur if a family waits to order a new supply at the last minute.

Emotional Barriers to Good Adherence

Children receive GH treatment because they have a long-term health condition but may develop a needle phobia with a fear of the pain of the injection combined sometimes with the noise of the injection device. A vicious cycle of events can develop and escalate in importance, predictably leading to missed injections. The anticipation of the injection and then its attempted administration can be very stressful. In the longer-term, a child might start to feel different to their peers, especially around friends, of whom not many will be having daily injections. Bullying and exclusion of the patient can occur. Peer pressure increases during adolescence, when additional stresses, such as exams, provide further opportunities to miss GH injections and for poor adherence to become habitual.

Benefits of Peer Support

Availability of communication with other patients having similar experiences can be very supportive and can significantly reduce stress and the sense of isolation. Peer support organisations such as the CGF can support and advise their own patients and the HCPs who are responsible for them. Many host social media groups, providing a 24/7 online community for chats, questions, discussions and mutual support. The CGF holds an annual convention, but with e-technology, geographical boundaries have diminished and Facebook groups, educational websites, mobile phone applications and helplines can all contribute to enhanced patient and family support.

Contributions of the Paediatric Endocrinologist Specialist Nurse to Practical GH Management

The roles of the paediatric endocrinology specialist nurse have developed at different rates in different countries. In the USA, UK, Canada, Australia and Scandinavia this nursing speciality has grown, with funding now established for positions in most university paediatric endocrinology departments (23). In other countries paediatric endocrinology nursing is much less developed. We will discuss roles and responsibilities related to short stature management and specifically GH adherence.

Paediatric endocrine specialist nurses are uniquely positioned to offer a high-valued support network to HCPs, patients and their families, by being the regular first point of contact at consultation visits. Relationships, incorporating the whole family, are established and built on trust, specialised knowledge and expertise that is pivotal for families when starting GH therapy. Involvement in the initiation of GH treatment is key to establishing a fruitful relationship with the patient. 'Ideal' and 'worst-case' scenarios regarding initiation of GH therapy are shown in Table 1. If possible, meeting the family before the medical consultation can be very beneficial. Obtaining knowledge of the medical history and whether the family has studied the diagnosis on the internet can also be very valuable.

Communication skills are important and as discussed above, training in motivational interviewing can play an

Table 1. Ideal and worst-case scenarios when starting growth hormone therapy from the point of view of a paediatric endocrinology nurse specialist

Features	Ideal case	Worst case
Correct diagnosis	Yes	No
Correct GH device chosen	Yes	No (inappropriate device chosen)
Patient keen on treatment	Yes	No
Patient keen and psychologically positive about starting treatment	Yes	No (needle phobia)
Parents, child and healthcare professional committed to GH therapy	Yes	No (family upset by injections)
Adequate information, advice and digitals tools	Yes	No

GH: growth hormone

essential role in the specialist nurse becoming an effective member of the growth management team and contributing to optimal GH adherence. Organising the patient's choice of GH brand and injection device is a further responsibility and needs to be based on specialist knowledge of the different GH devices.

Education in injection technique will logically lead to the establishment of a network of regular contacts and availability for the patient and family. Contact and support by phone and internet have become inherent in the nurse specialist's responsibilities. In terms of adherence, the use of electronic monitoring of injections with feed-back to the nurse and endocrinologist allows adherence to be examined, so that a human-eHealth partnership develops to support the family. At consultation visits, it is logically the nurse specialist who can take the lead in non-judgemental interviewing to investigate actual or potential non-adherence.

In the long-term, the paediatric endocrinology specialist nurse maintains support and positive relationships with the family and the patient. Everyone needs to continue to work together, ensuring encouragement and a combined committed goal of optimal response to GH therapy. Finally, by using a personalised approach, technology can be positively integrated into care and assist adherence and optimise outcomes.

Conclusion

The successful management of paediatric growth disorders, involving GH therapy, can be judged by the achievement of catch-up growth, followed by growth within the normal centile lines leading to an adult height within the genetic target of the family. Relatively few cases achieve this ideal triad and a combination of personalised input by medical and nursing HCPs and the use of technological tools can improve the chances of success. Understanding the personal psychological barriers to good GH adherence in each patient can be combined with the use of an electronic GH injection recorder to monitor and communicate accurate adherence data. Motivational interviewing and a non-judgemental approach are also beneficial. This human-eHealth partnership gives synergistic advantages and improves the likelihood of a clinically beneficial long-term growth outcome.

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ORIGINAL ARTICLE

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The Effects of Risk Behaviors and Orthorexic Behavior on Glycemic Control in Adolescents with Type 1 Diabetes

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What is already known on this topic?

Adolescents with chronic disease are as likely to exhibit risk-taking behavior as their peers. The risky behavior and disordered eating behavior of adolescents with type 1 diabetes (T1D), the most common chronic disease of adolescence, may result in serious negative consequences.

What this study adds?

This study investigated the effect of orthorexic eating behavior of adolescents with T1D on glycemic control (GC) for the first time. Assessing risk-taking behavior by gender, investigating the use of alcohol and tobacco by male adolescents with poor GC, and evaluating girls' mood at each visit to the outpatient clinic, have been suggested as beneficial.

Abstract

Objective: Adolescents with chronic disease are as likely to exhibit risk-taking behavior as their peers. The aim was to investigate the risk behaviors of adolescents with type 1 diabetes (T1D) and the effect of orthorexic eating behaviors (OEB) on glycemic control (GC).
Methods: This cross-sectional study was conducted with 107 adolescents with T1D, aged between 13-18 years and attending high school. The Risk Behavior Scale (RBS) and Orthorexic Behavior Scale (ORTO-11) were administered. A high RBS score indicates risky behavior; a low ORTO-11 score suggests a tendency to OEB. Participants hemoglobin A1c (HbA1c) status was used to assess GC: optimal GC (HbA1c ≤7%); or poor GC (HbA1c >7%).
Results: Among females, those with poor GC had significantly lower ($p = 0.031$) ORTO-11 scores than those with optimal GC, which was not the case in males. A significant correlation ($r = 0.358$, $p < 0.001$) was found between HbA1c and total RBS, eating habits subscale, and suicidal tendency subscale scores. Participants with poor GC had significantly higher eating habits subscale, alcohol use, and tobacco use subscale scores ($p < 0.05$). Among females, total RBS and suicidal tendency subscale score was found to be significantly higher in those with poor GC; among males, alcohol subscale score was found to be significantly higher in those with poor GC.
Conclusion: This study is the first to show the effect of the tendency for OEB on GC among female adolescents with T1D. The study showed that, along with inappropriate eating behaviors, adolescents with T1D should also be assessed for other risk behaviors to help achieve optimal GC.
Keywords: Adolescent, risk behaviors, orthorexic eating behavior, glycemic control, type 1 diabetes

Introduction

Adolescence is a period of rapid physical growth, sexual development, and psychosocial maturation. In the mid-adolescence period, which covers 13 to 17 years of age, peer norms take precedence over family norms, and the individual seeks peer-approval. In this period, an increase in impulsive behaviors is observed; feeling invincible, some adolescents may get involved in risky behaviors that may harm them. Throughout this process, change and maturation occurs in the adolescent brain. Compared with other age

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groups, adolescents are more susceptible to reward because of this developmental process of the brain. They may start smoking or using substances or engage in unsafe sexual behavior or unhealthy eating behaviors (1,2).

A 2010 study from the United States reported that 29.8% of high school students consumed alcohol, and 8.8% smoked. It was also found in the same study that 39.5% of adolescents were sexually active (3). A similar study from Asia in 2015 found the rate of smoking and alcohol consumption to be 22.3% and 27.9% (4), which were 32.3% and 18.7% in Turkey, respectively (5). The rate of suicide attempts was higher in adolescents than in other age groups (6). The higher rates may be due to psychological problems in adolescents such as anxiety and depression, stressful life (problems with peers or family, chronic illness, etc.) and personal characteristics (impulsivity, inadequate flexibility or resilience, etc.) (6). Adolescents often do not consider how they will be affected by risk behaviors, but this period usually passes without problems during healthy development. However, risk behaviors of adolescents with chronic diseases may have serious negative consequences (7). Adolescents exhibiting risk-taking behaviors were found to be less compliant to treatments (8). Type 1 diabetes (T1D) is the most common chronic disease of adolescence. In children with T1D, glycemic control (GC) is often found to be impaired during adolescence. Hormonal changes and physical growth during this period complicate metabolic control. Some studies emphasized the adverse effects of risk behaviors on GC in adolescents with T1D (9,10). Disordered eating behaviors (DEBs) are one of the risk behaviors in adolescents. DEBs include various behaviors to control body weight such as skipping meals, being a choosy-eater, vomiting after overeating, and improper exercise (11). DEBs may be asymptomatic in healthy adolescents; however, they may cause complications requiring immediate medical attention in adolescents with T1D. Previous studies found that adolescents with T1D have a higher rate of DEBs compared to their healthy peers and that the former group also had impaired GC (12,13). Orthorexia nervosa (ON) is defined as obsessive healthy-eating behavior and has not yet been defined or classified as an eating disorder (14). However, orthorexic eating behavior (OEB), tendency to orthoxic nervosa, might be risky for adolescents with T1D and negatively affect their GC. OEB has been previously investigated in healthy young people (15). The effect of OEB on GC has not been studied in adolescents with T1D. This study aimed to investigate the effect of risk behaviors and orthorexic tendencies on GC in adolescents with T1D.

Methods

The study was conducted with patients between the ages of 13-18 who came to the pediatric endocrinology outpatient clinic in October, November, and December 2018. The Ethics Review Board of the University of Health Sciences Child Health and Diseases Hematology - Oncology Training and Research Hospital approved the study protocol (protocol number: 2018-130). The study was carried out in accordance with the Helsinki Declaration. Written consent for participation was obtained from adolescents and their parents. Adolescents over the age of 13 years, who had been followed up with a diagnosis of T1D for at least six months, using insulin, and attending high school were invited to the study. Adolescents from middle-income families (at least one parent had a permanent job, adolescent had room and phone, and expressed that they can get the recommended food) who live with both biological parents were included in the study. Those from low-income (parents had no work or received social assistance) and high-income (children attended a private school or had more than one car) families were excluded from the study. Adolescents who were underweight or obese, had another chronic disease, or psychiatric illness, were on medication other than insulin for any reason, or were hospitalized in the last three months, were also excluded from the study.

A total of 119 patients who met the inclusion criteria for the study were invited to participate; 107 patients agreed to participate in the study. Twelve adolescents did not consent to participate in the study. Two of the participants did not complete the scales; therefore, the demographic characteristics were analyzed based on 107 participants, and the data derived from scales were evaluated for 105 participants. Participants had a routine physical examination, necessary laboratory tests, and treatments similar to other patients with T1D. Weight was measured in kilograms (kg) using an electronic scale (Scale-Seca 220, Hamburg, Germany), height was measured in centimeters (cm) using the Harpenden stadiometer (Seritex, East Rutherford, NJ, USA). The standard deviations (SD) of height, weight, and body mass index (kg/m^2) were calculated (16). Participants' GCs were based on their hemoglobin A1c (HbA1c) level. At the time of blood sample collection for HbA1c, the Risk Behavior Scale (RBS) and Orthorexic Behavior Scale (ORTO-11) scale were administered to the participants. All participants completed these scales. They were instructed to fill out the questionnaires and put the forms in an envelope. Envelopes were deposited in a sealed box that was opened at the time of data analysis to provide anonymity. There was no time limit given to complete the scales. The relationships between whether patients had optimal GC (≤7%, ≤53

mmol/mol) or poor GC (>7%, >53 mmol/mol) and the patients' age, age at diagnosis, and duration of disease were evaluated. The optimal-GC and poor-GC groups were also analyzed concerning their RBS and ORTO-11 scores.

Glycemic Control

Glycated hemoglobin (HbA1c) measures a patient's GC. HbA1c reflects the average serum glucose level in the period of the previous 3-4 months. HbA1c levels were analyzed by turbidimetric inhibition immunoassay using commercial kits (Beckman Coulter HbA1c) on clinical chemistry analyzer (Beckman Coulter AU 680, Brea, CA, USA). Clinical guidelines recommend an HbA1c level of no more than 7% (53 mmol/mol) for optimal GC (17,18). Based on these data, HbA1c values of ≤7% (53 mmol/mol) were taken to be the optimal GC, and values >7% (53 mmol/mol) were considered poor GC.

Risk Behaviors Scale

The RBS has been developed by Gençtanırım and Ergene (19) to evaluate the risk behaviors of high-school students in Turkey. The RBS is a self-report scale with 36 five-point Likert items (5 = strongly agree, 4 = agree, 3 = partially agree, 2 = disagree, 1 = strongly disagree) where total score ranges from 36 to 180. Higher total scores indicate more risk behaviors. The RBS consists of six subscales: anti-social behaviors (I get involved in physical fighting, I get involved in bullying, I am teasing my friends, etc.), alcohol use (I drink alcohol when I want, I relax when I use alcohol, I drink alcohol to feel good, etc.), tobacco use (I smoke, I can get smokes if I want, my best friend smokes, etc.), suicidal tendency (I feel helpless in the face of problems, I feel pessimistic, I have low self-confidence, I wake up unhappy in the morning), eating habits (I like to eat junk food, I drink soft drinks every day, I eat mostly fast food, etc.), and school dropout (I think about dropping out of or taking a break from school, being successful in school does not help me, I want to work instead of going to school, etc.). High scores in each subscale indicate increased risk behaviors in a related area. Exploratory factor analysis of the RBS found item factor loads between 0.49 and 0.83. For reliability, the internal consistency coefficient (Cronbach alpha) was 0.91 for the whole scale and varied between 0.70 and 0.87 for the subscales.

Orthorexia Nervosa Scale

The ORTO-15 scale was created by Donini et al (20) and was adapted to Turkish as the ORTO-11 by Arusoğlu et al (21). ORTO-11 is a Likert-type scale that includes 11 items (items 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, and 14) from ORTO-15. The participants were asked to respond to ORTO-11 and to indicate how often the expression in each item identified themselves (always = 1, often = 2, sometimes = 3, never = 4). The total ORTO-11 score ranges from 11 to 44; a lower score indicates a tendency for OEB. The psychometric properties of ORTO-15 were investigated by factor analysis during its adaptation to Turkish; internal consistency coefficient was calculated to reveal factor loads varying between -0.44 and 0.69 for various items. Only the items with factor loads ≥0.50 were included in the Turkish version to generate an 11-item ORTO-11. The Cronbach's alpha calculated for 15 items was 0.44 while it was 0.62 for the 11 items included in the Turkish language version.

Statistical Analysis

The data were analyzed using SPSS software, version 23.0 for Windows (IBM Corp., Armonk, NY, USA). Descriptive statistics were presented as mean ± SD, frequency, and percentage. Chi-square test was used to analyze categorical variables. The normal distribution of variables was tested using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov or Shapiro-Wilk tests). Equality of variance was checked with the Levene test. Student's t-test was used to compare the groups, and Pearson correlation coefficient was used to analyze the relationship between the variables when the parametric test prerequisites were met; Mann-Whitney U test and Spearman correlation coefficient were used otherwise. For all tests, the significance level was set at $p < 0.05$.

Results

Of the 107 adolescents diagnosed with T1D, 46.7% (n = 50) were female, and 53.3% (n = 57) were male. Other demographic data are presented in Table 1.

There was no significant difference between total RBS scores of males and females ($p > 0.05$). Nonetheless, the alcohol use subscale score was significantly higher for males ($p = 0.003$). On the contrary, the eating habits subscale scores were significantly higher in females ($p = 0.029$).

Table 1. Demographic data of participants

	Mean ± SD
Age (years)	15.27 ± 1.7
Age of diagnosis (years)	9.16 ± 4.02
Duration of disease (years)	6.09 ± 3.98
BMI-SDS	-0.16 ± 0.67
BMI (kg/m^2)	20.65 ± 1.8
HbA1c (%)	8.8 ± 1.92

HbA1c: hemoglobin A1c, BMI-SDS: body mass index-standard deviation score, SD: standard deviation

There was no significant difference between males and females regarding other RBS subscale scores ($p > 0.05$). Total ORTO-11 score was significantly lower in females ($t = -2.255$, $p = 0.026$).

The percentage of those who had a positive response to the item exploring smoking habits (strongly agree or agree) was 8.5% (n = 9), of which 33.3% (n = 3) were female and 66.7% (n = 6) were male. The percentage of those who had a positive response to the item exploring alcohol use (strongly agree or agree) was 4.7% (n = 5), all of whom were males.

A significant correlation was found between HbA1c and total RBS, eating habits subscale, and suicidal tendency subscale scores. No significant correlation was found between HbA1c and ORTO-11 score ($p > 0.05$) (Table 2). There was also no significant correlation between ORTO-11 score and total RBS score or RBS subscales scores ($p > 0.05$).

Of the participants, 18.7% (n = 20) had an HbA1c of ≤7%; 81.3% (n = 87) had an HbA1c of >7%. A significant relationship was found between HbA1c and patients' age, patients' age at diagnosis, and duration of disease (Table 3).

Those with an HbA1c of >7% had significantly higher eating habits subscale, alcohol use, and tobacco use subscale scores ($p < 0.05$). There was no significant difference between those with high or low HbA1c regarding total RBS and other subscale scores (Table 4).

No significant difference was found between those with an HbA1c of >7% or ≤7% regarding gender distribution ($\chi^2 = 1.920$, $p > 0.05$).

Table 2. Correlation analysis between Risk Behavior Scale and Orthorexic Behavior Scale-11 scale scores and hemoglobin A1c

	HbA1c	
	r	p
Total RBS score	0.358	< 0.001**
Anti-social behaviors	0.098	0.320**
Alcohol use	-0.119	0.226**
Tobacco use	-0.085	0.391**
Suicidal tendency	0.228	0.019*
Eating habits	0.654	< 0.001*
School dropout	0.089	0.368**
ORTO-11 score	0.134	0.168*

*Pearson correlation coefficient was used.

**Spearman correlation coefficient was used.

RBS: Risk Behavior Scale, ORTO-11: Orthorexic Behavior Scale, HbA1c: hemoglobin A1c

Table 3. Age, age of diagnosis and duration of disease of participants according to hemoglobin A1c level

	HbA1c (%)	Mean ± SD (years)	p
Age	≤7	16.05 ± 1.66	0.020
	>7	15.12 ± 1.66	
Age of diagnosis	≤7	11.32 ± 5.00	0.012
	>7	8.53 ± 3.57	
Duration of disease	≤7	4.74 ± 4.75	0.042
	>7	6.41 ± 3.74	

HbA1c: hemoglobin A1c, SD: standard deviation

Table 4. Risk Behavior Scale total and subscale scores and Orthorexic Behavior Scale-11 scale score according to hemoglobin A1c level

	HbA1c (%)	Median (min.-max.)	p
Total RBS	≤7	52.5* (38-85)	0.076*
	>7	59* (36-168)	
Anti-social behaviours	≤7	10* (7-22)	0.266*
	>7	11* (7-32)	
Alcohol use	≤7	7* (7-17)	0.013*
	>7	7* (7-35)	
Tobacco use	≤7	7* (6-30)	0.030*
	>7	6* (5-32)	
Suicidal tendency	≤7	8* (4-16)	0.248*
	>7	10* (4-19)	
Eating habits	≤7	8.5* (5-15)	< 0.001*
	>7	15* (5-24)	
School dropout	≤7	7* (7-17)	0.606*
	>7	7* (6-35)	
ORTO-11	≤7	26** (16-35)	0.668**
	>7	26** (14-38)	

*Mann-Whitney U test

**Independent T test.

min.: minimum, max.: maximum, HbA1c: hemoglobin A1c, RBS: Risk Behavior Scale, ORTO-11: Orthorexic Behavior Scale

Among female participants, those with an HbA1c of >7% had significantly higher total RBS, suicidal tendency subscale, and eating habits subscale scores ($p < 0.05$). Among females, the ORTO-11 score was significantly lower for those with an HbA1c of >7% ($p < 0.05$). The ROC curve was calculated and used to determine whether the ORTO-11 score was a usable variable in determining the HbA1c value above 7% in the girls in our sample group. ROC analysis showed the area under the curve was low (AUC = 0.54), the likelihood ratio (LR) + value (LR + value = 1.8) was "weak" and the sensitivity of the test was low (20%).

Among male participants, those with an HbA1c of >7% had significantly higher eating habits and alcohol use subscale scores in RBS ($p < 0.05$) (Table 5).

Discussion

This study investigated, for the first time, the relationship between GC and the tendency for OEB among adolescents

Table 5. Risk Behavior Scale total and subscale scores and Orthorexic Behavior Scale-11 score according to gender, related to hemoglobin A1c level

	HbA1c (%)	Female		Male	
		Median (min.-max.)	p	Median (min.-max.)	p
Total RBS	≤7	44.00* (38-64)	0.005*	47.00* (36-84)	0.749*
	>7	60.5* (42-98)		55.00* (36-168)	
Anti-social behaviours	≤7	9.00* (7-17)	0.178*	10.00* (7-22)	0.755*
	>7	11.50* (7-24)		10.00* (7-32)	
Alcohol use	≤7	7.00* (7-14)	0.909*	7.00* (7-17)	0.008*
	>7	7.00* (7-17)		7.00* (7-35)	
Tobacco use	≤7	6.00* (6-16)	0.352*	10.00* (6-30)	0.056*
	>7	6.00* (6-32)		6.00* (5-27)	
Suicidal tendency	≤7	7.00* (4-14)	0.047*	8.00* (4-16)	0.812*
	>7	10.00* (4-18)		8.00* (4-19)	
Eating habits	≤7	7.00* (5-15)	<0.001*	8.00* (5-15)	<0.001*
	>7	16.00* (5-22)		15.00* (5-29)	
School dropout	≤7	7.00* (7-11)	0.551*	7.00* (7-17)	0.535*
	>7	7.00* (7-14)		7.00* (6-35)	
ORTO-11	≤7	26** (16-31)	0.031**	26** (18-35)	0.499**
	>7	25.5** (14-35)		27** (18-38)	

*Mann-Whitney U test.

**Independent t test.

min.: minimum, max.: maximum, HbA1c: hemoglobin A1c, RBS: Risk Behavior Scale, ORTO-11: Orthorexic Behavior Scale

with T1D. Poor GC was shown to be associated with a tendency for OEB among female adolescents with T1D. In addition, this study indicated that adolescents' risk behaviors and HbA1c were correlated. The suicidal tendency was higher among females with poor GC while alcohol use and tobacco use as risky behaviours were higher in males. As expected, unhealthy eating habits were found to be more common in patients with poor GC in both genders.

Results indicated that healthy eating habits should be emphasized for adolescents with T1D at each follow-up meeting. Unfortunately, there is a tendency to eat fewer fruits and vegetables and to eat unhealthy foods in all adolescents (22,23). Studies have shown that adolescents with T1D have similar eating behaviors to their peers (eating sweets, drinking carbonated drinks, and eating junk food) (24,25). Similar to this study, a review study recounted that adolescents with T1D often did not comply with their diets, and their GC was adversely affected (26). Clinical guidelines emphasize that adolescents with T1D should comply with their diet in order to have optimal metabolic control (17,18).

ON, or OEB, is defined as an obsession with healthy eating at a pathological level and has not yet met the criteria for an eating disorder. ON was first described by Bratman (27). Unlike other eating disorders, weight control is not the primary aim in OEB; however, over-occupation with healthy-eating practices is at a level that may impair one's health (28). Similar to the other DEBs, a tendency for OEB was significantly higher among females than males (29). Portion control and calorie restriction, which are often advised for adolescents, might have increased female adolescents' attention to nutritional issues. A previous study with 48 adolescents and young adults (aged 7-19 years) with T1D in Turkey indicated an increased tendency for OEB (30). We found that, among female adolescents with T1D, GC was found to be more impaired in those with a tendency for OEB; therefore, one cannot claim that they have healthier eating habits. A review study reported that ON was usually accompanied by psychopathology (31). In our study, no relationship was found between ORTO-11 scores and a suicidal tendency among female adolescents. ROC curve analysis was used to determine whether the ORTO-11 score was a usable variable in predicting poor GC in the girls in our sample group. As a result of this analysis, the area under the ROC curve was low, the LR + value was "weak" and the sensitivity of our test was low. For these reasons, it is concluded that ORTO-11 score is not a usable variable in determining the likelihood of an HbA1c value above 7. However, in our study, girls with poor GC tend to be more prone to OEB, suggesting that further investigation of this issue would be beneficial.

Among female adolescents with T1D, those with poor GC were found to have more suicidal tendencies. The suicidal tendency subscale of RBS includes the statements "I feel helpless in the face of problems", "I feel pessimistic", "I have low self-confidence", and "I wake up unhappy in the morning", which reflect depressive feelings and thoughts. Depressive mood and low self-esteem were associated with suicidal ideation. In previous studies, it was also reported that depressive symptoms in adolescents with T1D had negatively affected their GC (32,33). The results of our study were consistent with those findings. Studies have indicated that negative mood is more common in female adolescents with T1D compared to their male counterparts (34,35,36). However, contrary to these studies, no significant difference was found between the suicidal tendency subscale scores of male and female adolescents in our study.

When males and female were evaluated separately, the suicidal tendency of females with poor GC was higher than those with optimal GC, whereas such a difference was not observed in males. Similarly, it has previously been reported that, among adolescents with T1D who had negative mood, females had a worse GC than males (35). In the pediatric endocrinology department where this study was conducted, psychiatric consultation is requested for every patient with poorly controlled diabetes. The significant association between poor GC and suicidal tendency that was identified, despite the exclusion of patients with any psychiatric diagnosis, is remarkable. This finding may suggest that the resilience in the face of chronic disease and compliance to treatment were worse among our female patients.

Similar to previous studies (10,37,38), it was found that alcohol consumption and smoking in adolescents diagnosed with T1D had a negative effect on GC in our sample. In contrast, a previous Turkish study found no relationship between GC and alcohol consumption or smoking in another study with adolescents with T1D (39). In our study, the rates of smoking and alcohol consumption rates were much lower than the rates in the studies that found a relationship (10,37,38). The relationship between smoking and alcohol use and serious health outcomes of adolescents diagnosed with T1D is well-known. Alcohol consumption of adolescents with T1DM increases the risk of hospitalization due to ketoacidosis. Even in small amounts, it can increase the risk of hypoglycemia within 1-2 days (11,40,41). Smoking in adolescents with T1DM has been shown to impair blood sugar regulation. In addition, tobacco use will aggravate diabetes and tobacco-related complications over time (42).

The lack of alcohol use among female adolescents in our study was probably related to cultural factors. In Turkey, the rate of alcohol consumption is much lower among healthy

female adolescents than their male counterparts (43). The age range of adolescents with poor GC in our study corresponds to the beginning of middle adolescence and high school. The process of adaptation to new friends and social environment may have influenced their eating behavior and GC. Their compliance with treatment may improve after 1-2 years of getting used to their new school and peers. Perhaps due to the developmental characteristics of adolescence, our rate of impaired GC patients was high (81.3% of the cohort). Sociocultural factors other than school life, like the influence of family and peer relationships, may also have affected their behavior. Adolescents diagnosed with T1D at a younger age and had a long duration of disease were found to have worse GC. Metabolic control interventions should be performed differently depending on age, duration of disease and age of onset of disease.

Study Limitations

Our study has several limitations. Improper sexual behavior is an important risk behavior, and the lack of data on the sexual behavior of adolescents in our study was a significant limitation. One of the risks that affect GC is the inappropriate injection of insulin; lack of data on adolescents' compliance with insulin treatment was also a significant limitation. A review study indicated that OEN was accompanied by various obsessive disorders (31). Although adolescents with existing psychiatric diagnoses were not included in the study, we did not investigate the presence of obsessive disorders and other psychopathologies in our study.

Conclusion

In conclusion, this study has demonstrated the importance of considering the relationship between GC and risk behaviors in adolescents with T1D. Higher incidence of OEB in female participants with poor GC suggests that adolescents with T1D should be evaluated for nutritional habits, including an obsession with healthy eating. Evaluating nutritional habits according to their development, assessing depressive symptoms repeatedly, and asking about alcohol and tobacco use based on gender may contribute to disease management in adolescents with T1D who have poor GC. The fact that bad eating habits are one of the most critical problems of adolescents with T1D necessitates the further investigation of this issue. Future studies should address the incidence of OEB and the psychopathologies that may accompany OEB and influence GC in adolescents with T1D.

Ethics

Ethics Committe Approval: The study was approved by the Ethics Review Board of the University of Health Sciences Child Health and Diseases Hematology - Oncology Training and Research Hospital (protocol number: 2018-130).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Demet Taş, Eda Mengen, Pınar Kocaay, Seyit Ahmet Uçaktürk, Design: Demet Taş, Seyit Ahmet Uçaktürk, Data Collection and Processing: Eda Mengen, Pınar Kocaay, Demet Taş, Seyit Ahmet Uçaktürk, Analysis and Interpretation: Eda Mengen, Pınar Kocaay, Demet Taş, Seyit Ahmet Uçaktürk, Writing: Demet Taş, Seyit Ahmet Uçaktürk.

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Intramuscular Short-term ACTH Test for the Determination of Adrenal Function in Children: Safe, Effective and Reliable

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What is already known on this topic?

Intravenous (iv) short synacthen test has commonly been used to detect adrenal insufficiency (AI) in childhood. However, the iv form of adrenocorticotropic hormone (ACTH) is not readily available in all countries.

What this study adds?

The intramuscular (im) form of ACTH test can be used as an alternative to iv form to detect cases with AI. The im form of ACTH test is safe and reliable test for suspected AI in childhood.

Abstract

Objective: Standard short adrenocorticotropic hormone (ACTH) stimulation test (SST) has traditionally been used for assessing adrenal gland fuction by intravenous (iv) application. However the iv form is not readily available in all countries, including Turkey. The aim of this study was to evaluate the effectiveness of the intramuscular (im) SST.

Methods: Patients underwent im SST with suspected adrenal insufficiency (AI) and hyperandrogenism. The SSTs were done with 250 mcg ACTH (Synacthen Depot ampul, concentration 1 mg/mL). The cases were divided into two groups: suspected AI (group 1 n = 87); and hyperandrogenism group (group 2 n = 124). Definite AI was defined as peak cortisol < 18 µg/dL, suspected AI as a peak cortisol of 18-21 µg/dL and normal result was defined as a peak cortisol ≥22 µg/dL.

Results: The mean age of the patients was 11.7 ± 5.2 years. In 164 patients (78%) all of the peak cortisol tests were normal (≥22 mcg/dL). The rates were 64% and 88% in group 1 and 2, respectively. Only 8.5% (n = 18) of all cases had an inadequate peak cortisol response of < 18 mcg/dL. On follow up, 15 patients whose peak cortisol was < 18 mcg/dL needed cortisol therapy. Of all cases 3.3% (n = 8) had 17-OHP ≥10 ng/dL. Clinical findings suggestive of non-classical congenital adrenal hyperplasia and/or mutation were found in six of these cases (75%). No local and systemic side effects or allergic reactions were observed in any patient.

Conclusion: IM ACTH SST is a safe, effective and reliable test in children with suspected AI. There were no local or systemic side effects, supporting the reliability of the im ACTH test.

Keywords: Adrenal insufficiency, intramuscular ACTH, childhood, reliability

Introduction

The clinical presentation of adrenal insufficiency (AI) is very variable. Severe overt adrenal failure is usually relatively easy to diagnose; however milder AI may be much more clinically challenging. Thus dynamic tests are extremely important in detecting subtle (intermediate) AI. It has been recommended to perform dynamic test in every suspected case (1). Although the insulin tolerance test (ITT) has been used as a gold standard test for the diagnosis of AI, it is used with caution because of the risk of seizures in patients with epilepsy and possible morbidity, especially in infants and in patients with cardiovascular diseases (1,2).

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The ITT may be replaced by a short adrenocorticotropin hormone (ACTH) stimulation test (SST), due to a better safety profile. However the ACTH preparations used for an intravenous (iv) SST test are not readily available in Turkey and require an official application for prescribing of overseas medicine. Due to this limitation, use of the intramuscular (im) form, which is easily accessible and cheaper, has been used to replace the iv preparation in order to evaluate the adrenal axis of patients without overt AI. The easily available im-ACTH preparations in Turkey include the original molecule, tetracosactide, known as Synacthen® Depot, which contains 50 units/mL of ACTH (Maalincrodot Speciality Pharmaceuticals Ltd, Ireland Limited College Business & Technology park, Curiserath, Blanchardstown, Dublin 15, Ireland).

The aim of this study was to evaluate the effectiveness of the SST using this im preparation and to correlate the findings with the clinical and acquired peak responses and basal cortisol results.

Methods

The characteristics and data of the patients including age, gender, complaints, laboratory results and other demographics, undergoing im-ACTH test between 2010 and 2018 were extracted from the hospital records system and retrospectively analyzed. The inclusion criteria were patients aged <18 years who were tested because of suspected AI and all patients who were admitted with hyperandrogenism and underwent SST to investigate the possibility of non-classical congenital adrenal hyperplasia (NCCAH).

The cases were divided into two groups: suspected AI (group 1); and hyperandrogenism group (group 2). Group 1 consisted of cases that had AI with low basal cortisol and poor cortisol response to ITT. Group 2 had presented with findings of virilization, such as premature adrenarche, hirsutism and a clinical suspicion of NCCAH (see Figure 1).

The SSTs were performed with 250 mcg ACTH (Synacthen Depot ampoule, concentration 1 mg/mL) in the patients over two years of age. Blood samples were taken for measurement of cortisol, dehydroepiandrosterone sulfate (DHEAS) and 17-hydroxyprogesterone (17-OHP) at 0, 30 and 60 minutes after administration of the im-ACTH. Cortisol and DHEAS concentrations were analysed on a Beckman Coulter analyser (Beckman Coulter 250 S. Kraemer Blvd, Brea 92821, USA) by immunoassay method and 17-OHP concentration was measured by using a radioimmunoassay (DIA Source Immunoassay SA, Belgium). AI was defined as peak cortisol <18 µg/dL, suspected AI as a peak cortisol of 18-21 µg/dL and a normal result was defined as a peak cortisol ≥22 µg/dL. Genetic analysis for 21 hydroxylase gene mutations were performed in patients with a peak 17-OHP >10 ng/dL. Sensivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. In patients with a definitive diagnosis indicated by the results of the ACTH SST, findings during clinical follow-up were also investigated.

Statistical Analysis

Statistical Package for Social Sciences version 22.0 (IBM Inc., Chicago, IL, USA) was used for all statistical analyses. Descriptive statistical analysis of the data was performed. Data distribution was assessed for normality using the

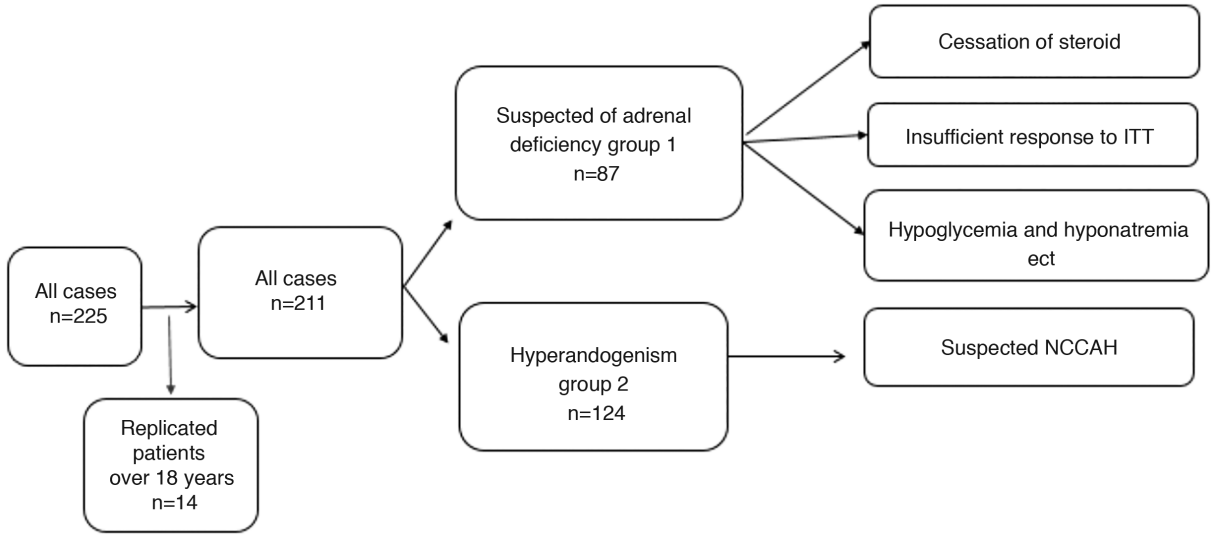

Figure 1. Classification of selected cases according to etiological factors

ITT: insuline tolerance test, NCCAH: non-classical congenital hyperplasia

Shapiro-Wilk test. Data distribution was non-parametric so groups were compared with Mann-Whitney U test. A $p < 0.05$ was considered significant.

The study was approved by the Ethics Committee of Ankara University, with the decision number: 02-139-19.

Results

Over the study period 225 patients who had undergone SST were evaluated. Fourteen patients were excluded because of duplicated patients or being aged over 18 years old. Thus the final number of study patients was 211. The mean age of the patients was 11.7 ± 5.2 years and 69.2% were female. Of the 211 patients, 87 had been assessed for suspected AI (group 1) while 124 patients had been assessed for hyperandrogenism (group 2). In 164 patients (78%) all of the peak cortisol tests were normal that is ≥22 mcg/dL (equivalent to ≥600 nmol/L). This proportion was 64% and 88% in group 1 and 2 respectively. In 29 (13.7%) patients the peak cortisol was 18-21 µg/dL (equivalent to 500-599.9 nmol/L). Only 8.5% (n = 18) of all cases had had an inadequate peak cortisol response of < 18 µg/dL (equivalent to < 500 nmol/L). Age of 11 patients were under 2 years. Their test endications were (one patient can have 2 indications for test) 5% of all patients. The distrubution was similar for two groups

Group 1 results: In group 1, peak cortisol response was < 18 mcg/dL in 15 (16%) patients, 18-21 µg/dL in 19 (21%) patients and ≥22 mcg/dL in 53 (60%) patients (Table 1). Of the patients treated in group 1, nine had pituitary insufficiency and three had developed AI after cessation of steroid therapy.

Three of the patients with a borderline peak cortisol of 18-21 µg/dL were diagnosed as multiple pituitary hormone deficiency (MPHD) and received treatment. Peak cortisol response was < 19 µg/dL in a further three. In the remaining 16 patients, peak cortisol response was 19- < 22 µg/dL and these patients were followed without treatment since there was no clinical finding suggesting cortisol replacement would be appropriate (see Figure 2).

Group 2 results: In group 2, peak cortisol response was < 18 µg/dL in three (3%) patients, 18-21 µg/dL in 10 (8%) and ≥22 µg/dL in 111 (60%) patients (Table 1). Three of the patients treated in group 2 with peak cortisol < 18 µg/dL were diagnosed with 17 hydroxylase deficiency and two patients had multiple organ deficiency.

Multiple organ dysfunction syndrome is altered organ function in an acutely ill patient requiring medical intervention to achieve homeostasis. One of the patients with a peak cortisol of 18-21 µg/dL was diagnosed as NCCAH. No patient was detected with a peak cortisol response above 19 mcg/dL who also exhibited clinical signs of AI. On follow up, no patient with a peak corisol of > 19 mcg/dL after im-ACTH test required treatment.

A total of 24 patients with a peak cortisol < 18 mcg/dL after ITT, were evaluated with im-ACTH test and four of 24 (16.7%) patients showed an inadequate cortisol response and treatment was started. Five patients had a peak cortisol response of 18-21 µg/dL but none of them had any clinical

Table 1. Demographic and biochemical characteristics of patients with intramuscular-adrenocorticotropic hormone test

	Group 1 (Suspected adrenal insufficiency) (n = 87)	Group 2 (Hyperandrogenism) (n = 124)	p
Decimal age (years)*	10 ± 5.4 (0.14-18.9)	12.9 ± 4.7 (0.32-23.8)	< 0.05
Sex (female/male)	44/43	102/22	
Basal cortisol (µg/dL)*	8.6 ± 4.2 (0.1-19)	15.9 ± 7.4 (3.9-52)	< 0.05
Stimulated cortisol (µg/dL)*	15.9 ± 7.4 (3.9-52)	29.3 ± 7.2 (3-53)	< 0.05
Peak cortisol			
< 18 µg/dL (n = 18)	16% (n = 15)	3% (n = 3)	
18- < 22 µg/dL (n = 29)	21% (n = 19)	8% (n = 10)	
≥22 µg/dL (n = 164)	60% (n = 53)	89% (n = 111)	
Basal 17-OHP (ng/dL)*	0.86 ± 0.9 (0.1-5)	0.86 ± 0.9 (0.1-5)	< 0.05
Stimulated 17-OHP (ng/dL)*	2.7 ± 1.7 (0.42-9.44)	5.1 ± 6.7 (1-50)	
Requiring treatment	18% (n = 16)	6% (n = 7)	
Not on treatment	84% (n = 71)	94% (n = 117)	

*: $p < 0.05$.

