JCRPE

Journal of Clinical Research in Pediatric Endocrinology

March 2019 | volume 11 | issue 1 | www.jcrpe.org | ISSN: 1308-5727

E-ISSN: 1308-5735

Official Journal of
Turkish Pediatric Endocrinology
and Diabetes Society

galenos yayınevi

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

Editor in Chief

Feyza Darendeliler
İstanbul University İstanbul Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
feyzad@istanbul.edu.tr ORCID-ID: orcid.org/0000-0003-4786-0780

Associate Editors

Abdullah Bereket
Marmara University Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
abdullahbereket@gmail.com ORCID-ID: orcid.org/0000-0002-6584-9043

Damla Gökşen
Ege University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
damla.goksen@ege.edu.tr ORCID-ID: orcid.org/0000-0001-6108-0591

Korcan Demir
Dokuz Eylül University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
korcandemir@gmail.com ORCID-ID: orcid.org/0000-0002-8334-2422

Samim Özen
Ege University Faculty of Medicine, Department of Pediatric Endocrinology, İzmir, Turkey
samim.ozen@ege.edu.tr
ORCID-ID: orcid.org/0000-0001-7037-2713

Serap Turan
Marmara University Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey
serap.turan@marmara.edu.tr ORCID-ID: orcid.org/0000-0002-5172-5402

Editorial Advisor

Olcay Neyzi
Emeritus Professor, İstanbul, Turkey
oneyzi@superonline.com

English Language Editor
Jeremy Jones, *Kocaeli, Turkey*

The paper used to print this journal conforms to ISO 9706: 1994 standard (Requirements for Permanence).
The National Library of Medicine suggests that biomedical publications be pirinted on acid-free paper (alkaline paper).

Reviewing the articles' conformity to the publishing standards of the Journal, typesetting, reviewing and editing the manuscripts and abstracts in English, creating links to source data, and publishing process are realized by Galenos.

Editorial Board

Galenos Publishing House
Owner and Publisher
Erkan Mor

Publication Coordinator
Burak Sever

Web Coordinators
Soner Yıldırım
Turgay Akpınar

Graphics Department
Ayda Alaca
Çiğdem Birinci
Gülşah Özgül

Project Coordinators
Eda Kolukısa
Hatice Balta
Lütfiye Ayhan İrtem
Sedanur Sert
Zeynep Altındağ

Project Assistants
Gamze Aksoy
Nurcan Acarçağ

Finance Coordinator
Sevinç Çakmak

Research&Development
Kerim Sancar Ölmez

galenos yayınevi

Contact
Address: Molla Gürani Mahallesi
Kaçamak Sokak No: 21 34093
Fındıkzade, İstanbul-Turkey
Phone: +90 (212) 621 99 25
Fax: +90 (212) 621 99 27
E-mail: info@galenos.com.tr
Publisher Certificate Number: 14521
www.galenos.com.tr

Printing at:
Üniform Basım San. ve Turizm Ltd. Şti.
Matbaacılar Sanayi Sitesi 1. Cad. No: 114
34204 Bağcılar, İstanbul, Türkiye
Phone: +90 212 429 10 00
Certificate Number: 42419
Date of printing: February 2019
ISSN: 1308-5727
E-ISSN: 1308-5735

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

AIMS AND SCOPE

The Journal of Clinical Research in Pediatric Endocrinology (JCRPE) publishes original research articles, reviews, short communications, letters, case reports and other special features related to the field of pediatric endocrinology. JCRPE is published in English by the Turkish Pediatric Endocrinology and Diabetes Society quarterly (March, June, September, December). The target audience is physicians, researchers and other healthcare professionals in all areas of pediatric endocrinology.

JCRPE is indexed in **EBSCO, SCOPUS, EMBASE, Engineering Village, Reaxys, Index Copernicus, CINAHL, ProQuest, GALE, Turk Medline, Tübitak Ulakbim TR Index, Index Medicus/PubMed, Turkiye Citation Index, PubMed Central (PMC), Science Citation Index-SCI-E and PubMed/MEDLINE.**

JCRPE has an impact factor 1.163 in 2017.

The journal is printed on an acid-free paper.

Permissions
Requests for permission to reproduce published material should be sent to the publisher.
Publisher: Erkan Mor
Adress: Molla Gürani mah. Kaçamak Sok. 21/1 Fatih, Istanbul, Turkey
Telephone: +90 212 621 99 25
Fax: +90 212 621 99 27
Web page: http://www.galenos.com.tr/en
E-mail: info@galenos.com.tr

GENERAL INFORMATION
Manuscripts must be written in English and must meet the requirements of the journal. Papers that do not meet these requirements will be returned to the author for necessary revision before the review. Manuscripts submitted to JCRPE are evaluated by peer reviewers. Authors of manuscripts requiring modifications have two months to resubmit a revised paper. Manuscripts returned after this deadline will be treated as new submissions. The journal is in compliance with the uniform requirements for manuscripts submitted to biomedical journals published by the International Committee of Medical Journal Editors (NEJM 1997; 336:309-315, updated 2001). Upon submission of the manuscript, authors are to indicate the type of trial/research and provide the checklist of the following guidelines when appropriate: Consort statement for randomized controlled trials (Moher D, Schultz KF, Altman D, for the CONSORT Group. The CONSORT statement revised recommendations for improving the quality of reports of parallel group randomized trials. JAMA 2001 ; 285 : 1987 - 91), the QUOROM statement for meta-analysis and systemic reviews of randomized controlled trials (Moher D, Cook DJ, Eastwood S, Olkin I, Rennie D, Stroup DF. Improving the quality of reports of meta-analyses of randomized controlled trials: the QUOROM statement. Quality of Reporting of Meta-Analyses. Lancet 1999; 354 : 1896 – 900) and the MOOSE guidelines for meta-analysis and systemic reviews of observational studies (Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis of observational studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008 – 12). Keywords are included according to MeSH (Medical Subject Headings) National Library of Medicine.

Once the manuscript is accepted to be published in The Journal of Clinical Research in Pediatric Endocrinology, it receives a Digital Object Identifier (DOI) number. Uncorrected full text files can be reached online via PubMed and Ahead of Print section of the journal's website (http://www.jcrpe.org/ahead-of-print).
All contents will be printed in black and white. The payment of $75 per article should be made if the author(s) wish their articles in color. For payment details, please contact the Editorial Office.

NEW
Article Publication Charges for accepted case reports is $100. Please contact the editorial office for detailed information by the following link: info@jcrpe.org

All other forms of articles are free of publication charge.

MANUSCRIPT CATEGORIES
All manuscripts must adhere to the limitations, as described below, for text only; the word count does not include the abstract, references, or figure/table legends. The word count must be noted on the title page, along with the number of figures and tables. Original Articles should be no longer than 5000 words and include no more than six figures and tables and 50 references.

Short Communications are short descriptions of focused studies with important, but very straightforward results. These manuscripts should be no longer than 2000 words, and include no more than two figures and tables and 20 references.

Brief Reports are discrete, highly significant findings reported in a shorter format. The abstract of the article should not exceed 150 words and the text/article length should not exceed 1200 words. References should be limited to 12, a maximum of 2 figures or tables.

