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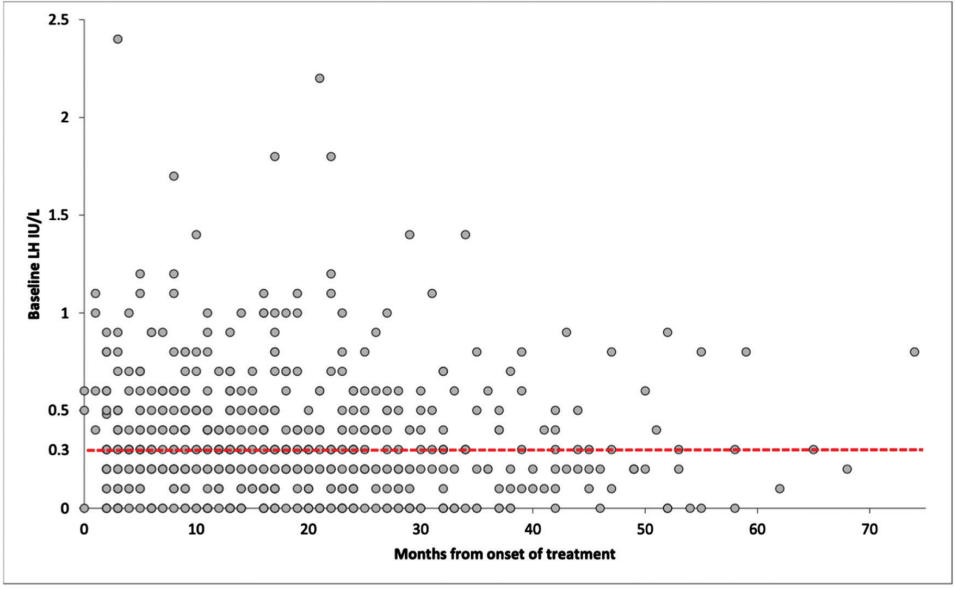

Pre-injection basal luteinizing hormone (LH) concentrations during gonadotropin-releasing hormone agonists (GnRHa) treatment for central precocious puberty. All samples were drawn just prior to the next GnRHa injection. The horizontal dashed line indicates the cut-off for a pubertal baseline LH concentration

Elevated Pre-injection Basal Luteinizing Hormone Concentrations are Common in Girls Treated for Central Precocious Puberty
Schubert S et al.
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REVIEW

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J Clin Res Pediatr Endocrinol 2021;13(2):124-135

Important Tools for Use by Pediatric Endocrinologists in the Assessment of Short Stature

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Abstract

Assessment and management of children with growth failure has improved greatly over recent years. However, there remains a strong potential for further improvements by using novel digital techniques. A panel of experts discussed developments in digitalization of a number of important tools used by pediatric endocrinologists at the third 360° European Meeting on Growth and Endocrine Disorders, funded by Merck KGaA, Germany, and this review is based on those discussions. It was reported that electronic monitoring and new algorithms have been devised that are providing more sensitive referral for short stature. In addition, computer programs have improved ways in which diagnoses are coded for use by various groups including healthcare providers and government health systems. Innovative cranial imaging techniques have been devised that are considered safer than using gadolinium contrast agents and are also more sensitive and accurate. Deep-learning neural networks are changing the way that bone age and bone health are assessed, which are more objective than standard methodologies. Models for prediction of growth response to growth hormone (GH) treatment are being improved by applying novel artificial intelligence methods that can identify non-linear and linear factors that relate to response, providing more accurate predictions. Determination and interpretation of insulin-like growth factor-1 (IGF-1) levels are becoming more standardized and consistent, for evaluation across different patient groups, and computer-learning models indicate that baseline IGF-1 standard deviation score is among the most important indicators of GH therapy response. While physicians involved in child growth and treatment of disorders resulting in growth failure need to be aware of, and keep abreast of, these latest developments, treatment decisions and management should continue to be based on clinical decisions. New digital technologies and advancements in the field should be aimed at improving clinical decisions, making greater standardization of assessment and facilitating patient-centered approaches.

Keywords: Short stature, height monitoring, bone age, cranial imaging, growth hormone treatment, prediction models

Introduction

One of the most important tools in monitoring the health of children is an assessment of how an individual child is growing relative to his/her peers. Linear growth monitoring of apparently healthy children can provide early indications of serious conditions (1), which may be identified through growth algorithms and the expertise of clinicians (2,3). If a child is noted to be particularly short, the cause should be determined and referral to a pediatric endocrinologist is likely, where a diagnosis can often be assigned from factors such as body proportions and clinical and family history (1,4). Although genetic abnormalities associated with short

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stature are continually being identified (5), a high percentage of cases remain idiopathic with no specified cause (6). While identified causes are often associated with defects in the growth hormone (GH)–insulin-like growth factor-1 (IGF-1) axis, a large proportion of cases do not involve GH or IGF-1 abnormalities, but may involve factors such as growth plate abnormalities (3,5,7).

GH therapy to improve linear growth is approved for a number of conditions associated with growth failure in children, although this varies to some extent by GH formulation and country (2,8,9,10,11). It is important for clinicians to identify ways of optimizing treatment; while starting GH treatment at a young age generally improves outcomes, for many indications, referral, diagnosis and GH initiation occur later than optimal (2,8). Artificial intelligence and machine-learning are revolutionizing diagnostic tools in all areas of medicine, including child growth and development. It remains important for pediatric endocrinologists to continually assess the innovative techniques that are becoming available with regard to identifying the nature of growth failure in children and how to tailor the management of the condition.

The present report is based on a meeting held in Rome, Italy, funded by Merck KGaA, Darmstadt, Germany, which discussed important new tools being developed and used by pediatric endocrinologists. Accurately identifying which children should be defined as short, what is the cause of the short stature, problems relating to bone maturation and how a child with growth failure will respond to treatment can all be aided by digital technologies, leading to improved clinical decisions, greater standardization and patient-centered approaches.

Growth Monitoring and Algorithms

Among children referred for evaluation of short stature, the prevalence of pathological conditions is only approximately 5%, with reported variation from 1.3% to 19.8% depending on the criteria considered (12,13,14). Thus, the majority of referrals show no pathology and diagnostic work-up may be carried out unnecessarily (3,15). Additionally, use of growth charts that are not population-specific can result in a large proportion of incorrect referrals for diagnostic work-up (14,16,17). Using appropriate growth references is of paramount importance when assessing a child's height because of the impact on eligibility for therapy (17). Wide variations are reported in population height measurements and the choice of auxological criteria for referral for short stature (12,13,14,18). Current protocols for growth monitoring frequently result in delayed diagnosis, and a consequent delay in the treatment of growth failure (19,20,21). Appropriate referrals will avoid unnecessary procedures and enable earlier diagnosis of the cause of growth failure and prompt initiation of therapy, thus improving outcomes and diminishing complications. Therefore, it is important that clinical practice should be optimized by standardization of growth monitoring, with validated, evidence-based protocols (2,12,13).

There is currently a lack of consensus on criteria for definition of abnormal growth. Seven different algorithms have been published in the last 20 years, involving around eight auxological parameters to be evaluated (22). However, the level of validation is low for each of the algorithms, with widely varying sensitivities and specificities. While height standard deviation score (SDS) is used in all algorithms, there is no consensus concerning the cut-off. A study across 23 European countries found height SDS cut-off ranged from -1.64 to -2.67 SDS and growth deflection or height velocity cut-off ranged from -0.50 to -2.32 SDS (18). Distance to target height SDS is the second most frequently used parameter, but different formulae may be used for calculation and there may be inaccuracies in measurements of parental heights. Other parameters were based on dynamic growth indicators, such as height deflection and height velocity SDS.

Comparison of children using the Dutch, Finnish and UK guidelines showed variations in sensitivity and specificity (14). Distance to target height provided the best specificity, particularly using the Dutch guidelines, and combination with height SDS provided effective growth monitoring. However, sensitivity was much lower and, even using combinations of up to four criteria, at least 20% of children with pathological growth failure may not be identified by these auxological measurements. Adding growth deflection may improve sensitivity and in the pre-pubertal period recent growth deflection should be considered a "red flag", leading to referral to a specialist clinic (14). Strict application of guidelines and cut-offs may lead to excessive numbers of referrals and other parameters may need to be added (15). In children younger than three years height/length measurements are often inaccurate; referral at this age should be based on extreme short stature or repeated measurements (23).

In children over three years of age, it was shown that electronic monitoring of growth, together with algorithms for assessment of auxology, could identify patients for referral for specialist care, and resulted in increased detection of pathological conditions associated with growth failure (24). In order for electronic health records to be clinically advantageous, good infrastructure and databases are required. With increasingly widespread collection,

monitoring of height of individual children in relation to their peers should become automatic and improve accuracy of referrals. Despite this availability, few countries have currently adopted such use of electronic records and automated growth algorithms. Electronic monitoring of height and weight has been used to assess obesity in children in Canada (25,26), but not linear growth and it was noted that better documentation by physicians was required. Currently, only the Scandinavian countries of Norway, Sweden and Finland have fully developed centralized systems for electronic recording and only Finland has implemented automated height growth analysis. However, it should be noted that factors such as being born small for gestational age, dysmorphic features or disproportionate short stature should also be assessed and appropriate algorithms be applied, emphasizing the importance at present of clinical judgment in making a diagnosis of the cause of growth failure.

Categorization of Diagnoses

When growth monitoring has indicated that a child has growth failure, the cause is determined from auxology, clinical history and biochemical assessments, and a diagnosis is ascribed. In order to analyze data on diagnoses from large populations it is necessary to transform the reported medical conditions into code numbers. These codes can then be used by health researchers, healthcare providers, government health programs, health insurance companies and others, for a variety of applications. A number of different systems have been designed to classify the diagnoses. The classification should allow inclusion of all patients, enable new etiologies and pathogenic aspects to be accommodated, define diagnoses accurately to prevent misclassification, follow one general principle, be easy to use and, optimally, serve the designed purpose (27). The International Classification of Diseases (ICD) was started in 1948 by the World Health Organization, and is now in version ten (http://apps.who.int/classifications/icd10/browse/2010/en). Its aim is to delineate all major disease groups, but diagnoses are not well defined, with too many conditions included together. For example, the term "endocrine, nutritional and metabolic disease" includes thyroid disorders, diabetes and disorders that are not really related to endocrinology, such as malnutrition and obesity. Thus, the system does not prevent misclassification. Online Mendelian Inheritance in Man (OMIM; https://www.omim.org/) can only be used for genetic disorders. Also, unless phenotypic features or clinical history indicate what genes to examine, in most children with short stature there is no specific "candidate gene" for targeted analysis.

The European Society of Pediatric Endocrinology Classification of Pediatric Endocrine Diagnoses aimed to define all pediatric endocrine disorders and included multiple specific sections, such as the one for all conditions involving short stature (27). This has now been superceded by the online International Classification of Pediatric Endocrine Disorders (ICPED), which is a comprehensive system of pediatric conditions developed by an international group of experts and endorsed by all pediatric endocrinology societies (28,29). The system incorporates the most recent versions of ICD-10 codes and OMIM numbering in order to be easily assimilated into hospital registries, and continues to be developed and added to. The online version is freely available (www.icped.org). While ICPED is used to a reasonable extent in the USA and Netherlands, its use in most other countries is limited. Worldwide standardization within pediatric endocrinology requires the use of ICPED for healthcare, economic and scientific purposes in order that electronic health records can be linked to diagnostic classification and coding systems.

Cranial Imaging

As part of the work-up for assigning a diagnosis for the cause of growth failure, cranial imaging is frequently included. However, the only consensus specifying which patients require cranial imaging were published in 2000 and have not been updated (30). They stated that patients with known or suspected intracranial tumors, optic nerve hypoplasia, septo-optic dysplasia or other structural or developmental anomalies should be assessed by magnetic resonance imaging (MRI) or computed tomography of the central nervous system. In patients with confirmed GH deficiency, pituitary height and/or volume, anatomy of the stalk and position of the posterior pituitary should be determined. The guidelines noted though that further normative data were required to improve the quality of assessment.

Standard MRI protocols include sagittal and/or coronal sections of 2-3 mm, with or without contrast medium, and pituitary height and/or volume, stalk anatomy and posterior pituitary position should be determined. Knowledge of the normal shape and volume of the pituitary is required to interpret the images and identify abnormalities (31,32). It is also advised that a survey of the entire brain should be carried out, such as use of fluid attenuation inversion recovery or diffusion-weighted imaging (32,33). MRI signals for the anterior pituitary and posterior pituitary are similar in the first few months of life, becoming different thereafter. The size/height of the anterior pituitary is 2.6-5 mm in the first postnatal 6 weeks, decreasing to 3 mm by two years and then increasing over time until puberty at 6-8 mm;

however, it should be noted that pituitary height at puberty is greater in females than in males. The position of the pituitary gland and connection with surrounding tissue are also important because abnormal development or migration of the pituitary may constitute some of the features of congenital hypopituitarism, confirming the requirement for assessing the surrounding brain from the imaging, along with the pituitary features.

Different imaging studies of patients with a diagnosis of hypopituitarism have shown normal pituitary in 0 to 86% of patients, hypoplastic anterior pituitary in 0 to 84%, and ectopic posterior pituitary in 4% to 100% (34). However, ectopic posterior pituitary was observed more frequently when patients had multiple pituitary hormone deficiencies (>50% in 15 of 18 studies) rather than isolated GH deficiency (<50% in 13 of 18 studies) (34). Abnormal pituitary features may help to predict the development of pituitary hormone deficiencies and the most likely genes involved (34,35). Thus, genetic studies can be better targeted by using MRI data together with associated phenotypic features, such as abnormalities of the brain, eyes and palate and additional central nervous system anomalies. Pituitary abnormalities also help in determining long-term status of GH deficiency; ectopic posterior pituitary or stalk abnormalities are associated with permanent GH deficiency whereas patients with isolated GH deficiency and a normal or small pituitary may have sufficient GH secretion at near-adult height and require re-testing (36,37).

In order for such predictions of genetics and endocrine status to be accurate, the MRI techniques must be as sensitive as possible. To enhance the contrast of MRI scans, intravenous gadolinium-based agents have routinely been administered to patients for more than 20 years and were generally considered safe (38,39,40). However, studies have indicated that gadolinium may be retained in the body, particularly in the brain when administered for pituitary imaging, so that after multiple administrations the contrast agents remained and deposits could be identified (40,41). This raised questions as to whether such deposits have harmful effects and indicated that further research was needed. The studies prompted new evaluations by regulatory bodies and warnings have been added to the labels for gadolinium formulations (42,43). This has led to new computer-aided techniques being developed, such as T2-DRIVE (driven equilibrium), whereby enhanced contrast can be attained without gadolinium administration (44). T2-DRIVE actually appears to be more accurate than gadolinium contrast and enables extremely reliable evaluation of pituitary size and identification of abnormalities. Thus, the technique can greatly improve the diagnosis and knowledge of the pathogenesis of non-tumoral hypothalamic-pituitary disorders.

Determination of Bone Age

Another part of the work-up for evaluation of growth failure is an accurate assessment of bone age, which is important because bone age delay or advancement is a useful diagnostic clue, and is used to predict adult height (45,46). Conventional assessments are known to be fraught with difficulties due to the many short-comings of the methods. There is no universally accepted method for manual assessment and great variability between individuals making the assessments. Healthcare personnel making the rating may differ in training, experience, motivation and alertness, and bias may be introduced if the rater knows the chronological age and clinical background of the patient. Variability can also occur due to ethnic differences, use of old/inappropriate reference data and lack of validation of methods; the original atlases were developed using data from particular ethnic and socio-economic groups (47).

It was recognized many years ago that computerized ratings could be better than conventional manual methods and a computer-assisted version of the Tanner-Whitehouse method was designed (48,49). However, it was not fully automatic, cumbersome and only ever used by a small number of research centers. Advances in computer technology allowed better methods to be developed and the first reports of the fully-automated BoneXpert method were published just over 10 years ago (50,51). The method uses X-radiographs of 15 bones in the wrist, hand and fingers, and originally did not use the carpals, which are considered to be less useful, although a new version does include carpals (52). The process interprets the shape, intensity and texture by principal component analysis and BoneXpert is currently the only medical device that has been certified for bone age determination.

Other systems have also recently been developed that use computer learning with deep convolutional neural networks, which do not require prior identification of features and calculations because these are part of the machine-learning process (53,54,55). While not fully automated or validated, such techniques are improving the accuracy, shortening the time required and increasing the cost efficiency of bone age assessments (56,57). A recent report provided evidence that an artificial intelligence, deep-learning, neural network method could estimate bone age with at least similar accuracy to expert radiologists and other existing automated models (58). These data for 12,611 hand radiographs, plus a further 1,425 validation data set, were used as part of a challenge issued by the Radiological Society of North America (RSNA)

to create new machine-learning techniques in medical imaging to accurately determine bone age (59). The ten best entries were considered to out-perform the model used originally in the data-set study, and the Toronto-based 16Bit system (www.16bit.ai) achieved first place (59,60). At present, there is only very limited understanding or control of deep-learning algorithms, making such techniques difficult to validate. However, eHealth technology is incorporating pediatric endocrinology into novel processes, enabling communication between technology experts and clinicians and ensuring assessments become more efficient and precise.

The BoneXpert system, which does not require deep-learning techniques, came fourth in the RSNA challenge, with less than 0.5% difference in performance from the 16Bit system. As BoneXpert is based on more traditional machine-learning techniques, it has some advantages over the novel deep-learning methods and is currently being used in over 150 clinics. It is validated for boys aged from 2.5 to 19 years and girls 2 to 18 years, although a new version extends the range to new-borns. BoneXpert can be used across multiple ethnicities and is consistent with all prevailing bone age scales (52,61,62,63). Precision is ≤0.18 years when comparing two concurrent X-radiographs, and accuracy is ≤0.72 years relative to experienced manual raters. It provides visual feedback on delineation of each bone and automatically rejects an image if the rating is at risk of being incorrect, giving the potential to replace manual rating, although radiologists may still check the image to look for findings such as skeletal dysplasias. It has been validated with an adult height prediction model, with root mean square deviation of predicted from observed of 2.8 cm for boys and 3.1 cm for girls (63). The technique can also be used to provide an index of bone health from the relationship of the cortical thickness to the length and width of the bones (64,65). These developments that have occurred in recent years in bone age measurements using artificial intelligence systems now allow much more objective evaluation. The models continue to be refined and validated and are providing much greater accuracy that in turn provides increased precision in assessment of adult height prediction.

Prediction Models

One of the still unresolved problems of GH treatment of a short child is how much height growth should be expected, in the short-term and long-term. Growth response has commonly been expressed as either observed height velocity (cm/year) or change in height SDS, based on normal reference data. The characteristics of height and height velocity show specific average patterns and changing degree of variance around the mean with age (66). Therefore, specifying a set figure, e.g. height SDS gain >0.5 during the first GH-treatment year (67), as a "normal" growth response is inadequate for children of different sex or age.

After it was recognized that the growth response to GH was correlated with several factors related to the treated children and the mode of treatment, the problem of a more complex response evaluation was approached by several groups in the early 1990s by means of growth prediction models (68,69). In principle, prediction models are mathematical algorithms based on empirical observations from large cohorts of GH-treated children with specific diagnoses (70). Prediction models aim to explain as much as possible of the growth response within a set period of time, with the least possible error. This requires taking into consideration a child's characteristics, such as diagnosis, age and sex, and the chosen treatment modality (dose, injection frequency, time on treatment). The incorporation of laboratory parameters such as IGF-1 concentration, and factors such as genetic and proteomic markers (70), may also be considered, but this requires their standardization before implementation in models suitable for wider clinical use.

Data from the large KIGS (Pfizer International Growth Study) surveillance database provided the basis for development of various growth prediction models, in children at differing pubertal stages and diagnoses (71,72). These models have been independently validated and are accessible through freely available software (https://igro-gh.com). GH dose is a prediction variable of relevance for the utility of these models in clinical practice, since dose is the only parameter that can be modified. Incorporating multiple pre-pubertal factors in the prediction models identified GH dose as the most important factor for patients with Turner syndrome or born small for gestational age. However, prediction analyses showed that in children with GH deficiency, disease severity is the most important predictor of growth during the first pre-pubertal year of treatment (73). The extent of responsiveness to GH observed during the first year of therapy is an indicator of the overall response.

Thus, early prediction of response to GH treatment of a child with growth failure potentially enables optimization and individualization of treatment in terms of efficacy and costs (70). Height velocity targets provide a simpler model to evaluate the appropriate response in children treated for the first two pre-pubertal years, by considering age, sex and diagnosis, but not the individual dose and other factors found relevant in prediction models (66,74).

The Gothenburg prediction model focused on children with idiopathic short stature or partial GH deficiency and assumed nothing about diagnosis (75). They compared a standard GH dose (43 µg/kg/day) with a dose adapted for predicted sensitivity (17-100 µg/kg/day) and, as expected, the variation in response was reduced by 32% with the individualized dose (76,77). The Cologne model uses both baseline and three-month data for prediction of subsequent response to GH, and includes baseline factors of IGF-1 level, deoxypyridinoline (as a marker of bone resorption) and bone age retardation, and height velocity in the first three months of treatment (78). However, as the bone resorption marker is rarely measured, the model has only been used to a limited extent. However, the Cologne model was recently shown to effectively predict first year response to GH treatment in patients with short stature homeobox-containing gene (*SHOX*) deficiency (79).

Baseline IGF-1 SDS is used in the Cologne model, whereas values both at baseline and during treatment have been used in other prediction studies in children with growth failure due to GH deficiency and other conditions (70,80,81,82). These models use multivariate linear regression to identify factors associated with response for inclusion in the models. However, artificial intelligence techniques of machine-learning and neural networks have been suggested to detect both linear and non-linear variables with no pre-conceived assumptions, and may prove more flexible and useful in clinical practice (83). Initial studies suggest that early growth response and IGF-1 concentration changes were among the most important predictors of long-term response (84). Accurate prediction of growth outcomes could help in educating patients and their families and managing their expectations. While applications (apps) designed for personal use that incorporate prediction models are being developed (85), accuracy and validation are so far unknown. Very few apps currently include education and links between patients, caregivers and healthcare professionals. A better understanding of how patients can use such apps is required and there remains an unmet need for assessment of quality and physician endorsement of such tools for use in clinical practice.

Interpretation of IGF-1 Data

Determination of IGF-1 level has many uses in children with growth failure because it has a long half-life in blood and a stable circadian concentration. Therefore, single daily measurements can be taken and a consensus guideline for measurement was developed (86). Current methods for measuring total IGF-1 concentration require separation from its binding proteins using two-step acidification and neutralization, with blocking of re-aggregation by adding an excess of IGF-2 (87). Nevertheless, different commercial assays are available and their reference intervals vary, so it is important to note which assay is used when comparing data (88,89). IGF-1 concentrations change with age and gender so that normative data have been determined from large numbers of healthy children and adolescents (87). This enables appropriate correction to provide SDS values, although the correct normative data should be used because diverse cohorts may give different reference measurements (90,91). Such differences may give clinically relevant variation when used to establish a diagnosis of GH deficiency (92). Clinical background of the patient should be considered when determining whether an IGF-1 assay is necessary and how the result should be interpreted; also the test may require repeating if clinical features and laboratory results are discrepant (93). Nutritional history should also be considered in evaluation of IGF-1 when assessing GH status as both short-term and chronic under-nutrition or over-nutrition can affect circulating concentration and body mass index should be considered when interpreting IGF-1 level (94).

IGF-1 SDS is used not only to aid in identifying the cause of growth failure, but also to assess whether, and how well, a short child will benefit from GH treatment (95). In patients with GH deficiency, normalization of IGF-1 SDS is not always required for a good response, particularly in patients with severe deficiency. A greater response is generally seen in those with the lowest baseline values. Using artificial intelligence neural networks on data for patients with non-acquired isolated GH deficiency, more severely reduced IGF-1 SDS at baseline was shown to be a significant indicator of GH response, both in the first year and for adult height (84). In evaluating IGF-1 level, the cause of GH deficiency should be considered, because children with acquired GH deficiency have higher IGF-1 SDS than those with non-acquired deficiency (96). In patients without obvious GH deficiency and who are classified as idiopathic short stature, IGF-1 SDS may be below normal for approximately 40% (87). During GH treatment, a low IGF-1 SDS may be due to low sensitivity, concomitant illness or malnutrition/malabsorption, or poor adherence with the therapy (96,97). A normalized IGF-1 SDS with low response may also suggest poor adherence, with GH correctly administered only for a few days before evaluation; better evaluation of continuous adherence may then be necessary, and addressing issues of adherence could improve response (98,99). Maintaining a normalized IGF-1 SDS, without excessive values, is also required from a safety point of view (95,100). While guidelines recommend a decrease in GH dose when IGF-1

SDS is > +2 (101), this may not always occur in practice, particularly if response is only assessed from height velocity (102). Maintaining a set IGF-1 SDS has also been used to adjust GH dose in studies of children with GH deficiency or idiopathic short stature and was shown to enable a better growth response (103). However, non-approved high doses of GH were used in some children when IGF-1 was titrated to +2 SDS in that study, and titrating to 0 SDS was subsequently shown to achieve a better long-term height gain per dose and was potentially safer (104).

Conclusion

Digital health and computer-based technologies are rapidly altering healthcare services to make medicine much more patient-centered and personalized. While there have been great advances in use of digital tools and use of artificial intelligence, the importance of this research is only just beginning to be recognized in the field of pediatric endocrinology and growth failure. Algorithms for height monitoring can now be integrated into electronic health records, which can increase the diagnostic yield and identify individual children who may have growth failure. However, the lack of accurate centralized health records in many countries is slowing this referral and diagnosis progress. At a more individual level, various digital health tools are being developed that can provide better identification of disorders and promote effective engagement between clinicians and patients. Growth monitoring applications for phones and tablet computers are being developed, based on longitudinal growth studies. Such technologies should form part of the overall clinical management of children with growth failure.

When short stature is identified, standardizing the diagnoses associated with pediatric endocrinology requires a comprehensive classification system, in which the organization of diagnoses is well defined in order to prevent misclassification. Computerization of health records enables transformation of identified medical conditions into code numbers, which can then be used by multiple organizations to analyze population data on health and healthcare. Most systems currently in use are either complicated or diagnoses are insufficiently defined and, therefore, do not fit the needs of the scientific, governmental and healthcare communities. ICPED is an online classification system that is simple, comprehensive and fulfills the needs of such users, can be standardized worldwide and incorporated into hospital registries. This would allow wider access and better use of electronic health records.

Assessment of the cause of growth failure in a child frequently includes cranial imaging and new techniques are being devised. A recent development is the routine inclusion of T2-DRIVE into sellar MRI protocols. In light of recent safety concerns regarding gadolinium contrast agents, the computer-aided technique is considered a valid alternative for pituitary imaging without gadolinium in patients with pituitary hormone deficiencies. T2-DRIVE is advocated for more accurate diagnosis of pituitary gland abnormalities since it has been shown to provide better contrast than gadolinium agents. However, it should always be noted that knowledge of the normal pituitary dimensions is required for interpretation of any technique and good clinical expertise remains vital for accurate diagnosis.

New computerized techniques for measurement of bone age are using machine-learning to become much more accurate and precise. As these are objective, rather than previous subjective manual ratings, they are much less variable, can be used for both short and tall children, and are adaptable to various ethnicities. The BoneXpert system, which uses machine-learning though not deep-learning, is the only medically certified and systematically validated technique. As well as providing rapid and accurate bone age measurements and adult height predictions, it also provides a bone health index that can be used for multiple medical conditions. However, automated techniques such as BoneXpert, should continually be compared with manual ratings in order that clinicians do not become totally dependent on them. The techniques are still developing, with mistakes occasionally occurring, and at present syndromes and anomalies cannot be automatically detected. Therefore, clinical experience and judgment is still required.

A variety of models to predict the response to GH treatment in individual children with growth failure have been developed and validated over time. However, they are still largely underused in assessment of growth potential. Various factors are included in different models, such as patient characteristics and treatment modalities, but incorporating further laboratory, proteomic and genetic predictors could potentially improve accuracy. Until recently, the models relied on multivariate regression analysis. However, newer methods are beginning to be developed that use deep-neural networks and machine-learning techniques, which can analyze non-linear as well as linear relationships and do not require *a priori* assumptions about importance of various factors. Prediction models are now also being incorporated into apps, to educate and help patients understand their condition. However, these require clarity in the models being used, expert validation and assessment by endocrinologists.

Some prediction models use IGF-1 SDS as a factor and IGF-1 SDS may also be used to evaluate response to GH treatment for short stature. Machine-learning techniques indicated that

baseline IGF-1 SDS is among the most important indicators of response to GH treatment. However, IGF-1 measurements should be considered with care because results can vary widely between different assays. Laboratories should ensure that appropriate normative data are used to determine SDS values, and pubertal stage of the child should be taken into consideration in addition to age, gender and nutritional history, when interpreting results. As there is imprecision in assays, borderline values for use in clinical diagnosis of short stature should be repeated using a second blood sample and clinical history assessed when evaluating IGF-1 level. Titrating GH dose to IGF-1 helps to take into account the sensitivity of treatment due to diagnostic factors and has been reported to provide better clinical outcomes.

Thus, rapid advances in computing and artificial intelligence technologies are providing many new tools for pediatric endocrinologists. Such tools are improving identification of short stature and enabling better diagnosis of causes of growth failure. Cranial imaging is becoming more accurate and sensitive, bone age and bone health can be evaluated more objectively, prediction of response to GH treatment is improving and use of IGF-1 measurement is becoming more consistent. However, this research has only really scratched the surface and development of new computer-learning techniques could be further explored in terms of digitalization and development of patients-centric solutions. New eHealth tools can help pediatric endocrinologists by making their clinical assessment and patient management more efficient. Most tools are currently designed to be used by clinicians, although future directions may need to explore new ways in which patients can access the technologies more directly. While clinicians need to keep abreast of all these new techniques, clinical decisions should always be based on their experience. However, these new digital technologies should provide better communications between clinicians and patients. Treatment decisions based on these new techniques should always be patient-centered, in order to personalize and optimize assessment of child growth and management of growth failure.

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Methylation Status of *GLP2R*, *LEP* and *IRS2* in Small for Gestational Age Children with and without Catch-up Growth

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What is already known on this topic?

In the last two decades, significant advances have been made in the understanding of the epigenetic impact in human growth and development. However, the different approaches and methodologies used do not clearly identify the genes potentially related to the changes and phenotypes observed in small for gestational age (SGA) children.

What this study adds?

This study presents simultaneous analysis of promoter methylation status of multiple genes that are not related to parental imprinting and that may play a role in the development of metabolic diseases in children with SGA. Investigation of the methylation status of leptin (*LEP*), glucagon-like peptide-2 receptor (*GLP2R*), insulin receptor substrate-2 (*IRS2*) in SGA patients showed no association between *IRS2* promoter methylation and the catch-up growth phenotype in this population. In addition, *GLP2R* and *LEP* were methylated in all samples. Children with catch-up should be routinely followed to perform timely diagnosis of possible metabolic impairments.

Abstract

Objective: In small for gestational age (SGA) children, catch-up growth could be influenced by methylation of several genes involved in metabolism. Epigenetics may influence the development of metabolic diseases in adulthood. To compare the methylation of leptin (*LEP*), glucagon-like peptide-2 receptor (*GLP2R*), insulin receptor substrate-2 (*IRS2*) in SGA patients with and without catch-up growth.

Methods: Observational prospective study of SGA children. Demographical and clinical variables were collected from clinical records and parents' questionnaire. Methylation status of *LEP*, *IRS2*, and *GLP2R* promoters was evaluated in DNA extracted from patient and one parent saliva samples.

Results: Forty-eight SGA patients were included. Twenty-six (54.2%) had catch-up growth phenotype and 22 (45.8%) did not. The median age was 5.2 years [RIC 4.1-6.8] without difference between groups ($p = 0.306$). The catch-up group had increased appetite (42.3% vs 9.1%, $p = 0.008$), family history of dyslipidemia (42.3% vs 27.3%) and diabetes (34.6% vs 22.7%) compared to non-catch-up group. Catch-up patients had significantly larger waist circumference compared to non-catch-up group (median 55 cm [RIC 52-58] versus median 49.5 cm [RIC46-52]; $p < 0.001$). *LEP* and *GLP2R* were methylated in all samples. IRS2 was methylated in 60% of SGA patients without difference between groups ($p = 0.520$).

Conclusion: There is no association between *IRS2* methylation and catch-up growth among SGA patients. *LEP* and *GLP2R* were methylated in all SGA patients. Gene methylation may be implicated in metabolic disease later in life. More studies should be performed to confirm this hypothesis.

Keywords: Low birth weight, infant, small for gestational age, epigenetics, methylation, DNA, insulin resistance

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Introduction

Small for gestational age (SGA) is defined as a newborn with weight, length, and/or head circumference below the 10th percentile according to sex and gestational age (1). In 2012, in low- and middle-income countries, approximately 23.3 million infants were born SGA (2). In Colombia in the last decade, the SGA rate increased from 70 to 90 SGA newborns per 1,000 live births (3).

SGA is associated with decreased lean mass, muscular mass, and bone mineral content. An SGA infant could have reduced protein, nitrogen and glycogen content in skeletal muscle and liver, due to decreased plasma glucose and insulin concentrations (4). These factors are associated with increased risk of hypoglycemia, hypocalcemia, polycythemia, hyper-viscosity, impaired thermoregulation, and immune dysfunction (5). SGA infants have a 20 times greater risk of mortality than healthy controls during the neonatal period (6). SGA also has poor long-term outcomes, including significantly increased risk of chronic cardiovascular and metabolic diseases compared with Appropriate for Gestational Age infants (7,8,9,10).

Additionally, the physiological response to SGA is evident in the growth pattern during the first two years of life, especially during the first 2-6 months. Approximately 15% of children with a history of SGA continue to present with low weight and height for their ages, associated with a non-catch-up growth phenotype (11). The remaining 85% show excessive catch-up growth, with height velocities that exceed the normal statistical limits for their age and/or maturity during the first three years of life following the prenatal period of growth inhibition (12).

The term "epigenetics" refers to heritable changes in gene expression that occur without requiring alterations in DNA sequences, including the expression of non-coding RNAs, DNA methylation, and histone modifications. Genomic imprinting is one of the most important and well-researched forms of epigenetic inheritance, during which the regulation of a gene or chromosomal region is dependent on the sex of the transmitting parent (13). Imprinted regions play vital roles during embryonic development and have been associated with low birth weight (LBW) and other phenotypes associated with abnormal weight, such as overgrowth syndromes (14). The epigenetic regulation of the genome is a critical facet of development. Several genes located in imprinted regions are associated with the control of embryonic growth, such as *IGF2, H19,* and *MEST* (15). However, most studies that have examined the genes in imprinted regions (15,16) have not been able to consistently or conclusively determine their associations with LBW (11).

The embryonic environmental characteristics of patients with SGA could induce changes at the epigenetic level that affect gene transcription and would be stable throughout life (5,14). Because of the importance of epigenetic regulations during human development, these changes could be associated with diseases and pathological phenotypes that present during childhood and adulthood in patients with a history of SGA. The aim of this study was to compare the methylation status of insulin receptor substrate-2 (*IRS2*), glucagon-like peptide 2 receptor (*GLP2R*), and leptin (*LEP*) genes in SGA patients with and without catch-up growth. These genes were chosen due to their importance in appetite control and their role in regulation of carbohydrate metabolism.

Experimental Subjects

This is an observational prospective study. SGA patients were selected from the pediatric endocrinology outpatient clinic. Patients with metabolic comorbidities or whose parents did not agree to enter the study were excluded. *LEP*, *GLP2R*, and *IRS2* were categorized according to whether the patient presented with a catch-up growth or non-catch-up growth phenotype during early childhood (before five years of age). This study was performed in accordance with the Declaration of Helsinki Good Clinical Guidelines. Written informed consent was obtained from all patients' parents before study participation. The institutional ethics review board of Fundación Valle del Lili approved the study (Act 084-2014).

Methods

Data and Setting

Cali is a city of 2.3 million inhabitants, and it is the capital of the Valle del Cauca Department in Southwestern Colombia. The natality rate in Cali was 12 per 1000 inhabitants in 2017 with an infant mortality rate of 9 per 1000 life newborns and 11% LBW (17). Fundación Valle del Lili is a tertiary care university hospital with a catchment area of approximately 10 million people.

Exposure Variables

Demographic and clinical variables were obtained from clinical records. The SGA criterion was defined as birth weight and birth height below the 10th percentile for gestational age (1). The parents or legal guardians of the patient were asked, through a survey, for information regarding demographic characteristics, lifestyle, and other variables such as a lack of appetite or a voracious appetite, hours per week of physical activity and abdominal circumference. In addition,

we asked for self-report of a parental clinical diagnosis of dyslipidemia, diabetes mellitus, arterial hypertension and cardiovascular disease.

To assess appetite, parents were asked to determine their child's appetite by choosing among three options: a) The child has very good appetite, eats everything on the plate and constantly asks for additional food (voracious appetite); b) The child takes time to eat his/her meals or snacks but eats what is recommended for his/her age (slow eater); and c) The child has low appetite, parents must insist on finishing eating or remain many hours without eating (lack of appetite).

Weight in kilograms and height in centimeters were measured at the time of evaluation, which allowed the classification of patients into two groups: children who presented with the catch-up growth phenotype; and children who presented with the non-catch-up growth phenotype.

Outcome Variables

Saliva samples of children and their parents were collected during clinical assessment using the Oragene-DNA OG500 saliva self-collection kit (DNA Genotek Inc. Ottawa, ON, Canada), and stored at 4 °C until DNA extraction and analysis. Catch-up growth was defined as the height velocity above the limits of normal for age for at least one year after a transient period of growth inhibition (18). The examined genes were selected based on their significant contributions to the anabolic metabolism of the pediatric population: *LEP, GLP2R* and *IRS2*.

DNA Methylation Analysis

DNA extraction was performed from oral fluid samples using the prepIT-L2P kit® (DNA Genotek Inc. Ottawa, ON, Canada), according to the manufacturer's instructions, at the Genomic Medicine Laboratory at Universidad Icesi. Extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientifics Waltham, MA, USA). The bisulfite conversion of DNA was performed using the Epitect kit® Fast Bisulfite Conversion (Qiagen Inc, Germantown, MD, USA). All samples were processed and analyzed in an anonymous manner.

Primers were designed, using the freely available, web-based software program Beacon Designer v.8.14 (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1), to cover the CG-rich regions, with amplifications in the target range of 400-600 bp (Supplemental Table 1). The promoter regions for *LEP, GLP2R* and *IRS2*, were examined, and 1 CpG site for each gene was selected for methylation measurements: CpG site 1, *LEP* promoter: 1120-1718; CpG site 2, *IRS2* promoter: 4256-4600; and CpG site 3, *GLP2R* promoter: 1108-1507.

Qualitative methylation analysis of genomic DNA was performed by real-time polymerase chain reaction (PCR) assay, using an EpiTect MethyLight PCR kit (Qiagen Inc., Germantown, MD, USA). This methylation-specific assay is comprised of two non-specific methylation primers and a Taqman probe, which specifically amplified methylated DNA within the gene locus. PCR was performed in a total volume of 25 µL, containing 12.5 µL EpiTect MethyLight PCR Master Mix (Qiagen Inc., Germantown, MD, USA), 1 µL (20 ng) bisulfite-converted genomic DNA, 2.5 µL 10× primer-probe mix, and 9 µL water. Real-time PCR was performed using a 7500 fast real-time PCR instrument (Applied Biosystems, CA, USA), with the following temperature profile: 20 min at 95 °C and 55 cycles of 15 sec at 95 °C and 30 sec at 60 °C.

Table 1. Demographic and clinical characteristics of the study population

Variable	Non-catch-up growth	Catch-up growth	p value
	n = 22	n = 26	
Male, n (%)	8 (36.4)	12 (46.2)	0.493
Age at recruitment (years)*	6.1 (4.4-6.8)	4.7 (4.0-6.7)	0.306
Gestational weeks at birth*	37 (37-38)	37 (37-39)	0.565
Appetite, n (%)			
Slow eater	10 (45.5)	3 (11.5)	0.008
Voracious eater	2 (9.1)	11 (42.3)	
Lack of appetite	10 (45.5)	12 (46.2)	
Hours of physical activity/per week*	9 (7-10)	7.5 (5-10)	0.555
Family history, n (%)			
Dyslipidemia	6 (27.3)	11 (42.3)	0.278
Diabetes mellitus	5 (22.7)	9 (34.6)	0.281
Arterial hypertension	8 (36.4)	12 (46.2)	0.348
Cardiovascular disease	6 (27.3)	7 (26.9)	0.615
Overweight	5 (22.7)	9 (34.6)	0.670
Obesity	3 (13.6)	4 (15.4)	0.608
***IRS2* gene methylation status, n (%)**			
Inconclusive	1 (4.5)	4 (15.4)	0.52[a]
Negative	7 (31.8)	7 (26.9)	
Positive	14 (63.6)	15 (57.7)	
Waist circumference*	49.5 (46-52)	55 (52-58)	< 0.001

*Median (interquartile range), [a]Chi-square test performed with inconclusive *IRS2* gene methylation status

EpiTect Control DNA (human), which was methylated and bisulfite-converted (Qiagen), was used as the positive control for methylation assays. CpG units that yielded data in more than 90% of samples passed the initial quality control step. Poor-quality data for each CpG site were excluded during the qualitative evaluation of methylation.

For each sample, a relative methylation value was determined using the ΔCт method and ΔΔCт method (19) and normalized against the ΔCт mean of EpiTect Control DNA. The ΔCт values for each sample were measured in triplicate. Samples were considered negative (non-methylated) in the study when more than two replicates showed cycle threshold (CT) values greater than 35 during the total DNA quantification assay. The area under the curve of the receiver operating characteristic was computed, using the trapezoidal rule.

Statistical Analysis

Dichotomous variables were reported as percentages and continuous data were reported as the median and interquartile range (IQR), or mean and standard deviation (SD) if normally distributed. Comparisons were made using the χ^2 or Fisher's exact test, for dichotomous variables, as appropriate. The Mann-Whitney U test was used for comparisons of continuous data. P values were considered significant at $p < 0.1$. The statistical analysis was performed using STATA® 14.0 (StataCorp, College Station, TX, USA) registered to Fundación Valle de Lili.

Results

Between November 2013 to January 2015, 48 children with a history of SGA were treated at the pediatric endocrinology clinic. None of the patients were excluded. Of these, 45 (93.7%) were born at term (37 weeks of gestational age or more), 28 (58.3%) were girls, and the median age at medical assessment was 5.1 years old (IQR 4.1-6.8). The demographic and clinical characteristics of these patients during infanthood are shown in Table 1.

Twenty-six patients had catch-up growth phenotype and 22 children did not. Catch-up patients were characterized as voracious eaters (42.3% vs 9.1%) and had higher waist circumferences (median 55 cm vs 49.5 cm) than patients presenting with the non-catch-up growth phenotype. No differences in the hours per week of physical activity were observed between groups. Family history of dyslipidemia, diabetes mellitus, and arterial hypertension were more common in the catch-up growth group than in the non-catch-up growth group.

The qualitative methylation-specific assay found that CpG sites associated with *GLP2R* and *LEP* were methylated in all samples. Methylation of the *IRS2* promoter was observed in 57.7% of the catch-up growth group and in 63.6% of the non-catch-up growth group ($p = 0.52$). In four children with catch-up growth group children and one non-catch-up growth child, the status of promoter methylation could not be determined (Supplemental Figure 1 and Supplemental Table 2).

Discussion

Catch-up growth acts as a compensatory mechanism for perinatal age, reducing morbidity. However, catch-up growth is also associated with adverse outcomes, including obesity, insulin resistance, glucose intolerance, type 2 diabetes mellitus, and cardiovascular disorders, in adulthood (12,20). We hypothesized that *IRS2* promoter methylation status may play a role in catch-up growth. However, no association was identified between *IRS2* promoter methylation and the catch-up growth phenotype in this population. In addition, *GLP2R* and *LEP* were methylated in all samples.

Few studies have examined the methylation status of genes in SGA infants. The largest study was conducted by Liu et al (21), in 2012, who measured the methylation status of *IGF2/H19*, *MEST,* and other imprinted genes, using a bisulfite pyrosequencing method on cord blood DNA from 508 infants, and found no significant differences in the methylation levels of the *MEST* differentially methylated region between LBW neonates and normal-weight neonates. No study reported in the literature included all of the genes that were examined in the present study and saliva samples used here are another differential factor.

Saliva is composed of more than 99% water, and also contains white blood cells and epithelial cells, which represent the cell types of the oral mucosa. Previous DNA methylation studies comparing profiles between tissue types within individuals have shown that regions of tissue-specific differential methylation mainly map to CpG poor regions and demonstrated that methylation profiles correlating positively between saliva and diverse tissue in question (22,23). The viability of saliva as an alternative for less accessible tissues, including brain, lung/bronchial epithelium, and peripheral blood mononuclear cells, and in a recent study, intestinal mucosa, has been demonstrated (23). The similar composition and function of mucosa between the oral mucosa and intestinal mucosa suggests that comparable methylation profiles between saliva and intestinal tissue might exist, and strengthens the idea that

saliva has the potential to be used as an alternative for more difficult to sample tissues (22).

Promoter methylation of *GLP2R* and *LEP* was observed in all samples, suggesting the population-wide down-regulation of *GLP2* and *LEP*. These genes have been physiologically associated with appetite control, satiety, and glucose homeostasis (24). Whether the methylation of these gene promoters reflects an adaptation response associated specifically with a history of SGA or whether methylation reflects a general tendency in all populations is currently unknown because the methylation status of these promoters has not been previously studied. Other studies have reported a reduction in the methylation status of the *LEP* promoter among obese patients and an increased methylation status among SGA children (25). Reynolds et al (26), in 2017, suggested that high-birth-weight babies showed an increased expression levels of obesity-related genes including lipoprotein lipase (*LPL*) and LEP receptor (*LEPR*). Studies in rodents have shown that treatment with *LEP* during late developmental stages in offspring, slows neonatal weight gain and reverses prenatal adaptations caused by stimuli that promote adulthood obesity (27), and *LEPR* expression increases in response to *LEP* insensitivity, as a compensatory mechanism to defend against obesity. *LEP* promoter methylation status approached 100% among the parental control samples, suggesting that *LEP* downregulation in the digestive tract is a generalized adaptation during adulthood.

We did not identify an association between *IRS2* promoter methylation and phenotype that could explain the trend toward insulin resistance that is commonly associated with the catch-up growth phenotype. IRS1 and IRS2 proteins play roles during the regulation of the insulin signal transduction pathway, through phosphorylation and binding with the insulin receptor. IRS1 is expressed predominately in skeletal muscle, whereas IRS2 is expressed in the liver, fat tissue, and skeletal muscle. The downregulation of these substrates has been associated with insulin resistance, altered secretion patterns in pancreatic β cells, and the development of diabetes mellitus (28). A recent study showed that the downregulation of IRS2 in an SGA murine model was associated with abnormal glucose metabolism (29). In a previous study of high-birth-weight babies, significantly elevated expression levels of *GLUT4* and *IRS2* mRNA were observed and were correlated with insulin resistance, as both proteins are stimulated by insulin and are associated with cellular glucose uptake (26).

However, our findings were not completely consistent with the results reported by previous studies examining the epigenetic dysregulation associated with SGA. Differences between study findings may be related to the methods used to assess methylation, the classification standards used to define SGA in different countries or districts, or differences in population characteristics. The limited sample sizes associated with studies of SGA populations also likely results in increased variation. Studies with larger sample sizes are necessary to replicate our findings. The causal relationship between gene methylation status and SGA should be examined further but our findings add to the emerging evidence that the methylation of genes associated with metabolic regulation may adversely impact fetal growth and development. Compounds that act as methyl group donors may influence the epigenetic regulation of specific genes, although the implications of these alterations remain unclear.

Study Limitations

Our study has a few limitations. Given the small sample size of our study, our results have low statistical power. Second, the use of saliva samples does not allow for comparisons with other published studies of epigenetics analysis in SGA. Lastly, this study was performed with patients followed in a pediatric endocrinology outpatient clinic. Therefore, the results could not be extrapolated to the general pediatric population.

Conclusion

Understanding potential epigenetic factors associated with the development of metabolic diseases could facilitate the early identification of at-risk populations, which could then be treated with early and adequate preventive intervention methods. The association between SGA and the development of metabolic diseases during adult life represents an opportunity to identify potential epigenetic characteristics and to establish targets for the prevention and treatment of metabolic diseases, which could benefit both these children and the general population. Our findings showed the widespread methylation of genes related to metabolic control, suggesting that the high metabolic risks that have been previously identified in multiple studies of SGA children may be due to epigenetic adaptations that occur *in utero,* and the epigenetic adaptations identified during postnatal life may represent non-specific events.

Further studies are necessary to better understand the development of metabolic pathologies among SGA children and whether the methylation patterns of key genes are associated with the development of short stature and metabolic syndromes among this population, and both comparisons among SGA children with different response

phenotypes and comparisons of both populations with the general population should be performed.

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Ethics

Ethics Committee Approval: The institutional ethics review board of Fundación Valle del Lili approved the study (Act 084-2014, date: 21.04.2014).

Informed Consent: Written informed consent was obtained from all patients' parents before study participation.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practicioners: Mario Angulo, Ximena García, Rodrigo Lemus, Concept: Mario Angulo, Design: Mario Angulo, Diana M. Dávalos, Harry Pachajoa, Data Collection or Processing: Ximena García, Rodrigo Lemus, Danielle Floyd-Aristizábal, Ana M. Aristizabal, Lorena Díaz-Ordoñez, Diana Ramírez-Montaño, Analysis or Interpretation: Mario Angulo, Lorena Diaz-Ordoñez, Harry Pachajoa, Diana Ramírez-Montaño, Laura Torres-Canchala, Literature Search: Diana Ramírez-Montaño, Laura Torres-Canchala, Writing: Diana Ramírez-Montaño, Laura Torres-Canchala.