17-OHP: 17-hydroxyprogesterone

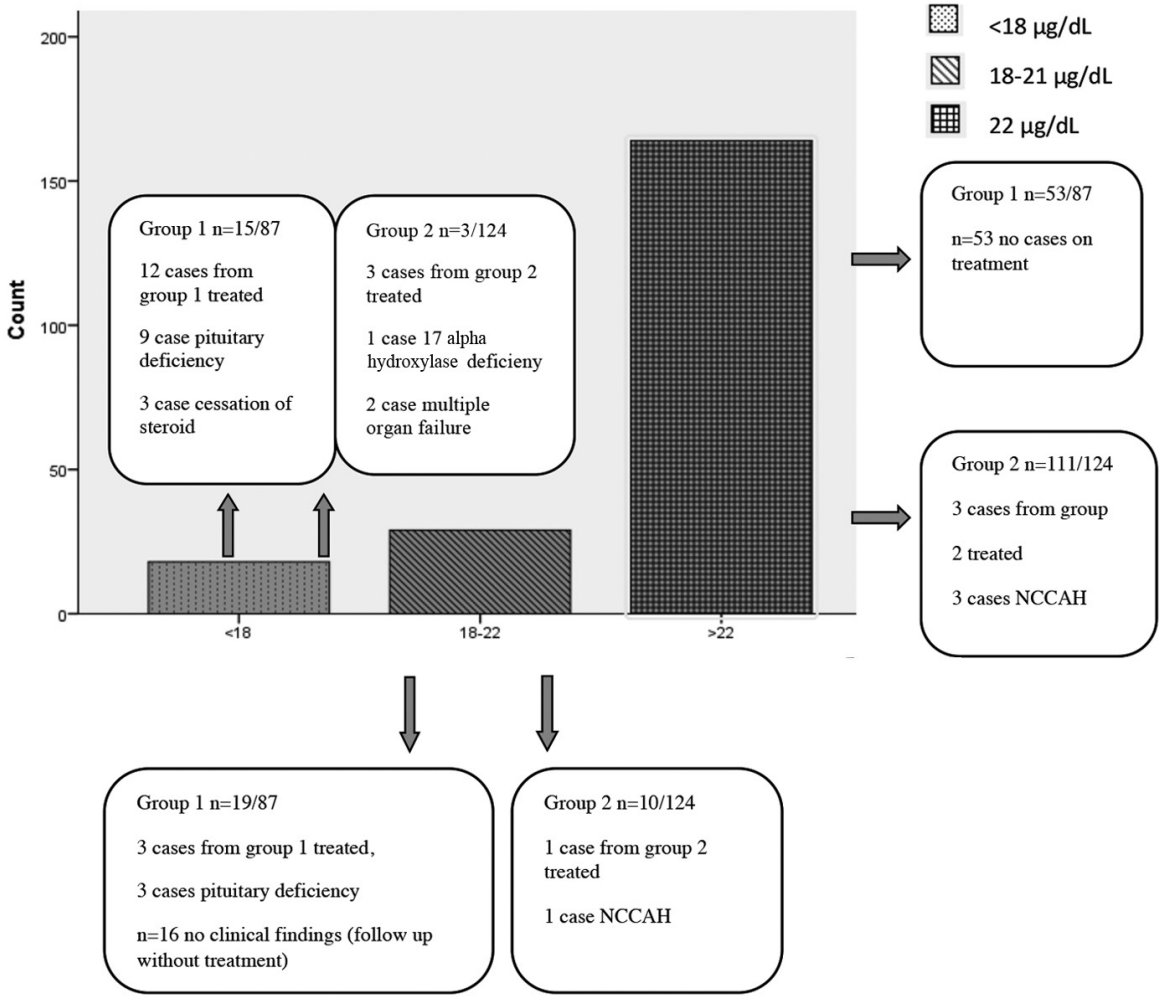

Figure 2. Number and treatment status of all cases according to peak cortisol level

ITT: insuline tolerance test, NCCAH: non-classical congenital adrenal hyperplasia

findings and/or risk factors for AI. Fifteen patients had a peak cortisol level ≥22 µg/dL. When the gold standard was taken as clinical diagnosis; when a peak cortisol <18 µg/dL was used to define AI the following results were obtained: sensitivity 93%; specificity 99%; PPV 93%; and NPV 99%. Similarly, when AI was defined as peak cortisol <22 µg/dL the same parameters were: sensitivity 100%; specificity 86%; PPV 40%; and NPV 100% (see Table 2).

On follow up, 15 patients needed cortisol therapy whose peak cortisol was <18 µg/dL. In the whole cohort 3.3% (n=8) had 17-OHP ≥10 ng/dL. NCCAH clinic findings and/or mutation were found in six of these cases. The clinical findings and the test results of the cases were consistent. Mutations were detected in the 21 hydroxylase gene in 6 of 8 (75%) patients who were diagnosed as NCCAH with 17-OHP >10 ng/mL in group 2 as follows: *V281L* mutation in four, Intron 2 mutation in two, and the nine most common mutations were not present in the remaining two. No local and systemic side effects or allergic reactions were observed in any patient undergoing im-ACTH test.

Discussion

There are no studies comparing im and iv ACTH tests for the detection of AI in childhood. The aim of this study was to assess the effectiveness of the im-ACTH test due to difficulty in obtaining iv-ACTH preparations in Turkey.

Due to limited access to IV Synacthen® (Novartis® Pharma AG 250 mcg/mL) clinicians have to make the diagnosis of AI with basal cortisol and ACTH. Although suspected cases are evaluated with basal ACTH and morning cortisol, this does not always provide a definitive diagnosis. The sensitivity of

Table 2. Threshold value after intramuscular-adrenocorticotropic hormone in patients with insulin tolerance test and peak cortisol < 18 µg/dL

	Adrenal insufficiency	No adrenal insufficiency
im-ACTH		
< 18 µg/dL	14	1
< 22 µg/dL	17	25
≥18 µg/dL	1	163
≥22 µg/dL	0	163

When peak cortisol < 18 µg/dL was used to define adrenal insufficiency; sensivity 93%, specificity 99%, positive predictive value (PPV) 93%, negative predictive value (NPV) 99%.

When peak cortisol < 22 µg/dL was used to define adrenal insufficiency; sensivity 100%, specificity 86%, PPV 40%, NPV 100%.

basal cortisol for the diagnosis of AI is only 60% (2). There are a number of reasons for this. These include immaturity of the hypothalamo-pituitary-adrenal (HPA) axis, very young patients, innaccurate sampling times for the morning cortisol which, because of diurinal cortisol variation, should be performed at a consistent time and thus differences between studies can be difficult to reconcile.

There are some adult studies describing assessment of the HPA axis with im-ACTH due to difficulties in obtainig the iv form and the poor reliability of the diagnosis of AI based on basal cortisol values. The efficacy of im-ACTH has been evaluated in detecting both primary and central AI cases in India (3). Gundgurthi et al (3), performed STT tests with im-ACTH (25 U im preformed with Acton). Two groups were identified; the validation group which included healthy volunteers, diabetes mellitus, hypothyroidism and AI and the study group, which included all patients who were tested because of a suspicion of AI. When the basal cortisol level was less than 3 µg/dL, it was diagnostic, but in the study, only 40% of those diagnosed with AI had basal cortisol levels below 3 µg/dL. In seven cases with a baseline cortisol value < 3 µg/dL, the delta, defined as the difference between basal and peak cortisol in the test, increased by 7 µg/dL on stimulation. In eight cases, there was less increase but basal cortisol level was found to be > 3 µg/dL. Subnormal increases have a similar sensivity to basal cortisol and have lower specificity and PPV. The authors concluded that the diagnosis of AI with im-ACTH form was superior to basal cortisol values and the HPA axis could be assessed with these tests in many health institutions. In addition, it was suggested that the im-ACTH test may be preferred because it was cheap and easily accessible.

Among our cases, 11 cases with basal cortisol ≤3 µg/dL indicating suspicion of AI were found. In only six of these eleven cases, peak cortisol value was < 18 µg/dL and only seven cases required treatment.

Although there are limited studies in children, there are studies investigating the effectiveness of im and iv forms of ACTH in adults. Kelestimur et al (4) evaluated twenty healthy adults who were tested with IV Synacthen® and two weeks later with Depot Synacthene R. Thus the authors evaluated peak cortisol acquisition times and cortisol increase rates in the same volunteers using both iv and im forms of ACTH. Samples were taken at 0, 30, 60, 90 and 120 minutes and the time to obtain cortisol increase and peak response were similar. They concluded that depot forms of ACTH for SST may successfully replace iv Synacthen®. In 90% of cases tested with iv Synacthen®, peak responses were obtained ≥60 minutes and with the im form peak responses were detected at ≤90 minutes. When the peak response was taken as 22 µg/dL, the peak response was obtained in 95% of the patients who received both the iv and im form at 90 minutes or before. The only difference was that peak responses were concentrated at 30 minutes with the iv form and at 60 minutes using the im form. Thus, regardless of the form of synacthen® used, there was no need to prolong the test to 120 minutes or more. In our study, blood was taken at 0, 30 and 60 minutes with the im form and peak responses were reached at 60 minutes. In our study, sampling was stopped after the 60 minute sample. We did not take a blood sample after 60 minutes.

Data obtained from adult studies are similar. Women of reproductive age (n = 29) were given ACTH, either iv or im, and cortisol and androgen precursors were tested and no difference was found between the stimulated peaks obtained with the two forms (5). In another study, using im and iv forms, cortisol increase was compared with serum levels every 15 minutes and peak cortisol levels were obtained for both iv and im-ACTH forms at 60 minutes and the two forms were reported to be no different (6).

There are also studies attempting to determine the lowest effective dose of im-ACTH for the stimulation of the HPA axis. In one study, 21 healthy volunteers, nine primary adrenal AI and ten secondary AI were given im-ACTH (250

μg/mL Synacthen® NovartisR Pharma AG) at a standard dose of 250 μg im into the deltoid muscle and subsequently blood, saliva cortisol and aldosterone concentrations were measured (7). Standard dose was used for the study because it was conducted in adults.

In this study the doses were varied and titrated so that 12.5, 25 and 250 μg doses were compared. In healthy humans, 30 minute cortisol response was the same after 25 and 250 μg im while 12.5 μg was not sufficient to detect AI. Neither did the 30 minute salivary cortisol concentration differ between the 25 and 250 μg im injections. However, there was no correlation between salivary and blood cortisol levels. The responses were similar with no change in serum and salivary cortisol levels after low (25 μg) and high dose (250 μg) im ACTH in patients with AI.

The effectiveness of the im-ACTH test in detecting central AI cases has also been investigated (8). Cases with short stature and at risk of having multiple hormone deficiencies and tested with ITT were evaluated. Twenty cases were identified because of ITT peak cortisol below <18 μg/dL. All cases who responded poorly to ITT were re-evaluated one week later with 25 U ACTH and cortisol responses in both tests were compared. Although the peak responses were similar, an adequate response was obtained by im ACTH in six cases although there was insufficient response by ITT. This was attributed to the injection of ACTH at supraphysiological doses. A further finding of this study was that when the cut-off value was taken as 18 mcg/dL, sensivity was 57% with a specificity of 94% whereas the sensitivity increases to 100% when the cut-off was 22 μg/dL although the specificity fell to 75%. Thus the diagnosis of AI with a peak cortisol <18 μg/dL by im ACTH was robust whereas high reliability was ruled out at peak values above 22 μg/dL. In our cohort, in 24 cases with ITT cortisol response <18 mcg/dL, when the im ACTH test was done subsequently, the peak response was between 18-21 μg/dL in five patients and above 22 mcg/dL in 15 of them (it means that five patients have 22 and above 22) Only four patients underwent hydrocortisone replacement. There are two possible explanations. Firstly, the dose used for im ACTH may be a supraphysiological dose for central AI detection. Secondly, there was no central AI in any of the patients with a peak cortisol level <18 mcg/dL by ITT (9). It is known that the ITT response is age-variable and threshold values are not standard for all ages. This is supported by the finding that higher peak values were obtained in healthy children under the age of 12 years (9). Our second hypothesis supports the lack of AI findings in any of our patients who had insufficient response with ITT and had adequate response with im ACTH test.

Study Limitation

The most important limitation of our study was that retest with iv ACTH could not be performed to evaluate the correlation with the im form of ACTH in our patients.

Conclusion

In conclusion we have shown that the im form of ACTH test in children with suspected AI is a safe, effective and reliable test. None of the patients with peak cortisol levels ≥22 μg/dL were found to have AI on long-term follow-up. In contrast, peak responses <18 μg/dL were diagnosed as AI. Thus a peak response above 22 μg/dL excluded the diagnosis of AI.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Ankara University with decision number: 02-139-19.

Informed Consent: It was not taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Merih Berberoğlu, Zeynep Şıklar, Concept: Merih Berberoğlu, Zeynep Şıklar, Design: Merih Berberoğlu, Zeynep Şıklar, Data Collection or Processing: Elif Özsu, Esra Bilici, Ayşegül Ceran, Rukiye Uyanık, Analysis or Interpretation: Zeynep Şıklar, Zehra Aycan, Elif Özsu, Literature Search: Elif Özsu, Esra Bilici, Ayşegül Ceran, Rukiye Uyanık, Tuğba Çetin, Writing: Merih Berberoğlu, Zeynep Şıklar, Elif Özsu.

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ORIGINAL ARTICLE

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Vitamin D Status Across Age Groups in Turkey: Results of 108,742 Samples from a Single Laboratory

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What is already known on this topic?

The extra-skeletal effects of vitamin D have caused an increase in interest in vitamin D. This interest has led to an increase in requests for serum 25-hydroxyvitamin D (25-OHD) levels. Previous reports have suggested a high prevalence of vitamin D deficiency in Turkey. Furthermore, high dose vitamin D treatment has been prescribed, even in cases with normal 25-OHD levels, in order to obtain some of the extra-skeletal effects of vitamin D.

What this study adds?

This study with a very large sample size aimed to assess vitamin D status in Turkey. Data from this study do not support the idea of a vitamin D deficiency pandemic in Turkey. Successful implementation of the vitamin D supplementation program appears to have overcome vitamin D deficiency for those under 1-year of age in Turkey

Abstract

Objective: The aim was to determine vitamin D status in the general population in Turkey between 2011 and 2016, and to evaluate the effectiveness of the national vitamin D supplementation programme.

Methods: Serum 25-hydroxyvitamin D (25-OHD) measurement data were retrieved from an internationally accredited laboratory, operating nationwide. A total of 108,742 measurements of 25-OHD were analyzed using the cut-off values of 0-11 ng/mL, 12-19 ng/mL, 20-49 ng/mL, 50-70 ng/mL and >70 ng/mL for vitamin D deficiency, insufficiency, sufficiency, possibly harmful and excess respectively.

Results: The mean ± standard deviation 25-OHD level was 21.6 ± 13.3 ng/mL. Mean 25-OHD concentrations by age groups were: 37.3 ng/mL, 30.1 ng/mL and 23.7 ng/mL for <1, 1-10 and 11-18 year old groups, respectively. Mean 25-OHD levels of children <1 year and 1-3 years of age were significantly higher than those found in other age groups. The prevalence of vitamin D deficiency (<12 ng/mL) was lowest in children at 1-3 years of age (5%). In subjects older than 18 years of age, mean 25-OHD levels were 18.2 ng/mL, 20.1 ng/mL, 21.9 ng/mL and 21.1 ng/mL for age groups 19-30, 31-50, 51-70 and >70 years, respectively.

Conclusion: Successful implementation of the national vitamin D supplementation programme, appears to have nearly eliminated vitamin D deficiency for children under 1-years of age. However, the positive impact of the vitamin D supplementation diminishes as children get older suggesting that supplementation may be required in the older children and adults. In addition, improved awareness of the benefits and risks of excess vitamin D should prevent unnecessary and excessive use of vitamin D supplements.

Keywords: Vitamin D, deficiency, National Prophylaxis Program, 1 year of age

Introduction

In the past 10 years, there has been growing interest in vitamin D deficiency, especially concerning its extra-skeletal effects. Serum 25-hydroxyvitamin D (25-OHD) measurements have become a part of routine health evaluations in both pediatric and adult populations, and more importantly, claims about a "vitamin D deficiency pandemic" have generated considerable debate associated with diverse definitions of deficiency (1). According to a recently published study evaluating data from 711,718 children from Britain, the incidence of vitamin D deficiency increased from 3.14/100,000 in 2000 to 261/100,000 in

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2014, amounting to a 15-fold increase after adjusting for population increases (2).

In Turkey, data are lacking on annual changes in the incidence of vitamin D deficiency. According to the "Intercontinental Marketing Services Health" database, in 2012, a total of 2,280,626 ampoules of vitamin D (containing 300,000 international units per ampoule) were sold in Turkey, with about 4 times increase to 8,376,319 ampoules in the first eight months of 2016. This is despite a nationwide vitamin-D-prophylaxis programme, including free provision of vitamin D drops (400 international units daily) to infants, which has successfully operated since 2005 (3).

This population-based study aimed to determine the vitamin D status in Turkey between 2011 and 2016, evaluate the effectiveness of the vitamin D supplementation programme which started in 2005, analyze the relationships among simultaneous serum measurements of the 25-OHD, parathyroid hormone (PTH) and alkaline phosphatase (ALP), and determine the prevalence of vitamin D deficiency by age, gender, year, region and season.

Methods

Data on serum 25-OHD measurements between January 2011 and December 2016 were retrieved from an internationally accredited private laboratory operating nationwide in Turkey.

No ethical approval was sought as this study was a retrospective study of previously collected data. In addition no consent form was issued although approval from the owners of the data was obtained.

Serum 25-OHD concentrations were measured with the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, Quattro Premier QE (Waters, MA, USA), as the sum of D2 and D3 levels. After adding internal standard (Deuterated stable isotope) to the calibrators (Chromsystems 3PLUS1 multilevel Serum calibrator set 25-OH-VITAMIN D3 and D2), controls (Chromsystems Level 1/2 25-OH-VITAMIN D3 and D2) and samples, extraction with hexane is performed. After extraction, the extracts dissolved in 70% methanol are injected into the LCMS/MS device. Acquity BEH 2.1x50 mm C8 1.7 µm column was used in the analysis. The measurement range of LC-MS/MS method for 25-OHD concentration was 2.5-100 ng/mL. Serum PTH concentrations were measured by an electrochemiluminescence immunoassay (ECLIA) and serum ALP concentrations were measured by a colorimetric assay using the Roche Cobas e601 and Cobas c501 modules (Roche Diagnostics, Mannheim, Germany), respectively, in accordance with the International Federation of Clinical Chemistry (IFCC) standardization.

A total of 108,742 random measurements of 25-OHD were analyzed according to age, gender, region, season and year, using the cut-off values of 0-11 ng/mL, 12-19 ng/mL, 20-49 ng/mL, 50-70 ng/mL and >70 ng/mL from the 2001-2006 data of the National Health and Nutrition Examination Survey (NHANES) (4). According to the Global Consensus Recommendations, a serum 25-OHD concentration of less than 12 ng/mL was considered to be diagnostic for vitamin D deficiency, while concentrations between 12-20 ng/mL were considered vitamin D insufficiency, and concentrations above 20 ng/mL but less than 50 ng/mL were considered normal (5). In addition, 25-OHD concentrations greater than 50 ng/mL were defined as 'possibly dangerous' according to the recommendations of the Centers for Disease Control and Prevention (CDC) (4). Of the total sample of 108,742, 17% (n=18,613) were samples obtained from patients younger than 18 years of age. Repeat measurements obtained from the same individuals at different times were excluded from the study.

The data were compared with those of the NHANES 2001-2006 reports. Moreover, correlations between 25-OHD and ALP or PTH were assessed when simultaneous ALP (n=10,139) or PTH (n=4,916) measurements were available.

Statistical Analysis

The statistical analysis was performed using R program. Outliers were discarded using Tukey's method with boundaries 2.5 times the interquartile range (IQR), which amounted to 25-OHD values between 0.25 ng/mL and 74.5 ng/mL. A total of 2040 observations were discarded, all of which were between 74.5 ng/mL and 1590 ng/mL. The Wilcoxon test was used for between-group comparisons of 25-OHD. The Kruskal-Wallis test followed by the Conover post-hoc test was used for multiple comparisons. The Spearman's correlation coefficient was used for bivariate correlations, and multiple comparisons were corrected using Bonferonni's method.

Results

The number of 25-OHD measurements was similar across years, with a female predominance (72.9%). Overall, the mean ± standard deviation 25-OHD concentration was 21.6 ± 13.3 ng/mL, being lower in women (21 ± 13.5 ng/mL) than in men (23.2 ± 12.5 ng/mL) ($p < 0.001$). The mean 25-OHD concentrations according to age groups were as follows: 37.2 ng/mL for <1 year of age, 27.1 ng/mL for 1-10

years of age, 19.2 ng/mL for 11-18 years of age, 18.25 ng/mL for 19-30 years of age, 20.1 ng/mL for 31-50 years of age, 21.9 ng/mL for 51-70 years of age, and 21.1 ng/mL for >70 years of age. The mean 25-OHD levels among children <1 year of age and 1-10 years of age were significantly higher compared with those found for the other age groups ($p < 0.001$). According to seasonal distribution, 25-OHD concentrations were higher in summer (23.8 ng/mL) and fall (24.5 ng/mL) than those measured in winter (19.1 ng/mL) and spring (19.6 ng/mL) ($p < 0.001$). Serum 25-OHD levels according to years, seasons, geographic regions, age groups, and gender are shown in Table 1 and Figure 1.

The prevalence of vitamin D deficiency (<12 ng/mL) was lowest in children under 1 year of age (7%) and 1-10 years of age (8%), while the highest rates of prevalence were noted in women (30%), in those 19-30 years of age (36%),

Table 1. The percentages of serum 25-hydroxyvitamin D levels according to age groups, gender, geographic regions, seasons, and years

25-OHD level intervals (ng/mL)						25-OHD (ng/mL) Mean ± SD (min-max)	Measurements (n)
	0-11	12-19	20-49	50-70	>70		
Age groups (year)							
<1	7	6	66	19	1	[a]37.2 ± 15.8 (2.5-74.3)	2903
1-10	8	22	67	4	0	[a]27 ± 11.7 (1-74.3)	10331
11-18	26	32	41	1	0	19.1 ± 10.4 (1-72.9)	5379
19-30	36	28	34	2	0	18.2 ± 11.8 (2-74.2)	11831
31-50	30	26	41	3	0	20.1 ± 12.5 (0.25-74.4)	36946
51-70	26	23	46	4	0	21.9 ± 13.4 (1.8-74.4)	30675
>70	33	19	43	4	0	21.1 ± 14.2 (1.8-74.5)	10677
Overall	27	24	45	4	0	21.6 ± 13.2 (0.25-74.5)	108742
Female	30	23	43	4	0	[b]21 ± 13.4 (0.25-74.5)	79293
Male	18	27	50	4	0	23.2 ± 12.5 (1-74.4)	29449
Regional distribution							
Central Anatolia region	24	26	47	3	0	21.9 ± 12.7	51754
Aegean region	22	22	51	4	0	23.5 ± 13.7	14566
Marmara region	30	22	44	4	0	21.6 ± 14	10420
Mediterranean region	26	23	47	4	0	22.2 ± 13.7	14289
Eastern Anatolia region	39	20	37	4	0	19.5 ± 13.9	376
Black Sea region	42	24	31	3	0	17.6 ± 12.7	8488
Southeastern Anatolia region	36	23	38	3	0	19.4 ± 13.4	8849
Seasonal distribution							
Spring	34	26	36	4	0	19.6 ± 13.3	29108
Summer	17	23	55	4	0	[c]24.5 ± 13.2	24384
Autumn	18	23	55	4	0	[c]23.8 ± 12.5	26813
Winter	36	25	36	3	0	19.1 ± 12.9	28437
Years							
2011	25	27	47	4	0	22.3 ± 13.4	16884
2012	33	24	39	3	0	19.8 ± 13.1	15827
2013	29	24	44	3	0	20.9 ± 13.1	20130
2014	29	24	44	4	0	21.2 ± 13.3	21076
2015	25	24	47	4	0	22.5 ± 13.4	16897
2016	21	25	50	4	0	22.9 ± 12.9	17928

25-OHD: 25-hydroxyvitamin D, min-max: minimum-maximum, SD: standard deviation.

[a]The mean 25-OHD levels among children <1 year of age and 1-3 years of age were significantly higher compared with those found for the other age groups ($p < 0.001$).

[b]The mean 25-OHD level was significantly lower in women than in men ($p < 0.001$).

[c]25-OHD levels were significantly higher in summer and fall than those measured in winter and spring ($p < 0.001$).

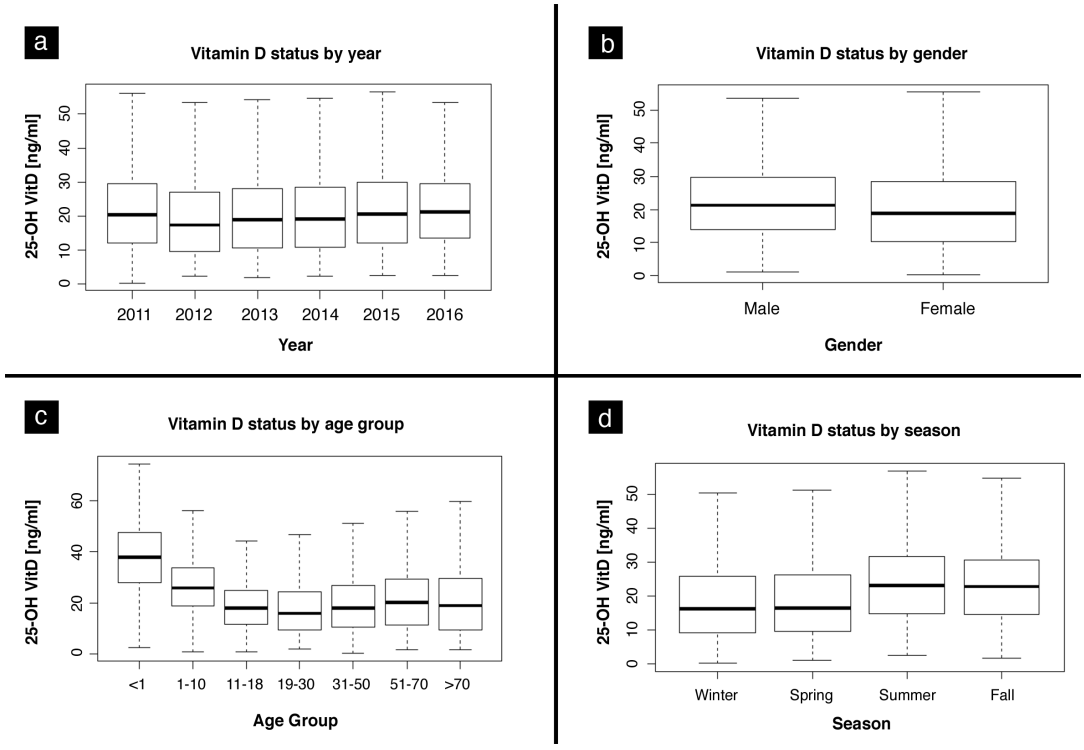

Figure 1. Distribution of serum 25-OHD levels according to years (a), gender (b), age groups (c), and seasons (d) (ng/mL)

25-OHD: 25-hydroxyvitamin D, VitD: vitamin D

in the Black Sea region (42%), and in winter (36%). The prevalence rates of vitamin D deficiency and insufficiency according to age groups and seasonal distribution are shown in Figure 2.

For the youngest age group (< 1 year), the prevalence of vitamin D deficiency was highest in the Mediterranean (15%), Black Sea (14%) and Southeast (15%) regions (Figure 3). For the youngest age groups (< 1 year), the rates of deficiency and insufficiency showed decreases over the years and were around 3-4% in 2015 and 2016 (Figure 4). When 25-OHD levels were classified according to the Endocrine Society's guideline (6) recommending cut-off levels of < 20, 20-30 and 30-100 ng/mL for deficiency, insufficiency and sufficiency, respectively, the rate of sufficiency was still highest in the youngest age group (Table 2).

ALP was simultaneously measured in 9.3%, and PTH was measured in 4.5% of the subjects undergoing serum 25-OHD measurements making correlation analyses possible. In correlation analysis, 25-OHD showed an inverse correlation with PTH in those under 1 year of age ($r = -0.28$, $p < 0.001$), and those between 11 and 18 years of age ($r = -0.38$, $p < 0.001$). It was shown that elevated PTH consistently dropped to a plateau when serum 25-OHD was at 20 ng/mL or higher overall (Figure 5). Correlation of serum 25-OHD

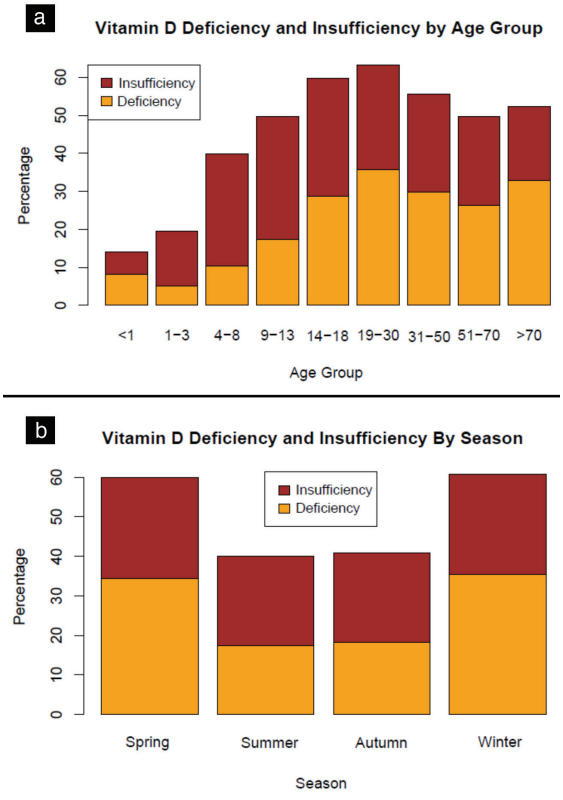

Figure 2. The prevalence rates of vitamin D deficiency and insufficiency according to age groups (a) and seasonal distribution (b) (%)

concentrations with ALP was significant only in the adult population (> 18 years) (r = -0.095538, p = 0.015). The mean PTH level was significantly elevated in the group with 25-OHD concentrations of 0-20 ng/mL (p < 0.0001). However, the mean ALP level was similar across the different vitamin D status groups (p > 0.05). Serum concentrations of ALP and PTH according to vitamin D status are shown in Table 3.

Discussion

Notwithstanding the fact that the present data are from subjects whose clinical features are unknown, 25-OHD measurements indicate the expected differences between genders and seasons in this cohort. More importantly, these results show the beneficial effects of the vitamin D supplementation programme in the 0-1 years-old age group in Turkey, serum 25-OHD levels being highest in the youngest age group, making these data reliable for evaluation. Furthermore, a high data count of 108,742 strengthens the statistical power of the study.

These findings demonstrate a relatively stable number of vitamin D test requests from physicians between 2011 to 2016. It is notable that physicians usually ordered 25-OHD tests without simultaneous ALP or PTH measurements. The growing interest in measuring and use of vitamin D supplementation mainly stems from concerns for the extra-skeletal effects of vitamin D and controversies on the thresholds of serum 25-OHD (7,8). Based on our

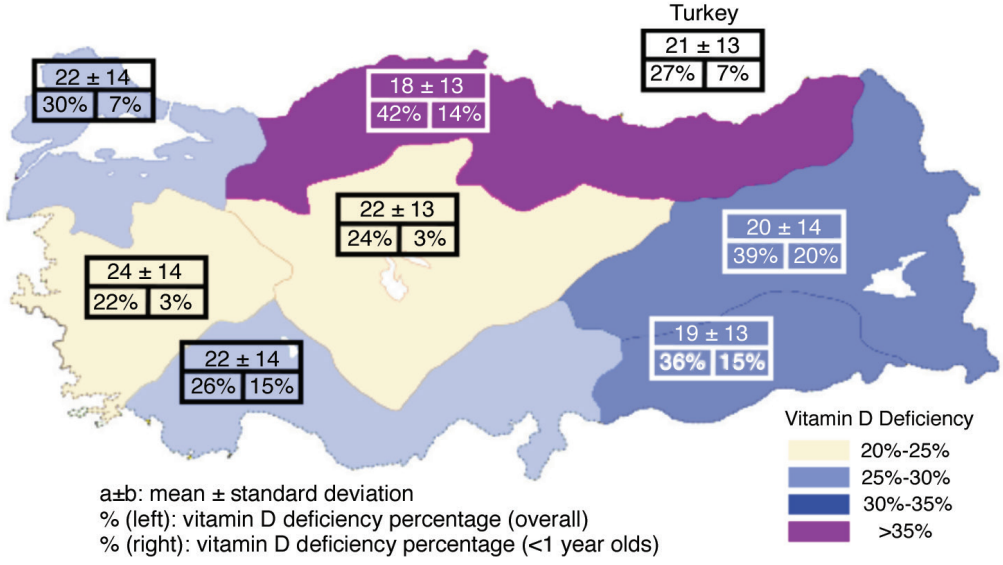

Figure 3. Regional distribution of the mean 25-hydroxyvitamin D levels (ng/mL) and the frequencies of vitamin D deficiency (overall and for < 1 year of age) (%)

Table 2. The percentages of vitamin D deficiency, insufficiency and sufficiency based on the Endocrine Society's recommendations in different age groups

25-OHD level intervals (ng/mL)	0-20	21-29	30-100
Age groups (year)			
< 1	14	16	70
1-10	29	35	36
11-18	58	30	12
19-30	63	23	14
31-50	56	26	18
51-70	50	27	23
> 70	52	23	24

25-OHD: 25-hydroxyvitamin D

Table 3. Comparison of the mean serum 25-hydroxyvitamin D levels in our study and the National Health and Nutrition Examination Surveys 2001-2006 according to age groups

Age groups	Mean 25-OHD level (ng/mL) (Turkey)	NHANES 2001-2006 the 50 percentile of 25-OHD levels (ng/mL)
< 1	37.26 ± 15.82	Not available
1-3	30.12 ± 11.95	27.8
4-8	23.70 ± 10.49	26.9
9-13	21.07 ± 9.86	24.8
14-18	18.75 ± 10.68	23.2
19-30	18.25 ± 11.83	22.2
31-50	20.17 ± 12.57	22.9
51-70	21.99 ± 13.47	23.2
> 70	21.11 ± 14.24	22.8

NHANES: National Health and Nutrition Examination Surveys, 25-OHD: 25-hydroxyvitamin D

observations, we think that, due to this interest, physicians often order a 25-OHD test as part of the routine health assessment, especially in women, although it is unlikely that a vitamin D deficiency-related clinical problem was the source of the request. This is the case not only for adults but also for children. In order to prevent unnecessary vitamin D testing in primary care, the Turkish Ministry of Health vitamin D Scientific Board published a guideline about the indications of vitamin D testing (9). In the UK, a 17-fold increase in vitamin D testing and prescription costs, from £1,647 in 2008 to £28,913 in 2014 per 100,000 patient-years has been reported (10), perhaps increased testing leads a dramatic increase in the diagnosis of vitamin D deficiency as well. The overvaluing of 25-OHD measurements without accompanying ALP or PTH measurements (11) and diagnosing 'vitamin D deficiency' based on diverse thresholds have resulted in an increased use of high-dose vitamin D (12). However, the Institute of Medicine has emphasized that studies concerning vitamin D and extra-skeletal problems, such as cancer, cardiovascular diseases, diabetes and autoimmune diseases, do not provide consistent evidence and that there is no need to determine varying vitamin D thresholds for these diseases, and thus to prescribe more vitamin D (7,8,13).

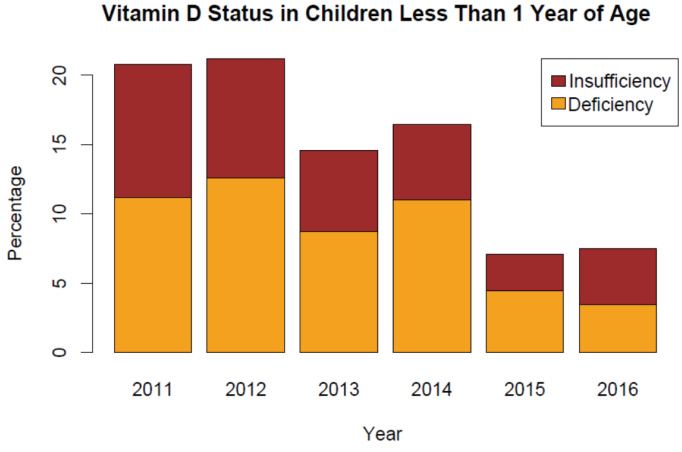

Figure 4. The prevalence rates of vitamin D deficiency and insufficiency for the youngest age group (< 1 year) according to years

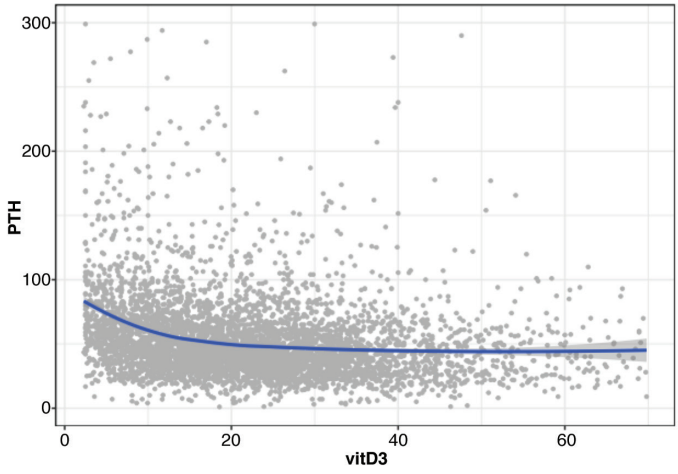

Figure 5. Relation between serum 25-hydroxyvitamin D concentrations and mean (±SE) serum concentrations of parathyroid hormone

SE: standard error, vitD: vitamin D, PTH: parathyroid hormone

Table 4. Mean serum levels of alkaline phosphatase and parathyroid hormone according to vitamin D status

25-OHD level (ng/mL)	ALP (IU/L)	PTH (pg/mL)
0-20	82 ± 68	**63.1 ± 61[a]**
20-30	90.4 ± 102	49.5 ± 53
30-50	109.2 ± 213	45 ± 28
50-70	111 ± 155	48.1 ± 48
> 70	89.2 ± 62.4	44.7 ± 31.7

PTH: parathyroid hormone, ALP: alkaline phosphatase, 25-OHD: 25-hydroxyvitamin D

[a]The mean PTH level was significantly elevated in the group with 25-OHD level of 0-20 ng/mL ($p < 0.0001$).

In the present study, the mean 25-OHD concentrations found for diverse age groups correspond to those reported in the NHANES 2001-2006 data (Table 4) (4). This similarity may be related to the fact that the samples were obtained from a private laboratory, serving people with relatively high socio-economic status. Nevertheless, when the same thresholds are used, a notably higher prevalence of vitamin D deficiency in Turkey becomes apparent (27% vs. 8%), mostly owing to adults with Vitamin D deficiency, which might be related to less exposure to sunshine due to clothing styles and less intake of dairy products in Turkey. Moreover, vitamin D insufficiency still appeared to be a health issue, particularly among the adults. The prevalence rates of vitamin D deficiency and insufficiency were found to be 27% and 24%, respectively, with increasing rates in the less economically developed regions such as Eastern Anatolia (39%) and Southeastern Anatolia (36%), and in the Black Sea region (42%) which has a relatively low number of sunny days, which supports that hypothesis. However, a very recent retrospective study (14) from the western part of Turkey showed the prevalence of vitamin D insufficiency and deficiency among children to be 21.3% and 44.8%, respectively. Although these proportions are higher than those in our study, the highest proportion having normal vitamin D status was found in the 0-1 years of age group, in keeping with our results.

Another interesting finding was that a 25-OHD level of greater than 50 ng/mL, which the CDC defines as "possibly harmful", was found in 4% of subjects compared with 1%

in the United States. Although we do not have definitive data to account for this discrepancy, this might be related to the recently increased use of vitamin D ampoules in Turkey which contain 300,000 IU of vitamin D per ampoule, instead of lower strength vitamin D preparations, such as drops. The reasoning for the increased use of ampoules is more rapid correction of vitamin D deficiency. Recently, a regulation was implemented by health authorities so that obtaining vitamin D in ampoule form requires prescription from a doctor, which might decrease hypervitaminosis D cases.

The most important finding of the present study is that in Turkey, 25-OHD levels are higher in the first year of life, with significantly lower rates of vitamin D deficiency and insufficiency compared with the other age groups. This favors the vitamin D supplementation programme, launched in 2005, for provision of free vitamin D supplementation to all newborn infants until 1 year of age at a daily dose of 400 U. This programme has been closely supervised by the Ministry of Health, and according to the most recent data, 96.6% of all newborn babies were given vitamin D drops. However, apart from the high success rate of this programme, the prevalence rates of vitamin D deficiency for those younger than 1 year of age still remain above 10% in the Eastern, Southeastern, Black Sea and Mediterranean regions, suggesting that more emphasis be placed on regional differences.

Several recommendations have been made for successful implementation of vitamin D supplementation programmes, which include informing families from birth, providing vitamin D support to all infants, providing vitamin D free of charge, and utilizing the family physician system to monitor usage of vitamin D (15). Turkey is among the European countries with at least 80% compliance with vitamin D supplementation and supervision programme and compared with practices in the UK, the active role of the Ministry of Health is of particular importance for the continuation of the program (15,16). However, despite the high success rate of the programme, some problems still remain. As high as 47% of pediatricians recommend or prescribe vitamin D at the end of the second week, and 19.9% discontinue vitamin D due to reasons such as the presence of a small fontanelle (17). According to Global Consensus recommendations, efforts should be continued to provide 400 international units of vitamin D to all newborns starting from the first day of life, regardless of nutritional status (5,18).

As expected, 25-OHD levels showed inverse correlation with PTH and ALP levels but this was most prominent after 14-19 years of age. ALP levels were not correlated with 25-OHD levels in the pediatric age group, possibly owing to a wide reference range and dynamic temporal changes in ALP concentrations that occur with age during infancy and childhood (19).

Study Limitations

The data presented in this study were from a private laboratory, which may be a somewhat inappropriate sample to represent a nationwide study. The other limitations were the lack of data regarding the time of day the samples were taken, clinical status, lifestyle and vitamin D intake. The low rate of concurrent PTH and ALP measurement caused difficulties in interpreting the severity of vitamin D deficiency. Despite these limitations, due to the high number of measurements, presence of samples from all regions of the country, and the fact that results show expected differences between genders, seasons and age groups, the authors feel that the present study provides important information on, and relevant insight into, vitamin D status in Turkey.

Conclusion

In conclusion, with the successful implementation of the vitamin D supplementation programme, Turkey seems to have overcome vitamin D deficiency for those under 1 year of age. However, the positive impact of the program does not continue beyond the first year of life, indicating that vitamin D supplementation may be required in older children and adults. On the other hand, evidence of unnecessary and excessive use of vitamin D supplements is of concern and increased awareness about excess as well as deficient vitamin D levels is also required.

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Ethics

Ethics Committee Approval: No ethical approval was sought as this study was a retrospective study of previously collected data. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent: As this is a retrospective study and it is based on recorded data.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Şükrü Hatun, Design: Şükrü Hatun, Data Collection or Processing: Ekin Deniz Aksu, Analysis or Interpretation: Ekin Deniz Aksu, Literature Search: Şükrü Hatun, Gül Yeşiltepe-Mutlu, Writing: Şükrü Hatun, Gül Yeşiltepe Mutlu, Abdullah Bereket.

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The Results of 16 Years of Iodization: Assessment of Iodine Deficiency Among School-age Children in Antalya, Turkey

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What is already known on this topic?

Despite highly effective salt iodization programs initiated by the World Health Organization, iodine deficiency continues to be a problem in some areas around the world.

What this study adds?