Clinical Reviews address important topics in the field of pediatric endocrinology. Authors considering the submission of uninvited reviews should contact the editors in advance to determine if the topic that they propose is of current potential interest to the Journal. Reviews will be considered for publication only if they are written by authors who have at least three published manuscripts in the international peer reviewed journals and these studies should be cited in the review. Otherwise only invited reviews will be considered for peer review from qualified experts in the area. These manuscripts should be no longer than 6000 words and include no more than four figures and tables and 120 references.

Case Reports are descriptions of a case or small number of cases revealing novel and important insights into a condition's pathogenesis, presentation, and/or management. These manuscripts should be 2500 words or less, with four or fewer figures and tables and 30 or fewer references.

Consensus Statements may be submitted by professional societies. All such submission will be subjected to peer review, must be modifiable in response to criticisms, and will be published only if they meet the Journal's usual editorial standards. These manuscripts should typically be no longer than 4000 words and include no more than six figures and tables and 120 references.

Letters to the Editor may be submitted in response to work that has been published in the Journal. Letters should be short commentaries related to specific points of agreement or disagreement with the published work.

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

Letters should be no longer than 500 words with no more than five complete references, and may not include any figures or tables.

Note on Prior Publication

The journal publishes original research and review material. Material previously published in whole or in part shall not be considered for publication. At the time of submission, authors must report that the manuscript has not been published elsewhere. Abstracts or posters displayed at scientific meetings need not be reported.

MANUSCRIPT SUBMISSION PROCEDURES

JCRPE only accepts electronic manuscript submission at the web site www.jcrpe.org

After logging on to the website www.jcrpe.org click 'online manuscript submission' icon. All corresponding authors should be provided a password and a username after providing the information needed. If you already have an account from a previous submission, enter your username and password to submit a new or revised manuscript. If you have forgotten your username and/or password, e-mail the editorial office for assistance. After logging on the article submission system with your own password and username please read carefully the directions of the system to provide all needed information. Attach the manuscript, tables and figures and additional documents.

All Submissions Must Include:

1. A cover letter requesting that the manuscript be evaluated for publication in JCRPE and any information relevant to your manuscript. Cover letter should contain address, telephone, fax and e-mail address of the corresponding author.

2. Completed Copyright Assignment & Affirmation of Originality form. This form should be filled in thoroughly and faxed to the JCRPE Editorial Office at +90 212 621 99 27.

3. Completed Disclosure of Potential Conflict of Interest Form. The corresponding author must acquire all of the authors' completed disclosure forms and fax them to the editorial office at +90 212 621 99 27.

Authors must complete the online submission forms. If unable to successfully upload the files please contact the editorial office by e-mail.

JCRPE does not charge any fee for article submission or processing.

MANUSCRIPT PREPARATION

General Format

The Journal requires that all submissions be submitted according to these guidelines:

- Text should be double spaced with 2.5 cm margins on both sides using 12-point type in Times Roman font.
- All tables and figures must be placed after the text and must be labeled.
- Each section (abstract, text, references, tables, figures) should start on a separate page.
- Manuscripts should be prepared as word document (*.doc) or rich text format (*.rtf).

Title Page

The title page should include the following:

- Full title
- Authors' names and institutions.
- Short title of not more than 40 characters for page headings
- At least three and maximum eight key words. Do not use abbreviations in the key words
- Word count (excluding abstract, figure legends and references)
- Corresponding author's e-mail and post address, telephone and fax numbers
- Name and address of person to whom reprint requests should be addressed
- Any grants or fellowships supporting the writing of the paper
- The ORCID (Open Researcher and Contributor ID) number of the all authors should be provided while sending the manuscript. A free registration can be done at http://orcid.org.

Structured Abstracts (According to the The Journal of the American Medical Association)

Original Articles should be submitted with structured abstracts of no more than 250 words. All information reported in the abstract must appear in the manuscript. The abstract should not include references. Please use complete sentences for all sections of the abstract. Structured abstract should include background, objective, methods, results and conclusion.

What is already known on this topic?
What this study adds?

These two items must be completed before submission. Each item should include at most 2-3 sentences and at most 50 words focusing on what is known and what this study adds.

Review papers do not need to include these boxes.

Introduction

The article should begin with a brief introduction stating why the study was undertaken within the context of previous reports.

Experimental Subjects

All clinical investigations described in submitted manuscripts must have been conducted in accordance with the guidelines in the Declaration of Helsinki and has been formally approved by the appropriate institutional review committees. All manuscripts must indicate that such approval was obtained and that informed consent was obtained from subjects in all experiments involving humans. The study populations should be described in detail. Subjects must be identified only by number or letter, not by initials or names. Photographs of patients' faces should be included only if scientifically relevant. Authors must obtain written consent from the patient for use of such photographs.

Clinical Trials Registration

For clinical trial reports to be considered for publication in the Journal, prospective registration, as endorsed by the International Conference of Medical Journal Editors, is required. We recommend use of http://www.clinicaltrials.gov.

Experimental Animals

A statement confirming that all animal experimentation described in the submitted manuscript was conducted in accord with accepted standards of humane animal care, according to the Declaration of Helsinki and Genova Convention, should be included in the manuscript.

Materials and Methods

These should be described and referenced in sufficient detail for other investigators to repeat the work. Ethical consent should be included as stated above.

The name of the ethical committe, approval number should be stated.

Results

The Results section should briefly present the experimental data in text, tables, and/or figures. Do not compare your observations with that of others in the results section.

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

Discussion
The Discussion should focus on the interpretation and significance of the findings with concise objective comments that describe their relation to other work in that area and contain study limitations.

Study Limitations
Limitations of the study should be detailed. In addition, an evaluation of the implications of the obtained findings/results for future research should be outlined.

Conclusion
The conclusion of the study should be highlighted.

Acknowledgments (Not Required for Submission)
An acknowledgment is given for contributors who may not be listed as authors, or for grant support of the research.

Authorship Contribution
The kind of contribution of each author should be stated.

References
References to the literature should be cited in numerical order (in parentheses) in the text and listed in the same numerical order at the end of the manuscript on a separate page or pages. The author is responsible for the accuracy of references.
Number of References: Case Report max 30 / Original Articles max 50
Examples of the reference style are given below. Further examples will be found in the articles describing the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (Ann Intern Med.1988; 208:258-265, Br Med J. 1988; 296:401-405). The titles of journals should be abbreviated according to the style used in the Index Medicus.

Journal Articles and Abstracts: List all authors. The citation of unpublished observations, of personal communications is not permitted in the bibliography. The citation of manuscripts in press (i.e., accepted for publication) is permitted in the bibliography; the name of the journal in which they appear must be supplied. Citing an abstract is not recommended.

Books: List all authors or editors.

Sample References

Papers Published in Periodical Journals: Gungor N, Saad R, Janosky J, Arslanian S. Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents. J Pediatr 2004;144:47-55.

Papers Only Published with DOI Numbers: Knops NB, Sneeuw KC, Brand R, Hile ET, de Ouden AL, Wit JM, Verloove-Vanhorick SP. Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. BMC Pediatrics 2005 doi: 10.1186/1471-2431-5-26.

Book Chapters: Darendeliler F. Growth Hormone Treatment in Rare Disorders: The KIGS Experience. In: Ranke MB, Price DA, Reiter EO (eds). Growth Hormone Therapy in Pediatrics: 20 Years of KIGS. Basel, Karger, 2007;213-239.