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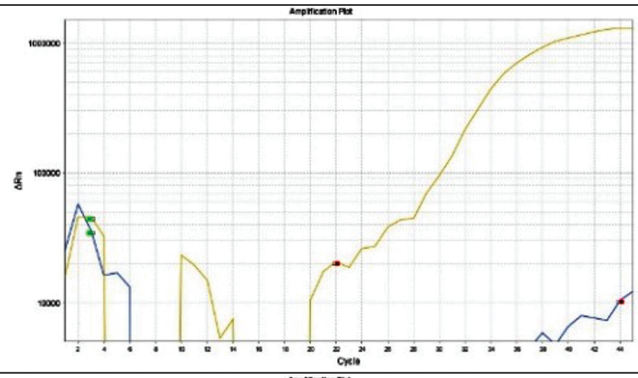
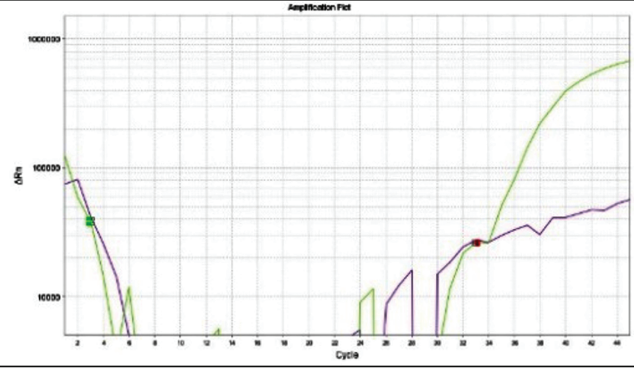
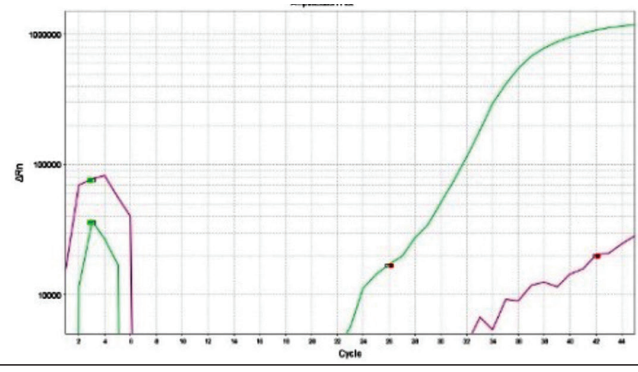
Supplemental Figure 1. Examples of quantitative polymerase chain reaction graphs obtained during the methylation assay

Gene	Forward	Result
LEP		Optimal
IRS2		
GLP2R		

Supplemental Table 1. Designed primers and probes for the evaluation of genomic DNA methylation

GEN	Designed primers and probe		
	Forward primers	**Probes**	**Reverse primers**
LEP	CAACCCCGCAATCTAAATCGAAAA	CGCACTACGAACCGCTCCCTCTAACC	GGTTTTGGACGTTAGGGAAGTTTA
IRS2	AAGTTTAATTGCGAGTAGTCGTCG	ACCGAATCGTCCGCCTACATCCACA	TCCAAAATAATCTCGTAAATATTCTACGC
GIP	CGCCCAAACTAACAAACAATAACG	CTCACTACAACCTCCGCCTACCGAATT	CGCCCAAACTAACAAACAATAACG
GLP2R	GAATTTTGAAGATTTCGTAGATTGTTTTAG	ACACCGCAAACAACCTCCTCTTACATTCC	AAATACATCTCTCTAACCGTCCAAA
IGF2	TTTTCGTTTTGTTTCGTCGTATATTCG	TAACCCTCCTACCGAACACTCCTCTACCA	TACTACGTATCGCAAACCGAACAA

Supplemental Table 2. Relative quantification for *LEP* in samples according to ΔΔCT

Block Type	96fast
Chemistry	TAQMAN
Experiment File Name	D:\Users\INSTR-ADMIN\Desktop\Epigenetica\First run.eds
Experiment Run End Time	2017-03-13 17:07:21 PM COT
Instrument Type	sds7500fast

Well	Sample name	Target name	Reporter	Quencher	C_T	CT mean	C_T SD	ΔC_T	$\Delta\Delta C_T$
A1	017	LEP	FAM	NFQ-MGB	32,48601	32,73349	0,309419	-0,86849	-34,2468
A2	017	LEP	FAM	NFQ-MGB	33,0804	32,73349	0,309419	-0,2741	-33,6524
A3	017	LEP	FAM	NFQ-MGB	32,63406	32,73349	0,309419	-0,72044	-34,0987
A4	017P	LEP	FAM	NFQ-MGB	31,29929	31,27273	0,110781	-2,05521	-35,4335
A5	017P	LEP	FAM	NFQ-MGB	31,36781	31,27273	0,110781	-1,98669	-35,365
A6	017P	LEP	FAM	NFQ-MGB	31,15108	31,27273	0,110781	-2,20342	-35,5817
A7	018	LEP	FAM	NFQ-MGB	32,19761	31,92159	0,379013	-1,15689	-34,5352
A8	018	LEP	FAM	NFQ-MGB	32,0777	31,92159	0,379013	-1,2768	-34,6551
A9	018	LEP	FAM	NFQ-MGB	31,48945	31,92159	0,379013	-1,86505	-35,2433
A10	004	LEP	FAM	NFQ-MGB	31,24569	31,59458	0,356612	-2,10881	-35,4871
A11	004	LEP	FAM	NFQ-MGB	31,95844	31,59458	0,356612	-1,39606	-34,7744
A12	004	LEP	FAM	NFQ-MGB	31,57961	31,59458	0,356612	-1,77489	-35,1532
B1	015	LEP	FAM	NFQ-MGB	32,59671	32,70707	0,100448	-0,75779	-34,1361
B2	015	LEP	FAM	NFQ-MGB	32,73132	32,70707	0,100448	-0,62318	-34,0015
B3	015	LEP	FAM	NFQ-MGB	32,79316	32,70707	0,100448	-0,56134	-33,9396
B4	015P	LEP	FAM	NFQ-MGB	30,79715	31,22849	0,37471	-2,55735	-35,9356
B5	015P	LEP	FAM	NFQ-MGB	31,41473	31,22849	0,37471	-1,93977	-35,3181
B6	015P	LEP	FAM	NFQ-MGB	31,4736	31,22849	0,37471	-1,8809	-35,2592
B7	016	LEP	FAM	NFQ-MGB	32,31133	32,43078	0,166969	-1,04317	-34,4215
B8	016	LEP	FAM	NFQ-MGB	32,35945	32,43078	0,166969	-0,99505	-34,3733
B9	016	LEP	FAM	NFQ-MGB	32,62157	32,43078	0,166969	-0,73293	-34,1112
B10	006	LEP	FAM	NFQ-MGB	31,14703	31,75572	0,527453	-2,20747	-35,5858
B11	006	LEP	FAM	NFQ-MGB	32,07829	31,75572	0,527453	-1,27621	-34,6545
B12	006	LEP	FAM	NFQ-MGB	32,04184	31,75572	0,527453	-1,31266	-34,691
C1	013	LEP	FAM	NFQ-MGB	32,23765	32,20446	0,058418	-1,11685	-34,4951
C2	013	LEP	FAM	NFQ-MGB	32,137	32,20446	0,058418	-1,21749	-34,5958
C3	013	LEP	FAM	NFQ-MGB	32,23872	32,20446	0,058418	-1,11578	-34,4941
C4	013P	LEP	FAM	NFQ-MGB	30,72647	31,1143	0,350237	-2,62803	-36,0063
C5	013P	LEP	FAM	NFQ-MGB	31,20893	31,1143	0,350237	-2,14557	-35,5239
C6	013P	LEP	FAM	NFQ-MGB	31,4075	31,1143	0,350237	-1,947	-35,3253
C7	014	LEP	FAM	NFQ-MGB	31,75308	31,81729	0,174822	-1,60142	-34,9797

C8	014	LEP	FAM	NFQ-MGB	32,01514	31,81729	0,174822	-1,33936	-34,7177
C9	014	LEP	FAM	NFQ-MGB	31,68365	31,81729	0,174822	-1,67085	-35,0491
C10	008	LEP	FAM	NFQ-MGB	30,90038	30,78209	0,248279	-2,45411	-35,8324
C11	008	LEP	FAM	NFQ-MGB	30,94911	30,78209	0,248279	-2,40539	-35,7837
C12	008	LEP	FAM	NFQ-MGB	30,49679	30,78209	0,248279	-2,85771	-36,236
D1	011	LEP	FAM	NFQ-MGB	32,95736	32,91446	0,096077	-0,39714	-33,7754
D2	011	LEP	FAM	NFQ-MGB	32,9816	32,91446	0,096077	-0,3729	-33,7512
D3	011	LEP	FAM	NFQ-MGB	32,8044	32,91446	0,096077	-0,5501	-33,9284
D4	011P	LEP	FAM	NFQ-MGB	31,99075	32,03038	0,114877	-1,36375	-34,742
D5	011P	LEP	FAM	NFQ-MGB	31,94057	32,03038	0,114877	-1,41393	-34,7922
D6	011P	LEP	FAM	NFQ-MGB	32,15983	32,03038	0,114877	-1,19467	-34,573
D7	012	LEP	FAM	NFQ-MGB	32,60457	32,40089	0,182291	-0,74993	-34,1282
D8	012	LEP	FAM	NFQ-MGB	32,25306	32,40089	0,182291	-1,10144	-34,4797
D9	012	LEP	FAM	NFQ-MGB	32,34504	32,40089	0,182291	-1,00946	-34,3878
D10	010	LEP	FAM	NFQ-MGB	31,85977	31,62498	0,29803	-1,49473	-34,873
D11	010	LEP	FAM	NFQ-MGB	31,28969	31,62498	0,29803	-2,06481	-35,4431
D12	010	LEP	FAM	NFQ-MGB	31,72548	31,62498	0,29803	-1,62902	-35,0073
E1	008P	LEP	FAM	NFQ-MGB	31,40598	31,49476	0,08228	-1,94852	-35,3268
E2	008P	LEP	FAM	NFQ-MGB	31,56846	31,49476	0,08228	-1,78604	-35,1643
E3	008P	LEP	FAM	NFQ-MGB	31,50984	31,49476	0,08228	-1,84466	-35,223
E4	009	LEP	FAM	NFQ-MGB	32,8835	32,91939	0,060303	-0,471	-33,8493
E5	009	LEP	FAM	NFQ-MGB	32,88566	32,91939	0,060303	-0,46884	-33,8471
E6	009	LEP	FAM	NFQ-MGB	32,98901	32,91939	0,060303	-0,36549	-33,7438
E7	009P	LEP	FAM	NFQ-MGB	31,8208	30,97832	0,732797	-1,5337	-34,912
E8	009P	LEP	FAM	NFQ-MGB	30,62545	30,97832	0,732797	-2,72905	-36,1073
E9	009P	LEP	FAM	NFQ-MGB	30,48873	30,97832	0,732797	-2,86577	-36,2441
E10	012P	LEP	FAM	NFQ-MGB	31,20582	31,18705	0,061365	-2,14868	-35,527
E11	012P	LEP	FAM	NFQ-MGB	31,23683	31,18705	0,061365	-2,11767	-35,496
E12	012P	LEP	FAM	NFQ-MGB	31,11849	31,18705	0,061365	-2,23601	-35,6143
F1	006P	LEP	FAM	NFQ-MGB	31,27403	31,15672	0,165998	-2,08047	-35,4588
F2	006P	LEP	FAM	NFQ-MGB	31,22935	31,15672	0,165998	-2,12515	-35,5034
F3	006P	LEP	FAM	NFQ-MGB	30,96679	31,15672	0,165998	-2,38771	-35,766
F4	007	LEP	FAM	NFQ-MGB	32,72506	32,83705	0,244365	-0,62944	-34,0077
F5	007	LEP	FAM	NFQ-MGB	32,66875	32,83705	0,244365	-0,68575	-34,064
F6	007	LEP	FAM	NFQ-MGB	33,11734	32,83705	0,244365	-0,23716	-33,6155
F7	007P	LEP	FAM	NFQ-MGB	31,28529	30,71506	0,499296	-2,06921	-35,4475
F8	007P	LEP	FAM	NFQ-MGB	30,3563	30,71506	0,499296	-2,9982	-36,3765
F9	007P	LEP	FAM	NFQ-MGB	30,50359	30,71506	0,499296	-2,85091	-36,2292
F10	014P	LEP	FAM	NFQ-MGB	32,07011	32,06537	0,17548	-1,28439	-34,6627
F11	014P	LEP	FAM	NFQ-MGB	31,88756	32,06537	0,17548	-1,46694	-34,8452
F12	014P	LEP	FAM	NFQ-MGB	32,23843	32,06537	0,17548	-1,11607	-34,4944
G1	004P	LEP	FAM	NFQ-MGB	31,11732	31,35745	0,237773	-2,23718	-35,6155
G2	004P	LEP	FAM	NFQ-MGB	31,36222	31,35745	0,237773	-1,99228	-35,3706
G3	004P	LEP	FAM	NFQ-MGB	31,5928	31,35745	0,237773	-1,7617	-35,14
G4	005	LEP	FAM	NFQ-MGB	32,95717	32,6046	0,306358	-0,39733	-33,7756
G5	005	LEP	FAM	NFQ-MGB	32,40327	32,6046	0,306358	-0,95123	-34,3295

G6	005	LEP	FAM	NFQ-MGB	32,45337	32,6046	0,306358	-0,90113	-34,2794
G7	005P	LEP	FAM	NFQ-MGB	31,71308	31,54452	0,22419	-1,64142	-35,0197
G8	005P	LEP	FAM	NFQ-MGB	31,29009	31,54452	0,22419	-2,06441	-35,4427
G9	005P	LEP	FAM	NFQ-MGB	31,63039	31,54452	0,22419	-1,72411	-35,1024
G10	016P	LEP	FAM	NFQ-MGB	30,58578	30,45538	0,382029	-2,76872	-36,147
G11	016P	LEP	FAM	NFQ-MGB	30,75513	30,45538	0,382029	-2,59937	-35,9777
G12	016P	LEP	FAM	NFQ-MGB	30,02522	30,45538	0,382029	-3,32928	-36,7076
H1	002P	LEP	FAM	NFQ-MGB	31,24798	31,14372	0,098846	-2,10652	-35,4848
H2	002P	LEP	FAM	NFQ-MGB	31,13182	31,14372	0,098846	-2,22268	-35,601
H3	002P	LEP	FAM	NFQ-MGB	31,05136	31,14372	0,098846	-2,30314	-35,6814
H4	003	LEP	FAM	NFQ-MGB	31,08652	31,20967	0,185724	-2,26798	-35,6463
H5	003	LEP	FAM	NFQ-MGB	31,11919	31,20967	0,185724	-2,23531	-35,6136
H6	003	LEP	FAM	NFQ-MGB	31,42329	31,20967	0,185724	-1,93121	-35,3095
H7	003P	LEP	FAM	NFQ-MGB	31,99791	31,84208	0,198175	-1,35659	-34,7349
H8	003P	LEP	FAM	NFQ-MGB	31,90929	31,84208	0,198175	-1,44521	-34,8235
H9	003P	LEP	FAM	NFQ-MGB	31,61904	31,84208	0,198175	-1,73546	-35,1138
H10	Control DNA metilado	LEP	FAM	NFQ-MGB	33,3545	33,3783	0,033653	0	-33,3783
H11	Control DNA metilado	LEP	FAM	NFQ-MGB	33,40209	33,3783	0,033653	0,047592	-33,3307
H12	Blanco	LEP	FAM	NFQ-MGB	Undetermined				

Analysis Type		Singleplex	**ΔC_T Mean** 33,3782959
Endogenous Control	LEP		
RQ Min/Max Confidence Level	95.0		
Reference Sample		002P	

SD: Standard deviation

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Growth Hormone Treatment and Papilledema: A Prospective Pilot Study

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What is already known on this topic?

Growth hormone (GH) replacement therapy is a risk factor for secondary pseudotumor cerebri. The incidence of this complication can vary depending on different GH indications.

What this study adds?

Children with intracranial hypertension could be asymptomatic, so the diagnosis should be based on fundus examination and not on patient's symptoms. In our series, at risk patients had GH deficiency and hypothalamic-pituitary anatomic anomalies or genetic or chromosomal diseases. A previous history of pseudotumor cerebri should be investigated.

Abstract

Objective: To investigate the incidence of pseudotumor cerebri syndrome (PTCS) in children treated with growth hormone (GH) in a paediatric hospital and to identify risk factors for this complication.

Methods: Prospective pilot study of paediatric patients treated with recombinant human GH, prescribed by the Paediatric Endocrinology Department, between February 2013 and September 2017. In all these patients, a fundus examination was performed before starting treatment and 3-4 months later.

Results: Two hundred and eighty-nine patients were included, of whom 244 (84.4%) had GH deficiency, 36 (12.5%) had short stature associated with small for gestational age, six (2.1%) had a mutation in the *SHOX* gene and three (1.0%) had Prader-Willi syndrome. Five (1.7%) developed papilledema, all were asymptomatic and had GH deficiency due to craniopharyngioma (n = 1), polymalformative syndrome associated with hypothalamic-pituitary axis anomalies (n = 2), a non-specified genetic disease with hippocampal inversion (n = 1) and one with normal magnetic resonance imaging who had developed a primary PTCS years before.

Conclusion: GH treatment is a cause of PTCS. In our series, at risk patients had GH deficiency and hypothalamic-pituitary anatomic anomalies or genetic or chromosomal diseases. Fundus examination should be systematically screened in all patients in this at-risk group, irrespective of the presence or not of symptoms.

Keywords: Growth hormone treatment, pseudotumor cerebri syndrome, idiopathic intracranial hypertension, papilledema, risk factors

Introduction

Pseudotumor cerebri syndrome (PTCS) is a condition defined by elevated intracranial pressure (ICP) in the absence of clinical, laboratory or radiologic evidence of infection, vascular abnormality, intracranial space-occupying lesion or hydrocephalus (1,2). It can be primary, or secondary if there are any identifiable risk factors. Primary PTCS most commonly occurs in obese adult women of childbearing age (12-32/100,000). However, it is a rare condition in childhood

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(0.5-0.9/100,000) with an identifiable risk factor in 53-77% of paediatric cases (3,4,5). Papilledema is a hallmark in PTCS, which is not a benign disorder; there is a risk of severe and permanent visual loss. Even patients with mild visual loss experience reduction in quality of life (6).

Growth hormone (GH) replacement therapy was first associated with PTCS by The Food and Drug Administration in 1993 (7). Since then, multiple publications have described this association. Reeves and Doyle (8) found that the prevalence in the GH treated population was approximately 100 times greater than in the general paediatric population. This complication usually occurs in the first weeks after initiating treatment (approximately 2-12 weeks) and the papilledema (optic disc swelling from raised ICP) resolves on stopping treatment (8,9).

Pseudopapilledema is an elevated optic disc with obscured margins that may occur in hyperopic eyes and in the presence of buried optic disc drusen, rather than from raised ICP. Optic disc drusen are acellular deposits located within the optic disc. Sometimes it can be very difficult to differentiate between pseudopapilledema and papilledema, requiring the opinion of a suitably experienced ophthalmologist and complementary tests (10).

The aim of this study was to investigate the incidence of PTCS in children treated with GH in a paediatric hospital and to identify risk factors for this complication. A secondary aim was to identify which children really need fundus examination during GH treatment in order to detect this complication.

Methods

A prospective pilot study was conducted in paediatric patients who started GH treatment from February 2013 to September 2017. Inclusion criteria were patients under 16 years of age prescribed GH by the Paediatric Endocrinology Department. Exclusion criteria were: absence of fundus examination prior to or at 3-4 months from starting treatment; severe optic disc atrophy where optic disc swelling would not develop; and cases where GH was prescribed by the Paediatric Nephrology Department. Predisposing pathology for intracranial hypertension was not considered an exclusion criterion, as fundus examinations were conducted before initiating treatment to rule out prior pathology of the optic nerve. The following variables were recorded: previous medical history; anthropometric data; and compliance with treatment.

Fundus was explored prior to and after 3-4 months of starting treatment or at any time when severe constant headache, vomiting or other presenting symptoms suggestive of intracranial hypertension arose. Prior fundus examination identified possible cases of pseudopapilledema and avoided possible confusion between this entity and newly developed papilledema in subsequent fundus follow-up examinations.

Fundus examination was carried out with indirect ophthalmoscopy and retinography was taken in cases of pseudopapilledema to facilitate follow up. In cases when papilledema was detected, GH was stopped and fundus examination was repeated four weeks later. If papilledema persisted, the patient was referred for neurological assessment, lumbar puncture and possible medical treatment with acetazolamide. Once papilledema resolved, if GH treatment was still considered necessary, treatment was re-initiated at a lower dose and progressively increased until the objective dose was achieved, with monthly ophthalmology visits until four months were completed at the target GH dose.

Cerebral magnetic resonance imaging (MRI) of the hypothalamic-pituitary axis was performed in all patients with GH deficiency, prior to treatment. Initial GH doses were as indicated: 0.028-0.035 mg/kg/day for patients with GH deficiency and small for gestational age (SGA), 0.042 mg/kg/day for those with mutations in the *SHOX* gene and 1 mg/m^2/day for patients with Prader-Willi syndrome (PWS).

Ethics approval was obtained from the Ethic Review Committee of the Hospital Universitario Vall d'Hebron (approval number: 383). All subjects (or their parents or guardians) gave their written informed consent.

Statistical Analysis

In this case series, simple descriptive statistics were sufficient to delineate our populations. Variables are expressed as number, mean and percent (%) or range, as appropriate.

Results

There were 306 patients to whom GH was prescribed by the Paediatric Endocrinology Department between February 2013 and September 2017. Only 289 patients were enrolled in this study, 17 were excluded because of loss of ophthalmological follow up.

The mean age was nine years (range 1-16) and 53% of the patients were male. The patients were categorized according to their indication for GH treatment: 244 patients (84.4%) with GH deficiency; 36 patients (12.5%) with short stature associated with SGA; six patients (2%) with a mutation in the *SHOX* gene; and three (1%) with PWS. Patients

diagnosed with chronic kidney disease were not included in the study as their indication for GH treatment and follow up was made by the Paediatric Nephrology Department.

In the first visit, prior to starting GH treatment, there were 546 eyes with normal optic discs, 16 eyes with pale optic discs, 11 eyes with pseudopapilledema, two eyes with optic disc and chorioretinal coloboma and one eye with dysplastic optic disc. Pale optic discs and optic disc and chorioretinal coloboma were secondary to the patient background pathology, suprasellar cerebral tumours and CHARGE anomaly respectively. In the follow up visit, five patients had optic disc swelling, suggesting papilledema, despite there being no symptoms of intracranial hypertension. None of the patients who presented with headache, who were visited urgently, presented with papilledema on fundus examination.

Table 1 outlines the characteristics of the five patients with papilledema. All these patients had GH deficiency: three had hypothalamic-pituitary axis abnormalities on brain MRI; two were congenital cases; and one was secondary to a suprasellar tumour. The patient with a suprasellar tumour had a ventriculoperitoneal shunt and was treated with external radiotherapy. The only patient with an isolated GH deficiency with normal MRI, had developed primary PTCS five years before and he was obese. The incidence of obesity in children without PTCS in our population is 10.3%.

The papilledema resolved on discontinuing GH treatment in four patients, whereas in the remaining patient, a lumbar puncture confirmed the diagnosis and was also therapeutic. The opening pressure was 25 cm H_2O. The glucose was 61 mg/dL (38-82); protein 20 mg/dL (15-45) and there were neither leukocytes nor red blood cells present in the cerebrospinal fluid (CSF). Medical treatment was not required. In four cases GH treatment was reintroduced at a lower dose with progressive incremental doses, without the reappearance of papilledema.

Discussion

GH treatment is a risk factor for developing secondary PTCS although the mechanism is little understood. Two hypotheses have been proposed. The first is that GH could have a physiological antidiuretic effect, causing retention of sodium and water and expansion in blood volume, and reducing CSF resorption by the arachnoid villi. The second hypothesis proposes that GH would cross the blood-brain barrier, resulting in raised cerebral levels of GH and its mediator, insulin-like growth factor 1, and finally increasing CSF production (11,12,13,14).

GH was initially obtained from human pituitary gland, and was given at lower doses and frequency, and prescribed in fewer clinical situations. Since 1985, when recombinant human GH became commercially available, more patients were treated with larger doses and more often. Since then, indications for GH treatment have increased, as well as the potential adverse effects (15).

Table 1. Characteristics of patients treated with growth hormone who developed papilledema

Patients	Gender/age (years)	Papilledema onset time (months)	W (kg)/H (cm)	GH dose (mg/kg/week)	Other pathology/MRI anomalies	Risk factors PTCS	Treatment PTCS
1	M/14	2	**71.4**/144.7	0.20	--	PTCS 2010, obesity	GH stopped
2	M/3	3	11.4/86	0.23	Polymalformative syndrome, pituitary gland anomaly and partial agenesis of corpus callosum on brain MRI	None	GH stopped
3	M/11	3	26.5/128.3	0.21	PDR Dysmorphic phenotype Incomplete hippocampal inversion on brain MRI	None	GH stopped + LP
4	M/10	3	36.4/132	0.19	Craniopharyngioma, radiation therapy	None	GH stopped
5	F/4	3	10.7/80.4	**0.32**	Microcephaly Pituitary gland compression by an arachnoid cyst Hypoparathyroidism PDR	None	GH stopped

F: female, GH: growth hormone, H: height, LP: lumbar puncture, M: male, MRI: magnetic resonance imaging, PDR: psychomotor development retardation, PTCS: pseudotumor cerebri syndrome, W: weight

Table 2. Distribution of patients with growth hormone deficiency according to our classification in subgroups

	GH deficiency subgroups	Nº patients	Nº papilledema
1	Isolated GH deficiency with normal H-P axis on MRI	164	1
2	GH deficiency (isolated or not) with abnormal H-P axis on MRI and/or RT	62	3
3	Genetic or chromosome disorders	18	1

GH: growth hormone, H-P: hypothalamic-pituitary, MRI: magnetic resonance imaging, RT: cranial radiotherapy

In our study, there were five patients with papilledema (1.7%), a higher incidence compared with the expected incidence in the general paediatric population (8,9,11,13). All of them had GH deficiency. Other indications for GH treatment did not appear to cause papilledema in this series, although these results may be biased due the small populations with these other indications.

As all the patients with papilledema were found to have GH deficiency, we analysed this group, classifying patients into three subgroups (Table 2):

1. Isolated GH deficiency (no other associated hormone deficiencies) with normal hypothalamic-pituitary axis anatomy on MRI.

2. Isolated GH deficiency or associated with other hormone deficiencies with altered hypothalamic-pituitary axis anatomy on MRI and/or past history of cerebral radiotherapy.

3. GH deficiency in patients with genetic or chromosomal diseases.

When considering patients who presented with papilledema, three completed criteria for subgroup 2, presenting with hypothalamic-pituitary axis abnormalities and external radiotherapy treatment in one case for a suprasellar tumour. These three patients in subgroup 2 had an intracranial hypertension, but not technically a PTCS, because the brain MRI was not normal. One other patient was classified as subgroup 3 for a non-specific genetic disease. Only one patient presented with an isolated GH deficiency with normal cerebral MRI, however this patient had had primary PTCS five years earlier. The patient with a suprasellar tumour also had a ventriculoperitoneal shunt, suggesting that the presence of a shunt is not protective against a rise in ICP induced by GH therapy.

For each GH indication, the incidence of this adverse effect can vary. Reeves and Doyle (8), reported higher incidences in patients with renal failure and Turner syndrome. Souza and Collet-Solberg (11), also detected a higher incidence in patients with chronic kidney disease. Darendeliler et al (16) proposed that patients with Turner syndrome, organic GH deficiency, PWS and chronic renal insufficiency might be more prone to develop papilledema when receiving GH. In our study, patients with renal disease were excluded, as the GH was not prescribed by the Paediatric Endocrinology Department. However, we have conducted a retrospective study of these patients with renal disease on GH treatment during the same time period and have also detected a higher incidence of papilledema, as described in the literature.

The association between the dose of GH and the risk of PTCS is not clearly established. Malozowski et al (15) reported that higher doses and increased frequency of administration since the introduction of recombinant human GH in 1985 may be contributing to the development of PTCS in some patients. However, Reeves and Doyle found no relationship between the GH dose and PTCS development (8). One of our patients received GH at the usual dose without complications, later on it was withdrawn due to lack of therapeutic effect. Interestingly, papilledema appeared three months after restarting GH at a higher dose. Patients with PWS and mutations in the *SHOX* gene, who received higher doses of GH did not present with papilledema, although these subgroups were small in this study. Even though there is no evidence that the GH dose is directly related with this complication, we recommend starting at lower dose and increasing it progressively, in order to minimize this complication.

Patients with a previous history of PTCS may experience recurrence at rates reported to vary between 6-22% (4,17,18,19). There is usually a triggering factor, such as weight gain or the introduction of known medications associated with secondary PTCS (20). In our series, the only patient with a previous history of PTCS, presented with papilledema. This patient was obese, which could have been a further risk factor for primary PTCS recurrence, however his weight had remained stable prior to starting GH treatment, at one month and at two months of treatment, when papilledema was diagnosed. For this reason, we believe it is important to identify patients initiating GH therapy with a previous history of PTCS because of the risk of recurrence. Also, consideration should be taken for starting treatment at a lower dose with progressive increases, accompanied by with careful follow-up including fundus examination during the first months of treatment.

Headache is a common symptom in the general paediatric population, and is also relatively frequent in patients on GH

therapy, being the third most prevalent side effect described in KIGS (Pfizer International Growth study database) (16). On the other hand, headache is the main manifestation of intracranial hypertension at any age. However, in the paediatric population, headache is less common and 33% of children with PTCS may be totally asymptomatic (21,22), and diagnosis is only made on the observation of papilledema on fundus examination. It is important to highlight that papilledema is a cause of visual morbidity, including irreversible vision loss, independent of the patient symptoms. In our series, all patients with papilledema were asymptomatic, whereas no patients examined urgently for headache presented with papilledema.

Stopping GH is usually enough to treat this complication. Once papilledema has resolved GH can be reintroduced at a lower dose and progressively increased until the required dose is achieved to prevent recurrence and optimum growth. In the four patients where GH was reintroduced there were no recurrences of papilledema.

Study Limitations

The strengths of our study are the prospective design and the number of patients included in it. As children may be asymptomatic, prospective studies are the only way to establish the real incidence of this complication. The main limitation of our study is that patients with kidney diseases were not included and this group of patients has been reported to be at greatest risk of this complication in a different series.

Conclusion

In this study, we have shown that GH therapy is a risk factor for intracranial hypertension and the at-risk group were patients with GH deficiency and hypothalamic-pituitary axis abnormalities on MRI or genetic or chromosomal diseases. Patients may be totally asymptomatic, so fundus examination should be systematically implemented in this at-risk group, irrespective of the presence or absence of symptoms.

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Ethics

Ethics Committee Approval: Ethics approval was obtained from the Ethic Review Committee of the Hospital Universitario Vall d'Hebron (approval number: 383, date: 17.05.2019).

Informed Consent: All subjects (or their parents or guardians) gave their written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste, Concept/Design: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste, Data Collection or Processing: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste, Analysis or Interpretation: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste, Literature Search: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste, Writing: Nieves Martín-Begué, Eduard Mogas, Charlotte Wolley Dod, Silvia Alarcón, María Clemente, Ariadna Campos-Martorell, Ana Fábregas, Diego Yeste.

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Is Waist-height Ratio Associated with Thyroid Antibody Levels in Children with Obesity?

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What is already known on this topic?

It is known that obesity influences thyroid functions. Recently, it was reported that thyrotropin levels were associated with body mass index standard deviation score and waist-height ratio (WHtR)-an indicator of central fat accumulation-in obese children. In adults with obesity thyroid autoantibody levels were higher than healthy subjects. However, in children with obesity, thyroid autoimmunity is not considered as prevalent as in adults, as autoimmune thyroiditis was reported in only 19.5% of obese children with hyperthyrotropinemia. Although a correlation between WHtR and thyrotropin-thyroxine levels was found in obese children, its association with thyroid autoantibody levels still remains unclear.

What this study adds?

In our study, children with obesity had higher concentrations of thyroid autoantibodies compared to healthy controls, although these levels remained below the cut-off for clinical significance. The obese patients with a WHtR >0.6 had higher thyroid antibody levels compared to those with a WHtR ≤0.6, even in the absence of autoimmune thyroid disease, and there was a positive correlation between WHtR and thyroglobulin-antibodies levels. These findings suggest that central adiposity influences thyroid autoantibody production in children with obesity.

Abstract

Objective: Obesity is known to affect thyroid function. Recently, waist-height ratio (WHtR) has been considered as a useful marker of subclinical hypothyroidism in obese cases, but its relation with thyroid autoimmunity still remains unclear. We evaluated the effect of body fat mass, WHtR, and metabolic parameters on thyroid autoantibody levels in children with obesity.

Methods: This was a cross-sectional study carried out with an obese [n=56, male/female (M/F): 29/26] and a healthy group (n=38, M/F: 19/19). All subjects underwent anthropometric measurements, laboratory investigations for thyroid function tests, thyroid peroxidase (TPO-ab) and thyroglobulin-antibodies (Tg-ab), transaminases, blood glucose, insulin levels, and lipids after overnight fasting; homeostatic model assessment for insulin resistance (HOMA-IR) was calculated for assessment of insulin resistance. Fat mass was estimated by multiple frequency bioimpedance analysis in the obese group, which was further divided into two subgroups according to the median of WHtR. All parameters were compared between the groups/subgroups.

Results: In the obese group, weight, height, body mass index (BMI), free triiodothyronine, thyrotropin, TPO-ab, insulin, low density lipoprotein-cholesterol, total cholesterol, alanine aminotransferase levels, and HOMA-IR were significantly higher than the controls group ($p<0.05$ for all). Median of WHtR was 0.6 in the obese group. In the "WHtR >0.6" subgroup (n=28), weight, BMI, fat mass, TPO-ab, Tg-ab, insulin and triglyceride levels were higher than WHtR ≤0.6 subgroup ($p<0.05$). A positive correlation was obtained between Tg-ab and WHtR (rho=0.28, $p=0.041$).

Conclusion: Euthyroid children with obesity and a WHtR >0.6 are likely to have higher thyroid antibody levels, and Tg-ab levels have a positive correlation with WHtR, which reveals an association of central adiposity with thyroid autoantibody levels in these cases.

Keywords: Free thyroxine, free triiodothyronine, thyroglobulin antibody, thyroid peroxidase antibody, thyroid stimulating hormone, waist-height ratio

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Introduction

Obesity leads to a number of metabolic and hormonal disturbances in children, such as thyroid dysfunction (1,2). Moderate elevation of thyrotropin (TSH) levels (subclinical hypothyroidism) is a common condition in children with overweight/obesity, with a prevalence ranging from 7% to 23%. However, prior studies offer conflicting results about thyroid hormone levels (2-12). Nonetheless, it is accepted that TSH and thyroid hormone levels usually normalize with weight loss, from which it was interpreted that this is an adaptive response to lipid accumulation (2,3,4).

Anthropometric measurements are mostly used to diagnose and evaluate obesity and its complications (1). To date, numerous studies have reported a positive correlation between body mass index (BMI) and TSH, and some follow-up studies have demonstrated that TSH level increased with weight gain and decreased with weight loss (2,3,4,5,6,7,8,9). Besides, some other anthropometric measurements have also been evaluated in several studies (1,4,5,6,8,9). In a study with a large cohort, TSH levels were reported to be associated positively with BMI standard deviation (SD) score (SDS) and waist/height ratio (WHtR), regardless of age, gender and pubertal stage. However, serum free thyroxine (fT4) concentrations were found to be associated only with WHtR (5). In another study, waist/hip ratio was considered as a predictor of increased free triiodothyronine (fT3) to fT4 ratio (9). These results suggest that central obesity could increase the risk of concurrent thyroid abnormalities in children with obesity (5,9).

Although TSH and thyroid hormone levels in children and adults with obesity have been evaluated in numerous studies, the effect of obesity and lipid accumulation on thyroid autoantibody levels still remains unclear in children (2,3,4,5,6,7,8,9,10,11,12). Obesity is well known to be a chronic inflammatory process (1). Elevated thyroid autoantibody levels, especially antithyroid peroxidase (TPO-ab), with hypothyroidism have been reported to be common in adults with obesity (10). In addition, increased leptin levels have been found to be associated with the presence of autoimmune thyroiditis disease (AITD) (10). However, results of studies of childhood obesity are slightly different, and non-autoimmune thyroiditis should be considered in the differential diagnosis before diagnosing AITD (3,12,13). It was reported that obese cases with hyperthyrotropinemia and hypoechogenicity on thyroid ultrasound, but without thyroid autoantibody seropositivity, had normal cytological findings in ultrasound-guided fine needle aspiration biopsy specimens, which indicates the important role of thyroid antibodies in a diagnosis of AITD in obese patients (13). AITD was detected in only 19.5% of children with obesity and hyperthyrotropinemia and in 7.4% of children with normal TSH levels (3,12). Moreover, it has been shown that TPO-ab level was positively associated with BMI (7).

Obesity and central fat accumulation lead to insulin resistance and dyslipidemia (1). In previous studies, TSH levels were found to correlate with fasting insulin (6), total cholesterol (6,11), triglyceride levels (6,8,11,14) and homeostatic model assessment for insulin resistance (HOMA-IR) (6). However, little is known about the association of thyroid antibody levels and metabolic disturbances in children with obesity.

The aim of this study was to evaluate the association of thyroid hormone and thyroid autoantibody levels with body fat mass, WHtR and metabolic parameters, such as lipid profile, fasting glucose and insulin levels in euthyroid children with obesity.

Methods

Study Population

In this cross-sectional study, 55 obese and 38 healthy children aged between 8 and 18 years were involved. The obese group consisted of subjects without additional endocrine/genetic disorders leading to obesity. The control group consisted of age-and sex-matched children who were admitted to the pediatric clinic for routine health screening. Cases who were previously diagnosed with overt or subclinical hypothyroidism, AITD, who had a family member with a diagnosis of AITD or who were receiving medication affecting energy metabolism, such as metformin, were excluded from the study.

Anthropometric Measurements and Puberty Staging

All anthropometric measurements were obtained by the same clinician on subject admission. Height was measured using a stadiometer (Holtain Limited, Crymych, Wales) to the nearest 0.5 cm with the subject having bare feet, eyes looking straight ahead and back against the wall. Weight was measured using an electronic scale (Tefal, France) sensitive to 100 g and BMI was calculated as weight in kilograms divided by the square of height in meters. The waist circumference (WC) was measured in the obese group as an abdominal circumference in the horizontal plane midway between the lowest rib and the superior border of iliac crest and at the end of normal expiration with a non-stretchable tape to the nearest 0.1 cm. The SDS and percentiles were calculated according to Turkish pediatric reference values previously reported by ÇEDD Çözüm/TPEDS Metrics (15,16). The patients with BMI percentile ≥95 for sex and age were

defined as obese (1). Puberty stage was reported using the method of Marshall and Tanner (17).

Assessment of Body Composition

Body composition including fat mass (kg) was estimated by multiple frequency bioimpedance analysis in the obese group. Measurements were performed by a single physician using a portable body bioimpedance spectroscopy device, the Body Composition Monitor (Fresenius Medical Care, Germany). Fat mass index (FMI) was calculated as the quotient of fat mass/height^2. Fat mass to weight ratio (fat%) was described as the quotient of fat mass/body weight x 100. All anthropometric measurements and body composition analyses were carried out at the same study visit and obtained after overnight fasting.

Blood Sample Collection

Serum specimens collected from the patient and control groups were stored at -80 °C until analysis. Routine biochemical tests were analyzed in the Central Biochemistry Laboratory, Cerrahpaşa Faculty of Medicine, İstanbul. Fasting blood glucose, total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol and triglyceride concentrations were measured by enzymatic colorimetric methods, while aspartate aminotransferase and alanine aminotransferase (ALT) were assayed by kinetic ultraviolet methods on a Roche Modular System (Cobas, Roche GmbH, Germany). Insulin concentration was measured by solid phase sandwich enzyme-linked immunosorbent assay (DRG instruments GmbH, Germany). Insulin resistance was assessed using HOMA-IR, which was calculated using the following standard formula: glucose (mg/dL) x insulin (μIU/mL)/405 (18).

Thyroid Function and Autoantibody Tests

For quantitative analysis of fT3 [DRG Instruments GmbH, Germany, Catalog No: enzyme immunoassay (EIA)-2385; intra-assay CV 3.6%, inter-assay CV 7.9%], fT4 (DRG Instruments GmbH, Germany, Catalog No: EIA-2386; intra-assay CV 4.26%; inter-assay CV 6.01%), and TSH (DRG Instruments GmbH, Germany, Catalog No: EIA-4171; intra-assay CV 5.7%; inter-assay CV 7.1%) commercial EIA kits were used, according to the manufacturer's guidelines.

A TSH level <5 μU/mL was defined as "normal"; and, reference intervals for fT4 and fT3 levels were 0.7-1.6 ng/dL and 1.71-3.72 pg/mL respectively (19). The patients were considered to be euthyroid if their serum fT4 and TSH levels were within normal range (19). TPO-ab and thyroglobulin-antibodies (Tg-ab) levels were measured by chemiluminescence method using the Roche Modular System (Cobas, Roche GmbH, Germany). Intra-assay CV and inter-assay CV for TPO-ab were 4.1 and 6.1 respectively. For Tg-ab, intra-assay CV and inter-assay CV were 2.1 and 4.6. A TPO-ab level >34 IU/mL and a Tg-ab level >115 IU/mL were described as "positive".

The study was approved by the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Ethical Committee (date: 04.02.2020 and ethics approval number: 21299). Patients were included in the study following a consent form signed by parents/caregivers. The information about the patients was kept confidential and used only for the purposes of the study.

Statistical Analysis

All data were assessed for normal distribution using Shapiro-Wilk test. Normally distributed data were presented as mean ± SD, and nonparametric data were presented as median (interquartile range). Between group comparisons were made using Fisher's exact test or Mann-Whitney U test, according to a normal or a nonparametric distribution of the tested variable. Pearson's correlation was used to investigate the relation between normally distributed quantitative data, while Spearman's rank correlation was used otherwise. Obese cases were divided into two different groups according to the median value of WHtR. The association between thyroid antibodies and WHtR were tested with univariate analysis in the obese group. Biostatistical analysis of the study results was performed by Statistical Package for Social Sciences software, version 21.0 (SPSS Inc., Chicago, IL, USA) and p values <0.05 were considered statistically significant.

Results

The study subjects consisted of obese [n = 55, male/female (M/F): 29/26] and healthy groups (n = 38, M/F: 19/19) with a mean age of 12.4 ± 2.6 years. A majority (78.5%, n = 73) of the patients were pubertal. Between the two groups, age, gender and pubertal stages were similar ($p>0.05$). Serum TSH, fT4, fT3 and thyroid autoantibody levels of all subjects were within normal ranges. Weight and weight SDS, BMI and BMI SDS were higher in the obese group compared to controls, as expected. In laboratory findings, fasting insulin, ALT, fT3, TSH, TPO-ab levels and HOMA-IR were higher, and fT4 was lower in children with obesity than the controls ($p<0.001$). Anthropometric measurements, metabolic parameters, thyroid function test and autoantibody levels are summarized in Table 1.

The mean value of the WC, WHtR, fat% and FMI of obese patients were 97.3 ± 13.6, 0.61 ± 0.06, 39.2 ± 5.7 and 16.6 ± 4.2, respectively.

Table 1. Comparison of anthropometric measurements, metabolic parameters, thyroid function test and autoantibody levels between the obese and control group

		Obese group (n = 55)	Control group (n = 38)	
Variable		**Mean ± SDS Median (IQR) (minimum-maximum)**	**Mean ± SDS Median (IQR) (minimum-maximum)**	**p**
Age, year		12.6 ± 2.6 (7.1-17.3)	12 ± 2.7 (7.3-17.5)	0.25*
Gender	Female	26 (47.3)	19 (50)	0.796**
n (%)	Male	29 (52.7)	19 (50)	
Puberty	Prepubertal	11 (20)	9 (23.7)	0.67**
n (%)	Pubertal	44 (80)	29 (76.3)	
Weight (kg)		76 (29.5) (31.2-122.9)	42.7 (24.1) (23-65)	**< 0.001***
Weight SDS		2.82 (1.17) (0.94-4.46)	-0.12 (1.44) (-1.23-1.18)	**< 0.001***
Height (cm)		158.7 ± 13.7 (125-188)	149.8 ± 15 (123.8-175)	**0.004***
Height SDS		0.86 ± 1.1 (-1.52-4.12)	0.01 ± 0.9 (-1.74-1.72)	**< 0.001***
BMI		29.9 (7.2) (20-37.2)	18.7 (5.2) (14.3-23.9)	**< 0.001***
BMI SDS		2.59 (0.81) (1.65-3.65)	-0.11 (1.44) (-1.83-1.14)	**< 0.001***
BMI percentile		99.5 (1.5) (95.1-99.9)	44 (52) (3.5-84)	**< 0.001***
fT3, pg/dL		4.02 (0.96) (1.0-6.23)	3.13 (0.96) (1.19-4.96)	**< 0.001***
fT4, ng/dL		1.02 ± 0.14 (0.71-1.39)	1.15 ± 0.16 (0.81-1.52)	**< 0.001***
TSH, IU/L		2.01 (1.06) (0.74-4.62)	1.46 (0.74) (0.51-4.18)	**< 0.001***
TPO-ab, IU/mL		13 (10) (5-29)	7 (6) (5-20)	**< 0.001***
Tg-ab, IU/mL		17 (12) (10-60)	15 (7) (8-26)	0.19***
Fasting glucose, mg/dL		90 (9) (66-100)	92 (6) (71-100)	0.81***
Fasting insulin, uU/mL		19 (13.5) (2.4-88.3)	8.1 (5.8) (1-20.6)	**< 0.001***
HOMA-IR		4.6 (3.4) (0.56-17.9)	1.9 (1.3) (0.7-3.22)	**< 0.001***
Triglyceride, mg/dL (n = 88)		86 (50) (21-188)	78 (46) (18-169)	0.09***
HDL-C, mg/dL (n = 88)		50 (14) (29-83)	60 (25) (4-84)	0.113***
LDL-C, mg/dL (n = 88)		93 (31) (48-187)	82 (35) (41-110)	**0.036***
Cholesterol, mg/dL (n = 88)		162 (40) (119-255)	150 (35) (106-178)	**0.041***
AST, IU/L (n = 91)		20 (6) (11-45)	22 (6) (12-31)	0.429***
ALT, IU/L (n = 91)		17 (12) (8-79)	15 (7) (7-25)	**0.001***

*: T-test, **: Chi-square test, ***: Mann-Whitney U test.
ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, SDS: standard deviation score, fT4: free thyroxine, fT3: free triiodothyronine, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, Tg-ab: thyroglobulin antibody, TPO-ab: thyroid peroxidase antibody, TSH: thyroid stimulating hormone

In, children with obesity , WHtR was significantly correlated with Tg-ab ($p = 0.041$, $r = 0.28$), triglyceride level ($p = 0.011$, $r = 0.35$), fat% ($p < 0.001$, $r = 0.52$) and FMI ($p < 0.001$, $r = 0.62$) (Figure 1).

Although the cut-off value of WHtR for predicting subclinical hypothyroidism has previously been proposed as 0.5 (5), in our cohort, there was no significant difference in thyroid antibody levels between the patients with a WHtR above and below 0.5 ($p > 0.05$). As no other cut-off value has been reported to predict an increase in thyroid antibody levels, the obese cases were divided into two different groups according to the median WHtR, which was 0.6: patients with a WHtR ≤0.6 and patients with a WHtR >0.6. As expected, in the WHtR >0.6 group, weight, weight SDS, BMI and BMI SDS were significantly higher, as well as fat% and FMI than the WHtR ≤0.6 group. Although no significant difference was obtained for TSH and thyroid hormone levels, both thyroid autoantibody levels were significantly higher in the WHtR >0.6 group. Among the metabolic parameters, triglyceride and fasting insulin levels were significantly higher in the WHtR >0.6 group ($p < 0.05$) (Table 2). Anthropometric measurements, metabolic parameters, thyroid function

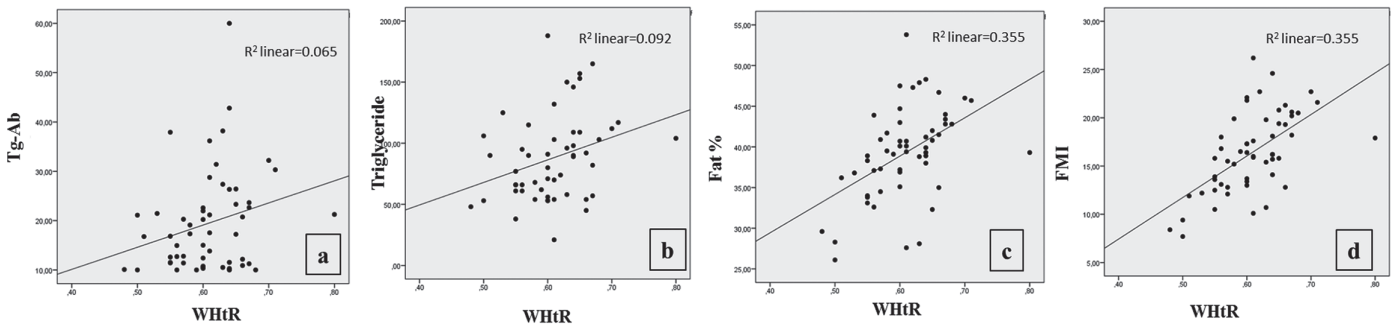

Figure 1. a. b. c. d. Waist/height ratio associated with thyroid antibody levels in obese children

WHtR: waist/height ratio, Tg-ab: thyroglobulin antibody, FMI: fat mass index

test and autoantibody levels of the two subgroups are summarized in Table 2.

Univariate analysis revealed a significant difference in TPO-ab and Tg-ab levels between WHtR ≤0.6 and WHtR >0.6 subgroups with an odds ratio (OR) of 0.262 and 0.275 respectively (Table 2).

Discussion

This study identified an association between WHtR and thyroid antibody levels, particularly Tg-ab. Thyroid antibody levels were higher in patients with obesity, concurrent with elevated WHtR, BMI and BMI SDS, fat mass, fasting insulin and triglyceride levels, which may suggest that lipid accumulation and particularly central obesity may influence thyroid autoantibody production.

In this study, fT3 and TSH levels were significantly higher and fT4 levels were significantly lower in euthyroid children with obesity compared to controls. Although there are some studies with diverse outcomes, our results are similar to those of Marwaha et al (7), who showed higher TSH and fT3 levels and lower fT4 levels in obese euthyroid children. Leptin effect, enhanced deiodination of thyroid hormones, blunted feed-back response of TSH release to circulating fT3, promoting action of TSH on adiposity and insulin resistance have all been considered as the probable causes of this condition (7).

Anthropometric measurements, such as BMI SDS and WHtR have been recently associated with TSH and thyroid hormones (4,5,6,8). Dahl et al (5) reported that TSH concentrations were associated positively with WHtR, and they concluded that the OR of exhibiting subclinical hypothyroidism was 1.8 when presenting with a WHtR >0.5. However, no cut off value of WHtR was reported to predict an increase in thyroid autoantibody levels. Our study revealed that children with obesity and a WHtR >0.6 (the median value of the obese group) had significantly higher TPO-ab and Tg-ab levels even in the absence of hyperthyrotropinemia with an OR of 0.262 and 0.275 respectively, and in these patients BMI, BMI SDS, fat% and FMI were significantly higher. Anthropometric and biometric measurements such as BMI, BMI SDS, fat%, FMI and WHtR have been associated with lipid accumulation, and changes in the concentrations of adipocytokines, such as leptin and some other inflammatory markers (1). It has previously been shown that leptin played a role in the regulation of the T helper-1 response and the proliferation of CD4+ and CD25+ cell clone involved in the apoptotic process leading to AITD (20,21). In adults, leptin concentration has also been associated with AITD regardless of bioanthropometric variables (10). In this study, a weak but significant, positive correlation between WHtR and Tg-ab level was identified, but no significant correlation was obtained between BMI and Tg-ab levels in obese children with obesity. Thus, we suggest that abdominal obesity may be a better predictor of thyroid autoimmunity than BMI in obese cases, which only reflects the obesity degree rather than its distribution.

Our results indicate that the concentrations of thyroid antibodies appeared to increase, despite the absence of thyroid dysfunction, hyperthyrotropinemia and autoantibody levels below the cut-off values. in children with obesity. Also, in children with obesity and a WHtR >0.6, thyroid antibody levels, and in particular Tg-ab, were higher and only Tg-ab levels were found to be correlated with central fat accumulation; no such association was found for TPO-ab levels.