This study demonstrates that the 16-years iodization program allowed Antalya to become an iodine sufficient region with 174.69 (119.17-242.83) µg/L median (interquartile range) urinary iodine concentrations.

Abstract

Objective: Iodine deficiency (ID) continues to be a problem around the world. This study investigated the prevalence of ID and goiter among school-age children in the city center of Antalya, Turkey. The aim was to investigate the effect of an iodization program, which had been running for sixteen years, on nutritional iodine status in this population.

Methods: A total of 1,594 school children, aged 6-14 years, were included in this cross-sectional study. ID was evaluated based on median [interquartile range (IQR)] urine iodine/creatine (UI/Cr) (µg/g) ratio and median (IQR) UI concentrations (UIC) (µg/L). UICs were measured using the Sandell-Kolthoff method. Goiter was determined by palpation and staged according to World Health Organization classification.

Results: Median (IQR) UIC was found to be 174.69 (119.17-242.83) µg/L, and UIC was found to be lower than 50 µg/L in 6.5% of the population. The median UI/Cr ratio increased from 62.3 to 163.3 µg/g and goiter rates had decreased from 34% to 0.3% over the 16 years of the program. However, 19% were still classified as ID (mild, moderate or severe) and, furthermore, 11.5% were classified as excessive iodine intake.

Conclusion: Comparison of two cross-sectional studies, carried out 16-years apart, showed that Antalya is no longer an ID region. However, surveillance should be continued and the percentage of ID and iodine excess individuals in the population should be monitored to avoid emerging problems.

Keywords: Iodine deficiency, prevalence, school-age children, Turkey

Introduction

Iodine is an essential trace mineral required for the synthesis of thyroid hormones that play a critical role in normal growth and neurodevelopment in fetal life, infancy and childhood. Iodine deficiency (ID) causes a wide spectrum of pathologies throughout human development, from fetal life into adulthood. The clinical manifestations of ID disorders (IDD) include miscarriage, stillbirth, neurologic and myxomatous cretinism, goiter, hypothyroidism, mental

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retardation, intellectual impairment and impaired physical development (1,2).

Despite the significant increase in the number of iodine-sufficient countries as a result of the universal, largely effective salt iodization programs initiated by World Health Organization (WHO) and the International Council on Iodine Deficiency Disorders (ICCIDD) in 1990, in 2012 insufficient iodine intake was still reported in 29.8% (246 million) of school-age children (SAC) globally (3).

Knowledge of the prevalence and degree of ID is important, in that it provides information about the effectiveness of iodine prophylaxis in the population. The best indicators of iodine nutrition (IN) in a population are the percentage of households using adequately iodized salt, the prevalence of goiter and the median urinary iodine (UI) concentration (UIC) among SAC or pregnant women (4).

The table salt iodization program was launched officially in Turkey in 1998. Erdoğan et al (5) reported a 31.8% goiter rate and a 58% moderate to severe ID in the Turkish population prior to mandatory iodization. Eight years after mandatory iodization, Erdoğan et al (6) reported that 27.8% of the population continued to suffer from moderate to severe ID and ID remained a serious public health problem in Turkey. Erdoğan et al (7) have suggested that more than a decade of iodine prophylaxis would be needed to eradicate goiter in a moderately iodine-deficient region.

To the best of our knowledge, the only study of community-based ID and the prevalence of goiter in Antalya, Turkey was conducted by Semiz et al (8) in 1999, prior to the salt iodization program. Semiz et al (8) reported mild-to-moderate ID in Antalya, Turkey with a 34% prevalence of goiter, and a median UI/creatine (UI/Cr) ratio of 62.3 (10.7-136.9) µg/g among SAC.

The aim in the present study was to evaluate ID, as determined by UI levels, and to evaluate the prevalence of goiter through physical palpation, in the city center SAC population of Antalya. The results were then compared with those of the study conducted by Semiz et al (8). In this way, the IN status of the population 16 years after the launch of mandatory salt iodization was evaluated.

Methods

A cross-sectional study was carried out between May and June 2015 and included 58 schools of the total 124 (46.8%) schools in the city center of Antalya, Turkey. For the study, 1,700 school children aged 6-14 years were selected from the total of 61,092 students attending the schools, using a probability proportional to population size (9) cluster sampling method. After excluding 108 participants with chronic disease, regular medication use or malnutrition, or those who could not provide urine samples under appropriate conditions, a total of 1,594 students were included in the study. None of the participants had been exposed to gadolinium, iodine or barium containing contrast material in the 96 hours prior to sample collection.

Written permission was taken from the Antalya Provincial Directorate of Health and the Antalya Province National Education Directorate. The study was approved by the Ethics Committee of Akdeniz University (decision no: 269 of 27.05.2015) and was supported by the Akdeniz University Scientific Research Projects Coordination Unit (project number: TSA-2016-962). Informed consent was obtained from the participants and their parents.

Early-morning spot urine samples were collected, ensuring no contamination, and goiter staging was assessed according to the WHO classification (grade 0: no goiter; grade 1: thyroid palpable but not visible; grade 2: thyroid visible with neck in normal position) by a pediatric endocrinologist. Urine samples were collected in deiodized test tubes, and were transferred immediately to the laboratory where they were stored at -20 °C.

The median UI/Cr (µg/g) and the median UIC (µg/L) were used in order to compare our results with the previous study conducted by Semiz et al (8) in Antalya and with other studies in literature, respectively. Severe ID was defined as median UIC < 20 µg/L, moderate ID as 20-49 µg/L and mild ID as 50-99 µg/L. Optimal IN was defined as median UIC 100-199 µg/L, more IN than required as 200-299 µg/L and excessive IN as > 300 µg/L (4).

Iodine levels in the urine were measured using the Sandell-Kolthoff method, as recommended by WHO and ICCIDD (4). Urine creatinine levels were measured spectrophotometrically using the Jaffe colorimetric method.

Statistical Analysis

The data was analyzed using Statistical Package for Social Sciences software, version 22 (IBM Inc., Chicago, Ill., USA). The values in the text are presented as median, and interquartile range (25^{th} and 75^{th} percentiles; IQR), unless otherwise stated. The statistical analysis was performed using parametric (Student's t-tests) or nonparametric (Mann-Whitney U tests) tests, when appropriate. Values of $p < 0.05$ were accepted as statistically significant.

Results

A total of 1,594 students, 839 male (52.6%) and 755 (47.4%) female, were included in the study. The mean ± standard deviation age of the participants was 10.6 ± 2.5 years.

The median (IQR) UIC was found to be 174.69 (119.17-242.83) µg/L among school children aged 6-14 years in the city center of Antalya, Turkey. The UIC was lower than 50 µg/L in 6.5% of the sample. The percentages of participants with mild, moderate, and severe ID and the IN status are shown in Table 1. Stage 1 goiter was detected in only five students (0.3%) upon physical examination.

When UI/Cr was evaluated for a comparison of the results with the 1999 study, the median (IQR) was found to be 163.3 (105.3-254.8) µg/g, and the UI/Cr ratio was found to be lower than 50 µg/g in 4.4% of the population using this method. The results of the comparison of the two cross-sectional studies carried out 16 years apart, that is before and after mandatory salt iodization, are presented in Table 2.

Discussion

In the most recent cross-sectional study it was shown that 16 years after mandatory introduction of table salt iodization, Antalya has become an iodine-sufficient region, with a median (IQR) UIC of 174.69 (119.17-242.83 µg/L) among SAC. The UI/Cr ratio increased from 62.3 to 163.3 µg/g, the proportion of the population with below 50 µg/L UIC was found to be only 6.5% and the goiter rate decreased from 34% to 0.3% in 16 years (Table 1, 2). Accordingly, the WHO targets of median UIC between 100 µg/L and 299 µg/L among SAC, not more than 20% of UIC samples below 50 µg/L and total goiter rate below 5%, were met in the region (4).

Despite these positive results, as is the case in all iodine-sufficient countries, Turkey is suffering from some ongoing and newly emerging problems that must be taken into careful consideration. As more than 90% of dietary iodine is excreted in the urine, UIC is an useful biomarker of the current iodine intake of the population, and is the main indicator in assessments of the iodine status of a population (4,10). However, it is known that UIC can show high intra-individual variability, which may lead to reports of high percentages of individuals with inadequate IN in iodine-sufficient populations where most of the iodine intake comes from iodized salt (3). In our study, although the median UIC was 174.69 µg/L, 19% of the population was classified as ID (mild, moderate or severe) (Table 1). Prevalence assessments from 2012 estimate that 75% of the children who are affected by low iodine intake live in iodine sufficient countries (3,11). Gordon et al (12) found that iodine supplementation improves cognitive function, even in mildly iodine-deficient children, and so the iodine sufficient countries should also monitor the percentage of affected individuals.

The second problem is that, although the iodine status of SAC is generally representative of the adult population, this does not hold true for pregnant women and newborns. In a recent study, Erdoğan et al (7) reported a median UIC of 117 µg/L and goiter prevalence of 1.3% among SAC in Ankara, Turkey's capital. Over a similar period, Oguz Kutlu and Kara (13) reported a median UIC of 80.5 µg/L and goiter prevalence of 15.4% among pregnant women in Ankara, and indicated that UIC was below 150 µg/L in 72.8% of pregnant women. In a recent large survey, the median UIC was found to be 73 µg/L among pregnant women in an iodine-sufficient metropolitan city. UIC was found to be below 50 µg/L in 36.6% of pregnant women and below 150 µg/L in 90.7% (14). There have been numerous studies to date highlighting the presence of ID in pregnant women and newborns in various countries where iodine appears to be adequate (15-17). These studies show that national iodization programs do not meet the increased iodine requirements of pregnant women. Accordingly, even iodine-sufficient countries should periodically screen these at-risk groups. In addition, there is a need for

Table 1. The distribution of iodine deficiency and iodine nutrition status in the city center of Antalya, Turkey

	Median UIC (µg/L)	n	%
Severe ID	< 20	38	2.4
Moderate ID	20-49	66	4.1
Mild ID	50-99	199	12.5
Optimal IN	100-199	654	40.5
More than required	200-299	454	28.1
Excessive IN	> 300	183	11.5

ID: iodine deficiency, IN: iodine nutrition, UIC: urinary iodine concentration

Table 2. Comparison of two cross-sectional studies, carried out one year and 16-years after introduction of a national salt iodization programme, in Antalya, Turkey

	1999*	2015	p
Median UI/Cr (µg/g)	62.3 (10.7-136.9)	163.3 (105.3-254.8)	**0.0001**
The UI/Cr < 50 µg/g	20% of population	4.4% of population	**0.0001**
Goiter prevalence	34%	0.3%	**0.0001**

*Ref. 8.

UI/Cr: urine iodine/creatine

mandatory iodine supplementation programs for pregnant and lactating women.

Another population vulnerable to ID are those people living in rural areas. In the 2009 survey, Erdoğan et al (6) found a significant difference among rural and urban areas in Turkey. There are many studies highlighting the difference in prevalence of ID levels in rural areas (18,19). Therefore, it should be noted that this study does not reflect iodine status of rural populations in Antalya.

In societies where iodine intake is now sufficient, the fourth, newly emerging problem is excessive iodine intake (median UIC greater than 300 µg/L). Recent national surveys found excessive iodine intake in 10 countries around the world (4). This is reflected in the increase in number of studies investigating excessive iodine intake. Katagiri et al (20) showed in a meta-analysis that chronic exposure to excess iodine is a risk factor for hypothyroidism. The mechanism behind hypothyroidism is thought to involve an adaptation of the thyroid gland to excess iodine uptake (21,22). In China, the prevalence of subclinical hypothyroidism and thyroid nodules was found to be 20% and 15.5%, respectively, in areas with excess iodine intake, although the prevalence of subclinical and overt hyperthyroidism in the ID group was higher than in the excess iodine group (23). It has been shown that, after the start of mandatory salt iodization, the incidence of autoimmune thyroid disorder has increased in many countries (24,25). In the present study, UICs were 200-299 µg/L (more than required) in 28.1% of the population and were >300 µg/L (excessive IN) in 11.5%. Accordingly, surveillance studies should be continued in iodine sufficient regions to prevent the growth of excess iodine as a problem.

Measuring the ratio of UI to creatinine for population screening is expensive, and can be misleading in some cases, such as in the presence of malnutrition, and so is no longer recommended (4,26). In the present study, however, in order to be comparable with a previous study conducted in Antalya, we evaluated both UI/Cr ratios as well as UICs.

Study Limitations

In our study, we detected goiter in 0.3% of the population upon physical examination. Although palpation is the traditional diagnostic method, the sensitivity and specificity of palpation are poor in mild to moderate ID areas. Percentages may be higher when thyroid size is measured by ultrasound. The strength of this study was its detailed demonstration of the outcome of 16 years of mandatory salt iodization in the same geographical region.

Conclusion

Our study shows that as a result of the application of an effective salt iodization program 16 years previously, Antalya is now an iodine sufficient region. Surveillance studies should be continued in SAC and in at-risk groups to ensure adequate iodine intake. The levels of ID and excess iodine intake in the population should be carefully monitored to avoid newly emerging problems.

Ethics

Ethics Committee Approval: The Ethics Committee of Akdeniz University (decision no: 269 of 27.05.2015).

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Gamze Çelmeli, Yusuf Çürek, Zümrüt Arslan Gülten, Concept: Gamze Çelmeli, İkbal Özen Küçükçetin, Sema Akçurin, Design: Gamze Çelmeli, Yusuf Çürek, Sema Akçurin, İffet Bircan, Data Collection or Processing: Gamze Çelmeli, Yusuf Çürek, Zümrüt Arslan Gülten, Analysis or Interpretation: Gamze Çelmeli, İkbal Özen Küçükçetin, Sebahat Özdem, İffet Bircan, Literature Search: Gamze Çelmeli, İkbal Özen Küçükçetin Zümrüt Arslan Gülten, Sebahat Özdem, Writing: Gamze Çelmeli, Sebahat Özdem, Sema Akçurin, İffet Bircan.

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Mutations Within the Transcription Factor *PROP1* in a Cohort of Turkish Patients with Combined Pituitary Hormone Deficiency

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What is already known on this topic?

The *PROP1* gene product is a critical transcription factor for development and maintenance of proper functioning of the anterior pituitary gland. To date, *PROP1* gene mutations are reported to be the most frequent genetic aetiology of combined pituitary hormone deficiency (CPHD) and these mutations are associated with progressive anterior pituitary hormone deficiencies.

What this study adds?

The frequency of *PROP1* gene mutations in a Turkish cohort of CPHD patients is reported. Pathogenic mutations were detected in 11 of 57 (19.3%) patients and gross deletions were present. A novel variant was discovered in two siblings. Clinical patient characteristics and treatment responses are also described.

Abstract

Objective: Mutations of the genes encoding transcription factors which play important roles in pituitary morphogenesis, differentiation and maturation may lead to combined pituitary hormone deficiency (CPHD). *PROP1* gene mutations are reported as the most frequent genetic aetiology of CHPD. The aim of this study was to describe the phenotypes of Turkish CPHD patients and define the frequency of *PROP1* mutations.

Methods: Fifty-seven CPHD patients from 50 families were screened for *PROP1* mutations. The patients were affected by growth hormone (GH) and additional anterior pituitary hormone deficiencies.

Results: All patients had GH deficiency. In addition, 98.2% had central hypothyroidism, 45.6% had hypogonadotropic hypogonadism, 43.8% had adrenocorticotropic hormone deficiency and 7.1% had prolactin deficiency. Parental consanguinity rate was 50.9% and 14 cases were familial. Mean height standard deviation score (SDS) and weight SDS were -3.8 ± 1.4 and -3.1 ± 2.0, respectively. Of 53 patients with available pituitary imaging, 32 (60.4%) showed abnormalities. None had extra-pituitary abnormalities. Eight index patients had *PROP1* gene mutations. Five sporadic patients were homozygous for c.301_302delAG (p.Leu102CysfsTer8) mutation, two siblings had exon 2 deletion, two siblings had complete gene deletion and two siblings were homozygous for the novel c.353A > G (p.Q118R) mutation. The frequency of the *PROP1* mutations was 16% in our cohort. Mutation rate was significantly higher in familial cases compared to sporadic cases (42.8% vs 11.6%; $p < 0.01$).

Conclusion: Phenotype of patients regarding hormonal deficiencies, pituitary morphology, presence of extra-pituitary findings, family history of CPHD and parental consanguinity are important for deciding which pituitary transcription factor deficiency should be investigated. *PROP1* mutation frequencies vary in different populations and its prevalence is high in Turkish CPHD patients.

Keywords: Combined pituitary hormone deficiency, hypopituitarism, pituitary transcription factors, *PROP1* gene

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Introduction

Combined pituitary hormone deficiency (CPHD) is defined as deficiencies of growth hormone (GH), thyroid-stimulating hormone (TSH), the gonadotropins-luteinizing hormone (LH) and follicle-stimulating hormone (FSH), prolactin (PRL) and adrenocorticotropic hormone (ACTH). Worldwide prevalence of CPHD is estimated as 1/8000 (1).

Both in human and mice, pituitary organogenesis and maintenance of its proper functioning necessitates the appropriate expression of a cascade of signalling molecules and transcription factors which are crucial for organ commitment, cell proliferation, patterning and terminal differentiation (2,3,4).

The genes that are related to these transcription factors are *PROP1*, *POU1F1 (PIT1)*, *LHX3*, *LHX4*, and *HESX1*. In 1998, Wu et al (5) identified homozygous or compound heterozygous inactivating mutations of *PROP1* gene as being associated with CPHD. To date, *PROP1* mutations are the most commonly reported genetic aetiology of CPHD in humans (4,5,6). Prophet of PIT-1 *(PROP1)* is a paired-like homeobox 1 gene, located on chromosome 5q35.3 and consists of three exons encoding for a 226-amino acid protein which is a late-expressed transcription factor (4). Mutations of *PROP1* gene cause autosomal recessively inherited CPHD and clinical phenotypes include GH, TSH, FSH/LH, PRL and rarely ACTH deficiencies together with morphological pituitary anomalies (4,7).

Phenotypes associated with *PROP1* gene mutations can be highly variable. Deficiencies of all pituitary hormones may be present with varying severity and at different ages. However, in all cases anterior pituitary function deteriorates over time (4,8). TSH and GH deficiencies have a tendency to occur in early childhood, whereas gonadotropin and corticotropin deficiencies manifest later in life (4,8). As *PROP1* is a "later-acting transcription factor", extra-pituitary manifestations are not observed (8). Magnetic resonance imaging (MRI) of the anterior pituitary gland shows normal or enlarged gland in early stages and pituitary involution in later stages whilst size and location of the posterior pituitary is normal and pituitary stalk interruption is not observed (1,4). Rarely, pituitary masses associated with *PROP1* gene mutations are reported (4,9,10). Point mutations, small and large deletions and insertions in the *PROP1* gene have been reported but there are no associations between any specific variants and specific regions or ethnicities (11,12,13).

The aim of this study was to define patient characteristics and to identify *PROP1* gene mutations in our CHPD patient cohort.

Methods

Study Design and Patient Selection

This retrospective cohort study was conducted in Çukurova University Research and Education Hospital including 57 patients with combined anterior pituitary hormone deficiency who attended the hospital between January 1997 and August 2019. Exclusion criteria for the patients were isolated GH deficiency (GHD), brain tumour, central nervous system surgery, cranial-neck irradiation, systemic chronic illnesses or chromosomal abnormalities.

Patients who were diagnosed with CPHD were analysed for *PROP1* mutations. The patients included in the study were all affected by GHD and at least one additional anterior pituitary hormone deficiencies including TSH, gonadotropins, ACTH or PRL. Diagnosis was based on clinical, laboratory and imaging investigations. Serum GH, insulin-like growth factor-1 (IGF-1), IGF-binding protein-3 and plasma ACTH concentrations were analysed by commercial kits using Siemens immulite 2000 immunoassay system and FSH, LH, oestradiol, cortisol, TSH, free thyroxine and PRL were analysed by commercial kits using Beckman Coulter Unicel Dxl 800 immunoassay system based on electrochemiluminescence immunoassay.

Genomic DNA was isolated from peripheral leucocytes. *PROP1* gene (transcript ID: ENST00000308304.2 and protein ID: O75360) was screened by polymerase chain reaction (PCR) amplifications of exons and neighbouring intronic regions. The PCR products were purified and directly sequenced using the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif., USA) in an ABI PRISM 3130 automatic sequencer (PE Applied Biosystems, Foster City, Calif., USA). DNA sequence data analyses were evaluated with DNA Sequencing Analysis Software-Sequencher 5.0 programme (http://genecodes.com/). All of the variants were investigated using 1000 genomes browser database (http://browser.1000genomes.org/index.html) and the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/clinvar) as to whether they were novel or previously reported. Subsequently, mutant variants were interpreted by *in silico* prediction tools such as Mutation Taster, SIFT and PolyPhen-2 (14,15,16).

Segregation analysis was performed only for the family of patients with the novel variant. It was not possible to test the parents of the patients with known pathogenic variants due to financial limitations.

PCR amplification of certain exons of the *PROP1* gene failed for initial DNA samples obtained from nine patients. Four

patients from two families gave consent for further testing and multiplex ligation dependent probe amplification (MLPA) assays were performed only for these patients. The other five patients were not included in the calculation of mutation frequency.

Patients from the same family are indicated with the same superscript letter.

The Ethics Committee of the Çukurova University Faculty of Medicine approved this study (approval: #TF2013LTP24), and written informed consent was obtained for each patient or from their legal guardians.

Statistical Analysis

Data obtained from this study were analysed using SPSS statistical software, version 23.0 (IBM Inc., Armonk, NY, USA). The distribution of data was evaluated with the Kolmogorov-Smirnov test. For numerical comparisons, the independent sample t-test or Mann-Whitney U tests were used for parametric and non-parametric distribution of the measured parameters, as appropriate. Descriptive statistics which were not normally distributed were presented as median and range. Frequency distributions and percentages were given for categorical variables.

Results

All 57 of the patients included to the study were affected by GHD and diagnosed in childhood. In addition, 56 patients (98.2%) had central hypothyroidism, 26 (45.6%) had hypogonadotropic hypogonadism, 25 (43.8%) had ACTH deficiency and four (7.1%) had PRL deficiency. More than two-thirds of the patients were male (68.4%). Median age age at diagnosis was 7.7 years (range: 3 months-19.8 years). Mean delay in bone age at diagnosis was 3.3 ± 2.4 years. More than half of the patients (n = 29; 50.9%) had parental consanguinity and 14 patients were familial cases. There was no history of perinatal asphyxia or difficult birth. None of the patients had any major dysmorphic findings. Height standard deviation score (SDS) at diagnosis was -3.8 ± 1.4. Weight SDS at diagnosis was -3.1 ± 2.0. IGF-1 SDS at diagnosis was -3.0 ± 1.5). Median age at the start of GH replacement treatment was 8.5 years (range: 3 months - 20 years). All of the patients received appropriate treatments for their hormonal deficiencies. Twelve patients achieved their final height and mean final height SDS for these cases was -1.0 ± 0.7. Final height and target height values for *PROP1* mutated patients are shown in Table 1.

Pituitary MRI was available for 53 patients. Twenty-one had normal pituitary MRI, 17 had pituitary hypoplasia, eight had hypoplasia of the adenohypophysis, three had ectopic neurohypophysis and three had pituitary adenoma. Patient 22 had pituitary adenoma, which resolved on follow-up and had transformed into anterior pituitary hypoplasia. None had extra-pituitary abnormalities on MRI.

Patients 14, 22, 41, 46 and 57 had homozygous deletion of c.301_302delAG in exon 2 of the *PROP1* gene. This mutation resulted in frame-shift and premature stop codon (p.Leu102CysfsTer8). These five patients had different combinations of anterior pituitary hormone deficiencies. All had GH and TSH deficiencies at the time of diagnosis. Four of these patients, who have reached the age of puberty, showed clinical and laboratory findings consistent with hypogonadotropic hypogonadism. Only one (patient 22) had ACTH deficiency and none had PRL deficiency (Table 1). Patients 14, 22, 41 and 46 responded quite well to the GH and levothyroxine supplementations and appropriate hormone replacement to induce secondary sex characteristics. Individual responses of the patients to GH replacement are shown in Table 1. Patient 57 was newly diagnosed and was recently started on GH replacement.

PCR amplification of second and third exons of *PROP1* gene had failed for DNA of patients 1^{a}-2^{a}, 7^{d}-8^{d}, 9^{e}-10^{e} and 15 all of whom had parental consanguinity. No pathogenic mutations were detected within exon 1 for these patients. MLPA assay was only performed for patients 1^{a} and 2^{a} from the same family and a homozygous deletion of exon 2 of the *PROP1* gene were detected in both siblings. These two brothers have GHD at the time of diagnosis and developed TSH deficiency after approximately one or two years. Both had delayed pubertal development and lack of secondary male sex characteristics due to hypogonadotropic hypogonadism. Eventually, both developed ACTH deficiency (Table 1).

PCR amplification of the whole *PROP1* gene had failed for DNA from patients 58^{f}-59^{f}. MLPA assays detected complete gene deletion in these siblings. The elder sister showed GHD at the age of two-and-a-half years and developed TSH deficiency four years later. When she reached the age of puberty, she developed both ACTH deficiency and hypogonadotropic hypogonadism. Her younger brother showed both TSH and GH deficiencies at diagnosis; he is currently prepubertal and is not affected by ACTH insufficiency (Table 1). Pituitary imaging revealed pituitary adenoma in patient 58^{f} but was normal in 59^{f}. Adenoma did not exhibit progression and remained stable.

Patients 3^{b} and 4^{b} from the same family with the same phenotype had homozygous c.353A > G (p.Q118R) variant in exon 3 of the *PROP1* gene (Figure 1). This novel variant was predicted to be disease-causing by *in silico* predictive

Table 1. Clinical features and genotype of the patients with *PROP1* gene mutations

Case	Sex	Parental cons.	Age at Dx (years)	Current age (years)	Peak GH, stim. (µg/L)	Onset of hormonal def. (years)					MRI of anterior pituitary	GH dose (mg/kg/week)	Height SDS (before Tx)	Growth vel. SDS (1st year of Tx)	Growth vel. SDS (2nd year of Tx)	Final Height (cm [SDS])	Target Height (cm [SDS])	Mutation
						GH	TSH	Gn.	ACTH	PRL								
1[a]	M	+	5.25	20.67	0.9	4	5.25	12	13.5	N/A	Normal	0.36	-4.39	10.83	6.13	168.7 [-1.09]	167.5 [-1.28]	Homozygous deletion of exon 2
2[a]	M	+	6.6	22.25	0.08	4	6	13	14.25	N/A	Normal	0.35	-4.77	9.97	5.15	166.2 [-1.4]	167.5 [-1.28]	Homozygous deletion of exon 2
3[b]	F	+	9.1	20.33	0.1	9.1	11.75	13	12	13	Normal	0.27	-3.77	4.08	2.18	164.5 [0.24]	164 [0.15]	**Homozygous c.662A > G (p.Q118R)**
4[b]	F	+	4.25	17	0.1	4.25	8.5	14	9	10	Adenoma	0.27	-5.11	6.09	3.51	159.2 [-0.66]	164 [0.15]	**Homozygous c.662A > G (p.Q118R)**
14	F	+	8.83	22	0.4	8.83	8.83	15	N/A	14.5	Normal	0.25	-4.1	3.44	1.3	160.4 [-0.41]	166.1 [0.51]	Homozygous c.301_302delAG (p.S101fsX9)
22	F	-	7.75	20.67	0.01	7.75	7.75	14	13.1	N/A	Adenoma/ hypoplasia	0.29	-3.67	4.92	2.34	162.2 [-0.15]	158.5 [-0.78]	Homozygous c.301_302delAG (p.S101fsX9)
41	F	+	6	18.42	2.3	6	6	14	N/A	N/A	Hypoplasia	0.3	-6.04	8.33	2.02	164.7 [0.32]	154.5 [-1.46]	Homozygous c.301_302delAG (p.S101fsX9)
46	F	-	7.75	19.75	0.5	7.75	7.75	14.5	N/A	13.5	Normal	0.31	-4.88	11.51	5.08	157.5 [-0.95]	164.5 [0.24]	Homozygous c.301_302delAG (p.S101fsX9)
57	M	+	4.33	4.5	0.6	4.33	3	N/A	N/A	N/A	Adenoma	N/A	-2.9	N/A	N/A	N/A	174.5 [-0.28]	Homozygous c.301_302delAG (p.S101fsX9)
58[f]	F	+	6.3	13	0.07	2.5	6.5	12	12	N/A	Normal	0.24	-1.23	5.86	5.54	N/A	168.5 [0.92]	Homozygous complete deletion of *PROP1* gene
59[f]	M	+	6	10.8	1.1	5	5	N/A	N/A	N/A	Adenoma	0.32	-2.93	7.85	4.39	N/A	181.5 [0.86]	Homozygous complete deletion of *PROP1* gene

*Novel mutations are shown in bold.

The patients who are from the same family are indicated with the same superscript letter. The patients who are from the same family are indicated with the same superscript letter ([a], [b] and [f]).

ACTH: adrenocorticotropic hormone, cons.: consanguinity, def.: deficiency, Dx: diagnosis, FSH: follicle-stimulating hormone, GH: growth hormone, Gn.:gonadotropins, LH: luteinizing hormone, MRI: magnetic resonance imaging, N: normal, N/A: not applicable, PRL: prolactin, SDS: standard deviation score, stim.: stimulated, TSH: thyroid-stimulating hormone, Tx: treatment, vel.: velocity, M: male, F: female

tools such as Mutation Taster, SIFT and PolyPhen-2 due to splice site changes and possibly affected protein features (14,15,16). Both parents, who were consanguineous, and a healthy sister of the patients were heterozygous for the same mutation. Both siblings had GHD at the time of diagnosis and a few years later they developed TSH deficiency. They showed hypogonadotropic hypogonadism and PRL deficiency in adolescence (Table 1). On physical examination, decreased body hair growth and pubic hair growth were marked in both siblings. Patient 4[b] had pituitary adenoma on pituitary MRI. On follow-up, she had visual impairment and consequently underwent pituitary surgery.

Discussion

In this study, *PROP1* gene mutations were detected in eight index patients from a cohort of 57 CPHD patients from 50 families. Segregation analysis of the variants in the pedigrees revealed three patients with the same pathogenic *PROP1* mutations. More than half of the patients with mutation were familial cases and positive mutation frequency was significantly higher in familial cases compared to sporadic cases (3/7 familial cases versus 5/43 sporadic cases, $p < 0.01$).

There are several reports of cohorts defining genetic aetiology of CPHD from different parts of the world. *PROP1* gene mutations are reported to be the most frequent amongst both sporadic and familial CPHD patients (4,6,8,17). However, the frequency was reported to vary widely between 0% and 70.1% from different populations (10,18,19,20,21). *PROP1* mutation frequencies among CPHD patients are highest in Eastern European populations especially Lithuanian, Polish and Hungarian, and also high in Portuguese, Russian and Brazilian cohorts (3,10,12,22,23,24,25,26,27,28). In contrast, *PROP1* mutation rates are usually low in Western and Southern European countries, Australia and in cases with Asian origin,

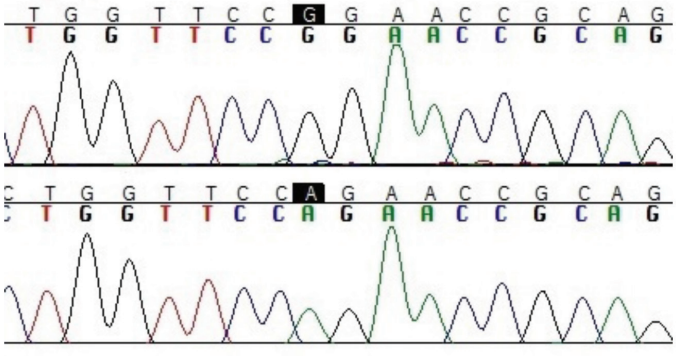

Figure 1. Sequencing electropherogram of patients 3[b] and 4[b]

especially in sporadic CPHD patients (3,6,18,19,20,21,29). *PROP1* gene mutations are not rare among Turkish CPHD patients (13,30). In 2014, Baş et al (30) screened 76 Turkish CPHD patients and the frequency of *PROP1* mutations was 21.8%. *PROP1* mutation frequency in this study was similar to our study. Kandemir et al (13) reported *PROP1* mutations in another Turkish cohort which was present in two familial patients while 51 sporadic CPHD patients were mutation negative. In our study, we detected *PROP1* mutations in 16% patients. Interestingly, Kandemir et al (13) detected lower *PROP1* mutation prevalence compared to our study. This might be attributed to dissimilarities in ethnicity, parental consanguinity rate and frequency of familial cases between these three Turkish cohorts. Overall evaluation of Turkish CPHD patients from previous studies together with the patients from our study gives an estimated frequency of *PROP1* gene mutations of 16.6% amongst Turkish CPHD patients. In addition to their study, De Rienzo et al (6) reviewed all CPHD cases retrospectively and postulated that *PROP1* gene mutations are responsible for 11.2% of all CPHD cases.

PROP1 mutation prevalence is higher in familial patients compared to sporadic cases in all cohorts (3,6,13,22,24,26,29,30,31,32). Parental consanguinity is known to increase the risk for autosomal recessive conditions. Thus, parental consanguinity would appear to be a risk factor for *PROP1* mutations (12,22,30). This hypothesis is supported by evidence from our study, with an overall parental consanguinity rate of 50.9% which increased to 81.8% amongst *PROP1* mutated patients. If the cases are sporadic, that is that there is a single affected individual in a family, and there is no parental consanguinity, the aetiology is more likely to be acquired rather than genetic (1,3,4).

The c.301_302delAG mutation was reported to be one of the most prevalent mutations of *PROP1* gene (2,8,10,11,26). This mutation is a two base pair deletion which results in a frameshift and early termination of the protein at codon 109. Dusatkova et al (2) investigated this variant and suggested that the reason for the high occurrence rate may be a founder effect rather than a variant hot spot (2). This assumption was made by haplotype analyses and the geographic distribution of the c.301_302delAG variant which was interpreted as suggesting an ancestral origin. Five of our patients had this variant and exhibited variable hormone deficiencies. Large deletions were detected in four patients. Many studies, in which CPHD patients from different populations including Turkish patients were screened for *PROP1* deficiency, reported homozygous deletions of the entire gene or particular exons (7,30,33).

For this reason, MLPA analysis should be a routine part of genetic investigation in MPHD patients.

The previously unreported p.Q118R substitution is interpreted as likely pathogenic considering the concordance of phenotype, parental consanguinity and segregation analyses of the variant. This variant is anticipated to be important as it is highly conserved in different orthologues. In addition, it is located in the homeobox domain (5). In 1998, Wu et al (5), identified p.F117I and p.R120C substitutions and they postulated that these variants allowed protein binding but with reduced affinity. As, p.Q118R variant is present in the sequence between these variants, it is assumed that this variant is also associated with pathogenicity due to altered protein function. In silico analyses with Mutation Taster, SIFT and PolyPhen-2 also indicate likely alteration of protein features and splice site changes (14,15,16).

Patients with *PROP1* mutations typically have clinical manifestations of GHD in early childhood. TSH and PRL deficiencies often coexist at the time of diagnosis. At the onset of puberty, patients usually do not exhibit secondary sexual characteristics due to hypogonadotropic hypogonadism. Rarely, some patients show pubertal changes and hypogonadotropic hypogonadism may develop later in adulthood. ACTH deficiency occurs variably as the patient grows older (1,4). As a result, these patients should be carefully monitored for occurrence of other anterior pituitary hormone deficiencies. It is postulated that this phenomenon of progressive hormone deficiency is due to dysfunction of *PROP1* in initiating pituitary stem cell migration and differentiation (34). Patients with *PROP1* mutations lack extra-pituitary manifestations (6,8,31). All of the *PROP1* mutated patients in our cohort had GH and TSH deficiency at the time of diagnosis in early childhood. Nine patients had hypogonadotropic hypogonadism when puberty should have been evident and the other two patients were prepubertal. The two siblings with the novel mutation had remarkably sparse body and pubic hair. ACTH deficiency was observed in half of the patients and the patients without ACTH deficiency are continuing to be monitored as usually ACTH deficiency is the last hormonal deficiency to occur, if it does. Onset age of progressive hormonal deficiencies differ in patients with the same mutations and even in familial cases in our cohort. A clear phenotype-genotype correlation has not been proposed in the literature, since progressive hormonal deficiencies occur at different chronologies even in individuals with the same genotype (4,17,24).

Response to GH treatment was satisfactory in our patient cohort and was similar to previous reports (32). Final height was achieved in nine of the *PROP1* mutated patients, all of whom had final height SDS in the mid-parental target height SDS range. This result was in agreement with previous reports (10,35,36).

MRI of the hypophysis commonly reveals pituitary hypoplasia or aplasia in these patients but occasionally pituitary hyperplasia evolving to hypoplasia and pituitary masses have been reported. (1,4,6,37,38). In contrast, ectopic posterior lobe and stalk abnormalities have not been observed (4). Interestingly, anterior pituitary MRI was normal in six patients. Three more patients had adenoma, two had hypoplasia and one initially had adenoma which evolved into pituitary hypoplasia. Pituitary morphology can change during follow-up of patients with *PROP1* gene mutation (9). None of our patients showed extra-pituitary manifestations on neuroimaging. Patients with adenoma have different genotypes; two had the common homozygous c.301_302delAG mutation, one had a novel mutation and one had complete gene deletion. Of interest, two of these cases were familial, and their siblings had normal pituitary gland upon MRI. With the exact genetic aetiology, patients with pituitary adenoma have the opportunity to avoid unnecessary invasive procedures (1).

Study Limitations

Five of the patients with failed PCR amplification were not available for further testing with MLPA analysis. There is a high probability that a large deletion may exist in the *PROP1* gene of these familial CPHD cases with parental consanguinity which would have increased the proportion of MPHD patients with *PROP1* mutations in our cohort. In this study, we were not able to test the parents of all patients with pathogenic mutations due to financial limitations. For future studies, patients without any mutations identified in the *PROP1* gene may be screened for the other genes of pituitary transcription factors and gene panels may be more cost-effective for this purpose.

Conclusion

It is crucial to screen GHD patients regularly for other anterior pituitary hormone deficiencies. With the exact genetic aetiology, the family is able to receive genetic counselling, unnecessary laboratory testing can be avoided and at the same time the opportunity of predicting the typical phenotype and developing hormonal deficiencies can be detected earlier. If the patients are familial and have parental consanguinity, genetic testing would be even more cost-effective.

Ethics

Ethics Committee Approval: The Ethics Committee of the Çukurova University Faculty of Medicine approved this study (approval: #TF2013LTP24)

Informed Consent: Written informed consent was obtained for each patient from their legal guardians.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Fatma Derya Bulut, Semine Özdemir Dilek, Damla Kotan, Eda Mengen, Fatih Gürbüz, Bilgin Yüksel, Concept: Eda Mengen, Fatih Gürbüz, Bilgin Yüksel, Design: Fatih Gürbüz, Bilgin Yüksel, Data Collection or Processing: Fatma Derya Bulut, Semine Özdemir Dilek, Damla Kotan, Analysis or Interpretation: Damla Kotan, Fatih Gürbüz, Bilgin Yüksel, Literature Search: Fatma Derya Bulut, Semine Özdemir Dilek, Damla Kotan, Eda Mengen, Writing: Fatma Derya Bulut, Semine Özdemir Dilek, Damla Kotan, Eda Mengen, Fatih Gürbüz, Bilgin Yüksel.

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Internipple Distance and Internipple Index in Prepubertal Turkish Girls

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What is already known on this topic?

Increased internipple distance is a clinical feature in some dysmorphic syndromes which may be subjectively evaluated by the physician during examination. Some studies have produced normative data for objective comparison of this parameter with "normal" after measurement and usually proportioning to chest circumference. However, these studies are scarce, and even fewer evaluate subjects beyond the neonatal period.

What this study adds?

There are no reference values available for a Turkish population. This study provides the first references for internipple distance and the internipple index in the country. In addition this reference data may serve internationally for school aged girls. A short overview of the literature is also given to illuminate the present status for these parameters.

Abstract

Objective: To determine internipple distance and internipple index in prepubertal Turkish girls.
Methods: The internipple distance and chest circumference of 667 healthy prepubertal Turkish girls aged 6 to 11 years were measured in a school screening program in Düzce. Measurements were performed at the end of expiration with a standard non-stretch tape measure graduated in millimeters with the arms hanging in a relaxed position on the sides of the body. The internipple distance was measured between the centers of both nipples, and chest circumference was measured across the internipple line. The internipple index was calculated by dividing the internipple distance (cm) x100 by the chest circumference (cm). Age specific internipple index reference curves were constructed and smoothed with the Lambda-Mu-Sigma method. Mean and standard deviations of internipple distance and internipple index were calculated according to decimal ages.
Results: Age was found to be positively correlated with internipple distance and chest circumference, while it was negatively correlated with internipple index. The reference values of internipple index, including 3rd, 10th, 25th, 50th, 75th, 90th, and 97th percentiles, and standard deviations were calculated for prepubertal girls.
Conclusion: The reference ranges provided by this study might be helpful for the evaluation of syndromic cases by serving as normative data for internipple index in prepubertal girls aged 6-11 years in Turkey although ethnic differences may affect applicability to other countries.
Keywords: Internipple distance, internipple index, Turkey

Introduction

Anthropometric evaluation of nipple placement may be helpful in diagnosing some syndromes. For instance, increased internipple distance is seen especially in Turner syndrome (TS), Noonan syndrome, fetal hydantoin syndrome, deletion 9p syndrome, Trisomy 8, Trisomy 18 and bilateral renal hypoplasia, while it may also be found in cerebro-oculo-facio-skeletal syndrome, Fraser syndrome, Bartsocas-Papas syndrome, Juberg-Hayward syndrome and Langer-Giedion syndrome (1). On the other hand, decreased

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internipple distance is generally reported in Jeune syndrome (asphyxic thoracic dystrophy) and cerebro-costo-mandibular syndrome (2).

TS is rather common with a frequency of one case per 2.500 live female births. However, diagnosis may be delayed due to the subtlety of dysmorphic features; in fact, it is not unusual for girls with TS to only be recognized in the prepubertal or pubertal period (3,4). TS should be considered in girls with short stature or primary amenorrhea. However, it is often overlooked in girls with short stature as shown research that found that up to 4% of girls who underwent genetic analysis due to short stature were found to have TS (5,6). Early diagnosis and prompt treatment are crucial in TS to prevent delayed intervention, growth disturbance, cognitive limitations, osteoporosis and to identify severe cardiac malformations (mostly aorta coarctation and bicuspid aortic valve) (7,8,9).