Books: Practical Endocrinology and Diabetes in Children. Raine JE, Donaldson MDC, Gregory JW, Savage MO. London, Blackwell Science, 2001;37-60.

Tables
Tables must be constructed as simply as possible. Each table must have a concise heading and should be submitted on a separate page. Tables must not simply duplicate the text or figures. Number all tables in the order of their citation in the text. Include a title for each table (a brief phrase, preferably no longer than 10 to 15 words). Include all tables in a single file following the manuscript.

Figures Legends
Figure legends and titles should be submitted on a separate page. Figure legends and titles should be clear and informative. Tables and figures should work under "windows". Number all figures (graphs, charts, photographs, and illustrations) in the order of their citation in the text. Include a title for each figure (a brief phrase, preferably no longer than 10 to 15 words).

Figures & Images
At submission, the following file formats are acceptable: AI, EMF, EPS, JPG, PDF, PPT, PSD, TIF. Figures may be embedded at the end of the manuscript text file or loaded as separate files for submission purposes.
All images MUST be at or above intended display size, with the following image resolutions: Line Art 800 dpi, Combination (Line Art + Halftone) 600 dpi, Halftone 300 dpi. See the Image quality specifications chart for details. Image files also must be cropped as close to the actual image as possible.

Units of Measure
Results should be expressed in metric units.

Validation of Data and Statistical Analysis
Assay validation: Bioassay and radioimmunoassay potency estimates should be accompanied by an appropriate measure of the precision of these estimates. For bioassays, these usually will be the standard deviation, standard error of the mean, confidence limits. For both bioassays and radioimmunoassays, it is necessary to include data relating to within-assay and between-assay variability. If all relevant comparisons are made within the same assay, the latter may be omitted. Statistical analysis should be done accurately and with precision. Please consult a statistician if necessary.

Proofs and Reprints
Proofs and a reprint order are sent to the corresponding author. The author should designate by footnote on the title page of the manuscript the name and address of the person to whom reprint requests should be directed. The manuscript when published will become the property of the journal.

Page and Other Charges

Archiving
The editorial office will retain all manuscripts and related documentation (correspondence, reviews, etc.) for 12 months following the date of publication or rejection.

Submission Preparation Checklist
As part of the submission process, authors are required to check off their submission's compliance with all of the following items, and submissions may be returned to authors that do not adhere to these guidelines.
1. The submission has not been previously published, nor is it before another journal for consideration (or an explanation has been provided in Comments to the Editor).
2. The submission file is in Microsoft Word, RTF, or WordPerfect document file format. The text is double-spaced; uses a 12-point font; employs italics, rather than underlining (except with URL addresses); and all illustrations, figures, and tables are placed within the text at the appropriate points, rather than at the end. Please do not send the manuscript in docx.
3. Where available, URLs for the references have been provided.
4. Upon acceptance of your manuscript for publication, a completed Copyright Assignment & Affirmation of Originality Form will be faxed to the JCRPE Editorial Office (Fax: +90 212 621 99 27)
5. The text adheres to the stylistic and bibliographic requirements outlined in the Author Guidelines, which is found in About the Journal.

INSTRUCTIONS TO AUTHORS

6. Completed Disclosure of Potential Conflict of Interest Form. The corresponding author must aquire all of the authors" completed disclosure forms and fax them, together, to the editorial office along with the Author Disclosure Summary.

Privacy Statement

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

Peer Review Process

1. The manuscript is assigned to an editor, who reviews the manuscript and makes an initial decision based on manuscript quality and editorial priorities.
2. For those manuscripts sent for external peer review, the editor assigns reviewers to the manuscript.
3. The reviewers review the manuscript.
4. The editor makes a final decision based on editorial priorities, manuscript quality, and reviewer recommendations.
5. The decision letter is sent to the author.

The Reviewer is Asked to Focus on the Following Issues:

1. General recommendation about the manuscript
How original is the manuscript?
Is it well presented?
How is the length of the manuscript?

2. Publication timing, quality, and priority
How important is the manuscript in this field?
Does it present original data?
Does it carry priority in publishing?

3. Specific questions regarding the quality of the manuscript
Does the title describe the study accurately?
Is the abstract informative and clear?
Do the authors state the study question in the introduction?
Are the methods clear?
Are ethical guidelines met?
Are statistical analyses appropriate?
Are the results presented clearly?
Does the discussion cover all of the findings?
Are the references appropriate for the manuscript?

4. Remarks to the editor
Accepted in its present form
Accepted after modest revisions
Reconsidered for acceptance after major changes
Rejected

5. Remarks to the author
What would be your recommendations to the author?
Conflict of interest statement for the reviewer (Please state if a conflict of interest is present)
For further instructions about how to review, see Reviewing Manuscripts for Archives of Pediatrics & Adolescent Medicine by Peter Cummings, MD, MPH; Frederick P. Rivara, MD, MPH in Arch Pediatr Adolesc Med. 2002;156:11-13.

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

Editorial

Review

Original Articles

CONTENTS

JCRPE Journal of Clinical Research in Pediatric Endocrinology
www.jcrpe.org

Case Reports

Brief Report

109 Erratum

CONTENTS

EDITORIAL

DOI: 10.4274/jcrpe.galenos.2019.2019.0001

J Clin Res Pediatr Endocrinol 2019;11(1):1-3

Editorial: Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey

Abdullah Bereket

Marmara University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, İstanbul, Turkey

Newborn screening (NBS) for congenital diseases is among the biggest achievements of modern medicine in the field of public health (1). As a secondary prevention tool, NBS is aimed at early detection of asymptomatic infants affected by certain inborn diseases, an accomplishment which will facilitate early diagnosis, thus with proper treatment, will prevent further complications and sequelae and ensure a better quality of life. Sixty years ago, Guthrie and Susi developed a method that allowed early detection of phenylketonuria, the first disease to be screened in the newborns (2). Later addition of thyroid function tests in the mid 1970s to NBS programs facilitated early detection and treatment of hypothyroidism, thus, largely eliminating the neurodevelopmental impairment from both of these congenital diseases. Since then, screening neonates for dozens of congenital diseases became a routine in most of the developed countries with the objective to initiate early treatment and prevent morbidity and mortality associated with them, depending on the sources for healthcare. Cost-effectivity is one of the important considerations when implementing NBS programs. The diseases screened should be frequently seen in that community, treatable when diagnosed early and result in irreversible sequelae if left untreated.

Turkey still has a high consanguinous marriage rate which results in higher incidence of recessively inherited congenital diseases (3). Nevertheless, implementation of NBS in Turkey was relatively late, the first neonatal screening attempts starting at Hacettepe University in the 1980s for phenylketonuria and sponsored by the Turkish Scientific and Research Centre, followed by hypothyroidism in 1990s (4,5). After these pioneering studies, a countrywide-universal screening program executed by the Ministry of Health finally initiated in 2006 which covered phenylketonuria and hypothyroidism in the beginning with gradual expansion of the program to include screening for biotinidase deficiency starting in 2008 and cystic fibrosis in 2015. The program is highly effective with a current coverage rate of 99% all over the country (6).