The TPO-ab and Tg-ab positivities have been reported to be 12-26% and 10.5% in healthy adults; and 15% and 14% in adults with obesity (22,23,24). The increase in thyroid autoantibodies may result from both AITD and obesity, "Does this finding complicate the diagnosis and management of AITD in children with obesity?", in other words, "Does the increase in antibody levels suggest AITD or is it just the result of obesity?". We suggest that it is unnecessary to

Table 2. Comparison of anthropometric measurements, metabolic parameters, thyroid function test and autoantibody levels between the WHtR ≤0.6 and WHtR > 0.6 subgroups

		WHtR ≤0.6 (n = 27) (minimum-maximum)	WHtR > 0.6 (n = 28) (minimum-maximum)		OR (95% CI)
Variable		**Mean ± SDS Median (IQR)**	**Mean ± SDS Median (IQR)**	**p**	
Age, year		12 ± 2.7 (7.1-15.8)	13.3 ± 2.4 (9.3-17.3)	0.054*	
Gender	Female	12 (22)	14 (25.5)	0.68**	
n (%)	Male	15 (27)	14 (25.5)		
Puberty	Prepubertal	6 (11)	5 (9)	0.686**	
n (%)	Pubertal	21 (38)	23 (42)		
Weight (kg)		69.4 (31.1) (31.2-111.3)	85.5 (30.5) (56-122.9)	**0.011*** **	
Weight SDS		2.37 (1.7) (0.94-4.34)	2.95 (0.8) (1.5-4.46)	0.083***	
Height (cm)		156.1 ± 15.3 (125-186)	161.1 ± 11.8 (142.2-188)	0.183*	
Height SDS		1.08 ± 1.28 (-1.52-4.12)	0.65 ± 0.9 (-1.35-2.15)	0.152*	
BMI		27.3 (4.6) (20-35.8)	32.9 (5.6) (26.7-37.2)	**< 0.001*** **	
BMI SDS		+ 2.2 (0.78) (1.65-3.48)	+ 2.83 (0.5) (1.98-3.65)	**0.001*** **	
BMI (%)		99.6 (1.5) (95.1-99.9)	99.8 (0.6) (97.6-99.9)	**0.001*** **	
fT3, pg/dL		4.02 (1.04) (1.0-6.23)	4.05 (0.86) (1.0-5.74)	0.395***	
fT4, ng/dL		1.01 ± 0.15 (0.77-1.34)	1.04 ± 0.13 (0.71-1.39)	0.55*	
TSH, IU/L		1.96 (0.88) (1.24-4.01)	2.03 (1.46) (0.74-4.62)	0.655***	
TPO-ab, IU/mL		11 (8) (5-29)	16 (10) (6-25)	**0.023*** **	**0.262**
Tg-ab, IU/mL		13 (10) (10-38)	22 (18) (10-60)	**0.009*** **	**0.275**
Fasting glucose, mg/dL		92 (10) (80-100)	89 (10) 66-100)	0.269***	
Fasting insulin, uU/mL		17.7 (10) (2.4-39.8)	26.7 (14.3) (6.5-88.3)	**0.029*** **	
HOMA-IR		3.9 (2) (0.56-8)	5.7 (3.9) (1.5-17.9)	0.076***	
Triglyceride, mg/dL (group 1 n = 26) (group 2 n = 26)		66 (36) (38-188)	98 (47) (21-165)	**0.019*** **	
HDL-C, mg/dL (group 1 n = 26) (group 2 n = 26)		51 (17) (35-83)	49 (13) (29-71)	0.213***	
LDL-C, mg/dL (group 1 n = 26) (group 2 n = 26)		95 (30) (48-187)	92 (34) (67-146)	0.833***	
Cholesterol, mg/dL (group 1 n = 26) (group 2 n = 26)		164 (43) (119-255)	159 (35) (128-213)	0.184***	
AST, IU/L (group 1 n = 26) (group 2 n = 28)		19 (6) (12-45)	21 (7) (11-40)	0.728***	
ALT, IU/L (group 1 n = 26) (group 2 n = 28)		15 (9) (9-79)	22 (14) (8-42)	0.112***	
Fat%		37.3 ± 5 (26.1-47.5)	41.1 ± 5.5 (27.6-53.8)	**< 0.001***	
FMI		14.5 ± 3.6 (7.7-22.1)	18.2 ± 3.9 (10.1-26.2)	**< 0.001***	

*: T-test, **: Chi-square test, ***: Mann-Whitney U test.
ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, SDS: standard deviation score, Fat%: Fat mass/weight ratio, FMI: Fat mass index, fT4: free thyroxine, fT3: free triiodothyronine, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, LDL-C: low-density lipoprotein cholesterol, Tg-ab: thyroglobulin antibody, TPO-ab: thyroid peroxidase antibody, TSH: thyroid stimulating hormone, WHtR: waist/height ratio, CI: confidence interval

routinely investigate thyroid antibody levels in children with obesity, especially when thyroid function tests are within normal limits. In cases of seropositivity, a certain distinction of AITD may be challenging, given that thyroid changes in ultrasound is a common finding in cases with obesity (12). Further long-term follow-up studies investigating if weight loss reverses the abnormal findings in ultrasound and leads to a decrease in levels of thyroid autoantibodies would shed light on this topic.

As expected, fasting insulin and triglyceride levels were significantly higher in the WHtR >0.6 subgroup compared to the WHtR ≤0.6 subgroup, as high WHtR is associated with high fat mass and central lipid accumulation (1,9). The relation between thyroid function tests and lipid profile in obese subjects shows variations in different studies. Unüvar et al (8) previously reported that triglyceride level was the strongest independent variable correlated with TSH level in children with obesity. Shalitin et al (14) found a positive correlation between TSH and triglyceride levels, whereas Reinehr at al (25) could not find any correlation between TSH and lipid profile in their study conducted with 246 children with obesity.

Despite a large number of studies on thyroid hormones and TSH in childhood obesity, there are few data concerning the association between thyroid autoantibody levels and metabolic parameters of obesity, such as insulin/glucose levels, HOMA-IR, and lipid profile. We did not find any correlation between these parameters and thyroid antibody levels. It has recently been shown that TPO-ab and Tg-ab levels were not significantly different in obese children with and without insulin resistance (9). In adults with obesity, no association was found between thyroid antibody positivity, insulin resistance and atherogenic dyslipidemia (23). However, Tamer et al (26) reported that TPO-ab levels positively correlated with triglyceride levels and WC, and negatively correlated with HDL-C levels in premenopausal women with Hashimoto thyroiditis, whereas Tg-ab level correlated with triglyceride and non-HDL-C levels. In these patients, no correlation was found between TSH levels and lipid profile. The investigators suggested that thyroid autoimmunity could be associated with hyperlipidemia, independent of thyroid function. The association between thyroid antibody levels and insulin resistance and/or lipid profile still remains unclear and further investigations in adults and children are needed.

Study Limitations and Strength

Ultrasound imaging of thyroid glands was not performed. However, this is a minor setback because thyroid changes on ultrasound may also be due to non-autoimmune thyroiditis, which has been associated with obesity in a pediatric population (12). Due to limited sample sizes and study design, we could not obtain a cut-off value of WHtR to predict the presence of thyroid autoimmunity. Nevertheless, univariate analysis showed a significant difference in TPO-ab and Tg-ab levels when we divided the obese subjects into two subgroups, according to the median WHtR value "0.6" in our cohort of children with obesity. Iodine levels were not studied which can also be effective in thyroid autoimmunity. Mediators such as leptin, adiponectin and resistin which may have a link between autoimmunity and fat accumulation were not studied.

The strength of the study is that the impact of lipid accumulation (estimated by bioelectrical impedance analysis) and central adiposity (described by WHtR) on thyroid function and autoantibodies were both evaluated, and their association with metabolic parameters was fully investigated.

Conclusion

This study showed that euthyroid children with obesity had higher concentrations of thyroid autoantibodies compared to controls, although these levels remained below the cut-off for clinical significance. In addition, the obese patients with a WHtR >0.6 had higher thyroid antibody levels compared to those with WHtR ≤0.6, even in the absence of AITD, and there was a positive correlation between WHtR and Tg-ab levels. These findings suggest that central adiposity influences thyroid autoantibody production in children with obesity. Further studies with larger number of participants are needed to determine the effect of central adiposity and its modulation of thyroid autoimmunity and the efficacy of the WHtR parameter in clinical practice concerning thyroid autoimmunity in obesity.

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Ethics

Ethics Committee Approval: The study was approved by the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Ethical Committee (date: 04.02.2020 and ethics approval number: 21299).

Informed Consent: Patients were included in the study following a consent form signed by parents/caregivers. The information about the patients was kept confidential and used only for the purposes of the study.

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Authorship Contributions

Surgical and Medical Practices: Bahar Özcabı, Gürkan Tarçın, Esma Şengenç, Feride Tahmiscioğlu Bucak, Concept: Olcay Evliyaoğlu, Bahar Özcabı, Design: Olcay Evliyaoğlu, Bahar Özcabı, Data Collection or Processing: Bahar Özcabı, Gürkan Tarçın, Esma Şengenç, Feride Tahmiscioğlu Bucak, Analysis or Interpretation: Bahar Özcabı, Olcay Evliyaoğlu, Literature Search: Bahar Özcabı, Gürkan Tarçın, Esma Şengenç, Feride Tahmiscioğlu Bucak, Olcay Evliyaoğlu, Writing: Bahar Özcabı, Gürkan Tarçın, Esma Şengenç, Feride Tahmiscioğlu Bucak, Oya Ercan, İbrahim Murat Bolayırlı, Olcay Evliyaoğlu.

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ORIGINAL ARTICLE

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Time to the Peak, Shape of the Curve and Combination of These Glucose Response Characteristics During Oral Glucose Tolerance Test as Indicators of Early Beta-cell Dysfunction in Obese Adolescents

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What is already known on this topic?

Oral glucose tolerance test (OGTT) is traditionally used to define glucose tolerance status based on 2-hour plasma glucose level. There is growing evidence that glucose curve characteristics, such as time of the glucose peak and shape of the glucose curve, may serve as indicators of beta-cell dysfunction.

What this study adds?

Late glucose peak, or a monophasic glucose curve during OGTT, are associated with impairment of beta-cell function in obese adolescents with normal glucose tolerance. Moreover, a combination of these glucose curve characteristics strongly predicts low oral disposition index.

Abstract

Objective: Characteristics of the glucose response during oral glucose tolerance test (OGTT) may reflect differences in insulin secretion and action. The aim was to examine whether timing of the glucose peak, shape of the glucose curve and their combination could be indicators of beta-cell dysfunction in obese/severely obese adolescents with normal glucose tolerance (NGT).

Methods: Data from 246 obese/severely obese adolescents who completed OGTT were reviewed. Out of 184 adolescents with NGT, 174 could be further classified into groups based on timing of the glucose peak (early/30 minutes vs late/≥60 minutes) and shape of the glucose curve (monophasic vs biphasic). Groups were compared with respect to insulin sensitivity (whole body insulin sensitivity index - WBISI), early-phase insulin secretion (insulinogenic index - IGI) and beta-cell function relative to insulin sensitivity (oral disposition index - oDI).

Results: Late glucose peak ($p = 0.004$) and monophasic glucose curve ($p = 0.001$) were both associated with lower oDI after adjustment for age, sex, puberty stage and body mass index z-score. Among obese/severely obese adolescents with NGT, those with coexistent late glucose peak and monophasic glucose curve had lower oDI than those with early glucose peak and biphasic glucose curve ($p = 0.002$). Moreover, a combination of late glucose peak and monophasic glucose curve was the most powerful predictor of the lowest oDI quartile [odds ratio (OR): 11.68, 95% confidence interval: 3.048-44.755, $p < 0.001$].

Conclusion: Late timing of the glucose peak, monophasic shape of the glucose curve and, in particular, a combination of those characteristics during OGTT may indicate early beta-cell dysfunction in obese/severely obese adolescents with NGT.

Keywords: Oral glucose tolerance test, beta-cell dysfunction, obese adolescents

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Introduction

Oral glucose tolerance test (OGTT) has been used traditionally to diagnose dysglycemia, according to fasting and 2-hour post-load glucose thresholds (1,2). However, even obese individuals with initially normal glucose tolerance (NGT) can eventually develop prediabetes and progress to type 2 diabetes (3,4,5). Impaired insulin secretion relative to insulin sensitivity, reflecting an early defect in beta-cell function, presents a key pathophysiological feature in those with the highest risk for disease progression (5). Identifying the predictors of beta-cell dysfunction in obese normoglycemic adolescents might be important for timely prevention of diabetes in youth (5,6). In recent years it has been investigated whether some features of the glucose response during OGTT, including time of the glucose peak and shape of the glucose curve, could be used as predictors of insulin secretion relative to insulin sensitivity.

Time of the glucose peak was found to be a reliably reproducible variable of the OGTT (7). It's role in prediction of beta-cell dysfunction has been studied in adult populations (8,9,10). Delayed timing of post-load glucose peak >30 minutes was associated with declining beta-cell function, worsening glucose tolerance over time (8) and a greater likelihood of prediabetes and diabetes (9,10). To our knowledge, association of glucose peak time and beta-cell function in youth has not been thoroughly investigated. So far, time of the glucose peak has been explored as potential predictor of beta-cell function and prediabetes risk in post-pubertal overweight/obese adolescent girls of diverse ethnicity (11) and recently in overweight/obese white and black adolescents (12).

A monophasic glucose curve shape has already been investigated as an early marker of beta-cell dysfunction and a risk predictor for type 2 diabetes in adults (13,14,15), pregnant women (16) and adolescents (17,18,19). In addition, a monophasic glucose curve predicted the risk for progression to type 1 diabetes among autoantibody-positive relatives of people with type 1 diabetes. Moreover, the risk in the monophasic group was increased with delayed timing of the glucose peak (20).

As time of the glucose peak and shape of the glucose curve reflect differences in insulin secretion and action, they deserve further investigation and validation in different populations, including obese youth. The joint ability of these two features of the OGTT to detect impaired beta-cell function is not sufficiently explored.

Therefore the aims of the present study were: 1) to investigate time of the glucose peak and shape of the glucose curve as independent predictors of beta-cell dysfunction and; 2) to explore their joint ability to detect impaired beta-cell function in obese/severely obese adolescents.

Methods

Participants

This retrospective analysis included data from 246 adolescents aged 10-18 years, referred for obesity to Department of Pediatric Endocrinology at the University Hospital Center Sestre milosrdnice, who subsequently completed 2-hour OGTT from January 2016 to March 2018. None of them was previously treated for obesity. Subjects taking any medication or having any systemic or endocrine disease, as well as those fulfilling the OGTT criteria for prediabetes or diabetes (1,2) were excluded (n=62). Out of 184 adolescents with NGT, 174 could be classified based on both time of the glucose peak and shape of the glucose curve, and their data were further analysed.

The study protocol was approved by the Ethics Committee of the University Hospital Center Sestre milosrdnice (approval number: 251-29-11-20-01-3). The requirement for informed consent was waived due to the retrospective nature of the study.

Anthropometric Measurements

Body weight was measured using a digital weighting scale to the nearest 0.1 kg, with subjects wearing only underwear. Standing height was measured with a Harpenden stadiometer to the nearest 0.1 cm. Body mass index (BMI) was calculated by dividing weight (kg) by height in metres squared (m^2). Obesity was defined as BMI ≥95th percentile for age and sex (21). Subjects were further classified as either obese if having a BMI ≥95th percentile and <120% of the 95th percentile for age and sex or severely obese if having a BMI ≥120% of the 95th percentile for age and sex or an absolute BMI ≥35 kg/m^2 (22), whichever was lower. The BMI z-score was calculated using US reference values (21). The pubertal stage was assessed using Tanner criteria (23).

Oral Glucose Tolerance Testing

After 10-12 hour overnight fast, a standard OGTT was performed with ingestion of the glucose load, 1.75 g/kg body weight, up to 75 g of glucose. Venous blood samples for measurement of plasma glucose and serum insulin were obtained at 0, 30, 60, 90 and 120 minutes. NGT was defined as fasting glucose <5.6 mmol/L and 2 hour glucose <7.8 mmol/L (1).

Classification of the Glucose Response-time of the Glucose Peak, Shape of the Glucose Curve

With respect to timing of the first glucose peak during OGTT, glucose response was dichotomized as maximal glucose occuring at 30 or ≥60 minutes (excluding 120 minutes due to inapplicability for the shape classification stated below). The glucose peak was considered either early (at 30 minutes) or late (≥60 minutes).

Shape of the glucose curve was classified as monophasic or biphasic (24). A monophasic curve was characterised by an increase of glucose to a maximum between 30-90 minutes, followed by a decrease until 120 minutes. The curve was classified as biphasic if glucose peaked at 30 or 60 minutes, followed by a nadir and a second peak by 120 minutes. The upward or downward change in plasma glucose between the time points was defined as glucose difference of ≥0.2 mmol/L to minimize fluctuations in glucose concentrations, which may be caused by the method of glucose analysis, rather than physiological reasons. Ten subjects with NGT who could not be classified according to the aforementioned criteria were excluded from further analysis (inability to determine glucose peak time (n = 3), curve shape (n = 3) or peak time and curve shape (n = 2), incessant increase (n = 1) and paradoxical glucose response (n = 1).

Calculations Derived from OGTT

Insulin sensitivity was assessed using the Matsuda index, an established measure of whole body insulin sensitivity index (WBISI), which has been validated against the euglycaemic-hyperinsulinaemic clamp, and calculated as: WBISI = 10 000/√ [(fasting glucose (mmol/L) x 18.02 x fasting insulin (mIU/L)) x (mean glucose (mmol/L) x 18.02 x mean insulin (mIU/L))] (25).

Early-phase insulin secretion was assessed using the insulinogenic index (IGI), which was calculated as the ratio of the incremental change of plasma insulin (mIU/L) to that of plasma glucose (mmol/L) during the first 30 minutes after glucose ingestion, as: $IGI = \Delta Insulin_{30}/\Delta Glucose_{30}$ x 18.02 (26).

In addition, in order to assess beta-cell function relative to insulin sensitivity, oral disposition index (oDI) was calculated as the product of WBISI and IGI. As a surrogate estimate of beta-cell function relative to insulin sensitivity, oDI can be applied to obese adolescents in studies where the applicability of clamp studies is limited due to feasibility, cost and labor intensiveness (27).

Protocol

Subjects were initially grouped based on whether time of the glucose peak occured early (at 30 minutes, P_{30}) or late (≥60 minutes, $P\geq_{60}$). Those two groups were compared with respect to WBISI, IGI and oDI.

Participants were subsequently grouped according to shape of the glucose curve as either monophasic (M) or biphasic (B) and compared with respect to WBISI, IGI and oDI.

Subjects were finally stratified into four groups, those with: (1) early glucose peak and monophasic curve shape ($P_{30}M$); (2) early glucose peak and biphasic curve shape ($P_{30}B$); (3) late glucose peak and biphasic curve shape ($P\geq_{60}B$); (4) late glucose peak and monophasic curve shape ($P\geq_{60}M$). Groups were compared with respect to WBISI, IGI and oDI.

Analytical Methods

Plasma glucose concentration was determined by the hexokinase method on the Abbott Architect c8000 chemistry analyzer (Abbott Diagnostics, USA). Serum insulin was assessed by the electrochemiluminescence immunoassay on the Cobas E601 analyzer (Roche Diagnostics, Germany).

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences 25.0.0.1. (IBM Inc., Armonk, NY, USA).

Descriptive statistics was used to describe the basic features of the sample in the study, using relative frequencies for categorical variables, and mean and standard deviation for continuous variables. Since some variables deviated from normal distribution, non-parametric descriptive parameters, median and interquartile range were also calculated. Normality of distribution was tested using Shapiro-Wilk test. To test statistical significance of differences between obese and severely obese group of participants, independent samples t-test and chi-square were calculated. ANOVA and ANCOVA adjusted for age, sex, Tanner stage and BMI SDS were used to determine the association of glucose peak timing, glucose curve shape and combined glucose curve features with insulin sensitivity, early-phase insulin secretion and beta-cell function relative to insulin sensitivity. Logistic regression was used to explore the relationship between predictor variables and the lowest oDI quartile. P values < 0.05 were considered statistically significant.

Results

Demographic Characteristics of Study Participants

There were no statistically significant differences between the groups of obese (n = 76) and severely obese (n = 98) adolescents with respect to age (p = 0.127), sex (p = 0.357) and puberty stage (p = 0.929) (Table 1).

OGTT-derived Indices of Insulin Sensitivity, Secretion and Beta-cell Function in Obese vs Severely Obese Adolescents

The group of severely obese adolescents had significantly lower WBISI (p = 0.002) and oDI (p = 0.019) than the obese group. At the same time, there was no difference in IGI (p = 0.420) between the groups (Table 1).

Prevalence of Glucose Curve Characteristics with Respect to Time of the Glucose Peak and Shape of the Glucose Curve

Early glucose peak and monophasic glucose curve were the prevalent morphological features identified, irrespective of the degree of obesity, while the most common curve characteristics with combined features were biphasic curve with early glucose peak and monophasic curve with late glucose peak (Table 2).

Among the participants, 57.5% (100/174) had a glucose peak at 30 minutes and 42.5% (74/174) at ≥60 minutes. Glucose curve was monophasic in 55.7% (97/174) and biphasic in 44.3% (77/174) of subjects. A biphasic curve with glucose peak at 30 minutes was identified in 36.2% (63/174), a monophasic curve with glucose peak at ≥60 minutes in 34.5% (60/174), a monophasic curve with glucose peak at 30 minutes in 21.3% (37/174), and a biphasic curve with glucose peak at ≥60 minutes in 8% (14/174).

In adolescents with glucose peak at 30 minutes, 63% (63/100) had biphasic glucose curve. In individuals with glucose peak at ≥60 minutes, 81.1% (60/74) had monophasic glucose curve.

In subjects with biphasic curve, 81.8% (63/77) had glucose peak at 30 minutes, while in those with monophasic curve, 61.9% (60/97) had glucose peak at ≥60 minutes.

More frequent association of early glucose peak and biphasic curve, as well as association of late glucose peak and monophasic curve was observed in both obese and severely obese adolescents (Table 2).

Relationship of Glucose Curve Characteristics and OGTT-derived Indices

Time of the Glucose Peak

After adjustment for age, sex, puberty stage and BMI z-score, glucose peak at ≥60 minutes was associated with lower oDI (p = 0.004) (Figure 1A, Table 3). There was no statistically significant difference in WBISI between the groups with glucose peak at 30 and ≥60 minutes (p = 0.302), while a trend towards lower IGI with glucose peak at ≥60 minutes did not reach statistical significance (p = 0.057) (Table 3).

Shape of the Glucose Curve

No difference between the groups with monophasic and biphasic glucose curve was observed with respect to WBISI (p = 0.784) after adjustment for age, sex, puberty stage and BMI z-score (Table 3). However, adolescents with monophasic curve had lower IGI (p < 0.001) and oDI (p = 0.001) (Figure 1B, Table 3).

Table 1. Demographic characteristics of study participants and oral glucose tolerance test-derived indices of insulin sensitivity, secretion and beta-cell function

	Obese	Severely obese	p value	Total
	n = 76 (43.7%)	n = 98 (56.3%)		n = 174 (100%)
BMI (kg/m^2)	29.50 ± 2.31 (24.5-36)	35.55 ± 4.74 (28-51.7)	< 0.001	32.8 ± 4.8 (24.5-50.9)
BMI z-score	1.94 ± 0.16 (1.61-2.21)	2.41 ± 0.19 (2.08-3.05)	< 0.001	2.21 ± 0.29 (1.61-3.05)
Age (years)	14.19 ± 2.12 (10-18)	13.72 ± 1.98 (10-17.6)	0.127	13.92 ± 2.05 (10-18)
Sex-M/F	30 (39.5)/46 (60.5)	46 (46.9)/52 (53.1)	0.357	76 (43.7)/98 (56.3)
Tanner stage				
I	8 (10.5)	10 (10.2)		18 (10.3)
II	16 (21.1)	19 (19.4)		35 (20.1)
III	7 (9.2)	13 (13.3)	0.929	20 (11.5)
IV	12 (15.8)	13 (13.3)		25 (14.4)
V	33 (43.4)	43 (43.9)		76 (43.7)
WBISI	2.49 ± 1.16 (0.67-6.52)	1.98 ± 0.95 (0.35-7.90)	0.002	2.20 ± 1.08 (0.35-6.52)
IGI	2. 44 ± 1.32 (0.03-6.52)	2.63 ± 1.66 (0.44-9.61)	0.420	2.55 ± 1.52 (0.03-9.61)
oDI	5.20 ± 2.23 (0.09-11.06)	4.42 ± 2.06 (0.60-11.68)	0.019	4.76 ± 2.16 (0.09-11.68)

Data are reported as n (%), mean ± standard deviation (range).
P values: chi-square for categorical variables, independent t-test for continuous variables.
M: male, F: female, BMI: body mass index, WBISI: whole body insulin sensitivity index, IGI: insulinogenic index, oDI: oral disposition index

Table 2. Prevalence of glucose curve characteristics in obese vs severely obese adolescents

Glucose curve characteristics	Obese n = 76	Severely obese n = 98	p value*
P_{30}	46 (60.5)	54 (55.1)	
$P\geq_{60}$	30 (39.5)	44 (44.9)	0.537
M	41 (53.9)	56 (57.1)	
B	35 (46.1)	42 (42.9)	0.759
$P_{30}M$	18 (23.7)	19 (19.4)	
$P_{30}B$	28 (36.8)	35 (35.7)	
$P\geq_{60}B$	7 (9.2)	7 (7.1)	0.727
$P\geq_{60}M$	23 (30.3)	37 (37.8)	

Data are reported as n (%); P_{30}: glucose peak at 30 minutes, $P\geq_{60}$: glucose peak at ≥60 minutes, M: monophasic glucose curve, B: biphasic glucose curve

*Significance of differences between obese and severely obese adolescents in each glucose curve characteristics frequency: chi-square statistics (Fisher's exact test for 2x2 tables)

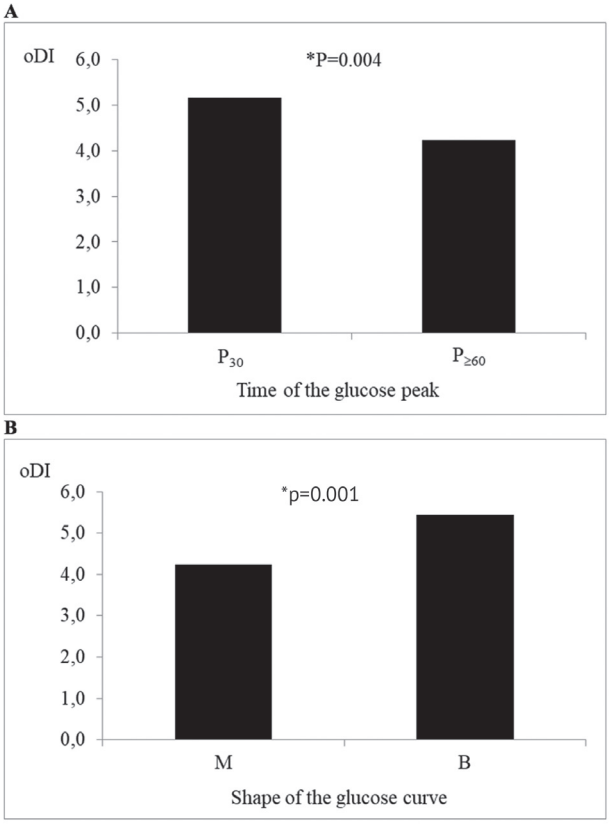

Figure 1. Comparison of average oral disposition index in groups with: (A) early vs late glucose peak; (B) monophasic vs biphasic glucose curve

*After adjustment for age, sex, puberty stage and body mass index z-score.

oDI: oral disposition index, P_{30}: glucose peak at 30 mintues, $P\geq_{60}$: glucose peak at ≥60 minutes, M: monophasic glucose curve, B: biphasic glucose curve

Time of the Glucose Peak and Shape of the Glucose Curve as Combined Glucose Curve Characteristics

Among four groups with combined glucose curve characteristics ($P_{30}M$, $P_{30}B$, $P\geq_{60}B$ and $P\geq_{60}M$), after adjustment for age, sex, puberty stage and BMI z-score, significant differences were found for IGI (p = 0.001) and oDI (p = 0.004). $P_{30}M$ group had lower IGI than $P_{30}B$ group, while $P\geq_{60}M$ group had both lower IGI and lower oDI than $P_{30}B$ group (Figure 2).

Time of the Glucose Peak and Shape of the Glucose Curve as Predictors of Low oDI

Among adolescents with oDI in the first (lowest) quartile, the proportion of those having glucose peak at ≥60 minutes was significantly higher than among adolescents with oDI in the third or fourth quartile, whose predominant glucose peak time was 30 minutes ($\chi^2 = 10.281$, df = 3, p = 0.016) (Figure 3A).

With regard to the shape of the glucose curve, proportion of subjects with monophasic glucose curve was significantly higher among adolescents with oDI in the first quartile, while participants with biphasic glucose curve were more prevalent among adolescents with oDI in the third or fourth quartile ($\chi^2 = 17.135$, df = 3, p = 0.001) (Figure 3B).

According to logistic regression, when age, sex, puberty stage and BMI z-score were included in the model, probability to have oDI in the lowest quartile was almost three times higher in adolescents with late (vs early) glucose peak [odds ratio (OR): 2.96, 95% confidence interval (CI): 1.3339-6.526, p = 0.007], almost five times higher in subjects with monophasic (vs biphasic) glucose curve (OR: 4.91, 95% CI: 1.856-12.977, p = 0.001), and almost twelve times higher in participants with combination of late glucose peak and monophasic glucose curve (vs combination of early glucose peak and biphasic glucose curve) (OR: 11.68, 95% CI: 3.048-44.755, p < 0.001).

Discussion

This study indicates that morphological characteristics of the glucose response curve during OGTT, including time of the glucose peak, shape of the glucose curve and combination of these features, may be informative of impaired beta-cell function in obese/severely obese youth with NGT.

There was no statistically significant difference in the prevalence of glucose curve characteristics, including time of the glucose peak, shape of the glucose curve or both features combined, between otherwise demographically comparable groups of obese/severely obese adolescents.

Table 3. Oral glucose tolerance test-derived indices in groups with early vs late glucose peak and biphasic vs monophasic glucose curve

OGTT-derived indices	Time of the glucose peak			Shape of the glucose curve		
	P_{30}	$P\geq_{60}$	*p value	B	M	*p value
WBISI	2.30 ± 1.13	2.08 ± 1.00	0.302	2.21 ± 1.06	2.20 ± 1.10	0.784
	(0.66-7.9)	**(0.35-4.94)**		**(0.66-5.13)**	**(0.35-7.9)**	
IGI	2.76 ± 1.62	2.27 ± 1.32	0.057	3.04 ± 1.72	2.16 ± 1.21	< 0.001
	(0.62-9.61)	**(0.44-7.13)**		**(0.77-9.61)**	**(0.44-7.13)**	
oDI	5.23 ± 2.16	4.15 ± 2.02	0.004	5.44 ± 2.07	4.25 ± 2.10	0.001
	(1.23-11.68)	**(0.68-10.57)**		**(1.64-11.3)**	**(0.68-11.68)**	

Data are reported as mean ± standard deviation (range); WBISI: whole body insulin sensitivity index, IGI: insulinogenic index, oDI: oral disposition index, P_{30}: glucose peak at 30 mintues, $P\geq_{60}$: glucose peak at ≥60 minutes, M: monophasic glucose curve, B: biphasic glucose curve, OGTT: oral glucose tolerance test
*After adjustment for age, sex, puberty stage and body mass index z-score

Through the literature search, we found no similar data comparing the prevalence of glucose curve characteristics in subjects with different obesity classes.

As expected, severely obese adolescents had lower WBISI, reflecting lower insulin sensitivity (28,29). Insulin secretion expressed as IGI did not differ significantly between the groups. However, beta-cell function was worse in severely obese adolescents, which is consistent with previous findings (30,31).

Prevalent features of the glucose response in our sample of obese/severely obese adolescents with NGT were early glucose peak and monophasic glucose curve. According to studies published to date, early glucose peak was detected in a minority of obese postpubertal adolescent girls with NGT or prediabetes (34/88) (11) and in approximately half of obese black and white adolescents with NGT or impaired glucose tolerance (IGT) (142/278) (12). A higher proportion of subjects with early glucose peak in the current study (100/174) is probably due to normotolerant glucose status of the included adolescents and is in agreement with cross-sectional analyses that have linked later time of the glucose peak with impaired glucose tolerance and type 2 diabetes (7,32). With regard to the prevalence of the monophasic glucose curve, our findings are in line with formerly published data for obese adolescents with NGT (33). The prevalence of combined glucose curve characteristics involving time of the glucose peak and shape of the glucose curve has not been thoroughly investigated. According to our results, 71% of participants had either early glucose peak with biphasic curve or late glucose peak with monophasic curve. In the study of Chung et al (9), normotolerant or prediabetic adults with glucose peak >30 minutes more often had monophasic glucose curve (78%), while those with glucose peak at 30 minutes had an equal chance of having either biphasic or monophasic curve (54% vs 45%). In the present study, adolescents with late glucose peak more frequently had monophasic glucose curve too (81%), but in subjects with early glucose peak a higher prevalence of biphasic curve was detected (63%). As younger persons are more likely to be characterized by a biphasic glucose response (19,24), our results suggesting a stronger association of early glucose peak and biphasic glucose curve may be atributed to younger age and normotolerant glucose status of the included subjects. In non-diabetic Latino adolescents, the biphasic group exhibited a higher percentage of "early responders" compared with the monophasic group (57% vs 32%) (18), and this trend was even more pronounced in our sample (82% vs 38%).

The time point after an oral glucose load at which the peak glucose concentration occurs has recently been shown to represent a reliably reproducible parameter of the OGTT, with 76% agreement on triplicate testing performed at three different days (7). In addition, time to glucose peak has already emerged as a potential predictor of beta-cell function in adults, while only scarce data related to adolesents currently exist. In adults, cross-sectional studies have linked a late glucose peak during OGTT with beta-cell dysfunction, impaired glucose tolerance and type 2 diabetes (7,9,10). A longitudinal study comprising 532 women in the first year postpartum, revealed that both a shift of the glucose peak to a later time point and a consistently delayed glucose peak were associated with declining beta-cell function and worsening of glucose tolerance status over a nine month period (8). In adults with newly diagnosed type 2 diabetes, time to glucose peak during OGTT was assessed before and after four weeks of intensive insulin therapy; a resultant improvement of beta-cell function was associated with a shift of the glucose peak to an earlier time point (7). Regarding the adolescent population, 54 overweight/obese postpubertal girls with late glucose peak had lower insulin sensitivity index ($p = 0.004$) and oDI ($p < 0.001$) than 34 girls with earlier glucose peak (11). Nolfe et al (33) also supported the concept that the more quickly the plasma glucose concentration returned to or below the

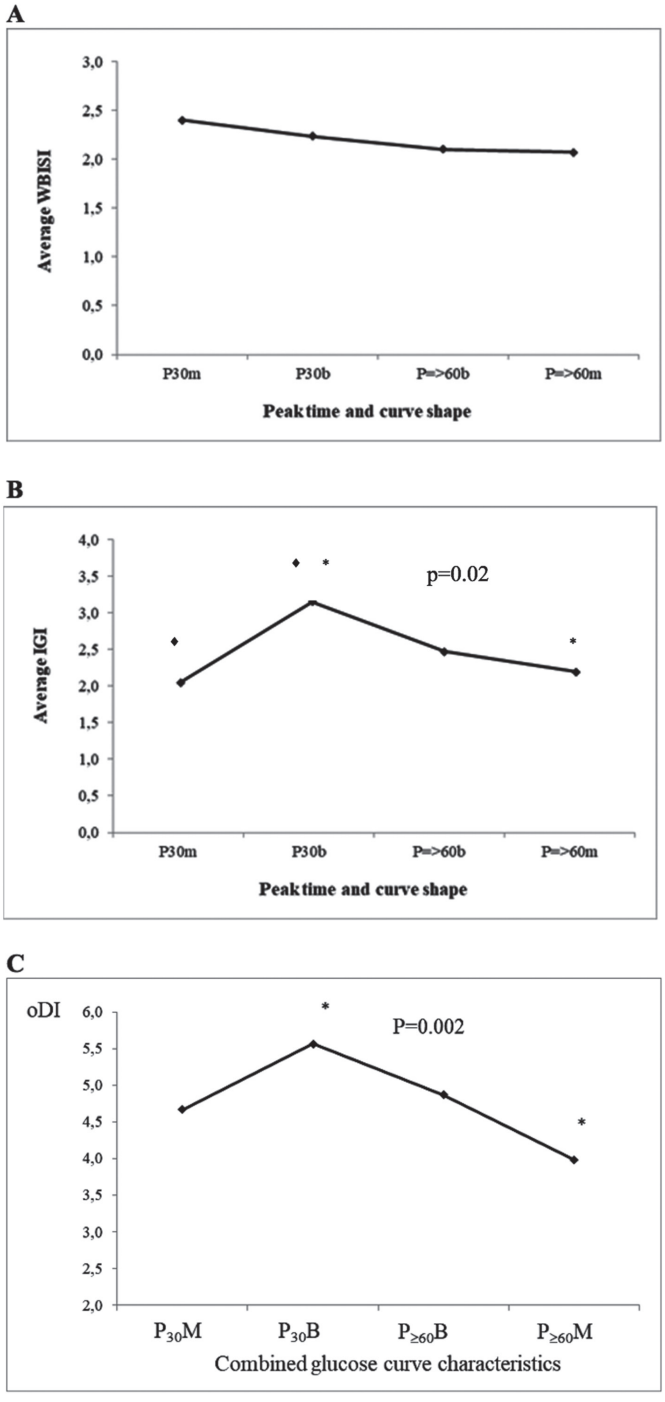

Figure 2. Comparison of average whole body insulin sensitivity index (A), insulinogenic index (B) and oral disposition index (C) in groups with combined glucose curve characteristics

♦*Statistically significant difference after adjustment for age, sex, puberty stage and body mass index z-score.

WBISI: whole body insulin sensitivity index, IGI: insulinogenic index, oDI: oral disposition index, $P_{30}M$: glucose peak at 30 mintues and monophasic glucose curve, $P_{30}B$: glucose peak at 30 minutes and biphasic glucose curve, $P\geq_{60}B$: glucose peak at ≥60 minutes and biphasic glucose curve, $P\geq_{60}M$ glucose peak at ≥60 minutes and monophasic glucose curve

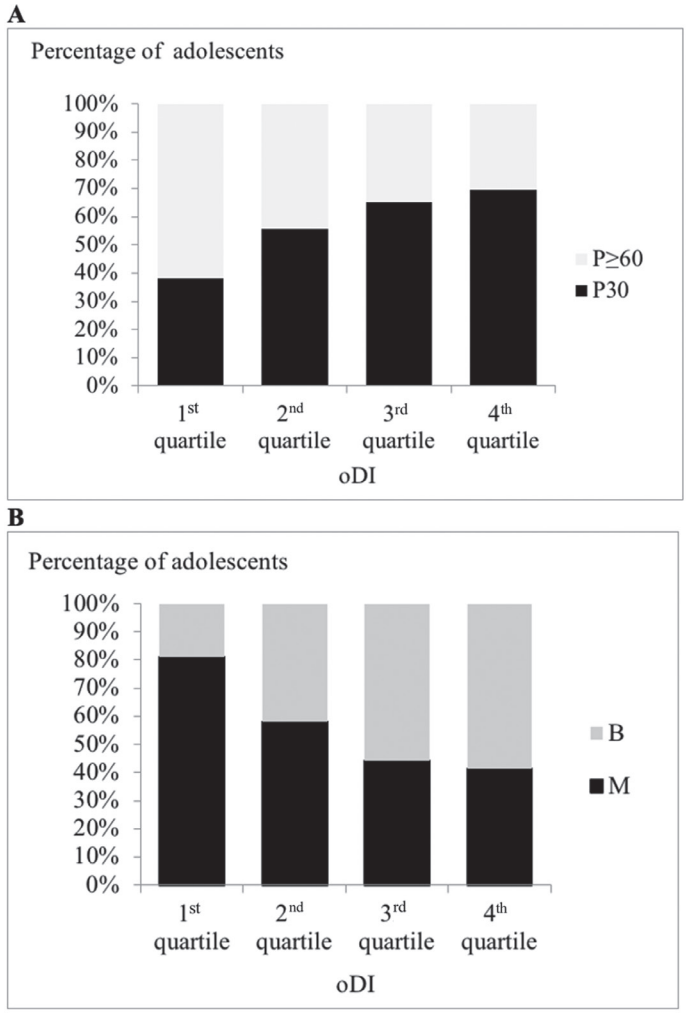

Figure 3. Percentage of adolescents with: (A) early vs late glucose peak in each quartile of oral disposition index (oDI); (B) biphasic vs monophasic glucose curve in each quartile of oDI

oDI: oral disposition index

fasting glucose level following glucose ingestion, which was associated with earlier glucose peak, the lower was the risk for future diabetes. In the present study, participants with late glucose peak had lower oDI than subjects with early glucose peak ($p = 0.002$). Our findings are in agreement with cross-sectional analyses linking delayed glucose peak with early defect in beta-cell function (8,9,10,11,12).

The significance of the glucose curve shape was first established in adults. Beta-cell function, adjusted for insulin resistance, was found to be significantly lower in non-diabetic individuals with monophasic glucose curve. Moreover, a monophasic glucose response was more prevalent among subjects with IGT than in individuals with NGT (13). Another study linked monophasic glucose curve shape with increased risk for type 2 diabetes. Over a 7-8 years follow-up, the conversion rate to type 2 diabetes in

prediabetic adults with monophasic glucose curve was twice that of subjects with a biphasic glucose response (14). Although questions regarding the curve shape stability were initially raised, recent data suggest that it is high (34). In the European Group for the Study of Insulin Resistance-Relationship between Insulin Sensitivity and Cardiovascular Disease cohort, 70% of participants presented with monophasic OGTT-glucose curve shape both at baseline and three years later (15). Besides that, persistence over time of a monophasic shape and switch from biphasic to monophasic shape was associated with increased risk of impaired glucose metabolism (15). Cross-sectional studies in adolescents (17,18), including clamp studies in obese youth of both sexes and all pubertal stages (19), found a monophasic glucose curve shape to be associated with significantly worse beta-cell function relative to insulin sensitivity. In our study, after adjustment for age, sex, puberty stage and BMI z-score, subjects with monophasic glucose response had lower oDI ($p = 0.001$) reflecting poorer β-cell function, which is in accordance with findings of other studies performed in youth (17,18,19).

The glucose curve shapes observed within a 2-hour window during OGTT are partially influenced by the time of the first glucose peak. Subjects with early peak are more likely to have biphasic, while those with late peak more often have monophasic curve shape. Cree-Green et al (11) found that peak glucose time was more predictive of β-cell function than shape of the glucose curve. Similar findings were published in an adult cohort with increased risk for type 2 diabetes (9).

To our knowledge, significance of combined glucose curve features in the detection of beta-cell dysfunction have not been investigated. Thus, we further categorised subjects according to the combination of both features, time of the glucose peak and shape of the glucose curve. Adolescents with late glucose peak and monophasic curve shape had lower oDI than those with early peak and biphasic curve shape ($p = 0.002$). Moreover, a combination of late glucose peak and monophasic glucose curve proved to be the strongest predictor of poor beta-cell function, as reflected by the highest risk of oDI in the lowest quartile (OR: 11.68, 95% CI: 3.048-44.755, $p < 0.001$). We found no data in the literature highlighting the fact that glucose normotolerant adolescents with the combination of late glucose peak and monophasic glucose response during OGTT are at increased risk for poor beta-cell function.

Study Limitations

In the current study, time of the glucose peak and shape of the glucose curve were determined by a single OGTT. Although recent studies in adults suggest glucose response pattern reproducibility and persistance over time (7,15), youth-specific investigation of glucose curve characteristics are needed. In addition, our classification of glucose curve response was based on 2-hour OGTT with standard 30-minutes sampling intervals. By using more frequent sampling intervals, it could be possible to capture more details and probably provide better information on beta-cell function. Another drawback of this study was the inability to assign all the adolescents with NGT to either early/late glucose peak or monophasic/biphasic glucose response group, due to the criteria needed for glucose response classification. However, the number of unclassified subjects was small ($n = 10$, 0.05%). Finally, factors which could influence the gastric emptying or differences in pre-test carbohydrate loading were not assessed in the present study.

Prospective longitudinal studies in obese adolescents are needed to confirm the predictive value of glucose response curve morphology with respect to deterioration of beta-cell function and progression from NGT to prediabetes or type 2 diabetes. In addition, lifestyle interventions in obese adolescents with poor beta-cell function should be investigated to understand if these interventions could shift the glucose peak to an earlier time point and/or glucose curve shape from monophasic to biphasic.

Conclusion

Early identification of subjects at high risk for type 2 diabetes among obese adolescents requires studies that focus on the initial stages of the disease, before the onset of any alterations in glucose tolerance. The present study confirms that obese adolescents with late glucose peak, as well as those with monophasic glucose response during OGTT, although normoglycemic, have reduced beta-cell function relative to insulin sensitivity. The risk of impaired beta-cell function is even more pronounced in obese youth with the aforementioned glucose curve features combined, making them a target population for intensive lifestyle intervention.

Acknowledgments

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Ethics

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the University Hospital

Center Sestre Milosrdnice (approval number: 251-29-11-20-01-3).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Marija Požgaj Šepec, Concept: Maja Cigrovski Berković, Gordana Stipančić, Design: Lavinia La Grasta Sabolić, Maja Cigrovski Berković, Gordana Stipančić, Data Collection or Processing: Lavinia La Grasta Sabolić, Analysis or Interpretation: Lavinia La Grasta Sabolić, Maja Cigrovski Berković, Gordana Stipančić, Literature Search: Marija Požgaj Šepec, Writing: Lavinia La Grasta Sabolić, Marija Požgaj Šepec, Maja Cigrovski Berković, Gordana Stipančić.

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ORIGINAL ARTICLE

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An Estimation of the Incidence of Thyroiditis Among Girls in Primary Care in Spain

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What is already known on this topic?

Current estimates of the incidence of thyroid dysfunction derive from adults and are limited for children and adolescents. The majority of thyroiditis cases occurs in females; an early diagnosis is relevant to prevent complications, mainly in childhood growth and maturation and metabolism of the nervous system.

What this study adds?

Among girls aged 9-18 years in Spain, the incidence of thyroiditis was estimated at 20.83-32.12/100,000 person years, and a temporal increase was also identified. Among thyroiditis cases, ≥50% were autoimmune, 43% not specified, and 3% referred to other types. Delayed diagnosis was suggested in 10% of the cases, which is important due to possible clinically significant consequences.

Abstract

Objective: As for other auto-immune processes, thyroiditis is monitored after vaccinations. The aim was to estimate the baseline incidence of thyroiditis among girls, before investigating papillomavirus vaccination as a potential risk factor.

Methods: Observational cohort study including girls aged 9-18 years and registered between 2002-2016 in the Spanish Primary Care Database for Pharmacoepidemiological Research. Girls were followed until a thyroiditis occurred, 19 years of age, left the cohort, died, or the study ended. Anonymized records were reviewed for diagnosis confirmation (endocrine discharge letter and/or free-text comments) in a random sample. Incidence rate (IR) per 10^5 person years (/10^5 py) was estimated.

Results: The cohort numbered 480,169 girls, of whom 641 had a record of thyroiditis: 346 autoimmune thyroiditis; 17 thyroiditis of other types; and 278 unspecified. Incidence of recorded thyroiditis increased with age, from 23.96 at age 9 years to 47.91 at age 14 years, and stabilized around 31.06-34.43 among girls aged 15-18 years. Of the 98 records reviewed, 60.2% were 'confirmed' cases, 32.7% 'possible' and 7.1% were discarded. After correction for discarded cases, IR = 20.83 'confirmed' cases, increasing to 32.12/10^5 py when 'confirmed' plus 'possible' cases were included. Between 2002-2005, incidences were lower (16.28 and 20.93 cases/10^5 py) than in the period 2007-2016 (21.17 and 33.78 cases/10^5 py) for 'confirmed' and 'confirmed' plus 'possible', respectively.

Conclusion: Two-thirds of the recorded thyroiditis included confirmatory evidence. The incidence of thyroiditis among girls increased with age and in the later period, and remained stable among girls aged 15-18 years.

Keywords: Thyroiditis, women, incidence, precision of recording, International Classification of Primary Care codes, ICD-9 codes, primary care electronic records, paediatric thyroid disease

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Introduction

Thyroid dysfunction is one of the most common endocrine disorders in clinical practice (1). The incidence is influenced by a number of factors, with age, sex, geographic factors or ethnicity known to be important (2).

Thyroid dysfunction is associated with morbidity and deleterious consequences in terms of coronary artery disease and cardiovascular mortality. In order to prevent complications, routine screening programs that facilitate appropriate timely treatment are recommended (3). Therefore, determining the epidemiology and distribution of thyroid dysfunction in the European population, as well as trends in population subgroups, is relevant for designing tailored public health policies and programs (3). Current estimates of the prevalence of thyroid dysfunction are largely derived from data in white middle-aged populations (1) and limited data are available in children. Autoimmune thyroiditis (AIT) is the most common thyroid disorder in the paediatric population, being either goitrous (Hashimoto's thyroiditis) or non goitrous (atrophic thyroiditis, also known as primary myxedema). In this population, the most common period at presentation is adolescence, but the disease may occur at any time, even in children under one year of age, and is more common in girls (4).

Thyroid hormones play an important role in childhood growth and are involved in the maturation and metabolism of certain organs, such as the skeleton and brain, influencing the process of myelination of the nervous system. As a consequence, some of the differences in children and adults presenting with Hashimoto thyroiditis occur because the hypothyroidism affects developing systems rather than matured systems and in children may result in short stature, decline in school performance, and retardation of development (5).

We are currently studying the possible role of human papilloma virus vaccination (HPVv) in the development of immune mediated disorders, including some forms of thyroiditis. In order to facilitate this study, it was planned to estimate the recording of thyroiditis among girls at ages when HPVv is given in Spain in order to confirm the precision (internal validation) and sensitivity (external validation) of the data in the Spanish Primary Care Database For Pharmacoepidemiological Research (BIFAP), as well as providing incidence rates (IR) of thyroiditis in this population.

Methods

Source of Data

In the current study, BIFAP was used as the data source (6). BIFAP is a longitudinal population-based database of anonymised electronic primary care records from 881 paediatricians and 5871 physicians of the Spanish public National Health System (S.N.S.). In Spain, around 98.9% of the population is registered with a local primary care paediatrician or physician (PCPs) of the S.N.S (7).

BIFAP contains information for 66% of the girls aged 10-19 years as registered in the Spanish census (7) from 7 out of 19 regions. Available data include patient age, sex, clinical events which are recorded using the International Classification of Primary Care (ICPC) and the International Classification of Diseases 9th revision (ICD-9) (8,9), anonymised PCPs free text notes, specialist referrals and discharge letters, prescriptions issued in primary care and their dispensation by pharmacies, vaccinations, laboratory test results and life-style factors, including body mass index, smoking status, and alcohol consumption.

BIFAP is fully funded by the Spanish Agency on Medicines and Medical Devices (10), is overseen by the Department of Health, and is maintained with the collaboration of the participant regions.

The current study is part of a broader project intended to study the use and safety of the HPVv. This study was funded by Instituto de Salud Carlos 3 through the project 'PI17/02300' (Co-funded by European Regional Development Fund) and the study protocol was approved by the Scientific Committee of BIFAP (Reference BIFAP_01_2016).

Study Design and Population

In this observational cohort study, based on information collected retrospectively, the population was composed of girls aged 9-18 years registered in BIFAP from 2002 (first year for standard quality in data collection in the database) to 2016 (last year of data available at the moment the study was performed), with PCP medical records for at least one year and without a previous diagnosis of thyroiditis. The start date of follow-up was defined as the last date of fulfilling all inclusion criteria.