It was claimed that widened internipple distance is "illusionary" in TS, but the study supporting this idea was conducted in 36 syndromic and 247 normal children of both sexes which are small samples (10). Moreover, though the contribution to the diagnosis is not well known, subjective evaluation of internipple distance is almost always recorded in the clinical records of such cases. Thus an objective comparison of this stigma may increase the quality of the clinical examination in several syndromes. The evaluation of TS is part of the daily practice of pediatric endocrinologists. In addition to the contribution reference data for internipple distance may make to diagnosis, it is important as well for research purposes. Therefore to attempt to accurately measure internipple distance in girls and compare with normative data might prove to be a useful tool. However, data regarding internipple index and internipple distance are very limited in the literature. Considering that racial differences significantly affect internipple distance and internipple index, our aim with this study was to determine reference values for internipple distance and internipple index in prepubertal Turkish girls.

Methods

The study was conducted between March-May 2009 in the context of a large school survey on 9.177 children in the 1st to 8th grade from 14 schools. The aim of this survey was to determine local height, weight and pubertal development data and finally to extract cases with short stature and puberty precox who were then invited to the hospital for a complete endocrine work-up (11). The schools were selected by stratified sampling method among 29 schools located in the city center of Düzce, Turkey, a small city located in North-West Anatolia, in pleasant wooded countryside. The stratification was made according to the socioeconomic level of the districts and the schools with highest student numbers were selected. A sample of 667 girls aged between 6-11 years were randomly selected from the schools and distributed as evenly as possible by age and group size in the present study. In every school the students came in order starting from the first to the sixth level. Each level was divided in four subgroups (classes). Approximately 50 girls in each school and nine from each level were targeted for screening. The first class to be sampled fulfilled the target number on most occasions. Birth date, date of physical examination, measured height, weight, internipple distance, chest circumference and pubertal scores according to Marshall and Tanner (12) were recorded and decimal age was calculated. Internipple distance and chest circumference were measured with a non-flexible tape at the end of expiration and with the arms hanging loosely by the sides. An experienced pediatric endocrinologist evaluated puberty and measured internipple distance and chest circumference. Height and weight were measured by a pediatrician. All measurements were made with light clothing, barefoot; internipple-chest measurements were made with naked upper body in a separate room. Internipple index was calculated using the following formula: Internipple distance (cm) x 100/chest circumference. Subjects with body mass index (BMI) above the 95th percentile, children with congenital anomalies or with a chronic disease were excluded from the study. Girls with thelarche were noted and excluded from the study (Tanner stage ≥B2).

The study protocol was approved by the Ethics Committee of Düzce University (protocol number: 2008.211/2189) and permission was obtained. Parents' written consent was obtained prior to the study and the procedures were in accordance with those outlined by the Declaration of Helsinki.

Statistical Analysis

The Statistical Package for the Social Sciences, version 22 (IBM Inc., Armonk, NY, USA) and the Lambda-Mu-Sigma (LMS) Chart Maker Pro version 2.54 software (13) were used for analyses. Descriptive statistics of the variables in the study were calculated. The normality assumption of continuous quantitative variables was checked with the Kolmogorov-Smirnov test. Mean, standard deviation, and minimum and maximum values were given as descriptive statistics of the variables in text and tables, since all quantitative variables met the normal distribution assumption. The relationships between quantitative variables were analyzed with the Pearson correlation test. The age-specific internipple index

reference curves were constructed and smoothed with the LMS method in which the final curves of percentiles were produced by three smooth curves represented as L (Lambda, skewness), M (Mu, median) and S (Sigma, coefficient of variation) (14). Reference values of internipple index, including 3rd, 10th, 25th, 50th, 75th, 90th, and 97th percentiles were determined. Statistical significance was accepted when p values were < 0.05.

Results

The mean age of the 667 individuals included in the study was 8.2 ± 1.2 (6.3-11.5) years. Descriptive statistics for weight, height, BMI, internipple distance, chest circumference and internipple index for each decimal age are given in Table 1 and Table 2. As the subjects ages increase the sample number in groups decreased as the proportion of pubertal cases increased.

The internipple index values were estimated with the LMS method according to age and are presented in Table 3 along with L (lambda-skewness), M (median) and S (coefficient of variation) values for each age group. The graphical presentation of results is shown in Figure 1. These latter analyses were made among 657 subjects after removing the 11 year-old age group.

Age was found to be positively correlated with weight ($r = 0.491$; $p < 0.001$), height ($r = 0.660$; $p < 0.001$), internipple distance ($r = 0.158$; $p < 0.001$) and chest circumference ($r = 0.412$; $p = 0.001$); whereas it was negatively correlated with internipple index ($r = -0.176$; $p < 0.001$). There was no relationship between age and BMI values ($r = 0.041$; $p = 0.295$). However, BMI values were found to be positively correlated with internipple distance ($r = 0.404$; $p < 0.001$) and chest circumference ($r = 0.628$; $p < 0.001$), while they were negatively correlated with internipple index ($r = -0.084$; $p = 0.030$). Internipple index values were also negatively correlated with weight ($r = -0.187$; $p < 0.001$) and height ($r = -0.190$; $p < 0.001$).

Discussion

The majority of studies assessing internipple index are comprised of patients in the newborn period (Table 4) (15,16,17,18,19,20,21). The newborn period is an important time and opportunity for recognizing dysmorphic syndromes. Therefore, these studies are valuable references but performing end-expiratory measurements in newborns and infants is almost impossible, while it is difficult in small children. Measurements performed with these limitations

Table 1. Mean anthropometric values of age groups

Age (years)	Weight (kg)			Height (cm)		BMI	
	n	Mean ± SD	Min-Max	Mean ± SD	Min-Max	Mean ± SD	Min-Max
6 (6-6.99)	122	22.3 ± 3.2	15.6-33.2	119.8 ± 4.9	106.1-133.4	15.5 ± 1.6	10.5-19.6
7 (7-7.99)	201	24.1 ± 3.3	17.2-36.8	123.6 ± 5.5	109.5-140.5	15.7 ± 1.5	11.9-19.5
8 (8-8.99)	190	26.0 ± 3.6	16.8-37.8	128.4 ± 5.7	116.1-154.4	15.8 ± 1.6	12.1-19.4
9 (9-9.99)	114	27.4 ± 4.3	12.0-39.0	132.4 ± 5.3	119.0-145.5	15.6 ± 2.0	12.9-19.4
10 (10-10.99)	30	29.3 ± 3.6	24.0-37.2	136.0 ± 6.3	122.9-150.0	15.8 ± 1.5	13.4-19.2
11 (11-11.99)	10	29.7 ± 2.5	25.8-33.8	136.5 ± 5.8	128.7-145.5	15.9 ± 1.3	14.4-19.1
Whole cohort	667	25.2 ± 4.1	12.0-39.0	126.5 ± 7.3	106.1-154.4	15.7 ± 1.6	10.5-19.6

Mean ± SD: mean ± standard deviation, Min-Max: minimum-maximum, BMI: body mass index

Table 2. Internipple distance and internipple indices with regard to age groups

Age (years)	n	Internipple distance (cm)		Chest circumference (cm)		Internipple index (%)	
		Mean ± SD	Min-Max	Mean ± SD	Min-Max	Mean ± SD	Min-Max
6 (6-6.99)	122	14.0 ± 1.3	11.1-20.0	57.5 ± 3.4	49.5-68.5	24.3 ± 2.1	19.7-33.1
7 (7-7.99)	201	14.4 ± 1.2	11.2-18.2	58.8 ± 3.3	51.0-67.5	24.4 ± 1.7	19.6-28.9
8 (8-8.99)	190	14.3 ± 1.2	11.5-18.4	60.3 ± 3.7	50.2-71.6	23.8 ± 1.7	18.9-28.8
9 (9-9.99)	114	14.6 ± 1.2	11.6-17.1	61.7 ± 3.9	51.7-75.5	23.7 ± 1.7	18.9-28.1
10 (10-10.99)	30	14.8 ± 1.2	12.8-17.3	62.9 ± 3.8	57.0-72.6	23.5 ± 1.7	20.7-28.2
11 (11-11.99)	10	14.6 ± 1.4	12.4-16.5	63.4 ± 3.1	59.7-69.1	23.0 ± 1.7	20.6-25.3
Whole cohort	667	14.3 ± 1.2	11.1-20.0	59.7 ± 3.9	49.5-75.5	24.0 ± 1.8	18.9-33.1

Mean ± SD: mean ± standard deviation, Min-Max: minimum-maximum, BMI: body mass index

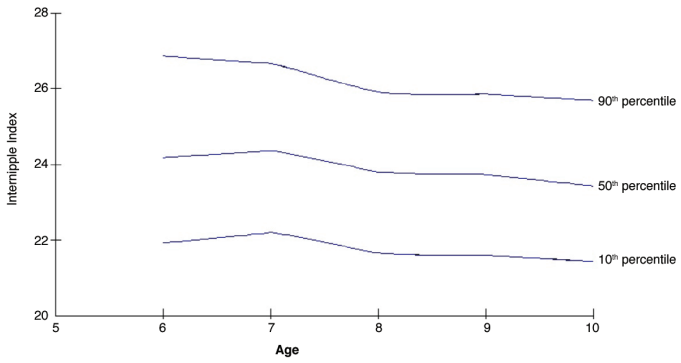

Figure 1. Graph showing the internipple index values (10th, 50th and 90th percentile), calculated using the Lambda-Mu-Sigma method, according to age

may cause erroneously high chest circumference and thus decreasing internipple index values. Therefore, studies with larger subject numbers are more reliable as measurement errors are balanced by the number of subjects.

Unfortunately, there are very few studies in older age groups (10,19,22). All identifiable studies involving older age groups have been summarized below. Chest measurement is easier in school aged girls since they cooperate quite well with instructions. However when puberty starts, the contribution of breast tissue to this parameter changes individually. Therefore, measurement is the easier to standardize and less prone to error in prepubertal girls. Tanner stage 1 girls were chosen for the present study resulting in the age distribution of our study. This age group is well representative of the

Table 3. Smoothed age-specific internipple index percentile values of prepubertal girls aged 6 to 10 years

				Percentiles								
Age	**L**	**M**	**S**	**3rd**	**5th**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**	**97th**
6 (6-6.99)	-0.733	24.181	0.079	21.0	21.4	21.9	22.9	24.2	25.5	26.9	27.7	28.3
7 (7-7.99)	0.315	24.368	0.072	21.2	21.6	22.2	23.2	24.4	25.6	26.7	27.4	27.8
8 (8-8.99)	1.193	23.796	0.070	20.6	21.0	21.6	22.7	23.8	24.9	25.9	26.5	26.9
9 (9-9.99)	1.037	23.729	0.070	20.6	21.0	21.6	22.6	23.7	24.9	25.9	26.5	26.9
10 (10-10.99)	-0.354	23.427	0.071	20.6	20.9	21.4	22.3	23.4	24.6	25.7	26.4	26.9

L: Lambda-Skewness, M: median, S: coefficient of variation

Table 4. Internipple distances and internipple indices in other studies

Study authors (years)	**Place**	**Subjects n**	**Internipple index (%) Mean ± SD**
Pelz (20) (1972)	Germany	390 (newborn-18 years)	23.09 ± 4.1 M 23.41 ± 1.9 F
Collins (10) (1973)	Australia	247 (2-10 years)	Internipple distance: 11.2 ± 1.8 M 11.6 ± 2
Feingold and Bossert (22) (1974)	United States	2403 (0-14 years)	26.4 ± 1.6
Mehes and Kitzveger (18) (1974)	Hungary	600 (0-5 days)	25.1 ± 2.9
Chen et al (1) (1974)	Michigan, USA	472 (0-3 days)	25 ± 0.018
Sivan et al (15) (1983)	Israel	198 (term-preterm)	23.2 (27 weeks) 24.5 (41 weeks)
Ejiwunmi et al (17) (1984)	Nigeria	278 (term neonates)	22.88 ± 1.94
Leung et al (19) (2004)	China	3290 (0-18 yrs.)	Neonates: 26.4 ± 1.6 M 26.3 ± 2 F Child: 23.8 ± 1.2 M 23.8 ± 1.4 F
Fok et al (16) (2005)	Hong Kong	10,339 (24-42 weeks)	Internipple distance: 8.4 (41 weeks) M 8.2 (41 weeks) F
Feingold and Bossert (22) (1974)	India	1 077 (term neonates)	27.1 ± 3.5 F 27.0 ± 3.5 M

F: female, M: male, yrs.: years, SD: standard deviation

age interval in which a great majority of TS patients attend clinics.

Feingold and Bossert (22) screened 2.403 children (2006 Caucasian, 206 Black and 43 Asian) between the newborn period and 14 years of age for many anthropometric indices. They reported that internipple index was the highest in the newborn period, with the lowest values between 15 months and seven years of age. This finding was partly supported by a study by Chen et al (1) who also found that internipple index was maximum at birth and decreased until two years of age. Furthermore, in a study by Leung et al (19) internipple index decreased steadily from 1 to 18 years of age. Pelz (20) claimed that the intermammillary index, which is identical to internipple index, does not change with age prior to puberty.

In our study, this gradual decrease in internipple index was evident. Notably, the difference between the 3rd and 97th percentiles was the biggest in the age 6 group, it decreased somewhat in the age 7 group, but stabilized in the 8-10 groups. These groups show minor differences in percentiles between 5 and 95, which is perhaps ignorable. Correlation analyses was used to investigate the internipple index-age relationship and there was a negative correlation between these two parameters. There was also a negative correlation between both weight and height with internipple index. BMI was also negatively correlated with internipple index but this relationship was much weaker. Weight and height generally increase with age in childen although BMI is derived from these two parameters, so would be expected to remain relatively constant throughout the ages groups studied, given proportional increases in height and weight. In the study of Leung et al (19) the decrease in internipple index with age was relatively stable between the ages of 6 to 11 years. When we compared our 6-11 age results with the corresponding results of Leung et al (19), we found that internipple index values in in our study group were increasing with decreasing age by more than 1%, the variation in the same age groups was around 0.1% in the study of Leung et al (19). We can speculate that they couldn't catch this age pattern with their limited subject numbers in each age group.

There are different views on the influence of ethnicity on internipple index measurements (1,16,19,20,21,22). In addition to ethnicity, methodologies and measurement devices may also contribute to these differences in internipple distance. On review of the literature, it was evident that Chinese neonates had higher internipple indexes than those reported in US, Nigerian and Hungarian cohorts (1,17,18).

The contribution of increased internipple distance to the diagnoses of Turner and Noonan patients was questioned in some earlier studies (1,10). Chen et al (1) found that internipple distance in patients with TS (n = 40) was significantly different (p = 0.001) from normal after adjusting for height but not for age. However, the difference between TS and unaffected subjects was less striking when compared to chest width or circumference (p = 0.01), but still significant Collins (10) suggested that an increased internipple distance is a subjective clinical impression owing to the illusion created by the body shape and short stature in Turner as well as Noonan syndrome of note, samples sizes in these two studies were quite small and their hypotheses should be tested in larger groups of Turner and Noonan patients in comparison to normal subjects. Since height is effective on both the objectively measured internipple index as shown in our study and on perceived internipple distance as claimed in Collin's study, it might be reasonable to compare Turner subject's height age with normative data, rather than chronological age.

Study Limitations

Sample numbers in older age groups were reduced due to the increasing proportion of pubertal cases.

Conclusion

Although the internipple index provides a method of assessment that is objective in comparison to physical examination alone, and could increase the chances of early diagnosis in many syndromes, especially in TS, it is apparent that racial and ethnic differences may cause variations in assessment. Therefore, appropriate reference intervals should be used. In this study, normal values of internipple distance and internipple index were obtained for the Turkish population in prepubertal girls aged between 6-11 years. Despite the possibility of contribution of ethnic differences to a variation in internipple index, our data might be used as reference values for other countries with largely Caucasian populations, until local normative values are available. Regarding our local population, the missing neonatal reference standards might be helpful additional data when future studies of internipple distance are performed.

Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of Düzce University (protocol number: 2008.211/2189) and permission was obtained.

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Seda Erişen Karaca, İlknur Arslanoğlu, Concept: İlknur Arslanoğlu, Design: İlknur Arslanoğlu, Data Collection or Processing: Seda Erişen Karaca, İlknur Arslanoğlu, Analysis or Interpretation: Şengül Cangür, Literature Search: Seda Erişen Karaca, İlknur Arslanoğlu, Writing: Seda Erişen Karaca, İlknur Arslanoğlu.

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ORIGINAL ARTICLE

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Validity of Six Month L-Thyroxine Dose for Differentiation of Transient or Permanent Congenital Hypothyroidism

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What is already known on this topic?

Setting a lower thyroid stimulating hormone (TSH) referral cut-off value in neonatal screening programs is increasingly common. However, this had resulted in referral of neonates with lower TSH concentrations and an increase in transient mild TSH elevation. Identifying infants who are likely to have transient TSH elevation and therefore withdrawing replacement therapy earlier would be of clinical benefit.

What this study adds?

L-thyroxine replacement dose requirement at the sixth month of therapy may be a good marker for predicting those with transient elevated TSH in patients with an eutopic thyroid gland.

Abstract

Objective: The tendency to reduce thyroid stimulating hormone (TSH) referral cut-off values in congenital hypothyroidism (CH) neonatal screening programs has resulted in an increase in the incidence of CH, but also the referral of infants with mild transient elevation of TSH. Therefore, there is a need to develop markers for differentiation of transient elevated TSH and permanent CH as early as safely possible to avoid unnecessary treatment. The aim was to evaluate sixth-month L-thyroxine (LT4) dose as a predictive marker for differentiation of transient elevated TSH and permanent CH.

Methods: Data of patients who had been followed after referral from the neonatal screening programme between the year 2010 and 2019 in a tertiary pediatric endocrine centre were examined retrospectively.

Results: There were 226 cases referred, of whom 186 (82.3%) had eutopic thyroid gland, and 40 (17.7%) had dysgenetic gland. In patients with a dysgentic gland there was a non-significant tendency to have lower diagnostic free thyroxine concentration but significantly higher TSH compared with those with eutopic gland ($p = 0.44$ and $p = 0.023$, respectively). Patients with thyroid dysgenesis required higher initial and six month LT4 doses compared with those with eutopic glands ($p = 0.001$). Receiver operator curve analysis showed the optimum cut-off value for LT4 at six months for transient vs. permanent CH was 2 µg/kg/day (sensitivity 77% and specificity 55%), regardless of etiology. Similarly, in patients with eutopic glands the optimum cut-off value for LT4 dose at six months for permanent vs. transient patients was 2 µg/kg/day (sensitivity 72% and specificity 54%).

Conclusion: Results suggest that LT4 requirement at six months of therapy may be a good marker for predicting transient TSH elevation in patients with eutopic thyroid gland, thus facilitating the decision to halt LT4 therapy.

Keywords: Congenital hypothyroidism, transient, permanent, six month L-thyroxine dose

Introduction

Thyroid hormones are critical for normal brain development during the intrauterine period and early infancy. Therefore, delay in the diagnosis and treatment of congenital hypothyroidism (CH) will lead to severe neurological and psychiatric disorders (1,2). Indeed, CH is considered to be the most common cause of preventable mental retardation (1,3,4).

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Neonatal screening for identification of infants suspected of having CH has improved the early diagnosis and immediate treatment of CH. The majority of screening programs use blood spot (capillary) thyroid stimulating hormone (TSH) concentration to identify infants requiring urgent assessment as central hypothyroidism accounts for only 2-5% of all neonates with CH (4,5). The referral cut-off value for TSH in neonatal screening programs has gradually declined from 20-50 μIU/mL at screening programme inception to lower values (1,4,6). Determination of lower cut off values has led to an increase in the incidence of CH from 1/3000-4000 (4), to 1/2000-3000 (1,7).

The number of referrals from screeing programmes has been increasing as the TSH cut-off values have reduced, while the incidence of permanent CH has not changed much (8,9). Therefore, developing criteria for differentiation of transient TSH elevation and permanent CH is important from the point of management. In this study, the clinical characteristics of the patients diagnosed with CH over a 10-year period were evaluated. The case characteristics were assessed to identify possible parameters that would help to differentiate the transient abnormalities and permanent CH at the diagnosis or during follow-up.

Methods

Data of the patients who were being followed with the diagnosis of CH between January 2010 and January 2019 in Pediatric Endocrinology Outpatient Clinic of Health Sciences University, Diyarbakir Gazi Yasargil Training and Research Hospital were examined retrospectively. The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 2019/334). Exclusion criteria were: patients diagnosed in another centre and were on LT4 replacement but with incomplete data about their initial LT4 dose and/or pretreatment thyroid function tests (TFTs); those lost to regular follow up; and those not yet three years of age.

The gender, age of diagnosis (in days), parental consanguinity, family history of thyroid disorders and complaints at presentation were recorded. The most recent TSH, free thyroxine (FT4), free tri-iodothyronine concentrations and height standard deviation (SD) score (SDS) and weight SDS, as well as LT4 doses of the patients at diagnosis, at the sixth month of the treatment, at the end of the treatment and during follow-up were noted. Results of thyroid imaging, including ultrasonography (USG) and ^{99m}Tc scintigraphy, were recorded.

Patients whose thyroid gland was of normal size and location on thyroid USG and/or thyroid scintigraphy were defined as eutopic CH. Thyroid dysgenesis was defined as cases with gland hypoplasia, ectopia, hemi-agenesis or complete agenesis (Dysgenetic CH). A trial of L-thyroxine (LT4) cessation was undertaken in all patients who had a eutopic thyroid gland, had reached the age of three years and no longer required dose increases due to continuing elevation of TSH, implying increasing LT4 requirement. TSH and FT4 were measured four weeks following cessation of LT4 treatment. Cases in whom TFTs were normal four weeks after cessation of LT4 and remained stable over the ensuing six months were considered to have transient eutopic CH. Cases who required reintroduction of LT4 replacement (TSH > 10 μIU/mL) were defined as permanent eutopic CH (Figure 1) (8).

TSH and FT4 levels were analyzed on the Abbott Architect i8000 device (Abbott Park, Illinois, United States) using the electrochemiluminescence immunoassay "ECLIA" method. Normal range was considered to be 0.35-4.94 μIU/mL for TSH level and 0.70-1.48 ng/dL for FT4 level.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences for Windows, version 16 (IBM Inc., Chicago, IL, USA). Continuous variables were presented as mean ± SD, or median (interquartile range), whereas categorical variables were presented as count and

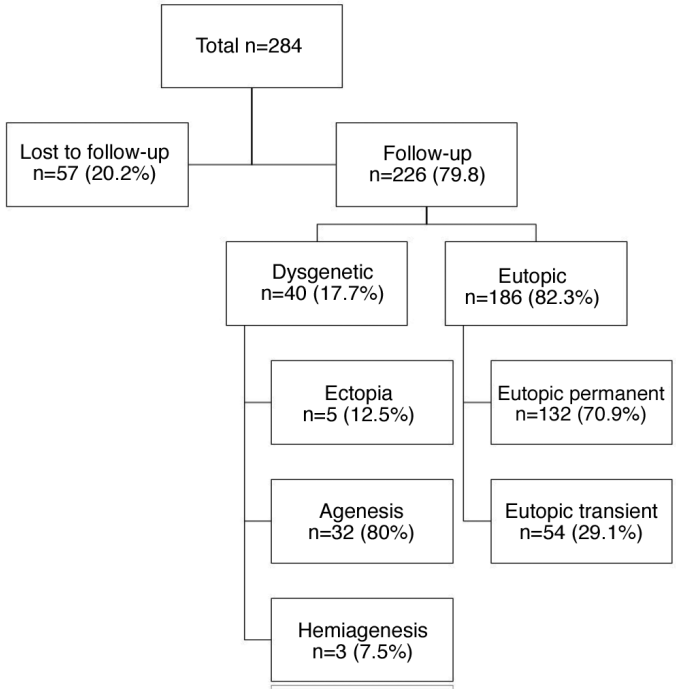

Figure 1. A flow-diagram of all patients with congenital hypothyroidism

percentage (%). For evaluation of normality of distribution of the data, Shapiro-Wilk tests were used. For comparison of normally distributed data Student's t-test was applied, whereas for comparison of non-normally distributed data Mann-Whitney U test were applied. A receiver operating characteristics (ROC) curve analysis was performed for determination of the best cut-off value for LT4 dose at the six month of the treatment between permanent and transient CH. A p value <0.05 was considered to indicate statistical significance.

Results

In total, 284 patients diagnosed with CH within the first six months of life were eligible for inclusion. Fifty-eight patients were excluded from the study, as they were lost to follow-up (Figure 1). The remaining 226 patients (123 female) were included. The mean age of diagnosis was 41.18 ± 39 (range 4-180) days. At first assessment, the mean TSH level was 81.79 ± 35 mIU/mL and the mean FT4 level was 0.55 ± 0.33 ng/dL. Starting L thyroxine dose was 7.04 ± 2.64 µg/kg/day (Table 1). Etiological evaluation of cases revealed that 186 out of 226 (82.3%) cases were diagnosed with eutopic CH, whereas 40 (17.7%) were diagnosed with thyroid dysgenesis. Of the patients with eutopic CH; 132 (71%) had permanent and 54 (29%) had transient CH (Figure 1). The patients identified as transient TSH elevation with eutopic gland constituted 54/226 (23.9%).

Table 1. Patient's clinical and laboratory findings

Diagnosis age (day)*	44.18 ± 39 (4-180)
Gender (male/female)**	123 (54.2%)/103 (45.8%)
Consanguinity**	126 (55.5%)
Birth weight (gr)*	3113.39 ± 554.87 (1470-4500)
First TSH (µIU/mL)*	81.79 ± 35.61 (10.20-315.30)
- FT4 (ng/dL)*	0.55 ± 0.33 (0.01-1.75)
- FT3 (ng/dL)*	3.08 ± 1.54 (0.03-6.49)
L-thyroxin dose at diagnosis (µg/kg)*	7.04 ± 2.64 (0.9-10.0)
Sixth month TSH (µIU/mL)*	2.46 ± 3.6 (0.01-23.9)
- FT4 (ng/dL)*	1.28 ± 0.51 (0.5-4.19)
L-thyroxine dose at six months (µg/kg)*	2.71 ± 1.19 (0.01-8.30)
L-thyroxine cessation age (month)*	27.04 ± 14.42 (3-93)
Permanent CH/transient CH**	171 (75.3%)/54 (24.2%)

*Mean ± standard deviation (minimum-maximum).

**n (%).

TSH: thyroid stimulating hormone, FT4: free thyroxine, FT3: tri-iodothyronine, CH: congenital hypothyroidism

While the FT4 concentrations measured at the time of the diagnosis were not statistically different between patients with eutopic and dysgenetic glands ($p=0.44$), TSH levels were significantly higher in cases with thyroid dysgenesis ($p=0.023$). Initial and sixth-month LT4 doses of cases with dysgenetic CH were significantly higher compared to eutopic CH patients ($p=0.001$) (Table 2).

When cases of transient and permanent CH were compared, no difference was determined in any parameter except for the LT4 dose at the sixth month and discontinuation of the treatment (Table 2).

Of the permanent cases, 54 (58%) had eutopic CH and 40 (42%) had dysgenetic CH. The ratio of consanguinity in permanent cases was similar in both groups (55.5%). LT4 dose at the sixth month was higher in cases with permanent CH (2.92 ± 1.2 µg/kg) then in those with transient CH (2.13 ± 0.88 µg/kg) ($p<0.001$) (Table 2). Doses at the sixth month were higher in dysgenetic cases compared to the eutopic cases (3.26 ± 1.1 µg/kg; 2.60 ± 1.18 µg/kg,

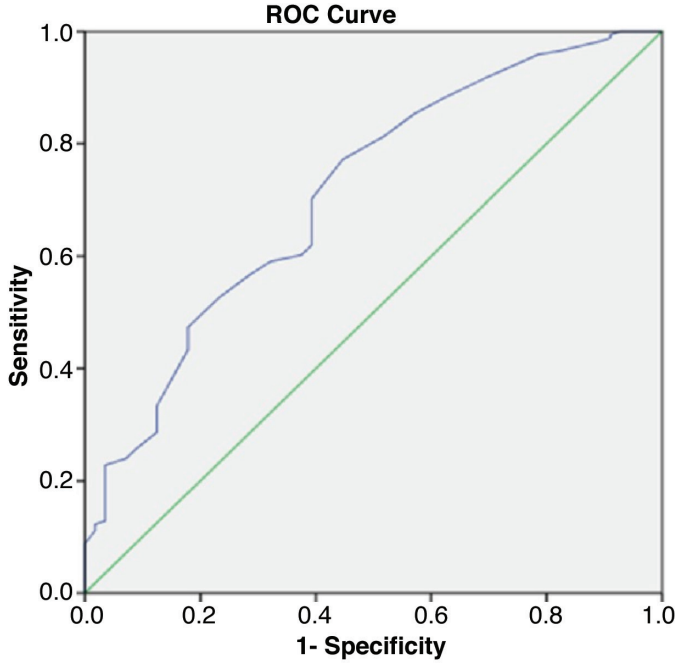

Figure 2. The receiver operating characteristics analysis for L-thyroxine dose at the 6th month for eutopic transient vs. permanent congenital hypothyroidism

ROC: receiver operating characteristics

Table 2. Comparisons between groups parameters**

Time	CH group	Permanent	Transient	p	Dysgenetic	Eutopic	p	Permanent eutopic	Transient eutopic	p
At diagnosis	TSH (µIU/mL)	82.59 ± 34.5	78.99 ± 39.1	0.51*	93.37 ± 20.09	79.32 ± 37.6	0.023*	79.1 ± 37.1	79.54 ± 39.2	0.93*
	FT4 (ng/dL)	0.55 ± 0.31	0.57 ± 0.21	0.98*	0.51 ± 0.4	0.56 ± 0.2	0.44*	0.56 ± 0.31	0.54 ± 0.21	0.64*
	L-thyroxine dose (µg/kg/day)	7.12 ± 2.52	6.78 ± 2.8	0.4*	8.23 ± 2.09	6.79 ± 2.6	< 0.01*	6.81 ± 2.6	6.72 ± 2.83	0.84*
At six months	TSH (µIU/mL)	1.44 ± 2.4	1.06 ± 0.91	< 0.05†	2.68 ± 7.22	1.2 ± 1.67	< 0.05†	1.31 ± 2.04	1.06 ± 0.92	> 0.05†
	FT4 (ng/dL)	1.1 ± 0.8	1.19 ± 0.32	> 0.05†	1.3 ± 0.96	1.1 ± 0.3	< 0.05†	1.1 ± 0.31	1.19 ± 0.33	> 0.05†
	L-thyroxine (µg/kg/day)	2.92 ± 1.2	2.13 ± 0.82	< 0.001*	3.26 ± 1.1	2.6 ± 1.1	0.001*	2.8 ± 1.22	2.14 ± 0.81	< 0.001*
At treatment cessation	L-thyroxine (µg/kg/day)	2.64 ± 1.39	1.39 ± 0.66	< 0.001*	3.54 ± 1.33	2.09 ± 1.22	< 0.001*	2.35 ± 1.29	1.41 ± 0.66	< 0.001*
	Age (month)	-	27.04 ± 14.4	-	-	-	-	-	26.98 ± 14.5	

**Data are presented as mean ± standard deviation, while comparison was performed using.

*Independent sample t-test or †Mann-Whitney U test according to the distribution of the data.

CH: congenital hypothyroidism, TSH: thyroid stimulating hormone, FT4: free thyroxine

respectively) (p = 0.001). In the ROC analysis, regardless of the aetiology, the optimum cut-off value for LT4 dose at the sixth month for transient vs. permanent CH was 2 µg/kg/day [area under curve (AUC): 0.713; sensitivity 77%; specificity 55%; p < 0.001]. The optimum cut-off value for LT4 dose at the sixth month for eutopic permanent vs. eutopic transient CH was also 2 µg/kg/day (AUC: 0.677; sensitivity 72%; specificity 54%; p < 0.001). The positive predictive value of the treatment dose of 2 µg/kg/day at six months in patients with eutopic CH was 59.2% (Figure 2).

Discussion

In the present study 226 patients diagnosed with CH were evaluated and a high rate of transient cases was observed. The sixth-month LT4 doses were lower in transient cases and it is suggested that this parameter may function as a marker for differentiating permanent and transient CH.

Although a nation-wide neonatal screening program is available in Turkey, the age of presentation of our cases (44.18 ± 39 days) was a bit late than expected. This was attributed to the fact that the majority of patients were from rural areas and probably have some difficulties in accessing their results of screening as well as in admission to our tertiary pediatric endocrine centre. While in some studies, a female predominance has been reported, some others, showed a male predominance (8,9,10,11,12,13,14). In the present study, there was a slight female predominance.

Regarding the aetiology of CH, 186 out of 226 cases (82.4%) had eutopic thyroid gland. Of which 132 (71%) cases had permanent CH. Patients with eutopic thyroid gland account for (54%) of cases with permanent CH (Figure 1). When considering the aetiology of permanent CH, in previous studies, thyroid dysgenesis was reported to cause 75-85% of permanent CH cases which was followed by dyshormonogenesis cases with eutopic glands (5,13). However, a considerable shift has been observed in the incidence rate as well as the ratio of transient and permanent cases with a change in the cut-off values which had been used for neonatal screening programs (5,8,15). In our series an increase in the number of cases with transient hypothyroidism can be attributed to moderate iodine deficiency, while the high rate of permanent cases may be attributed to the high rate of consanguinity (55%) which may be associated with increased risk of autosomal recessive dyshormonogenesis (5,16,17,18).

Prompt and correct diagnosis and differential diagnosis of cases admitted from neonatal screening program are critically important for avoiding missing cases as well as introducing the most appropriate treatment. Indeed,

unnecessary and overtreatment may associate with an economic burden and poor neurodevelopmental outcome (3,19). Nevertheless, the presenting hormonal features are usually overlapping and do not allow to make a differential diagnosis of transient and permanent CH. In keeping with this, transient CH accounts for 55% of our cases and cases with transient and permanent CH did not have statistically significant different TSH and FT4 levels (Table 2). There was also no difference between permanent and transient CH in cases with a eutopic thyroid gland. However, when dysgenetic and eutopic CH were compared, dysgenetic cases had higher mean TSH and lower mean FT4 levels (Table 2). Although this data may help to estimate dysgenetic cases, this is not a reliable method as these values may overlap frequently. There are also studies reporting that TSH level at assessment is higher in patients with permanent CH compared to transient CH (20).

Evaluation of LT4 doses in dysgenetic/eutopic, total transient/permanent and eutopic transient/eutopic permanent CH, revealed a significant difference in doses required at the age of six months (Table 2). ROC analysis revealed an optimal cut-off value of 2 µg/kg/day for LT4 dose at the sixth month, for differentiation of cases with eutopic transient and eutopic permanent CH, with a sensitivity of 72% and a specificity of 54% (Figure 2). Similarly, Oron et al (21) have reported a cut-off value for sixth-month LT4 dose of 2.2 µg/kg in retrospective analysis of 142 cases. However, in the study of Saba et al (22) the cut-off value for treatment doses at the sixth month of 49 transient and 43 permanent CH patients with a eutopic thyroid gland was reported as 3.2 µgr/kg/day, with a sensitivity of 71% and a specificity of 79%. The mean time of discontinuation of treatment of transient cases was 1.5 years of age. The treatment dose at the sixth month was higher than the cut-off value we reported for our eutopic transient and permanent CH patients. For our cases, mean FT4 at the sixth month was 1.28 ± 0.51 and the mean time of discontinuation of treatment in eutopic transient cases was 26 months. The discrepancy between the cut off values in our and Saba's study could be attributed to higher LT4 doses they have used in their patients.

In a multi-centre retrospective study evaluating LT4 doses of cases with CH for 12 years, LT4 doses received per kg of body weight was shown to gradually decrease every year, starting from the sixth month of age (21). By the 12th year, while eutopic CH cases were receiving a mean dose of 1.7 µg/kg/day, patients with ectopic gland and agenesis required a dose of 2.1 and 2.2 µg/kg/day, respectively, with no statistically significant difference (21). LT4 dose at the sixth month was lower in cases with eutopic CH compared to the other two groups. In the study by Unüvar et al (10) in which they compared cases with permanent CH and hyperthyrotropinemia, they did not observe any difference between the groups other than the required LT4 dose at the 1st year (4.79 ± 2.09 and 3.46 ± 1.23 µg/kg/day, respectively). In a study of 204-case series from a neighbouring country, Iran, a statistically significant difference was reported in only total LT4 doses between transient and permanent CH (40.0 + 12.77 µg/day and 48.3 + 47.64 µg/day, respectively) (9).

Messina et al (23) reported that LT4 doses at 1st, 2nd and 3rd years were predictive in early differentiation of transient and permanent CH. They reported that predictive cut-off values for transient eutopic CH were 1.7 µg/kg/day, 1.45 µg/kg/day and 0.98 µg/kg/day, respectively with a sensitivity of 100%. However, they did not report the dose at the sixth month.

There are limitation of present study. Firstly, as we recruited cases retrospectively we could not access and take into account the birth weight and clinical status of cases. We also have not data about maternal and fetal iodine status as well as breastfeeding status. In addition, in our laboratory the upper detectable limit for TSH were 100 µIU/mL and TSH levels above 100 µIU/mL was noted as 100 µIU/mL in patients' hospital records. This might caused an underestimation and overlap of TSH levels between TSH levels of cases.

Conclusion

In conclusion, setting a lower TSH level as cut-off for neonatal screening programs is increasingly common. This has resulted in diagnosing more transient elevated neonatal TSH and mild hypothyroidism. There is therefore a need to investigate criteria which may identify babies in which it is safe to stop replacement thyroxine therapy earlier. LT4 doses required at the sixth month may be a useable marker for predicting transient-permanent eutopic CH patients. As a delay in discontinuation of treatment and overtreatment may be associated with worse neurological outcome, increased anxiety for both families and physicians, as well as for insurance systems, the results presented here may contribute to an earlier clinical decision regarding discontinuation of therapy. Larger studies adjusting for iodine status of the population, breast feeding status of the included infants and clear diagnostic criteria for dysgenetic, dyshormonogenetic and other babies with gland *in situ* are warranted to investigate if evaluating LT4 doses required at the sixth month is safe and sensitive enough to distinguish cases with transient and permanent eutopic CH.

Ethics

Ethics Committee Approval: The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 2019/334).

Informed Consent: As it is a retrospective study, an informed consent form was not received.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Muhammet Asena, Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Concept: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Design: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Data Collection or Processing: Muhammet Asena, Murat Öcal, Analysis or Interpretation: Muhammet Asena, Meliha Demiral, Edip Unal, Literature Search: Mehmet Nuri Özbek, Meliha Demiral, Edip Unal, Writing: Mehmet Nuri Özbek, Hüseyin Demirbilek, Muhammet Asena.

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ORIGINAL ARTICLE

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Increased Incidence of Type 1 Diabetes in Children and No Change in the Age of Diagnosis and BMI-SDS at the Onset - is the Accelerator Hypothesis not Working?

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What is already known on this topic?

An association between increase in body mass index-standard deviation score (BMI-SDS) and younger age of type 1 diabetes manifestation has been postulated. This is known as the accelerator hypothesis.

What this study adds?

An increase of type 1 diabetes incidence in the paediatric population of Lesser Poland was not associated with younger age of diagnosis, nor higher BMI-SDS.

Abstract

Objective: One of the hypothesized reasons for the observed increase in type 1 diabetes incidence in children is weight gain, causing accelerated disease development in predisposed individuals. This so-called accelerator hypothesis is, however, controversial. The aim was to analyze whether, in the ethnically homogeneous population of Lesser Poland, an increase in the number of cases of diabetes among children was associated with younger age and higher body mass index-standard deviation score (BMI-SDS) at the time of diagnosis.
Methods: Retrospective data analysis from medical records of all patients < 14 years (n = 559; 50.6% male), with newly diagnosed type 1 diabetes, in Lesser Poland between 1st January 2006 and 31st December 2017 (11 years).
Results: The incidence ratio ranged significantly ($p < 0.001$) from the lowest in 2006 (11.2/100,000/year) to the highest in 2012 (21.9/100,000/year). The mean age of diagnosis was 8.2 ± 3.5 years. There was no trend in decreasing diagnosis age ($p = 0.43$). The mean BMI-SDS was -0.4 ± 1.2. Almost all children (91.6%) presented with BMI-SDS within the normal range at the time of diagnosis, with only 2.7% of cases being obese and 5.7% underweight at the moment of diagnosis. There was no clear trend at all in BMI-SDS over the study period.
Conclusion: These results do not corroborate an increase of type 1 incidence in paediatric population being associated with younger age of diagnosis and higher BMI-SDS. This implies that the *accelerator hypothesis* does not hold true in the study population.
Keywords: *Accelerator hypothesis*, body mass index, children, type 1 diabetes

Introduction

Type 1 diabetes is one of the most common chronic diseases in children and adolescents worldwide (1). Its increasing incidence, especially in industrially developed countries, makes it necessary to look for and define potential risk factors. Some recent studies point to a possible contribution of childhood overweight and obesity to the development of type 1 diabetes at a younger age (2,3). Obesity is a

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well-documented risk factor for type 2 diabetes, but there are some studies indicating an association between the increase of body weight in children and adolescents and the increase in the incidence rate of type 1 diabetes in these age groups (2,3). That postulated association has been called the *accelerator hypothesis* (3,4,5). One of the arguments for the existence of such a relationship is the fact that in countries where the incidence of type 1 diabetes is increasing in the younger age groups, a simultaneous increase in the incidence of obesity in the general pediatric population was found. The number of obese children and adolescents worldwide has increased tenfold in the last 40 years, and childhood obesity, defined as equal to or greater than the 95th percentile of body mass index (BMI), has been recognized as an epidemic by the World Health Organization (6,7). The *accelerator hypothesis* identifies three processes which may accelerate the apoptosis of pancreatic beta cells: constitution, insulin resistance and autoimmunity. According to that hypothesis, weight gain causes an increase in insulin resistance which leads to a deterioration in control of blood glucose level. The rising blood glucose level accelerates beta-cell apoptosis directly via glucotoxicity, and indirectly by inducing immunogenicity (3,4,5,6,7,8). The authors of the *accelerator hypothesis* postulate that the pathogenesis of type 1 and type 2 diabetes may be, to some extent, similar and that overweight and insulin insufficiency are associated with both types of diabetes (4,5). According to the hypothesis, excessive body weight in a child who is predisposed genetically to the development of type 1 diabetes accelerates the process of beta cell destruction leading to an earlier occurrence of an overt deficit of insulin (3,6,8). This theory has been confirmed by some studies strongly supporting the association between BMI and earlier diagnosis of type 1 diabetes (6,9,10,11). Nevertheless, it is not universally accepted, as there are other studies that have contradicted these findings and do not support such a relationship (12,13,14,15). The starting point for the current study was a significant increase in the incidence of type 1 diabetes in the young age groups in the Lesser Poland region (from 5.2/100,000/year in 1987 to 21.9/100,000/year in 2012), which was demonstrated in our previous paper (16). Simultaneously, there was evidence of an increase in obesity incidence in the general paediatric population over the same time period and in the same geographic area (17).