Classical congenital adrenal hyperplasia (CAH) is the most common form of of primary adrenal insufficiency in childhood and is a potentially life-threatening condition that requires accurate diagnosis and urgent treatment with glucocorticoid and mineralocorticoid replacement (7). CAH occurs in 1:13,000 to 1:15,000 live births. The most common form of CAH is 21-hydroxylase enzyme deficiency (21-OHD) which constitutes 90 to 95% of all cases of CAH. Approximately 75% of infants with classical 21-OHD have the severe salt-wasting form of the disease that, if not promptly diagnosed and treated, can cause death in early infancy from shock, hyponatremia, and hyperkalemia. While, affected female newborns have ambiguous genitalia, male infants appear normal and and may easily be overlooked.

In 2002 the Joint Lawson Wilkins Paediatric Endocrine Society/European Society for Pediatric Endocrinology Working Group recommended biochemical screening for CAH in the newborn period. Neonatal screening for CAH is effective in detecting the salt-wasting form and thereby reducing mortality (8).

In this issue of JCRPE, Güran et al (9) present the first pilot study of NBS for 21-OHD in Turkey in nearly 40,000 neonates to estimate incidence of CAH in Turkey and to assess the characteristics and efficacy of the adopted newborn CAH screening strategy. The study was carried out under the authority of the Turkish Directorate of Public Health, currently the main organisation responsible for nationwide NBS programs. The screening protocol included

Address for Correspondence: Abdullah Bereket MD, Marmara University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, İstanbul, Turkey
E-mail: abdullahbereket@gmail.com **ORCID ID:** orcid.org/0000-0002-6584-9043

Conflict of interest: None declared
Received: 10.01.2019
Accepted: 10.01.2019

one sample two-tier testing with measurement of 17α-hydroxyprogesterone (17-OHP) the most abundant substrate for the CYP21 enzyme by fluoroimmunoassay in the first step in dried blood spots obtained on the 3-5th days of life followed by steroid profiling in the same dried blood spots using liquid chromatography-tandem mass spectrometry (LC-MS) method to measure 17-OHP, 21-deoxycortisol, cortisol, 11-deoxycortisol and androstenedione as a second-tier test in those with positive initial screening. The babies with a steroid ratio (21-deoxycortisol + 17-OHP)/cortisol of ≥0.5 were referred to pediatric endocrinology clinics for further diagnostic assessment. They found that of the 38,935 infants tested, 2265 (5.82%) had second-tier testing, and 212 (0.54%) were referred for clinical assessment. Final analyses showed that six cases of CAH were picked up (four males, two females). Four cases were identified as salt-wasting 21-OHD (two males, two females), one male baby had simple virilizing 21-OHD, and one male baby had 11-OHD CAH. The incidence of classical 21-OHD in the screened population was 1:7,787.

Incidence ratio obtained in this study is among the highest detected so far, confirming the necessity of including 21-OHD screening in the extended NBS program in Turkey. This high incidence is mostly due to the high rate of consanguineous marriages in Turkey (overall 22%, increasing to 34% in South East Anatolia region) giving rise to higher incidence of homozygous biallelic mutations in this population together with compound heterozygotes for two or more different mutant *CYP21A2* alleles (3). Not surprisingly, 3 of 5 patients identified in the current study were homozygous carriers of biallelic mutations causing classical 21-OHD. Furthermore, the high carrier rate for classical CAH in the general population is another reason for this relatively higher incidence of classical CAH in Turkey. Finally, the high efficiency of the screening strategy owing to relatively lower cut offs, hence not missing any case, might have also contributed to the high incidence obtained in the study.

At present, NBS for 21-OHD is carried out in more than 50 countries worldwide. The majority of screening programs use a single screening test which might result in some cases to go undiagnosed even with screening (10). To improve the efficacy of screening, some screening programs reevaluate samples with borderline first-tier test results with a second-tier test and some implement repeat screening in this situation. Because of the high false-positive rate of immunoassay methods, LC-MS was recommended as a second-tier test (11). Mass spectrometry is an analytical technique that allows the identification and quantification of compounds in a biological sample according to the mass/charge ratio. This methodology makes possible the simultaneous measurement of several metabolites and, consequently, ratio measurements of one analyte in respect to another are also possible, which improves the specificity of the screening. In 21-OHD screening, as done in this study, some programs measure the concentration of different hormones (17-OHP, 21-deoxycortisol, and cortisol) as a second-tier test on samples with a positive first-tier test result (12). Some US states mandate organic solvent extraction prior to immunoassay of dried blood spots in order to increase specificity. A search for the best screening approach which detects patients earlier without increasing false negative cases is an ongoing challenge in this area.

Single sample, two-tier screening strategy as used in this study, carries some risk in missing CAH cases with delayed rise in 17-OHP, but obtaining a second sample at a later time would further complicate the screening and cause further delay in obtaining results. As in all screened diseases, a high level of suspicion in patients presenting with signs and symptoms of CAH should still be maintained even if the baby has false-negative screening results. Furthermore, meticulous search for any adrenal-related crises/deaths in the screened area/population is important for evaluation of the efficacy of screening strategy, an activity which requires reliable hospital and/or registry records.

Ideally, screening results should be available before approximately two weeks of age, when salt-wasting typically becomes evident. However, in the current study, the initiation of hydrocortisone treatment ranged between 10 to 30 days of life in 4 cases with salt-wasting 21-OHD and the duration from birth to clinical evaluation of abnormal screening test results of false positive cases was 25.8 ± 6.4 days, results which indicate a delay. Fortunately, none of the patients (except one with hyponatremia) were in critical condition despite this delay. The authors rightly identified the reasons for this delay and suggested possible solutions (accelerating sample transport times, performing both steps of screening in a single central laboratory so that second-tier testing can be performed on the same day upon positive first screening, defining an alarm level of 17-OHP in the first tier and sending newborns with 17-OHP above this level directly for clinical evaluation without waiting for second tier results etc.) to optimize screening program, which is the main objective of doing pilot studies.

For any screened disease, recall rate should be in a reasonable range since referral of these babies to clinics for further confirmation of the diagnosis and initiation of treatment require considerable work, time and cost. This is particularly important for countries where the healthcare system is overloaded and the sources are limited. In

addition, parents of infants with positive screens may suffer significant psychological distress until the final diagnosis is reached. In that respect, recall rate was higher (5.82% in the first and 0.54% in the second tier testing) despite two-tier screening approach in this pilot study compared to previous studies which reported a figure between 0.002% and 1.2% (13). This is due to lower cut-off values the authors used for the first step 17-OHP FIA measurements as well as the second step (21-S + 17-OHP)/F ratio to increase the sensitivity of this pilot study. However, based on the obtained results, elevating cutoff values two-fold would seem to reduce false positives dramatically without decreasing sensitivity. (taking 20 instead of 10 ng/mL for the first tier 17-OHP and taking (21-S + 17-OHP)/F ratio ≥1 instead of ≥0.5) thus potentially eliminating this problem in the future extended NBS program in Turkey. This observation is in line with that reprted by Janzen et al (12) who found that none of the cases with CAH had a (21-S + 17-OHP)/F ratio < 1. It appears that using the above-mentioned cut-off values may enable less labour intensive, and more efficient screening strategy for CAH with a better cost-benefit profile.