Thyroiditis Automatic Identification

Girls were followed from the start date until a recorded ICPC/ICD-9 code for thyroiditis diagnosis (listed in Annex 1) was identified, they reached 19 years of age, left the cohort, died, or the study ended (31 December 2016).

According to the description of the thyroiditis codes, cases were categorized as autoimmune (including Hashimoto, Graves-Basedow or Graves' disease), unspecified, and other types which included acute, sub-acute, chronic, silent or viral (Annex 1).

In order to assess a potential temporal change in the incidence of thyroiditis over the period of the study, the

analysis was replicated in two periods 2002-2005 (previous to the marketing approval of the HPVv by regulatory agencies) and 2007-2016 (once HPV vaccination was scheduled for girls and female adolescents). The HPVv were approved for marketing in Spain during the last months of 2006. Thus the year 2006 was excluded from this time trend analysis, as interrupted time, in order to clearly distinguish between those two periods.

Review of Thyroiditis Records and Diagnosis Precision

The anonymized clinical profiles of a random sample of girls with thyroiditis identified automatically were manually reviewed to retrieve:

- Cases with 'confirmatory' diagnosis information, such as a letter to/from a referral (mostly to an endocrinologist) or hospital, or free-text comments mentioning a clear diagnosis (as gold standard);

- Cases with information which led to rejection of the diagnosis, which included prevalent cases, confirmation of an alternative diagnosis or only reference to family history;

- Cases lacking information to confirm or disregard the diagnosis. These were considered 'possible' cases.

The sample was randomised by simply assigning a random number to each identified girl through the 'random' function in Excel program (i.e. [= RAND ()]). Then, in order to extract the amount of profiles required to review, we just filtered those random numbers by random ranges.

In order to adjust for a delay from diagnosis to the recording or a delay in the diagnosis from the moment the first symptoms were consulted, the date of first prescriptions of (anti-)thyroid hormones, propranolol (used as a symptomatic treatment to control adrenergic symptoms) (11) as well as the ICPC/ICD coding linked electronically to thyroiditis by the PCP were extracted. Among them, the earliest date was used as thyroiditis onset, for the purposes of this study.

Statistical Analysis

In order to calculate the positive predictive value (PPV), we defined as gold standard the scenario where all 'possible' cases were in fact false positives, and broad gold standard as the scenario where all 'possible' cases were true positives. Thus, PPV for the gold standard was the proportion of 'confirmed' thyroiditis cases over all patients automatically identified and PPV for the broad gold standard included also 'possible' thyroiditis cases. PPV was calculated also by type of thyroiditis, calendar period, and the presence of a thyroid peroxidase (TPO) test in the patient records.

The sensitivity of records with a detailed type of thyroiditis and TPO test to detect all cases was estimated for the gold standard, dividing the number of 'confirmed' cases in each category by the total 'confirmed' cases. Similar calculations were performed for broad gold standard including both 'confirmed' and 'possible' cases.

The so-called *observed* (12) IR of thyroiditis per 100,000 person-years (/10^5 py) was calculated by dividing the number of recorded thyroiditis cases by the total number of person-years of follow-up in the study cohort, overall and by age group.

The expected 'confirmed' and 'possible' cases were estimated by applying PPV to the observed thyroiditis. The so-called *true* (12) IRs were then estimated by using the number of expected 'confirmed' or 'confirmed' plus 'possible' thyroiditis cases for both scenarios, overall and by age groups. IRs were also estimated for the two calendar periods considered, i.e. from 2002 to 2005 and from 2007 to 2016.

Results

Description of Thyroiditis Records and Incidence Estimations

In total, 480,169 girls who met the initial inclusion criteria were identified, of whom 641 had a recorded thyroiditis diagnosis, and 40.9% of them having a TPO test recorded (Figure 1). The distribution of 641 thyroiditis records by calendar year is shown in Table 1. The type of thyroiditis was given as 'autoimmune' in 346 cases (53.98%), unspecified in 278 cases (43.37%) and other types in 17 cases (2.65%) the latter category including acute (n = 15), viral (n = 1) and silent thyroiditis (n = 1). The IR of recorded thyroiditis was 34.59/10^5 py, being 18.67/10^5 py for AIT, 15.00/10^5 py for unspecified thyroiditis and 0.92/10^5 py for other types of thyroiditis.

The number of girls with recorded thyroiditis and the *observed* IRs at each age are shown in Figure 2. The IR increased with age from 23.96 (among girls aged 9 years) to 47.91 (among girls aged 14 years) per 10^5 py, and decreased, remaining stable (between 31.90-34.43 per 10^5 py), in girls aged 15-18 years.

A manual review was performed for a random sample of 98 out of the 641 identified thyroiditis records (15.3%) and 59 of them were classified as 'confirmed' thyroiditis cases (PPV = 60.2%), 32.7% (n = 32) as 'possible' cases and 7.1% (n = 7) were discarded. The reason for ruling out seven cases included prevalent cases (n = 4), suspected thyroiditis ruled out in subsequent visits (n = 2) and term related only to the patient's family history of thyroiditis (n = 1). The PPV

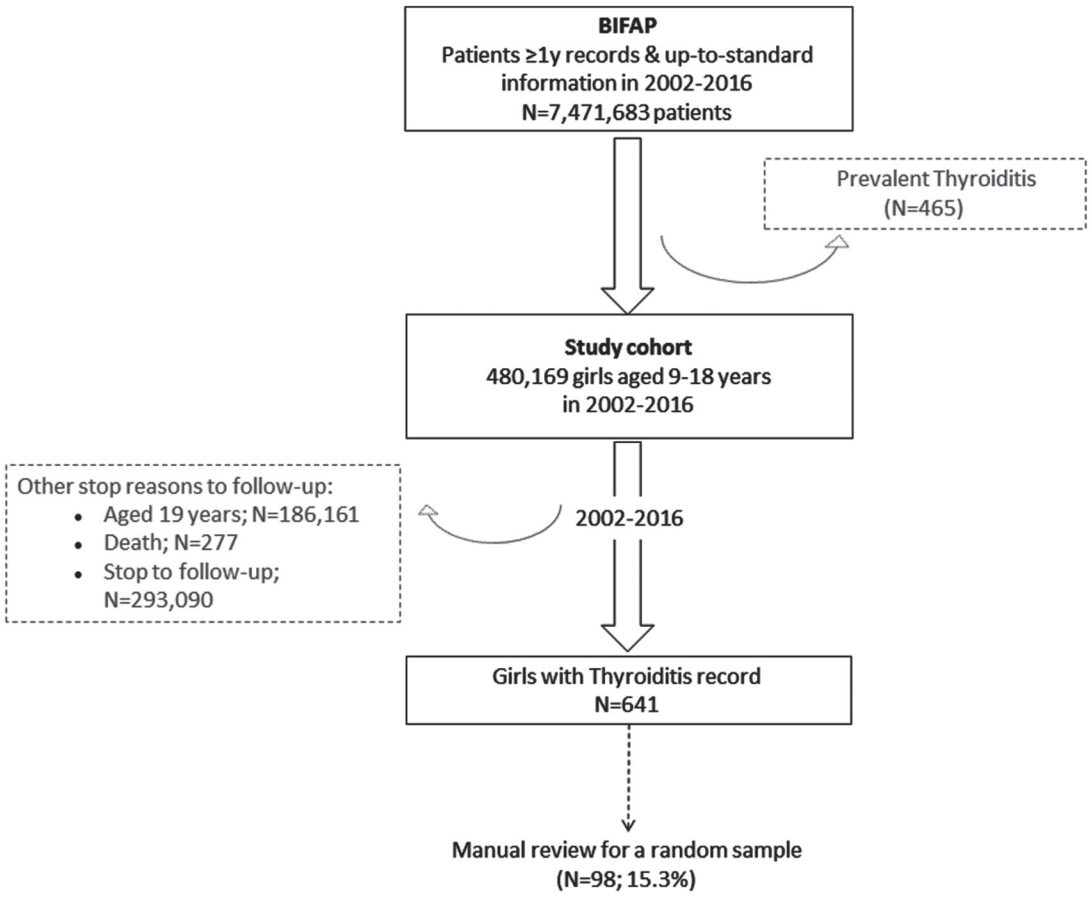

Figure 1. Study population ascertainment and follow-up until thyroiditis recording

was similar among recorded AIT (60.0%) and thyroiditis of unspecified type (61.7%), and slightly higher among patients with recorded TPO test results (65.1%) than among patients without TPO test results recorded (56.4%) (see Table 2). PPV was 92.9% for the broad gold standard, being 92.0% for AIT, and 93.6% for unspecified type.

Extrapolating these proportions to the 641 cases of thyroiditis recorded, it was estimated that 386 'confirmed', 209 'possible' and 46 discarded thyroiditis cases would occur in the study cohort if all records were manually reviewed. Thus, the *true* IR was 20.83 'confirmed' thyroiditis cases per 10^5 py, which increased to 32.12/10^5 py when 'possible' thyroiditis cases were also included. The IR increased with age from 9 to 14 years, and decreased thereafter, being stable for girls aged 15-18 years, as shown in Figure 2. For the period 2002-2005 the crude IR (16.28 and 20.93 per 10^5 py) was lower than for the period 2007-2016 (21.17 and 33.78 per 10^5 py) for 'confirmed' and 'confirmed' plus 'possible' cases, respectively.

Of the 641 thyroiditis cases recorded, 64 had a prescription (n = 57) and/or signs compatible with the disease (n = 49) registered before the diagnosis of thyroiditis was included, with a median of 591 days that could suggest a delay in thyroiditis recording. Prescriptions included levothyroxine (in 45 girls), anti-thyroid drugs (thiamazole or carbimazole; n = 13) and propranolol (n = 11). Most propranolol prescriptions had linked ICPC mentioning hyperthyroidism, bocio/thyrotoxicosis, or Graves-Basedow disease. Other beta-blockers were not prescribed. Linked signs and free-text comments to the record of thyroiditis were related to the thyroid status examination and included requested lab test (or its results) as well as signs compatible with thyroiditis.

Discussion

The current study provides valuable information on how thyroiditis is recorded among girls in primary healthcare records and on the incidence of the disease in such a population in Spain. Among the recorded thyroiditis episodes, few false positives (7%) were found, while two-thirds were 'confirmed' and a third had a thyroiditis record without additional evidence. After correcting for false positives, the IR did not change markedly compared

Table 1. Distribution of newly diagnosed thyroiditis cases by calendar year

Year of thyroiditis record	Number of thyroiditis cases	Proportion (%)
Total	641	100.00
2002	3	0.47
2003	14	2.18
2004	7	1.09
2005	23	3.59
2006	28	4.37
2007	33	5.15
2008	36	5.62
2009	39	6.08
2010	38	5.93
2011	41	6.40
2012	74	11.54
2013	77	12.01
2014	72	11.23
2015	79	12.32
2016	77	12.01

For the estimation of the global incidence of thyroiditis between 2002 and 2016, each calendar year was included. For the estimation of the potential incidence change over the years, two periods were designated, i.e. 2002-2005 and 2007-2016, while an interrupted period, 2006 when vaccination began, was also designated

to the *observed* IR (from 34.59 to 32.12 cases/10^5 py), but decreased to 20.83 cases/10^5 py when only cases with confirmatory information were included (gold standard). The *true* incidence of thyroiditis peaked in girls aged 13-14 years, as expected, and increased around 5-13 times from the period 2002-2005 to the period 2007-2016.

A previous precision study on the recording of new-onset autoimmune conditions (including thyroiditis and others) in primary healthcare databases, also reported confirmation rates in the same range (31-40%) (13). Gold standard information for thyroiditis diagnosis may be rather complex to be defined using secondary sources of data. For instance, the values of an hormonal test including thyrotropin (thyroid-stimulating hormone), free thyroxine (FT4), triiodothyronine (FT3) and anti-TPO antibody (anti-TPO-Ab) are commonly used for diagnosis (11,14,15,16) and may be commonly recorded in the databases, as found with BIFAP. However, the criteria for the diagnosis of thyroiditis based on hormonal tests, may be different according to patients' age and sex, type of test, medical setting or region. Therefore, specific guidelines may be needed. Our data suggest a slight increase in the PPV among clinical profiles including TPO results (65.1% with TPO vs 56.4% without TPO in gold standard; 93.03% with TPO vs 92.72% without TPO in broad gold standard) but did not add value to the global PPV (60.2% and 92.2%, respectively). Thus, TPO test record as a 'yes/no' response is not valuable as a component for future automatic algorithms for exploring thyroiditis cases.

Regarding the date of thyroiditis recording, few patients (10%) had previous prescriptions of anti-thyroid drugs, thyroid hormone supplementation or propranolol, or past medical comments linked electronically to thyroiditis by the PCP. These factors were recorded at a median of 1.6 years before the diagnosis, suggesting a substantial delay in the recording of thyroiditis versus diagnosis or a delay in the diagnosis compared with the initial presentation for consultation about symptoms. This delay must be taken into account when using this information for evaluating risk factors. The finding of anti-thyroid drugs or thyroid hormone supplementation provided absolute reassurance of the case, since these drugs are only used in the treatment of thyroid diseases (3).

Few papers report on the incidence of overall thyroiditis in children and adolescents, being more frequently focused on specific types of thyroiditis, such as AIT, Hashimoto or Graves' disease or related symptoms. For instance, a study performed in the UK using the information recorded in the Clinical Practice Research Datalink GOLD database (similar to BIFAP) estimated an incidence of 5.52 AIT/10^5 py among 130,000 females aged 9-18 years for the period 2008-2010, increasing to 8.3 for females aged 18-25 years (17). In that study, incidences ranged from 6.17 [95% confidence interval (CI): 1.68-15.80; n = 4] to 23.18 (12.98; 38.24; n = 15) 'confirmed' cases per 100,000 female-years among females with different profiles, increasing up to 27.76 (95% CI: 16.45-43.87; n = 18) and 40.18 (95% CI: 26.25; 58.88; n = 26), when 'confirmed' and 'possible' cases were included (17). Although CIs are of scarce precision, due to the small number of cases observed, the results from our study overlap with those calculations with estimates of 20.83 'confirmed' and 32.12 'confirmed' plus 'possible' /10^5 py. The incidence of Graves' disease, which accounts for 95% of hyperthyroidism in children (18), has been estimated at 27.8/10^5 py (95% CI: 13.9-49.7) for females aged 10-14 years in a Hong Kong study in 1994-1998 (19), with a male to female ratio of 1:9.7 and lower among younger girls aged 5-9 years (i.e. 7.6/10^5 py [95% CI: 1.6 to 22.3]). Similarly, in the US (13) 26 new-onset cases of Graves' disease per 10^5 py were found among women aged 9-26 years as well as 81 Hashimoto cases per 10^5 py. Also, the incidence of thyrotoxicosis increased with age, from 0.1 (among girls aged < 5 years), 0.6-0.98 (among girls aged 5-9 years) to 3-3.4/10^5 py (in adolescents girls aged 10-14 years) in Denmark (20), the UK and Ireland (21), observing

a female preponderance from childhood (versus incidences found among boys: 0.1, 0.1-0.3 and 0.48-1.1, respectively).

As observed, a broad range of IRs has been reported, probably due to different methods for data collection (voluntary participation of paediatricians, the use of electronic health records or the performance of surveys), the period covered or the populations' characteristics, including geographical area, clinical setting or health system, among others.

Estimations of hyperthyroidism, hypothyroidism or childhood thyrotoxicosis occurrence, regardless of the aetiology, are more commonly found in the literature. Such incidences were below 3 per 100,000 in Sweden (22,23), increasing over the years (23). In a French study, the number of cases of treated hyperthyroidism for Graves' disease, due to Graves' disease in 95% (18), also increased with age, being estimated at 7.89 per 100,000 among girls aged 10-14 years and 21.53 per 100,000 among girls aged 15-17 years (24). We found similar IR for thyroiditis of any type.

The slight increase in the crude IR found in the second period studied (2007-2016) versus the previous period (2002-2005) is in line with the reported temporal increase of hyperthyroidism or autoimmune disorders among children (25,26,27). However, changes along the years in the recording habits of participating PCP (that can be related to getting used to the computer record systems or to other factors), thyroiditis diagnosis criteria, regions collaborating, the age of participants or a less precise rate due to a lower population size in the former period (N = 129,835 girls) versus the latest one (N = 423,960) as well as other unknown factors may have also contributed to observe a difference between the two periods that might be lower or even confounded. In order to confirm any time trend, further control of the yearly incidences by confounders, is waranteed.

Complications associated with either overt or subclinical thyroid dysfunction cannot be neglected, being especially severe in patients with cardiovascular diseases, in postmenopausal women, or in women antedating pregnancy. Since the incidence in children and adolescents appears significant, early diagnosis is relevant to prevent complications associated with thyroiditis.

The strengths of the current study include the large number of girls participating, representing the paediatric female population attended in primary care. In addition, the study identified incident cases of thyroiditis and provided a new estimation of the incidence that will enable future studies of risk factors and health interventions among girls and adolescent women in Spain. Finally, the case finding algorithm for thyroiditis cases will enable the identification of cases with a known precision over the entire population registered in the database.

Study Limitations

Some limitations of the current study must be mentioned. First, no PCP or specialists were contacted to confirm the correct recording and diagnoses of thyroiditis. Secondly, although commonly recorded in the medical profiles, thyroid function tests were not straightforward to interpret and thus use as confirmation criteria. For this reason, we relied on written evidence of the diagnosis itself (through a letter to/from a referral or hospital, or free-text comments) to confirm it rather than using the diagnostic criteria. Since that information was less frequently available in the database, we decided to review a small sample size of cases (15% of the total potential cases identified). We are aware that, even though that sample was randomly selected, its small size could have affected the precision of the predictive values. Finally, the incidence may have been underestimated due to the fact that there was little recorded evidence for case confirmation. For this reason, the incidence including both, 'confirmed' and 'possible' thyroiditis was calculated and reported. The real incidence should be somewhere in between, assuming few false negatives (i.e. cases of thyroiditis not recorded in the clinical profiles).

Conclusion

This new estimation of the incidence of thyroiditis among girls suggests that cases increased with calendar years and age, and remained stable among girls aged 15-18 years in a Spanish population. Of the thyroiditis cases, more than half were autoimmune, few had other aetiology and for the remaining, the type of thyroiditis was not reported. A delayed diagnosis was suggested in a few patients. Further research about risk factors triggering thyroiditis among paediatric patients, especially if some of these risk factors prove to be preventable, is warranted.

Acknowledgement

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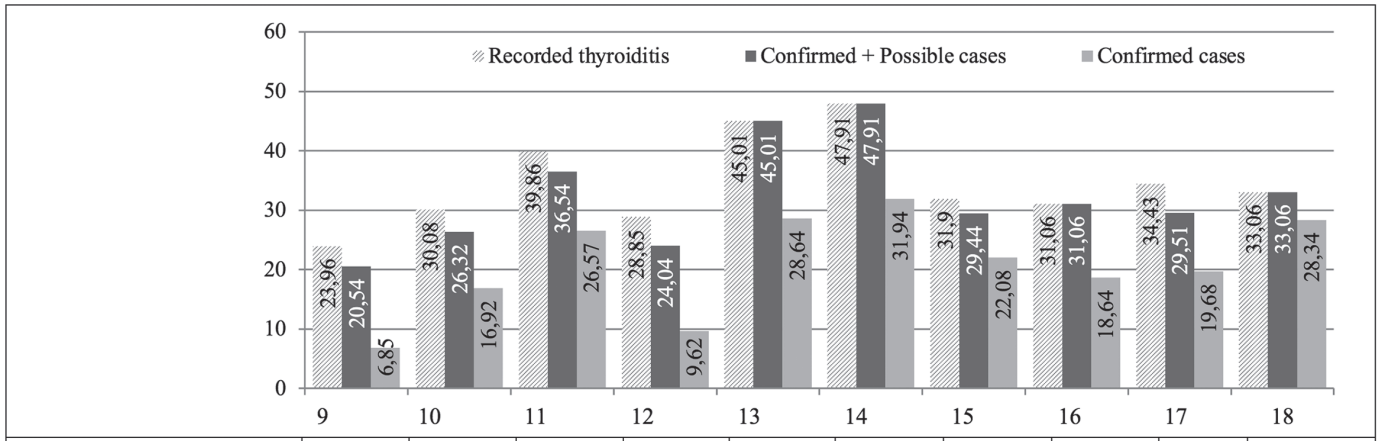
Table 2. Validity parameters of manually reviewed thyroiditis records versus gold standard information recorded in free-text comments, overall, by aetiology and by calendar period, among girls aged 9-18 years between 2002 and 2016 in the Spanish primary healthcare database BIFAP

								Gold standard for incident thyroiditis[¶]				Broad gold standard for incident thyroiditis[¶]		
		Thyroiditis detected automatically	*Observed* Reviewed *IR/10^5 py*		Confirmed incident thyroiditis	Possible incident thyroiditis	Thyroiditis discarded (always false positives)	PPV of incident thyroiditis	Possible assumed as false positives	*True* IR/10^5 py	Sensitivity over all confirmed	PPV of incident thyroiditis	*True IR/10^5 py*	Sensitivity over all confirmed + possible
Overall	First ever recorded thyroiditis	641	**34.59**	98	59	32	7 (7.1%)	**60.2%**	**32.7%**	**28.83**	-	**92.9%**	**32.12**	-
Recorded aetiology	Autoimmune thyroiditis	346	18.67	50	30	16	4 (8.0%)	60.0%	32.0%	11.22	50.8%	92.0%	17.22	50.5%
	Unspecified type	278	15.00	47	29	15	3 (6.4%)	61.7%	31.9%	9.26	49.2%	93.6%	14.04	48.4%
	Other thyroiditis type	17	0.92	1	0	1	0 (0.0%)	0.0%	100.0%	0.92	0.0%	100.0%	0.00	1.1%
Period	2002-2005	47	**23.26**	10	7	2	1 (10.0%)	70.0%	20.0%	**16.28**	-	90.0%	**20.93**	-
	2006	Interrupted time by study design												
	2007-2016	566	**36.48**	81	47	28	6 (7.4%)	58.0%	34.6%	**21.17**	-	92.6%	**33.78**	-
	TPO as potential component for case finding automatic algorithm													
TPO test	Recorded TPO test	262	NA	43	28	12	3 (7.0%)	65.1%	27.9%	NA	47.5%	93.0%	NA	44.0%
	Not recorded TPO test	379	NA	55	31	20	4 (7.3%)	56.4%	36.4%	NA	52.5%	92.8%	NA	56.0%

[¶]Gold standard is the scenario when all 'confirmed' cases of incident thyroiditis are true positive and 'possible' cases are in fact false positives. Broad gold standard is the scenario when both 'confirmed' and 'possible' cases of incident thyroiditis are true positives.
Percentages for PPV, false positives and possible proportions apply over all the thyroiditis reviewed.
NA: Non-applicable; the availability of the TPO test (i.e. whether or not recorded at anytime in the girls clinical profiles) were assessed only as potential component for improving the validity index of the case-finding automatic algorithm, thus incidences of thyroiditis by TPO were not estimated.
TPO: thyroid peroxidase, IR: incidence rate, PPV: positive predictive value



Recorded thyroiditis, N=641	45	56	74	54	84	88	59	57	63	61
Expected* N no-cases	6	7	6	9	0	0	5	0	9	0
Expected* N possible	26	18	19	27	31	29	14	23	18	9
Expected* N confirmed	13	32	49	18	53	59	41	34	36	52

*The expected number of thyroiditis cases for incidence calculations were the results of applying the proportion of 'confirmed', 'possible' and discarded cases by age among 98 reviewed thyroiditis to the total number of automatically identified thyroiditis cases. Gold standard is the scenario when all 'confirmed' cases of incident thyroiditis are true positive and 'possible' cases are in fact false positives. Broad gold standard is the scenario when both 'confirmed' and 'possible' cases of incident thyroiditis are true positives

Figure 2. Distribution of recorded and expected thyroiditis cases according to confirmatory information and incidence rate per 10^5 py among girls aged 9-18 years between 2002 and 2016 in Spanish primary healthcare database (BIFAP)

Ethics

Ethics Committee Approval: The investigators had access to secondary use of only fully anonymized data, and under this condition, no specific ethics review was required according to Spanish law.

Informed Consent: Not applicable according to Spanish law.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: Elisa Martín-Merino, Data Collection or Processing: Elisa Martín-Merino, Belén Castillo Cano, Analysis or Interpretation: Elisa Martín-Merino, Aida Moreno-Juste, Belén Castillo Cano, Mar Martín Pérez, Dolores Montero Corominas, Literature Search: Elisa Martín-Merino, Aida Moreno-Juste, Belén Castillo Cano, Mar Martín Pérez, Writing: Elisa Martín-Merino, Aida Moreno-Juste, Belén Castillo Cano, Mar Martín Pérez, Dolores Montero Corominas.

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Annex 1. ICPC and ICD-9 BIFAP specific description, and string text for automatic identification of thyroiditis

Classification	Description of classification code¥	Type of thyroiditis			Number of cases detected automatically
		Autoimmune	Unspecified	Other	
ICPC-BIFAP specific					**269**
T85.1	BASEDOW, ENF. DE §	X			38
T99.16	HASHIMOTO, ENFERM. DE	X			21
T99.23	TIROIDITIS		X		166
T99.32	TIROIDITIS DE QUERVAIN			X	
T99.39	TIROIDITIS AUTOINMUNE	X			42
T99.40	TIROIDITIS CRONICA AUTOINMUNE	X			1
T99.41	TIROIDITIS SUBAGUDA			X	1
ICD-9-BIFAP specific					**171**
242.00:2	ENFERMEDAD DE GRAVES BASEDOW	X			13
245:0	TIROIDITIS		X		1
245.0:0	TIROIDITIS AGUDA			X	12
245.1:0	TIROIDITIS SUBAGUDA			X	1
245.1:1	TIROIDITIS DE QUERVAIN			X	

245.2:0	TIROIDITIS LINFOCITICA CRONICA	X			109
245.2:2	TIROIDITIS CRONICA AUTOINMUNE	X			
245.2:5	ENFERMEDAD DE HASHIMOTO	X			
245.2:4	TIROIDITIS DE HASHIMOTO	X			
245.2:1	TIROIDITIS CRONICA LINFOCITICA	X			
245.2:3	TIROIDITIS AUTOINMUNE	X			
245.3:5	ESTRUMA FIBROSA			X	
245.3:1	TIROIDITIS INVASIVA			X	
245.3:2	TIROIDITIS LEÑOSA			X	
245.3:4	TIROIDITIS DE RIEDEL			X	
245.3:3	TIROIDITIS CRONICA FIBROSA			X	
245.3:0	TIROIDITIS FIBROSA CRONICA			X	
245.4:0	TIROIDITIS IATROGENICA			X	
245.8:0	OTRAS TIROIDITIS CRONICAS Y TIROIDITIS CRONICAS NO ESPECIF			X	
245.9:0	TIROIDITIS NO ESPECIFICADA		X		35
245.9:1	TIROIDITIS		X		
String text	**Searching algorithm (Like, Owa or M # of separated words)**				**201 (80.1% of them were recorded in Description of ICPC classified as Endocrine/Metabolic and Nutritional, i.e. T-codes)**
TIROIDITI	L		X		
HASHIMOTO	O	X			
HASIMOTO	O	X			
GRAVES BASEDO	M 2	X			
ESTRUMA FIBROSA	M 2			X	
ENF GRAVES	M 2	X			
E. GRAVES	M 2	X			
GRAVES TIROID	M 7	X			
BASEDOW	O	X			
BASEDO	O	X			
TIROIDITI AUTOINMUNE	L	X			
TIROIDITI CRONICA LINFOCITI	L	X			

*Codes or string text were searched in tables of Diagnosis, Clinical Antecedents or Clinical Determinants. Additionally, string text were not selected when referred to familiar antecedents, lack of certainty, negation, discarding and doubt through computer algorithms.
¥Description of codes is reported in Spanish language as recorded in BIFAP.
ICD-9: International Classification of Diseases 9th revision, ICPC: International Classification of Primary Care

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Clinical Characteristics of 46,XX Males with Congenital Adrenal Hyperplasia

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What is already known on this topic?

Severely virilized patients with 46,XX congenital adrenal hyperplasia (CAH) can be raised as males but the number of these patients is small and data on their follow-up is lacking.

What this study adds?

Most (65.9%) of the 46,XX CAH cases raised male were diagnosed after two years of age. In 46,XX CAH cases, hysterectomy and bilateral salpingoopherectomy, genital corrective surgeries and testicular prosthesis operations were performed in a very wide age range.

Abstract

Objective: To retrospectively evaluate the follow-up data in patients with 46,XX congenital adrenal hyperplasia (CAH) who were raised male.

Methods: A national database was created. The data of patients were asked to be recorded in the data form.

Results: The median (range) age of diagnosis was three (0.1-18.3) years in 44 patients. Twenty nine cases were diagnosed after the age of two years. Most (95.4%) cases were stage 4-5 virilized. Hysterectomy and bilateral salpingoopherectomy, at a median age of 7.25 (2.4-25.3) years, was performed in 35 cases. Testicular prostheses were placed in 11 (25%) cases at a median age of 11.2 (2.8-17) years. The median final height was 149.2 (132.8-172) cms in 38 patients, including simple virilizing (n = 18), salt-wasting (n = 6), and 11-beta hydroxylase (n = 12). Of the 16 patients above the age of eighteen, university education was completed in 25%.

Conclusion: It was seen that most (65.9%) of the 46,XX CAH cases raised male were diagnosed after two years of age. In these cases, hysterectomy and bilateral salpingoopherectomy, genital corrective surgeries and testicular prosthesis operations were performed in a very wide age rage.

Keywords: 46,XX, congenital adrenal hyperplasia, final height

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Introduction

According to consensus guidelines published in 2006, severe virilization during the neonatal period should not be considered a criterion for male sex determination, and 46,XX congenital adrenal hyperplasia (CAH) cases should be raised as females (1). This recommendation is supported by the fact that most of these patients identify as females and moreover, they retain their sexual and reproductive function (2). However, the age at diagnosis, age of initiation of glucocorticoid treatment, and degree of virilization were not considered. Some studies that considered these factors proposed that severely virilized patients with 46,XX CAH may be raised as males (3,4,5). Gender determination at birth and appropriate family and social support may be important in shaping sexual identity (5,6). Lee et al (4) suggested that the social and cultural environment is important to the gender identity of severely virilized 46,XX CAH patients. Gender dissatisfaction in adolescence and adulthood is high among patients with brain virilization caused by perinatal androgen exposure, particularly in girls with a delayed CAH diagnosis (7). Daae et al (8) performed a systematic review of the literature, investigating sexual orientation in individuals with CAH. This recently published review included 30 studies investigating sexual orientation in patients with CAH assigned female at birth (46,XX) (n = 927) or assigned male at birth (46,XY and 46,XX) (n = 274). A majority of assigned females with CAH self-identified (defined themselves) as heterosexual, but figures varied widely across studies (40-100%). Results indicate that fewer assigned females reported homosexual (3-20%) or bisexual orientation (3.4-37%). The rates of non-heterosexual orientation were higher in assigned females with CAH than controls, whereas no individuals with CAH assigned male (46,XY or 46,XX) expressed any non-heterosexual orientation.

Patients with CAH are often diagnosed late in countries without a newborn screening program for the disorder. Such patients tend to be raised as males by their families. Even virilized CAH patients diagnosed early may be raised as males in male-dominant societies. Follow-up data on 46,XX CAH patients raised as males are insufficient. Experiences in 46,XX CAH patients raised as males are mostly in the form of case reports. In this study, it was planned to evaluate 46,XX CAH cases followed up with this diagnosis in our country. Therefore, this study aimed to create a national database of 46,XX CAH patients including retrospectively collected diagnostic and follow-up data.

Methods

This study included 46,XX patients with CAH raised as males (21 hydroxylase and 11 beta hydroxylase-deficient CAH patients; analyzed as a single group given their similar clinical characteristics). The diagnosis of CAH was made based on medical history, physical examination, and cytogenetic and hormonal analyses. The diagnosis of salt-wasting 21 hydroxylase deficiency was made on the basis of findings of salt-wasting, ambiguous genitalia and elevated 17-hydroxyprogesterone levels. The diagnosis of simple virilizing 21 hydroxylase deficiency was made on the basis of findings of ambiguous genitalia and elevated 17-hydroxyprogesterone levels without salt wasting. The diagnosis of 11 beta hydroxylase deficiency was made on the basis of findings of ambiguous genitalia and elevated 11-deoxycortisol levels. Cytogenetic studies confirmed the karyotype to be 46,XX. Patients with a history of additional chronic systemic disease or chronic drug use for reasons other than CAH were excluded from the study.

National-scale projects in Turkey, such as the current study, are supported by the Pediatric Endocrinology and Diabetes Association. After receiving approval from the Pediatric Endocrinology and Diabetes Association, the details of the project, including the start and end dates for data collection (July 11, 2018 and January 31, 2019, respectively), were sent to all participating pediatric endocrinology clinics via e-mail. The clinics were also provided with access to online data entry forms (cedd.saglik-network.org site). The data form (Table 1) is provided in the supplementary material. Adult endocrinology clinics were not invited to take part in the study.

Diagnostic and follow-up data were obtained retrospectively from patient records. The requirement for informed consent was waived due to the retrospective nature of the study.

Table 1. Data form

Age of diagnosis
Height/height sds at diagnosis
Weight/weight sds at diagnosis
Virilization Prader stage at diagnosis
Steroid treatment type at diagnosis
Age of hysterectomy and bilateral salpingoopherectomy
Age of first genital corrective surgery
Number of genital corrective surgeries
Testicular prosthesis placement age
Steroid treatment compliance
Testosterone treatment start age
Testosterone treatment compliance
The presence of breast development
Age of last follow-up
Duration of follow-up
Final height
Education status
Working condition

The genital virilization levels of the patients were evaluated by pediatric endocrinologists at the time of diagnosis and staged according to the Prader classification (9).

This study was approved by Zekai Tahir Burak Women Health Training and Research Hospital Clinical Research Ethics Committee (approval number: 15/2018, date: 06.03.2018).

Statistical Analysis

Statistical analysis was done using Statistical Package for the Social Sciences, version 22 (IBM Inc., Armonk, NY, USA). For evaluation of the normality distribution of the data, the Shapiro-Wilk test was used. The statistical significance was investigated using t-test for numerical variables, Mann-Whitney U test for non-normal distributions, cross table, Pearson chi-square test and Fisher's exact test for categorical variables. A $p < 0.05$ was considered statistically significant.

Results

Total Number and Distribution of Patients

Data for 44 patients from seven Pediatric Endocrinology Centers were analyzed. In seven Pediatric Endocrinology Centers, the total number of patients with 46,XX CAH was 439. Of these 439, 44 (10%) were raised as male. Of the 44 CAH cases, 30 (68.1%) were CYP21A2-deficient CAH and 14 (31.8%) were CYP11B1-deficient. Of the CYP21A2 deficient CAH cases, 10 (33.3%) were of the salt-wasting type and the remainder were of the simple virilizing type. The characteristics and clinical findings of the patients at diagnosis and the final follow-up are given in Table 2.

Age and Virilization Stage at Diagnosis

The median (range) age at diagnosis was 3 (0.1-18.3) years. Fifteen patients (34%) were diagnosed at <2 years of age, while 29 (65.9%) were diagnosed at >2 years of age. Eight patients were diagnosed during the neonatal period. Only two cases (4.6%) were Prader stage 3 (one case early diagnosis 11 beta hydroxylase deficiency, one case late diagnosis simple virilizing 21 hydroxylase deficiency); the remaining 42 cases (95.4%) were stage 4 (n=13) or 5 (n=29).

Surgeries

Hysterectomy and bilateral salpingoopherectomy was performed in 34 cases, at a median age of 7.25 (2.4-25.3) years. Early diagnosed cases were operated at a median of 7.8 (3.5-12) years while late diagnosed cases were operated at 7.8 (2.4-25.4) years. In six cases (17%), these procedures were performed at ≤3.5 years of age.

Table 2. Clinical follow-up characteristics of 46,XX congenital adrenal hyperplasia patients raised male

	Clinical characteristics
Age of diagnosis, median (range) in years	3 (0.1-18.3)
Age of last follow-up mean ± SD (range) in years	14.9 ± 5.7 (1-24.9)
Duration of follow-up mean ± SD (range) in years	10 ± 6.1 (0.1-24)
Distribution of patients by age of diagnosis	Newborn (n = 8) <2 years (n = 7) Pre-schooler (n = 18) School-aged (n = 5) Adolescents (n = 5) Adult (≥18 years) (n = 1)
Virilization Prader stage in diagnosis	Stage 3 (n = 2) (4.5%) Stage 4 (n = 13) (29.5%) Stage 5 (n = 29) (65.9%)
Median (range) age of hysterectomy and bilateral salpingoopherectomy (years) (n = 34)	7.25 (2.4-25.3)
Number of patients who had more than one operation	n = 13 (38.2%)
Median (range) testicular prosthesis placement age (n = 11) (years)	14 (2.8-17)
Steroid treatment compliance	Good (n = 19) (42.2%) Poor (n = 26) (57.7%)
Testosterone treatment start age, mean ± SD (range) (years) (n = 21)	14 ± 1.85 (10-17)
Testosterone treatment compliance	Good (n = 18) (85.7%) Poor (n = 3) (14.2%)
Median Final height (cm) (n = 38)	149.2 (132.8-172)

SD: standard deviation

Genital reconstructive surgery was performed in 12 (27.2%) patients; two of these patients were Prader stage 3, and 10 were Prader stage 4. Genital reconstructive surgery was performed once in four cases, twice in six cases, and four times in two cases. The first genital corrective surgery was performed at the a median of 8.5 (2-14.5) years.

A testicular prosthesis was placed in 11 (25%) cases. The testicular prosthesis was replaced at a median age of 11.2 years (2.8-17) years which varied by period of diagnosis: late diagnosis 15.5 (14-17) years and early diagnosed cases 9.8 (2.8-14) years. Breast development occurred in 12 cases with bilateral oophorectomy, four of whom underwent a mastectomy. Eight of the patients did not consent to surgery.

Treatment

All patients were started on glucocorticoid replacement treatment at the time of diagnosis which consisted of

hydrocortisone and hydrocortisone-equivalent steroid treatment in 30 and 14 patients, respectively. However, steroid treatment compliance was low, at 56.8%.

Testosterone treatment was started in 21 cases. The mean age at initiation of testosterone treatment was 14 ± 1.85 years, with a range of 10-17 years, and the mean duration of the treatment was 4.76 ± 2.63 years. Testosterone depot forms were started at a dose of 50 mg/4 weeks. The dose was increased by 50 mg over six months, and the full dose of 250 mg was reached in two years. Only three cases (14.2%) were unsuitable for testosterone treatment. Testosterone treatment was started in 13 patients at the age of >14 years. The age of initiation of testosterone treatment in eight patients was between the ages of 10-14 years. Testosterone treatment was not started in eight cases despite an age >14 years.

Duration of Follow-up and Final Height

The median age at the last examination was 15.3 years and the median follow-up duration was 10.7 (0.1-24) years. A total of 28 (63.6%) patients were aged >14 years. Thirty-eight patients (18 patients with simple virilizing 21 hydroxylase deficiency, six patients with salt wasting 21 hydroxylase deficiency, 14 patients with 11 beta hydroxylase deficiency) reached their final height during the follow-up. The median final height was 149.2 (range: 132.8-172) cm.

Education and Job

Of the 16 patients aged >18 years, two were primary school graduates, four were high school students, six were high school graduates, two were university students, and two were university graduates. University education was completed by 25% of the patients.

Regarding employment type, one patient was a shepherd, one had an assembly job, one was an office apprentice, one was an "asphalt worker" and one was a chemical engineer. Only 5 of the 10 adult patients (two primary school graduates, six high school graduates, and two university graduates) had a job with medical insurance.

Discussion

This study is the largest case series of 46,XX CAH patients raised as males reported to date. Although it has been recommended by physicians that patients diagnosed as newborns or during early infancy be raised as girls, families may desire to raise their child as a boy in male-dominated societies. In our study, the majority of the 46,XX CAH patients raised as males were diagnosed after the age of two years, but approximately one-third were diagnosed at an earlier stage. The upbringing of 46,XX CAH patients diagnosed early and identified as male may depend on the culture of the country of birth. In the literature, the majority of 46,XX CAH patients raised as males were diagnosed late (4,10,11,12,13). Few patients are diagnosed before the age of two years (3). In our cohort, it was recommended that female patients diagnosed at <2 years of age be raised as females, particularly those diagnosed during the neonatal period. However, some families insisted on raising their child as a male. Although family preferences and sociocultural factors are important, virilization also plays a role in the decision to raise these patients as males. In studies from countries with different sociocultural contexts, most patients with 46,XX CAH raised as males are Prader stage 4 or 5 (3,10,13,14). In our study, 95.5% of the patients were Prader stage 4 or 5; only two (4.5%) were Prader stage 3. According to the literature, few Prader stage 2-3 patients are raised as males, similar to our study (12,15,16).

According to the current consensus, surgeries leading to irreversible infertility should not be performed in patients with sex development disorders until their sexual identity is clear (17). However, hysterectomy and bilateral salpingoopherectomy are almost always performed during childhood in 46,XX CAH patients (3,13,14,15). In our series, the median age of patients who underwent hysterectomy and bilateral salpingoopherectomy was 7.25 but varied widely from 2.4 to 25.3 years. In eight (22.8%) patients, hysterectomy and bilateral salpingoopherectomy were performed at the age of ≤3.5 years. These surgeries were performed at the age of 25.5 years in a patient diagnosed with 46,XX CAH when he was 18 years and four months old. This patient had not presented to the endocrine department prior to being diagnosed with 46,XX CAH. He was diagnosed with an undescended testicle at the ages of five and eight years, but no further examinations were performed. In 33 other patients, hysterectomy and bilateral salpingoopherectomy were performed before the age of 15 years. About 68% of the cohort were aged <10 years, which is the average age of onset of puberty, and the surgery was performed without a full assessment of sexual identity. Hysterectomy and bilateral salpingoopherectomy operations should be performed only after the sexual identity of the child becomes clear, that is when the individual can adequately express their desire in that respect.

A testicular prosthesis was placed in 11 patients (25%) in our series and the median (range) age of implantation was 11.2 (2.8-17) years. Similar to the literature, the rate of testicular prosthesis placement was low and the age at placement varied (3,13,14,15). There is no standard age for testicular prosthesis placement, and no recommendations

regarding changing the testicular prosthesis of a patient to one of a different size (3,13,14.15). Therefore, this is left to the discretion of the managing clinicians.

Breast development is particularly obvious during puberty in 46,XX CAH patients raised as males. Breast development may also occur in patients who are unsuitable for steroid treatment and breast surgery may be required in these cases (10,13). In our series, breast development occurred in 12 patients with mastectomy being performed in four of these cases. Hysterectomy and bilateral salpingoopherectomy were performed in 10 of 12 cases with breast development. This suggests that treatment incompatibility may have an etiological role. It should be emphasized, both to the patients and their families, that patients who need a large number of genital corrective operations must comply with treatment to prevent the requirement for an additional mastectomy.

Compliance with testosterone treatment was generally good among our patients. However, steroid compliance was poor and approximately half of the patients were unsuitable for steroid treatment. Without steroid treatment, 46,XX CAH patients may exhibit increased levels of androgens, potentially making them feel better. However, to avoid the negative effects associated with a lack of steroid treatment (short stature, risk of adrenal crisis at any age), the importance of treatment compliance should be impressed upon patients and their families.

Thirty-eight patients reached their final height during the follow-up. The median final height was 149.2 cm (range: 132.8-172 cm), which is too short for individuals to continue living as men. In all children with CAH, boys and girls, androgen excess causes accelerated bone maturation and growth and reduces adult height. In these children, hydrocortisone replacement therapy is very challenging: overdosing results in growth inhibition and excessive weight gain, whereas underdosing results in accelerated bone maturation and short adult height (18). A meta-analysis reviewed adult height data until 2008 and confirmed height loss; the mean adult height in salt wasting and simple virilizing patients was -1.38 standard deviation (SD)-score (-1.56 to -1.2) (19). Bretones et al (20) monitored French CAH children from the pre-screening era and found that patients had a shorter adult height than the general population mean: -1.2 SD (156.7 cm) in girls and -1 SD (168.8 cm) in boys. In comparison with the general French population, short AH (<-2 SD score) was seen in 24% of the cohort (22.5% of girls and 26% of boys) and presented with a dramatically advanced 8-year bone maturation that strongly influenced the risk of short adult height. Several authors have found a better height outcome in patients who received fludrocortisone (21). A higher pubertal hydrocortisone dose was associated with a slightly higher risk of short adult height. This may be due to the well-known negative effect of excessive glucocorticoid doses on growth (21,22). High hydrocortisone doses may reflect a poorly controlled disease either because of disease severity or secondary to poor compliance to the treatment. The adult heights achieved are actually below the average and 20% of adult CAH patients have a short adult height (below -2 SD) (20). The final height achieved in patients with 46,XX CAH is very important, particularly if they are to be raised as males, given its psychosocial effects. Woelfle et al (11) reported a patient who attempted suicide due to short stature. Families of 46,XX CAH patients raised as males should be informed that their children will not be fertile, and that they will be short. In these cases, early and long-term growth hormone treatment and aromatase inhibitors may be beneficial (23). If growth hormone treatment is to be used (which should only be for a protracted period), planning should begin at the youngest possible age.

Follow-up of 46,XX CAH cases raised as males is important during adulthood to assess quality of life. Further studies on this subject are needed. Only 25% of the 16 cases aged >18 years in this study received a university education. According to the data of the Statistical Institute in our country, the ratio of faculty or high school graduates to the population was 15.7%. Thus the educational achievement rate of these patients was not lower than the general population.

All patients were followed up in the pediatric endocrine clinic. Clinics facilitating transition to adulthood should be established for 46,XX CAH patients aged >18 years raised as males. A multidisciplinary approach should be taken, with follow-up throughout life to assess endocrine function and psychiatric status.

The main strengths of our study were the inclusion of a large number of 46,XX CAH patients raised as males, and the availability of follow-up data. It is important to note that this study included a sample drawn from clinics nationwide, such that the outcomes should be generalizable.

Study Limitations

A limitation of our study was that some of the patient data were obtained retrospectively. Also, comparing 46,XX CAH patients raised as females with those raised as males during the same period would have provided more valuable information.

Conclusion

No guidelines for the care and management of 46,XX CAH patients raised as males are available. The results of our

study, which is the largest case series of 46,XX CAH patients raised as males conducted to date, can be summarized as follows. The 46,XX CAH patients raised as males were diagnosed late, and included cases of advanced virilization. Surgeries that eliminated the potential for fertility were mostly performed without a full assessment of gender identity. Testicular prosthesis placement rate, and the age at placement, were highly heterogeneous. The onset of testosterone treatment was late, and the rate of treatment was inadequate. Steroid treatment compliance was poor, and the final height of most patients was short for males. We recommend that irreversible surgeries that impair the possibility of fertility should be avoided unless an explicit self-consent is obtained. A consensus should also be sought regarding testicular prostheses, and the importance of steroid hormone treatment compliance to final height should be emphasized to 46,XX CAH patients raised as males. Sexual identity assessments should be performed periodically, and clinics should be established to facilitate the transition to adulthood. Finally, given the importance of sharing experience, adult follow-ups visits should be scheduled.

Ethics

Ethics Committee Approval: This study was approved by Zekai Tahir Burak Women Health Training and Research Hospital Clinical Research Ethics Committee (approval number: 15/2018, date: 06.03.2018).

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

Peer-review: Externally peer-reviewed.

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Associations Between Antioxidant Vitamin Status, Dietary Intake, and Retinol-binding Protein 4 Levels in Prepubertal Obese Children After 3-month Weight Loss Therapy

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What is already known on this topic?

Oxidative stress conditions in obese subjects are associated with abnormal serum pro- and anti-inflammatory adipokines. Antioxidant vitamins may be important factors in the treatment and prevention of obesity and obesity-related disorders.

What this study adds?

An increased risk of vitamin E deficiency due to decreased vitamin E intake may occur in children losing weight during lifestyle intervention. Changes in body mass index value may influence changes in retinol-binding protein 4 concentrations and consequently the vitamin A status in prepubertal obese children after therapy.

Abstract

Objective: Adiposity is associated with increased oxidative stress, leading to changed fat-soluble vitamin concentrations. The aim of this study was to determine whether weight loss alters fat-soluble vitamin status and whether these alterations are associated with dietary intake, anthropometric parameters and adipokines in obese children.
Methods: Vitamin A and E concentrations were measured using high-pressure liquid chromatography in 60 obese children before and after weight loss therapy. Retinol-binding protein 4 (RBP4), leptin, soluble leptin receptor (sOB-R), and high molecular weight adiponectin concentrations were determined by immunoenzymatic assays.
Results: The intake of vitamin E was lower in obese children with weight loss after therapy ($p = 0.038$). In this group, an increase was found in the vitamin A/lipids ($p = 0.022$) and the vitamin E/lipids ($p = 0.008$) ratios but due to the reduction in triglyceride levels. In the obese group, changes in vitamin E level were positively correlated with changes in dietary vitamin E ($p = 0.017$) and the leptin/sOB-R ratio ($p = 0.046$). Changes in vitamin A level were positively correlated with changes in dietary vitamin A ($p = 0.001$) and RBP4 concentration ($p = 0.023$). Associations between changes in RBP4 level with the changes in body mass index (BMI) ($p = 0.011$) and total cholesterol concentration ($p = 0.023$) but not with changes in vitamin A concentration were found in the obese after therapy.
Conclusion: An increased risk of vitamin E deficiency may occur in children losing weight during lifestyle intervention. Changes in BMI value may influence changes in RBP4 concentrations and consequently the vitamin A status in obese children after therapy.
Keywords: Vitamin A, vitamin E, retinol-binding protein 4, prepubertal period, weight loss therapy

Introduction

Obesity is associated with a subclinical inflammatory condition characterized by an increase of proinflammatory adipokines, which may contribute to increase oxidative stress (1,2). Scientific evidence suggests that antioxidant vitamins may be important factors in the treatment and prevention of obesity and obesity-related disorders. Fat-

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soluble vitamins, which act as free radical scavengers, belong to the most important components of the antioxidant cell defence system. Vitamin A (retinol) is a micronutrient required for growth and development, conceivably affecting lipid metabolism, energy regulation and body composition (3). Vitamin E is most commonly found in the form of alpha-tocopherol and acts as a peroxyl scavenger, thus preventing membrane lipid oxidation (4). Some studies in young populations have shown positive associations between these vitamin levels and obesity (5) but other studies suggest that fat deposition, chronic inflammation and oxidative stress are associated with deficiencies in fat-soluble vitamin concentrations (6,7). Lower values of fat-soluble vitamins in American and European populations of obese children compared with normal-weight controls were observed (8,9). These alterations may also result from an unbalanced diet in overweight and obese individuals. It was observed that dietary antioxidant intakes were lower among some populations of obese children (10,11), however in others they did not differ between normal-weight, overweight, and obese children (12). Diet is an important factor in the modulation of oxidative stress and the inflammatory process, and an antioxidant-rich diet is associated with a reduced risk of diseases resulting from obesity (13,14).