Objective

The aim of the study was to analyze whether, in the ethnically homogeneous population of Lesser Poland, an increase in the number of cases of diabetes among children was associated with younger age and higher BMI-standard deviation (SD) score (SDS) at the time of diagnosis.

Methods

Retrospective data was extracted from medical records of all patients under the age of 14 years, with newly diagnosed type 1 diabetes in the Krakow region (former *województwo krakowskie*) between 1st of January 2006 and 31st of December 2012 and in the whole of Lesser Poland between 1st of January 2013 and 31st of December 2017 and analyzed. The analysis included children with type 1 diabetes only; patients with other types of diabetes were excluded. Type 1 diabetes was defined as acute-onset diabetes presenting with ketoacidosis and/or symptoms of polyuria, polydipsia and weight loss, complete insulin dependence within <1 year from diagnosis, or positive anti-glutamic acid decarboxylase or anti-IA2 test on diabetes diagnosis. All data were collected in one centre for the region; the Department of Pediatric and Adolescents Endocrinology, Chair of Pediatrics, Jagiellonian University Medical College, which is the reference centre for the region.

Body weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, using a stadiometer (Harpenden, UK) and a balanced scale (Seca 700, Germany). All measurements were done during the hospitalization at the moment of diabetes diagnosis, after normalization of the general condition and rehydration. As the standard of reference for calculating BMI-SDS, normal values from the local population were used (18). Incidence rate was evaluated based on the data from the Central Statistical Office (Polish: Glowny Urzad Statystyczny) for the population of the region which was subject to analysis (19).

Statistical Analysis

Statistics data are presented as means with SD or medians and quartiles for continuous variables, or counts and percentages for categorical variables. Univariate and multiple regression models were used to test the association between BMI-SDS, age of diagnosis and calendar year of diagnosis. P values <0.05 were considered significant. All calculations were performed using the Microsoft Excel and SAS 9.3 software.

Ethics

The study was conducted in accordance with the requirements of ethics, with particular regard to the protection of sensitive data. No additional consent from the bioethics committee was required due to the retrospective nature of the studies. Parents (legal guardians) and study

participants gave informed consent for the later use of anonymised data.

Results

There were 559 (50.6% male) cases of type 1 diabetes diagnosed before age 14. The incidence ratio ranged significantly ($p < 0.001$) from the lowest in 2006 (11.2/100,000/year) to the highest in 2012 (21.9/100,000/year) (Figure 1). The median (interquartile range) age of diagnosis was 8.4 (5.4 to 11.1) years, and the mean age was 8.2 ± 3.5 years. The mean BMI-SDS was -0.4 ± 1.2, indicating type 1 diabetes onset in individuals somewhat leaner than the reference population. This reflects the weight loss characterizing the onset of diabetes. During the study period, there was no trend of decreasing age at the time of diagnosis (Figure 2, $p = 0.43$). Almost all children (91.6%) presented with BMI-SDS within the normal range at the time of diagnosis, with only 16 (2.7%) of cases being obese and 5.7% underweight at the moment of diagnosis (Table 1). There was no association between calendar year of diabetes diagnosis and BMI-SDS ($p = 0.87$, see Figure 3). A significant relationship was observed between the age of diabetes diagnosis and BMI-SDS. An increase in BMI-SDS by 1 SD was associated with the development of the disease 0.54 years of age later (Figure 4) and this association remained unchanged after adjusting for sex and calendar year of diabetes diagnosis ($p < 0.001$). This association is the reverse of what would be expected from the accelerator hypothesis.

Discussion

The incidence of type 1 diabetes, as well as overweight and obesity in children are increasing in Poland (13,17). The present study investigates whether these phenomena are interrelated or only co-exist in the same time and place. This continuous and longitudinal study is the first such study conducted in our country. According to the current definitions, type 1 diabetes mellitus is an autoimmune chronic disease in children and adolescents determined by insulin insufficiency, while type 2 diabetes mellitus is a metabolic disorder in adults and the elderly population, associated with obesity and insulin resistance (13). The *accelerator hypothesis* attempts to unify both types of diabetes as the same insulin secretion disorder, but with a different background (3,6,8). The rate of beta cell damage and insulin loss in type 1 diabetes seems to be associated with more susceptible genotypes and the influence of undefined environmental factors. To date there are more than 60 genomic loci identified, but human leukocyte antigen 6p21 has the strongest association with type 1 diabetes development, and islet autoimmunity develops in about 5% of people with that genetic predisposition (19,20). Contrary to the well-defined genetic background, most of the environmental factors contributing to beta cell loss remain unidentified (21). One of the problems that has been investigated is the possible impact of overweight on the acceleration of insulin insufficiency. In recent years, many trials have been conducted to prove this theory. Studies conducted in different ethnic settings worldwide reported conflicting results (inverse, positive or lack of correlation between body weight and the development of type 1 diabetes). A Norwegian cohort study pointed to an increased risk

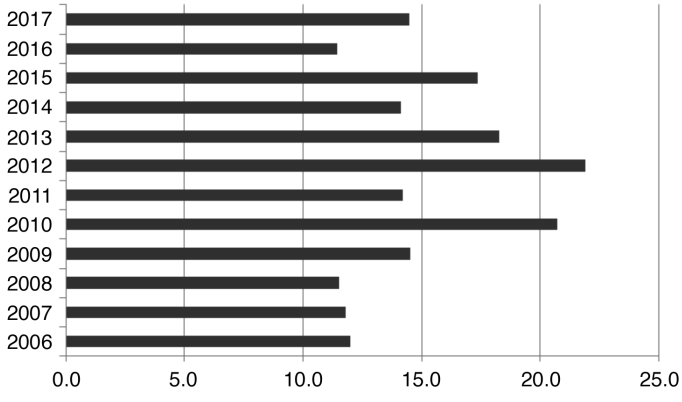

Figure 1. Incidence rates (per 100,000) for type 1 diabetes in Krakow and Lesser Poland region (Krakow only up to 2012 then figures represent the whole of Lesser Poland)

Table 1. The occurrence of obesity (body mass index-standard deviation score > 2.0) in patients with newly diagnosed type 1 diabetes in sequencial years of observation

Year	Number of patients with obesity (BMI-SDS > 2.0) (number of all newly diagnosed type 1 diabetes cases)
2006	0 [21]
2007	1 [20]
2008	2 [21]
2009	0 [22]
2010	0 [23]
2011	0 [27]
2012	0 [22]
2013	5 [97]
2014	1 [75]
2015	2 [92]
2016	1 [61]
2017	4 [78]

BMI-SDS: body mass index-standard deviation score

of autoimmunity in individuals with high-risk genotype with a weight gain of over 15 kg within the first year of life and/or maternal BMI during pregnancy over 30 kg/m^2 (19). Even stronger evidence supporting the *accelerator hypothesis* was provided by the Southeastern Wisconsin study that revealed a significant inverse correlation between BMI and age of diagnosis (9). In a European study, Knerr et al (10) also demonstrated that elevated BMI has an impact on younger age of diabetes onset. Slightly more cautious conclusions were drawn from the study by Dabelea et al (11) that indicated an inverse correlation but only in children with already reduced beta cell function indicated by fasting C-peptide level below the median. In our study, we did not confirm an association between higher BMI-SDS and age at the moment of diagnosis. Indeed, in younger children there was a small association in the opposite direction. Similar observations were also made by authors investigating this phenomenon in various parts of the world. Over 20 years of observation of Australian children under 16 years of age with type 1 diabetes, Islam et al (13) reported that the number of overweight and obese children has remained relatively stable, despite an increase in the incidence rate of type 1 diabetes. Moreover, Derraik et al (12) showed that the mean BMI-SDS of newly diagnosed type 1 diabetes over the period 1990-2009 in New Zealand did not alter in comparison to the general population. Also in our group the incidence of obesity at the moment of type 1 diabetes diagnosis was comparable to the general population living in the same geographic area (17). The prevalence of overweight and obesity is not regulary screened among Polish children. There is no national registry, therefore the precise, reliable data is not available. Nevertheless, in recent years several studies have been published on the prevalence of overweight and obesity in children and adolescents living in different regions of Poland. However, mainly due to differences in research methodology, their results are quite divergent. For example, in Gdańsk, in the North of Poland, obesity was found in 1.5-7.5% of subjects, depending on sex and age at the time of the study (22). In a study conducted among seven-year-olds in a city of Wrocław, in Lower Silesia, South-Western Poland, obesity was found to vary between 10.7-26.6% of children, depending on the place of residence (23). In South-Eastern Poland in 2012, the prevalence of obesity in preschool children was 10.8% (24). In the region covered by our study, the prevalence of obesity in adolescents aged between 14-18 years old is 4.2% (17). To some extent the prevalence of excess weight may be obscured by the fact that anthropometric measurement were taken

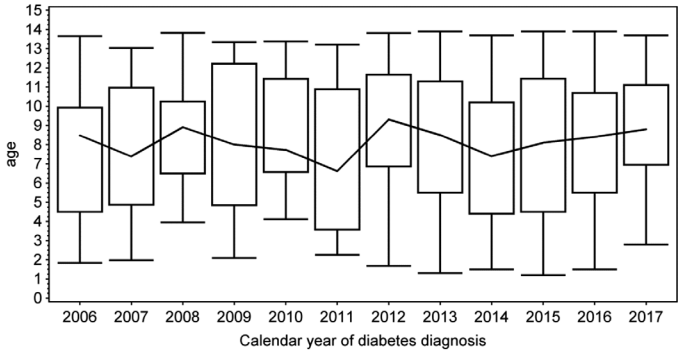

Figure 2. Box plot of mean age at the time of type 1 diabetes diagnosis in sequencially analyzed calendar years. The upper and lower box boundaries indicate quartiles and the line across the centre of the graph joins the medians. The whiskers indicate the range of age in each year

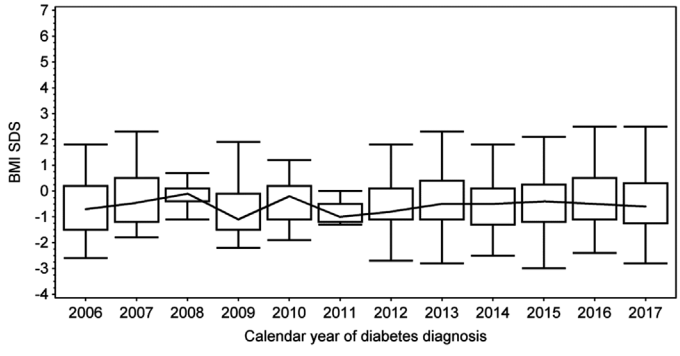

Figure 3. Box plot of mean age at the time of type 1 diabetes diagnosis in sequencially analyzed calendar years. The upper and lower box boundaries indicate quartiles and the line across the centre of the graph joins the medians. The whiskers indicate the range of age in each year

BMI-SDS: Body mass index-standard deviation score

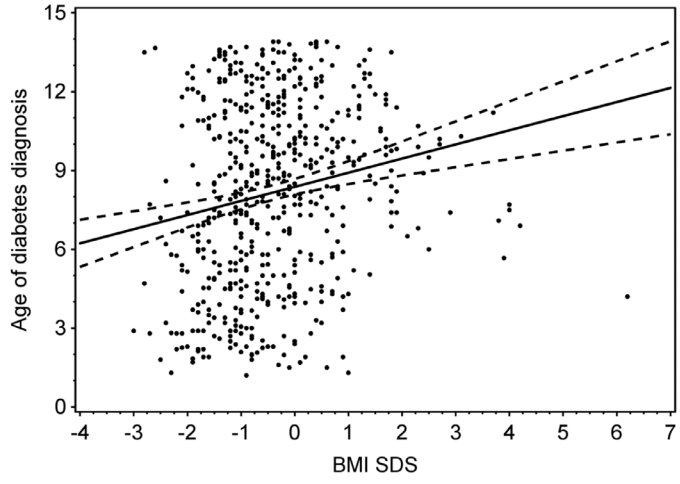

Figure 4. Box plot of mean body mass index-standard deviation score (BMI-SDS) at the time of type 1 diabetes diagnosis in sequencially analyzed calendar years. The upper and lower box boundaries indicate quartiles and the line across the centre of the graph joins the medians. The whiskers indicate the range of BMI-SDS in each year

at diabetes diagnosis. The effect of dehydration may be ruled out, but some degree of loss of body mass has persisted at that time. That is an undoubted limitation of this study, as well as other similar papers. All the weight measurement took place after the diagnosis of the disease on the basis of clinical symptoms. Although the body weight values analyzed came from measurements made after rehydration, resolution of acidosis and improvement of general condition, they cannot take into account the full weight loss associated with lypolysis of adipose tissue before the onset of diabetes. In fact, such an analysis is not possible, because in the first phase type 1 diabetes occurs without clinically overt symptoms and it is not possible to accurately determine its onset, and thus accurate determination of BMI-SDS at the time of actual onset of the disease is also not possible. We were unable to use body weight from before diabetes onset, as regular and standardized measurement were never taken on regular basis in healthy children. Since weight loss would affect all study subjects, we do not expect that this issue has altered the relationships between age of diabetes diagnosis and BMI-SDS. Recently published data from over 360,000 British children and young adults (<25 years old) observed during 1994-2013 did not show any significant correlation between BMI and type 1 diabetes incidence. Interestingly, the authors pointed to a slightly higher incidence rate in overweight, but not in obese children (14). Attempts to include additional ethnic factors showed that the *accelerator hypothesis* is not universal (15). Interestingly, we found that BMI-SDS was significantly higher in the older age groups. An increase in BMI-SDS by 1 SD was associated with the development of the disease 0.54 years of age later. This novel observation seems to be particularly important if we take into consideration the possible impact of puberty on insulin secretion. The relationship between obesity, puberty and type 2 diabetes incidence is clear and well documented (25). During puberty, growth hormone and cortical secretion increases, causing physiological insulin resistance (2). Therefore type 2 diabetes almost never occurs in children before puberty. Our results point to a potential contribution of increased body weight during puberty on the age of type 1 diabetes manifestation. Perhaps, the *accelerator hypothesis* may therefore partly explain the incidence of type 1 diabetes in the period of puberty. However, this would be very difficult to prove but warrants further investigation.

Study Limitations

The main limitation of our study is the inclusion of a relatively small group of participants. However, taking into consideration that we analyzed all new cases of type 1 diabetes under the age of 14 in the homogeneous Lesser Poland population, the results can be considered valuable. To obtain more reliable data, it would be advisable to perform a similar analysis for a national cohort or, optimally, a multinational study.

Conclusion

These results do not corroborate an increase of type 1 incidence in a paediatric population being associated with younger age of diagnosis and higher BMI-SDS. This implies that the *accelerator hypothesis* does not hold true in the studied population.

Ethics

Ethics Committee Approval: Ethics committee approval was not necessary for this kind of study, which was an analysis of anonymous retrospective data from Children's University Hospital database.

Informed Consent: Parents (legal guardians) and study participants gave informed consent for the later use of anonymised data.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Barbara Wasyl-Nawrot, Małgorzata Wójcik, Design: Barbara Wasyl-Nawrot, Małgorzata Wójcik, Data Collection or Processing: Barbara Wasyl-Nawrot, Małgorzata Wójcik, Analysis or Interpretation: Jan Skupień, Małgorzata Wójcik, Literature Search: Barbara Wasyl-Nawrot, Małgorzata Wójcik, Writing: Barbara Wasyl-Nawrot, Małgorzata Wójcik, Jan Skupień, Joanna Nazim, Jerzy B. Starzyk.

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ORIGINAL ARTICLE

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Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: Outcomes of Extended Pilot Study in 241,083 Infants

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What is already known on this topic?

Classical congenital adrenal hyperplasia (CAH) occurs in 1:13,000 to 1:15,000 live births. 21-hydroxylase enzyme deficiency (21-OHD) occurs in 90 to 95% of all cases of CAH. In contrast to 21-OHD, the carrier rate for 11β-OHD is fairly low, the rate of compound heterozygosity of *CYP11B1* mutations in the pathogenesis is less frequent and the estimated overall frequency is 1 in 100,000 live births. However, the prevalence of 11β-OHD is relatively higher in the Middle East and North Africa. Neonatal screening for CAH is effective in detecting the salt-wasting form and thereby reducing mortality. The estimated incidence of classical 21-OHD was 1:7,787 in an initial pilot study of newborn screening for CAH, in which 38,935 neonates were screened in Turkey.

What this study adds?

The incidence of classical 21-OHD and 11β-OHD CAH in Turkey in the screened population of 241,083 neonates was 1:15,067 and 1:60,270, respectively. Turkish neonatal CAH screening led to the early and effective diagnosis of 21-OHD and 11β-OHD by the use of steroid profiling as a second-tier test

Abstract

Objective: Turkish Directorate of Public Health introduced the first pilot screening program for congenital adrenal hyperplasia (CAH) in four Turkish cities in 2017, and in 2018 extended the program, with a slight change in screening strategy, to fourteen cities. To evaluate the performance of the extended study and update previously reported outcomes.

Methods: Retrospective, descriptive study. Neonates of ≥32 gestational weeks and ≥1500 gr birth weight from fourteen cities, born between May-December 2018, were included. Screening protocol included one sample, two-tier testing as applied in the previous pilot study. In the first step, 17α-hydroxyprogesterone (17-OHP) was measured by fluoroimmunoassay in dried blood spots (DBS) obtained at

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3-5 days of life. Cases with positive initial screening underwent second tier testing by steroid profiling in DBS using liquid chromatography-tandem mass spectrometry to measure 17-OHP, 21-deoxycortisol (21-S), cortisol (F), 11-deoxycortisol and androstenedione. The babies with a steroid ratio (21-S + 17-OHP)/F of ≥0.7 (increased from ≥0.5 in the earlier pilot study) were referred to pediatric endocrinology clinics for diagnostic assessment.

Results: In the evaluated period, 241,083 newborns were screened. 12,321 (5.11%) required second-tier testing and 880 (0.36%) were referred for clinical assessment, twenty of whom were diagnosed with CAH (10 females, 10 males). Sixteen were diagnosed as classical 21-hydroxylase deficiency (21-OHD) CAH (12 with salt-wasting and four with simple virilising CAH), and four cases were identified with 11β-OHD CAH. No case of salt-wasting CAH was missed by neonatal screening (sensitivity was 100%). The incidence of classical 21-OHD and 11β-OHD in the screened population was 1:15,067 and 1:60,270, respectively.

Conclusion: Turkish neonatal CAH screening effectively led to earlier diagnosis of 21-OHD and 11β-OHD, using steroid profiling as a second-tier test. This will result in improved care of these patients in the future.

Keywords: Neonatal screening, congenital adrenal hyperplasia, second-tier, steroid profiling, incidence, 11β-hydroxylase deficiency

Introduction

The most common cause of congenital adrenal hyperplasia (CAH) is 21-hydroxylase deficiency (21-OHD), which accounts for about 95% of CAH in most populations. Severe deficiency of the 21-hydroxylase enzyme leads to life-threatening adrenocortical insufficiency in both sexes and varying degrees of pathology of the external genitalia in females, and it is associated with high mortality during the first days of life and increased risk for incorrect sex assignment. Neonatal screening for 21-OHD CAH is effective in detecting the severe forms (classical 21-OHD) and reducing mortality and is helpful in correct sex assignment of female cases. To this end, the Turkish Directorate of Public Health (TDPH) introduced the first Turkish newborn screening (NBS) programme for CAH in 2017 in four Turkish cities as a pilot study. The incidence of classical 21-OHD in the screened population was estimated at 1:7,787, which was high compared to many other countries (1,2). However, this incidence figure required validation in a larger number of babies from nationwide screening.

The NBS programme for CAH adopted a one sample, two-tier testing protocol in the 2017 pilot. This resulted in a recall rate for clinical assessment of 0.54% after a positive second tier test. In the pilot study the second tier test consisted of measuring 17-hydroxyprogesterone (17-OHP), 21-deoxycortisol (21-S), cortisol (F), 11-deoxycortisol and androstenedione using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The ratio of (21-S + 17-OHP)/F was calculated and a cut-off value for this ratio of ≥0.5 resulted in referral to pediatric endocrinology clinics for formal assessment. In 2018, the TDPH extended the screening the programme to fourteen cities including a slightly higher second-tier cut-off of ≥0.7 in screening strategy in order to decrease recall rate and cost of screening without missing classical 21-OHD cases.

Although 11β-OHD is the second most common cause of CAH (5-8%) with an incidence of about 1:100,000, in 2017 one male 11β-OHD CAH case was identified among 38,935 neonates screened. The prevalence of 11β-OHD is reported to be relatively high in the Middle East and North Africa (3). An advantage of the Turkish second-tier screening protocol is that it includes 11-deoxycortisol measurement which is a diagnostic steroid for 11β-OHD CAH, in addition to 17-OHP, 21-S, cortisol and androstenedione. Hence, the adopted second-tier CAH screening strategy would facilitate identification of 11β-OHD cases in addition to those with 21-OHD.

In this study, the results of the Turkish extended NBS programme for CAH were evaluated and the previous outcomes of the earlier pilot study have been updated.

Methods

The extended screening programme for CAH occurred between March and December 2018, in fourteen cities (Konya, Adana, Kayseri, Samsun, Ankara, Gaziantep, Diyarbakır, Mersin, Kahramanmaraş, Elazığ, Erzurum, Malatya, Trabzon, and Van) as directed by TDPH. The CAH screening algorithm was the same as used in 2017 (1) with the exception of an increased steroid profile ratio cut-off for second-tier testing (Figure 1). Heel-prick blood samples were studied, as previously described (1). Initial CAH screening was based on the measurement of 17-OHP in dried blood spot (DBS) on filter paper by fluoroimmunoassay (Labsystems Diagnostics, Finland). Cut-off values for 17-OHP were based primarily on gestational age and birth weight. 17-OHP values of 10 ng/mL and 15 ng/mL have been used as cut-off points for newborn babies ≥36 gestational weeks (gw) and/or ≥2500 grammes (gr) birth weight, and for newborn babies between 32-36 gw and/or 1500-2500 gr birth weight, respectively. If the 17-OHP level was above the cut-off level in the first-tier test, the filter paper was directly analyzed by LC-MS/MS for a steroid profiling assay. This simultaneously analyzes 17-OHP, 21-deoxycortisol (21-S), cortisol (F), androstenedione

(4AS) and 11-deoxycortisol (11-S). Normal values for babies 32-36 weeks and/or 1500-2500 gr were; 17-OHP: < 8 ng/mL, 21-S: < 1.5 ng/mL, F: > 50 ng/mL, 4AS: < 4.5 ng/mL and 11-S: < 1.8 ng/mL. Normal ranges for babies ≥36 weeks and/or ≥2500 gr were; 17-OHP: < 1.5 ng/mL, 21-S: < 1.5 ng/mL, F: > 50 ng/mL, 4AS: < 4.5 ng/mL and 11-S: < 1.8 ng/mL. Although all steroids were evaluated in each baby, a steroid ratio ≥0.7 and/or an elevation of 11-S (> 10 ng/mL) were considered as the main criteria for referral for formal clinical evaluation for CAH.

Final calculations of true-positive (TP), false-positive (FP), true-negative (TN) and false-negative (FN) screening results were made. Efficiency of screening protocol was assessed with positive predictive value (PPV), sensitivity and specificity calculated by the following formulas: PPV = TP/(TP + FP); sensitivity = TP/(TP + FN); specificity = TN/(TN + FP).

Ethics

The parents were informed about NBS. Heel-prick blood samples were collected from live-born babies after written consent from the parents was obtained. The study was carried out with the written permission of the Scientific Committee of the TDPH.

Statistical Analysis

Statistical evaluation was performed using GraphPad Prism® V5.0 software (GraphPad Software Inc., San Diego, California, USA). The results for each steroid are reported as mean, standard deviation (SD) or as median (interquartile range) in the text. The 99.8% and 99.5% values of 17-OHP are shown for healthy babies in order to define healthy cut-off values.

Results

A total of 241,083 neonates underwent CAH screening. Of those, 220,367 (91.4%) were ≥36 gw and ≥2500 gr birth weight. There were 16,919 babies (7.0%) between 1500-2500 gr birthweight and 11,017 babies (4.5%) were born between 32-36 gw. In addition 7,220 (2.9%) of the babies were born between 32-36 gw and had a birthweight of 1500-2500 gr.

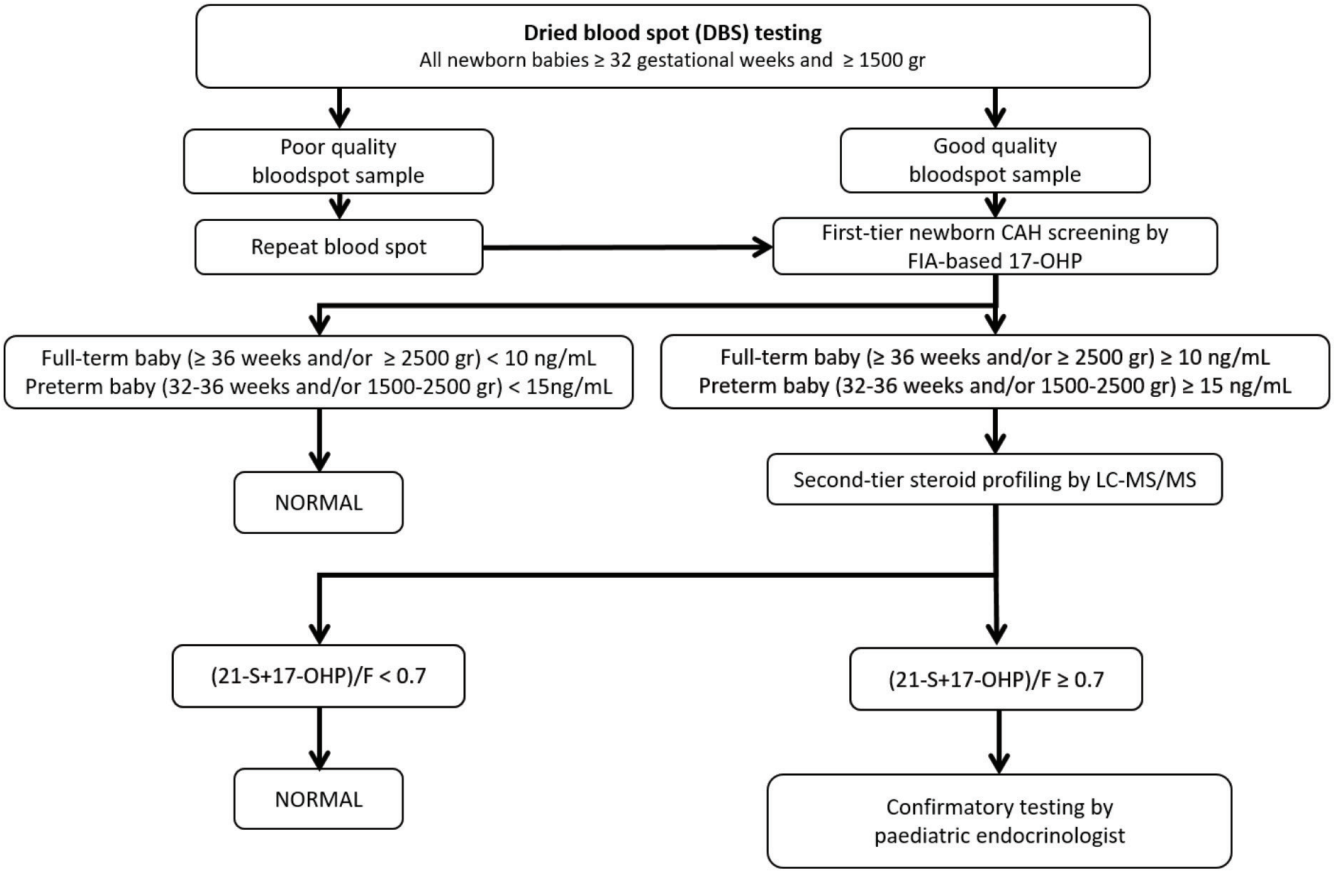

Figure 1. Flowchart for extended neonatal congenital adrenal hyperplasia screening initiated by the Turkish Directorate of Public Health [17-hydroxyprogesterone (17-OHP) conversion factor from ng/mL to nmol/L: multiply by 3.02]

CAH: congenital adrenal hyperplasia, FIA: fluoroimmunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, 17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol

Results of first-tier 17-OHP measurement in DBS from the healthy newborn population (those without CAH) are summarized in Table 1. The 99.8 and 99.5 percentile values for capillary 17-OHP concentration for healthy babies are shown in order to define healthy cut-off values with greater sensitivity (4).

In total 12,321 (5.1%) babies had second-tier testing by LC-MS/MS steroid profiling using a single DBS. During screening the majority of babies that failed to pass the first-tier screen were born between 32-36 gw and/or 1500-2500 gr birthweight and required second-tier testing in comparison to those with a birthweight of ≥2500 gr and/or gestational age ≥36 weeks (Table 2).

Eight hundred and eighty babies, who failed to pass second-tier testing were referred to paediatric endocrinology clinics for further evaluation, which corresponds to an overall recall rate of 0.36%.

Table 3 shows the distribution of second-tier testing values of babies referred for further analysis and results are summarized with respect to gestational age and birth weight. The highest proportion of babies requiring formal assessment had (21S + 17-OHP)/F ratio of between 0.7-1.

An increased level of 17-OHP was observed in the first tier testing of cases with 11β-OHD. On second-tier testing the cases with elevated 11-S (>10 ng/mL) (5) were referred to clinics with the potential diagnosis of 11β-OHD. These cases were further evaluated for 11β-OHD after referral to pediatric endocrinologists.

Consequently, 20 babies were diagnosed with CAH (10 females, 10 males). Sixteen were diagnosed with 21-OHD CAH. Of these sixteen 12 cases had salt-wasting and four cases had simple virilising 21-OHD CAH and four cases were identified with 11β-OHD CAH. No patients with salt-wasting CAH were missed by neonatal screening and thus the sensitivity was 100%. The incidence of classical 21-OHD and 11β-OHD in the screened population was 1:15,067 and

Table 1. Fluoroimmunoassay based 17-hydroxyprogesterone values of screened population according to birth weight and gestational age

17-OHP ng/mL [nmol/L]	1500-2500 gr	≥2500 gr	32-36 gw	≥36 gw	32-36 gw + 1500-2500 gr	≥36 gw + ≥2500 gr
	n = 16,919	n = 224,164	n = 11,017	n = 230,066	n = 7,220	n = 220,367
Mean	7.07	3.62	8.95	3.62	10.08	3.56
	[21.21]	[10.86]	[26.85]	[10.86]	[30.24]	[10.68]
SD	7.42	2.56	8.49	2.55	9.29	2.41
	[22.26]	[7.68]	[25.47]	[7.65]	[27.87]	[7.23]
Min-max	0.05-93.69	0.00-90.00	0.05-93.69	0.00-90.00	0.05-93.69	0.00-90.00
	[0.15-281.07]	[0.00-270.00]	[0.15-281.07]	[0.00-270.00]	[0.15-281.07]	[0.00-270.00]
Median	4.72	3.11	6.19	3.11	7.01	3.09
	[14.16]	[9.33]	[18.57]	[9.33]	[21.03]	[9.27]
IQR	2.98-8.01	2.15-4.39	3.85-11.08	2.15-4.39	4.36-12.56	2.14-4.35
	[8.94-24.03]	[6.45-13.17]	[11.55-33.24]	[6.45-13.17]	[13.08-37.68]	[6.42-13.05]
99.5% p	46.49	15.99	51.93	15.77	55.19	14.89
	[139.47]	[47.97]	[155.79]	[47.31]	[165.57]	[44.67]
99.8% p	56.28	21.07	61.08	21.39	65.39	19.21
	[168.84]	[63.21]	[183.24]	[64.17]	[196.17]	[57.63]

17-OHP: 17-hydroxyprogesterone, min-max: minimum-maximum, SD: standard deviation, gw: gestational week, gr: grammes, IQR: interquartile range 25th-75th percentile.

SI units are given in brackets

Table 2. Rate of second-tier testing among babies based on birth weight and gestational weeks

	1500-2500 gr	≥2500 gr	32-36 gw	≥36 gw	32-36 gw + 1500-2500 gr	≥36 gw + ≥2500 gr
Number of babies	16,919	224,164	11,017	230,066	7,220	220,367
Second-tier testing n (%)	3,130 (18)	9,191 (4)	3,021 (27)	9,300 (4)	2,373 (32)	8,543 (3)

gw: gestational week, gr: grammes

1:60,270, respectively. None of these babies was premature and none had a low birth weight. The definitive diagnoses of CAH cases identified and screening results are presented in Table 4. The screening results of patient number 6 were not available, although the patient was registered as CAH and hospital files recorded that he was referred after a positive screening test. His genetic results were obtained from the molecular diagnostic laboratory and were consistent with salt-wasting 21-OHD. After hydrocortisone and fludrocortisone treatments were started, the patient was lost to clinical follow-up.

The mean ± SD (range) duration from birth to clinical evaluation of abnormal second-tier screening results of the cases was 17.35 ± 5.64 (7-54) days.

Overall PPV, sensitivity and specificity of the current screening protocol for 21-OHD CAH was calculated as 1.9%, 100% and 99.7%, respectively. There were no FN case (Table 5).

Discussion

In this retrospective analysis of extended pilot study of neonatal CAH screening, the incidence in Turkey of 21-OHD and 11β-OHD was determined as 1:15,067 and 1:60,270, respectively. We have analysed the characteristics and efficacy of the extended NBS for CAH and revisited our previous outcomes to enhance screening performance for the upcoming nationwide NBS for CAH in Turkey.

The incidence of classical CAH is approximately 1:14,000 to 1:18,000 in most populations (2), which is similar to our data in this extended CAH screening programme. The incidence of classical 21-OHD was estimated to be 1:7,787 in the initial pilot study, in which 38,935 neonates were screened. The high prevalence of CAH was attributed to a high rate of consanguinity in that study. However, in retrospect the overestimation is more probably due to low number of neonates included in the pilot. In addition, the rate of consanguinity differs in different regions of Turkey. Although the carrier rate for classical 21-OHD is fairly stable (~2%) in the general population, the prevalence of classical 21-OHD may change with the rate of consanguinity. Therefore, we would be able to determine more accurate prevalence of classical CAH once a nationwide NBS for CAH is established in Turkey.

In contrast to 21-OHD, the carrier rate for 11β-OHD is fairly low, the rate of compound heterozygosity of CYP11B1 mutations in the pathogenesis is less frequent and estimated overall frequency is 1 in 100,000 live births (3). However, it has been reported that the prevalence of 11β-OHD is relatively higher in the Middle East and North Africa (3). In this study, the incidence of 11β-OHD was found to be 1:60,270, which is comparable to the approximate incidence of 1:40,000 in the earlier pilot study (1). Based on the more recent findings, real life data on the incidence of 11β-OHD in Turkey shows that the incidence is twice as common as the estimated overall frequency reported by Khattab et al (3) The diagnosis 11β-OHD CAH is generally delayed and can cause significant morbidities including arterial hypertension, precocious pseudopuberty, genital virilization and testicular adrenal rest tumors. Inclusion of 11-deoxycortisol in our second-tier CAH screening strategy, made it possible to identify cases with 11β-OHD in addition to cases with 21-OHD. Early diagnosis and treatment of these cases may reduce the morbidity.

There was no mortality due to unrecognized classical CAH among screened cohort in the extended NBS programme. However, one of the failings of the pilot study was the delay in recall of positive screening results. In the extended screening programme the mean ± SD duration from birth to clinical evaluation of abnormal screening test results of false positive cases was reduced to 17.35 ± 5.64 from 25.8 ± 6.4 days in the pilot. Nevertheless and despite the fact that there has been no mortality to date, this reduction in time to clinical evaluation does not suggest improved safety for the screening program as a salt-wasting crisis may develop due to the long current recall time. As seen in our current and previous data, all cases with salt-wasting 21-OHD had significantly elevated concentrations of 17-OHP

Table 3. Distribution of babies based on (21-deoxycortisol + 17-hydroxyprogesterone)/cortisol ratio adjusted for gestational age and birth weight

(21S + 17-OHP)/F ratio	1500-2500 gr	≥2500 gr	32-36 gw	≥36 gw	32-36 gw + 1500-2500 gr	≥36 gw + ≥2500 gr
0.7-1.0	164	329	170	323	136	295
1.0-2.0	147	162	149	160	126	139
2.0-5.0	35	19	33	21	31	17
≥5.0	7	17	5	19	4	16
Total (n)	**353**	**527**	**357**	**523**	**297**	**467**

21-S: 21-deoxycortisol, 17-OHP: 17-hydroxyprogesterone, F: cortisol, gw: gestational weeks, gr: grammes

Table 4. Biochemical and molecular characteristics of congenital adrenal hyperplasia (CAH) patients diagnosed in extended pilot study of neonatal CAH screening in Turkey

		First-tier Screening by FIA	Second-tier Screening by LC-MS/MS							
Case no	Karyotype	17-OHP (ng/mL)	Cortisol (ng/mL)	Androstenedione (ng/mL)	11-Deoxycortisol (ng/mL)	21-Deoxycortisol (ng/mL)	17-OHP (ng/mL)	(21-S + 17-OHP)/F ratio	Diagnosis	Molecular defect
1	46, XY	90.00	10.70	20.90	1.37	41.90	321.00	36.29	21-OHD (SW)	NA
2	46, XX	90.00	5.44	7.70	0.28	27.23	104.40	24.19	21-OHD (SW)	*CYP21A2* c.293-13C > G (homozygous)
3	46, XX	90.00	8.10	9.15	1.18	28.93	220.00	30.73	21-OHD (SW)	NA
4	46, XY	46.60	2.33	3.98	0.25	43.21	41.47	36.34	21-OHD (SW)	*CYP21A2* p.Gln319* (c.955C > T) + p.Arg317* (c.949C > T)
5	46, XX	95.00	9.04	9.19	1.91	22.94	73.60	10.67	21-OHD (SW)	NA
6	46, XY	NA	NA	NA	NA	NA	NA	NA	21-OHD (SW)	*CYP21A2* p.Arg357Trp (c.1069C > T) (homozygous) + p.Pro454Ser (c.1360C > T) (homozygous)
7	46, XX	90.00	9.04	44.97	5.56	9.14	234.36	26.93	21-OHD (SW)	*CYP21A2* p.Pro30Leu (homozygous) + E6 Cluster (homozygous)
8	46, XX	95.00	6.80	43.77	9.32	0.84	287.35	42.38	21-OHD (SW)	NA
9	46, XY	90.00	6.38	34.28	0.86	24.56	168.37	30.23	21-OHD (SW)	*CYP21A2* p.Gln319* (c.955C > T) (homozygous)
10	46, XY	90.00	13.20	41.00	5.57	18.40	267.10	21.62	21-OHD (SW)	*CYP21A2* del8bp (homozygous) + I2A/C656G (homozygous)
11	46, XY	90.00	4.32	9.13	1.09	4.99	129.90	31.22	21-OHD (SW)	*CYP21A2* I2A/C656G (homozygous)
12	46, XY	90.00	5.10	1.75	0.22	1.28	300.73	59.21	21-OHD (SW)	NA
13	46, XY	29.32	15.52	0.53	0.56	8.64	8.64	1.11	21-OHD (SV)	*CYP21A2* p.Arg357Trp (c.1069C > T) + p.Arg480Trp (c.1438C > T)
14	46, XY	73.70	21.40	1.02	1.09	3.67	22.70	1.23	21-OHD (SV)	NA
15	46, XX	39.59	6.38	0.91	0.95	4.78	5.97	1.68	21-OHD (SV)	NA

Table 4. Continued

Case no	Karyotype	First-tier Screening by FIA	Second-tier Screening by LC-MS/MS							Molecular defect
		17-OHP (ng/mL)	Cortisol (ng/mL)	Androstenedione (ng/mL)	11-Deoxycortisol (ng/mL)	21-Deoxycortisol (ng/mL)	17-OHP (ng/mL)	(21-S + 17-OHP)/F ratio	Diagnosis	
16	46, XX	38.22	4.28	2.55	0.40	20.10	34.76	12.79	21-OHD (SV)	NA
17	46, XX	46.74	0.10	12.90	55.91	0.36	4.96	53.2	11β-OHD	*CYP11B1* c.1201 -9C > A (homozygous)
18	46, XY	15.02	0.69	23.85	109.25	0.21	0.76	1.40	11β-OHD	*CYP11B1* p.Arg448Cys (c.1342 C > T), (homozygous)
19	46, XX	69.02	3.23	30.13	52.50	0.36	5.62	1.85	11β-OHD	NA
20	46, XX	11.58	24.82	9.13	91.92	0.09	1.29	0.05	11β-OHD	*CYP11B1* p.Arg141Ter (c.421C > T), + p.Asn394Argfs*37 (c.1180_1181insGA)

FIA: fluoroimmunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, 17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol, 21-OHD: 21-hydroxylase deficiency, 11β-OHD: 11β-hydroxylase deficiency, SW: salt wasting, SV: simple virilising, NA: Not available.

Conversion factors to SI units: 17-OHP, ng/mL × 3.02 → nmol/L; 4AS, ng/mL × 3.49 → nmol/L; F, ng/mL × 2.75 → nmol/L; 11-S, ng/mL × 2.88 → nmol/L

Table 5. Summary of the results of screening protocol for congenital adrenal hyperplasia in Turkey (March to December, 2018)

Outcome parameter	Result
Total infants screened (n)	241,083
Detection rate of 21-OHD	1:15,067
Detection rate of 11β-OHD	1:60,270
True positives (n)	20
True negatives (n)	241,063
False positives (n)	788
False negatives (n)	0
Positive predictive value for 21-OHD CAH (PPV) (%)	1.9
Sensitivity (%)	100
Specificity (%)	99.7

CAH: congenital adrenal hyperplasia, 21-OHD: 21-hydroxylase deficiency, PPV: positive predictive value

at the first step of screening (≥90 ng/mL in almost all cases). Therefore, we suggest that a remarkably high 17-OHP value on first-tier testing (90 ng/mL), which is strongly suggestive of 21-OHD CAH, should be sufficient to directly recall neonates with such elevated 17-OHP concentrations. This approach would reduce the time to formal assessment and may reduce the risk of mortality due to salt wasting crises, at least in cases with severe 21-OHD.