Thanks to the LC-MS method, another "bonus" of this pilot study was diagnosing a male newborn with classical 11 hydroxylase deficency, who might be the first patient with 11-OHD identified directly during NBS for CAH. Second-tier LC-MS also measures 11-S in addition to 17-OHP, 21-S, cortisol and 4AS, which is specifically diagnostic for 11-hydroxylase deficiency. Thus, the method of steroid profiling has a potential to distinguish other rare forms of classical CAH, beyond 21-hydroxylase deficiency. Even though the hormones measured in LC-MS/MS based panels are not specifically diagnostic for the rare forms of CAH, perturbations in simultaneous steroid measurements have the potential to provide preliminary information suggesting the need for further evaluation. This is particularly important for countries like Turkey where "rare" forms of classic CAH are common due to high rate of consanguinity.

Analysis of the data from the current study is a big step towards optimization of the 21-OHD screening in Turkey. For no doubt, the incidence of 21-OHD obtained in this study calls for inclusion of 21-OHD screening in the NBS panel in Turkey. It would be interesting to see the figures after the implementation of countrywide screening in the years to come.

References

1. Centers for Disease Control and Prevention (CDC). Ten great public health achievements - United States, 2001-2010. MMWR Morp Mortal WKLY Rep 2011;60:619-623.
2. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics 1963;32:338-343.
3. Koc I. Prevalence and sociodemographic correlates of consanguineous marriages in Turkey. J Biosoc Sci 2008;40:137-148.
4. Ozalp I, Coskun T, Tokol S, Demircin G, Mönch E. Inherited metabolic disorders in Turkey. J Inherit Metab Dis 1990;13:732-738.
5. Yordam N, Calikoğlu AS, Hatun S, Kandemir N, Oğuz H, Teziç T, Ozalp I. Screening for congenital hypothyroidism in Turkey. Eur J Pediatr 1995;154:614-616.
6. Keskinkılıç B. Neonatal screening programs. Clin Biochem 2014;47:692. Epub 2014 May 18
7. Güran T. Latest Insights on the Etiology and Management of Primary Adrenal Insufficiency in Children. J Clin Res Pediatr Endocrinol 2017;9(Suppl 2):9-22. Epub 2017 Dec 27
8. Clayton PE, Miller WL, Oberfield SE, Ritzén EM, Sippell WG, Speiser PW; ESPE/ LWPES CAH Working Group. Consensus statement on 21-hydroxylase deficiency from the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society. Horm Res 2002;58:188-195.
9. Güran T, Tezel B, Gürbüz F, Selver Eklioğlu B, Hatipoğlu N, Kara C, Şimşek E, Çizmecioğlu FM, Ozon A, Baş F, Aydın M, Darendeliler F. Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: A Pilot Study with 38,935 Infants. J Clin Res Pediatr Endocrinol 2018. [Epub ahead of print]
10. Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. Pediatrics 2012;130:1261-1268. Epub 2012 Oct 15.
11. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, Meyer-Bahlburg HF, Miller WL, Montori VM, Oberfield SE, Ritzen M, White PC; Endocrine Society. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2010;95:4133-4160.
12. Janzen N, Peter M, Sander S, Steuerwald U, Terhardt M, Holtkamp U, Sander J. Newborn screening for congenital adrenal hyperplasia: additional steroid profile using liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 2007;92:2581-2589. Epub 2007 Apr 24
13. Gidlöf S, Falhammar H, Thilen A, von Döbeln U, Ritzen M, Wedell A. Nordenström A. One hundred years of congenital adrenal hyperplasia in Sweden:a retrospective, population based cohort study. Lancet Diabetes Endocrinol 2013;1:35-42. Epub 2013 Feb 26

REVIEW

DOI: 10.4274/jcrpe.galenos.2018.2018.0036

J Clin Res Pediatr Endocrinol 2019;11(1):4-12

Neonatal Hypopituitarism: Approaches to Diagnosis and Treatment

Selim Kurtoğlu[1,2], Ahmet Özdemir[1], Nihal Hatipoğlu[2]

[1]Erciyes University Faculty of Medicine, Department of Pediatrics, Division of Neonatalogy, Kayseri, Turkey
[2]Erciyes University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Endocrinology, Kayseri, Turkey

Abstract

Hypopituitarism is defined as a decreased release of hypophyseal hormones, which may be caused by disease of the pituitary gland disease or hypothalamus. The clinical findings of neonatal hypopituitarism depend on the causes and on presence and extent of hormonal deficiency. Patients may be asymptomatic or may demonstrate non-specific symptoms, but may still be at risk for development of pituitary hormone deficiency over time. Patient history, physical examination, endocrinological, radiological and genetic evaluations are all important for early diagnosis and treatment. The aim of this paper was to present a review of etiological factors, clinical findings, diagnosis and treatment approaches in neonatal hypopituitarism.

Keywords: Diagnosis, hypophysis, hypothalamus, neonatal hypopituitarism, treatment

Introduction

The pituitary gland is the central regulator of growth, metabolism, reproduction and homeostasis. It consists of a frontal lobe (adenohypophysis), a posterior lobe (neurohypophysis) and a middle lobe. Growth hormone (GH), follicle stimulating hormone (FSH), luteinising hormone (LH), thyroid stimulating hormone (TSH), prolactin (PRL) and adrenocorticotropic hormone (ACTH) are released from the frontal lobe and arginine vasopressin (AVP) and oxytocin from the posterior lobe. The frontal and middle lobes consist of ectoderm and the posterior lobe consists of neural ectoderm (1). A series of transcription factors are involved in pituitary gland formation. Neonatal hypopituitarism may occur due to developmental defects of the pituitary gland, genetic mutations, and perinatal and neonatal events (Table 1) (1,2,3,4,5,6,7,8). Genetic mutations causing hypopituitarism and their sub-groups are shown in Tables 2 and 3 (1,9,10,11,12,13,14). The incidence of congenital hypopituitarism is estimated to be between 1/4000-1/10,000 (15).

Hypopituitarism findings may not be present in the neonatal period and may occur with different, non-specific, clinical presentations. Also, the sensitivity of laboratory methods may not be satisfactory for newborns (13). Nevertheless, it is possible for neonatologists to reach a diagnosis by focusing on some clues.

Neonatal Clinical Findings in Congenital Hypopituitarism Cases

It is interesting that newborns with congenital hypopituitarism have normal birth weight and height (16). Clinical presentations in hypopituitary newborns occur due to combined or total hypophyseal hormone deficiency. Ocular findings, midline defects and genital abnormalities may also be detected in these patients (Figures 1, 2, 3).