High oxidative stress conditions in obese subjects are associated with raised levels of serum pro-inflammatory adipokines, such as leptin, and decreased anti-inflammatory adipokines, such as adiponectin especially its high molecular weight (HMW) form, which can influence insulin sensitivity (1). In obese patients chronic endoplasmic reticulum stress decreases the amount of soluble leptin receptor (sOB-R) due to diminished membrane leptin receptor (OB-R) expression. In consequence, endoplasmic reticulum stress can limit the inhibitory effect of the leptin receptor on leptin function, inducing a leptin resistant state in obesity. Oxidative stress is also observed in obese children with increased levels of retinol-binding protein 4 (RBP4), a hepatocyte and adipocyte derived adipokine, thus potentially providing the link between obesity and its consequences due to the induction of mitochondrial dysfunction and vascular oxidative damage (15). RBP4 is also a plasma retinol transporter that carries retinol from the liver to the periphery, while very little plasma RBP4 is derived from adipose tissue. In obese subjects, RBP4 levels decrease during weight loss intervention and this reduction is correlated with the magnitude of the decrease in inflammatory markers, triglycerides, insulin, and the Homeostasis Model Assessment index (16). However, studies concerning the relation between RBP4 concentrations and vitamin A intake and plasma concentration are inconclusive (17,18). Therefore, the aim of this study was to determine: (a) the status of vitamins A and E in prepubertal obese and non-obese children; (b) whether weight loss after a 3-month lifestyle intervention alters fat-soluble vitamin status; and (c) whether these alterations are associated with dietary intake, anthropometric parameters, and pro- and anti-inflammatory adipokines in obese children.

Methods

Patients

One hundred and five prepubertal children, aged 5-10 years, were recruited from a group of consecutive patients seeking dietary counseling in the Department of Nutrition at the Institute of Mother and Child in Warsaw. The obese group consisted of 60 children with a body mass index (BMI) Z-score of >2. The exclusion criteria were: pubertal and postpubertal period; the presence of endocrine disorders or genetic syndromes, including syndromic obesity; acute or chronic medical conditions; intake of medications that could affect growth, pubertal development, nutritional or dietary status; and refusing consent to participate. The control group consisted of 45 prepubertal non-obese children (BMI Z-score $<-1\ +1>$) within the same age range as the obese group with an adequate nutritional or dietary status according to the recommendations of Kułaga et al (19) and Jarosz (20). This control group consisted of children without either acute or chronic disorders, were not taking any medications that could affect their development or nutritional or dietary status. Pubertal stage was determined according to the Tanner scale. All obese and non-obese children were Tanner stage 1. The study was performed in accordance with the Helsinki Declaration for Human Research, and the study protocol was approved (No.9/18) by the Ethics Committee of the Institute of Mother and Child in Warsaw, Poland. All parents gave their informed consent for the study.

Dietary Intervention

The weight loss therapy used in this study was based on a calorie restricted diet of 1200-1400 kcal/day (10-30% caloric deficit) and was composed of 20% protein, 30% fat and 50% carbohydrates for approximately 10% weight loss in three months (21). Patients had 3-5 meals every day. For each obese child, parents received nutritional guidelines including: (a) low-energy diet plan, including examples of daily menu and portion sizes of some products; (b) daily recommended consumption of products from various groups in grams, paying special attention to the sources of vitamins, including A and E, and minerals in accordance with the daily requirement depending on age; and (c) a list of products with different energy values.

Two weeks before the child was due to visit the department of nutrition, a 14-day food diary was completed at home using a questionnaire and brought to the Institute. The parents had previously been trained by a nutritionist to provide reliable estimates of diary intake. In the nutrition department, nutritionists carried out an interview concerning the family and environmental conditions of the children, their nutritional behavior and food preferences, and checked the diary in the presence of the child and his/her parents. The nutritionist asked for detailed information about the foods and drinks recorded, such as portion sizes and preparation methods. When necessary the portion size records were corrected during the visit. This was done by the nutritionist on the basis of an interview with the parents using a photo album of products and dishes presenting meal portion sizes (22). The three-day methodology was used according to the guide on nutrition research to assess the intake in the children's dietary habits (23). Two weekdays and one weekend day before (visit T0) and after three months of therapy (visit T3), were entered into nutritional analysis software (Dieta 5®, National Food and Nutrition Institute, Warsaw, Poland) to evaluate the average daily energy intake and the percentage of energy intake from protein, fat and carbohydrates, as well as vitamin (A, E) intakes in the children's diets (24). The data for each child were compared to the recommendations for the appropriate age and gender. The age- and sex-specific percentages of Estimated Energy Requirement (EER) for total energy intake, Estimated Average Requirement (EAR) for vitamin A, and adequate intake (AI) for vitamin E were calculated for each obese child before and after the three-month therapy according to the recommendations of Jarosz (20). In addition, these values were calculated once for each non-obese child. The participants in the present study did not receive supplements, except for standard supplementation with vitamin D.

Anthropometric Measurements

Body height was measured using a standing stadiometer and recorded with a precision of 1 mm. Body weight was assessed, unclothed, to the nearest 0.1 kg with a calibrated balance scale. BMI was calculated as body weight divided by height squared (kg/m^2). The BMI of each individual was converted to the BMI Z-score for the child's age and sex using Polish reference tables (19). Body composition was measured by dual-energy X-ray absorptiometry using Lunar Prodigy (General Electric Healthcare, Madison, WI, USA) with pediatric software, version 9.30.044. All subjects were measured with the same equipment, using standard positioning techniques.

Biochemical Analyses

Venous blood samples were collected between 8:00 and 10:00 am after an overnight fast, and centrifuged at 1000 x g for 10 min at 4 °C. Serum specimens were stored at -70 °C prior to assay. Vitamin A and E in serum were determined using an Ultra High Performance Liquid Chromatography system (Nexera LC30A, Shimadzu, Kyoto, Japan) integrated with a 190-800 nm UV-VIS detector (SPD20AV, Shimadzu, Kyoto, Japan) (25). The detection of alpha-tocopherol and retinol was carried out at 290 nm and 320 nm, respectively. The retinyl acetate, alpha-tocopherol and all trans retinol (Sigma Aldrich, St Louis, MO, USA) were used as a standard. The concentrations of vitamins were expressed in µmol/L.

A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to determine the concentration of RBP4 (Immundiagnostic AG, Bensheim, Germany), which had an inter-assay variation (CV%) of less than 10%. The concentrations of leptin and sOB-R were also measured by commercial ELISA kits (DRG Diagnostics, Marburg, Germany). Inter-assay CVs were 5.3% and 3.3% for leptin and sOB-R, respectively. Serum levels of HMW adiponectin were determined using an ELISA kit (ALPCO Diagnostics, Salem, NH, USA). Inter-assay variation was 5.7% for HMW adiponectin. Total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol, and triglyceride levels were measured using standard methods (Roche Diagnostics, Switzerland, Basel). To reduce inter-assay variance, the samples obtained before and after therapy were analyzed in one assay.

Statistical Analyses

Statistical analysis was performed using IBM SPSS v.25.0 software (SPSS Inc., Chicago, IL, USA). The results are presented as mean ± standard deviation for normally distributed data or medians and interquartile range (25th-75th percentiles) for non-normally distributed variables. The Kolmogorov-Smirnov test was used for evaluating distribution for normality. Differences in anthropometric characteristics and biochemical parameters of obese and non-obese children were assessed using Student's t-test for normally distributed data and the non-parametric Mann-Whitney test for non-normally distributed variables.

Changes in BMI Z-score, body composition and biochemical parameters were expressed as delta variables, calculated by subtracting the values at baseline (T0) from the values measured after three months of therapy (T3). Obese children were categorized into two subgroups according to the magnitude of BMI Z-score change during the intervention: children with weight loss (ΔBMI Z-score ≤-0.5, n = 37); and children without weight loss (ΔBMI Z-score > -0.5, n = 23).

The correlation coefficients between changes in the serum concentrations of vitamins A, E and RBP4 and changes in the anthropometric, dietary and biochemical parameters after therapy were calculated using Spearman's correlations.

The univariate and stepwise forward linear regression with the changes of serum concentration of RBP4 as dependent variables were used to examine the potential impact of the anthropometric and biochemical variables. A $p < 0.05$ was considered to be statistically significant.

Results

The median value of BMI was over 50% higher in obese patients than in controls (Table 1). The obese children had a four-fold greater fat mass, a two-fold higher percentage of fat mass, approximately 30% greater lean mass, and a 50% greater total bone mineral content compared with non-obese subjects. The values of vitamin A, RBP4, and the RBP4/vitamin A ratio were significantly higher in obese than non-obese children by 20%, 35%, and 25%, respectively. The levels of vitamin E and the vitamin E/lipids (the sum of total cholesterol and triglycerides) ratio were similar in both study groups. In obese children the leptin/sOB-R ratio was more than 10-fold higher but the value of HMW adiponectin was significantly lower by 30% compared to controls.

The daily energy intake was significantly higher in obese children compared to controls before the intervention (Table 1). The obese children had higher intake of carbohydrate and fat than normal-weight subjects, but the proportions of protein, fat, and carbohydrates in the daily energy intake were similar in both groups. The diet of obese children contained a higher intake of vitamin E than that of normal-weight children ($p < 0.02$). However, the median value of AI percentage for vitamin E in obese and non-obese children were lower by about 10% and 20%, respectively, than the recommended value for this vitamin by Jarosz (20). A similar intake of vitamin A and percentage of EAR for this vitamin were observed in both studied groups and these values were appropriate for the age group (20).

In the whole obese group before therapy, positive relations between vitamin A levels and dietary vitamin A ($r = 0.411$; $p < 0.01$), total cholesterol ($r = 0.318$; $p < 0.02$) and LDL-cholesterol levels ($r = 0.269$; $p < 0.05$) were found. The value of vitamin E correlated negatively with BMI value ($r = -0.347$; $p < 0.01$), leptin level ($r = -308$; $p < 0.02$) and positively with total cholesterol ($r = 0.420$; $p < 0.001$) and LDL-cholesterol ($r = 0.330$; $p < 0.02$).

After the three-month intervention, a significant increase was found in the vitamin A/lipids and the vitamin E/lipids ratios in patients with weight loss (ΔBMI Z-score ≤-0.5) (Table 2). These parameters did not change in the group without weight loss after therapy. In obese children with weight loss the leptin/sOB-R ratio decreased and the HMW adiponectin level increased but leptin/sOB-R ratio was still higher ($p < 0.001$) and the HMW adiponectin level was lower ($p < 0.01$) compared with the control group.

The group of children who lost weight reduced their energy intake and percentage of EER during the therapy ($p < 0.001$), as opposed to the group without weight loss (Table 2). In addition, the percentage of energy intake from proteins increased significantly in obese children with weight loss, whereas the percentage of energy intake from fat increased significantly in obese children without weight loss after intervention. In contrast to children without weight loss after therapy, in children with weight loss the intakes of carbohydrate and fat were significantly lower during this intervention.

The values of vitamin A intake and the EAR percentage for this vitamin after the three-month therapy were lower but statistically insignificant in both groups of obese children (Table 2). The intake of vitamin E and the AI percentage for this vitamin were significantly lower ($p = 0.038$ and $p = 0.02$, respectively) in children with weight loss in contrast to those without weight loss. In addition, median values of the AI percentage for vitamin E in the group with and without weight loss were lower than the recommendation by about 25% and 10%, respectively (20).

Changes in vitamin A level were positively correlated with changes in dietary vitamin A and vitamin E, concentrations of vitamin E, RBP4, leptin, and lipids (Table 3). Changes in vitamin E level were positively correlated with changes in dietary vitamin E, the leptin/sOB-R ratio, and lipid levels. Changes in RBP4 level after the three-month therapy were positively correlated with changes in BMI value, concentrations of vitamin A, leptin and lipids.

In the stepwise forward linear regression with changes in RBP4 level after three months of therapy as a dependent variable and changes in BMI values, vitamin A, leptin and total cholesterol concentrations as independent variables, only changes of BMI values ($p < 0.02$) as well as changes in total cholesterol concentrations ($p < 0.05$) in the obese group after therapy were found to be statistically significant (Table 4).

Discussion

Fat-soluble vitamin deficiency is more common in children than adults, probably because they have limited stores

Table 1. Demographic and biochemical characteristics and dietary intake of prepubertal obese and non-obese children

Parameter*	Obese children N = 60	Non-obese children N = 45	p value
Age (years)	7.9 ± 1.4	7.3 ± 1.8	0.066
Boys (%)	50.0	50.0	0.820
Height (cm)	134 ± 10	123 ± 11	**< 0.001**
Height Z-score	1.00 ± 1.10	-0.27 ± 0.98	**< 0.001**
Weight (kg)	41.8 (36.6-55.0)	23.0 (18.8-28.4)	**< 0.001**
BMI (kg/m^2)	24.1 (21.8-26.5)	15.4 (14.5-16.3)	**< 0.001**
BMI Z-score	3.19 (2.41-4.51)	-0.36 (-0.85-0.03)	**< 0.001**
Body composition			
Fat mass (%)	41.7 ± 5.1	20.9 ± 8.2	**< 0.001**
Fat mass (kg)	16.1 (13.6-23.0)	4.1 (2.8-6.6)	**< 0.001**
Lean mass (kg)	24.3 ± 4.4	18.2 ± 3.5	**< 0.001**
Total BMC (kg)	1.2 (1.0-1.5)	0.8 (0.7-1.0)	**< 0.001**
Biochemical parameters			
Vitamin A (µmol/L)	1.47 (1.20-1.87)	1.21 (1.07-1.34)	**0.041**
RBP4 (mg/L)	22.3 ± 6.2	16.4 ± 4.3	**< 0.001**
RBP4/Vitamin A	0.745 ± 0.295	0.588 ± 0.192	**0.012**
Vitamin A/lipids (µmol/mmol)	0.29 (0.21-0.35)	0.24 (0.22-0.27)	**0.199**
Vitamin E (µmol/L)	17.7 (13.4-22.8)	18.7 (16.3-20.4)	**0.521**
Vitamin E/lipids (µmol/mmol)	3.53 (2.57-4.18)	3.60 (3.29-4.16)	0.199
Leptin/sOB-R	0.80 (0.35-1.44)	0.06 (0.03-0.11)	**< 0.001**
HMW adiponectin (µg/mL)	3.70 ± 1.60	5.20 ± 2.70	**0.002**
Total cholesterol (mg/dL)	171.0 ± 28.3	172.7 ± 24.7	0.755
HDL-cholesterol (mg/dL)	51.1 ± 11.3	64.4 ± 12.6	**< 0.001**
LDL-cholesterol (mg/dL)	101.0 (93.0-121.0)	99.5 (82.0-117.0)	0.216
Triglycerides (mg/dL)	81.5 (57.5-122.8)	54.5 (43.0-76.3)	**< 0.001**
Dietary intake**			
Energy (kcal/day)	1634.5 (1381.6-2004.2)	1488.7 (1244.6-1722.2)	**0.020**
Energy (of EER %)	93.5 (76.1-110.9)	86.9 (68.4-111.3)	0.329
Protein (of energy intake %)	13.8 ± 2.7	14.7 ± 2.9	0.104
Carbohydrate (of energy intake %)	52.4 ± 7.7	51.8 ± 6.3	0.662
Fat (of energy intake %)	33.8 ± 6.8	32.5 ± 5.6	0.833
Protein (g/day)	60.5 ± 21.6	53.9 ± 15.1	0.105
Carbohydrate (g/day)	246.2 ± 89.0	203.1 ± 60.2	**0.010**
Fat (g/day)	68.4 ± 25.9	56.7 ± 18.1	**0.014**
Vitamin A (µg/day)	723.0 (515.6-1192.1)	684.5 (441.6-882.0)	0.108
Vitamin A (EAR%)	193.8 (145.5-340.6)	193.9 (137.5-260.0)	0.226
Vitamin E (mg/day)	6.4 (4.9-8.9)	5.6 (3.8-6.7)	**0.016**
Vitamin E (AI%)	88.6 (64.2-119.5)	80.2 (55.7-102.5)	0.053

*Results are presented as means ± standard deviations for normally distributed data, or medians and interquartile ranges (25th-75th percentiles) for non-normally distributed variables.
**Recommended daily energy and nutrients intakes (4-6/7-9 years) according to Jarosz (20): energy (1400/1800 kcal/day), protein (10-20%), carbohydrate (45-65%), fat (20-35%), protein (16/23 g/day), carbohydrate (130/130 g/day), fat (31-54/40-70 g/day), vitamin A (300/350 g/day), vitamin E (6/7 g/day).
BMC: bone mineral content, BMI: body mass index, HMW: adiponectin, high molecular weight adiponectin, sOB-R: soluble leptin receptor, RBP4: retinol-binding protein 4, HDL: high-density lipoprotein, LDL: low-density lipoprotein; lipids, the sum of total cholesterol and triglycerides, EER: Estimated Energy Requirement, EAR: Estimated Average Requirement, AI: adequate intake

and grow rapidly, which promotes the appearance of the symptoms of this vitamin deficiency (8). Some authors observed vitamin A insufficiency in obese children and adolescents due to an unbalanced diet (6), but others found

Table 2. Demographic and biochemical characteristics and dietary intake of prepubertal obese children with and without weight loss after therapy

	Obese children with weight loss (ΔBMI Z-score ≤-0.5), n = 37				Obese children without weight loss (ΔBMI Z-score > -0.5), n = 23			
Parameter*	T_0	T_3	Δ (IQR)	$p_{\Delta=0}$	T_0	T_3	Δ (IQR)	$p_{\Delta=0}$
Age (years)	8.3	8.8	0.3 (0.2-0.3)	**< 0.001**	7.5	7.8	0.3 (0.2-0.3)	**< 0.001**
Boys (%)	48.6				52.2			
Height (cm)	137	138	2 (1-2)	**< 0.001**	130	131	2 (1-2)	**< 0.001**
Height Z-score	1.04	0.97	0.24 (-0.38-0.32)	0.411	0.75	0.76	0.29 (0.02-0.34)	**0.046**
Weight (kg)	45.3	40.6	-3.0 [(-4.9)-(-1.8)]	**< 0.001**	37.8	39.3	0.7 (-0.2-1.9)	**0.016**
BMI (kg/m^2)	24.1	22.5	-2.1 [(-3.2)-(-1.5)]	**< 0.001**	24.1	24.2	0.0 (-0.5-0.2)	0.529
BMI Z-score	3.17	2.11	-1.03 [(-1.45)-(-0.63)]	**< 0.001**	3.21	3.09	-0.01 (-0.20-0.07)	0.604
Biochemical parameters								
Vitamin A (μmol/L)	1.48	1.44	0.07 (-0.17-0.31)	0.458	1.49	1.73	0.08 (-0.25-0.47)	0.397
RBP4 (mg/L)	23.2	19.9	-1.3 (-7.5-3.0)	0.065	19.3	19.2	-1.3 (-3.2-2.6)	0.432
RBP4/Vitamin A	0.72	0.65	-0.07 (-0.22-0.11)	0.062	0.56	0.54	-0.07 (-0.17-0.08)	0.134
Vitamin A/lipids (μmol/mmol)	0.29	0.30	0.03 (-0.02-0.09)	**0.022**	0.29	0.34	0.05 (-0.05-0.12)	0.082
Vitamin E (μmol/L)	16.7	16.5	0.4 (-1.7-4.2)	0.192	18.4	18.7	0.9 (-6.4-4.5)	1.000
Vitamin E/lipids (μmol/mmol)	3.28	3.35	0.42 (-0.14-0.99)	**0.008**	3.74	3.80	0.43 (-0.27-0.81)	0.157
Leptin/sOB-R	0.84	0.29	-0.46 [(-0.92)-(-0.11)]	**< 0.001**	0.69	0.57	-0.11 (-0.27-0.30)	0.687
HMW adiponectin (μg/mL)	3.50	4.00	0.30 (0.00-1.2)	**0.002**	3.60	3.80	0.1 (-1.2-0.8)	0.876
Total cholesterol (mg/dL)	161.5	159.0	-4.5 (-23.8-10.8)	0.328	171.0	165.0	-13.0 [(-26.0)-(-1.0)]	**0.016**
HDL-cholesterol (mg/dL)	50.5	51.5	1.0 (-6.0-6.8)	0.765	51.0	46.0	-1.0 (-7.0-3.0)	0.544
LDL-cholesterol (mg/dL)	98.5	98.5	-5.5 (-20.8-6.8)	0.124	113.0	105.0	-13.0 (-21.0-9.0)	0.168
Triglycerides (mg/dL)	91.0	71.5	-8.0 (-58.5-6.8)	**0.020**	72.0	78.0	2.0 (-27.0-29.0)	0.946
Dietary intake								
Energy (kcal/day)	1653.8	1199.2	-438.5 (-831.7-0.0)	**0.001**	1925.6	1760.7	-58.6 (-449.7-0.0)	0.438
Energy (of EER%)	91.9	66.6	-26.2 [(-46.2)-(-3.7)]	**< 0.001**	107.0	97.8	-3.3 (-28.5-0.0)	0.438
Proteins (of energy intake %)	13.9	17.2	2.0 (-0.2-5.9)	**0.014**	13.4	14.3	0.5 (0.0-2.3)	0.375
Carbohydrates (of energy intake %)	52.8	51.2	-0.1 (-5.4-4.8)	0.849	56.0	50.0	-5.1 (-10.0-0.0)	0.078
Fat (of energy intake %)	32.6	31.4	-2.8 (-8.7-5.0)	0.166	31.7	36.1	4.7 (0.0-7.7)	**0.047**
Protein (g/day)	61.7	48.9	-5.13 (-27.1-9.7)	0.092	61.7	58.2	0.0 (-6.3-0.8)	0.906
Carbohydrate (g/day)	252.0	154.1	-39.9 [(-96.7)-(-8.6)]	**0.002**	266.7	250.1	-19.0 (-98.9-0.0)	0.203
Fat (g/day)	61.9	43.4	-21.3 (-49.9-0.0)	**0.002**	69.1	69.1	0.0 (-3.9-3.9)	0.938
Vitamin A (mg)	616.4	538.2	-75.1 (-373.6-175.4)	0.427	732.2	644.2	0.0 (-447.2-167.7)	0.906
Vitamin A (EAR%)	172.3	153.8	-35.9 (-120.5-50.1)	0.329	244.1	186.8	0.0 (-127.8-55.9)	0.938
Vitamin E (mg)	6.9	5.2	-1.5 (-4.5-0.3)	**0.038**	5.6	6.0	0.1 (0.0-1.5)	0.219
Vitamin E (AI%)	88.6	74.3	-22.1 (-63.8-4.4)	**0.020**	86.4	90.1	1.22 (0.0-25.6)	0.219

*Results are presented as means ± standard deviations for normally distributed data, or medians and interquartile ranges (25th-75th percentiles) for non-normally distributed variables.
BMI: body mass index, HMW: adiponectin, high molecular weight adiponectin, sOB-R: soluble leptin receptor, RBP4: retinol-binding protein 4, HDL: high-density lipoprotein, LDL: low-density lipoprotein; lipids, the sum of total cholesterol and triglycerides, EER: Estimated Energy Requirement, EAR: Estimated Average Requirement, AI: adequate intake, IQR: interquartile range

elevated serum retinol associated with dyslipidemia, BMI, adiposity and abdominal fat mass (7,26,27). Similar to Albuquerque et al (26), we found higher concentrations of vitamin A and well as vitamin A/lipids and RBP4/lipids in

Table 3. Associations between changes in fat-soluble vitamin and retinol-binding protein 4 concentrations and changes in anthropometric and biochemical parameters in obese children after three-month therapy (Δ)

Parameter*	Δ Vitamin A (μmol/L)	Δ Vitamin E (μmol/L)	Δ RBP4 (mg/L)
Δ BMI	0.242 (0.070)	0.155 (0.250)	0.314 **(0.018)**
Δ Vitamin A (mg)	0.589 **(0.001)**	0.169 (0.389)	0.154 (0.444)
Δ Vitamin A (EAR%)	0.609 **(0.001)**	0.172 (0.380)	0.147 (0.466)
Δ Vitamin A (μmol/L)	-	0.556 **(0.000)**	0.312 **(0.023)**
Δ Vitamin E (mg)	0.545 **(0.003)**	0.447 **(0.017)**	0.151 (0.453)
Δ Vitamin E (AI%)	0.603 **(0.001)**	0.394 **(0.038)**	0.172 (0.392)
Δ Vitamin E (μmol/L)	0.556 **(0.000)**	-	0.189 (0.176)
Δ RBP4 (mg/L)	0.312 **(0.023)**	0.186 (0.176)	-
Δ Leptin	0.278 (**0.036**)	0.274 **(0.039)**	0.306 **(0.022)**
Δ sOB-R	-0.136 (0.312)	-0.047 (0.728)	-0.172 (0.206)
Δ Leptin/sOB-R	0.222 (0.097)	0.265 **(0.046)**	0.219 (0.106)
Δ HMW adiponectin (μg/mL)	0.019 (0.890)	0.044 (0.750)	0.173 (0.208)
Δ Total cholesterol (mg/dL)	0.466 **(< 0.001)**	0.529 **(< 0.001)**	0.339 **(0.011)**
Δ HDL-cholesterol (mg/dL)	0.417 **(0.001)**	0.218 (0.107)	0.400 **(0.002)**
Δ LDL-cholesterol (mg/dL)	0.277 **(0.039)**	0.423 **(0.001)**	0.285 **(0.035)**
Δ Triglycerides (mg/dL)	-0.034 (0.802)	0.128 (0.346)	0.124 (0.366)

*Results are presented as Spearman's rho (p value).
BMI: body mass index, HMW: adiponectin, high molecular weight adiponectin, sOB-R: soluble leptin receptor, RBP4: retinol-binding protein 4, HDL: high-density lipoprotein, LDL: low-density lipoprotein

Table 4. Stepwise forward linear regression for changes in retinol-binding protein 4 and changes in anthropometric and biochemical parameters after three-month therapy (Δ)

Variable	Univariate analysis			Multivariate analysis		
	β	95% CI for β	p value	β	95% CI for β	p value
Δ BMI	1.610	(0.464; 2.755)	0.007	1.494	(0.356; 2.631)	0.011
Δ Vitamin A (μmol/L)	3.582	(0.637; 6.527)	0.018	-	-	-
Δ Leptin	0.140	(0.008; 0.271)	0.037	-	-	-
Δ Total cholesterol (mg/dL)	0.104	(0.042; 0.165)	0.001	0.072	(0.010; 0.133)	0.023

BMI: body mass index, CI: confidence interval

obese children than in normal-weight children, but these values for vitamin A were within the reference range (0.8-2.8 μmol/L) (28). In addition, both study groups differed significantly in terms of body composition (mainly fat mass), diet, and biochemical parameters, including the lipid profile. Although we found that the median of total energy intake was below the percentage of EER for obese and non-obese children, there were nevertheless differences in the diet composition of both groups. Obese children consumed significantly more fat and carbohydrates than children with normal body weight. In addition, we found differences in lipid profile between both groups. Obese children had higher values of triglycerides and lower values of HDL-cholesterol than the control group. The changes in the lipid profile, associated with abdominal obesity, often accompany childhood obesity (26,27).

Data on vitamin E concentration in obese children are also contradictory, as both lower and normal levels have been observed (7,26,29). Gunanti et al (7) found an association between lower serum concentrations of vitamin E and high adiposity in Mexican-American children (8-15 years of age). However, we observed similar values of vitamin E as well as vitamin E/lipids ratio in obese and non-obese children, despite the differences in body composition and lipid profile of these two groups and these vitamin E concentrations were above the deficiency cut-off value of 12 μmol/L in both groups. Overweight, abdominal obesity and lipid profile markers have been described in Brazilian adolescents (26). According to Albuquerque et al (26), vitamin E status was not associated with dyslipidemia in these patients.

In children who lost about 10% of their body weight after three months of lifestyle intervention, we observed

a significant improvement in their dietary composition, i.e. a reduction in total energy intake and a reduction in carbohydrate and fat intakes. In patients who complied with the recommendations, we observed favorable changes, not only in the values of anthropometric parameters, but also in biochemical parameters, such as the levels of triglycerides or the adipokines which were investigated. Similar to Guerendiain et al (30), we observed higher vitamin E/lipids and vitamin A/lipids ratios in patients with weight loss due to the significant reduction in triglyceride concentration. The increases in lipid-corrected alpha-tocopherol and retinol plasma levels in Spanish adolescents were associated with a reduction in adiposity and a clinically significant improvement in cardio-metabolic profiles (30).

Some authors suggest that obese children consume too little vitamin E despite their apparently elevated circulating a-tocopherol concentrations (8). According to Murer et al (31), vitamin E supplements in adequately nourished obese children decrease oxidative stress markers, suggesting that obese children routinely consume inadequate amounts of antioxidants to prevent oxidative stress. In fact, an increase in LDL oxidation as a consequence of increased oxidative stress and reduced antioxidant defences was observed in obese children (32,33). In our study, higher intake of vitamin E was found in obese compared with non-obese children, but no relationship between intake and serum concentration of vitamin E was identified in obese patients. According to Traber (8), circulating alpha-tocopherol concentrations do not correlate strongly with dietary alpha-tocopherol intakes in children. Although the median value for serum vitamin E did not change for both studied subgroups (weight loss, without weight loss) after therapy, there was a positive association between changes in vitamin E consumption and serum concentration in the whole obese group after lifestyle intervention. In addition, children who lost weight consumed significantly less vitamin E after therapy than obese children who did not lose weight. This may suggest that during a lifestyle intervention a vitamin E deficit may appear in patients losing weight. Geiker et al (34) and Hamułka et al (35) observed a lowered alpha-tocopherol status in obese adults after a 6-8 week weight loss intervention. These results, coupled with overweight and low alpha-tocopherol intake, suggest that there is an increased risk of oxidative stress diseases in individuals on a reduced diet.

López-Domènech et al (36) showed that weight loss attenuates inflammation and insulin resistance and promotes the amelioration of chronic endoplasmic reticulum stress and mitochondrial dysfunction in human obesity. Our obese children, even those who significantly lost weight after three months of therapy, still had excess weight and an unbalanced pro- and anti-inflammatory adipokine profile compared to normal-weight children. Children with weight loss after therapy had a significantly lower value of the leptin/sOB-R ratio but this ratio was still higher than the controls. Leptin has been reported to promote the accumulation of reactive oxygen species (ROS) in endothelial cells and vascular smooth muscle cells as well as to have a pro-atherogenic effect on macrophages via an oxidative stress-dependent pathway in diabetes (37). In addition, the HMW form of adiponectin, which exhibits anti-inflammatory, anti-atherogenic, and insulin-sensitizing properties, had higher values in obese children with weight loss after therapy, but these were still lower than found in normal-weight children. It is known that the exposure of adipocytes to high ROS levels suppresses the expression and secretion of adiponectin, which probably explains the findings seen in our children after the intervention (38). We also found that a greater decrease in vitamin E concentration was associated with a greater decrease in the value of the leptin/sOB-R ratio after therapy in obese children, which with longer weight loss intervention, may disrupt vitamin E status. Moreover, vitamin E concentrations may be inadequate for normal liver function in obese individuals due to the occurrence of oxidative stress and the dysregulation of liver lipoprotein secretion (39). Therefore, adequate vitamin E status is important in obesity to maintain healthy liver function and prevent the appearance and/or progression of fatty liver and serious forms of this disease.

Hepatic metabolism is the main regulator of both vitamin E as well as vitamin A in the body. RBP4, including RBP4, which distributes vitamin A from the liver to the cells, play an important role in vitamin A metabolism (40). Previous studies have shown that a low-caloric diet considerably decreased RBP4 concentrations in children and adults (41,42). However, the effect was dependent on the amount of weight lost, as well as the quality and diversity of the diets (43). In our study, only a slight decrease in RBP4 levels and the RBP4/vitamin A ratio was observed and an unchanged vitamin A intake was present in obese children with weight loss after three months of therapy. In addition, no correlations were found between changes in RBP4 levels and changes in vitamin A intake as well as vitamin A concentrations in the obese group after therapy. Some authors found a positive association between RBP4 concentrations and vitamin A intake in non-obese Spanish and obese Iranian women (18,44). However, a RBP4 promoter polymorphism studied in a murine model of type 2 diabetes was not associated with retinol intake (17). According to Canas et al (45), RBP4

concentrations were not influenced by the consumption of fruit and vegetable juice concentrates in prepubertal obese boys.

In our analysis, the significant impact of the changes in BMI value and total cholesterol concentration were associated with changes in RBP4 levels in obese children after the three-month therapy. It seems that long-term weight loss therapy in obese children may affect RBP4 concentration, which may result in lower vitamin A levels in tissues. Trasino et al (46) demonstrated that even with adequate dietary vitamin A, obesity dramatically reduces vitamin A levels and signaling in multiple organs of mice and humans. So, one cannot exclude that lifestyle intervention may modulate the vitamin A status in the organs of obese subjects, due to a decrease in RBP4 concentrations associated with a decrease in BMI value during therapy.

Study Limitations

This study has several potential limitations. First, the number of participants was a limitation of this study. However, the study groups were homogenous in terms of developmental period. All participants were in the prepubertal period, and obese as well as non-obese children were characterized anthropometrically and metabolically. Second, the ELISA kit used in the study does not differentiate between holo- and apo-RBP4 but evaluates the total RBP4 concentration. Apo-RBP4 is defined as RBP4 that is not bound to retinol, whereas retinol-bound RBP4 (holo-RBP4) is associated with transthyretin in plasma to prevent the loss of RBP through kidney filtration (47). For a more accurate determination of the associations, apo- and holo-RBP4 should be analyzed separately in relation to the anthropometric and biochemical parameters. In further research, the analysis of these relationships should be undertaken. Finally, the lifestyle intervention period in this study was only three months and a study of a long-term intervention is needed to verify the relationship between fat-soluble vitamin status, dietary intake and biochemical parameter levels in relation to clinical outcomes.

Conclusion

A significant effect of the changes in BMI value and total cholesterol concentration on changes in RBP4 levels in prepubertal obese children after a three-month life-style intervention was demonstrated. It seems that weight loss during long-term lifestyle intervention may affect vitamin A status, due to a decrease in RBP4 concentrations associated with a decrease in BMI value in treated obese children. Moreover, children who lost weight consumed significantly less vitamin E during therapy than obese children who did not lose weight. Therefore, the increased risk of oxidative stress due to the vitamin E deficiency may occur in patients losing weight during long-term therapy. We suggest the necessity of further monitoring of antioxidant vitamin levels in obese children during weight loss therapy and possible supplementation, especially of vitamin E, due to the risk of vitamin deficiency resulting from decreasing vitamin intake in these patients.

Ethics

Ethics Committee Approval: The study was performed in accordance with the Helsinki Declaration for Human Research, and the study protocol was approved (No.9/18) by the Ethics Committee of the Institute of Mother and Child in Warsaw, Poland.

Informed Consent: All parents gave their written informed consent for the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Joanna Gajewska, Design: Magdalena Chełchowska, Data Collection or Processing: Jadwiga Ambroszkiewicz, Witold Klemarczyk, Analysis or Interpretation: Joanna Gajewska, Katarzyna Szamotulska, Halina Weker, Literature Search: Jadwiga Ambroszkiwicz, Halina Weker, Writing: Joanna Gajewska, Magdalena Chełchowska.

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Initial Basal and Bolus Rates and Basal Rate Variability During Pump Treatment in Children and Adolescents

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What is already known on this topic?

While recommendations for basal rate profiles in adolescents and adults have been published before, at present there is no general consensus on how to start basal rate profiles in different age groups, and which additional factors should be considered. In general there are two different methods used. Total basal dose is divided by 24 to give the average basal rate per hour. Basal rates are increased or decreased according to fasting blood sugars. In the second method, basal insulin requirement is adjusted according to the requirement during the day.

What this study adds?

At the initiation of insulin pump therapy, the basal rates should not be set equally during the day. The basal rates should be initiated at a specific day rhythm for the age group.

Abstract

Objective: Pump-treated children with type 1 diabetes (T1DM) have widely differing basal insulin (BI) infusion profiles for specific periods of the day. The pattern of BI requirements depends on the timing and magnitude of cortisol and growth hormone secretion within each age group. In adolescents and young adults, a decreased insulin sensitivity is seen, particularly in the early morning (dawn phenomenon) and to a lesser extent, in the late afternoon (dusk phenomenon). Different approaches exist for the inititation of basal rates. However, there is a lack of evidence-based recommendation, especially in young children. Usually the basal rates are set equally throughout day and night or the day is divided into tertiles. The aim of this study was to analyze the change of the initial, equally distributed, BI rates over the first year of standard insulin pump therapy.

Methods: A total of 154 patients with T1DM, aged between 0 and <21 years at diagnosis, from a single center were documented. Patients were divided into five age groups according to age at pump initiation: group 1, <5 years (n=36); group 2, 5-8 years (n=20); group 3, 8-15 years (n=74); group 4, 15-18 years, (n=19); and group 5, >18 years, (n=5). Distribution of hourly basal rates at the initiation of the pump and at the end of first year were evaluated.

Results: Median (range) age and diabetes duration was 14.46 (1.91-26.15) and 7.89 (1.16-17.15) years, respectively. Forty-four percent were male, 56% were female. Mean total insulin dose/kg in the whole cohort at the initiation and after one year of pump therapy was 0.86±0.23 U/kg and 0.78±0.19 U/kg, respectively and differed significantly between each age group ($p<0.001$; $p<0.001$). Mean daily basal rate/kg showed significant differences between the five groups ($p<0.001$). Circadian distribution of BI differed markedly among the five age groups.

Conclusion: At the initiation of insulin pump therapy, circadian profiles by age group should be taken into account in pediatric patients to optimize basal rate faster and more easily.

Keywords: Type 1 diabetes, insulin infusion pump therapy, basal insulin, basal rates

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Introduction

Continuous subcutaneous insulin infusion (CSII) therapy is an effective and flexible method of insulin delivery, associated with improved glycemic control in children with type 1 diabetes mellitus (T1DM) (1). CSII improves metabolic control and in addition offers more flexible and more precise insulin delivery than multiple daily insulin (MDI) therapy, while increasing the quality of life of children and adolescents (2,3,4,5,6,7,8). While recommendations for basal rate profiles in adolescents and adults have been published before, at present there is no general consensus on how to start basal rate profiles in different age groups, and which additional factors should be considered. In general, there are two different methods used. The first is total basal dose (TBD) when the dose is divided by 24 to give the average basal rate per hour. Basal rates are increased or decreased according to fasting blood glucose (9). In the second method, basal insulin (BI) requirement is adjusted according to the requirement during the day as defined by Bachran et al (8,10).

The aim of this study was to analyze the change of the initial, equally distributed, BI rates over the first year of standard insulin pump therapy.

Methods

Patients with T1DM who were switched to CSII from a single center were documented. Participants were all on MDI therapy before CSII therapy and were counting carbohydrates. Rapid acting aspart insulin was used for insulin pumps. Total dose of insulin at the initiation of pump therapy was calculated according the mean hemoglobin A1c (HbA1c) over the last year and was reduced by 10% if >64 mmol/mol (>8%), by 20% if between 53-64 mmol/mol (7-8%) and 30% if <53 mmol/mol (<7%). Forty percent of the total insulin dose was calculated as the BI dose. According to departmental recommendations, basal rates were equally distributed hourly at the initiation of therapy, and rates are changed based on pre-meal capillary blood glucose levels and, when needed, with a fasting test over a period of six to eight hours. After initiation of CSII therapy, basal dose changes were made according to the needs of the children and adolescents. Patients were evaluated every three months, and data on HbA1c, weight, TBD and bolus insulin (IU/kg) doses were recorded. Participants were divided into five age groups according to insulin requirements and chronological age at pump initiation as follows: group 1, <5 years; group 2, 5 to 8 years; group 3, 8 to 15 years; group 4, 15 to 18 years; and group 5, >18 years (n=5).

Data collection was approved by the institutional review board of Ege University and is in accordance with the Declaration of Helsinki (approval number: 20-5.1T/29, date: 08.07.2020). The children with T1DM and their parents signed a written informed agreement and consent form, respectively, when they were enrolled in the study. Young adults >18 years of age signed informed agreement and consent form.

Statistical Analysis

Analysis were carried out using Statistical Package for the Social Sciences for Windows, version 25.0 (IBM Inc., Armonk, NY, USA). Descriptive statistics are reported using mean ± standard deviation for normally distributed variables, and median (range) for skewed data. Groups are compared by independent samples t-test for normally distributed variables and the Mann-Whitney U test for skewed data. Trends across more than two groups were analyzed using linear polynomial contrasts (ANOVA). A $p < 0.05$ was considered statistically significant.

Results

Patient records of 154 T1DM children and adolescents, aged <21 years at diagnosis, between 2004 and March 2020 with a follow-up of >1 year on insulin pump therapy were evaluated. The sex ratio was 87 (56.5%) girls and 67 (43.5%) boys. Patient numbers in the five groups were: group 1 (n=36); group 2 (n=20); group 3 (n=74); group 4 (n=19); and group 5 (n=5). Median age and diabetes duration of the study group was 14.46 (1.91-26.15) years and 7.89 (1.16-17.15) years, respectively. Basic characteristics of the five different age groups are presented in Table 1. The mean total daily insulin dose (TDD) increased from the youngest to the oldest age group until the end of puberty. At initiation and at the end of the first year of pump therapy, insulin dose/kg was different in each age group ($p < 0.001$; $p < 0.001$) (Table 2). Also, the mean daily basal rate/kg showed substantial differences between the five groups with the highest basal requirement in group 4 ($p < 0.001$) (Table 2).

Median total insulin dose/kg at initiation and after one year of pump therapy was 0.86 ± 0.23 IU/kg and 0.78 ± 0.19 IU/kg in all children with T1DM respectively. The mean BI requirement/kg at pump initiation and after the first year of therapy according to age groups are given in Table 2.

The circadian distribution of BI differed markedly among the five age groups (Figure 1). In groups 3 and 5, BI requirement was highest between 06:00 and 09:00 but in group 4 the highest requirement was seen between 18:00 and 21:00. The lowest requirement was between 10:00

Table 1. Patient characteristics and daily insulin requirement by age group at the initiation of pump therapy (mean ± standard deviation, percentage)

	0-5 age (n = 36)	5-8 age (n = 20)	8-15 age (n = 74)	15-18 age (n = 19)	> 18 age (n = 5)
Age (years)	2.85 ± 1.47	6.83 ± 0.94	11.62 ± 2.12	16.27 ± 0.85	19.83 ± 1.43
Boys (%)	42	50	43	37	60
T1DM duration (years)	0.76 ± 0.92	2.38 ± 1.37	4.10 ± 3.08	4.60 ± 3.20	5.70 ± 3.23
Basal insulin (%)	36.47 ± 9.08	38.75 ± 7.67	38.45 ± 8.58	42.89 ± 14.59	38.40 ± 7.95
Basal insulin (IU/kg/d)	0.25 ± 0.07	0.30 ± 0.08	0.34 ± 0.09	0.32 ± 0.11	0.24 ± 0.06
TDI (IU/day)	12.12 ± 4.70	24.46 ± 6.96	44.73 ± 18.76	46.33 ± 12.94	44.69 ± 11.64
TDI (IU/kg/d)	0.69 ± 0.14	0.81 ± 0.12	0.89 ± 0.19	0.76 ± 0.16	0.63 ± 0.08

DM: diabetes mellitus, TDI: total daily insulin dose

Table 2. According to five age groups. The median basal insulin requirement per kilogram of body weight

Age group/n	Pump initiation BI (U/kg/d)		First year BI (U/kg/d)		p
	Median	Min/max	Median	Min/max	
0-5/36	0.22	0.04/0.72	0.26	0.10/0.43	0.019
5-8/20	0.30	0.18/0.57	0.30	0.12/0.52	0.794
8-15/74	0.30	0.09/0.87	0.33	0.17/0.66	0.019
15-18/19	0.32	0.12/0.50	0.34	0.10/0.53	0.334
> 18/5	0.25	0.13/0.26	0.22	0.18/0.36	0.500
p*	< 0.001		< 0.001		

p: initial and first year basal rates in the same age group.
p*: basal rates among age groups.
BI: basal insulin, Min/max: minimum/maximum

and 13:00 in all groups. Prepubertal children (group 1 and group 2) displayed a high peak between 22:00 and 01:00 h ($p < 0.001$ and $p = 0.007$ respectively) at the end of first year of therapy. While median (range) HbA1c was 7.5% (4.1-11.3) on the third month of pump therapy, it decreased to 7.1% (5.3-11.4) at the end of the first year after circadian rhythm was achieved ($p = 0.001$).

Discussion

CSII use in adolescents, children, and especially preschool children, is associated with improved glycemic control (6,8,11,12). Other than achieving metabolic control, CSII has beneficial effects on psychosocial factors, physical performance, protection from long-term complications and hypoglycemia (13). Insulin requirement at the time of pump initiation depends upon the insulin dose on MDI, the level of glycemic control and the weight of the patient. According to the consensus statement from the European Society for Pediatric Endocrinology, in children with good glycemic control and a low frequency of hypoglycemia, the total dose may need to be reduced by 10-20%. In a patient who has been experiencing frequent hypoglycemia, the dose should be reduced by 20% (6). According to Danne et al (14), in children with good glycemic control and a low frequency of hypoglycemia, the total dose has to be reduced by approximately 10% if using soluble regular human insulin in the pump. In case of frequent hypoglycemia, the dose should be reduced by 20%. Alemzedah calculated his daily total dose as "Total dose = Body weight x 0.74" in his research with 14 children with T1DM (15). In our institution, total dose of insulin at the initiation of pump therapy is calculated according glycemic control based on HbA1c of the patient and is reduced by 10% if above 64 mmol/mol (> 8%), by 20% if between 53-64 mmol/mol (7-8%) and 30% if below 53 mmol/mol (< 7%). After calculating the basal dose as 40% of total insulin, we divided the TBD equally into 24 hours.

Studies show that total insulin dose decreases in the first year after CSII therapy. Colino et al (16) showed a decrease from 0.89 to 0.73 UI/kg/day ($p < 0.001$) in TDD at the end of the first year of pump therapy. In contrast, Ahern did not show a decrease in TDD after 12 months of insulin pump use (3). A randomized study by Doyle showed that after 16 weeks of therapy, the CSII group had a significant decrease in TDD (5). In our research we showed a decrease from 0.86 to 0.78 UI/kg/day ($p < 0.01$) in TDD at the end of the first year of pump therapy.

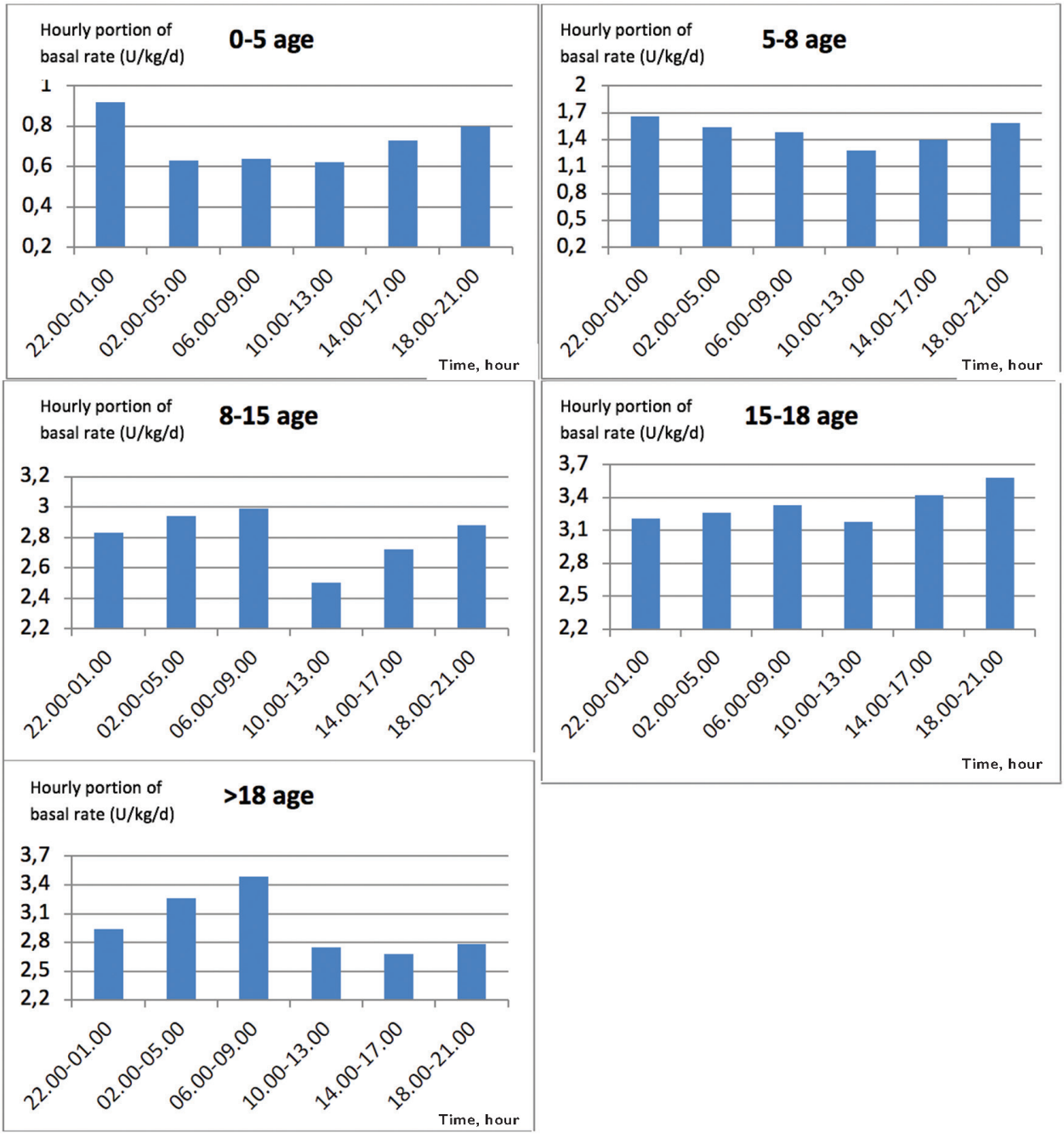

Figure 1. The mean circadian distribution of the total basal rate showed different profiles in the five age groups

In a cross-sectional, international survey of CSII in 377 children and adolescents with T1DM, the TDD of insulin was lower in the younger age groups and increased with puberty (17). In our study, the results were similar. The total insulin dose per kilogram was highest during adolescence (group 4).

CSII is the most physiological method of insulin delivery, simulating the pattern of insulin secretion with a continuous adjustable 'basal' delivery (18). Guidelines for insulin dosing basal/bolus ratio, have been established for adults with T1DM. However, these guidelines are not appropriate for children (6). According to Danne et al (14), as during injection therapy, approximately 30-40 %, rarely up to 50 %, of the TDD accounts for the basal rate. According to Hanas approximately 40-50 % of the daily insulin requirement should be the basal rate but some children with T1D may need up to 60 % (19,20). In our study, at the end of the first year, the mean basal rate of all cases was 38 % and was similar to other studies.

In children BI requirements are different in different age groups, especially in children younger than seven years of age, as well as in children who are in different stages of puberty (21). Klinkert et al (22) found that adolescents require the highest insulin doses, both as total and basal. Due to the balance of insulin and its counter-regulatory hormones, mostly the action of growth hormone, insulin requirement rises throughout puberty. According to the consensus statement from the European Society for Pediatric Endocrinology, the average TDD per kilogram of body weight should be 0.2-0.4 IU for toddlers, 0.4-0.6 IU for prepubertal children, and 0.8-1.2 IU for adolescents (6). In our study, median TDD basal per body weight was 0.18 IU/kg for 0-5 age group, 0.39 IU/kg for prepubertal children, and 0.71 IU/kg for adolescents ($p < 0.01$).