The recall rate during extended NBS was calculated and compared to the previous pilot study. By increasing the cut-off value for the (21-S + 17-OHP)/F ratio from ≥0.5 to ≥0.7, it was possible to reduce the recall rate from 0.54% to 0.36%. Importantly, this change did not lead to any missed cases with salt-wasting 21-OHD or 11β-OHD in the screened population. Supporting our previous analysis, 493/880 babies (56%) had (21-S + 17-OHP)/F ratio < 1 while this ratio ranged between 10.7-42.3 in salt-wasting 21-OHD cases. The lowest ratio value observed was 1.11 in a patient with simple virilizing 21-OHD and only one 11β-OHD case had a ratio < 1. Therefore, if 1 had been used as the cut-off for (21-S + 17-OHP)/F ratio, the recall rate would decrease by 56% without missing any classical 21-OHD cases.

Over the past 20 years screening programs for CAH have reported variable results for recall rates ranging between 0.002-1.2%, for PPV ranging between 0.1-60% and for sensitivity ranging between 75-100% (5,6,7,8,9,10,11). Despite higher costs, recall rates are lower and PPVs are better with two-tier screening programs. Therefore, two-tier testing may be more appropriate, particularly

in populations with high birth rates and/or high rates of consanguinity. Employing LC-MS/MS as second tier test and the use of cut-off values adjusted for gestational age and birth weight are other important measures to improve PPV (12).

Study Limitations

The prevelance of 21-OHD and 11-OHD was estimated among approximately 240,000 babies screened in this pilot study. This figure may change and may need to be recalculated after nationwide NBS is established in Turkey.

Conclusion

In conclusion, the current NBS strategy for CAH is efficient in identifying cases with both classical 21-OHD and 11β-OHD, which may allow for improved care of these patients and reduce morbidity in the future. However, further improvements to reduce recall time and the recall rate of abnormal screening test results is warranted.

Ethics

Ethics Committee Approval: The study was carried out with the written permission of the Scientific Committee of the TDPH.

Informed Consent: Heel-prick blood samples were collected from live-born babies after written consent from the parents was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: Meltem Çakır, Ayşehan Akıncı, Zerrin Orbak, Mehmet Keskin, Beray Selver Eklioğlu, Alev Ozon, Mehmet Nuri Özbek, Gülay Karagüzel, Nihal Hatipoğlu, Fatih Gürbüz, Filiz Mine Çizmecioğlu, Cengiz Kara, Enver Şimşek, Firdevs Baş, Murat Aydın, Concept: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Design: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Data Collection or Processing: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Analysis or Interpretation: Tülay Güran, Başak Tezel, Alev Ozon, Feyza Darendeliler, Literature Search: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Writing: Tülay Güran.

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ORIGINAL ARTICLE

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Evaluation of the Final Adult Height and Its Determinants in Patients with Growth Hormone Deficiency: A Single-centre Experience from the South-Eastern Region of Turkey

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What is already known on this topic?

Patients who the growth hormone (GH) treatment is started in the prepubertal period are known to achieve better final adult height (FAH) standard deviation score (SDS) and delta height SDS than those started in the pubertal period.

What this study adds?

The present study provides the data for height outcome of a large series of patients who reached the final height with GH therapy in a single center using a fixed dose GH therapy regimen. There was no difference between pubertal and prepubertal patients for FAH-SDS and delta height SDS with this fixed dose GH therapy. Commencement of GH therapy in the prepubertal period was not found to be associated with a better height outcome.

Abstract

Objective: The aim was to determine the final adult height (FAH) achieved by recombinant human growth hormone (rhGH) treatment, the factors affecting FAH and the success of attaining the genetic potential.

Methods: Data of 133 patients treated with rhGH therapy were reviewed retrospectively. Patients were grouped according to diagnosis, either isolated GH deficiency (IGHD) or multiple pituitary hormone deficiency (MPHD), and by sex, and pubertal status at the beginning of treatment.

Results: The mean age of initiation of treatment was 12.3 ± 2.18 years, and the mean duration of rhGH treatment was 3.65 ± 1.5 years. The mean height standard deviation score (SDS) at diagnosis was -3.11 ± 0.75 SD. All patients received a standardized GH dose of 0.033 mg/kg/day. Mean FAH-SDS was -1.8 ± 0.77 and delta height-SDS (the change in height SDS between the beginning and end of treatment) was 1.28 ± 0.94 SD. FAH-SDS was -1.79 ± 0.86 SD in males; -1.82 ± 0.64 in females (p = 0.857); -1.94 ± 0.71 at the beginning of treatment in pubertal patients and -1.68 ± 0.81 in prepubertal patients (p = 0.056); -1.84 ± 0.89 in patients with IGHD and -0.47 ± 0.2 in patients with MPHD (p>0.05). In multiple regression analysis, First year delta height-SDS was the most predictive factor for both FAH-SDS and delta height-SDS.

Conclusion: The majority of our patients achieved a final height compatible with their genetic potential as well as population standards when treated with rhGH even having started at a relatively late age. First year delta height-SDS was a predictive factor for FAH.

Keywords: Isolated growth hormone deficiency, multiple pituitary hormone deficiency, growth hormone treatment, final height, puberty

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Introduction

The goal of growth hormone (GH) therapy is to increase the growth rate in the short term and to improve the final height which, in turn, affects the psychosocial status of the person in the long-term (1). GH also has a positive effect on cardiac function, skeletal structure and body composition due to its impact on bone, lipid, protein and glucose metabolism (2). With the introduction of recombinant GH (rhGH), GH treatment has begun to be applied in a growing number of cases of GH deficiency (GHD), as well as in various diseases with short stature other than GHD (3). Chronic renal failure, Turner syndrome, small for gestational age birth history, idiopathic short stature, SHOX gene mutation, and Prader Willi syndrome are indicated for using different doses of GH (4).

GH is administered subcutaneously with a dose of 0.025-0.1 mg/kg/day (3,5) for six or seven days per week until the final adult height (FAH) is achieved. Factors affecting response to GH therapy have been reported to be frequency, dose, duration of treatment, adherence to treatment, age at onset of treatment, birth length, height standard deviation score (SDS) at the beginning of treatment, parental height, and the first-year response to GH treatment (6,7,8). The growth rate is particularly high in the pubertal period, but there is limited time available for rhGH treatment in pubertal patients. Therefore, a higher dose of rhGH is recommended for patients during puberty (9,10). The aim of the present study was to determine the FAH, factors affecting FAH and the genetic potential of FAH in a group of patients with both isolated GH deficiency (IGHD) and multiple pituitary hormone deficiency (MPHD) with rhGH treatment at a standardized dose of 0.033 mg/kg/day.

Methods

Study Population

In the present study, we recruited 133 patients with IGHD and MPHD who reached FAH from among the 557 patients who had received rhGH treatment in the Pediatric Endocrinology Department of Gazi Yaşargil Training and Research Hospital, a tertiary pediatric endocrine centre, between 2010 and 2018 (Figure 1). The study was performed in accordance with the rules of Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 4.7.2019/7305). The clinical and laboratory findings of the patients were retrospectively reviewed from hospital files.

GH initiation criteria were; to have a height below -2 SD, an annual growth velocity below 25th percentile for age and sex, a GH peak below 10 ng/mL on two GH provocation tests (clonidine and L-dopa), bone age retarded more than 2 SD in prepubertal patients and having open epiphysis in pubertal patients (11). Patients who had skeletal dysplasia, chromosomal disorder including Turner syndrome and Noonan syndrome, systemic disease, intracranial tumour, surgical intervention, radiotherapy and hormone deficiency secondary to chemotherapy were excluded. The patients were grouped into IGHD and MPHD according to the diagnosis and were also grouped into those who started treatment in the prepubertal and pubertal periods. All patients received subcutaneous 0.033 mg/kg/day GH

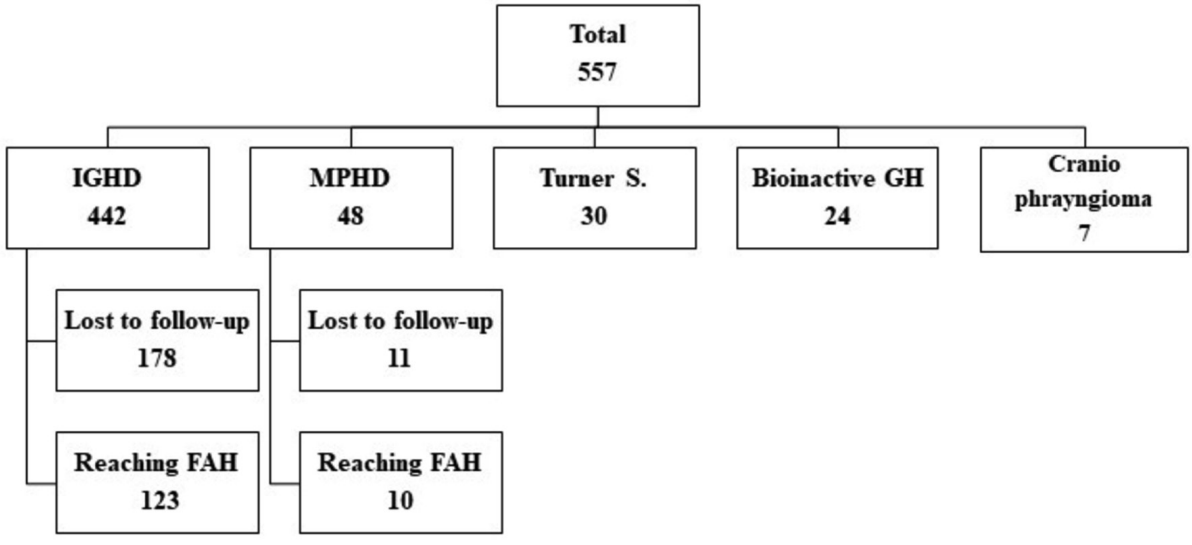

Figure 1. A flowchart of patients with a diagnosis of growth hormone (GH) deficiency who received GH treatment and their follow up

GH: growth hormone, IGHD: idiopathic GH deficiency, MPHD: multiple pituitary hormone deficiency, FAH: final adult height

treatment every day. In patients with MPHD, other deficient hormones were also replaced. Puberty was defined as breast development ≥ Tanner stage 2 in girls and testicular volume ≥4 mL in boys. Testicular volume was evaluated using Prader orchidometer. According to the rules determined by the social security institution in our country, GH treatment is discontinued when the height reaches 155 cm in girls and 165 cm in boys. Besides, during the follow-up of GH treatment, patients who had a height velocity less than 2 cm in nine months and/or whose chronological age was greater than 17 and/or a bone age greater than 14 in females and 15 in males had their GH treatment stopped (6). FAH-SDS, mid parental height (MPH) (mean parental height ± 6.5 cm) of all patients were calculated according to growth charts developed for Turkish children (12). Predicted adult height (PAH) SDS was calculated according to the Bayley-Pinneau method for children older than seven years and the Roche-Wainer-Thissen methods for children younger than seven years. Parent-specific lower limit of height SDS was calculated as (0.5× MPHSDS) -1.73 (13). Delta height SDS was the difference between height SDS at the beginning of the treatment and FAH-SDS. First-year delta height SDS was the difference between height SDS at the beginning of treatment and height SDS at the end of the first year of treatment.

Statistical Analysis

The Statistical Package for the Social Sciences, version 24.0, (IBM Inc., Armonk, NY, USA) was used for statistical analyses. Data were displayed as mean ± SD or median [25-75 interquartile range (IQR)]. Kolmogorov-Smirnov and Shapiro-Wilk tests were used for normality distribution of the data. In order to compare the data, an independent sample t-test was used in the normally distributed groups, and non-parametric tests were used in the non-normally distributed groups. To evaluate the relationship between FAH-SDS and delta height SDS with PAH-SDS, MPH-SDS, first year delta height SDS, treatment duration, age and GHD diagnostic parameters [height SDS, weight SDS, body mass index (BMI) SDS, GH peak, insulin-like growth factor-1 (IGF1) SDS, IGF binding protein-3 (IGFBP3) SDS] Pearson correlation analysis and multiple regression analysis was performed. A p value < 0.05 was considered statistically significant.

Results

A total of 133 patients (54 females; 40.6%) were included in the study. At the beginning of the treatment, 63 (47.4%) of the patients were pubertal and 70 were prepubertal. In addition 123 (92.5%) had IGHD, and 10 (7.5%) had MPHD. Birth weight was lower than -2 SDS in 15 patients. Pretreatment mean height SDS was -3.11 ± 0.75 and ranged from -6.1 to -1.71 SD. Height SDS was below -2 SD in all patients except for a patient with MPHD who had a height SDS of -1.71, who also suffered from recurrent hypoglycemia attributed to the GH deficiency which resolved after rhGH commencement. All patients with a height-adjusted weight below -2 SDS and/or BMI-SDS < -2 were also evaluated for protein-energy malnutrition. In our study, 85 of the patients were underweight according to height-adjusted weight SDS and 16 had BMI-SDS < -2. These patients were followed for at least a one year period with appropriate calorie intake. Patients who had low IGF SDS and low growth velocity in the first year of follow-up, despite appropriate calorie intake, were evaluated for GHD. Mean peak GH in the GH stimulation test was 5.32 ± 2.41 ng/mL which ranged from 0.2 to 9.8 SD, mean IGF SDS was -1.2 ± 1.04 and ranged from -3.4 to 2.1 SD. In total 80.4% of the patients were above the parent-specific lower limit for final height and 70% were within the normal range according to the population. Anthropometric measurements, laboratory values, and comparisons between the groups are shown in Table 1. The mean age of onset of GH treatment and the mean bone age was lower in girls than in boys ($p < 0.001$) (Table 1). There was no statistically significant difference between boys and girls in height SDS, weight SDS, BMI-SDS, GH peak, FAH-SDS, MPH-SDS, PAH-SDS, and delta height SDS (the change in height SDS between the beginning and end of treatment) (Table 1).

At the time of the treatment, age and bone age of pubertal patients were significantly higher than those of prepubertal patients ($p < 0.001$) (Table 1). There was no statistically significant difference between the pre-pubertal and pubertal groups in terms of height SDS, weight SDS, BMI-SDS, peak GH, FAH-SDS, MPH-SDS, PAH-SDS and delta height SDS (Table 1) (Figure 2).

GH peak, IGF1 SDS, IGFBP3 SDS, PAH-SDS were significantly lower in patients with MPHD compared to those with IGHD (Table 1). There was no statistically significant difference between the two groups for height SDS, weight SDS, duration of treatment, FAH-SDS, MPH-SDS, delta height SDS (Table 1) (Figure 3).

IGF1 levels were below -2 SDS in 25 patients, initially. There was no statistically significant difference between the FAH-SDS of patients who had an IGF1 below or above -2 SDS. However, delta height SDS was higher in the group with an IGF1 below -2 SDS.

FAH-SDS had a negative correlation with age and bone age at the beginning of treatment, and a positive correlation

Table 1. Comparison between groups

	Male Mean ± SD	Female Mean ± SD	p*	Pubertal Mean ± SD	Prepubertal Mean ± SD	p*	IGHD Median (IQR)	MPHD Median (IQR)	p†	All Mean ± SD
Age year	13.1 ± 1.72	10.7 ± 2.06	< 0.001	13.2 ± 1.46	11.12 ± 2.24	< 0.001	12 (3.1)	7.58 (2.08)	>0.05	12.3 ± 2.18
Bone age	9.95 ± 2.29	8.2 ± 2.21	< 0.001	10.6 ± 1.49	7.98 ± 2.39	< 0.001	9 (4)	3.75 (1.25)	>0.05	9.21 ± 2.4
Height SDS	-3.12 ± 0.82	-3.11 ± 0.64	0.96	-3.13 ± 0.69	-3.1 ± 0.8	0.83	-3.15 (1.07)	-3.9 (1.2)	>0.05	-3.11 ± 0.75
Weight SDS	-2.43 ± 1.07	-2.47 ± 0.85	0.81	-2.47 ± 0.99	-2.43 ± 0.98	0.81	-2.35 (1.3)	-2.72 (0.8)	>0.05	-2.45 ± 0.98
BMI-SDS	-0.93 ± 1.04	-0.99 ± 1.06	0.76	-1.05 ± 1	-0.87 ± 0.9	0.314	-1.01 (1.02)	-0.27 (1.1)	<0.05	-0.96 ± 1.04
GH peak (ng/mL)	5.34 ± 2.40	5.29 ± 2.48	0.906	5.76 ± 2.25	4.91 ± 2.51	0.044	4.85 (3.43)	0.8 (0.1)	<0.05	5.32 ± 2.41
IGF1 SDS	-1.18 ± 1.01	-1.26 ± 1.09	0.69	-1.21 ± 0.85	-1.2 ± 1.17	0.95	-1.05 (1.13)	-2.37 (1.14)	<0.05	-1.2 ± 1.04
IGFBP3 SDS	-1.36 ± 0.65	-1.13 ± 0.83	0.097	-1.17 ± 0.66	-1.33 ± 0.78	0.22	-1.3 (0.96)	-2.28 (1.8)	<0.05	-1.26 ± 0.73
Tx-time (year)	3.44 ± 1.4	3.93 ± 1.62	0.07	2.79 ± 0.91	4.44 ± 1.52	< 0.001	3.7 (2.04)	7.5 (5.33)	>0.05	3.65 ± 1.5
FAH-SDS	-1.79 ± 0.86	-1.82 ± 0.64	0.857	-1.94 ± 0.71	-1.68 ± 0.81	0.056	-1.84 (0.89)	-0.47 (0.2)	>0.05	-1.8 ± 0.77
MPH-SDS	-1.29 ± 0.47	-1.28 ± 0.59	0.944	-1.24 ± 0.61	-1.36 ± 0.43	0.27	-1.1 (0.72)	-0.66 (0.24)	>0.05	-1.3 ± 0.54
PAH-SDS	-1.46 ± 1.11	-1.81 ± 1.04	0.074	-1.67 ± 1.05	-1.54 ± 1.13	0.52	-1.8 (1.05)	-0.22 (0.1)	<0.05	-1.6 ± 1.09
FAH-MPH-SDS	-0.37 ± 0.96	-0.52 ± 0.76	0.39	-0.45 ± 0.84	-0.4 ± 0.9	0.77	-0.43 (0.87)	-0.15 (1.03)	>0.05	-0.21 ± 1.18
FAH-PAH-SDS	-0.34 ± 1.29	-0.73 ± 0.97	0.11	-0.29 ± 1.04	-0.13 ± 1.28	0.46	-0.15 (1.1)	-1.0 (1.81)	>0.05	-0.42 ± 0.87
Delta height SDS	1.28 ± 1.05	1.29 ± 0.78	0.948	1.18 ± 0.83	1.37 ± 1.03	0.46	1.3 (0.79)	3.43 (1.4)	>0.05	1.28 ± 0.94
First year delta height SDS	0.35 ± 0.4	0.5 ± 0.46	0.36	0.35 ± 0.33	0.47 ± 0.51	0.128	0.39 (0.41)	0.71 (0.62)	>0.05	0.42 ± 0.43

*Independent t-test, †Mann-Whitney U test, IQR: interquartile range, Tx: treatment, SDS: standard deviation score, GH: growth hormone, IGHD: idiopathic GH deficiency, MPHD: multiple pituitary hormone deficiency, FAH: final adult height, PAH: predicted adult height, MPH: mid-parental height

with height, weight, and first-year delta height SDS. Delta height SDS was negatively correlated with bone age, height, and weight, IGF SDS, IGFBP3 SDS, and positively correlated with first-year delta height SDS and treatment duration (Table 2). In multiple regression analysis, PAH-SDS, weight SDS, first year delta height SDS were predictive factors for both FAH-SDS and delta height SDS. A first year 1 SD increase in delta height SDS was found to be associated with a 0.68 SD increase in FAH-SDS (35% of variability, 0.18 of error SD) and 0.63 SD increase in delta height SDS (52% of variability, 0.2 of error SD). Besides, the duration of treatment and bone age at the time of the diagnosis was associated with FAH-SDS, whereas they were not associated with delta height SDS (Table 3).

Discussion

In the present study, the FAH achieved with GH therapy was evaluated and the factors affecting FAH in a group of patients who received 0.033 mg/kg/day GH treatment for IGHD and MPHD every day were investigated. Patients with MPHD had achieved a better final height (mean FAH-SDS was -0.47 SD) compared to cases with isolated IGHD (mean FAH-SDS was -1.84 SD).

In studies conducted in our country, by Yordam et al (14) and Kurnaz et al (15), FAH-SDS was reported to be -2.06 and -1.8 respectively, in patients with IGHD who received GH therapy with similar duration and treatment doses (Table 3). In an international study in 1619 patients with IGHD the FAH-SDS was reported to be -1.4 SD (6). Cappa et al (16), Rachmiel et al (17), Straetemans et al (18), Carel et al (19), and Thomas et al (20) reported -0.86, -1.04, -1.74, -1.6, -0.8 FAH-SDS in patients with IGHD, respectively. In all these studies, although the GH treatment dose was similar or lower than our study in IGHD groups, FAH-SDS were better than in our study, and delta height SDS was similar (Table 4). In these studies, the age of onset of treatment was earlier and the duration of treatment was longer. In our country, the duration of treatment is shorter due to the discontinuation of treatment when the height

reaches 155 cm in females and 165 cm in males as a rule determined by social security institution. Better response with lower treatment doses in other studies suggested that treatment dose may not an essential factor affecting FAH-SDS (8). However, the average age of treatment was earlier in these studies which may achieved a better response with lower doses. In patients with MPHD, treatment response and final height have been reported to be better than in patients with IGHD (15,21). However, although the treatment response to MPHD is better, FAH-SDS can be similar to that seen in patients with IGHD (GHD) due to more severe GHD, shorter initial height, and the presence of other hormone deficiencies accompanying GHD (22). In agreement with previous reports, both delta height SDS and FAH-SDS were higher in patients with MPHD than patients with IGHD (6,14,15,16,18). This was attributed to younger age for the onset of the treatment, longer duration of treatment, lower bone age, and lower IGF1 SDS as well as peak GH values in the GH stimulation test. IGF1 SDS and peak GH value in GH stimulation test were lower in patients with MPHD ($p < 0.05$). However, the difference between delta height SDS and FAH-SDS had not

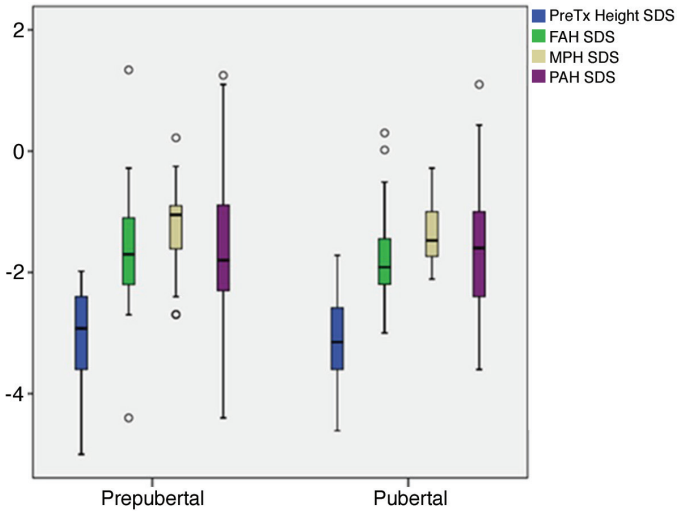

Figure 2. Comparison of final adult height and predictive variables outcomes in prepubertal and pubertal groups (lines within the boxes indicate the median, the limits of the boxes indicate the 25th and 75th percentiles, and the extensions of the boxes indicate the minimum and maximum)

FAH: final adult height, MPH: multiple pituitary hormone, PAH: predicted adult height, SDS: standart deviation score, Tx: treatment

Table 2. Correlation between final adult height standart deviation score (SDS), delta height SDS and other parameters

	FAH-SDS		Delta height SDS	
	r	p	r	p
PAH-SDS	0.248	**0.005**	-0.106	0.241
MPH-SDS	0.127	0.202	-0.046	0.647
Age	-0.228	**0.009**	-0.237	0.208
Bone age	-0.224	**0.01**	-0.237	**0.007**
Height SDS	0.291	**0.001**	-0.577	**< 0.001**
First year delta height SDS	0.293	**0.001**	0.499	**< 0.001**
Tx time	0.14	0.11	0.213	**0.014**
GH peak	-0.028	0.075	-0.143	0.103
IGF1 SDS	-0.022	0.81	-0.203	**0.026**
IGFBP3 SDS	-0.01	0.91	-0.287	**0.002**

Tx: treatment, SDS: standart deviation score, GH: growth hormone, FAH: final adult height, PAH: predicted adult height, MPH: mid-parental height, IGF1: insulin-like growth factor-1, IGFBP3: IGF binding protein-3

Table 3. Multiple linear regression analysis on final adult height standart deviation score (SDS) and delta height SDS

FAH-SDS					**Delta height SDS**			
$R^2 = 0.357$					**$R^2 = 0.523$**			
$p < 0.001$					**$p < 0.001$**			
Variable	**β**	**SE**	**t**	**p value**	**β**	**SE**	**t**	**p value**
PAH-SDS	0.237	0.097	2.45	**0.016**	0.275	0.108	2.55	**0.013**
MPH-SDS	0.17	0.138	1.28	0.202	-0.033	0.146	-0.22	0.822
Age	-0.01	0.007	-1.34	0.182	0.000	0.009	0.04	0.969
Bone age	0.188	0.085	2.21	**0.029**	0.161	0.102	1.58	0.118
Height SDS	0.161	0.160	1.00	0.318	-0.95	0.182	-5.27	**< 0.001**
First year delta height SDS	0.684	0.181	3.77	**< 0.001**	0.632	0.2	3.05	**0.003**
Tx time	0.014	0.006	2.42	**0.017**	0.008	0.006	1.28	0.202
GH peak	0.01	0.032	0.317	0.752	-0.001	0.036	-0.033	0.974
IGF1 SDS	-0.01	0.077	-0.124	0.902	-0.123	0.086	-1.42	0.159
IGFBP3 SDS	-0.018	0.113	-0.16	0.873	0.018	0.121	0.149	0.882

Tx: treatment, SDS: standart deviation score, GH: growth hormone, FAH: final adult height, PAH: predicted adult height, MPH: mid-parental height, IGF1: insulin-like growth factor-1, IGFBP3: IGF binding protein-3, SE: standard error, IGF1: insulin-like growth factor-1, IGFBP3: IGF binding protein-3

Table 4. Comparison of final adult height outcome in previously reported studies

	GHD cause	n	GH dose mg/kg/week	Tx time (year)	FAH-SDS	MPH-SDS	FAH-MPH SDS	Delta SDS
Current study	IGHD	123	0.21	3.7	-1.84	-1.1	-0.43	1.3
	MPHD	10	0.21	7.5	-0.47	-0.66	-0.15	3.4
Kurnaz et al (15)	IGHD	162	0.20	3.3	-1.8	-2.3	0.5	1.4
	MPHD	33	0.20	7.4	-1.6	-1.4	-0.2	2.6
Yordam et al (14)	IGHD	25	0.23	3.8	-2.06	-1.1	NA	NA
	MPHD	12	0.23	4.6	-1.7	-1.01	NA	NA
Darendeliler (KIGS) et al (6)	IGHD	1619	0.2	7.8	-1.4	-1.3	0.0	1.6
	MPHD	554	0.2	10.5	-1.1	-0.8	-0.3	2.6
Cappa et al (16)	IGHD	41	0.23	5.3	-0.86	NA	-0.17	1.1
	MPHD	18	0.17	8.3	-0.6	NA	0.02	2
Racmiel et al (17)	IGHD	96	0.18	5.4	-1.04	-0.49	-0.54	NA
Straetemans et al (18)	IGHD	90	0.2	9	-1.74	-1.6	-0.42	1.6
	MPHD	37	0.2	10.9	-1.35	-1.09	-0.46	2.6
Carel et al (19)	IGHD	1232	0.14	7.9	-1.6	-1.1	NA	1.1
Thomas et al (20)	IGHD with spontanous puberty	49	0.21/0.23	5.2	-0.8	-0.8	0.0	NA
	IGHD with induced puberty	12	0.21/0.23	4.7	-0.0	-0.1	0.1	NA

NA: not available, Tx: treatment, SDS: standart deviation score, GH: growth hormone, IGHD: idiopathic GH deficiency, MPHD: multiple pituitary hormone deficiency, FAH: final adult height, PAH: predicted adult height, MPH: mid-parental height

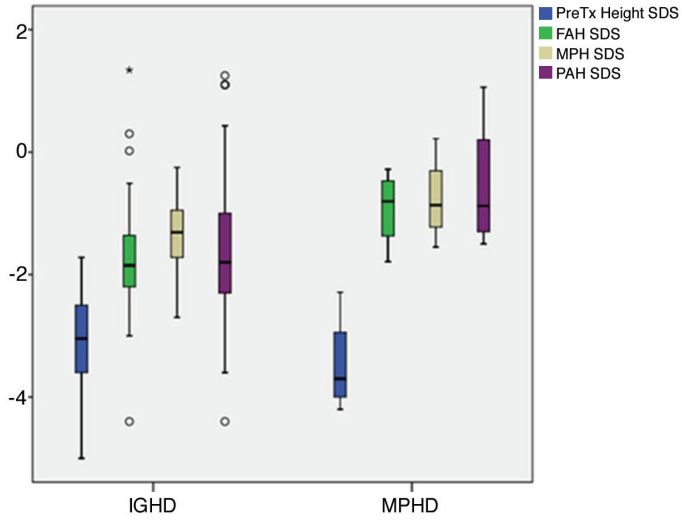

Figure 3. Comparison of final adult height and predictive variables outcomes in idiopathic growth hormone deficiency and multiple pituitary hormone deficiency groups (lines within the boxes indicate the median, the limits of the boxes indicate the 25th and 75th percentiles, and the extensions of the boxes indicate the minimum and maximum)

FAH: final adult height, MPH: multiple pituitary hormone, PAH: predicted adult height, SDS: standart deviation score, Tx: treatment, IGHD: idiopathic growth hormone deficiency, MPHD: multiple pituitary hormone deficiency

reached a statistical significance, presumably due to the small number of patients with MPHD.

Although early diagnosis and treatment with GH can provide a final height consistent with MPH, patients' final height usually remained below the average of the population (6,17). Similarly, in our study, although 80.4% of the patients had reached a final height above parent-specific lower limits, 70% of the patients had a FAH-SDS above -2 SD according to the growth charts determined for the Turkish population. The rate of achieving a FAH consistent with genetic potential has been reported from to be between 81% and 92% (6,15,17,20). The factors affecting the achievement of final height compatible with genetic potential have been reported to include treatment compliance, treatment dose and age of initiation. MPH is one of the important factors affecting the final height in children receiving GH treatment. Treatment response in GHD patients with short parents was also low (23). However, it is important to distinguish between normal children with genetic short stature and patients with GHD whose parents are short. In our study, mean MPH-SDS was -1.3, which was achieved in 80% of the patients diagnosed with IGHD. In studies evaluating the GH treatment response, the mean FAH-MPH has been reported to be between 0.0 and -0.6 SD (6,14,15,16,17,18,19,20). In our study, FAH-MPH-SDS

was -0.43 SD in IGHD and -0.15 SD in MPHD, and the findings were consistent with the literature.

About half of our patients were at an age at which puberty would normally be expected to start when GH treatment was started. Although the chronological age and bone age were higher in the pubertal group and the duration of GH treatment was longer in the prepubertal group, there was no statistically significant difference between the two groups in terms of FAH-SDS and delta height SDS. Similar to our study, Kurnaz et al (15) did not detect a difference between FAH-SDS in pubertal and prepubertal patients but found that delta height SDS was higher in pubertal patients. Cacciari et al (24) reported that pubertal patients had greater SDS gain than prepubertal patients (24). This suggests that GH therapy initiated in puberty has a synergistic effect with the pubertal growth spurt, resulting in a better treatment response than expected. These results indicated that apart from growth factors, growth during puberty may be affected by other individual factors such as the age of onset and rate of pubertal progression (25). However, appropriate GH treatment, even when initiated at the pubertal ages, could help to achieve a final height compatible with genetic height potential.

In our study, PAH-SDS, weight SDS, and first year delta height SDS were predictive factors for both FAH-SDS and delta height SDS. Duration of treatment and bone age were the only predictive factors for FAH-SDS. Among these parameters, the most significant factor in predicting height gain at the end of the treatment was first-year delta height SDS. First year response of GH treatment, is one of the most important predictor of FAH (15,26). During the first year of GH treatment, 1 SD increase in delta height SDS was associated with 0.68 SD increase in FAH-SDS and 0.63 SD increase in delta height SDS in our study. In a study; first year responsiveness was the second relevant predictors of near adult height in IGHD and MPHD (coefficient B: 0.4 and 0.3 respectively) (6).

MPH-SDS has been reported as an important predictor of final height and patients with short parents have a poorer response to treatment (6). In our case, MPH-SDS was not associated with either FAH-SDS or delta height SDS. The high rate of consanguineous marriages in our region may suggest that familial GHD may also be common. Therefore, although we could not evaluate the parents in terms of GHD, achieving a better final height compared to their genetic potential in our patients can be attributed to a high rate of missed GHD in the parents.

Study Limitations

The present study has some limitations which may affect the FAH and delta FAH-SDS. About half of the 557 patients with GH deficiency and GH treatment who had been referred to our clinic from other centres but had become lost to regular follow-up. Therefore, the number of patients who reached FAH was low (Figure 1). Moreover, in the majority of cases, the treatment had to be discontinued before achieving the maximum achievable final height, due to the rules of the social security which funds GH therapy, that the final height of 155 cm for girls and 165 cm for boys were assigned as criteria for stopping the treatment. In addition, we could not evaluate height gain before and after puberty separately in patients in whom GH treatment had been commenced in the prepubertal period.

Conclusion

In conclusion, the majority of our patients have achieved a final height compatible with their genetic potential and population standards even starting at a later age. First year delta height SDS was found to be the most predictive factor for FAH. Interestingly, the commencement of GH therapy in the prepubertal period was not found to be associated with a better height outcome. Recognition of these factors and individualization of the treatment accordingly will help to optimize the long-term response to GH treatment.

Ethics

Ethics Committee Approval: The study was performed in accordance with the rules of Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 4.7.2019/7305).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Concept: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Design: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Data Collection or Processing: Birsen Baysal, Rıza Taner Baran, Analysis or Interpretation: Meliha Demiral, Edip Unal, Literature Search: Mehmet Nuri Özbek, Meliha Demiral, Edip Unal, Writing: Meliha Demiral, Mehmet Nuri Özbek, Hüseyin Demirbilek.

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SHORT COMMUNICATION

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Children with Hashimoto's Thyroiditis Have Increased Intestinal Permeability: Results of a Pilot Study

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What is already known on this topic?

Increased intestinal permeability (IIP) was shown to precede the onset of several autoimmune disorders. Although Hashimoto's thyroiditis (HT) is the most common autoimmune disorder, the role of IIP in its pathogenesis had received little attention. Exact disease mechanisms are poorly elucidated in HT.

What this study adds?

Children and adolescents with HT had increased zonulin levels compared to their age, gender and body mass index matched peers with congenital hypothyroidism. Higher zonulin levels suggested IIP in these patients.

Abstract

Increased intestinal permeability (IIP) precedes several autoimmune disorders. Although Hashimoto's thyroiditis (HT) is the most common autoimmune disorder, the role of IIP in its pathogenesis had received little attention. Zonulin plays a critical role in IIP by modulating intracellular tight junctions. Rise of serum zonulin levels were shown to indicate IIP in human subjects. In this case-control study, we examined the hypothesis that patients with HT have IIP. We studied 30 children and adolescents with HT, and 30 patients with congenital hypothyroidism (CH) matched for age, gender and body mass index (BMI). Serum zonulin levels, free thyroxine (fT4), thyroid stimulating hormone (TSH), anti-thyroglobulin antibody and anti-thyroid peroxidase antibody were measured. Zonulin levels were significantly higher in patients with HT than patients with CH (59.1 ± 22.9 ng/mL vs. 43.3 ± 32.9 ng/mL, $p = 0.035$). In patients with HT, zonulin levels were positively correlated with weight ($r = 0.406$, $p = 0.03$), BMI ($r = 0.486$, $p = 0.006$) and levothyroxine dose ($r = 0.463$, $p = 0.02$). In patients with CH, zonulin levels were positively correlated with age ($r = 0.475$, $p = 0.008$), weight ($r = 0.707$, $p < 0.001$), BMI ($r = 0.872$, $p < 0.001$) and levothyroxine dose ($r = 0.485$, $p = 0.007$). After adjusting for age, weight, TSH and fT4 levels, serum zonulin was only associated with levothyroxine dose in patients with HT ($R^2 = 0.36$, $p = 0.05$). In patients with CH, only weight was associated with zonulin levels ($R^2 = 0.62$, $p < 0.001$). In conclusion, higher zonulin levels in children and adolescents with HT suggested IIP in these patients. Additionally, the association between zonulin levels and levothyroxine dose might imply a relationship between serum zonulin and disease severity.

Keywords: Autoimmune thyroiditis, congenital hypothyroidism, zonulin, increased intestinal permeability

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Introduction

Both genetic predisposition and environmental factors play some role in triggering Hashimoto thyroiditis (HT), but the exact mechanisms are still not fully understood. Increased intestinal permeability (IIP) was shown to be a constant and early feature of several autoimmune disorders (1). Although HT is the most common autoimmune disorder worldwide, the role of IIP in its pathogenesis had received little attention.

Human zonulin regulates intestinal permeability by disassembling the tight junctions (TJ) (2). Zonulin was shown to play a key role in IIP, when up regulated (3). Increased zonulin levels were reported to be correlated with IIP *in vivo* and changes in claudin-1, claudin-2, and myosin *IXB* gene expression (4). Higher zonulin expression was reported in the intestinal tissues of patients with many autoimmune disorders (4). Increased serum zonulin levels were detected in human subjects during the pre-diabetic stage and preceded the onset of type 1 diabetes (4). In a rat model, zonulin-dependent IIP was shown to precede the onset of type 1 diabetes by 2-3 weeks (3). Moreover, oral administration of the zonulin inhibitor (AT-1001) to these rats blocked autoantibody formation, zonulin-mediated IIP, and finally reduced the incidence of diabetes (3). AT-1001 competitively blocks the apical zonulin receptor and prevents the opening of the TJ (2).

In this study, the hypothesis that patients with HT have IIP was investigated. To this end, blood zonulin levels were examined in patients with HT and patients with congenital hypothyroidism (CH) were used as a control group. To the best of our knowledge this is the first study which examines zonulin levels in patients with HT.

Methods

This was a case-control study in a group of 30 consecutive children and adolescents with HT, and 30 age-, gender- and BMI-matched patients with CH. The diagnosis of HT was based on positive anti-thyroid peroxidase antibody (TPO), anti-TG (antibodies against thyroglobulin) levels and the presence of a typical thyroiditis ultrasound pattern. Obese patients and patients with acute or other chronic diseases were excluded from the study. Informed consent was obtained in accordance with a protocol approved by the Institutional Review Board of Sadi Konuk Training and Research Hospital (2017-207).

Anthropometric Measurements

Heights were measured in the standing position with bare feet, using Harpenden equipment (Holtain, United Kingdom), and an electronic scale sensitive to 0.1 kilogram (Seca, Germany) was used for weight measurements. Body mass index (BMI) was calculated according to the standard formula. Height, weight and BMI z-scores standard deviation score (SDS) were calculated according to national standards (5). Obesity was defined as a BMI-SDS ≥2 SDS.

Laboratory Evaluation

Venous blood samples were collected for zonulin, free thyroxine (fT4), thyroid stimulating hormone (TSH), anti-TPO, and anti-TG levels. Samples were immediately processed and sera were stored at -80 °C for subsequent batch analysis. Zonulin levels were measured by human zonulin enzyme-linked immunosorbent assay kit (ELISA, Elabscience, Houston, Texas, USA). Levels of fT4, TSH, anti-TPO and anti-TG were measured by electrochemiluminescence immunoassay (ECLIA, Cobas e602, Roche Diagnostics, Basel, Switzerland).

Thyroid ultrasound results were retrieved from the patient's files.

Statistical Analysis

The Shapiro-Wilk test was used to determine whether variables were normally distributed. Data were presented as mean ± SDS. Comparisons were made by using independent samples t-test or χ^2 test. Pearson analysis was used to determine correlations between zonulin levels and other clinical parameters. Separate linear regression models were created to examine the associations of blood zonulin levels in patients with HT and CH. All statistical analyses were conducted with Statistical Package for the Social Sciences version 15.0 (IBM Inc., Chicago, IL., USA). Statistical significance was defined as $p \leq 0.05$.