Generally these patients present with non-specific findings although clinical findings may become evident over time. All newborns suspected of hypopituitarism must be assessed for optic nerve hypoplasia, midline defects or syndromes; even those in whom the initial endocrine evaluations are normal (17,18,19). In premature infants diagnosis is difficult due to problems commonly associated with prematurity including hypothalamus-pituitary axis immaturity and limited information on normal values for newborns and contraindication of stimulation tests. It is reported that only 23% of cases are diagnosed with postnatal problems such as hypoglycemia, hyponatremia or recurrent sepsis in the neonatal period (20). Non-specific

Address for Correspondence: Ahmet Özdemir MD, Erciyes University Faculty of Medicine, Department of Pediatrics, Division of Neonatalogy, Kayseri, Turkey
Phone: +90 352 207 66 66 **E-mail:** dr.ozdemir@yahoo.com **ORCID ID:** orcid.org/0000-0002-7572-069X

Conflict of interest: None declared
Received: 01.02.2018
Accepted: 08.05.2018

symptoms such as hypoglycemia, neuroglycopenia-related lethargy, apnea, jitteriness, convulsions, inability to gain weight, hyponatremia unaccompanied by hyperkalemia, temperature instability, recurrent sepsis, hemodynamic instability, neonatal cholestasis and prolonged jaundice are observed in the neonatal period (21,22,23). In addition to hypoglycemia, lack of thymic involution and fluid intolerance are striking in cortisol deficiency (24). Cortisol deficiency-related hypoglycaemia, as a result of isolated or combined-type ACTH deficiency, is severe. Heart failure constitutes a vital risk in newborns with the *LHX4* mutation-related multiple hormone deficiency. Heart failure in these newborns can be resolved with thyroxine and hydrocortisone treatment and the hypoglycemia can be treated successfully with GH (25). Cortisol increases bile flow and cortisol deficiency leads to problems in bile acid synthesis and transport and eventually cholestasis. Cholestasis occurs generally in the first 13 days of life. Transaminase levels increase after 2-4 weeks but GGT remains within normal ranges (26). In cholestasis cases, liver biopsy, usually performed before hypopituitarism diagnosis, reveals canalicular cholestasis. Mild portal eosinophilic infiltration is demonstrable on histopathology. If there is a delay in diagnosis, transaminase levels continue to increase, while cholestasis recovers in 6-10 weeks if treatment is started after diagnosis (27,28). ACTH deficiency is present in over 50 % of cases with ocular and frontal brain abnormalities. Temperature instability and

Table 1. Causes of neonatal hypopituitarism

Congenital causes
Maternal hyperglycaemia,
Congenital infections (syphilis, toxoplasmosis),
Hypothalamus-pituitary development defects,
Midline defects, cleft lip/palate,
Genetic mutations.
Perinatal-neonatal causes
Birth trauma-asphyxia (pituitary stalk junction),
Neonatal sepsis,
Hemochromatosis (transient).

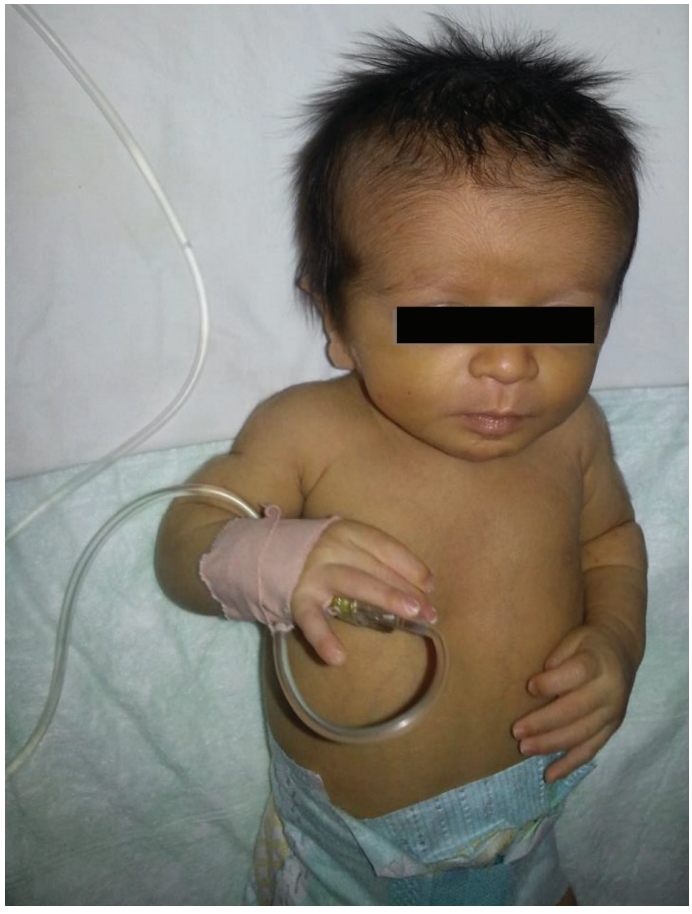


Figure 1. Facial appearance in the presence of neonatal growth hormone deficiency (from the files of Erciyes University Faculty of Medicine, Department of Neonatology)

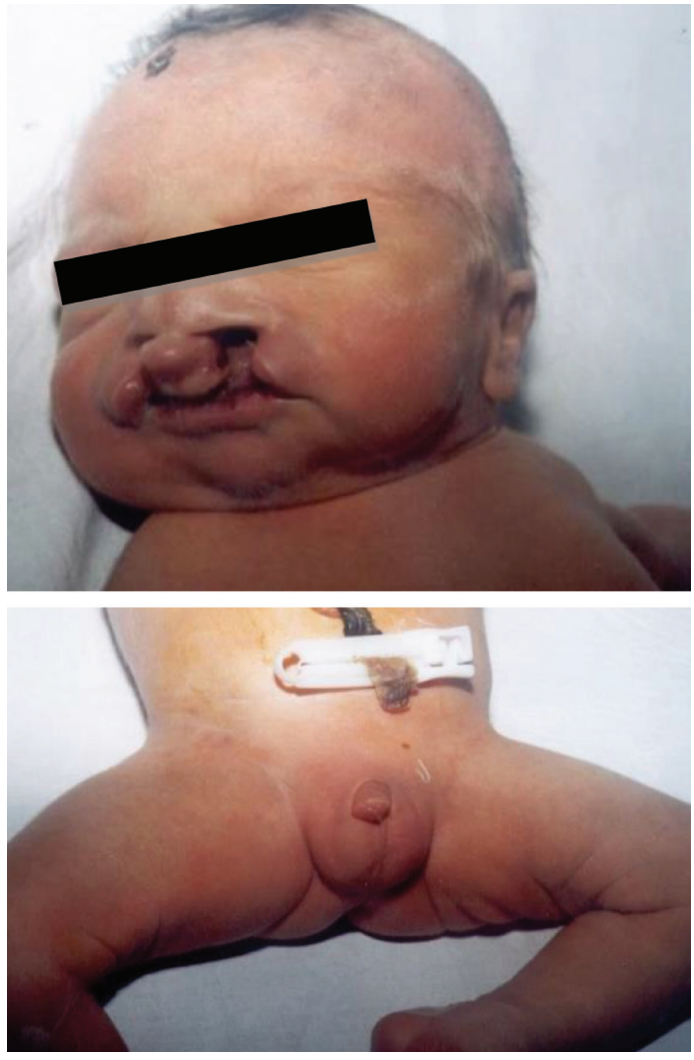


Figure 2. Midline defect with cleft palate-lip and micropenis (from the files of Erciyes University Faculty of Medicine, Department of Neonatology)

prolonged physiological jaundice are also usually present in cases with neonatal TSH deficiency. The development of female genitalia is independent of hormone secretion; hence congenital hypogonadotropic hypogonadism (HH) is not expected to affect the normal development of female external genitalia (29). Micropenis is defined according to a -2.5 standard deviation cut-off from the mean value. Values under 1.5 cm at gestational age 30 weeks, 2 cm at 34 weeks and under 2.5 cm in term infants are defined as micropenis (30). Optic nerve hypoplasia or corpus callosum agenesis-related nystagmus may be observed in infants (31,32). Polyhydramniosis, polyuria, weight loss, anxiety, demand for water instead of formula, signs and symptoms of dehydration and hypernatremia are observed in cases of diabetes insipidus (33).