The pattern of BI requirements depends on the timing and magnitude of cortisol and growth hormone secretion within each age group (23). According to studies to date, basal rate profiles should be programmed in hourly intervals, according to the patient's circadian variation in insulin sensitivity (6,8,14,19,23). Schreiner and Boyer (23) reported that, under twenty years of age, BI requirement often begins peaking before midnight, maintains at a relatively high level throughout the night, drops through the morning hours, and gradually increases from noon to midnight. Although no statistical difference was found in BI requirement between age groups, many adolescents experienced a mid-day "decrease" rather than a significant "increase" in BI requirement in this study. Twenty-four hour pattern of peaks and troughs was remarkably similar in the age group < 10 years and the 11-20 age group. In the study of Nicolajsen (24), children with T1DM between the ages of 3-9 years had higher basal rates late at night (10:00 pm-12:00 am), while the oldest age group had a slight increase in basal rates in the early morning (3:00 am-7:00 am) (18). The reason for the reversed dawn phenomenon in the younger age group is unclear. An emptying of the gastric contents after falling asleep could be one explanation. Gastric emptying is slow during sleep, but rapid eye movement (REM) sleep is associated with faster gastric emptying. Children who take "afternoon naps" reach REM sleep faster after sleep onset than pre-adolescents who have discontinued their afternoon naps. In two studies done in adolescents and young adults, decreased insulin sensitivity was seen, particularly in the early morning (dawn phenomenon) and to a lesser extent, in the late afternoon (dusk phenomenon). This leads to a typical two-wave basal rate profile (14). In other studies prepubertal children needed a higher basal rate late in the evening and it is common for the basal rate requirement to be higher earlier in the night (midnight to 3.00 a.m) than later on (3.00-7.00 am) (19). The PedPump Study group noted that younger children often need more BI between 21.00 and 24.00 h (6). In our study, the circadian distribution of the total basal rate showed characteristic profiles in the five age groups. Younger age groups had higher basal rates (10.00 pm-01.00 am), while the oldest age group had a slight increase in basal rates in the morning (6.00 am-9.00 am). Our study, like other studies, supports the high insulin requirement at early-night in the prepubertal period. We believe that adjusting the hourly BI doses according to the need instead of constant adjustment will provide faster blood glucose normalization in a pediatric population. However, since there is no consensus on basal dose adjustment according to age groups, further studies are needed in this area.

Study Limitations

This study was single center. An improvement in study design would be to conduct it as a multicenter study. This would increase the number of children with T1DM, would allow for a comparison between children with T1DM on the same hourly BI dose versus those with their BI dose adjusted to their circadian rhythm in a crossover design, which should result in more robust conclusions.

Conclusion

At the initiation of insulin pump therapy, the basal rates should not be set equally during the day but should be initiated at a specific day rhythm for the age group. Our results indicate that it is simply not reasonable to expect BI needs to be met by a flat rate of insulin delivery for 24 hours.

Ethics

Ethics Committee Approval: The study was approved by Ege University Faculty of Medicine, Ethics Committee (protocol number: 20-5.1T/29, date: 08.07.2020).

Informed Consent: Written informed consent was obtained from all participants or their parents/guardians.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Damla Gökşen, Concept: Günay Demir, Design: Günay Demir, Data Collection or Processing: Günay Demir, Yasemin Atik Altınok, Damla Gökşen, Samim Özen, Şükran Darcan, Analysis or Interpretation: Günay Demir, Damla Gökşen, Literature Search: Günay Demir, Yasemin Atik Altınok, Damla Gökşen, Samim Özen, Şükran Darcan, Writing: Günay Demir, Damla Gökşen.

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Elevated Pre-injection Basal Luteinizing Hormone Concentrations are Common in Girls Treated for Central Precocious Puberty

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What is already known on this topic?

Gonadotropin-releasing hormone agonists (GnRHa) reduce gonadotropic activity and are efficient in suppressing pubertal progression in precocious puberty. Pituitary suppression during GnRHa therapy is optimally assessed by GnRH stimulation.

What this study adds?

Pre-injection basal luteinizing hormone (LH) remains at pubertal concentrations in the majority of girls during GnRHa therapy in spite of lack of pubertal progression, a significant decline in bone age and height standard deviation score. Even after shortened intervals with subcutaneous administration of leuproreline 3.75 mg in girls suspected to have progressive pubertal development during treatment of central precocious puberty (CPP), pre-injection basal LH did not drop to prepubertal concentrations in 86% of patients.

Abstract

Objective: A consensus on how to monitor girls with central precocious puberty (CPP) during gonadotropin-releasing hormone agonist (GnRHa) treatment is lacking. Increased, unstimulated basal luteinizing hormone (LH) concentrations have been suggested to indicate lack of suppression. The aim was to evaluate pre-injection basal LH concentrations during GnRHa (leuprorelin 3.75 mg) treatment every four weeks in girls with CPP.

Methods: Medical records were reviewed for girls with CPP treated at a single center from 2014-2019. Clinical characteristics and laboratory findings during treatment were systematically recorded.

Results: A total of 587 GnRHa pre-injection basal LH concentrations were analyzed in 74 girls. Basal LH was pubertal (≥0.3 IU/L) in 53.5% of blood samples and 87.8% of all girls had a pubertal basal LH concentration at least once. A GnRH test (n = 29) was repeated in 23 girls due to suspicion of clinical progression, elevated basal LH or recordable estradiol concentrations. None had a stimulated LH > 3.1 IU/L. The predictability of treatment suppression (specificity) of basal LH concentrations was 12.0% when compared to repeated GnRH stimulation tests. Despite shortening the GnRHa injection interval to three weeks, basal LH concentrations remained pubertal in 85.7% girls. A significant reduction in height standard deviation score ($p < 0.001$) and bone age advance ($p < 0.001$) was observed during treatment.

Conclusion: Pre-injection basal LH remains at pubertal concentrations during treatment with leuprorelin 3.75 mg in girls with CPP. Clinical monitoring of pubertal progression is preferable to routine basal LH concentrations. Repeat GnRH stimulation testing should be regarded as the gold standard.

Keywords: Girls, precocious puberty, luteinizing hormone, gonadotropin-releasing hormone agonist, gonadotropin-releasing hormone test

Introduction

Central precocious puberty (CPP) in girls is often idiopathic, in up to 90% of affected girls (1,2). Gonadotropin-releasing hormone (GnRH) agonists (GnRHa) are used to suppress pubertal development by stimulating GnRH receptors continuously, causing pituitary desensitization and reduced

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gonadotropic activity (3,4,5). The use of GnRHa has increased in recent years (6,7). However, there is still no consensus on how to monitor the pituitary suppression during GnRHa treatment (8). It has been suggested that increased basal luteinizing hormone (LH) concentrations indicate lack of suppression (8,9,10). In case basal LH concentrations provide the same information as a GnRH stimulation test, monitoring would be less time consuming, costly and less invasive for the patients (11).

The aims of this study were to assess if pre-injection basal LH concentrations are reliable as a proxy for clinical progression of puberty during GnRHa treatment, and to test if basal LH concentrations are in accordance with GnRH stimulated LH concentrations under GnRHa suppressive treatment.

Methods

A review was undertaken of a cohort of girls followed at the Department of Pediatrics, Aalborg University Hospital, Denmark and who were treated with leuprorelin acetate 3.75 mg injections every four weeks for CPP. The electronic patient system (Clinical Suite 2017, DXC technology, Tysons, Virginia, USA) was searched using the International Classification of Diseases-10 codes for CPP (DE228A) and associated diagnosis [early puberty (DE301), hormonal dysregulation in puberty (DE309), premature thelarche (DE308A)]. All girls with the above-mentioned codes, who attended the Department of Pediatrics at Aalborg University Hospital between January 2014 and September 2019, were identified (Figure 1).

Only girls who had a pubertal response (stimulated LH >5 IU/L) at time 30 minutes during a GnRH stimulation test (gonadorelin 0.1 mg intravenous) and who started subcutaneous injections with leuprorelin acetate 3.75 mg at four-weekly intervals were included (Figure 1). Previous medical history was extracted from the medical notes retrospectively from the first contact.

Data were collected systematically from the electronic patient records by two investigators (AH, SS) according to a predefined protocol. A third researcher was consulted in unclear cases (TL). At first visit: age at presentation, Tanner stage (12), presenting symptoms, family pubertal history, height and weight were noted. Dates and results of the diagnostic GnRH stimulation tests and of repeated GnRH stimulation tests were obtained. Dates, GnRHa pre-injection basal gonadotropin concentrations and estradiol concentrations were noted. Hormonal blood samples and clinical examinations were undertaken regularly at 3-6 months intervals by pediatricians. A pre-injection basal

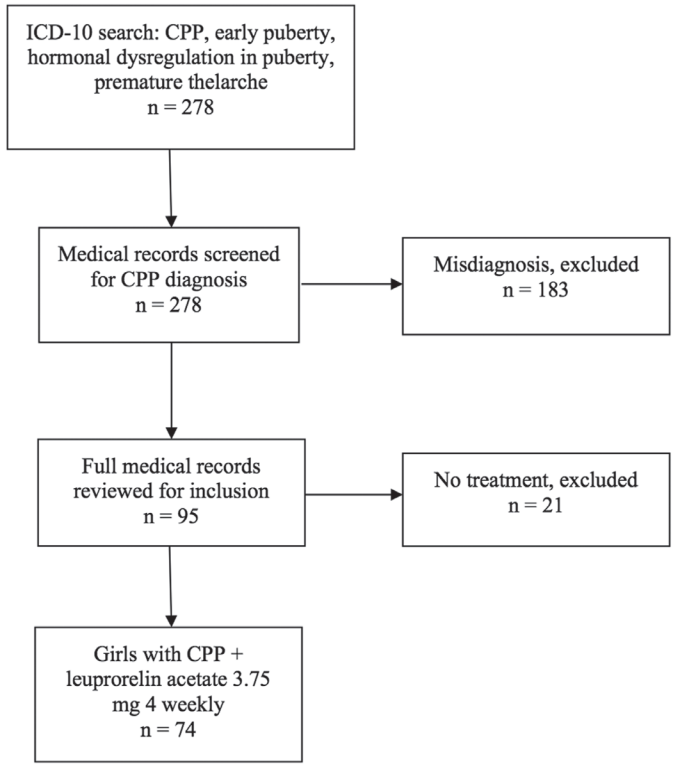

Figure 1. Flowchart showing patient inclusion and exclusion criteria in gonadotropin-releasing hormone agonists treated girls with central precocious puberty

ICD-10: International Classification of Diseases-10, CPP: central precocious puberty

LH ≥0.3 IU/L was considered pubertal. A stimulated LH/follicle stimulating hormone (FSH) ratio >1 was interpreted to indicate breakthrough of hypothalamic suppression. During follow-up, bone age (BA), brain magnetic resonance imaging (MRI) and hormonal blood samples (pre-injection basal gonadotropins, estradiol) were recorded. At the final clinical visit, age, height, weight and treatment status were noted. Standard deviation (SD) scores (SDS) of height and body mass index (BMI) were calculated based on Danish reference data (13).

Heights and weights were measured in clinic by a specialist endocrine nurse using a stadiometer with a precision of 0.1 cm or 0.1 kg, respectively. BAs were assessed according to Greulich & Pyle using BoneExpert Software (Visiana Aps, Denmark) on X-ray images of the left hand and wrist. LH, FSH, and estradiol were analyzed on Roche-Cobas 8000® immunochemistry module (Roche, Mannheim, Germany) by electro-chemiluminescense immunoassay. Limit of detection was 0.1 IU/L for LH and 20 pmol/L for estradiol. Interserial coefficient of variation at the detection limits were <20%. Interserial coefficients of variation were 5.2%

at 0.5 IU/L and 2.0% at 6.2 IU/L for LH and 11.0% at 360 pmol/L for estradiol. The laboratory is ISO 15189 accredited.

The study protocol was approved by the hospital management (journal no 2019-005812-58) as required by Danish law.

Statistical Analyses

Descriptive data were presented as mean ± SD or median (range) according to normal or non-parametric distribution. A paired t-test for parametric data was used to compare two variables in the same individual. An unpaired t-test was used for comparison of two independent groups. A Mann-Whitney U test was applied to compare non-parametric data. Pearson correlation coefficient was used to calculate the correlation between two parametric variables. Predictability of treatment suppression by basal LH concentrations was assessed by comparison of prepubertal basal LH concentrations among girls with fully suppressed GnRH responses (LH < 5 IU/L and LH/FSH ratio < 1) who had a second stimulation test. A p value of < 0.05 was considered significant.

Results

Population Characteristics

Inclusion criteria were fulfilled by 74 girls (Table 1). The mean presenting age in hospital was 8.0 ± 1.2 years. Mean age at onset of treatment was 8.2 ± 1.3 years. Within the time period the medical course was complete for 55 girls and the average duration of treatment was 2.8 ± 1.2 years until age 11.2 ± 0.7 years. The remaining 19 girls still had ongoing treatment at completion of the study.

Two girls (2.7%) had reached menarche at the first visit and they were clinically described to be at Tanner stage B3 and B4. Special circumstances with mental retardation or cerebral palsy influenced the decision to treat in two cases. Concern about psychosocial stressors related to early puberty contributed to the decision of treatment in 16 girls (21.6%). A family history of early puberty was confirmed by 18 families (24.3%), and another eight girls (10.8%) had an increased risk due to international adoption.

Average height SDS (Table 1) at first visit was above mean for age but proportional to average BMI SDS. At final visit height SDS approached the mean for age whereas age adjusted BMI SDS had increased ($p < 0.001$) (Table 1). BA was examined in all girls at the first clinical visit and the mean BA was 1.2 ± 1.1 years ahead of the chronological age (CA). Consecutive BAs were undertaken in 52 girls (70.3%) and the BA advance regressed and approached CA over time (0.5 ± 1.0 years) ($p < 0.001$) (Table 1).

Table 1. Clinical and radiological characteristics at first and final clinical visit

	First visit n = 74 Mean ± SD	Final visit n = 74 Mean ± SD
Age (years)	8.0 ± 1.2	10.7 ± 1.2
Age-treatment (years)	8.2 ± 1.3	11.2 ± 0.7*
BA advancement (years)	1.2 ± 1.1	0.5 ± 1.0**
Height (SDS)	0.9 ± 1.6	0.6 ± 1.6
BMI (SDS)	0.7 ± 1.1	1.2 ± 1.0
Presenting symptoms [n (%)]		
Breast development	68 (91.9%)	
Growth acceleration	34 (46.0%)	
Menarche	2 (2.7%)	
Adrenarche (hair, sweat, acne)	46 (62.2%)	
Mood swings	18 (24.3%)	

*Age at final injection. Calculated on 55 girls who stopped gonadotropin-releasing hormone agonists injections within the study period.
**Calculated on 52 girls who had consecutive BA.
BMI: body mass index, BA: bone age, SDS: standard deviation (SD) score

Brain MRIs were undertaken in 58 girls (78.4%) at a mean age of 7.7 ± 1.2 years (range 3.0 to 9.7).

The oldest girl had a rapid pubertal development with menarche at age 9.7 years at Tanner stage 3, for which she started GnRHa therapy.

Six girls (10.3%) with a mean age of 6.2 ± 2.3 years (range 3.0 to 9.3) had abnormal findings, including hamartomas or sequalae from brain trauma (Table 2). One girl, aged 9.3 years, had multiple MRI scans (ID 1, Table 2); the one presented here was closest to the time when leuprorelin was started (due to psychological reasons). Four girls had incidentalomas (Table 2).

Pre-injection Basal LH Concentrations

During treatment with GnRHa, 587 blood samples (7.9 per girl, range 1 to 20) were analyzed for pre-injection basal LH, FSH and estradiol. Basal LH was ≥0.3 IU/L in 314 samples (53.5%) and 65 girls (87.8%) had a basal LH ≥0.3 IU/L at some point in time. There was no declining temporal trend of pre-injection basal LH concentrations during treatment ($r = 0.09$) (Figure 2). Basal LH concentrations were ≥1.1 IU/L (range 1.1 to 2.4) in 10 girls at least once during treatment. Three of these girls had a repeat GnRH stimulation test of which one had a LH/FSH ratio > 1 and consequently the GnRHa dosing interval was reduced to 3 weeks.

Repeat GnRH Stimulation Tests

A GnRH stimulation test was repeated (n = 29) in 23 girls (31.1%) (six girls had two tests). Four girls (17.4%) had a ratio of LH/FSH > 1, but none had a stimulated LH > 3.1

IU/L. Unstimulated basal LH concentrations drawn prior to GnRH injection were 0.2 IU/L, 0.4 IU/L, 0.7 IU/L and 1.1 IU/L, respectively.

The remaining 25 GnRH stimulation tests showed LH/FSH ratios < 1 and LH peaks ≤2.9 IU/L. Three tests were preceded by pre-pubertal basal LH concentrations (< 0.3 IU/L) and 22 tests had pubertal basal LH concentrations ≥0.3 IU/L (median 0.4 IU/L, range 0.3 to 2.2 IU/L) at least once prior to the test. Thus, the predictability of proper treatment suppression (specificity) according to pre-injection basal LH was 12.0% (Figure 3).

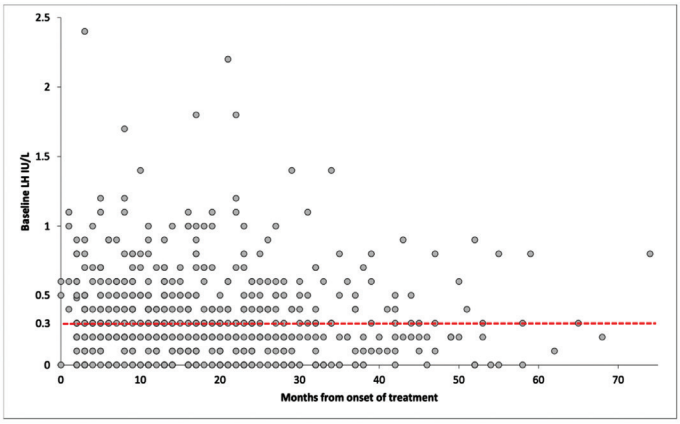

Figure 2. Pre-injection basal luteinizing hormone (LH) concentrations during gonadotropin-releasing hormone agonists (GnRHa) treatment for central precocious puberty. All samples were drawn just prior to the next GnRHa injection. The horizontal dashed line indicates the cut-off for a pubertal baseline LH concentration

LH: luteinizing hormone

There was no significant difference (p = 0.354) in median basal LH concentrations between tests with LH > FSH (17.4%) (0.6 IU/L, range 0.2-1.1) and suppressed tests (0.4 IU/L, range 0.3-2.2).

The mean time from the diagnostic GnRH stimulation test to the first repeated test was 1.7 ± 0.9 years (range 0.4 to 3.8). The likelihood of having a pubertal response on repeat GnRH testing was poorly correlated with the time from diagnosis to repeated testing (r = 0.4). Likewise, the CA was not associated with an increased risk (r = 0.1). When comparing the groups of girls with and without a repeat GnRH stimulation test, there was no significant difference in BMI SDS at first (p = 0.255) and last contact (p = 0.248).

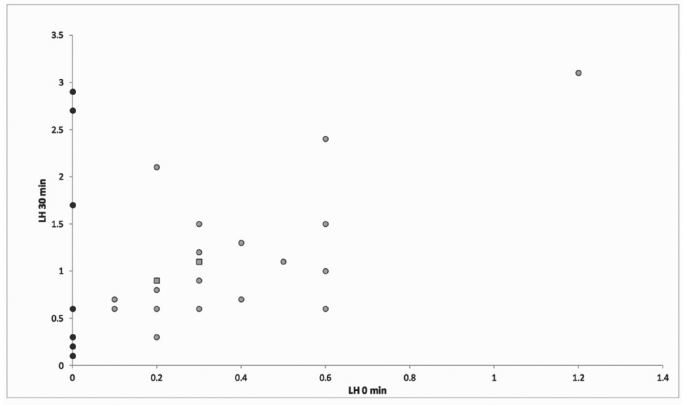

Figure 3. Luteinizing hormone (LH) concentrations (IU/L) at time 0 and 30 minutes at repeat gonadotropin-releasing hormone stimulation retesting. LH concentrations < 0.1 are marked as 0 (dark blue circle). Two tests had equal concentrations (square)

Table 2. Cerebral magnetic resonance imaging in girls (n = 10) with abnormal findings

ID	Age at MRI (years)	MRI	Pre-injection LH (range)	Peak LH*	Medical intensification
1	9.3	Supracellular pilocytic astrocytoma near the pituitary gland.	< 0.1	-	-
2	8.1	Tuber Cinereum hamartoma	0.2 to 0.8	-	+
3	6.0	Microadenoma. Sequalae after meningitis	< 0.1 to 0.5	-	+
4	3.0	Tuber Cinereum hamartoma. Microadenoma	< 0.1 to 1.4	-	-
5	4.7	Sequelae after subdural hematoma	0.1 to 1.2	-	-
6	6.3	Radiotherapy cause of esthesioneuroblastoma	< 0.1 to 0.2	-	-
Incidentalomas					
7	8.8	Enlarged pituitary stalk	0.5 to 1.8	3.1	-
8	8.0	Microadenoma	< 0.1 to 0.3	0.6	-
9	7.8	Microadenoma	0.2 to 0.5	-	-
10	6.9	Microadenoma	0.1 to 1.7	-	-

*Girls who had a repeat gonadotropin-releasing hormone stimulation test during therapy.
MRI: magnetic resonance imaging, LH: luteinizing hormone

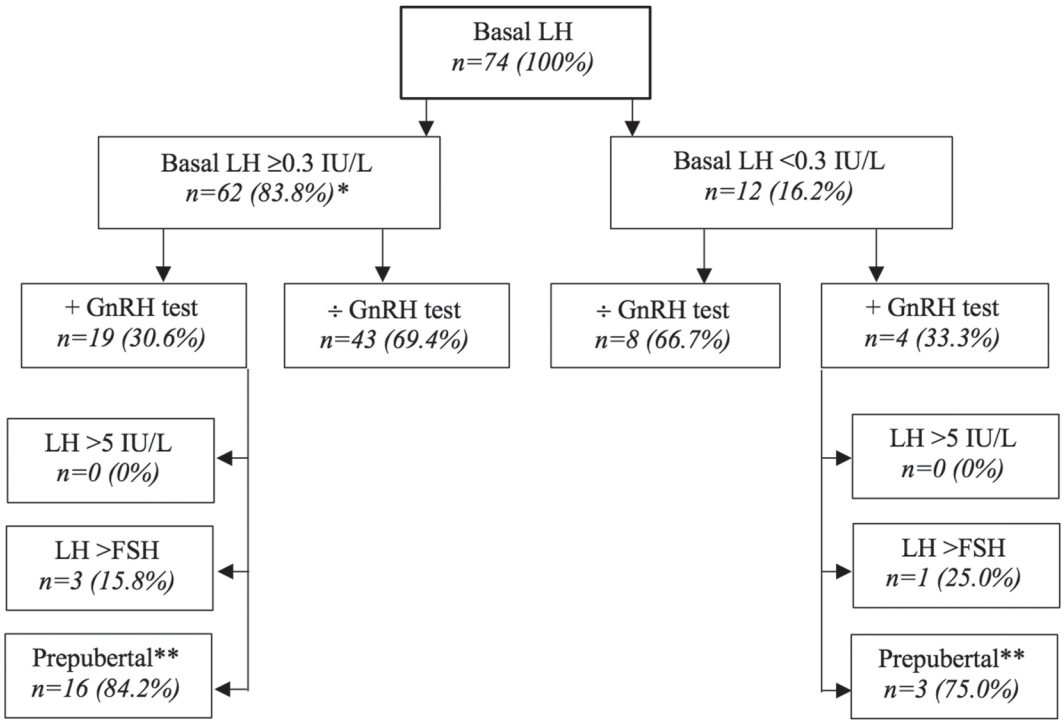

Figure 4. Girls split according to pre-injection basal luteinizing hormone concentrations prior to repeat gonadotropin-releasing hormone agonists (GnRH) testing (+ had repeat GnRH test/– had no repeat GnRH test). Girls who had a repeat GnRH test (n = 23) are only represented once. If more than one repeat test was undertaken (n = 6 girls) the first test in time was used unless overruled by a pubertal response at the second test (n = 1)

**N = 62 girls had LH ≥0.3 IU/L minimum once, up until the first repeat GnRH test (another three girls developed LH concentrations ≥0.3 IU/L after the first GnRH test and are not included in the figure).*

***Prepubertal: LH < 5 IU/L and LH < FSH.*

LH: luteinizing hormone, FSH: follicle stimulating hormone

During repeat GnRH testing a poor correlation between basal and stimulated LH concentrations was observed (r = 0.4) (Figure 4).

Estradiol

In 33 of 74 girls (44.6%) estradiol was detectable (≥20 pmol/L) at some point in time. The estradiol concentrations were significantly higher at diagnosis (median 100 pmol/L, range 30 to 320) than during treatment (median 40 pmol/L, range 20 to 380) (p < 0.001). Estradiol was > 100 pmol/L in two samples during treatment. One resulted in a repeat GnRH test (estradiol 320 pmol/L, peak LH/FSH 0.6/0.6 IU/L). The second girl with an increased estradiol (estradiol 380 pmol/L, basal LH 0.2 IU/L) stopped therapy shortly after at age 12.3 years. There was a trend towards a more advanced BA in girls with a detectable estradiol concentration at diagnosis compared to those with no detectable estradiol (p = 0.095) (Figure 5). This observation was not present at the end (p = 0.944) (Figure 5).

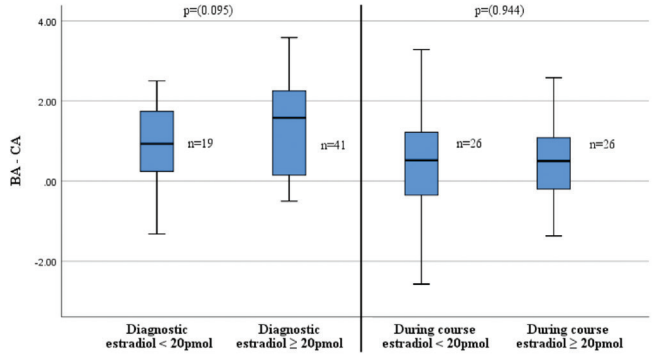

Figure 5. Bone age advancement compared with estradiol (< 20 pmol versus ≥20 pmol) at diagnosis and at the end of therapy

BA: bone age, CA: chronological age

The four girls who had a LH/FSH ratio > 1 on repeat GnRH stimulation testing never had detectable estradiol concentrations during treatment. Eight girls with prepubertal GnRH test responses on retesting previously had detectable

estradiol concentrations, also when discounting initial, possibly unsuppressed, concentrations sampled within the first three months of GnRHa treatment.

Intensification of Treatment

Treatment was intensified by reducing the GnRHa dosing interval to three weeks in 17 girls (22.3%). Most often this decision was based on multifactorial variables such as increased pre-injection basal LH concentrations, stimulated LH/FSH ratios >1 or recordable concentrations of estradiol but in some cases also due to the impression of clinical progression with regards to breast development. The mean time from onset of treatment to the first intensification was 1.3 years (range 0.2 to 4.4).

Basal LH concentrations (n=59) were sampled in 14 girls who had an increment in the GnRHa dosing interval to three weeks, after which point none had signs of breast development. The majority of the samples (n=44, 74.5%) in 12 girls (85.7%) persistently had a pre-injection basal LH ≥0.3 IU/L (median 0.4, range 0.3 to 1.8 IU/L). Two of three girls with a basal LH concentration <0.3 IU/L prior to intensification developed pubertal pre-injection basal LH concentrations ≥0.3 IU/L after shortening the dosing interval.

Discussion

In this large cohort of 74 girls with CPP, pre-injection basal LH remained at pubertal concentrations during GnRHa therapy, in spite of a lack of clinical pubertal progression (breast development), and a significant decline in BA and height SDS.

All girls were followed consecutively and 87.8% of girls had pre-injection basal LH concentrations ≥0.3 IU/L at some point in time during GnRHa therapy. Even after medical intensification, basal LH did not drop to prepubertal concentrations, but remained as high as 1.8 IU/L. Thus, elevated concentrations of LH did not indicate insufficient pituitary suppression as the girls never showed signs of breast tissue development, BA advancement or had increased growth velocity. In line with this report, Wiromrat and Panamonta (9) found that in spite of pubertal basal LH concentrations during GnRHa treatment, clinical measures such as Tanner stage, BA and decreased growth velocity indicated sufficient pituitary suppression. Other smaller studies in girls treated with a 50 mg histrelin implant have also reported elevated LH concentrations during treatment (14,15).

One study in girls treated with a 50 mg histrelin implant suggested that continuous low-concentration LH secretion tapered off over time as basal LH concentrations decreased during the course of therapy (14). We did not observe this temporal decline in LH concentrations, similar to findings in another study using leuprorelin 3.75 mg (9). Whether the shorter half-life of leuprorelin 3.75 mg allows breakthrough gonadotropic activity at GnRHa trough concentrations towards the next injection remains speculative. Growth velocity and pubertal progression, however, did not advance, indicating that any breakthrough at hypothalamic/pituitary level was not of clinical significance, supporting the efficacy of the leuprorelin dose.

The majority (86.2%) of our repeat GnRH stimulation tests (n=25) were anteceded by pubertal LH concentrations (0.3-2.2 IU/L) but on repeat stimulation none had a peak LH >3.1 IU/L.

Consequently, basal LH concentrations had a low specificity of only 12.0%, incorrectly suggesting that girls were not biochemically suppressed during GnRHa treatment when compared to the repeat GnRH stimulation tests. The same observation has been found in other studies in girls treated with histrelin implants (14,15). These findings indicate that, clinicians need not be concerned about elevated LH concentrations during GnRHa therapy, in our series reaching as high as 2.4 IU/L, if there are no other indicators of pubertal progression, such as breast development, BA and increased growth velocity.

Lee et al (16) found that basal LH concentrations <0.60 IU/L and 0.75 IU/L predicted 70.0% and 60.0%, respectively, of girls sufficiently suppressed during GnRHa treatment. A higher cut-off for basal LH identifies more girls with breakthrough gonadotropic activity (increased sensitivity) but with a reduced specificity (correctly suppressed girls) meaning that caution not to overlook unsuppressed girls should be warranted as the cut-off rises (16).

We, like others (9,14,15), question the advantage of including routine basal LH concentrations as a monitoring strategy for pituitary suppression during GnRHa therapy. Consecutive clinical assessment assisted by growth velocity and BA is likely superior as a first line strategy. In case of doubt about progression of puberty, which may be the case during assessment of breast development in a girl with an increasing BMI, analysis of a basal LH may assist in deferring the suspicion, if not elevated. As overtreatment, which has socioeconomic costs (4,6) and increase the burden of unnecessary painful injections (6), should be avoided, our results support the recommendation that GnRH stimulation testing should be considered the gold standard to evaluate suppression during GnRHa treatment (8).

Weight gain during GnRHa treatment has already been highlighted and rise in BMI SDS was also observed in our group (17,18).

A tendency towards a more pronounced advancement of BA at diagnosis was seen in girls who had a recordable estradiol concentration compared to girls with no detectable estradiol. This may likely reflect the maturing effect of estradiol on bone (19). The difference was insignificant and the question of whether this was a true trend, due to inaccuracy of paraclinical measurements or an underpowered study remains unanswered.

Eight girls with prepubertal responses on repeat GnRH stimulation testing had detectable estradiol during GnRHa treatment. Estradiol concentrations were not in accordance with pre-injection basal LH, nor clinical pubertal progression. It is widely known that low and fluctuating concentrations of estradiol around initiation of puberty make them difficult to measure (20,21), and we question the reliability of estradiol measurements in girls during GnRHa treatment (22).

Study Limitations

Due to the retrospective design of our study, suspicion of clinical pubertal progression was not necessarily confirmed by a repeat GnRH stimulation test prior to medical intensification.

In addition, we encountered only four girls with a LH/FSH > 1 during GnRH retesting, which did not add to the evaluation of suppression. Thus, a comparison of biochemically unsuppressed children to suppressed children was not possible, which is ultimately needed to answer the question at what concentration unstimulated basal LH may indicate reversal of pituitary suppression.

The electro-chemiluminescense immunoassay used to analyze LH concentrations had a detection limit of 0.1 IU/L, and was thus not as sensitive as other assays (15). However, we aimed to assess the highest concentrations of LH, for which reason this did likely not affect out results.

Estradiol was inappropriately elevated in two cases. Although our estradiol analyses were undertaken in the same laboratory on an electro-chemiluminescense immunoassay, tandem mass-spectrometry, which is more accurate, particularly when analyzing small concentrations, was not used.

Conclusion

Basal LH concentrations often remain at pubertal concentrations during GnRHa treatment, but does not necessarily reflect insufficient gonadotropic suppression. The current study emphasized that routine clinical monitoring of girls during GnRHa therapy is preferable to routine pre-injection basal LH concentrations. In cases with dubious clinical progression, a low basal LH may defer the suspicion. A repeat GnRH stimulation test however, is to be considered if doubt persists. Finally, we suggest that estradiol concentrations should not be monitored routinely in girls treated for CPP.

Ethics

Ethics Committee Approval: The study protocol was approved by the hospital management at Aalborg University Hospital (journal no: 2019-005812-58) as required by Danish law.

Informed Consent: The study was undertaken as a quality improvement study and requires no informed patient consent according to Danish law.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept/Design: Stefanie Schubert, Amalie H. Hvelplund, Søren Hagstroem, Tina Lund Leunbach, Data Collection or Processing: Stefanie Schubert, Amalie H. Hvelplund, Tina Lund Leunbach, Analysis or Interpretation: Stefanie Schubert, Amalie H. Hvelplund, Aase Handberg, Søren Hagstroem, Tina Lund Leunbach, Literature Search: Stefanie Schubert, Amalie H. Hvelplund, Tina Lund Leunbach, Writing: Stefanie Schubert, Amalie H. Hvelplund, Aase Handberg, Søren Hagstroem, Tina Lund Leunbach.

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CASE REPORT

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Adrenoleukodystrophy in the Differential Diagnosis of Boys Presenting with Primary Adrenal Insufficiency without Adrenal Antibodies

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What is already known on this topic?

The cerebral adrenoleukodystrophy form (CALD) of ALD is characterized by progressive inflammatory demyelination resulting in loss of neurological function and early death. Approximately 70-80% of boys have impaired adrenal function at the time of ALD diagnosis.

What this study adds?

Despite appropriate screening, some ALD cases may still be missed when relying on very-long chain fatty acid (VLCFA) levels alone, since some mutations may result in initial normal levels of VLCFA. The need for early CALD diagnosis in order to initiate timely treatment mandates awareness of the potential need for repetitive VLCFA screening of boys with unexplained, antibody-negative, primary adrenal insufficiency.

Abstract

Adrenoleukodystrophy (ALD) is an X-linked, metabolic disorder caused by deficiency of peroxisomal ALD protein resulting in accumulation of very-long chain fatty acids (VLCFA), primarily in the adrenal cortex and central nervous system. Approximately 35-40% of boys with ALD develop cerebral ALD (CALD), which causes rapidly progressive cerebral demyelination, loss of neurologic function, and death. Approximately 70-80% of boys with ALD have impaired adrenal function prior to the onset of neurologic symptoms. We present a boy who had recurrent episodes of hypoglycaemia from age two years and was diagnosed with adrenal insufficiency without adrenal antibodies at age 5.5 years. Following initial normal VLCFA levels, subsequent VLCFA analysis demonstrated elevated C26 fatty acids consistent with peroxisomal dysfunction and suggestive of ALD, which was confirmed via molecular genetic analysis of the *ABCD1* gene. Brain imaging at age 7 suggested cerebral involvement and the child underwent successful allogeneic hematopoietic stem cell transplantation. At last assessment (11.5 years old), he was performing as expected for age. This case highlights the importance of pursuing a diagnosis when clinical suspicion remains, and the significance of VLCFA analysis for patients with adrenal insufficiency without adrenal antibodies in securing an ALD diagnosis. Subsequent brain imaging surveillance can detect early, pre-symptomatic cerebral disease, allowing for timely treatment and successful arrest of cerebral disease progression.

Keywords: Adrenal insufficiency, adrenoleukodystrophy, very-long chain fatty acids, X-linked

Introduction

Adrenoleukodystrophy (ALD) is a rare, X-linked, metabolic disorder caused by mutations in the *ABCD1* gene that result in deficiency of peroxisomal membrane ALD protein and accumulation of very-long chain fatty acids (VLCFA) in tissues and plasma (1,2). The most severe phenotype is the cerebral form (CALD), which develops in 35-40% of at-risk male children typically between the ages of 3 and

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12 years (1). CALD rapidly progresses with inflammatory cerebral demyelination, loss of neurological function, and early death.

ALD was added to the recommended uniform screening panel in the United States in 2016, but newborn screening (NBS) remains limited, both within the US and worldwide (3). Therefore, early identification of ALD remains challenging due to nonspecific initial symptoms that overlap with other medical and developmental issues. Mutations in the *ABCD1* gene are associated with genetic forms of primary adrenal insufficiency (PAI) (4,5,6); therefore PAI in boys in the absence of adrenal antibodies can be an early "red flag" for a potential ALD diagnosis. Unexplained PAI may be the only clinical sign prior to the onset of neurologic symptoms in boys ultimately diagnosed with CALD (7,8).

Early detection of CALD is critical since allogeneic hematopoietic stem cell transplantation (HSCT) has the potential to stabilize cerebral disease progression, but is only effective if performed in the early stages of cerebral disease (9,10). While not possible to determine which children with ALD will develop CALD based on genotype, plasma VLCFA levels, or family history (11,12), monitoring at-risk boys for magnetic resonance imaging (MRI) abnormalities can successfully detect changes indicative of CALD prior to the onset of neurological symptoms (13,14). HSCT is only indicated once imaging detects white matter changes indicative of progression to CALD, and current management guidelines recommend repeating MRIs in pre-symptomatic boys with ALD every six months between the ages of 3 and 12 years (15). Given this need for early ALD diagnosis and monitoring for CALD, ALD should be included in the differential diagnosis of boys presenting with PAI without adrenal antibodies.

The following case presentation highlights the clinical course in a boy who was diagnosed with ALD through presentation with PAI and who, upon progression to CALD, received a timely and successful allogeneic stem cell transplant.

Case Report

A white male infant was born at term to non-consanguineous parents. There were no antenatal or perinatal concerns and early childhood was reported to be normal with normal growth parameters. No hyperpigmentation was ever noted. At 2 years of age he was admitted to a paediatric resuscitation bay via the local GP walk-in service following a 24 hour vomiting illness with subsequent collapse, convulsion and fever with acidosis (pH 7.279, base deficit 9.2 mmol/L), cardiac compensation (pulse 161/minute with mean peripheral blood pressure 84 mm Hg) requiring 20 mL/kg saline bolus, and hypoglycaemia (glucose 1.1 mmol/L; 19.82 mg/dL) which responded rapidly to 5 mL/kg 10% dextrose. He also had elevated urine ketones (+ + + on urine dipstick test), and his plasma cortisol measured after normalisation of glucose, was 320 nmol/L. Bedside sodium was 131 mmol/L and potassium 3.2 mmol/L. He recovered promptly with rehydration and dextrose infusions with intravenous antibiotics. He was discharged 48 hours later after a full infection screen, including lumbar puncture, was negative, and was tolerating oral food and fluids, with a presumptive diagnosis of hypoglycaemia following prolonged vomiting with a febrile convulsion.

At 2.5 years, he had an episode of multiple febrile convulsions, and again presented with hypoglycaemia (glucose 1.2 mmol/L; 21.62 mg/dL; sodium 137 mmol/L; potassium 3.8 mmol/L) and elevated urine ketones (+ + + on urine dipstick test). Plasma insulin (46 pmol/L) and C-peptide (315 pmol/L) were found to be inappropriately elevated at the time of hypoglycaemia. Plasma cortisol was 470 nmol/L. The hypoglycaemic episodes were initially ascribed to idiopathic ketotic hypoglycaemia. In view of the multiple episodes of hypoglycaemia and hyperinsulinaemia during this most recent admission, investigations included a 24-hour glucose profile and controlled 17-hour fast, both of which did not demonstrate any hypoglycaemia but normal suppression of insulin and mobilisation of ketone bodies at the end of the fast. Random plasma cortisol concentrations were 281 and 226 nmol/L at 12:00 and 18:00 hours respectively.

A subsequent hypoglycaemic event (glucose 1.4 mmol/L; sodium 139 mmol/L; potassium 3.9 mmol/L) with vomiting at age 5.2 years was associated with a plasma cortisol of 553 nmol/L. The supervising clinician changed at this time, and because of recurrent unexplained hypoglycaemia, a standard synacthen (tetracosactide) test was performed and showed adrenal insufficiency [Table 1 cortisol and adrenocorticotropin (ACTH) test 1]. A repeat synacthen test was performed at age 5.5 years in view of the relatively high cortisol at the time of the last hypoglycaemia event and confirmed these findings (Table 1, cortisol and ACTH test 2). Adrenal antibodies were negative, and the boy was commenced on standard hydrocortisone replacement at 10.0 mg/m^2/day in divided doses.

In view of the adrenal failure and negative adrenal antibodies, an analysis of VLCFA levels was ordered (using UKAS accredited laboratories), although due to family reasons there was a delay of several months in performance of these tests. VLCFA testing typically assesses abnormalities in three parameters: the level of hexacosanoic acid (C26:0), and the ratio of hexacosanoic acid to tetracosanoic acid

Table 1. Results of tests evaluating cortisol, adrenocorticotropin, and very-long chain fatty acid levels

Test					
Standard short synacthen					
	Time (mins)	**Test 1**		**Test 2**	
Cortisol nmol/L	0	178		226	
(Normal: rise above 550 nmol/L with an increment of > 200 nmol/L)	30	**163**		**201**	
	60	**157**		**200**	
ACTH ng/L (normal < 50)		**1106**		**522**	
VLCFA analyses					
	Laboratory A		**Laboratory B**		
	Test 1	**Reference**	**Test 2**	**Test 3**	**Reference**
C22 (µmol/L)	NA	NA	43.3	47.6	33.2-96.3
C24 (µmol/L)	NA	NA	63.0	68.3	25.2-71.4
C26 (µmol/L)	NA	NA	**3.32**	**3.72**	0.23-1.79
C24/C22 ratio	0.95	< 1.4	**1.45**	**1.43**	0-1.01
C26/C22 ratio	0.52	< 0.7	**0.077**	**0.078**	0-0.026
Phytanate (µmol/L)	NA	NA	2.89	6.11	0-15.00
Pristanate (µmol/L)	NA	NA	0.24	0.55	0-2.00

Bold numbers indicate parameters outside of normal references range.
NA: not available, ACTH: adrenocorticotropin, VLCFA: very-long chain fatty acid

(C26:0/C24:0) and to docosanoic acid (C26:0/C22:0) (2). Initial VLCFA tests at age 6.5 years showed normal C26/C22 and C24/C22 levels (Table 1, VLCFA Laboratory A test 1). Continued clinical suspicion and negative adrenal antibodies prompted repeat VLCFA tests using a different laboratory at 7 and 7.5 years of age (Table 1, Laboratory B, test 2 and 3) that showed elevated C26 fatty acids with moderately raised C26/C22 ratio, mildly raised C24/C22 ratio, and normal phytanic and pristanic acid levels (pristanic and phytanic acids are branched chain fatty acids that undergo oxidation in peroxisomes, and are typically elevated in peroxisome biogenesis disorders in the Zellweger spectrum, but not in ALD). These findings were consistent with a disturbance in peroxisomal function suggestive of ALD rather than a peroxisomal biogenesis disorder. Subsequent molecular genetic analysis of the *ABCD1* gene identified a previously reported pathogenic hemizygous mutation (c.1849C > T, [p.Arg617Cys]), which on family testing was confirmed to be maternally inherited. The child was commenced on Lorenzo's oil (oleic acid and erucic acid 4:1, such that 20% of energy requirements in diet came from Lorenzo's oil) available for use in UK with provision for monitoring on a non-research basis, and a low fat diet at 7.5 years of age following the second confirmatory VLCFA assessment.

At 7.5 years of age, baseline MRI brain scan identified features possibly consistent with cerebral ALD (Figure 1), with signal abnormality in the splenium of the corpus callosum and subtle changes in the deep parietal white matter, but no convincing gadolinium enhancement (an indicator of active inflammation and hallmark of cerebral disease). The MRI Loes score, a 34-point scale used to measure the extent of demyelinating brain lesions (16), was assessed as 2 with bilateral splenium of corpus callosum and bilateral parietal white matter central change. A repeat MRI after three months showed some progression of the lesion in the splenium, with patchy signal change in the parietal lobes and mild prominence of cerebellar folia and sulci. Loes score was now assessed as 3, with additional mild symmetrical cerebellar atrophy. Although the splenium lesion had progressed in size, this did not alter the Loes scoring of the already bilateral splenium lesion. Neurological examination at this stage was normal. The child had a history of temper tantrums but no other significant behavioural or psychological problems. In view of the MRI findings suggestive of progressing cerebral ALD, allogeneic HSCT was indicated.

At age eight years, he underwent 10/10 human leukocyte antigens-matched, unrelated, allogeneic HSCT with pre-transplant conditioning consisting of a combination of busulfan (1.9 mg/kg to a target cumulative area under the curve of 80 mg/L/hr), fludarabine (40 mg/m^2 x 4 doses) and alemtuzumab (0.2 mg/kg x 5 doses). The total white cell dose was 3.68x10^8/kg with a CD34 count of 7.03x10^6/kg and CD3 count 0.36x10^8/kg. There were no significant peri-transplant complications. Transplant outcome was excellent with 100% engraftment in myeloid lineage and stable mixed chimerism in T-cells (82% donor). Brain MRI imaging following transplant showed initial progression of the lesion

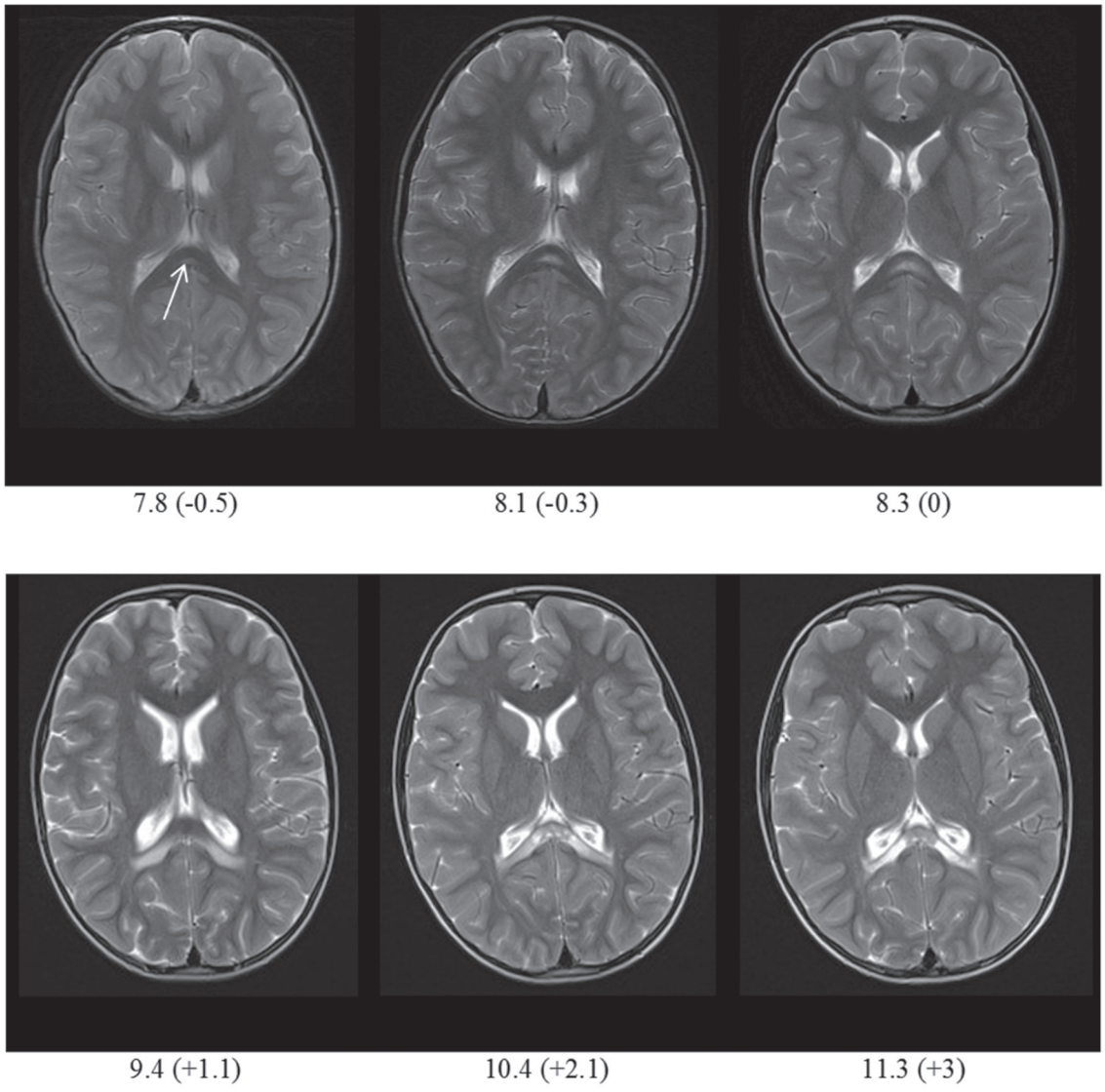

Figure 1. Brain magnetic resonance imaging, T2-weighted axial images. Top row: pre-transplant, bottom row post-transplant. Interval of scan in years (years before [-] or after [+] transplant). Arrow indicates increased signal lesion in splenium of corpus callosum that progressively enlarged pre-transplant, further progressed at 1.1 years post-transplant, but subsequent stabilisation

in the splenium at one year post-transplant, but subsequent stabilisation with no further progression through three years post-treatment (Figure 1). At this time the Loes score remained 3.

At last assessment at 11.5 years of age, the boy was attending mainstream secondary school where he was performing very well academically. He was on the 25th percentile for weight and 50th for height, and was being assessed for delayed puberty as possible late sequelae of either the transplant conditioning or primary testicular dysfunction due to ALD. He continued to receive hydrocortisone replacement but has not required mineralocorticoid replacement. Neurological examination revealed moderately brisk reflexes in the lower limbs but normal tone and power, and no gait disturbance.

Discussion

Recognition of PAI without adrenal antibodies in this boy with recurrent hypoglycaemic episodes prompted repeated analysis of VLCFAs and identification of the underlying ALD diagnosis before the onset of neurological symptoms. MRI surveillance detected early pre-symptomatic cerebral disease and permitted timely allogeneic HSCT, which was successful in arresting the progression of the cerebral disease. Glucocorticoid replacement requirements, stress-dosing guidelines, and monitoring for mineralocorticoid deficiency for patients with ALD are generally the same as those in other forms of PAI (17), and the patient continues on hydrocortisone replacement therapy without the need for mineralocorticoid supplementation currently.

Mineralocorticoid deficiency is less common in ALD (7,18), in part due to accumulation of VLCFA preferentially in the zona fasciculata and zona reticularis, with a relative sparing of the zona glomerulosa.

Since there is no ability to predict which boys diagnosed with ALD will develop CALD, vigilant MRI monitoring must be implemented in order to detect brain changes indicative of progression to CALD and ensure early HSCT (15). Allogeneic HSCT has been shown to have a beneficial effect on clinical indices of disease and long-term survival, but outcomes are more favourable if transplant is performed in the early stages of cerebral disease (10,19,20).