Results

Patients' Characteristics

Serum zonulin levels were significantly higher in patients with HT than patients with CH (Table 1, Figure 1). Age, gender, weight SDS, height SDS and BMI-SDS were not different between the groups (Table 1). Serum fT4 levels and daily levothyroxine dose were higher in patients with CH, but TSH levels were not different between the groups (Table 1). Anti-TPO and anti-TG levels were higher in patients with HT (Table 1). Thyroid ultrasonography in patients with HT revealed heterogeneous parenchyma in 17 patients and combined heterogeneous and pseudo-nodular parenchyma in 13 (Table 1). In patients with CH, ultrasonography showed agenesis of the thyroid gland in 15 patients, heterogeneous

Table 1. Comparison of the clinical and laboratory parameters of the patients with Hashimoto thyroiditis and with congenital hypothyroidism (all values are means ± standard deviations, except if otherwise stated)

	Hashimoto thyroiditis n = 30	Congenital hypothyroidism n = 30	p value
Age (years)	12.6 ± 2.7	11.3 ± 3.4	0.11
Gender (F/M)	25/5	22/8	0.35
L-thyroxine dose (µg/kg/day)	1.2 ± 0.84	1.9 ± 0.59	**< 0.001**
Weight SDS	-0.32 ± 1.3	0.01 ± 1.1	0.30
Height SDS	-0.40 ± 1.1	-0.20 ± 1.1	0.42
BMI-SDS	-0.11 ± 1.2	0.09 ± 1.3	0.54
fT4 (ng/dL)	1.28 ± 0.2	1.45 ± 0.2	**0.004**
TSH (mIU/L)	6.5 ± 5.9	7.7 ± 6.3	0.37
Anti-TPO (IU/mL)	268.2 ± 201	11.4 ± 3	**< 0.001**
Anti-TG (IU/mL)	641.4 ± 1076	12.3 ± 3.1	**< 0.001**
USG			
Normal	0	12	
Agenesis	0	15	
Heterogeneous	17	3	**< 0.001**
Heterogeneous and Pseudo-nodular	13	0	
Zonulin (ng/mL)	59.1 ± 22.9	43.3 ± 32.9	**0.035**

Anti-TG: anti-thyroglobulin antibody, Anti-TPO: anti-thyroid peroxidase antibody, BMI: body mass index, F: female, fT4: free thyroxine, L-thyroxine: levothyroxine, M: male, SDS: standard deviation score, TSH: thyroid stimulating hormone, USG: ultrasonography

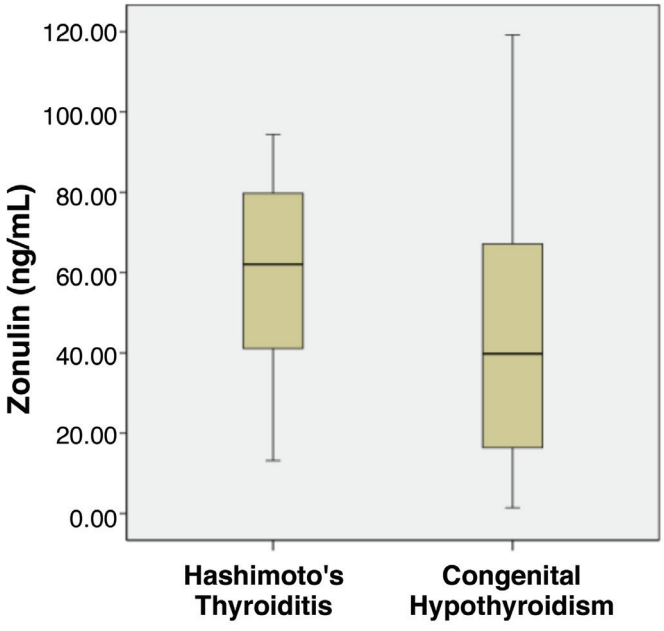

Figure 1. Serum zonulin levels (ng/mL) in patients with Hashimoto thyroiditis and congenital hypothyroidism

parenchyma in three and normal thyroid gland in 12 patients (Table 1).

Correlation Analyses

In patients with HT, zonulin levels were positively correlated with weight ($r = 0.406$, $p = 0.03$), weight SDS ($r = 0.377$, $p = 0.04$), BMI ($r = 0.486$, $p = 0.006$), BMI-SDS ($r = 0.419$, $p = 0.02$) and levothyroxine dose ($r = 0.463$, $p = 0.02$). There was no significant correlation between zonulin levels and anti-TPO or anti-TG levels ($r = -0.174$, $p = 0.4$ and $r = 0.295$, $p = 0.1$, respectively).

In patients with CH, there were strong positive correlations between zonulin levels and age ($r = 0.475$, $p = 0.008$), weight ($r = 0.707$, $p < 0.001$), weight SDS ($r = 0.532$, $p = 0.002$), BMI ($r = 0.872$, $p < 0.001$), BMI-SDS ($r = 0.681$, $p < 0.001$) and levothyroxine dose ($r = 0.485$, $p = 0.007$).

Regression Analyses

In patients with HT, serum zonulin was only associated with levothyroxine dose after adjusting for age, weight, TSH and fT4 levels, but with a borderline p-value ($R^2 = 0.36$, $p = 0.05$).

When the patients with CH were put into the same regression model, there was no significant association between zonulin level and levothyroxine dose ($p = 0.4$). However, serum zonulin was strongly associated only with weight in these patients ($R^2 = 0.62$, $p < 0.001$).

Discussion

Intestinal mucosa is the largest contact site for the host's immune system and exterior antigens, such as food antigens, bacteria, pathogens, and toxins (6). Increase in permeability can cause an exposure of sub-mucosal immune cells to these various antigens and may lead to the development of autoimmune disorders (6,7). Indeed, previous studies have shown that the increased permeability is a key pathogenic component rather than an epiphenomenon of autoimmune disorders (3,4). It was hypothesized that intestinal permeability is also increased in HT (8), but to the best of our knowledge, to date, no study has addressed this issue in these patients. Only in a letter to the editor, Sasso et al (9) described ultrastructural morphological changes of distal duodenum enterocytes in four patients with HT. They also reported IIP, evaluated by a lactulose/mannitol test, in these four patients (9). In our study, it has been demonstrated that children and adolescents with HT had increased zonulin levels compared to their age, gender and BMI matched peers with CH.

Intestinal TJ create gradients for the optimal absorption of nutrients and control tolerance/immunity balance to

non-self antigens (10). Zonulin, a physiological modulator of TJ, is involved in trafficking of macromolecules and, therefore, in balance between tolerance and immune response (2,11). Sturgeon et al (12) identified zonulin as a master regulator of intercellular TJ. They showed that, there was IIP present in zonulin transgenic mice compared to wild-type mice, which was associated with upregulation of zonulin gene expression. Moreover, treatment with AT1001 (Larazotide acetate) reduced intestinal permeability both *in vivo* and *ex vivo* and reverted morbidity and mortality in these zonulin transgenic mice. Their data demonstrated that zonulin-dependent small intestinal barrier dysfunction is an early step leading to the break of tolerance with subsequent development of colitis (12). Similarly, Arrieta et al (13) reported that IL10 gene-deficient mice treated with AT-1001, showed a marked decrease in small intestinal permeability and a clear reduction of colitis severity. In addition, several clinical trials reported a potential beneficial effect of AT-1001 in patients with celiac disease (14). In a study of 339 type 1 diabetic patients and their first degree relatives, Sapone et al (4) showed that patients with type 1 diabetes and their relatives have elevated serum zonulin levels that correlate with IIP associated with altered genetic expression of intestinal TJ proteins. Zonulin upregulation was shown to precede the onset of diabetes, providing a possible link between IIP, environmental exposure to non-self antigens, and the development of autoimmunity in genetically susceptible individuals for type 1 diabetes (4). Our finding of increased zonulin levels in patients with HT may trigger future studies that may lead to potential new therapies for HT.

Increased zonulin levels have been reported with aging and obesity (15). It has been suggested that IIP may play a critical role in the development of age-related and obesity-related inflammation and comorbidities. Similarly, we showed that, serum zonulin levels were strongly correlated with age, weight and BMI, in our study group.

Serum zonulin levels were also positively correlated with levothyroxine dose both in patients with CH and HT. Moreover, in patients with HT, zonulin level was only associated with levothyroxine dose after adjusting for age, weight, TSH and fT4 levels. By contrast, there was no such association in CH patients. This finding might imply a relationship between serum zonulin levels and disease severity in HT, because in patients with CH, levothyroxine dose is mainly based on the amount of residual thyroid tissue/function, age, body weight, and thyroid hormone levels (16). However, in patients in HT, there is another associate of the levothyroxine dose; degree of the thyroid damage by autoimmune mechanisms (17). We did not find a significant correlation between anti-TPO or anti-TG antibodies and serum zonulin levels. However, the contribution of anti-TPO antibodies to thyroid damage compared to T-cell and cytokine-mediated apoptosis in HT is minor and anti-TG antibodies do not cause thyroid cell destruction (18,19). Thyroid damage in HT is mostly due to an apoptotic processes combined with CD8 + cell mediated cytotoxicity, changes in cell junctions, and complement activation (18,19).

Study Limitations

This was a pilot study with small numbers of children. In addition, we did not have a healthy control group to compare zonulin levels in children and adolescents who had normal thyroid function and therefore would not be on levothyroxine therapy.

Conclusion

Higher zonulin levels in children and adolescents with HT suggested IIP in these patients. Further research is needed to expand these results in larger cohorts to gain more insight into the pathogenesis of HT and to lead to potential new treatments.

Ethics

Ethics Committee Approval: Institutional Review Board of Sadi Konuk Training and Research Hospital (2017-207).

Informed Consent: Informed consent was obtained in accordance with the protocol approved by the ethics committee.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Banu Küçükemre Aydın, Melek Yıldız, Abdurrahman Akgün, Neval Topal. Concept: Banu Küçükemre Aydın, Erdal Adal, Hasan Önal, Design: Banu Küçükemre Aydın, Erdal Adal, Hasan Önal, Data Collection or Processing: Banu Küçükemre Aydın, Melek Yıldız, Abdurrahman Akgün, Neval Topal. Analysis or Interpretation: Banu Küçükemre Aydın, Melek Yıldız, Literature Search: Banu Küçükemre Aydın, Melek Yıldız, Abdurrahman Akgün, Neval Topal, Erdal Adal, Hasan Önal, Writing: Banu Küçükemre Aydın, Melek Yıldız, Abdurrahman Akgün, Neval Topal, Erdal Adal, Hasan Önal.

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CASE REPORT

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A Duplication Upstream of *SOX9* Associated with *SRY* Negative 46,XX Ovotesticular Disorder of Sex Development: A Case Report

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What is already known on this topic?

The 46,XX ovotesticular disorder of sex development (DSD) is rarely observed in humans. It is known that excessive expression of *SRY*-related high mobility group box 9 *(SOX9)* is the cause of SRY-negative 46,XX ovotesticular DSD in the absence of *SRY*.

What this study adds?

This is the first case reported in Turkey, exhibiting SOX9 duplication in a patient with SRY-negative 46,XX ovotesticular DSD.

Abstract

The 46,XX ovotesticular disorder of sex development (DSD) is rarely observed in humans. This disorder is generally described as ambiguous genitalia with the presence of ovarian and testicular tissues in different gonads or in the same gonad. Almost no subjects with 46,XX ovotesticular DSD have sex-determining region of the Y chromosome *(SRY)* gene. It is known that excessive expression of SRY-related high mobility group box 9 *(SOX9)* is the cause of *SRY*-negative 46,XX ovotesticular DSD in the absence of *SRY*. Here, we analyzed our *SRY*-negative case with 46,XX ovotesticular DSD. In an array comparative genomic hybridization study using a peripheral blood sample from the patient, a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) in the region of 17q24.3 containing *SOX9* was detected. This is the first case reported from Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD.
Keywords: 46,XX ovotesticular disorder of sex development, *SRY*-negative, *SOX9*

Introduction

Normal gonadal differentiation and sex development depend on the synchronization of the pathways reflecting the effects and interactions of certain genes, transcription factors, and hormones in the genetic sex presence determined by the chromosomal structure. First, ovarian or testicular development from a primitive gonad occurs and then differentiation of internal and external genital structures take place. Modifications of these complex gene regulatory networks or deterioration of the gene expression regulating fetal gonadal development lead to disorders of sex development (DSD) (1,2).

These disorders include a heterogeneous abnormality spectrum in which the chromosomal, genetic, gonadal, hormonal or phenotypical aspects of sex are atypical. The gap in terminology provides a frame for approaching differential diagnosis in a patient. DSD categories include sex chromosome DSDs, such as 45,X/46,XY; ovotesticular DSD; 46,XY DSDs, such as disorders of testicular development, disorders of androgen synthesis and action, and XY sex reversal; and 46,XX DSDs such as masculinization of the XX individual and XX sex reversal. The incidence of DSDs is approximately 1 in every 4000 infants (1).

Usually ovarian differentiation proceeds in the normal way in the 46,XX fetus (1). However, in rare cases, 46,XX gonads can either differentiate in testes, which is known as 46,XX testicular DSD (previously termed XX male), or may cause a condition causing ovarian and testicular tissue to couple in the same individual, known as 46,XX ovotesticular DSD (previously termed true hermaphroditism) (3). Ovotesticular DSD is a rare DSD in which an individual generally has

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ambiguous genitalia and ovarian and testicular tissues are present in separate gonads or in the same gonad; two third of these have the 46,XX karyotype. Molecular studies have revealed that almost 90% of 46,XX patients with ovotesticular DSD were negative for the sex-determining region of the Y chromosome (*SRY*) gene (4). In these *SRY* negative individuals, testicular development may depend on the presence of an additional dose or excessive expression of an autosomal gene which influences male sexual differentiation such as SRY-related high mobility group box 9 (*SOX9*) (5).

To date, the definitive mechanism of testicular differentiation in 46,XX ovotesticular DSD has not been explained. Three putative mechanisms have been proposed to explain testicular determination: (i) Hidden mosaicism with a cell line carrying Y; (ii) translocation of Y-material from paternal Y to the X chromosome including the *SRY* gene; (iii) in a gene which is autosomal or connected to X, defects allowing testicular determination in the absence of *SRY* - *SOX9* is one of these genes (4).

SOX9 duplication is not a common cause of testicular development in cases with *SRY*-negative 46,XX testicular or ovotesticular DSD (6). Only three studies have been published exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD (4,5,7). This study is the first case reported from Turkey, exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD. In addition, a literature review of *SRY*-negative 46,XX ovotesticular DSD is presented and the role of *SRY* and *SOX9* in testicular development is discussed.

Case Report

A three-year and four-month-old male patient was brought due to uncertainty in the genital region. The patient was born through normal vaginal delivery with a birth weight of 3450 g, equivalent to a standard deviation score (SDS) of 0.28, and a birth length of 50 cm (-0.05 SDS) in the 39th gestational week, and this was the 4th pregnancy of the 32-year-old, healthy mother. The patient, whose atypical genitalia was discovered after birth, was examined because of possible DSD in an external center. The parents are non-consanguineous and family members exhibited no clinical manifestations. The family is of Turkish origin. Peripheral blood chromosome analysis was assessed as 46,XX. Medical history when he was 36 days old, showed: serum testosterone (T): 86.69 ng/dL (normal range, 75-400), dihydrotestosterone (DHT): 7.09 ng/dL (normal range, DHT decreases rapidly in the first week, then increases to 12-85 ng/dL between 30-60 days. Levels then decrease gradually to prepubertal values by seven months. Prepubertal children <3 ng/dL). When he was four months old, basal 17-hydroxyprogesterone (17-OHP) concentration was 1.4 ng/mL (normal range, 2 ng/mL), on ACTH stimulation test a peak cortisol response of 22.3 µg/dL and peak 17-OHP concentration of 4.9 ng/mL were observed and adrenal insufficiency was excluded. His hormonal evaluation when he was eight months old showed: Serum T concentration of 12.9 ng/dL (normal range, <3-10); estradiol concentration of <1 ng/dL (normal range, <1.5); follicle-stimulating hormone (FSH) concentration of 0.28 mIU/mL (normal range, 0.16-4.1), luteinizing hormone (LH) concentration of 0.03 mIU/mL (normal range, 0.02-7.0) and prolactin concentration of 10.16 ng/mL (normal range, 3-18). After human chorionic gonadotropin (hCG) stimulation performed to assess testicular functions at the age of one year, serum T was 129.7 ng/dL (normal value, 65-250), and it was considered a sufficient response. Afterward, the patient discontinued his follow-up in the external center.

At the time of writing the patient is three years and four months old. On physical examination body weight was 12.6 kg (-1.69 SDS), height was 89.5 cm (-2.4 SDS) and his head circumference was 50 cm (-0.35 SDS). There was no dysmorphism, scoliosis or skeletal dysplasia. Asymmetry was observed in his genital examination and phallus was 3.9 cm, cordis was observed, there was a single narrow urethral orifice opening to the phallus base. A 1 mL gonad was palpated in the labioscrotal fold on the right, a 1 mL gonad was palpated in the inguinal canal proximal on the left. In Figure 1, the external genital structure is shown. The most recent hormonal assessment of the patient revealed; serum T <1 ng/dL (normal range, <3-10); estradiol <1.2 ng/dL (normal range, <1.5 ng/dL); FSH 0.87 mIU/mL (normal range, 0.26-3.0), LH 0.53 mIU/mL (normal

Figure 1. The genital photograph of the patient was taken at the age of three years and four months

range, 0.02-0.3) and serum anti-Müllerian hormone (AMH) concentration of 19 ng/mL (normal range, 48.0-83.2). The hCG test was repeated with 1500 units of hCG given over three consecutive days. After the test, the serum T was 40 ng/dL (normal value, 65-250 ng/dL).

On pelvic ultrasound tissue was evident in the left inguinal canal with the appearance of ovarian tissue; dimensions were approximately 14x10x5 mm containing millimetric cystic areas. In addition there was testicular tissue with a homogeneous internal structure at the level of the hemiscrotum-labium majus on the right with dimensions 5x7x10 mm. In the left rectovesical area there was an apparent rudimentary structure with a front-back diameter of 5 mm. On abdominal and pelvic magnetic resonance imaging examination, the right testis located in the scrotum and its dimensions were approximately 13x8x11 mm. At the level of the inguinal canal proximal on the left, a structure was observed which again appeared to be ovarian tissue with the dimensions of 15x10 mm containing T2 hyperintense cystic areas that resembled follicular cysts (Figure 2, 3). There is a structure with the appearance of a rudimentary uterus, dimensions 7x5x17 mm, in the left posterior-inferior region (see Figure 4). A biopsy was taken from the wedge and central sections of the bilateral gonads. These gonadal tissues were examined histochemically.

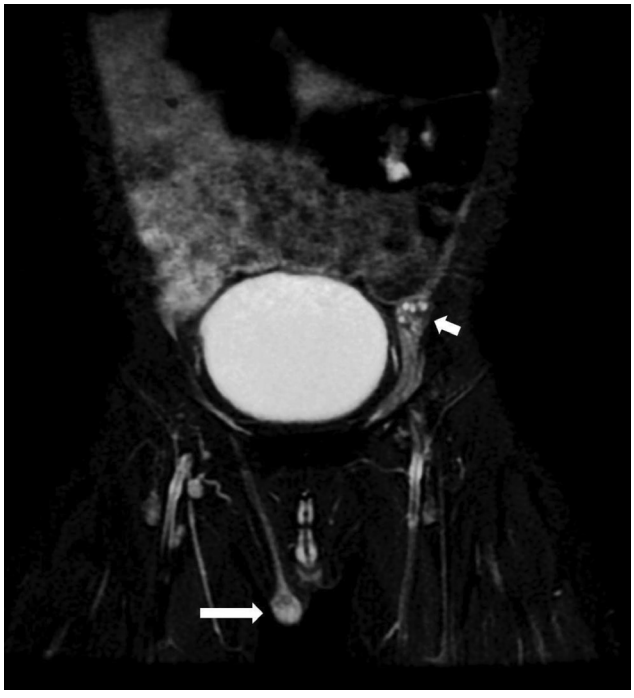

Figure 2. Coronal fat-suppressed T2-weighted image of the magnetic resonance sequence shows testicular tissue in the right scrotum. There is no testicular tissue in the scrotal area on the left. Ovarian tissue with millimetric follicular cysts is present in the left inguinal region

Histologically the right gonad was reported to be testis parenchyma containing a seminiferous tubule structure and the left gonad was reported to be ovarian tissue containing follicular structures at different maturation stages (see Figure 5). The *SRY* gene was not present in peripheral blood leucocytes by fluorescence *in situ* hybridization and polymerase chain reaction (PCR). Moreover, the *SRY* gene was found to be absent on analysis of the gonad biopsy material. In the array comparative genomic hybridization (CGH) study using DNA extracted from the peripheral blood sample (Agilent 8x60K array, Santa Clara, Ca, USA), a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3 (Figure 6). This duplication covers the region from the 1.1 Mb upstream of the *SOX9* gene (including the promoter region) to the 3' UTR region. Furthermore, the array CHG confirmed that there were two copies of the X chromosome and the Y chromosome was absent. Unfortunately blood samples taken from the parents were insufficient and it could not be confirmed whether this variant was *de novo* as blood samples could not be taken again.

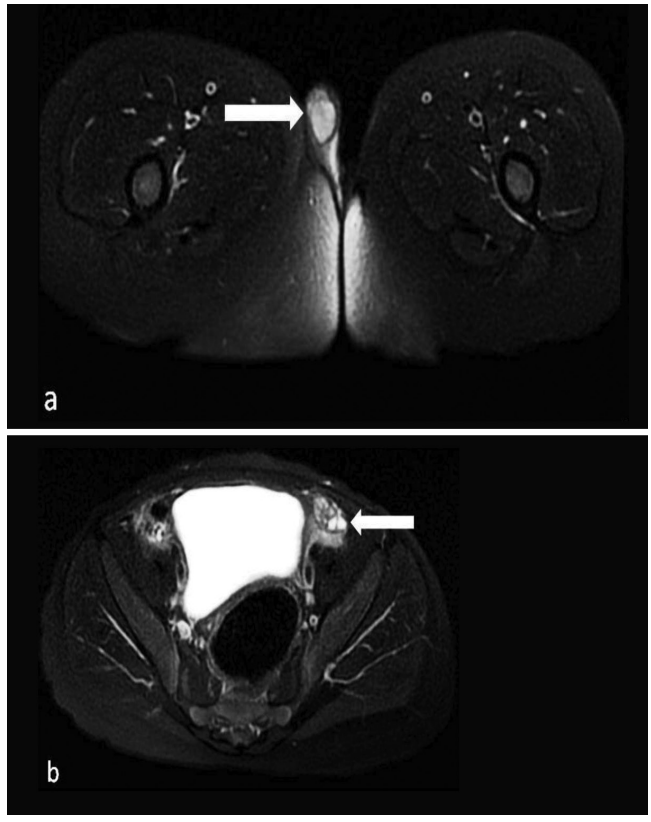

Figure 3. Axial fat-suppressed T2-weighted image of the magnetic resonance sequence shows testicular tissue in the right scrotum. Normal epididymis and spermatic cord are observed in association with this testicular structure (a). Ovarian tissue with millimetric follicular cysts is present in the left inguinal region (b)

The patient was evaluated in the sex research commission. As a result of the psychiatric evaluation of the patient, it was reported that the patient's selection of games and toys complied with the male sex and he selected playmates and clothes in compliance with the male sex according to the information given by the family, and as per the clinical conclusion reached, the patient's development of sexual identity was male-oriented. As a result of the patient's upbringing as a boy, and the family's agreement with male-oriented corrective operations together with the medical and psychiatric evaluations, a decision was made to proceed with male-oriented corrective operations.

Discussion

Ovotesticular DSD is defined as the presence of ovarian tissue with follicles and testicular tissue with seminiferous tubules in the same individual. Ovotesticular DSD is a rare DSD, with variable prevalence and karyotypes in different parts of the world. Although an ovotestis is the most commonly identified gonad in ovotesticular DSD, there may be an ovary on one side and a testis on the other (1). All the studies agree that 46,XX is the most common karyotype observed in blood samples of patients with ovotesticular DSD and the frequency varies between 65% and 90%. In the remaining cases, there is a Y chromosome (46,XY, 46,XX / 46,XY or other mosaic) that explains the development of the testicular tissue (3).

When the testicular tissue differs in a 46,XX *SRY*-negative gonad, two different mechanisms have been proposed: increased expression of the pro-testis genes or insufficient expression of the provarian/anti-testis genes (3).

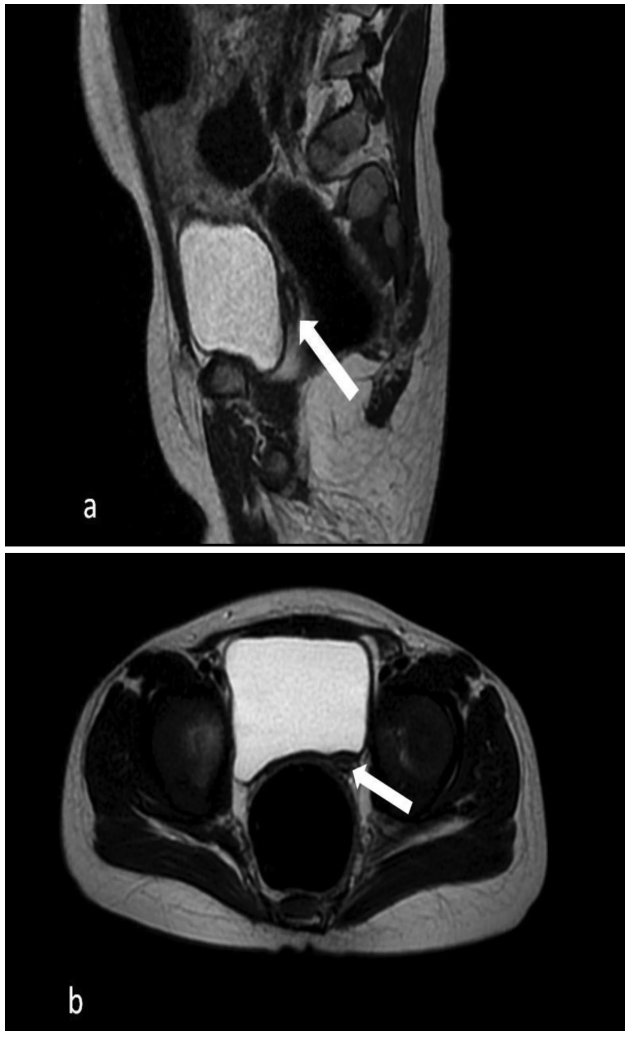

Figure 4. Rudimentaty uterus is shown on the sagittal T2 (a) and axial (b) T2-weighted magnetic resonance images

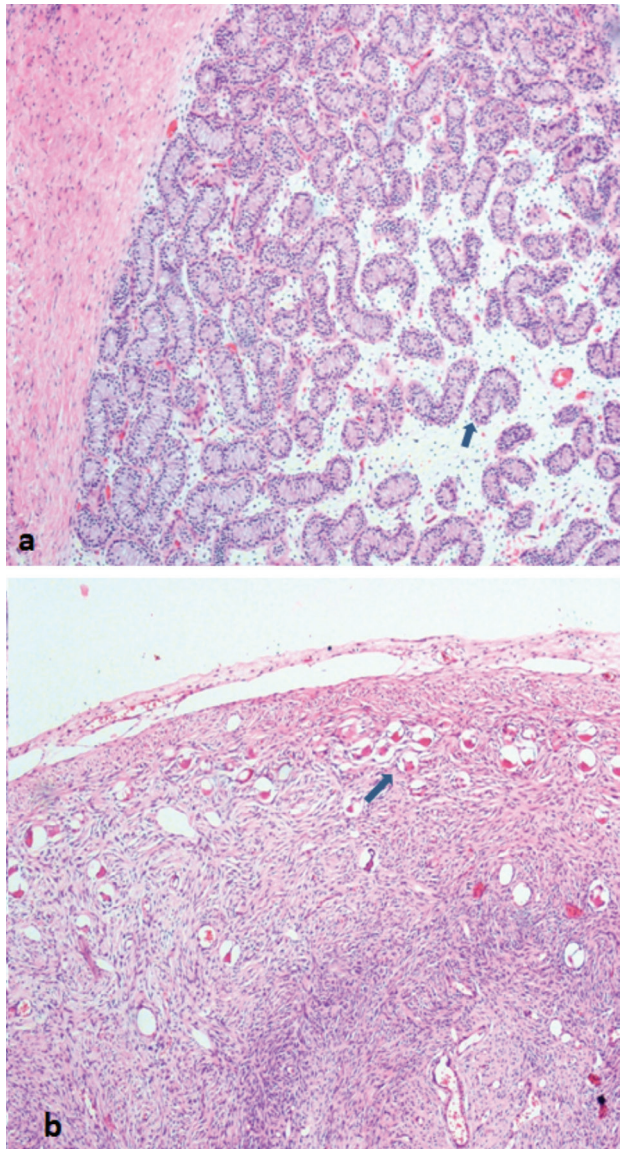

Figure 5. Testicular tissue with seminiferous tubules structure (a). Ovarian tissue containing follicle structures at different stages of maturation (b)

The binary switch responsible for testicular development is the *SRY* gene, located on the short arm of the Y chromosome. The *SRY* protein contains a high-mobility group (HMG) domain and is encoded by a single exon gene. The *SRY* protein is expressed in pre-Sertoli cells, where it triggers a molecular switch to induce Sertoli cell differentiation, thus initiating the process of male sexual differentiation. A threshold *SRY* level must be achieved at a critical time during gestation to establish male sexual differentiation. Otherwise, the ovarian differentiation pathway is activated.

Available data suggest that steroidogenic factor-1 (*NR5A1*) promotes *SRY* expression (1). *SRY* expression is independent of the presence of germ cells. *SRY* increases the expression of the SRY-related HMG box-containing-9 (*SOX9*) gene. *SOX9* is a member of the SRY-related HMG domain gene family located at chromosome 17q24.3-17q25 (1). *SOX9* is expressed in various tissues including chondrocytes and testes. Furthermore, it is found in the bile duct, central nervous system, hair follicles, heart, lungs, pancreas, and retina (3). *SOX9* is highly expressed in Sertoli cells where it functions to promote Sertoli cell differentiation. Phenotype-genotype studies of humans and mice demonstrate that *SOX9* expression is a crucial step, downstream of *SRY*, in testis development. Upstream from the *SOX9* transcription start site, there appears to be a testis-specific enhancer element (*hTES*). *SOX9* then re-regulates fibroblast growth factor 9 and prostaglandin D2, and a positive feedback cycle is established to regulate *SOX9*, which gradually becomes independent of *SRY*. *SOX9* is responsible for the Sertoli cell specification, which in turn leads to initiation of testicular differentiation and AMH production is triggered (1,3).

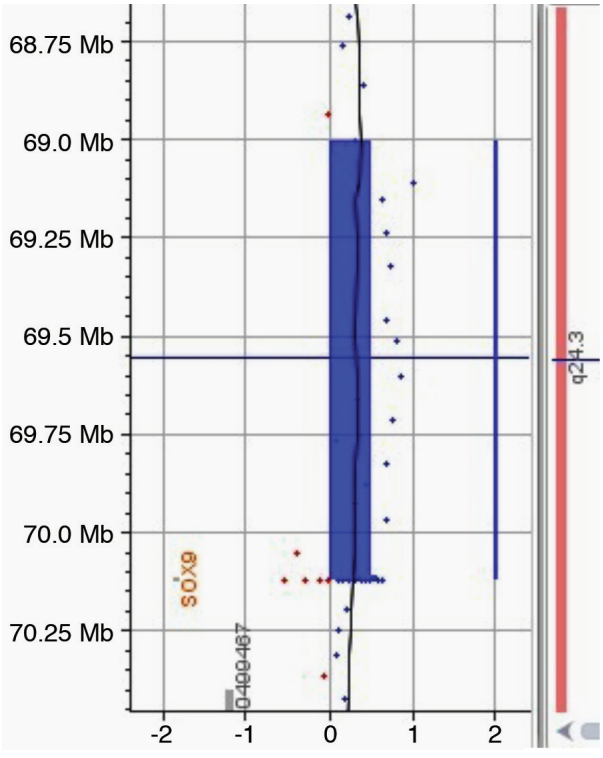

Figure 6. Patient result; in the comparative genomic hybridization study conducted on the peripheral blood DNA, a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3. This duplication covers the region from 1.1 Mb upstream of the *SOX9* gene (including the promoter region) to the 3' UTR region

Most of the *SOX9* duplications were identified in 46,XX testicular DSD patients (4). Firstly, Huang et al (8) (1999) reported an individual who had *SRY*-negative 46,XX testicular DSD and duplication of the 17q chromosome. To date, there have been only three studies of individuals with *SRY*-negative 46,XX ovotesticular DSD and duplication upstream of *SOX9*. Firstly, Benko et al (5) (2011) identified upstream duplications of *SOX9* in three cases with *SRY*-negative 46,XX ovotesticular DSD. These patients exhibited genital virilization to various degrees; one had gonads as bilateral ovotestis, one had ovarian remnant on the left and testis on the right, and the third patient had a streak gonad with partial ovarian differentiation on the right and ovotestis on the left. Molecular studies showed a different level of duplication in each patient. The region about 500 kb upstream of *SOX9* and covering 78 kb is accepted as the sex determination region (RevSex region). These authors asserted that the upstream region duplication of *SOX9* observed in 46,XX DSD patients had one or more regulating elements, which are critical for gonadal development (5). Secondly, Kim et al (7) (2015) reported two patients with 46,XX ovotesticular DSD. An upstream duplication of *SOX9* was found in both patients (including the RevSex region). Lastly, López-Hernández et al (4) (2018) conducted a molecular study on 10 unrelated patients with *SRY*-negative ovotesticular DSD. In only one patient, they found an heterozygosis duplication around 581 kb in the 5' upstream region, including almost all the coding region of *SOX9*. This patient was six months old and brought up as a girl; one of the gonads was an ovary, the other one was an ovotestis.

SOX9 duplication was also detected in 46,XX DSD studies. Vetro et al (9) (2015) analyzed 19 individuals with 46,XX DSD. In a patient with ambiguous external genitalia and 46,XX ovotesticular DSD, they detected a copy containing the gene-desert region upstream of *SOX9*, including the RevSex region. Recently, Ohnesorg et al (10) (2017) reported an individual with 46,XX with ovotesticular DSD with a heterozygous duplication upstream of *SOX9* encompassing a minimal region of 248 kb at 17q24.3.

Consequently, these studies demonstrate the importance of *SOX9* copies in male sexual differentiation with breasts and show that this is a key gene in testicular differentiation. We analyzed our *SRY* negative case with 46,XX ovotesticular DSD, the Y chromosomal sequence was not found in our patient. Therefore, testicular differentiation occurred in our patient in the absence of *SRY*. In the array CGH study a duplication of 1114 kb (Hg19 coordinates: chr17:69006280-70120619) was detected in the region of 17q24.3. This duplication covers the region from 1.1 Mb upstream of the *SOX9* gene (including the promoter region) to the 3' UTR region.

Duplication in the region of 17q that contains *SOX9* is not a common cause of testis development in subjects with *SRY*-negative 46,XX ovotesticular DSD. Seeherunvong et al (6) (2012) analyzed 30 *SRY*-negative people including 23 with 46,XX testicular DSD and seven with 46,XX ovotesticular DSD. They investigated the possible copies of *SOX9* and duplication of the *SOX9* region in 17q was not detected in any subject. Rajender et al (11) used PCR and microsatellite analysis in order to examine a person with *SRY*-negative 46,XX testicular DSD. However, they could not detect a microduplication of *SOX9* (8). In a similar study conducted on twins, one of whom had 46,XX testicular DSD and the other one had 46,XX ovotesticular DSD, neither had *SOX9* duplication (12). Lastly, Temel et al (13) referred to a large family with nine members who had 46,XX testicular or 46,XX ovotesticular DSD. None of the affected individuals in the family group had *SOX9* duplication.

Ovotesticular DSD requires the presence of seminiferous cords and ovarian follicles together with oocytes. The clinical picture does not differ from other types of DSD, there is a range extending from a male phenotype with mild hypospadias and cryptorchidism to a female phenotype with clitoromegaly and minor labial fusion. Internal genitals are usually associated with external virilization and Müllerian derivatives are observed in less virilized patients. Moreover, ultrasonographic evaluation may be deceptive as a diagnostic tool in neonates or infants. Histological analysis of the gonads is mandatory for diagnosis (3). The phenotype of our patient was male-dominant and he had a gonad as the testis and another gonad as ovarian tissue together with the rudimentary Müllerian structure.

T and AMH levels are generally between the normal male and female ranges (14,15). Estradiol levels can reflect the amount of functional ovarian tissues in neonates and teenagers. Gonadotropins may be increased or normal (12,14), the ovarian estrogen effect is reflected in these cases as the testicular tissue is dysgenetic in most conditions. Although the serum T concentration in mini-puberty and the serum T concentration following the hCG test, which was conducted at the age of one year, were adequate, the serum T concentration was substantially decreased in the later hCG test. In addition, the AMH concnetration was found to be under the age specific reference range. This suggested that function of the testicular tissue decreased as the patient became older.

Sex assignment is a problem in these patients and opinions are similar to those expressed in other forms of DSD (16). In patients with ovotesticular DSD, the ovarian tissue can be normal enough to produce oocytes. For this reason, a clinical aim may be the preservation of ovarian tissue and female assignment can be preferred. However, male sex was assigned to our patient. It was concluded that this selection resulted from the patient's development of male sexual identity as a result of his social upbringing as a boy, the family's willingness for male sex and psychiatric evaluation.

Partial gonadectomy requires specific interpretation. For children raised male, the ovarian part must be removed before the age of puberty, in order to prevent estrogen increase resulting in gynecomastia or other heterosexual pubertal development characteristics, and also the cystic folliclular complications that might emerge as a response to high FSH. In rare cases, it has been reported that male patients with a medical history of hypospadias and cryptorchidism presented with cyclic haematuria in puberty (17). In patients raised female, testicular tissue must be removed to prevent virilization in puberty. Regarding the risk of tumor growth in the testicular part, it was reported to be low, even if the tissue was dysgenetic, possibly because the Y chromosomal sequences are not available (18). For our case, a male-oriented corrective operation was planned due to the male sexual assignment.

Conclusion

The *SOX9* gene is considered as the target of *SRY* and thus induces a gene expression resulting in the testicular assignment. Studies have demonstrated the importance of *SOX9* copies in male sexual differentiation with breasts and show that this is a key gene in testicular differentiation. Duplication in the region of 17q that contains *SOX9* is a rare cause of testis development in subjects with *SRY*-negative 46,XX ovotesticular DSD. Such DSDs are very rare and require a careful systematic and sensitive approach to diagnosis and management of the diagnostic and ethical challenges. This case is the first report from Turkey of a patient exhibiting *SOX9* duplication in *SRY*-negative 46,XX ovotesticular DSD.

Ethics

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Eda Mengen, Seyit Ahmet Uçaktürk, Gülsüm Kayhan, Concept: Eda Mengen, Seyit Ahmet Uçaktürk, Design: Eda Mengen, Seyit Ahmet Uçaktürk, Data Collection or Processing: Eda Mengen, Seyit Ahmet Uçaktürk, Gülsüm Kayhan, Analysis or Interpretation: Eda Mengen, Seyit Ahmet Uçaktürk, Gülsüm Kayhan, Literature Search: Pınar Kocaay, Eda Mengen, Seyit Ahmet Uçaktürk, Writing: Eda Mengen.

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CASE REPORT

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Early Onset Diabetes in Two Children due to Progeria, a Monogenic Disease of DNA Repair

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What is already known on this topic?

Less is known about type 2 like diabetes mellitus in children and adolescents with progeria-syndrome although they have a high risk of developing diabetes mellitus.

What this study adds?

Early and regular screening for diabetes mellitus are mandatory. Treatment with metformin at an early stage should be recommended to prevent early symptoms of diabetes and potentially delay the clinical course of progeria

Abstract

Progeria syndrome is a rare disorder in childhood which causes accelerated systemic aging. Due to the accelerated aging process, disorders which normally occur only in old age will appear in these children at a much younger age. We report two children with progeria syndrome, in whom fulminant diabetes mellitus manifested at a very early age.
Keywords: Progeria syndrome, diabetes mellitus, metformin, prevention.

Introduction

Progeria syndrome is a group of very rare genetic disorders which are characterized by premature aging and classified by various names based on causative etiology: Hutchinson-Gilford progeria syndrome (HGPS), Néstor-Guillermo progeria syndrome, atypical progeria syndromes, restrictive dermopathy, mandibuloacral dysplasia, Werner syndrome (WS), Bloom syndrome, Rothmund-Thomson syndrome, Cockayne syndrome (CS), xeroderma pigmentosum, trichothiodystrophy, Fanconi anaemia, Seckel syndrome, ataxia telangiectasia (AT), AT-like disorder, cerebroretinal microangiopathy with calcifications and cysts, and Nijmegen breakage syndrome.

Children affected by progeria syndrome appear normal at birth, but the clinical manifestations become apparent in the first few years of life. Manifestations of progeria syndrome include failure to thrive, dermatologic, musculoskeletal, and neurologic abnormalities and eventually life-limiting cardiovascular disease can occur. Additionally, they may have audiologic, dental, and ophthalmologic issues that impair their lives. Less is known about metabolic complications in children with progeria syndrome. In WS, also known as adult progeroid syndrome, type 2 like diabetes mellitus is one of the clinical manifestations of the disease and attention must give to the differential diagnosis (1).

We report two patients, a boy and a girl, with progeria syndrome in whom fulminant diabetes mellitus manifested very early.

Case Reports

Case 1

Boy with Cockayne Syndrome

The boy was born per section on the 38th week of pregnancy with a birth weight of 2,250 g (1st percentile, z-score -2.42),

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41 cm length (< 1st percentile, z-score -4.39) and 31 cm head circumference (< 1st percentile, z-score -2.79). Within the first year delayed motor development, particularly of the gross motor skills, and delayed linguistic development became evident.

At the age of 22 months the boy was first seen in our social pediatric department due to severe psychomotor retardation, microcephaly and high-grade dystrophic macrosomia. In the following years CS was diagnosed in an external department. CS is one of the progeroid syndromes. At the age of seven years he was severely handicapped with hepatopathy, leukodystrophy and a spastic tetraparesis affecting predominantly the lower limbs (see Table 1). Nutrition was administered via a percutaneous endoscopic gastrostomy. Five days before presenting with diabetes in our department his antiepileptic medication was changed to Levetiracetam. During the previous day he had become progressively weaker and was unusually restless throughout the night with abnormal arm movements.

He presented acutely at the age of seven years with severe hyperglycemia due to hyperglycemic - hyperosmolar syndrome (HHS). This syndrome is characterized by extreme elevations in serum glucose concentrations, hyperosmolality without significant ketosis and a high mortality (2).

The criteria for HHS include: Plasma glucose concentration > 600 mg/dL (33.3 mmol/L), venous pH > 7.25, arterial pH > 7.30, serum bicarbonate > 15 mmol/L, slight ketonuria, absent to mild ketonemia, effective serum osmolality > 320 mosmol/kg and altered consciousness (e.g., obtunded, aggressive) or seizures.

Table 1. Main characteristics of both patients with Progeria syndrome

	Case 1	Case 2
Gender	**Male**	**Female**
Age at diabetes manifestation (years)	7	14
Initial blood sugar (mg/dL / mmol/l)	925 / 51.3	324 / 17.9
Initial HbA1c (%)	7.3	8.6
Initial C-peptid (mg/dL)	9	20.24
Initial serum osmolarity (mosm/kg)	345	-
Initial insulin (mU/L)	-	500
Type of progeria syndrome	Cockayne-syndrome	HGPS
Additional disorders	Hepatopathy Leukodystophy Spastic tertraparesis	Severe coronary heart disease Hyporegenerated anemia
Age of death (years)	9	17

HGPS: Hutchinson-Gilford-Progeria-syndrome

At that time his weight was 8700 g (approx. 12 kg below the third percentile), his length 86 cm (approx. 30 cm below the third percentile) and hypertensive RR-values with 174/147 mmHg. The initial plasma glucose level was 925 mg/dL, pH 7.4, HbA1c 7.3% (56.28 mmol/mol), C-peptide 9 ng/mL and serum osmolality 345 mosmol/kg (275-305 mosmol/kg) (see Table 1). As a result of high insulin sensitivity at that stage and to avoid rapid drop in blood sugar with standardized insulin treatment and development of hypernatremia, the patient was managed on the intensive care unit with meticulous rehydration and a gradual, slow reduction in plasma glucose. There insulin was administered intravenously with great care at 0.025-0.05 IE/body weight/hour. After emergency treatment and clinical recovery, the blood glucose levels could be adequately controlled in relation to tube feeding using a rapid acting human insulin three times daily to the feedings and long acting insulin analogues, which could be stopped subsequently. His blood sugar target under treatment was 150-200 mg/dL (8.3-11.1 mmol/L). Under antihypertensive treatment with captopril his RR-values were stable.