Diagnostic Approaches in Neonatal Hypopituitarism

Patient and family history: A careful and detailed medical history should be obtained including information on consanguineous marriage, index cases, traumatic/breech birth and possible neonatal central nervous system infection.

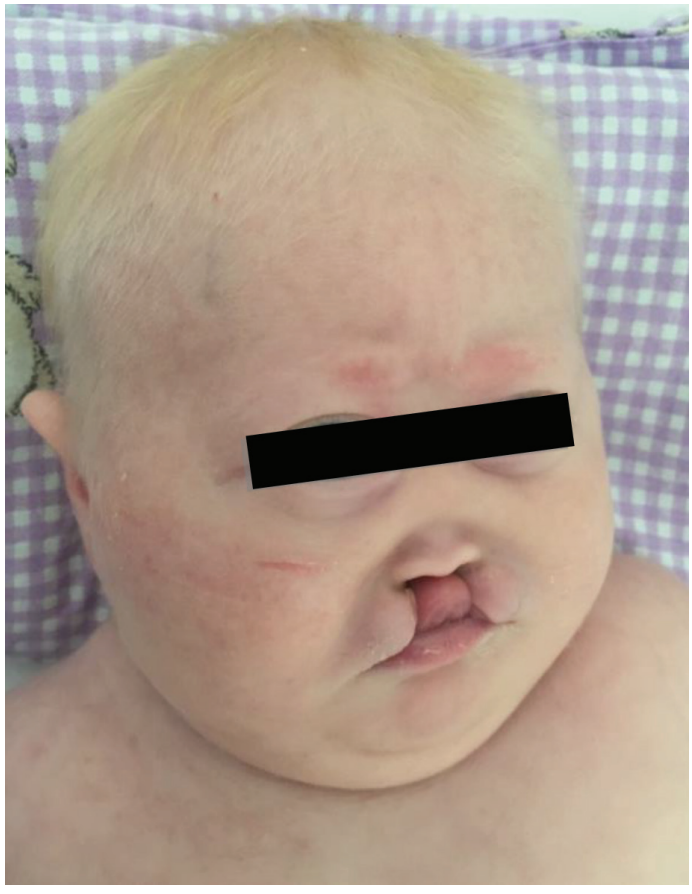


Figure 3. A case of holoprosencephaly with cleft palate/lip and anterior and posterior pituitary insufficiency (from the files of Erciyes University Faculty of Medicine, Department of Neonatology)

Physical examination findings and symptoms: Height, weight and head circumference should be measured in the newborns. Fontanelle size, eyes, cleft palate/lip, hepato-splenomegaly, lymphadenopathy, jaundice and malformations are assessed. Presence of microphallus and undescended testicles are noted in the genital examination.

Syndromes accompanied by hypophyseal deficiency are listed in Table 4 (1).

Endocrine Evaluation

Pituitary-adrenal axis: ACTH deficiency may be life threatening. Quick action is important, especially with asymptomatic midline defects. Circadian rhythm in cortisol secretion does not mature in the first six postnatal months. Thus, cortisol should be measured every hour of the day instead of only in the morning (34). Mehta et al (21) interpret cortisol values below 175 nmol/L (6.34 micrograms/dL) at 8 o'clock in the morning as deficiency. Multiple random cortisol measurements are not suitable for premature and ill infants and cortisol measurement by induced hypoglycemia is not recommended. However, cortisol measurement may be useful in addition to insulin and GH measurement in infants with hypoglycemia at presentation. Cortisol deficiency is accepted to be present if cortisol response remains below 12.67 micrograms/dL in hypoglycemic infants (35). While a standard ACTH test is easy and safe, the sensitivity is approximately 80% (10). False negative results can occur even in infants with ACTH deficiency (36). A corticotropin releasing hormone test can be performed to determine ACTH deficiency in infants. However, normative values in cases of central hypothyroidism and midline defects are not known and the test is contraindicated in ill infants (37). As the circadian rhythm matures, a cortisol value of 175 nmol/L (6.34 micrograms/dL) at 8 o'clock in the morning excludes ACTH deficiency if the cortisol level is above 540 nmol/L (19.56 microgram/dL) at the 30th minute with a low dose ACTH test. The specificity of the test was found to be 100% but the sensitivity was 69% (20).

TSH deficiency: In cases of central hypothyroidism, low or normal TSH level despite a low FT4 level is striking. Central hypothyroidism is diagnosed if the free T4 level is below 0.8 ng/dL and TSH is normal or slightly elevated (38). It should be kept in mind that severe infection, sick thyroid syndrome or dopamine infusion can cause low TSH levels in infants (39). Early diagnosis is important in central hypothyroidism cases since other hormone deficiencies may be concurrent in as high as 78% of cases (1). Hypoglycemia and neonatal

Table 2. Mutations and characteristics of genes involved in pituitary gland development

Transcription factor gene	Type of inheritance	Hormone deficiencies	MRI findings	Other findings
POU1FI (PIT-1)	AR, AD	GH, TSH, PRL	APH	-
PROP1	AR	GH, TSH, LH, FSH, PRL late ACTH deficiency	APH,	Transient AP hyperplasia
HESX1	AR, AD	IGHE, KPHE	APH, EPP	Septo-optic dysplasia Corpus callosum agenesis
LHX3	AR	GH, TSH, LH, FSH, PRL ACTH may be deficient	APH, N,	Neck rotation limited Short cervical spin Sensorineural deafness
LHX4	AD	GH, TSH, ACTH in addition to FSH, LH	APH, EPP	Cerebellar anomalies
SOX2	AD (*de novo*)	HH, GH deficiency	APH	Anophthalmia, microphtalmia, esophageal atresia, genital tract anomalies, hypothalamic hamartoma, diplegia, sensorineural deafness
SOX3	X-linked	Isolated or combined GH deficiency	APH, EPP	Mental retardation
OTX2	AD	Isolated GH or GH, TSH, PRL, FSH, LH deficiency	N, APH, EPP	Bilateral anophthalmia or severe microphthalmia
TBX19 (T-PIT)	AR	ACTH	N	Neonatal hypoglycemia
PC1	AR	HH, ACTH	N	Hypoglycemia, obesity
DAX-1	X-linked	HH, adrenal hypoplasia	N	-
GLI2	AD	Panhipopituitarism	-	Holoprosencephaly
GLI3	AD	Panhipopituitarism	-	Pallister-Hall syndrome
FGF8	AD	Hipopituitarism	-	Holoprosencephaly
FGFR1	AD	Hipopituitarism	Pituitary hypoplasia	Corpus callosum agenesis, ocular defects
IGFSF1	X-linked	TSH, GH, PRL deficiency	-	Macroorchidism
Holoprosencephaly related *SHHT*, *GIF* and *SIX3*	-	Isolated GH or combined	Pituitary stalk suture or fine stalk, small adenohypophysis and ectopic neurohypophysis	Cholestasis, single upper incisor tooth
PROKR2	AR, AD	GH, TSH, ACTH	APH, EPP	Neonatal hypoglycemia, micropenis
PITX2	AD	LH, FSH	APH	Anterior eye chamber, dental hypoplasia, craniofacial dysmorphism, protuberant umbilicus