PAI can result from genetic or acquired diseases that affect adrenal function. Adrenal insufficiency in ALD may arise as a result of abnormal VLCFA accumulation that alters the viscosity of adrenocortical cell membranes, and prevents the stimulatory effects of ACTH on adrenocortical cells by inhibiting ACTH receptor binding (21). Impaired activity of ACTH leads to primary atrophy of the adrenal cortex with resulting cortisol deficiency. ALD is estimated to account for up to 5% of unexplained PAI cases (5,6). Among boys with ALD, approximately 70-80% have impaired adrenal function at the time of diagnosis (7,8,18).

In retrospective analyses of boys with ALD, PAI was the presenting and only sign in 25-37% of patients (8,18). There were delays of between 1 and 10 years between diagnosis of PAI and diagnosis of ALD for approximately half of the boys in the study. Delays in ALD diagnosis were associated with higher Loes scores at the time of evaluation for HSCT, progression of disease after HSCT, and decreased survival (8). These data emphasize that all boys with unexplained PAI should be screened for ALD.

Published diagnostic guidelines for PAI include recommended VLCFA testing for males with PAI who are negative for 21-hydroxylase (21-OH) antibodies, and concurrent testing for adrenal antibodies and VLCFA through accredited laboratories in preadolescent boys (22). However, despite these guidelines and older cases or case series highlighting the need to screen children and adult males with unexplained PAI for ALD (23,24,25), a recent study highlights the continued need for education that PAI in the absence of adrenal antibodies should be a "red flag" for a potential ALD diagnosis. Age appropriate ranges for VLCFA need to be used. Furthermore, other confounding factors, such as diets rich in rapeseed or mustard seed oils, which can potentially be associated with false negative results, need to be considered when performing VLCFA tests. Results from a pilot educational program intended to encourage reflex VLCFA testing in cases of PAI with negative 21-OH antibodies suggest that gaps in reflex testing remain (26).

Although NBS programs will assist in early identification and surveillance of boys with ALD (27), the potential for delayed diagnosis persists where NBS is not available and where absence of family history precludes family screening.

Conclusion

This case demonstrates that some ALD cases may still be missed when relying on VLCFA levels alone, since some mutations may not only result in initial normal levels of VLCFA but varying levels of adrenal insufficiency requiring clinical vigilance and suspicion for a potential underlying VLCFA abnormality. The need for early diagnosis in order to initiate current and future treatments mandates pursuit of a clear diagnosis and awareness of the need to screen boys with unexplained PAI for ALD.

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Ethics

Informed Consent: The patient's family provided informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Concept: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Design: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Data Collection or Processing: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Analysis or Interpretation: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Literature Search: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison, Writing: Michael R. Ryalls, Hoong-Wei Gan, James E. Davison.

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CASE REPORT

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Three Patient Kindred with a Novel Phenotype of Osteogenesis Imperfecta due to a *COL1A1* Variant

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What is already known on this topic?

Osteogenesis imperfecta (OI) is a heterogeneous connective tissue disorder characterized by reduced bone mass and increased bone fragility. Peak fracture rates in OI occur during the toddler and adolescent years, decline during adulthood, and increase again after age 55 years.

What this study adds?

We describe a kindred including three members who presented with a unique phenotype of OI, presumably due to a proline-to-leucine missense variant in the *COL1A1* gene. All three members had a pattern of prenatal bone deformities, followed by multiple, nontraumatic long bone fractures within the first two years of life and then an absence of nontraumatic fractures thereafter. To our knowledge, a clinical phenotype of OI characterized by cessation of nontraumatic fractures after the first two years of life has not been described previously.

Abstract

Osteogenesis imperfecta (OI) is characterized by fractures and progressive bone deformities. Fracture rates peak during the toddler and adolescent years and decline during adulthood but do not stop entirely. We describe a kindred, the affected members of which were the mother and two sons, who presented with an apparently unique phenotype of OI. Our patients demonstrated a pattern of prenatal bone deformities followed by multiple, nontraumatic long bone fractures within the first two years of life and then an absence of nontraumatic fractures thereafter. No extra-skeletal manifestations have been noted to date. The mother did not receive bisphosphonate therapy but had no nontraumatic fractures after the age of five months. Intravenous bisphosphonate therapy was started for both sons within two months of birth, with the most recent infusions at age 18 months and 28 months in Patients 2 and 3, respectively. Two patients harbored a variant of uncertain significance in the *COL1A1* gene. This heterozygous variant, c.3548C > T; p.(Pro1183Leu), is listed in the OI Variant Database as affecting only one other individual with osteopenia. We describe three family members with a unique presenting phenotype of OI, characterized by cessation of nontraumatic fractures after the first two years of life.

Keywords: Fragility fractures, collagen, child, bisphosphonates, bone density

Introduction

Osteogenesis imperfecta (OI) is a heterogeneous connective tissue disorder characterized by reduced bone mass and increased bone fragility (1,2). The disorder is primarily caused by variants in the genes involved in the synthesis or post-transcription modification of type 1 collagen (3). Type

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1 procollagen is composed of two proα1(I) and one proα2(I) chains that are encoded by the *COL1A1* (OMIM #120150) and *COL1A2* (OMIM #120160) genes, respectively (2). The triple-stranded procollagen molecule is assembled and then secreted into the extracellular space, where the propeptides are enzymatically cleaved to form mature collagen (4). Although there are numerous other OI-related genes, most cases of OI (85-90%) are caused by variants in the *COL1A1* and *COL1A2* genes.

Typically, point variants affecting glycine residues in the triple-helix structure of type 1 collagen have been predicted to disrupt protein folding, resulting in OI (5). The substitution of leucine for proline (Pro) in exon 49 of the *COL1A1* gene is listed in the OI Variant Database but to date, it has been associated with only one individual affected by osteopenia and it has been designated as a variant of unknown pathogenicity (6).

The severity of OI varies from mild, to progressively deforming, to perinatally lethal (7). Peak fracture rates in OI occur during the toddler and adolescent years, decline during adulthood, and increase again after age 55 years (8). However, to our knowledge, a clinical phenotype of OI characterized by cessation of nontraumatic fractures after the first two years of life has not been described previously.

We describe a kindred of three members who presented with a unique phenotype of OI, presumably due to a Pro-to-leucine missense variant in the *COL1A1* gene. All three members had a pattern of prenatal bone deformities followed by multiple, nontraumatic, long bone fractures within the first two years of life and then an absence of nontraumatic fractures thereafter.

Case Reports

We describe a nonconsanguineous family of European descent, in which three members were affected by OI (Table 1, Figure 1). Informed verbal consent was obtained from the mother.

Patient 1: Mother

The mother received the diagnosis of OI when she was 23 years old, after the birth of her second child (Patient 2), whose prenatal ultrasound suggested OI. The mother had nine nontraumatic long bone fractures, primarily femoral, before the age of five months. Her prenatal ultrasound findings were unknown. She did not receive bisphosphonate treatment but did not have any other nontraumatic fractures after five months of age. She had normal sclerae, dentition, hearing, echocardiogram and stature (159.8 cm, 25th percentile) (9). She had no spinal or cranial deformities, no joint hypermobility and did not bruise easily. We were unable to obtain data regarding her bone turnover markers and bone mineral density (BMD). She had an unaffected 17-year-old daughter. As of manuscript writing, Patient 1 was 39 years old.

Patient 2: Older Sibling

Patient 2 was the second child of Patient 1 and an unaffected father. The father's height was 188 cm (94th percentile) (9). At five months gestation, a prenatal ultrasound showed evidence of bowing of both femurs. Patient 2 was born at 38 weeks gestation via caesarean delivery without respiratory distress at birth. His birth weight was 4.28 kg (90th percentile), length was 52 cm (50th percentile) and head circumference was 38 cm (90th percentile) (9). At age three days, during circumcision, he had a right femur fracture (Figure 1). Subsequently, he had fractures of bilateral humeri and femurs at six days of life and sustained a fracture of the left femur at age four weeks. There was minimal or no trauma associated with any of these fractures.

A diagnosis of OI was considered and pamidronate infusions were initiated (0.75 mg/kg per day on two consecutive days, every two months with a total of eight infusions) from the age of six weeks through 18 months at another center. He tolerated the infusions well. Age-related developmental milestones were normal. At age three months, he was at the 95th percentile for length and 97th percentile for weight. His sclerae, hearing and dentition were normal. He had no spinal or cranial deformities, no joint hypermobility, and did not bruise easily. At age 14 months, his lumbar spine BMD was 0.51 g/cm^2 and left hip BMD was 0.57 g/cm^2 (reference range not available for this age group) (10). He had normal serum calcium (10.6 mg/dL; reference range 9-11 mg/dL) and total alkaline phosphatase (274 U/L; reference range 150-420 U/L).

Given the paucity of fractures (no fractures since age four weeks, despite being very active), pamidronate therapy was discontinued at age 18 months. As of manuscript writing, he was 17 years old, had normal ambulation and no deformities. A follow-up BMD was not available for him. His height was at the 75th percentile and weight was at the 95th percentile. His corrected midparental height was at the 65th percentile.

Patient 3: Younger Sibling

Patient 3 was born at 39 weeks gestation to Patient 1 and a different father. The father's height was 170 cm (17th percentile) (9). At 26 weeks gestation, an ultrasound showed bowing of both femurs. Patient 3 was born via caesarean delivery, without respiratory distress at birth.

Table 1. Clinical characteristics of kindred cases

	Patient 1 Mother	Patient 2 Older sibling	Patient 3 Younger sibling
Current age	39 y	17 y	36 m
Current height (percentile)	25	75	27
Fractures, age	R femur, <5 m L femur, <5 m	R femur, 3 d R humerus, 6 d L humerus, 6 d R femur, 6 d L femur, 6 d L femur, 4 w	L 9th rib, 1 d R 8-10th rib, 2 w R tibia, 5 w R femur, 5 w L femur, 5 w R femur, 6 w L femur, 6 w L tibia, 15 w L5 compression, 21 m
Total fractures, n	9	6	11
BMD (g/cm²), age	N/A	14 m Lumbar spine: 0.51 L hip: 0.57	**12 m** Lumbar spine: 0.44 TBLH: 0.34 **28 m** Lumbar spine: 0.54 TBLH: 0.46
Bisphosphonate treatment	No	Yes Pamidronate	Yes Zoledronic acid
Total infusions, n	-	8	6
Age at last infusion	-	18 m	28 m
Sclerae	Normal	Normal	Normal
Dentition	Normal	Normal	Normal
Hearing	Normal	Normal	Normal
Echocardiogram	Normal	N/A	N/A
Spinal deformity	None	None	None
Extremity deformity	None	None	None
Joint mobility	Normal	Normal	Normal
Easy bruising	None	None	None
Genotype	c.3548C>T; p.(Pro1183Leu) Heterozygous	c.3548C>T; p.(Pro1183Leu) Heterozygous	N/A

y: years; m: months, d: days, w: weeks, R: right, L: left, L5: 5th lumbar vertebrae, BMD: bone mineral density, N/A: not available, TBLH: total body less head

His birth weight was 3.8 kg (75th percentile), length was 48 cm (25th percentile) and head circumference was 36.5 cm (65th percentile). Radiographic studies on the first day of life confirmed left posterior ninth rib fracture and femoral bowing bilaterally (Figure 2). Wormian bones were noted along the lambdoid sutures. He had nontraumatic fractures of the eighth through tenth ribs on the right side at two weeks, right tibia and bilateral femur fractures at five weeks, bilateral femur fractures at six weeks (distinct locations each time) and left tibia fracture at 15 weeks.

Physical examination showed that he had white sclerae and no dysmorphic features. His hearing screen was normal. Serum ionized calcium (5.89 mg/dL; reference range 3.9-6.0 mg/dL), phosphorus (6.3 mg/dL; reference range 4.5-9.0 mg/dL), total alkaline phosphatase (250 U/L; reference range 150-420 U/L) and 25-hydroxy-vitamin D (37 ng/mL; reference range ≥20 ng/mL) concentrations were normal. Zoledronic acid infusions were initiated every three months from age three weeks to nine months (0.0125 mg/kg for the first dose, 0.025 mg/kg for the next three doses, each infused over 60 minutes). The fracture at age 15 weeks occurred after he had received two infusions of zoledronic acid. Given the paucity of further symptoms of OI and the fact that his mother and older sibling had no fractures after

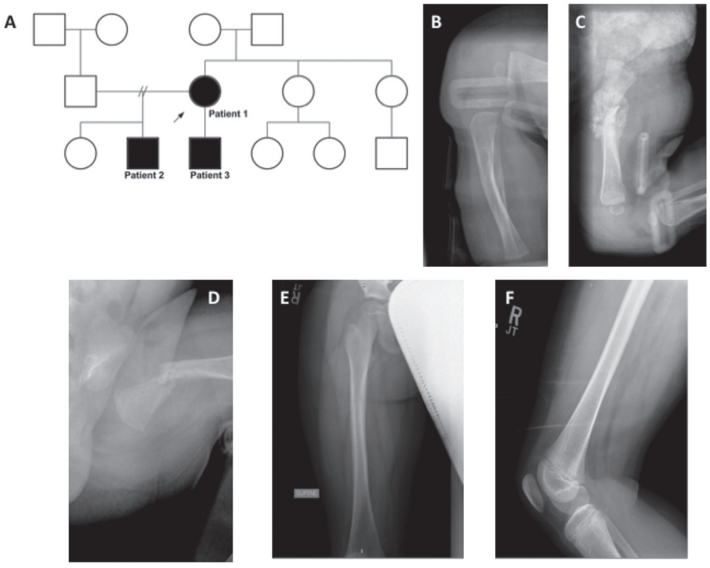

Figure 1. (A) Pedigree chart. The index case is indicated by the arrow. (B-F) Radiographs of Patient 2. (B) Bowing of midright tibial shaft at age three weeks, lateral view. (C) Abundant callus formation around a healing midshaft right femoral fracture at age three weeks, lateral view. (D) Acute proximal-shaft left femoral fracture at age four weeks, anteroposterior view. (E) Right femur anteroposterior view at age 10 years. (F) Right femur lateral view at age 10 years

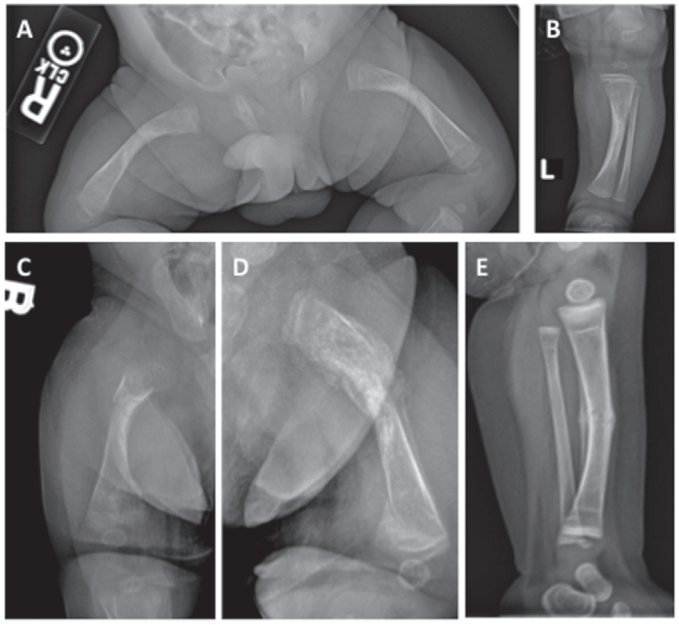

Figure 2. Radiographs of Patient 3. (A) Proximal femoral shaft bowing, right greater than the left, at age two weeks, anteroposterior view. Note the mild diffuse osteopenia. (B) Left tibial midshaft bowing at age two weeks, anteroposterior view. (C) Proximal right femoral fracture at age six weeks, lateral view. (D) Periosteal reaction and callus formation around a healing proximal left femoral fracture sustained at age six weeks, lateral view. (E) Transverse midshaft fracture of left tibia at age 15 weeks, lateral view

the first few months of life, bisphosphonate therapy was discontinued when he was nine months old.

At age 21 months, he was noted to have a new mild compression of the anterior-superior endplate of the 5th lumbar vertebral body (L5). He also had a mildly displaced oblique fracture of left distal tibial metaphysis due to moderate trauma (his leg was caught on the edge of a slide while sitting on his father's lap) at age 25 months. He was treated with two additional infusions of zoledronic acid at 21 months and 28 months (a total of six infusions). He has not sustained additional low-trauma fractures since. The mild L5 vertebral body deformity was less apparent on subsequent imaging seven months later.

He achieved normal developmental milestones, including appropriate dentition for age. Baseline BMD was obtained at 12 months (lumbar spine 0.44 g/cm^2; total body excluding head 0.34 g/cm^2; reference range not available for this age group) (10). A follow-up BMD at age 28 months after six zoledronic acid infusions revealed an improvement of 23.7% in spinal bone density (0.54 g/cm^2) and 36.5% in total body, excluding head, bone density (0.46 g/cm^2), compared to baseline. As of manuscript writing, he was 36 months old and at 27th percentile for length and 81st percentile for weight. His corrected midparental height was at the 20th percentile.

DNA Sequencing and *in silico* Analysis

Clinical information was collected from the patients and abstracted from the medical records. Conformation-sensitive gel electrophoresis (CSGE) was performed by Matrix DNA Diagnosis (New Orleans, LA, USA). The variant was described using the Human Genome Variation Society nomenclature (11). The pathogenicity of the identified variant was analyzed using the *in silico* prediction software, Alamut Visual (missense predictors: Align GVGD v.2007, SIFT v.6.20 and PolyPhen-2 HumVar; splicing algorithms: SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer and HSF) (12).

Results

Identification of the *COL1A1* Variant

Given the concern for OI in Patient 2, CSGE was performed for the *COL1A1-* and *COL1A2-* coding exons. The CSGE analysis identified a heterozygous missense alteration in exon 49 of the *COL1A1* gene resulting in a C to T nucleotide substitution that converted a Pro (CCT) to a leucine (CTT), [NM_000088.3:c.3548C > T; p.(Pro1183Leu), (RefSeqGene NG_007400.1:g.19734C > T)].

The patient's mother was heterozygous for the same variant and no variations were present in the father. The variant was classified as 'variant of uncertain significance'. *In silico* analysis was equivocal for potential pathogenicity of this variant (Align GVGD - Class C65 deleterious; SIFT - deleterious; PolyPhen-2 - benign). No significant changes were noted in canonical splice sites.

Discussion

We describe a kindred with a unique presenting phenotype of OI. The OI was presumably attributable to a missense variant c.3548C > T; p.(Pro1183Leu) that replaced Pro with leucine in the Y position of the glycine-Xaa-Yaa sequence of the proα1(I) chain (exon 49 of *COL1A1*). The same variant has been reported previously in only one individual [c.3548C > T; p.(Pro1183Leu)] (6). The referral reason for testing that individual was reported as symptoms of OI and a strong family history of recurrent femoral fractures and osteopenia. The variant was designated to be of unknown pathogenicity, and further family studies to clarify pathogenicity were requested. As of manuscript writing, that patient was 49 years old, and updates on the patient and results from family studies could not be obtained (personal communication, Dr. Meena Balasubramanian, 2018). As more individuals with this specific variant are identified, the clinical phenotype of this form of OI will become clearer.

Lim et al (13) described a patient with Ser40Trp variant in *IFITM5* gene, who presented with multiple fractures in the prenatal period. She remained fracture free after birth with normal BMD. She had blue sclerae, progressive lower limb deformities during childhood and severe short stature (standard deviation -3.5). Her mother, who did not have a history of fractures, was noted to have somatogonadal mosaicism for this variant and became pregnant with a second child with multiple prenatal fractures, found to have the same variant. This is in contrast with our patients who had normal sclerae, absence of postnatal limb deformities, normal stature and a variant in the *COL1A1* gene, although with similar phenotypic bone deformity pattern.

Type 1 procollagen helical domains contain a repetitive glycine-Xaa-Yaa sequence, where Xaa and Yaa are often the amino acids Pro or hydroxyproline (Hyp) (14,15). Some evidence suggests that Pro and Hyp are responsible for the thermal stability of the triple-helix structure (16). Bryan et al (17) hypothesized that a missense variant in the highly stable region of the collagen molecule, such as Gly-Pro-Hyp, will lead to a greater disruption and more severe clinical consequences than the same missense variant in a less stable region. Collagen chains with these abnormal molecules are overmodified and secreted inefficiently, which disrupts helix stability (18).

Various factors have been proposed to explain how the substitution of one amino acid for another affects collagen processing and the phenotype of OI, including the coordinates of the variant and its position relative to the C-terminus of the propeptide (5,14,19). The proximity of a variant to the C-terminus of the molecule is important because assembly of the collagen triple-helix chain begins at the C-terminal end and propagates toward the N-terminal end (20). Our kindred harbored a variant close to the C-terminus, at amino acid position 1183 on exon 49 of the *COL1A1* gene. Exon 49 is 283 base pairs long (cDNA base numbers 3532-3814) and encodes the last 15 amino acids of the triple-helix region (amino acids 1178-1192), the entire C-telopeptide (amino acids 1193-1218) and part of the C-propeptide domain (amino acids 1219-1271). A small number of point variants in the C-terminal propeptide have been identified as causing varying clinical phenotypes of OI, ranging from mild to lethal and high bone-mass OI (18,19,21). Although missense variants in the C-propeptide are known to impair or prevent proα chain assembly in the endoplasmic reticulum (19), the outcome of each substitution may differ, depending on the specific gene transcript.

For patients with milder forms of OI, the incidence of fracture declines with age but does not stop entirely (8). Interestingly, cessation of OI associated fractures after the first two years of life, as seen in our patients, has not been described in the literature in association with *COL1A1* variants. The pathophysiology resulting in this phenotype remains uncertain. There is evidence that posttranslational modification of procollagen is temporally regulated, and this regulation may be crucial for its folding, secretion, and extracellular matrix assembly (22). We speculate that a sequential improvement in these processes during the first two years of life in our patients resulted in paucity of fractures thereafter.

Finally, the long-term benefit of intravenous bisphosphonate therapy in our patients remains unclear, particularly because Patient 1 did not receive this therapy and did not sustain any nontraumatic fractures after five months of age. Patient 2 received his last infusion of pamidronate at age 18 months. At age 17 years, he was of normal adult height and remained fracture free, despite an unrestricted, active lifestyle. We speculate that prolonged bisphosphonate therapy may not provide additional benefit to those with this form of OI, although our sample size and duration of follow-up is too small to draw a definitive conclusion.

Our case series has certain other limitations including lack of genetic testing for variants in other OI-related genes such as *IFITM5*, which have been associated with a phenotype similar to that seen in our patients. Patient 1 and Patient 2 underwent genetic testing almost 17 years ago. To our knowledge, at that point in time, typical OI genetic testing included primarily *COL1A1* and *COL1A2* genes. Currently, we do not have the funding available to update genetic testing for Patient 1 and Patient 2, or send genetic testing for Patient 3 for research purposes. It would have entailed a huge economic burden on the family and the genetic testing results were unlikely to influence clinical decision making.

Conclusion

We describe a three-patient kindred with a unique phenotype of OI, presumably due to a variant in the *COL1A1* gene. The OI phenotype of affected individuals included a pattern of mild-moderate bone deformities prenatally and multiple nontraumatic fractures limited to the first two years of life. We speculate that the Pro-to-leucine substitution in close proximity to the C-propeptide domain might have influenced folding of the triple helix or helix stability. Given the small number of patients in our kindred, the full range of phenotypes associated with this variant remains to be established. Whether antiresorptive therapy is beneficial later in life also remains unclear.

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Ethics

Informed Consent: Informed verbal consent was obtained from the mother.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Concept: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Design: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Data Collection or Processing: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Analysis or Interpretation: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Literature Search: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben, Writing: Nidhi Gupta, Seth W. Gregory, David R. Deyle, Peter J. Tebben.

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CASE REPORT

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Two Subsequent Metachroneus Solid Tumors: Oncocytic Variant Adrenocortical Carcinoma and Rhabdomyosarcoma of Childhood: Case Report and Literature Review

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What is already known on this topic?

Adrenocortical carcinoma can be related to other cancers.

What this study adds?

Sequence analysis should be performed if fluorescence *in situ* hybridization analysis is negative. Analysis of Hounsfield unit density of the tumor on imaging can help to distinguish malign from benign. The Lin-Weiss-Bisceglia system should be used instead of the Weiss system for the oncocytic variant.

Abstract

Most cases of malignancies appear to be sporadic, but some syndromes are associated with malignancies with germline variants. Herein, a child with an unusual association of oncocytic variant adrenocortical carcinoma (ACC) and rhabdomyosarcoma (RMS) was presented. An 18-month-old-boy was admitted with virilization of the genital area, penis enlargement and erection, which had begun six months earlier. Serum total testosterone (457 ng/dL; NR < 10), androstenedione (3.35 ng/mL; NR < 0.5) and dehydroepiandrosterone-SO_4 (206 mcg/dL; NR < 35) were above the normal ranges. Right adrenal mass was detected. After adrenalectomy, histopathological examination revealed an oncocytic variant ACC. Three-month after surgery, he then presented with 6x8 cm sized swelling of the left leg. Histopathological examination revealed embryonal RMS. Testing for tumor protein (TP53) variant by DNA sequence analysis was positive; however; fluorescence *in situ* hybridization analysis was negative. After chemotherapy and local radiotherapy, the patient is in good condition without tumor recurrence. Only about one-third of these tumors have a variant of TP53. This status also applies to other genetic variants related to cancer. However, a significant association of malignancies strongly suggests a problem in tumor suppressor genes or new variants. Another as yet unidentified suppressor gene may also be present and effective in this locus. The occurrence of ACC as a part of a syndrome and positive family history of malignancies in patients are clinically important. These patients and their families should be scanned for genetic abnormalities. The patient with ACC should be followed-up carefully for other tumors to detect malignancy early.

Keywords: Child, adrenocortical carcinoma, oncocytic variant, rhabdomyosarcoma, TP53

Introduction

Oncocytic variant adrenocortical carcinoma (ACC) is an extremely rare tumor, with an incidence of 0.72 per million of population annually (1,2). In our country, ACCs account for 6.9% of carcinomas and other malignant epithelial tumors, and 0.19% of childhood cancers (3). Only 147 cases with adrenal oncocytic neoplasm have been reported in the literature (4). Although most cases of ACCs are sporadic, ACCs have an association with hereditary cancer syndromes such as Li-Fraumeni syndrome (LFS) and Beckwith-Wiedemann syndromes (BWS) (5). The pathogenesis of sporadic ACCs is less well understood. Tumor suppressor genes have a

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significant role in the development of cancer (6). Breast cancer, soft tissue and bone sarcoma, brain tumors, Wilms' tumor, neuroblastoma, and hepatoblastoma are some types of cancer that are associated with ACCs. However, well-known oncogenes/tumor suppressor variants are not relevant to each malignancy. Environmental factors and decreased penetrance of a particular gene defect cannot be excluded (6).

In this report, we described clinical features of an oncocytic variant ACC presenting as peripheral precocious puberty, and in whom embryonal rhabdomyosarcoma (RMS) developed as a second malignancy. Moreover, the genetic variant is discussed.

Case Report

An 18-month-old-boy was admitted at the department of pediatric endocrinology with virilization of the genital area, penis enlargement and erection, which had begun six months earlier. Informed consent was obtained from the parents of the patients. There were no relevant features in his personal nor his family history. There is no consanguinity.

Anthropometric Evaluation

At presentation his height was 86 cm (78th percentile), weight 14.5 kg (95th percentile), chronological/height ages were 1.61/1.88 years old, height standard deviation score (SDS)/target height/target height SDS were +0.80/165 cm/-1.65, respectively. The gap between height SDS and target height SDS was likely due to accelerated growth velocity. Although the patient had phallic enlargement and pubarche for six months, his testicular size was consistent with the prepubertal period with 2 mL/2 mL. Hemihypertrophy was not detected.

Laboratory Evaluation

Biochemical and complete blood count tests were within normal limits. His bone age was advanced and compatible with three years. Serum analytes wereall in the normal range as follows: follicle-stimulating hormone = 0.3 IU/L (NR 0.3-4.6), luteinizing hormone = 0.12 IU/L (NR 0.1-6.0), 17-OH progesterone = 0.5 ng/mL (NR 0.1-0.9), T4 = 0.96 ng/dL (NR 0.81-1.73), thyroid stimulating hormone = 2.84 mIU/mL (NR 0.8-6.26), adrenocorticotropic hormone = 21.7 pg/mL (NR 0-46), cortisol = 10.7 mcg/dL (NR 4.3-22.4), beta-human chorionic gonadotropin < 0.2 IU/mL (NR 0-10), alpha-fetoprotein = 2.1 ng/mL (NR 0-8.1) and urinary vanillylmandelic acid = 2.5 mg/g creatinine (NR < 18). However, serum total testosterone 457 ng/dL (NR < 10), androstenedione 3.35 ng/mL (NR < 0.5) and dehydroepiandrosterone (DHEA)-SO_4 206 mcg/dL (NR < 35) were all above the normal range.

Radiologic Evaluation

Abdominal ultrasonography (USG) revealed right adrenal, 22x17 mm-sized mass. Scrotal ultrasound was normal. Magnetic resonance imaging (MRI) revealed a 16x14x17 mm mass, which was isointense with muscle in T1 and hyperintense in T2 images. Abdominal computed tomography (CT) showed a 16x11 mm nodular, hypodense mass of 35 Hounsfield units (HUs).

Surgery, Pathology and Hormonal Changes

The patient underwent adrenalectomy, and the mass was resected in one piece. Histopathological examination revealed oncocytic variant ACC with a weight of 50 g and a volume of 2 cm^3. Histologically, the surgical border of the tumor, which was limited to the adrenal gland and capsule of the adrenal gland was intact. Neoplastic tissue had an expansile pattern. Normal adrenocortical cell components were seen in some sections and mixed with neoplastic cells. Retroperitoneal lymph node involvement and vascular invasion were not seen. Immunohistochemical analysis was positive for adrenal cortical specific markers "MART-1 and inhibin", neuroendocrine and adrenal medulla/cortex marker "synaptophysin", and negative for adrenal medulla stain "chromogranin" and marker of Schwann cell "solubility in 100% saturated ammonium sulfate at neutral pH (S100)", and renal epithelial marker "epithelial membrane antigen (EMA)". The Ki-67 proliferation index was 10%. Twenty mitoses were counted per 50 high power fields with the help of cell proliferation marker "Phospho-Histone H3 (PHH3)" (Figure 1). Serum total testosterone, androstenedione and DHEA-SO_4 levels decreased to < 10 ng/mL, < 0.3 ng/mL and 102 mcg/dL at the 12th postoperative hour and < 10 ng/mL, < 0.3 ng/mL and 57 mcg/dL at the 24th postoperative hour (Table 1).

Treatment

Positron emission tomography (PET)-CT and thoraco-abdominal CT were reported normal as part of the staging workup. Staging of this oncocytic variant ACC, according to the Children's Oncology Group (7) classification was evaluated as stage 1. No further therapy was planned due to the group 1 staging (complete resection and tumor volume < 200 cm^3 and negative markers after surgery) according to TREP (Rare Tumors in Pediatric Age) (8). No further medical treatment was given to the patient. He was followed-up closely with physical examination and USG.

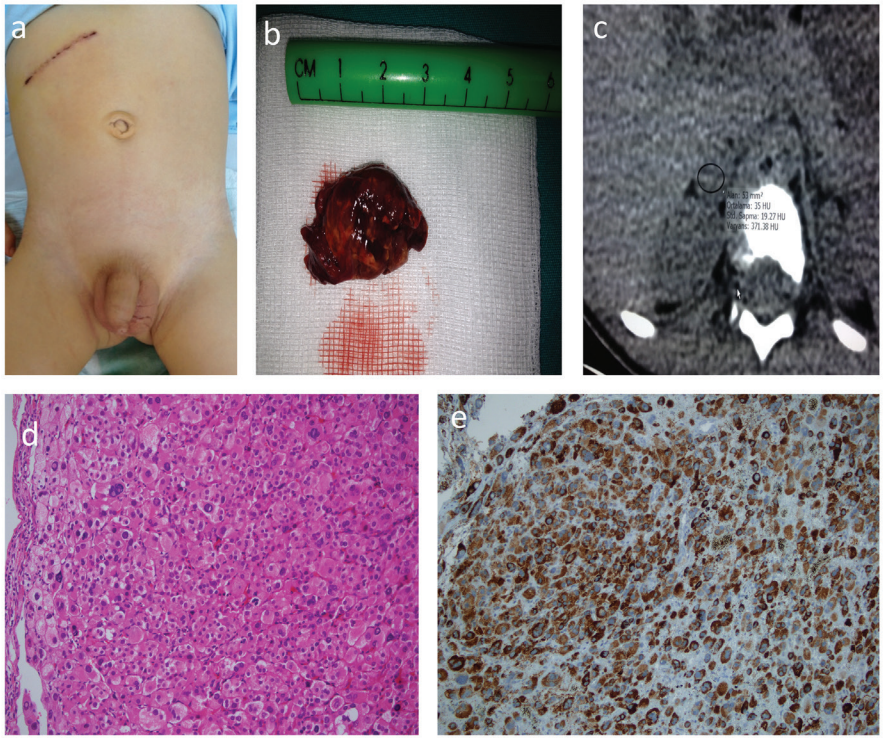

Figure 1. a) Enlargement of phallus, stage 2 pubic hair development. b) Abdominal computed tomography showing right adrenocortical carcinoma. c) The mass after adrenelectomy. d) Oncocytic adrenocortical carcinoma, oxyphilic cell population, hematoxylin-eosin, immunohistochemical expression, (magnification x200). e) Oncocytic adrenocortical carcinoma, Melan-A (MART-1) immunohistochemical expression, (magnification x200)

Evaluation After Relapse

Three months after adrenalectomy, the child represented with swelling of the left leg with a few weeks of history. A painless, immobile, rough, 6x8 cm swelling between the proximal anteroposterior femoral and the inguinal region was detected. MRI scanning revealed 9x4x3.5 cm mass, which was isointense with muscle in T1, hyperintense in T2 images with dense contrast enhancement. The mass compressed to the superficial femoral vein and surrounded the femoral artery for 200/360 degrees. A tru-cut biopsy was performed. The pathology result was reported as compatible with metastasis of oncocytic variant ACC without immunohistochemistry. After extensive resection of the mass, histopathological examination revealed embryonal RMS, which was 8.5x5x4 cm, with a grey-white color, and solid (Figure 2). Histologically, the surgical border of the tumor, which was limited to muscle was intact with 5% focal necrosis. Lymphovascular invasion was not detected. Immunohistochemical analysis was positive for desmin, Myo-D1, and muscle-specific-actin. MART-1, synaptophysin, S100, inhibin, chromogranin, CK7, CK20, MPO, and EMA were negative. The Ki-67 proliferation index was 70% (Figure 2). PET-CT and thoraco-abdominal CT evaluation were found to all be normal as part of the staging workup. Lymph node and pulmonary involvement were not detected. The extent of the RMS was defined according to the Intergroup RMS study (IRS) 3 staging system. The patient was evaluated as pretreatment stage 3, postoperative group 1, and low subset B risk.

Table 1. Abnormal hormanal levels changes of patient before and after operation

	Preoperative	Postoperative 12th hour	Postoperative 24th hour
Total testosteron (ng/dL)	**457**	< 10	< 10
Androstenedion (ng/mL)	**3.35**	< 0.3	< 0.3
DHEA-SO_4 (mcg/dL)	**206**	102	57

DHEA-SO_4: dehydroepiandrosterone

Genetic Evaluation

A pedigree chart was made. No consanguinity was present. Karyotype analysis of the patient was normal (46; XY). Testing for tumor protein (TP53) variant by DNA sequence analysis was positive in the peripheral blood sample, but fluorescence *in situ* hybridization was negative.

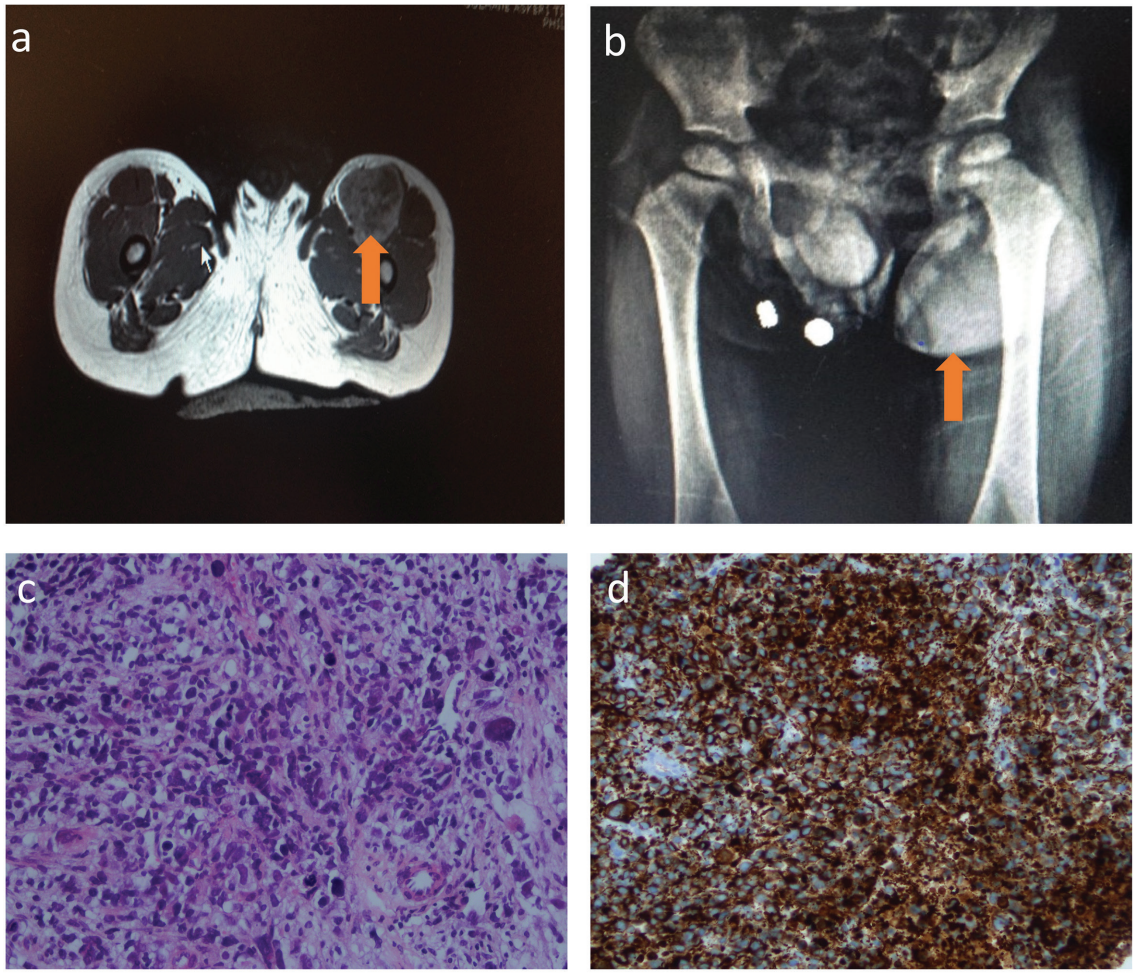

Figure 2. a) Femoral magnetic resonance imaging showing left side rhabdomyosarcoma. B) X-ray graphy showing left femoral mass. c) Rhabdomyosarcoma, hematoxylin-eosin, (magnification x400). d) Rhabdomyosarcoma, Desmin positivity, (magnification x200)

Treatment

The patient was treated with chemotherapy consisting of vincristine, cyclophosphamide, and actinomycin-D according to the protocol POG D-9602-VAC. Revision of pathological samples was performed again. Sarcomatous component of the oncocytic variant ACC was not found. The patient was evaluated ten weeks after starting the VAC regimen, and he was in remission at both the ACC and RMS sites. His disease was discussed with the radiotherapy department for local therapy of extremity RMS. Radiation oncology refused to give radiation therapy owing to remission of disease and because the patient was aged under three years. At the 24th week of treatment, a 2x2 cm sized, inguinal mass in the left sartorius muscle, which was enhanced after injection of contrast agent, and compatible with lymphadenopathy was detected with MRI. The mass was excised, and the pathological result was compatible with RMD metastasis. The patient was evaluated as relapse and progression under chemotherapy treatment, and the treatment was changed with ifosfamide, carboplatin, etoposide (ICE) regimen. After three cycles, he was in remission. Radiotherapy was given for local control of RMS. After the sixth cycle of ICE, the patient was in good condition with no tumor recurrence, and treatment was stopped. However, he came back six months later with left leg pain due to a mass and the mass-effect on the femoral artery and nerve. Thrombosis and malign mesenchymal tumour protocol and sorafenib were started. Surgeons recommended amputation due to nerve and lymphatic invasion of the tumor. However, the patient family refused the amputation. He died, due to progressive disease, 2.5 years after diagnosis.

Discussion

Although most cases of malignancies appear to be sporadic, some syndromes that are associated with malignancy or malignancies can be detected in oncology practice. Even if an association is not found, unusual associated malignancies can be evaluated for germline variants. In this study, we

aimed to describe an association of cancers with no family history in a child with oncocytic variant ACCs and RMS with the help of genetic evaluation.

Patients with LFS, BWS, and multiple endocrine neoplasia type 1 (MEN-1) are at risk for developing certain types of cancers, such as ACC and RMS (9). LFS especially is associated with a 40% risk of malignancy before the age of 16 years, high mortality rates, and second primary malignancies, and is an autosomal disorder (10,11). *TP53* tumor suppressor gene on chromosome 17p13 in LFS, *GNAS 1* variant and abnormalities of 11p15.5 in BWS and variants of the *MEN-1* gene on chromosome 11q13 in MEN-1 may be detected in some patients with malignancy (12,13,14,15). Six per cent of patients with second malignancies and no familial features of LFS had a germline *TP53* variant in a sample of 59 patients (16). Germline *TP53* variants with no familial features of LFS are identified in 50-80% of children with ACC and 10% with RMS (17,18). A variant related to malignancy may not be detected, as was the case in our patient. In this study, an extensive pedigree was obtained. No family history of any other malignancies was documented in this family. Our patient did not have clinical features of BWS and McCune-Albright syndrome. Thus, we decided to evaluate genetically because of sporadic malignancy.

ACC is an extremely rare tumour. It accounts for 0.2% of childhood cancers (12). In our country, ACC accounts for 6.9% of carcinomas and other malignant epithelial tumours, and 0.19% of childhood cancer (3). Although most cases of ACCs appear to be sporadic, some have been described as a component of several hereditary cancer syndromes (9). Virilization can be seen owing to increased DHEA and DHEA-SO_4 production (13). In this case, DHEA-SO_4, androstenedione, and testosterone were all above the reference range contemporary with virilization. The absence of hormonal hyperactivity is associated with poor prognosis because of the advanced stage of the tumor at diagnosis (14). The hormonally active status of this case was very significant in early diagnosis together with the evident virilization. The levels of total testosterone, androstenedione, DHEA-SO_4 were highly elevated at the time of diagnosis. After surgery, the levels of these hormones decreased to an average level in the 24th hour. The survival rates of stage 1 are higher than others (15). Our patient was followed-up without treatment after surgery. The primary site was normal in the follow-up.

Oncocytic variant ACC is a rare disease with low incidence. More than 80% are benign or with low malignant potential. It has been described in only 147 cases between 1986 and 2013 (4,16,17). Eighty per cent were detected incidentally owing to non-functional adrenal mass (4). In addition to this, a sarcomatous component may be present (1). RMS as a second cancer developed in the follow-up. RMS represents 6.5% of childhood cancers, and 52.9% of soft tissue sarcomas (3). Somatic variants of the *TP53* gene can be seen in as many as 50% of cases. However, germline variants are much less common and tend to be associated with a lower age (average age 22 months) at presentation (18,19). Although this case was hormonally active, the sarcomatous component was not found.

There is no definitive pattern on CT scan or MRI (4). On the CT Hounsfield Scale, adenoma/hyperplasia and carcinoma are assigned a value of 16.2 ± 13.6 and 36.9 ± 4.1, respectively (20). Specificity and sensitivity of PET-CT are $> 95\%$ (21). The mass was compatible with non-adenoma owing to a Hounsfield Scale value of 35 HU. PET-CT was performed after surgery. Evaluation of disease with PET-CT was normal in staging workup.

Clinicopathological, oncocytic variant ACC differs from conventional ACC. There is no preference for males or females. It is smaller and lighter than conventional ACC and tends to hold the left side. The oncocytic variant has rare mitoses including no atypia, low rate of necrosis, fibrosis, and venous, sinusoidal, and capsular invasion (22). These features were mostly compatible with the pathology and clinical features in our case, except the high mitotic rate and involvement of the right side.

Proposed major criteria including a mitotic rate of more than five mitoses per 50 high power fields, any atypical mitoses or venous invasion, and minor criteria including large size/weight (> 10 cm and > 200 g), necrosis, capsular invasion or sinusoidal invasion, have been investigated for distinguishing malignant tumors by Bisceglia et al (23). Defining criteria for oncocytic tumors have been outlined that include predominantly cells with eosinophilic and granular cytoplasm, a high nuclear grade, and a diffuse architectural pattern. The presence of one major criterion indicates malignancy, 1-4 minor criteria indicating uncertain malignant potential (borderline) and the absence of all major and minor criteria are indicative of a benign mass. In the case presented the mitotic rate was more than five mitoses per 50 high power fields. When mitosis was evaluated with PHH3, 20 mitoses were counted per 50 high power fields. Venous, capsular, and sinusoidal invasions and necrosis were not detected. Thus the ACC tumor in our patient met all defining criteria. The mass < 100 g was compatible with a good prognosis (24). Our patient was evaluated as stage 1 because his adrenal mass under < 100 g with total excision. However, it was assessed as a malign mass owing to the one major criterion present, that was a high mitotic rate.

The molecular pathogenesis of sporadic ACCs is less well understood. Activation of proto-oncogenes and oncogenes on chromosome 4, 5, and 12, and inactivation of tumor suppressor genes on chromosome arms 1p and 17p may be related to progression from adenoma to carcinoma (25). Loss of heterozygosity (LOH) at 17p13 is common, but only about one-third of these tumors are associated with a variant of TP53. However, T*P53* might not be the only or major tumor suppressor gene at 17p related to ACCs. Another suppressor gene, which is as yet unidentified, may be present and effective in this locus and there is evidence to support this hypothesis (26). ACCs are associated with multiple somatic gene alterations and thus it is difficult to identify the exact genetic changes (27). Amplification of the steroidogenic factor-1 gene as well as germline *TP53* variant in Southern Brazil, LOH of 11p15 with overexpression of insulin-like growth factor-2 as well as other growth-related tumor suppressor genes at this locus may explain this (28,29).

Study Limitations

Unfortunately, there was only resource enough to investigate *TP53* in this case, which is the limitation of our study. However, the *TP53* variant was positive in sequence analysis.

Use of imaging techniques that use ionizing radiation, such as PET and CT scans, during the follow-up of this small child at risk of other malignancies was inadvisable. Therefore, MRI and abdominal USG were used to avoid repeated irradiation after the second malignancy. External radiotherapy to the leg after 10-weeks from the beginning of chemotherapy was not given by the radiotherapy department owing to remission of the disease. However, it was agreed to provide radiotherapy after the recurrence of the disease for local control.

The occurrence of ACC as a part of a syndrome is clinically significant because of the choice of treatment, caution with radiotherapy in patients with LFS, individualized screening for other cancers in these syndromes with mammography, colonoscopy, and identification family members at risk (30).

Conclusion

A multidisciplinary team approach, including oncology, surgery, endocrinology, pathology, radiation oncology, and genetic counselling is necessary. The cost-effectiveness of cancer screening with colonoscopy is not considered controversial for well-defined common cancers such as colon cancer, but no data are available for ACCs. Genetic evaluation should be suggested for patients with a second primary cancer. An unusual association of malignancies with the absence of a positive family history of malignancies strongly suggests a problem in tumor suppressor genes or new variants. The present case with an initial ACC tumor and a subsequent RMS tumor had a *TP53* variant and inactivated *TP53* is present in about half of all human cancers. Whole genome analyses will provide important information on the development of ACCs and secondary cancer in the future.

Ethics

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Erman Ataş, İrem Ayşe Atasoy, Nihal Durmaz, Concept: Onur Akın, Erman Ataş, Ömer Kartal, Design: Erman Ataş, Nihal Durmaz, Data Collection or Processing: Erman Ataş, İrem Ayşe Atasoy, Analysis or Interpretation: Onur Akın, Erman Ataş, Nihal Durmaz, Literature Search: Erman Ataş, İrem Ayşe Atasoy, Ömer Kartal, Writing: Onur Akın, Erman Ataş, İrem Ayşe Atasoy.

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CASE REPORT

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Clinical and Genetic Characteristics of Patients with Corticosterone Methyloxidase Deficiency Type 2: Novel Mutations in *CYP11B2*

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What is already known on this topic?

Corticosterone methyloxidase deficiency type 2 is an autosomal recessive disorder which presents with salt loss and failure to thrive in early childhood. It is caused by inactivating mutations of *CYP11B2*. To date, approximately 56 mutations have been identified in the *CYP11B2* gene.

What this study adds?

We describe four Turkish patients from two families who have clinical and hormonal features compatible with corticosterone methyloxidase deficiency and had inherited novel *CYP11B2* mutations.

Abstract

Corticosterone methyloxidase deficiency type 2 is an autosomal recessive disorder presenting with salt loss and failure to thrive in early childhood and is caused by inactivating mutations of the *CYP11B2* gene. Herein, we describe four Turkish patients from two families who had clinical and hormonal features compatible with corticosterone methyloxidase deficiency and all had inherited novel *CYP11B2* variants. All of the patients presented with vomiting, failure to thrive and severe dehydration, except one patient with only failure to thrive. Biochemical studies showed hyponatremia, hyperkalemia and acidosis. All patients had normal cortisol response to adrenocorticotropic hormone stimulation test and had elevated plasma renin activity with low aldosterone levels. Three patients from the same family were found to harbor a novel homozygous variant c.1175T > C (p.Leu392Pro) and a known homozygous variant c.788T > A (p.Ile263Asn) in the *CYP11B2* gene. The fourth patient had a novel homozygous variant c.666_667delCT (p.Phe223ProfsTer35) in the *CYP11B2* gene which caused a frame shift, forming a stop codon. Corticosterone methyloxidase deficiency should be considered as a differential diagnosis in patients presenting with hyponatremia, hyperkalemia and growth retardation, and it should not be forgotten that this condition is life-threatening if untreated. Genetic analyses are helpful in diagnosis of the patients and their relatives. Family screening is important for an early diagnosis and treatment. In our cases, previously unreported novel variants were identified which are likely to be associated with the disease.

Keywords: Aldosterone synthase deficiency, salt wasting, *CYP11B2* gene, corticosterone methyloxidase type 2, failure to thrive

Introduction

Aldosterone is a steroid hormone synthesized by corticosterone methyloxidase (CMO) and secreted from the zona glomerulosa of the adrenal cortex. CMO catalyzes the final three steps in aldosterone synthesis (11β-hydroxylase, 18-hydroxylase, and, lastly, 18-methyloxidase), as the most important steps in aldosterone biosynthesis, which takes place only in the zona glomerulosa (1,2). In humans, two 11β-hydroxylase isoenzymes are encoded by two genes

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located on the long arm of chromosome 8 (3). *CYP11B1* expression is primarily controlled by adrenocorticotropic hormone (ACTH), which acts through a specific G-protein-coupled receptor to increase levels of cyclic adenosine monophosphate. *CYP11B2* is mainly regulated by angiotensin 2 and potassium. The promoter region of both genes is strikingly different, underlining the fact that both genes are differently regulated on the transcriptional level, leading to two dissimilar types of disease. Both types CMO type 1 deficiency (OMIM 203400) and CMO type 2 deficiency (OMIM 610600) have similar signs and symptoms but can be distinguished by laboratory testing. These conditions can be differentiated by the presence of insufficient or excessive 18-OH-corticosterone. In CMO 2 deficiency, despite high levels of 18-hydroxycorticosterone (18-OHB), aldosterone levels remain low or normal. These patients have a low ratio of corticosterone to 18-OHB (4).