Case 2

Girl with Hutchinson-Gilford Progeria Syndrome

The girl had an existing diagnosis of HGPS when she first presented to our department at the age of 14 years with severe coronary heart disease and hyporegenerative anemia requiring regular blood transfusions. The diagnosis of HGPS has not been genetically proven as the parents refused genetic tests. At first presentation her weight was 13.6 kg (26 kg below the third percentile), her length was 120 cm (31 cm below the third percentile) and her RR-value was 95/55 mmHg. She showed tachycardia with a pulse rate of 120 per minute and oxygen saturation of 92%. As part of the initial investigations she was found to have elevated blood glucose levels; plasma glucose 324 mg/dL (18 mmol/L), HbA1c 8.6% (70.49 mmol/mol), C-peptide 20.24 ng/mL and insulin 500 mU/L (see Table 1). As the patient had already been receiving palliative care, a decision was made in conjunction with the parents that no further active treatment, neither insulin nor metformin, would be administered.

Both children have subsequently died. Their mortality was not related to diabetes.

Discussion

The two children described above had progeria syndrome due to different etiologies. Clinically the girl had HPGS and the boy CS. Both children developed partly fulminant type 2-like diabetes mellitus, which is yet not known in these young patients. Only in WS, the adult form of progeria syndrome, diabetes mellitus due to severe insulin resistance has been reported as a possible clinical manifestation. The possible mechanism of insulin resistance includes reduced insulin receptors in fat cells, loss of signal transduction after the binding of normal insulin to normal receptors and a defective post-receptor step (1). In addition, dysregulation of adipocytokine may be another mechanism for the development of diabetes mellitus in WS patients.

HPGS is due to a mutation in the lamin A *(LMNA)* gene that leads to the production of a truncated and toxic form of LMNA, called progerin (3). Progerin accumulates and triggers growth impairment, lipodystrophy, dermal and bone abnormalities and cardiovascular changes, leading to a shortened lifespan. There is a major rationale for targeting progerin at different levels. Attempts to develop treatment in HGPS associated with progerin accumulation may thus rely on a multi-targetted approach, including decreased progerin production, increased degradation, or downstream noxious cascades (4).

In 2011, the RNA-binding protein SRSF1 (serine/arginine-rich splicing factor 1) was shown to affect alternative splicing of *LMNA* in human HGPS primary fibroblasts and mouse LMNA fibroblast (5). A recent whole-genome transcription analysis has revealed that SRSF1 expression is regulated by the anti-diabetic drug metformin (6). In a current study it has been demonstrated that metformin reduced progerin expression by regulating SRSF1 expression and altering the pathological phenotypes of HGPS cells. After treatment with 5 mmol/L of metformin, a decrease in SRSF1 protein of up to 40% could be demonstrated (3). Therefore, it may be interesting to explore the therapeutic potential of metformin in patients with this form of progeria.

Laminopathies, due to mutations in *LMNA*, encoding A type-lamins, can lead to premature ageing but also to lipodystophic syndromes, showing that these diseases may have related physiopathological mechanisms (7). Lipodystrophy syndromes are frequently associated with hormonal and metabolic derangements resulting in severe comorbidities that depend on the subtype, extent of fat loss, age and gender. Many complications of lipodystrophy are secondary to deficient adipose mass, resulting in ectopic lipid storage in the liver, muscle, and other organs and causing severe insulin resistance. Insulin resistance leads to diabetes, hypertriglyceridemia, polycystic ovarian syndrome and non-alcoholic fatty liver disease (8).

The male patient had proven CS resulting in a severe progeria syndrome. Very recently inherited defects in DNA repair have been identified as the underlying cause (9). DNA maintenance is emerging as a central factor in a multitude of diseases and loss of genomic integrity leads to severe multisystem syndromes. Loss of transcription-coupled repair, which occurs with mutations in twofold excision repair of cross-complementing genes (*ERCC6* and *ERCC8*) results in CS, which is characterized by progressive cachexia, severe growth retardation and leukoencephalopathy. Gradually our understanding of the clinical spectrum of the progeroid syndromes is becoming clearer. Clinical trials of treatment for these monogenic DNA-repair disorders may well be the key to intervention in other diseases associated with genomic damage and perhaps even for aging itself (9).

Due to the therapeutic potential of metformin, children and adolescents with progeria syndrome should be screened for diabetes from a very early age onwards and treated with metformin. Metformin is a well-known anti-diabetic drug, which has demonstrated a good safety profile in millions of patients over the past two decades. In children and adolescents with type 2 diabetes, metformin is the recommended first line therapy and is superior to treatment with sulfonylureas (10,11). Metformin acts through adenosine monophosphate (AMP) kinase in liver, muscle, and fat tissue, with a predominant action on the liver. Hepatic glucose output is reduced by decreased gluconeogenesis. Insulin-stimulated glucose uptake is increased in muscle and fat. Long-term use is associated with a 1-2% reduction in HbA1c (11).

Recently, new pathways in addition to AMPK activation – as discussed above – were discovered, which would explain the additional positive properties of metformin (12). The potential use of metformin as an anti-aging drug and its effect on progerin expression may be interesting to explore in the future.

Unfortunately, neither child was treated with metformin. In the case of the male patient, the evidence had not been published concerning the potential effects of metformin on both the metabolic and disease-progressive course. Treatment with metformin was refused by the female patient and her family because of the palliative care status.

However, for other children with progeria syndrome who are at risk of developing early type 2-like diabetes, treatment with metformin at an early stage should be recommended.

As a result early symptoms of diabetes may be prevented and the clinical course of progeria potentially delayed.

Conclusion

Less is known about type 2-like diabetes mellitus in children and adolescents with progeria-syndrome, although they have a high risk of developing diabetes mellitus. Therefore, early and regular screening for diabetes mellitus are mandatory in these patients. Treatment with metformin at an early stage should be recommended which may prevent early symptoms of diabetes and potentially delay the clinical course of progeria.

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Ethics

Informed Consent: It was not taken.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Martin Holder, Concept: Martin Holder, Design: Martin Holder, Data Collection or Processing: Martin Holder, Analysis or Interpretation: Martin Holder, Valerie Schwitzgebel, Literature Search: Martin Holder, Writing: Martin Holder, Valerie Schwitzgebel.

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CASE REPORT

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Ectopic Posterior Pituitary, Polydactyly, Midfacial Hypoplasia and Multiple Pituitary Hormone Deficiency due to a Novel Heterozygous IVS11-2A>C(c.1957-2A>C) Mutation in the *GLI2* Gene

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What is already known on this topic?

Patients with *GLI2* mutation usually present with multiple pituitary hormone deficiency (MPHD) accompanied by ectopic posterior pituitary, polydactyly and midfacial hypoplasia. Heterozygous mutations in *GLI2* cause a wide range of clinical phenotypes ranging from asymptomatic cases to more severe clinical phenotypes including Culler-Jones syndrome and holoprosencephaly (HPE) or HPE-like syndrome.

What this study adds?

A patient is reported with a novel heterozygous IVS11-2A>C(c.1957-2A>C) mutation in the *GLI2* gene which expands the mutation database. Extremely distinct phenotypical expression and incomplete penetrance of heterozygous *GLI2* mutations may cause MPHD to skip a generation and thus delay or missed diagnosis of these life-threatening hormonal disorders. The response to growth hormone (GH) replacement may be excellent. It is suggested that a trial of GH therapy in cases of *GLI2* mutation with GH deficiency may be beneficial.

Abstract

A novel heterozygous IVS11-2A>C(c.1957-2A>C) mutation in the *GLI2* gene is reported. There was an extremely distinct phenotypical expression in two siblings and their father. The index case was a boy who developed cholestasis and hypoglycaemia in the neonatal period. He had bilateral postaxial polydactyly, mid-facial hypoplasia, high palatal arch, micropenis, and bilateral cryptorchidism. Laboratory examination revealed a diagnosis of multiple pituitary hormone deficiency. There was severe anterior pituitary hypoplasia, absent pituitary stalk and ectopic posterior pituitary on magnetic resonance imaging which suggested pituitary stalk interruption syndrome with no other midline structural abnormality. Molecular genetic analysis revealed a novel heterozygous splicing IVS11-2A>C(c.1957-2A>C) mutation detected in the *GLI2* gene. His father and a six-year-old brother with the identical mutation also had unilateral postaxial polydactyly and mid-facial hypoplasia although there was no pituitary hormone deficiency. This novel heterozygous GLI2 mutation detected appears to present with an extremely variable clinical phenotype, even in related individuals with an identical mutation, suggesting incomplete penetrance of this *GLI2* mutation.

Keywords: Growth hormone deficiency, polydactyly, *GLI2* mutations, multiple pituitary hormone deficiency

Introduction

The sonic hedgehog (SHH) signalling pathway regulates differentiation, proliferation, tissue polarity, stem cell population, and carcinogenesis of the notochord and floor plate in the developing spinal cord (1,2). The SHH signalling pathway is mediated by three related zinc-finger transcription factors (GLI1, GLI2, and GLI3) which are members of the GLI-Kruppel family. GLI2 is an activating zinc-finger transcription factor which plays a crucial role in the development of the diencephalon and distal extremities

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during embryogenesis. It is encoded by the *GLI2* gene, a large polymorphic gene, that is mapped to 2q14.2. Therefore, it is very likely that analysis will show variants of uncertain significance (VUS). Homozygous deletion of both *GLI1* and *GLI2* results in complete absence of the pituitary gland (3). Heterozygous mutations of the *GLI2* gene cause a variety of clinical phenotypes, ranging from asymptomatic cases to more severe clinical phenotypes including Culler-Jones syndrome and holoprosencephaly (HPE) or HPE-like syndrome. Culler-Jones syndrome is a clinical spectrum of multiple pituitary hormone deficiency (MPHD), ectopic posterior pituitary, and postaxial polydactyly with or without midline defects and developmental delay (3). HPE presents with a more severe clinical spectrum with additional midline structural abnormality and forebrain cleavage defects. To date, about 25 different pathogenic *GLI2* mutations have been identified (4). Heterozygous *GLI2* mutations can be inherited in an autosomal dominant fashion or *de novo* (51 % maternal, 40 % paternal, and 9 % *de novo*) (5). Herein, we report a novel heterozygous IVS11-2A > C(c.1957-2A > C) mutation in the *GLI2* gene in two siblings and their father from a non-consanguineous marriage, suggesting an extremely distinct phenotypical expression and incomplete penetrance.

Case Report

Index Case

The proband was a male patient who was born after 40 weeks uneventful gestation via spontaneous vaginal delivery, with a birth weight of 3700 gr. The parents were not consanguineous. Family history revealed that one of his brothers, his father and paternal grandfather had polydactyly and atypical facial appearance with no known hormonal disorders. He had postaxial polydactyly, mid-facial hypoplasia, high palatal arch, micropenis and bilateral cryptorchidism. At the age of two months, he developed cholestasis and hypoglycaemic episodes. Growth hormone (GH), cortisol, and insulin concentrations were measured from critical blood samples which revealed a diagnosis of congenital MPHD (Table 1). Hypoglycaemia and cholestasis resolved with replacement of hydrocortisone and sodium L-thyroxine (L-T4). He had severe anterior pituitary hypoplasia, absent pituitary stalk and ectopic posterior pituitary with no other midline structural abnormality on pituitary magnetic resonance imaging (MRI). A surgical orchidopexy was performed. Diagnosis of GH deficiency was confirmed at the age of one year, and GH replacement therapy was commenced at another paediatric endocrine centre.

The patient was admitted to our hospital for the first time when he was 2.1 years old. He had been on GH replacement therapy for one year. His weight was 9 kg [-3.3 standard deviation score (SDS)] and height was 69 cm (-5.4 SDS). During follow up at our clinic response to the GH therapy was excellent (see Figure 1). At his most recent follow-up visit when he was 10-years-old, his height was 133.5 cm (-0.46 SDS), weight was 28.7 kg (-0.51 SDS), body mass index was

Table 1. Biochemical and hormonal characteristics of the index case and affected relatives

	Index case (two months)	Father (38 years)	Brother (six years)	Lab normal (for index case)
Na (mEq/L)	140	**138**	**137**	135-145
K (mEq/L)	4.5	**4.2**	**3.9**	3.5-5.5
Glu (mg/dL)	17	**97**	**85**	60-100
ALT (IU/L)	24	**38**	**44**	0-40
AST (IU/L)	34	**31**	**43**	0-40
GGT (IU/L)	501			10-61
Total bilirubin (mg/dL)	6.4	**1.1**	**0.8**	0-1.2
Direct bilirubin (mg/dL)	4.8	**0.3**	**0.2**	0-0.3
LDH (IU/L)	309	**181**	**192**	180-430
Calcium (mg/dL)	9.6	**9.2**	**9.5**	8.8-10.8
Phosphorus (mg/dL)	5.3	**4.1**	**3.9**	3.5-5.5
ALP (IU/L)	940	**110**	**147**	150-1076
Cortisol* (µg/dL)	1	**7.2**	**7.2**	5-25
GH* (ng/mL)	0.059	**N/A**	**N/A**	-
Insulin (mIU/L)*	< 2	**N/A**	**N/A**	-
fT4 (ng/dL)	0.4	**1**	**1.25**	0.8-1.9
TSH (IU/L)	0.84	**2.3**	**2.16**	0.4-8.6
Prolactin (ng/mL)	1.99	**7**	**14.5**	3-11
FSH** (mIU/mL)	0.05	**8**	**0.54**	0.7-11.4
LH** (mIU/mL)	0.1	**5.2**	**0.06**	0.8-7.6
Testosterone** (ng/dL)	< 20	**450**	**N/A**	12-21
IGF1 (ng/mL)	< 25	**467**	138	15-109

*Growth hormone (GH), cortisol and insulin were measured from critical blood sample collected during hypoglycaemia. Therefore, GH and adrenocorticotropic hormone deficiency considered due to inappropriate response.

**Gonadotropin [follicle-stimulating hormone (FSH), luteinizing hormone] and testosterone levels were considered low as these were collected during minipuberty.

LH: luteinizing hormone, ALT: alanin aminotransferaz, AST: aspartat transaminaz, GGT: gamma-glutamyl transferase, LDH: lactate dehydrogenase, ALP: alkaline phosphatase, TSH: thyroid stimulating hormone, IGF1: insulin-like growth factor 1, fT4: free thyroxine, N/A: not available

16.1 kg/m^2 (-0.4 SDS). He had no signs of puberty. He had bilateral postaxial polydactyly, mid-facial hypoplasia, high palatal arch and moderate developmental delay. He was on L-T4 (2.6 µg/kg/day), GH (with a dose of 0.033 mg/kg/day), hydrocortisone and antiepileptic therapy for focal epileptic seizures.

The patient's brother was six-years old with a weight of 20.7 kg (-0.01 SDS), and height was 116.2 cm (0.01 SDS). He had normal sized, pre-pubertal testes with no history of undescended testis. He had left postaxial polydactyly and mid-facial hypoplasia with no pituitary hormone deficiency. The patient's father was 38-years-old and his adult height was 166 cm. He also had left postaxial polydactyly and mid-facial hypoplasia with no pituitary hormone deficiency (Table 1). Cranial MRI was not performed in the father and sibling as they had no evidence of pituitary dysfunction.

Molecular Genetic Analysis

Genomic DNA was extracted according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). All coding exons of the *GLI2* gene and their flanking splice site junctions were amplified using in-house designed PCR primers (available upon request). These were subsequently sequenced by the MiSeq next-generation sequencing (NGS) platform (Illumina Inc., San Diego, CA, USA). The libraries were prepared with the NexteraXT kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions. Next-generation sequencing was carried on MiSeq (Illumina Inc., San Diego, CA, USA). Sequences were aligned to the hg19 genome within MiSeq Reporter software (Illumina Inc., San Diego, CA, USA). The data were visualized with IGV 2.3 (Broad Institute; http://exac.broadinstitute.org/) software. Sanger sequencing analysis was performed for confirmation of the variant detected at NGS analysis.

In silico prediction tools (MutationTaster and Human splicing finder) were used for evaluation of the novel unpublished variant. The variant was classified based on the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines (6).

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Local Ethical Committee. Written informed consent was obtained from the participants and their legal guardians.

A novel heterozygous IVS11-2A > C(c.1957-2A > C) mutation in intron 11 of the *GLI2* gene was identifid in the proband (Figure 2). His father and six-year-old brother, who both had postaxial polydactyly and facial dysmorphism with no hormonal deficiency, were also heterozygous for the identical mutation. The unaffected mother and sister had normal alleles. This variant was listed neither in the 1000 genomes

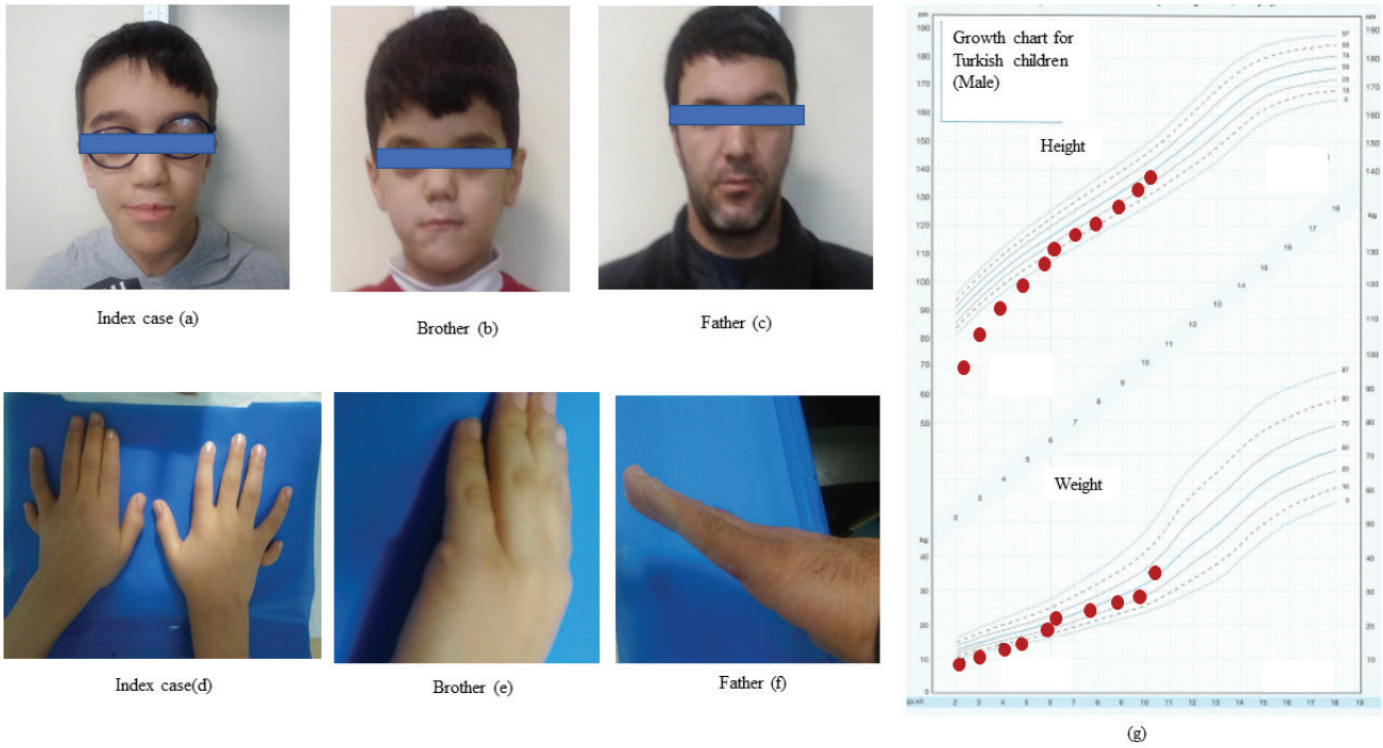

Figure 1. Facial dysmorphism and polydactyly in the index case, brother and father (a-f). Good response to recombinant human growth hormone therapy in the index case (g)

nor in the ExAC database (http://browser.1000genomes.org/index.html, http://exac.broadinstitute.org/, respectively). This mutation in *GLI2* disrupted the intron 11 acceptor splice-site and this was predicted to result in aberrant splicing, and thus synthesis of a truncated protein.

Discussion

Herein, a patient is presented with congenital MPHD, midfacial hypoplasia, bilateral postaxial polydactyly, anterior pituitary hypoplasia and ectopic posterior pituitary due to a novel heterozygous splicing mutation IVS11-2A > C(c.1957-2A > C) in the *GLI2* gene. Clinical features were similar to Culler-Jones syndrome. Although his father and brother with the identical heterozygous mutation had similar physical dysmorphisms, including postaxial polydactyly and mild facial hypoplasia, they had no hormonal deficiency (Table 2).

The heterozygous IVS11-2A > C(c.1957-2A > C) mutation is predicted to cause a splicing defect that would result in aberrantly spliced transcripts, and thus the synthesis of a truncated protein. *GLI2* mutations leading to a truncated protein usually cause panhypopituitarism, polydactyly and midfacial hypoplasia, which were present in our index case. Interestingly, pituitary dysfunction was not detected in the proband's father and brother, both of whom had the identical mutation, suggesting incomplete penetrance and variable expressivity (3,5,7,8). Distinct clinical phenotypes in subjects with identical heterozygous *GLI2* mutations have previously been reported and suggested as evidence for incomplete penetrance and variable expressivity (3,9). The variable expression of the *GLI2* gene mutations has been attributed to the combination of genetic, environmental and epigenetic factors or contribution of the other genes involved in the SHH pathway, which include *SHH*, *ZIC2*, *SIX3*, *PTCH1*, *GLI3* and *TGIF* genes (5,9,10,11).

The largest cohort with *GLI2* variants was reported by Bear et al (5) where a *GLI2* variant was detected in 112 of 400 patients with HPE spectrum, endocrine disorders or craniofacial anomaly. Of these 112, 43 were found to have a truncating mutation (frameshift, nonsense, or large deletion) and 69 were reported to have a VUS (5). The clinical characteristics of cases with *GLI2* mutations reported so far are shown in Table 3 (Supplementary file).

The clinical spectrum of mutations in *GLI2* may vary from asymptomatic individuals to polydactyly, functional

Table 2. Clinical characteristics of index case were different from father and brother with identical *GLI2* mutation and similar to Culler-Jones syndrome

Symptoms	Index case	Father	Brother	Culler-Jones syndrome
Mutation	IVS11-2A > C (c.1957-2A > C)	IVS11-2A > C (c.1957-2A > C)	IVS11-2A > C (c.1957-2A > C)	-
Inheritance pattern	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Facial dysmorphism	+	+	+	+ /-
Polydactyly	Bilateral postaxial polydactyly	Unilateral postaxial polydactyly	Unilateral postaxial polydactyly	Unilateral/bilateral post-axial polydactyly
Cranial midline defect	-	-	-	-
Forebrain cleavage defect	-	-	-	-
Anterior pituitary hypoplasia	+	N/A	N/A	+ /-
Posterior pituitary abnormality	Ectopic posterior pituitary	N/A	N/A	Ectopic posterior pituitary
Pituitary stalk	Interrupted	N/A	N/A	+ /-
GH deficiency	+	-	-	+ /-
TSH deficiency	+	-	-	+ /-
ACTH deficiency	+	-	-	+ /-
Gonadotropin deficiency	+	-	-	+ /-
Prolactin deficiency	+	-	-	+ /-
ADH deficiency	-	-	-	+ /-
Genitourinary system abnormality	Micropenis, cryptorchidism	-	-	+ /-
Developmental delay	+	-	-	+ /-

GH: Growth hormone, TSH: thyroid stimulating hormone, ACTH: adrenocorticotropic hormone, N/A: not available

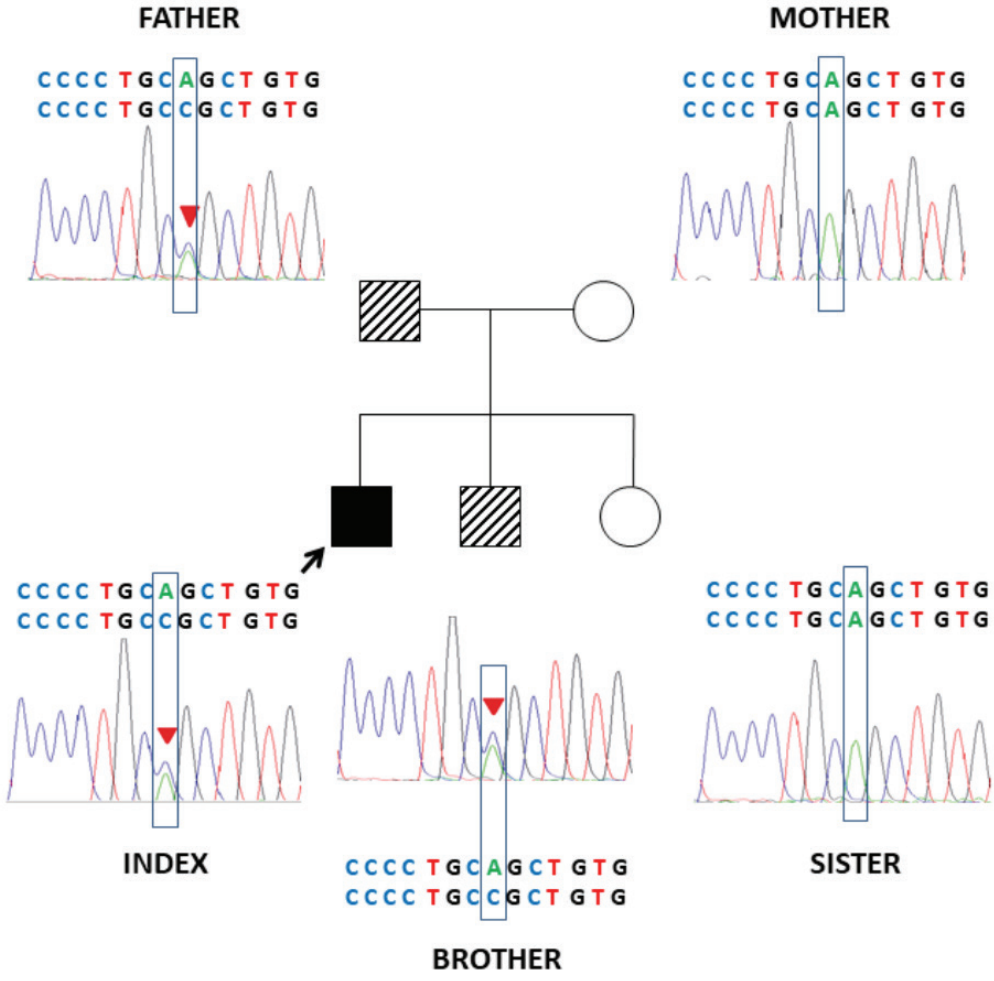

Figure 2. Family pedigree and electropherogram of heterozygous IVS11-2A > C(c.1957-2A > C) mutation in the *GLI2* gene. Full-black filled box indicates index case with Culler-Jones syndrome phenotype, shaded boxes indicate father and brother who are also heterozygous for the identical mutation with incomplete phenotype, empty boxes indicate mother and sister with wild type

and structural abnormality in the pituitary gland, facial dysmorphism, Culler-Jones syndrome, HPE-like syndrome, and frank HPE (4,8). In addition, renal problems such as renal hypoplasia/dysplasia, urethral stricture and cardiac problems such as ASD/VSD have been reported in patients with *GLI2* mutations (4,8). HPE is the most common anterior brain anomaly and HPE is characterized by incomplete separation of cerebral hemispheres and underdeveloped midbrain structures. However, the mutations in *GLI2* are rarely associated with an HPE phenotype (7,12). Indeed, in the study of Bear et al (5) only three of the 112 (2.7%) patients with *GLI2* mutations, had HPE (13). Also, neuroanatomical anomalies, such as agenesis of the corpus callosum, abnormal cerebral periventricular venous system and abnormal gyri have been reported in patients with *GLI2* mutations (8,14,15,16,17). In contrast to the literature, our patient had severe anterior pituitary hypoplasia, MPHD, and ectopic posterior pituitary with no features of HPE or HPE like syndrome. Pituitary stalk interruption syndrome (PSIS) is a congenital anomaly of the pituitary gland characterized by small or absent anterior pituitary lobe, interrupted or absent pituitary stalk, and ectopic posterior pituitary lobe (18). PSIS may be associated with isolated or syndromic features (18). Mutations in genes encoding transcription factors in signalling pathways, especially *GLI2* variants, have been reported in PSIS, which is consistent with our case (18,19).

Pituitary dysfunction due to *GLI2* mutations may vary from idiopathic GH deficiency to MPHD, with or without ADH deficiency (3,5). Our index case had biochemical and hormonal features of complete anterior pituitary hormone deficiency including GH, thyroid-stimulating hormone,

Table 3 (Supplementary file). Clinical and genetic characteristics of cases with mutations in *GLI2* gene

Reference	Proband's age/gender	Consanguinity	Pituitary imaging	Polydactyly	Pituitray insufficiency	Intellectual disability	Other clinical findings	Mutation
Present case	10-year-old/ male	No	Ectopic posterior pituitary, anterior pituitary hypoplasia, absent of pituitary stalk	Bilateral post-axial polydactyly	ACTH, GH, TSH, FSH, LH, PRL	Yes	Facial dysmorphism	Paternal c.1957-2A > C
Babu et al (19)	4.9-year-old/ female		Hypoplasia of the pituitary gland	No	GH	No	No	Maternal p.Pro386Leu
	2-year-old/ female		Anterior pituitary hypoplasia	Post-axial polydactyly	GH, TSH and ACTH	No	Cranio-facial abnormalities, bilateral renal hypoplasia	Maternal p.Tyr575His
	3.5-year-old/ male		Normal	No	GH, TSH and ACTH	No	No	p.Ala593Val
	3-year-old/ male		Anterior pituitary hypoplasia	No	ACTH, GH, TSH, FSH, LH, PRL	No	No	*De novo* p.Arg1226X
	16.6-year-old/female		Stalk interruption syndrome with ectopy of the neurohypophysis and hypoplasia of the anterior pituitary	No	GH, TSH and ACTH	Yes	Congenital heart disease renal hypoplasia with bladder - ureteral reflux, labiopalatoschisis, mental retardation, deafness and visual impairment	*De novo* p.Val1111Gfs*19
Kordaß et al (8)	25-year-old/ female	No	Abnormal temporal myelinization	No	No	No	Facial dysmorphism, microcephaly, ASD/ VSD, multi cystic kidney, scoliosis, growth hormone neurosecretory dysfunction	Paternal heterozygous deletion 2q14.2q14.3
Shirakawa et al (4)	15-year-old/ male	No	Ectopic posterior lobe	Bilateral finger and toes	GH	Yes	Renal hypoplasia/ dysplasia, ASD uretral stricture/renal failure, midfacial hypoplasia	*De novo* heterozygous frameshift c.3369delg
Martín-Rivada et al (21)	12-year old/ male	No	Absence of pituitary stalk and posterior pituitary	Bilateral postaxial	GH, TSH, ACTH, FSH, LH	Yes	Bilateral labial cleft, facial dysmorphism, bilateral cryptorchidism, micropenis	*De novo* c.2125del heterozygous frameshift
Valenza et al (11)	6-year-old/ female	N/A	Anterior pituitary agenesis	Bilateraly postaxial	Panhypopituitarism	N/A	Facial dysmorphism, prominent forehead 2-3 finger syndactyly single median maxillary incisor choanal atresia	Paternal c.3493delc heterozygous deletion

Table 3 (Supplementary file). Continued

Reference	Proband's age/gender	Consanguinity	Pituitary imaging	Polydactyly	Pituitray insufficiency	Intellectual disability	Other clinical findings	Mutation
Juanes et al (23)	4-year-old/ female	No	Ectopic posterior lobe Absent pituitary stalk	No	GH	Yes mild	Right cleft lip and palate Facial dysmorphism, hypoplastic nostrils, hypotelorism, mildfacial asymmetry	p.arg231gln Heterozygous missense
	14-year-old/ male	No	Posterior pituitary lobe and stalk were absent	No	GH, TSH, ACTH, FSH, LH	No	N/A	p.arg226leu hetrozygous missense
Kevelam et al (10)	9-year-old/ female	No	Ectopic neurohypophysis	No	GH, TSH	No	Bilateral cleft lip and palate left isomerism mild midface hypoplasia	Paternal heterozygous 2q14.2 deletion
França et al (3)	7-year-old / female	No	Ectopic posterior pituitary lobe Asymmetric brain hemispheres	Bilateral postaxial	GH, TSH, ACTH, PRL, FSH, LH	Yes	Seizures vesicouretral reflux	Maternal heretrozygous frameshift c.2362_2368del
	4.5-year-old/ male	No	Ectopic posterior pituitary lobe	No	GH, ACTH	No	Cleft lip and palate, flat nasal brige unilateral crptorchidism	Paternal heterozygous frameshift c.2081_2084del
	8-month-old/ male	No	Posterior pituitary lobe not visible hypoplastic anterior pituitary	No	GH, ACTH, TSH, ADH	Yes	Seizures	Maternal heterozygous c.1138g > t
Kremer Hovinga et al (9)	12-year-old/ male		Ectopic posterior pituitary lobe	Bilateral postaxial	Panhypopituitarism	N/A	Hypotelorism, single median incisor mid uretral stenosis-urethral valves cryptorchidism ribbed palatum durum	Paternal hetreozygous c.3676c > t nosense
Bertolacini et al (7)	4-year-old/ male	N/A	Normal	No	N/A	No	High forehead, flat facial profile, facial dysmorphism, right cleft lip	Heterozygous c.803 c > t 3' utr
	3-month-old/ female	N/A	Normal	Right preaxial	N/A	No	Bilateral cleft lip/palate, flatface, maxillary hypoplasia	Maternal heterozygous c.4663t > c
	28-year-old/ female	N/A	Normal	Bilateraly postaxial	N/A	No	Hypotelorism, long and flat profile, mid line cleft, broad nasal tip, agenesis of pre-maxilla, long philtrum	Maternal heterozygous c.1530_1531insc

Table 3 (Supplementary file). Continued

Reference	Proband's age/gender	Consanguinity	Pituitary imaging	Polydactyly	Pituitray insufficiency	Intellectual disability	Other clinical findings	Mutation
	3-month-old/ female	N/A	Semi-lobar HPE	Bilateral postaxial	N/A	Yes	Microcephaly, large cleft lip/palate involving partially premaxilla	Maternal c.864_866delcc
	5-year-old/ male	N/A	Normal	No	N/A	No	Facial asymmetry, abnormal odelled ears with skin tags, tessier cleft number 7 at right, abnormal temporomandibulary joint	c.1886g > a
	5-month-old	N/A	Normal	No	N/A	No	Facial asymmetry with hypoplastic left side left anophthalmia, abnormal modelled ears preauricular skin tag tessier cleft number 7 at left	*De novo* c.4558g > a
Antich et al (14)	8-month-old/ male	No	Corpus callosum agenes	Yes	N/A	Yes	Cleft lip and palate, facial dysmorphism, Low-set ears, microretrognathia, imperforate anus, VSD, hydronephrosis	*De novo* 2q14-q14 heterozygous
Lucas et al (24)	Newborn female	N/A	N/A	No	N/A	No	Cleft lip and palate, facial dysmorphism hypertelorism, low set ears, premature cranial synostosis	*De novo* 2q14-q21 heterozygous
Frydman et al (16)	2-year-old female	No	Corpus callosum agenesis	No	N/A	Yes	Cleft lip and palate, persistent disease activity, microphthalmia, low set ears	2q14-q21 heterozygous
Davis et al (15)	29 month-old/female	No	Corpus callosum agenesis, dandywalker malformation	No	N/A	Yes	Cleft lip and palate, facial dysmorphism poorly developed auricules, epicanthic fold, ASD, seizures, ovarian dysgenesis	*De novo* 2q13-q21 heterozygous
Baker et al (25)	15-year-old/ male	No	N/A	No	N/A	Yes learning difficulties	Thoracolombar kyphoscoliosis, pectus carinatum, facial dysmorphism mild aortic root dilatation	Paternal 2q14.1-22.1 heterozygous

Table 3 (Supplementary file). Continued

Reference	Proband's age/gender	Consanguinity	Pituitary imaging	Polydactyly	Pituitray insufficiency	Intellectual disability	Other clinical findings	Mutation
Gustavsson et al (26)	22-year-old/ male	No	N/A	Left postaxial	GH	No	Hypospadias, double sided ureters, undescended testes, oral polyposis deep vein thrombosis	*De novo* 2q14.2-22.1 hemizygosity
Peng et al (17)	18 week of gestation	N/A	Ventriculomegaly	Vo	GH	Yes	Facial dysmorphism, single incisor febrile convulsion undescended testes	*De novo* 2q14.2-21.3 heterozygous

GH: growth hormone, TSH: thyroid stimulating hormone, ACTH: adrenocorticotropic hormone, LH: luteinizing hormone, LDH: lactate dehydrogenase, N/A: not available, PRL: prolactin, HPE: holoprosencephaly, FSH: follicle-stimulating hormone

adrenocorticotropic hormone (ACTH), prolactin, follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) (Table 1). The most common pituitary hormone deficiency is GHD (20). Although the response to rhGH replacement has been reported to be poor in some cases with *GLI2* mutations, an excellent response to rhGH replacement was observed in our case and has been reported previously. This suggests that clinicians should consider a trial of rhGH therapy in cases with *GLI2* mutation who have GHD (Figure 1) (3,8,21). In addition, hypoglycaemia, cholestasis, recurrent seizures and intellectual disability have been reported in patients with *GLI2* mutations as a consequence of ACTH and GH deficiency (22). Hypoglycaemic episodes and cholestasis in our case resolved after replacement of hydrocortisone and with rhGH therapy. We also attributed the seizures and moderate developmental delay evident in our case to neonatal hypoglycaemic episodes due to ACTH and GH deficiency. While the presence of micropenis in our case may be attributed to GH deficiency, he also had cryptorchidism and inappropriately low FSH, LH and testosterone levels during mini-puberty, suggesting concomitant gonadotropin deficiency. Despite having an ectopic posterior pituitary on pituitary-imaging he had no diabetes insipidus at presentation and this has not developed to date during follow-up.

Conclusion

In conclusion, extra-pituitary findings may provide clues for the diagnosis of particular gene mutations including *GLI2*, *HESX1*, *LHX4*, *SOX3*, and *OTX2* which are involved in the development and differentiation of the pituitary gland resulting in a variety of pituitary hormone deficiencies. In cases presenting with MPHD accompanied by ectopic posterior pituitary, polydactyly and midfacial hypoplasia, a diagnosis of *GLI2* mutation should be considered. Furthermore, extremely distinct phenotypical expression and incomplete penetrance of heterozygous *GLI2* mutations may be associated with MPHD skipping a generation and thus delay or missed diagnosis of these life-threatening hormonal disorders. In light of this genetic analysis of either asymptomatic or symptomatic relatives for *GLI2* gene mutations and evaluation of carriers for panhypopituitarism is warranted.

Ethics

Informed Consent: The subject and his parents have given their written informed consent to publish their case, in accordance with the Declaration of Helsinki.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Design: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Data Collection or Processing: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek, Analysis or Interpretation: Meliha Demiral, Edip Ünal, Ceren Damla Durmaz, Serdar Ceylaner, Literature Search: Meliha Demiral, Edip Ünal, Writing: Meliha Demiral, Hüseyin Demirbilek, Mehmet Nuri Özbek.

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The Results of 16 Years Iodization: Assessment of Iodine Deficiency Among School-age Children in Antalya, Turkey

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Keywords: Iodine, urinary iodine concentration, children, Turkey

Dear Editor,

This letter is regarding the recent publication of "The results of 16 years iodization: Assessment of iodine deficiency among school-age children in Antalya, Turkey" by Çelmeli et al (1) (2020). The study consisted of 1594 children aged 6-14 years who were asked to provide spot urine samples for the determination of urinary iodine concentration (UIC) after informed consent form was obtained from the parents of the children (1). In addition, goiter rate was also assessed in the study (1).

Iodine deficiency is one of the most common micronutrient deficiencies which can be prevented with the implementation of universal salt iodization programme (2). However, like any other health intervention programmes, the monitoring and evaluation of universal salt iodization programmes are needed to conducted regularly. This is particularly important to ensure that timely information is obtained so that a population will not be getting too much or too little iodine from the universal salt iodization programmes. Also, this would allow for an adjustment of iodized salt concentration to meet the recommended iodine intake in populations (3). In the study, the authors reported a median UIC of 175 µg/L with 19% of children categorised as mild-to-moderate iodine deficiency. Mild-to-moderate iodine deficiency has been shown to affect the learning ability and cognition of children (2). Therefore, if these children remain to be iodine deficient without appropriate corrective measures taken, these children are mostly likely to have a lower cognition and intelligence quotient (2).

Median UIC is the recommended method by World Health Organization, UNICEF (United Nations Children's Fund) and IGN (Iodine Global Network) to assess iodine status in populations (2). Urinary iodine in spot urine samples can also be expressed as iodine-to-creatinine ratio (I/Cr) ratio and estimated 24-hour urine iodine excretion (2). Although the collection of spot urine samples for the determination of UIC is relatively easier to be performed, UIC has high intra- and inter-individual variation (2). In addition, UIC is also subjected to hydration status (2). Therefore, Cr has been proposed to be included in the assessment of UIC in order to minimize the effect of hydration status (4). However, relating UIC to Cr might be unnecessary because this adjustment might introduce another bias from Cr and increase the cost of assessing iodine status using UIC. This is because when a population is malnourished or having low protein intake, the excretion of Cr is usually low and this can confound I/Cr ratio. There are no acceptable criteria for determining iodine sufficiency using I/Cr ratio. In addition, the use of iodized salt in populations should be reported. A low coverage of iodized salt might put populations at risk of iodine deficiency in future (3).

Ethics

Peer-review: Internally peer-reviewed.

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