CMO deficiency (CMOD) type 2 is a rare disorder with unknown prevalence. A particularly high population density of CMOD type 2 was identified in Iranian Jews from the city Isfahan (5), but the disease has been documented throughout Europe and North America (6,7).

CMOD can cause nausea, vomiting, dehydration, low blood pressure, extreme tiredness (fatigue) and muscle weakness, associated with hyponatremia, hyperkalemia and metabolic acidosis. Severe cases of CMOD can result in seizures and coma. Affected infants often exhibit failure to thrive. The signs and symptoms of the disorder typically become milder or disappear by adulthood.

Case Reports

Family 1

Family 1-1

A six-month-old boy was admitted with salt loss and failure to thrive and moderate dehydration. He is the first child of consanguineous parents (Figure 1a), born with a birth weight of 2900 g and length of 50 cm. Physical examination revealed growth retardation, cachectic appearance, and decreased subcutaneous adipose tissue. His height and weight standard deviation scores (SDS) were -1.64 and -2.16, respectively. External genital appearance was normal. He had no hyperpigmentation. Blood pressure was normal (95th percentile = 99/55 mmHg) (Table 1). He had hyponatremia and hyperkalemia despite elevated renin and normal aldosterone levels (Table 2). His plasma 18-OHB level and 18-OHB to aldosterone ratio were increased (Table 2). All the other adrenal hormones including 17-hydroxyprogesterone (17-OH), androstenedione, total testosterone, (dehydroepiandrosterone-sulfate) and cortisol and his ACTH concentrations were within normal limits. He was diagnosed as isolated aldosterone deficiency and, thus, salt and fludrocortisone treatments were initiated. In his follow-up, his electrolytes and anthropometric measurements had normalized (height SDS -0.5, weight SDS 0.28). Genetic analysis revealed two different homozygous variants in the *CYP11B2* (NM_000498.3) confirming the diagnosis of CMOD type 2. The first variant changes thymine to adenine at nucleotide 788 (c.788T > A), resulting in an isoleucine-to-asparagine substitution at codon 263 (p.Ile263Asn) (8). The latter variant is novel and changes thymine to cytosine at nucleotide 1157 (c.1157T > C), resulting in a leucine to proline substitution at codon 392 (p.Leu392Pro). Consanguineous parents were heterozygous for both variants without any clinical findings. The variants NM_000498.3:c.1175T > C (p.Leu392Pro) and NM_000498.3:c.788T > A (p.Ile263Asn) were evaluated by American College of Medical Genetics (ACMG) criteria and classified as Variant of Unknown Significance.

Family 1-2

A two-year-old sibling of the first case was admitted with growth retardation. He was born at gestational week 38, with a weight of 3050 g and length of 50 cm. His medical records revealed a history of hyponatremia and hyperkalemia at the age of thre months, which did not persist in later follow-up. At admission, although his electrolytes were within normal limits, his height and weight SDS were -1.99 and -2.14, respectively (Table 1). Following fludrocortisone treatment, his growth characteristics normalized (height SDS 0.3, weight SDS -0.1). Genetic analysis revealed the same variant as that of his siblings.

Family 1-3

A three-month-old girl, sister of cases 1 and 2, was admitted due to poor weight gain. She was born at normal gestational age (38 + 5 weeks) with a birth weight of 2800 g and length of 49 cm. Her physical examination revealed normal female external genital development but decreased subcutaneous adipose tissue. Her height and weight SDSs were -2.82 and -1.62, respectively (Table 1). Her blood pressure was 77/50 mmHg (95th percentile 98/53 mmHg). She had hyponatremia, mild hyperkalemia, increased renin, and normal aldosterone levels (Table 1). Following fludrocortisone treatment, adequate weight gain and height velocity were achieved and laboratory findings normalized (height SDS -0.4, weight SDS 0.08). Genetic analysis revealed the same variant as that of her siblings.

Table 1. Clinical features of the patients, initial findings at presentation, laboratory results, genetic analyses and treatments

	Case 1	Case 2	Case 3	Case 4
Chronological age	10.9	6.5	4.6	18.7
Gender	Male	Male	Female	Male
Age at the time of diagnosis (months)	6	24	3	3
Consaginious marriage	Yes	Yes	Yes	No
Clinical presentation	Moderate dehydration, failure to thrive	Growth retardation	Poor weight gain	Failure to thrive
Weight (gr)/SDS	6.3/-2.16	4900/-2.14	4300/-2.82	4500/-2.73
Height (cm)/SDS	65/-1.64	58.5/-1.9	58/-1.62	58/-1.66
Pubertal stage at diagnosis time	1	1	1	1
Blood pressure at diagnosis time (mmHg)	70/40	80/50	77/50	80/55
Treatment	Fludrocortisone	Fludrocortisone	Fludrocortisone	Fludrocortisone
Genetic analyses	Missense mutation in CYP11B2 c.1175T > C (p.L391H) and c.788T > A (p.I263N)	Missense mutation in CYP11B2 c.1175T > C (p.L391H) and c.788T > A (p.I263N)	Missense mutation in CYP11B2 c.1175T > C (p.L391H) and c.788T > A (p.I263N)	c.666_667delCT (p.F223PfsTer35) mutation in the *CYP11B2* gene

SDS: standard deviation scores

Table 2. Laboratory findings at the time of diagnosis and steroid levels of cases in aldosterone synthesis pathways

	Case 1	Case 2	Case 3	Case 4	Reference value
Na (sodium) mmol/L	124	138	126	122	135-145
K (potassium) mmol/L	6.6	4	5.6	6.8	3.5-5.2
Renin (uIU/mL)	500	265	> 5500	1680	4.4-46.1
Aldosterone (ng/dL)	60	< 3.7	5.6	40	5-90
ACTH (pg/mL)	24	37	30	10	6-46
Kortizol (mcg/dL)	8	19.9	15	15.7	2.8-23
Urea (mg/dL)	34	33	36	27	5-20
Creatinine (mg/dL)	0.5	0.6	0.2	0.6	0.5-1.0
Corticosterone (pmol/L)	8700	4440	6200	5760	2308-4327
18-OH corticosterone (pmol/L)	5200	7060	5200	4050	137.9-3323
Aldosterone (pmol/L)	65.86	166	49	54	138.7-2497
18-OH corticosterone/aldosterone	78	42.5	106.1	75	2.3-6.0 Type 1: not applicable Type 2: > 10 (usually ~ 100)
Corticosterone/18-OH corticosterone	1.67	0.62	1.19	1.42	Type 1 > 40 Type 2 < 10

18-OH: 18-hydroxycorticosterone, ACTH: adrenocorticotropic hormone

Family 2

Family 2-1

A three-month-old-boy was brought to our clinic due to failure to thrive and vomiting. He was the first child of non-consanguineous healthy parents (Figure 1b), born with a weight of 3400 g and length of 50 cm. On physical examination, mild dehydration and decreased subcutaneous adipose tissue were observed. His weight was 4500 g (SDS -2.73) and height was 58 cm (SDS -1.66) (Table 1). Laboratory investigation showed hyponatremia, hyperkalemia, increased plasma renin activity and low serum aldosterone concentration. Adrenal steroids and ACTH levels were within normal limits (Table 2). A diagnosis of isolated aldosterone deficiency was established and 0.1 mg of fludrocortisone per day was initiated. A rapid weight gain, normalization

of serum electrolytes, and normalization of plasma renin activity were achieved. As isolated aldosterone deficiency was the probable diagnosis, further investigations were not performed at that time.

In follow-up, he was reassessed at the age of 18 years and blood sample was sent for genetic analysis, which revealed a novel homozygous c.NM_000498.3:c.666_667delCT (p.F223PfsTer35) mutation in the *CYP11B2* gene causing a frame shift and forming a stop codon, detected by Next Generation Sequencing. This variant, which was classified as pathogenic by ACMG criteria as it is a null variant, is predicted to result in replacing phenylalanine 223 with a proline, shifting the reading frame, and terminating at position Ter35 (p.Phe223ProfsTer35) (Figure 2). The patient is now 18.3 years old, receiving fludrocortisone treatment, and his height and weight SDS are 0.12 and 0.59, respectively, and normal for age.

Genomic DNA was extracted from peripheral blood samples of the patients. Genetic analyzes were performed by next generation sequencing (Miseq, Illumina, SanDiego, USA) following manufacturers instructions.

Discussion

Aldosterone deficiency is very rare and is a life-threatening condition when not treated. Clinical presentation of CMOD varies by age. Since ions cross the placental barrier, despite congenital enzyme deficiency, there are no symptoms during fetal life (6). Infants with a mineralocorticoid synthesis defect may show signs of salt-wasting within the first few days or weeks of life. These findings may include vomiting, dehydration, hypovolemia, hyponatremia, hyperkalemia and metabolic acidosis. In children diagnosed in early childhood, growth failure, nutritional problems, mild dehydration and electrolyte disturbances are observed. Miao et al (9) reviewed 44 patients in the published literature and compared characteristics of cases with CMOD type 1 and type 2. Clinical features showed no significant difference in CMOD type 1 and 2. Failure to thrive, recurrent vomiting and dehydration were the most common symptoms in these patients. Although electrolyte disorder normalizes by the age of four years, growth retardation continues throughout childhood. Adults are generally asymptomatic, but they cannot tolerate severe salt loss. They are usually recognized in family screenings.

In the present study, age of diagnosis varied between three months and two years. Our cases have some clinical similarities and some differences to previously reported CMOD cases. Our three cases from the first family presented with vomiting, severe dehydration, hyponatremia and hyperkalemia, and one case, whose brother was diagnosed previously, was asymptomatic and presented only with growth retardation. Almost all patients, as in our cases, clinically improve with age, even if clinical severity among individuals may vary widely.

Mineralocorticoid deficiency causes hyponatremia and hyperkalemia by causing excessive sodium excretion and potassium retention in the renal distal tubule and cortical collection channel. In untreated infants with CMOD, serum sodium level is generally between 120-130 mmol/L and serum potassium level is between 6.0-8.5 mmol/L (10). In accordance with the literature, in our patients, initial sodium and potassium levels were between 122-126 mmol/L and 5.6-7 mmol/L, respectively. All of our cases had high plasma renin activity and normal aldosterone levels (Table 2). Plasma renin activity is significantly increased in affected infants and young children (up to 100-fold normal) but can be normal in adults.

Two types of CMOD have been identified and these syndromes have the same clinical features but differ in the profiles of secreted steroids. Type 2 deficiency can be easily diagnosed by a marked increase in the ratio of 18-OHB

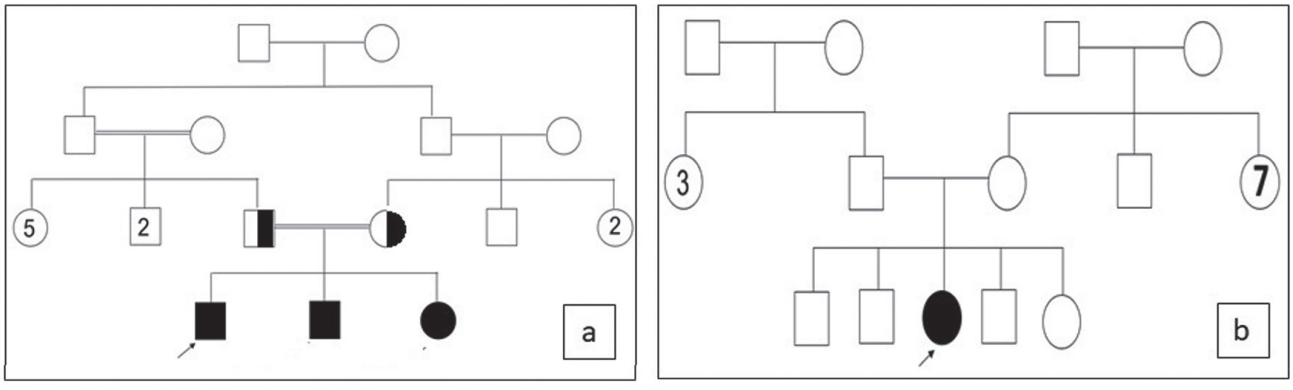

Figure 1. Pedigrees of families; a) Pedigree of family 1; b) Pedigree of family 2

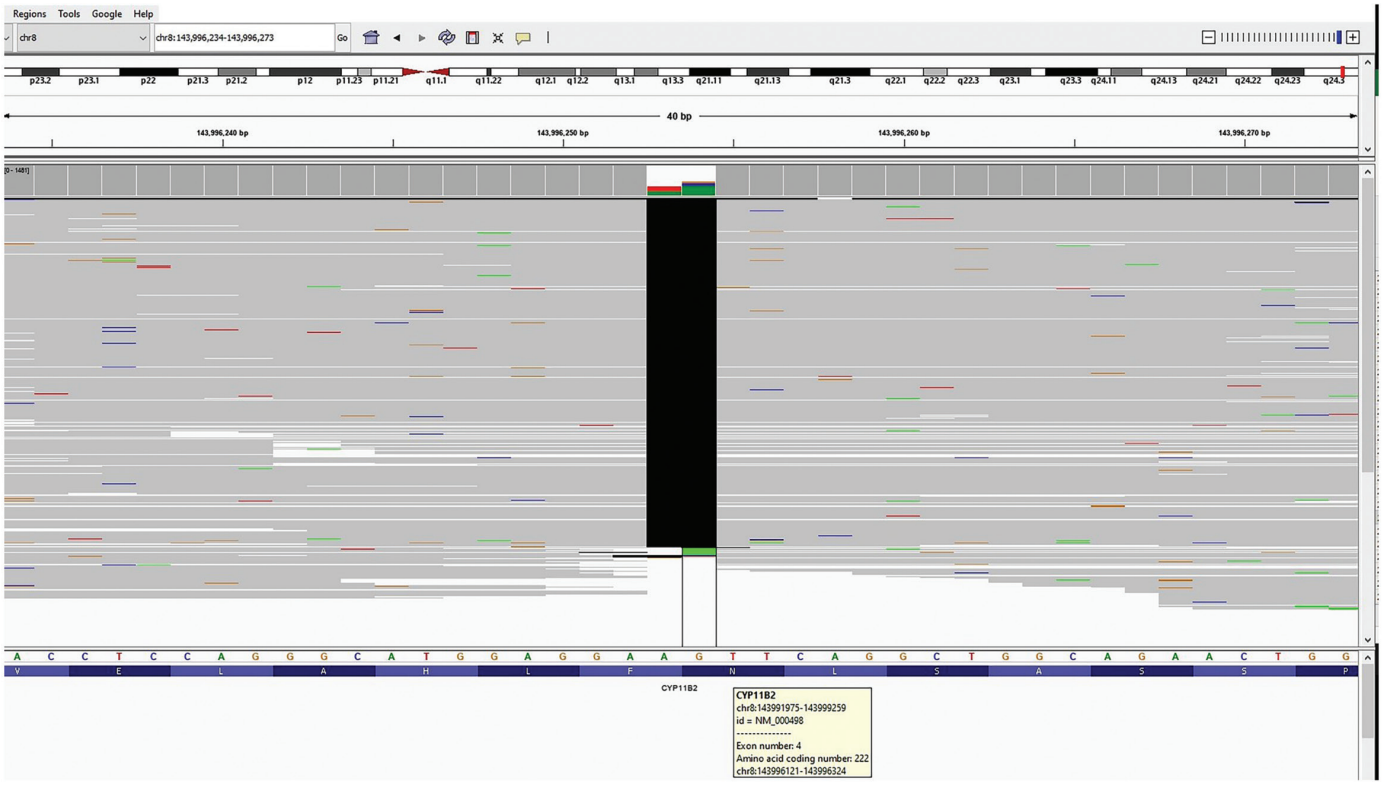

Figure 2. Image of genetic analysis of the patient "family 2-1"

to aldosterone in urine or serum (usually 100-fold). This ratio does not vary by age in affected individuals despite improving clinical features.

Steroid profiles of our patients are given in Table 2 and increased 18-OHB to aldosterone ratios in urine or serum were consistent with type 2 CMOD. This ratio is not useful in the diagnosis of CMOD type 1 because very low levels of aldosterone make the ratio insignificant (11).

The most common disorder in patients presenting with hyponatremia, hyperkalemia and vomiting is congenital adrenal hyperplasia (CAH). CAH should be excluded because the defects of aldosterone synthesis are often seen as a part of cortisol production failure. Bizzarri et al (12) reported their ten-year-experience in infants presenting with hyponatremia and salt loss. Only 2 of 51 patients had aldosterone deficiency due to a *CYP11B2* gene defect, and the majority (37.5%) was diagnosed with CAH. The lack of ambiguous genitalia in our female patient, normal basal 17-OH progesterone levels or increased levels of renin and 18-OH progesterone helped to differentiated CMOD from CAH in our patients.

Another disorder to consider in the differential diagnosis is CMOD type 1. Patients with CMOD type 1 also present with similar clinical findings. High 18-OHB levels and 18-OHB to aldosterone ratios differentiated our patients from CMOD type 1, characterized by the presence of inadequate 18-OHB.

Pseudohypoaldosteronism (PHA) is another disease to be considered in the differential diagnosis. The underlying pathogenesis for PHA are unresponsive aldosterone receptor or overactive Na-Cl co-transporter in the distal nephron. These patients do not improve on treatment with fludrocortisone due to resistance to aldosterone (8), but in our patients with CMOD, clinical findings improved with fludrocortisone treatment.

To date, approximately 56 mutations have been identified in the *CYP11B2* gene. Primary hypoaldosteronism can be caused by different defects in *CYP11B2*, such as nonsense/missense, splicing, regulatory and frame shift mutations, gross deletions and complex rearrangements (data from Human Gene Mutation Database) (13,14). Missense/nonsense mutations constitute the largest proportion of these mutations (Approximately 70%) (15). However, in this case report, we found two novel and one previously reported variant in the *CYP11B2* gene.

Three siblings were homozygous for two substitution variants. A novel variant, C.1175T>C (p.Leu392Pro) resulted in a leucine to proline substitution at codon 392.

The other variant is another substitution *CYP11B2* variant located in exon 4. This variant changes thymine to adenine at nucleotide 788 (c.788T > A), resulting in an isoleucine to asparagine substitution at codon 263. These variants were not detected in GnomAD exomes and GnomAD genomes databases. We also checked our own 2500 exome data and we could not find these variants. As the clinical picture of our patients clearly fits with the disorder, these variants were classified as "likely pathogenic". One of these two variants or both of them may be pathogenic. The other possibility is that these two variants may be pathogenic when present together on the same allel.

The c.788T > A (p.Ile263Asn) variant was first described in 2016 by Üstyol et al (16). This pathogenic variant has so far been reported only in Turkish patients supporting Turan et al (8) who suggested an ethnic specificity of the variant. As all cases carrying this variant were reported to have the clinical features of CMOD type 2, this variant is more likely to be pathological. So far, no functional enzymatic studies of this variant have been conducted, but the clinical presentation correlated well with previous studies. The parents of our three siblings are heterozygous for the same variants. In the genetic study of case 4, a novel homozygous two base pair frame shift mutation (c.666_667delCT) was found in *CYP11B2*, resulting in a stop codon. Due to this premature stop codon this variant is very probably related to the disease. However, functional analysis of genes should still be performed to determine the functional outcome of the loss in gene product.

Clinical symptoms of different severity can be observed in patients with the same mutation. Twelve patients from eight families, reported in 1977, had the same mutation but there was a marked range in clinical severity, which varied from an asymptomatic state in adulthood to acute salt-wasting crisis in infancy, detected only by biochemical profile. So researchers concluded that individual differences in the degree of severity do not reflect the allele variant (5). Instead, they indicate the effects of other genetic loci or non-genetic factors (17).

Fludrocortisone replacement is necessary to correct the deficiency. The response to mineralocorticoid replacement and salt supplementation (dramatic catch-up growth, no further diarrhea or vomiting and normalized appetite) confirmed the diagnosis. Salt wasting, possibly due to insulin-like growth factor-1 suppression or reduced extracellular fluid volume and could be a factor leading to impaired growth (9).

Some studies suggest that mineralocorticoid therapy should be given for linear growth, despite normal serum electrolytes (18,19). Clinical improvement in growth rate with mineralocorticoid therapy in reported cases with no ion deficits but growth failure, also support this view. This condition can be explained by chronic salt wasting. Prospective studies have shown poor linear growth when both rats and humans are fed sodium deficient diets. The Na-H antiporter, present in many types of cell membranes, is an important mediator of cell growth and proliferation by its action in alkalinizing the cell interior (20).

Salt-wasting improves with aging and the majority of the cases can be asymptomatic in adulthood, even if not treated, with normal electrolyte levels (20,21). There are some reasons why the mineralocorticoid requirements decrease with age. Firstly, mineralocorticoid receptors are poorly expressed in the renal epithelium of newborns and this increases with age. Secondly, newborn diets (breastfeeding) have low sodium content, and dietary sodium intake increases with age (9). Other reasons mentioned before including increased sodium reabsorption due to mature renal tubules and alternative pathway of mineralocorticoid biosynthesis (12). In follow-up, patients should be evaluated carefully because the need for treatment decreases with increasing age and keeping the same dosage of fludrocortisone may lead to hypokalemia and hypertension (18). Our patients continue to be treated but with reduced doses.

Conclusion

CMOD should be considered in the differential diagnosis in patients presenting with hyponatremia, hyperkalemia and growth retardation and it should not be forgotten that this condition is life-threatening if not treated. Genetic analyses are beneficial for diagnosis of the patients and other relatives at the risk of salt loss and failure to thrive. The same variants may result in a varying severity of clinical findings in different patients, even within the same family. Thus, family screening is important for early diagnosis and treatment.

Ethics

Informed Consent: We state that the subjects and their parents have given their written informed consent to publish their cases, in accordance with the Declaration of Helsinki.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Hande Turan, Oya Ercan, Saadet Olcay Evliyaoğlu, Design: Hande Turan, Gürkan Tarçın, Oya Ercan, Saadet Olcay Evliyaoğlu, Data Collection or Processing: Hande Turan, Aydilek Dağdeviren Çakır, Yavuz Özer, Bahar Özcabi,

Serdar Ceylaner, Oya Ercan, Saadet Olcay Evliyaoğlu, Analysis or Interpretation: Hande Turan, Saadet Olcay Evliyaoğlu, Serdar Ceylaner, Literature Search: Hande Turan, Aydilek Dağdeviren Çakır, Yavuz Özer, Gürkan Tarçın, Writing: Hande Turan, Gürkan Tarçın, Oya Ercan, Saadet Olcay Evliyaoğlu.

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CASE REPORT

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A Case of Familial Male-limited Precocious Puberty with a Novel Mutation

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What is already known on this topic?

Familial male-limited precocious puberty (FMPP) is a very rare cause of precocious puberty seen exclusively in males, which results in early onset puberty, rapid skeletal maturation and compromised final adult height, if not treated. Very limited data is available regarding treatment and there are no consensus guidelines.

What this study adds?

We report a novel mutation in the luteinizing hormone/chorionic gonadotropin receptor *LHCGR* gene causing FMPP in a very young child who presented with puberty at six months of age and is being successfully treated. Our report adds to the very scarce information available about this condition.

Abstract

Familial male-limited precocious puberty (FMPP), also known as testotoxicosis, is a rare cause of precocious puberty in males. It is caused by a mutation in the luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) gene, resulting in the receptor being constitutively activated. This causes excessive production of testosterone, leading to precocious puberty in males. Generally, boys present with signs of puberty, such as pubic hair growth, acne, and increased height velocity around the age of 2-4 years old. Like any other cause of precocious puberty, the goal of treatment is to prevent virilization and also delay closure of the epiphyseal plates to maintain adult height potential. Treatment, therefore, is aimed at decreasing the effects of testosterone, as well as stopping the conversion of testosterone to estrogen. Little is known about the long-term effects of treatment because the disorder is so rare. However, studies using bicalutamide and anastrozole have been promising. In this report, we present a boy with FMPP with a novel mutation in the *LHCGR* gene, who has been responding well to therapy using both drugs.

Keywords: Familial male-limited precocious puberty, bone age, short stature, adult height

Introduction

The luteinizing hormone/chorionic gonadotropin receptor (LHCGR), a G-protein coupled receptor, is present on multiple cell types, including ovarian theca cells, granulosa cells, and testicular Leydig cells (1). Under normal conditions, it is activated by LH and human chorionic gonadotropin (hCG). It is important for normal sexual development and production of sex steroids (1). The gene for the LHCGR is located on chromosome 2p21, and is the genetic basis of familial male-limited precocious puberty (FMPP), also known as testotoxicosis.

An activating mutation in the *LHCGR* gene may occur *de novo* or may be inherited in an autosomal dominant manner. The commonly reported genetic mutation is due to a substitution of an A to a G, which causes a change from aspartate to glycine, in position 578 of the LHCGR (2). This small change causes the protein to be constitutively

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activated without any input from LH, leading to excessive sex hormone production.

Interestingly, activating *LHCGR* mutations only affect phenotype in males. There are two proposed theories as to why females are not affected: LH and follicle stimulating hormone are both required to stimulate hormone synthesis, and that the activation of LH receptor (LHR) alone would not cause symptoms (2). Additionally, it is proposed that the degree of dysfunction is simply not high enough to cause symptoms (3). Regardless, the excessive production of testosterone causes early signs of puberty, such as pubic hair growth, increased penile length and acne, from direct effects of testosterone and dihydrotestosterone on the testosterone receptor, as well as increased height velocity with significant bone age advancement from conversion of androgens to estrogen by the enzyme aromatase. Boys generally present with symptoms around the age of 2-4 years old. Without treatment, rapid skeletal maturation occurs with a negative impact on final adult height.

Treatment goals are twofold: to decrease the effects of testosterone, typically through the use of anti-androgen agents; and to inhibit the conversion of testosterone to estrogen through the use of aromatase inhibitors. Multiple different medications have been trialed, but most recent studies show promising results using bicalutamide (an anti-androgen agent), and anastrozole (a third-generation aromatase inhibitor) (4,5,6,7,8). Little is known about the long-term effects of treatment because the disorder is so rare. However, studies using bicalutamide and anastrozole have shown improved height potential (5,6). In one study, final adult height was only 0.4 standard deviations below that of the general US male population (6).

Here, we present a boy with FMPP, with a novel mutation in the *LHCGR* gene, who has been responding well to bicalutamide and anastrozole therapy with marked decrease in growth velocity, pubertal progression and skeletal advancement.

Case Report

A 16-month-old male presented to our pediatric endocrinology clinic due to concerns about precocious puberty. His parents noticed presence of pubic hair at six months of age and also reported rapid growth acceleration. Parents denied exposure to creams, gels, or medications containing testosterone and there was no known history of early puberty or short stature in the family. Upon presentation, his height was over the 99th percentile while his target height was at the 50th percentile. Physical examination revealed prepubertal testes with pubic hair at Tanner stage 2. A bone age was obtained by his pediatrician, that was reported as 12-18 months at a chronologic age of 12 months.

Initial lab evaluation revealed normal serum levels of 17-hydroxyprogesterone, androstenedione and dehydroepiandrosterone sulfate with a slightly elevated serum LH level [0.7 mIU/mL (prepubertal <0.3 mIU/mL)], and a significantly elevated serum testosterone level [289 ng/dL (<5 ng/dL)]. A leuprolide stimulation test was performed that ruled out central precocious puberty and an adrenocorticotropic hormone stimulation test was also performed that ruled out congenital adrenal hyperplasia. His baseline serum testosterone during the test was 550 ng/dL. A magnetic resonance imaging study of the abdomen and pelvis was reported to be normal. Scrotal ultrasound was obtained that revealed pre-pubertal testes with no testicular masses but testicular microliths were noted bilaterally. An α-fetoprotein level was initially elevated, but repeat was normal, and serum β-hCG level was normal. Serum insulin-like growth factor-1 and thyroid function were also normal. Rapid skeletal maturation was noted with his bone age advanced to six years at a chronological age of 21 months and to eight years by 26 months of age. At 26 months of age, his stretched penile length was 11 cm. At this time, the likely differential diagnoses were FMPP and McCune-Albright syndrome. LHR testing was therefore obtained, which revealed a novel heterozygous missense mutation in the *LHCGR* gene (c.1733A>C; p.Asp.578Ala). The mutation was reported to be a variant of unknown significance though likely in the pathological end of the spectrum by variant analysis with SIFT and PolyPhen.

Based on the patient's classic presentation along with the genetic testing results, he was diagnosed to have FMPP and started on treatment with anastrozole 1 mg daily and bicalutamide 25 mg daily at two years and nine months of age. There was some parental hesitation in starting treatment, which delayed it by a few months.

The patient has been tolerating treatment well for over two years at the time of writing with a significant improvement in his linear growth, skeletal maturation and final height prediction (see Figure 1). The bone age to chronological age ratio has decreased from 4.5 at treatment initiation to 3.2 at two years after treatment. His predicted adult height as has improved from significantly below the 3rd percentile to 162.5 cm which is at the 3rd percentile. Annual scrotal ultrasound testing has revealed stable testicular microliths. Testes have remained prepubertal on exam with prepubertal early morning serum LH levels and prepubertal response to annual leuprolide stimulation testing. After approximately 14 months of treatment, he developed minimal gynecomastia bilaterally, which is a known side effect of androgen receptor antagonists.

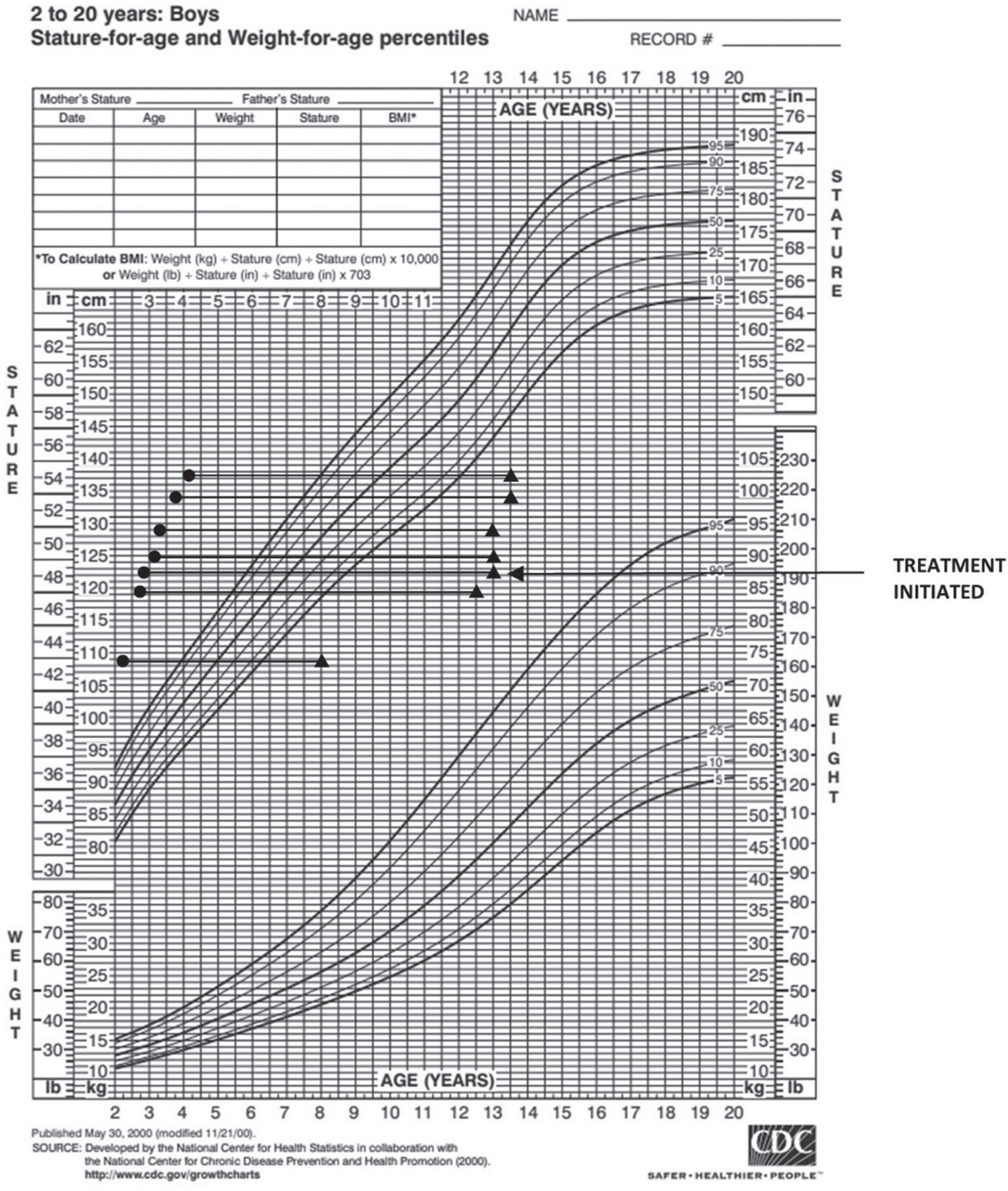

Figure 1. Patient's growth chart indicating height measurements with corresponding bone age readings before and after treatment initiation

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Discussion

FMPP is a rare cause of peripheral precocious puberty, exclusively affecting males. Affected males present with early onset of puberty, typically by the age of three years, accompanied by accelerated skeletal maturation resulting in premature epiphyseal fusion and compromised final adult height. The overall goal of treatment in FMPP is the same as for any cause of precocious puberty - to minimize virilization and maximize adult height potential.

In the short term, treatment efficacy is monitored using two criteria: physical examination to assess clinical progression; and bone ages to assess skeletal maturation and growth potential. The rarity and differences in phenotype of the condition make it difficult to do widespread clinical trials. Treatment has, however, evolved over the years but there is still no consensus on the optimal treatment.

Prior to the discovery that FMPP was due to an activating mutation of the *LHCG* receptor gene, gonadotropin-releasing hormone (GnRH) agonists were the primary treatment. Once the pathophysiology was better understood, anti-androgen medications such as Medroxyprogesterone acetate and cyproterone initially, and ketoconazole later, became the mainstay for treatment. The former two medications failed to show an improvement in height potential and the latter was associated with serious side effects, such as hepatotoxicity and adrenal suppression (9).

It was during this time that adding aromatase inhibitors was trialed in order to prevent early growth plate closure (8,10,11). Spironolactone (a weak anti-androgen) and testolactone (an aromatase inhibitor) were trialed with moderate success, and resulted in improvements in skeletal maturation, acne, aggressive behavior, and spontaneous erections (10,11). However, in one study, all boys ended up with secondary central precocious puberty (10). The development of central precocious puberty was likely due to continued elevated poor androgen receptor blockade (6,12,13). The fact that patients needed to discontinue spironolactone during gastro-intestinal illnesses, and the fact that it requires multiple daily doses, likely lead to poor compliance. All patients then required addition of deslorelin (a GnRH agonist) (10). Since that trial, more specific and potent medications have become available.

Most recent studies have shown good outcomes with anti-androgens such as bicalutamide and third generation aromatase inhibitors such as anastrozole (4,6,7,8). These treatments, in conjunction with one another, have shown better adult height potential preservation than either alone (5). In one study, anti-androgens alone did not prevent early growth plate closure (5), and in another, anastrozole was ineffective in treating continued aggressiveness (7). Bicalutamide and anastrozole offer the additional advantage of a prolonged half-life that allows for convenient once-daily dosing while other anti-androgen agents would require multiple daily doses.

The data is somewhat variable in regards to long-term outcomes, but results are promising. Adult height is improved following treatment, though adults with FMPP are still slightly below average in height (5,6). Long-term effects on fertility are still unknown, however one study did not show any differences in sperm count between patients taking anastrozole and controls (14). Patients seem to have preserved fertility in case reports (15). Until long-term data on adult height, fertility and bone health are available from a larger sample of patients, this combination therapy should be used judiciously. Endocrinologists should also keep in mind that the cost of therapy using bicalutamide and anastrozole is relatively more expensive than previous treatments (4).

Some studies have attempted to establish an association between phenotype and genotype (16,17), which may alter treatment strategy due to variability in medication effectiveness. The traditional mutation in FMPP is Asp578Gly, resulting in an activation of LHCGR (2). However, other mutations have been known to cause FMPP (13). Approximately 55% of these "other" activating mutations occur at the 578 position, changing the aspartate residue a tyrosine, histidine, glycine, or glutamine (1).

To our knowledge, our patient has a novel mutation in the *LHCGR* gene, with a change from an A to a C, changing aspartate to alanine in the 578 position. Our patient has been responding well to therapy, likely because he has a mutation in the "typical" spot. However, this new mutation could explain some of the phenotypic differences in our patient compared to the typical FMPP patient. Typically, FMPP presents at the age of 2-4 years. Our patient had initial symptoms much earlier than that at six months of age, which has only been documented in a handful of cases (13).

What remains unknown is what long-lasting effects remain from these increased hormone levels. It is known that testosterone has effects on the brain and on behavior, and increased levels can lead to aggressiveness, attention deficit hyperactivity disorder and developmental issues (3). Though duration of the androgen exposure may be related to level of aggressiveness in children (18), and this aggressiveness tends to disappear with treatment (11), there are no studies examining the long-term effects.

Additionally, our patient had bilateral testicular microlithiasis. Typically found incidentally, testicular microliths occur with a prevalence of 2.4-5.6% in asymptomatic pediatric patients (19). Their cause is unknown, but it is thought to be a degenerative process in the seminiferous tubules leading to accumulation of cellular debris, and then accumulation of glycoproteins surrounding the debris, which then calcifies (20,21). It is noted that testicular microliths do not typically affect Leydig cells, and maybe associated with Sertoli cell dysfunction (19). However, there is some evidence that microliths are associated with Leydig cell hyperplasia in rats (22). They are seen more frequently in children with Down syndrome and associated with testicular torsion, varicocele, retractile testes, cryptorchidism, inguinal hernias, and testicular neoplasms, though there is no evidence that they are premalignant (19,20).

The microliths in our patient may well be an incidental finding. However, testicular changes have been observed in FMPP in more than one case. Premature differentiation in all major testicular cell types, seminomas, and germ cell neoplasia *in situ* (13,15), and in particular, Leydig cell hyperplasia (2,3,13), have all been reported. Given the recent findings of microliths being associated with Leydig cell hyperplasia, and the known association between FMPP and Leydig cell hyperplasia, there may be a link between the two (2). Despite this, there has only been one other report of testicular microlithiasis (13).

Conclusion

FMPP is a rare disorder that is still being studied. No treatment guidelines have been established for this condition. Definitive evidence of effective treatment modalities has been weak, primarily because of the limited number of reported cases, small sample sizes, and short-term outcomes. However, this case study adds to the literature by demonstrating a novel mutation that responded well to a combination of bicalutimide and anastrozole.

Ethics

Informed Consent: Yes, from patient's mother.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Shilpa Gurnurkar, Concept: Shilpa Gurnurkar, Data Collection or Processing: Shilpa Gurnurkar, Emily DiLillo, Mauri Carakushansky, Literature Search: Shilpa Gurnurkar, Emily DiLillo, Mauri Carakushansky, Writing: Shilpa Gurnurkar, Emily DiLillo, Mauri Carakushansky.

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The My Friend Diabetes Camp was Held Online in Turkey This Year Due to the COVID-19 Pandemic

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Keywords: Type 1 diabetes, camp, virtual, COVID-19

Dear Editor,

Due to the coronavirus disease-2019 (COVID-19) pandemic currently affecting the whole world, the 23rd *My Friend Diabetes Camp* this year took place on a virtual platform from 16 to 21 August. The My Friend Diabetes Camp has been taking place in July on the shores of Lake İznik every year since 1997. the camp staff-which include dedicated physicians, nurses, dietitians, and psychologists, all actively engaged in improving the health of diabetic children, as well as medical school students and young guides with type 1 diabetes (T1D)-come together with up to 90 diabetic children (8-18 years of age) from all over Turkey (1).

The aim of the online camp also is to ensure that children and families would become friends with diabetes, create a new "normal", continue their lives with hope for the future and obtain the most up-to-date information on diabetes. The program of the six-day camp included sessions on social and psychological issues, medical recommendations on nutrition, especially carb counting, new technologies, experiences of sporters with T1D, conversation with young people and families with diabetes, exercise and art, mindfulness sessions, kitchen workshop and finally a forum for the children with T1D and their families.

The online camp was delivered on Instagram and Youtube through eighty people acting as moderators or speakers. These platforms allowed the camp activities (which accumulated the emotions, thoughts and experiences from the İznik, Diyarbakır and Uludağ My Friend Diabetes Camps) to be shared across the country. The camp program and all of the activities were (and still are), freely accessible through https://www.instagram.com/arkadasimdiyabetonlinekamp/ and https://www.youtube.com/channel/UCdlWmUqqL7Iom_3ksWHuf-Q.

The camp activities were launched with famous writer and museologist Sunay Akın giving a speech entitled "*Journey to the World of Children*", in which he presented the Toy Museum. By 6 pm, on the first day of the camp, 5,187 people were following the camp on Instagram and 1849 people had signed up as participatants. The camp activities were uploaded to Instagram TV and have had almost 62,000 views. We shared the stories of 237 people who messaged us alongside postings of videos of 24 people with T1D introducing themselves and the pictures of 19 children with T1D. In addition, it was possible to reflect the environment of the camps to the audience through a documentary about the İznik and Diyarbakır camps (1,2), a recorded presentation and 5 videos showing pictures of camps from previous years.

The online camp provided an excellent opportunity for up-to-date and comprehensive training on T1D. Conversations usually focus on the following key topics:

- Becoming friends with diabetes and remaining optimistic; not creating a drama out of diabetes; ensuring everyone is aware that people with T1D can lead a normal and successful life; resolving problems in working life.

- Spreading the knowledge that the COVID-19 pandemic does not pose a different risk for people with T1D compared

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to other children, that they should be treated like their peers, and to ensure that they return to school when the time comes like other children (3).

- Providing a safe family environment for children, not overwhelming children especially adolescents, reminding participants of the temporary nature of problems in adolesence.

- Not blaming everything on diabetes and preventing burnout in children and worried families.

- The importance of pre-meal insulin administration time and insulin administration locations (the pre-meal bolus dose should be injected 10-15 minutes prior to the meal, the abdomen and arms should be used for fast-acting insulins, the hips and legs should be used for long-acting insulins) (4).

- The importance of measuring glucose level 2 hours after a meal in order to ensure that the bolus insulin dose was sufficient, as well as measuring figures such as insulin/carbohydrate ratio which are used when calculating the dose (4).

- Changing attitudes on issues such as diet, carbohydrate count, the idea that snacks are compulsory or routine for children with T1D, removing or minimizing snacks with individual meal plans according to their glycemic responses, and not choosing milk for snacks at night.

- Avoiding unscientific opinions such as following a gluten-free and/or very low-carbohydrate diet, and instead following the recommendations of diabetes teams.

- Frequent and, if possible, continuous glucose monitoring, the use of sensors immediately after diagnosis; taking advantage of new technologies as soon as possible.

- Increasing state contribution to diabetes technologies, especially sensors, and ensuring equal access to technology, especially for children, and launching a strong campaign in this regard.

- Administering a correction dose when necessary and as often as possible (if the glucose value is > 150 mg two hours after the last fast-acting insulin dose); keeping the glucose in the range of 70-180 mg at least 70% of the day, and in the range of 70-145 mg at least 50% of the day (4).

- Keeping hemoglobin a1c at most 7%, below 6.5% if possible (4).

- Caring about hyperglycemia at least as much as hypoglycemia, or even more so.

- The fear of having a low blood glucose level often causes the patient to begin the evening with high glucose, which negatively affects the release of hormones that will increase low glucose when necessary. Trying to keep the glucose levels of people with T1D similar to those who do not have diabetes, and relieving the fear of hypoglycemia. Not taking/giving additional carbohydrates on a routine basis, unless necessary, after correcting low blood glucose by taking/giving simple carbohydrates (juice, sugar cubes or glucose tablets).

- Thinking of regular exercise as a "third insulin" and making it a part of daily life.

- Understanding the "mathematics" of diabetes and learning to give insulin like the pancreas.

- People with T1D need to evaluate their own data for at least two weeks and pay attention to when their average glucose is below 150 mg, listen to themselves and master the management of their diabetes through their own experiences.

- Updating people with T1D and their families' information on diabetes, for example by following the www.arkadasimdiyabet.com website.

- Being positive and supporting children with diabetes and their families around us; participating in solidarity networks.

- Reinforcing the opinion that the most important steps to be taken in diabetes care in our country are: to strengthen diabetes teams, to resolve the problem of the lack of dieticians and psychologists, and to support team members in terms of their personal rights.

The *My Friend Diabetes Online Camp*, which was supported by 9 international diabetes experts and researchers via video records (Ragnar Hanas, Eda Cengiz, Kaan Aktürk, Aaron Kowalski, Michael Riddell, Bruce King, Carmel Smart, Megan Paterson, Michelle Neylan), was announced to all members of the International Society for Pediatric and Adolescent Diabetes through its August news bulletin (5). The closing evening ended with music by pianist Gülce Sevgen, who has T1D, and a forum attended by guests from different parts of Turkey and the USA. While the COVID-19 pandemic caused many negative impacts, it also opened new horizons in innovating the online platform. The "My Friend Diabetes Online Camp" presented an opportunity for nationwide interaction and was a perfect example of this innovation. Nonetheless, it is still our greatest wish to be able to deliver our "My Friend Diabetes Camps" on-site next year.

Ethics

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Şükrü Hatun, Gül Yeşiltepe-Mutlu, Tuğba Gökçe, Ecem Can, Serra Muradoğlu, Elif Eviz, Kardelen Cemhan, Concept/Design: Şükrü Hatun, Gül Yeşiltepe-Mutlu, Tuğba Gökçe, Ecem Can, Serra Muradoğlu, Elif Eviz, Kardelen Cemhan, Writing: Şükrü Hatun, Gül Yeşiltepe-Mutlu, Tuğba Gökçe, Ecem Can, Serra Muradoğlu, Elif Eviz, Kardelen Cemhan.

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J Clin Res Pediatr Endocrinol 2021;13(2):248

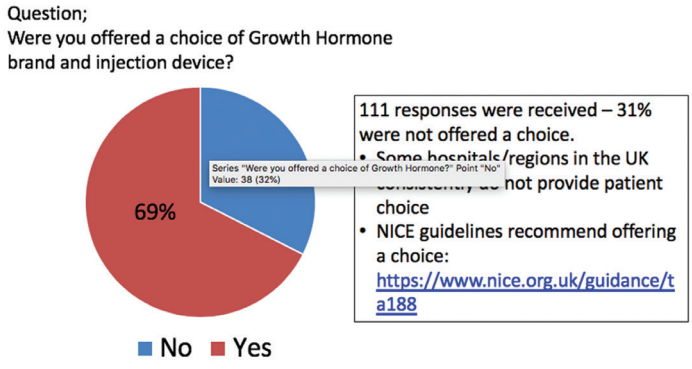
DOI: 10.4274/jcrpe.galenos.2019.2019.0153

Child J, Davies C, Frost K, McDermid E, Pidcock R, Weinman J, Savage MO. Managing Paediatric Growth Disorders: Integrating Technology Into a Personalised Approach. J Clin Res Pediatr Endocrinol 2020;12:225-232.

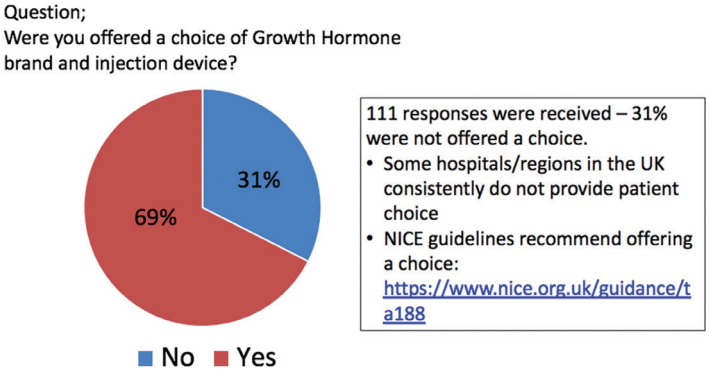
The mistake and the correction of the aforementioned article have been demonstrated in the following list:

The sentence in the gray area on Figure 1 should not appear on the image. The image has been corrected by removing the gray area as follows.

Incorrect Figure 1



Corrected version of the Figure 1



DOI: 10.4274/jcrpe.galenos.2021.e002

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Sethi A, Foulds N, Ehtisham S, Ahmed SH, Houghton J, Colclough K, Didi M, Flanagan SE, Senniappan S. Heterozygous Insulin Receptor (INSR) Mutation Associated with Neonatal Hyperinsulinemic Hypoglycaemia and Familial Diabetes Mellitus: Case Series. J Clin Res Pediatr Endocrinol 2020;12:420-426.

The mistake and the correction of the aforementioned article have been demonstrated in the following list:

The mistake has been made inadvertently. The reference cited in the 16th reference of the article is a withdrawn article. By the authors, instead of the 16th reference "Caruso M, Miele C, Oliva A, Condorelli G, Oriente F, Riccardi G, Capaldo B, Fiory F, Accili D, Formisano P, Beguinot F. The IR1152 mutant insulin receptor selectively impairs insulin action in skeletal muscle but not in liver. Diabetes 2000;49:1194-1202.", "Eckstein SS, Weigert C, Lehmann R. Divergent Roles of IRS (Insulin Receptor Substrate) 1 and 2 in Liver and Skeletal Muscle. Curr Med Chem 2017;24:1827-1852." has been corrected by citing.

The 16th reference in the manuscript recently:

16. Caruso M, Miele C, Oliva A, Condorelli G, Oriente F, Riccardi G, Capaldo B, Fiory F, Accili D, Formisano P, Beguinot F. The IR1152 mutant insulin receptor selectively impairs insulin action in skeletal muscle but not in liver. Diabetes 2000;49:1194-1202.

The 16th reference in the manuscript replaced with the prior mentioned:

16. Eckstein SS, Weigert C, Lehmann R. Divergent Roles of IRS (Insulin Receptor Substrate) 1 and 2 in Liver and Skeletal Muscle. Curr Med Chem 2017;24:1827-1852.

DOI: 10.4274/jcrpe.galenos.2021.e003

J Clin Res Pediatr Endocrinol 2021;13(2):250

DOI: 10.4274/jcrpe.galenos.2020.2019.0182

Güran T, Tezel B, Çakır M, Akıncı A, Orbak Z, Keskin M, Selver Eklioğlu B, Ozon A, Özbek MN, Karagüzel G, Hatipoğlu N, Gürbüz F, Çizmecioğlu FM, Kara C, Şimşek E, Baş F, Aydın M, Darendeliler F. Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: Outcomes of Extended Pilot Study in 241,083 Infants. J Clin Res Pediatr Endocrinol 2020;12:287-294.

The mistake and the correction of the aforementioned article have been demonstrated in the following list:
The expression in parentheses in the last sentence of the first paragraph on page 290 of the article is written as follows inadvertently.

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 (https://www.ncbi.nlm.nih.gov/pubmed/?term = Hayashi % 20 G % 5BAuthor % 5D&cauthor = true&cauthor_uid = 22218447) (4).

It is arranged as follows by removing the expression in the parentheses.

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).