AR: autosomal recessive, AD: autosomal dominant, GH: growth hormone, TSH: thyroid stimulating hormone, PRL: prolactin, APH: anterior pituitary hypoplasia, LH: luteinising hormone, FSH: follicle stimulating hormone, ACTH: adrenocorticotropic hormone, EPP: ectopic posterior pituitary, N: normal, MRI: magnetic resonance imaging, HH: hypogonadotropic hypogonadism

Table 3. Isolated growth hormone deficiency subtypes

Type	Gene	Heredity	Phenotype
1A	*GH1*	Autosomal recessive	Postnatal severe growth failure, very low GH level, antibody development with treatment
1B	*GH1, GHRHR*	Autosomal recessive	Milder growth insufficiency, GH is present but no antibody develops with treatment
2	*GH1*	Autosomal dominant	Growth insufficiency, hypoplastic pituitary, other hormone deficiencies may be added
3	*BTK*, *SOX3* or other genes?	X-linked	Agammaglobulinemia and mental retardation are accompanying features

GH: growth hormone

hepatitis can develop if hypothyroidism is diagnosed late and mortality is reported to approach 14% in these patients (36). The necessity of a TRH test for diagnosis is controversial (40,41).

Gonadotropin deficiency: Micropenis alone or together with undescended testicles in boys is observed in isolated HH or in multiple hypophyseal hormone deficiencies. Secretion of postnatal FSH and LH occurs in normal male newborns and testosterone levels increase with a peak in the 4-10th weeks and start to decrease around the sixth month. High gonadotropin levels may last for up to two years in girls. This series of events is called mini-puberty (42). If LH is <0.8 IU/L and total testosterone is <30 ng/dL in male infants between postnatal day 15 and six months, central hypogonadism is diagnosed (43). In female infants, central hypogonadism is diagnosed if FSH is <1.0 IU/L between day 15 and two years (44,45,46,47). HH can be diagnosed with gonadotropin releasing hormone and human chorionic gonadotropin (hCG) tests in infants (48,49). Basal FSH and LH are low in infants with HH and a blunt gonadotropin response is seen after the test (37). Attention should be paid to penile growth and testicular descent in infants tested with hCG (50).

GH deficiency: It should be kept in mind that GH level is high in the neonatal period (51,52). GH can be measured directly in the neonatal period although a decrease in the GH level and an increase in insulin-like growth factor-1 (IGF-1) and IGF binding protein-3 (IGFBP-3) levels are observed in the neonatal period, starting from birth. Kurtoğlu et al (53) reported that in term infants GH levels decreased and IGF-1 and IGFBP-3 levels showed a gradual increase (Table 5). Binder et al (54), reported GH levels in the neonatal period in children who were later detected to have GH deficiency. They concluded that in the postnatal first week, a level of 7 ng/mL reflected GH deficiency and this level showed very good sensitivity and specificity-100% and 98% respectively. The same group also observed that neonatal GH deficiency was present in cases who had multiple hormone deficiency and malformations, but that isolated GH deficiency was not detected in newborns (55). It has also been reported that random GH measurement may be useful in the first 14 days in newborns (56).

Although GH stimulation tests are not recommended in infants under 12 months old, GH levels measured in infants with hypoglycemia may yield useful clinical clues to diagnosis, though the specificity is low (57,58). A GH value of <7.7 ng/mL in infants with hypoglycemia has been suggested as a criterion of GH deficiency (35). It has been reported that a glucagon stimulation test may be used in infants younger than 12 months of age (59). When glucagon is injected at a dose of 0.03 mg/kg and samples are taken at basal, 45, 90, 120, 150 and 180 minutes, the GH level is normally expected to be above 10 ng/mL.

PRL deficiency: PRL concentration is low in cases with *POU1F1*, *LHX3*, *OTX2* and *IGFSF-1* gene mutations and in cases of panhypopituitarism. Values below 31 ng/mL in the first 30 postnatal days and 24 ng/mL between the 30th-60th days are accepted as hypoprolactinemia (60). Breast tissue should not be palpated before taking blood and it should be confirmed that no medicine affecting PRL level has been taken. PRL levels may be low in infants given dopamine as an inotropic agent in the neonatal period (61).

Diabetes insipidus: The definition for polyuria in diabetes insipidus is a daily urine output >2 liters/m^2, which corresponds to a volume of 150 mL/kg/day in the newborn (62). The suggested criterion for polyuria of 4 mL/kg/h in

Table 4. Some syndromes with pituitary insufficiency

PHACE(S)	Posterior fossa anomalies (Dandy-Walker cyst), hemangiomas of the face and neck, arterial malformations, cardiac defects, eye anomalies, sternal defects (sternal cleft, supraumblical raphe)
Rieger	Malformations in the anterior chamber of the eye, pretuberant umbilicus, abnormal teeth, mental retardation
Johannson-Blizzard syndrome	Microcephaly, exocrine pancreatic dysfunction, recto-urethral anomalies, hypothyroidism
Pallister-Hall syndrome	Polydactyly, imperforate anus, hypothalamic hamartoblastoma

Table 5. Weekly growth hormone, insulin-like growth factor-1 and insulin-like growth factor binding protein-3 values in the neonatal period (given as means ± standard deviation). The p value shows significant change over time

Parameter	0-7 days	8-14 days	15-21 days	22-30 days	p value
GH (ng/mL)	13.6 ± 5.68^a	9.38 ± 4.06^b	8.73 ± 3.19^c	7.91 ± 5.57^b	<0.001
IGF-1 (ng/mL)	55.4 ± 49.6^a	69.6 ± 46.6^{ab}	82.3 ± 70.0^{ab}	89.5 ± 47.6^b	0.026
IGFBP-3 (ng/mL)	2043 ± 572^a	2352 ± 777^{ac}	3002 ± 856^{bc}	3133 ± 1150^{bc}	<0.001

GH: growth hormone, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor binding protein-3.

$^{a, b, c}$:Shared different letters represent statistically significant differences and same letters represent similarity on the same line

children corresponds to a urine volume of > 6 mL/kg/hour in the neonatal period (63). In most cases of diabetes insipidus presenting in the neonatal period, anatomical defects or autosomal dominant-recessive genetic causes are present. Central diabetes insipidus presentation is also observed in cases with septo-optic dysplasia, corpus callosum agenesis and holoprosencephaly (64). Very rarely, tumours located in the posterior hypophysis and surgical intervention for craniopharyngioma cause diabetes insipidus. Symptoms such as polyuria, polydypsia (excessive drinking of water rather than formula), weight loss, growth deficiency and persistent hypernatremia despite giving fluid and dilute urine may be striking. Plasma and urine osmolarity measurements in the early hours of the morning may help in ascertaining a diagnosis (1). Serum osmolarity < 270 mosm/kg and urine osmolarity > 600 mosm/kg draw suggest an alternative diagnosis rather than diabetes insipidus. A water deprivation test is risky in the neonatal period and can only be done in special centers (33). However, a nasal desmopressin test may be performed. On the 8th and 24th hours after the application of 0.012 mL of desmopressin (1.2 microgrammes; 1 mL = 100 microgram), observation of a decrease in serum sodium and osmolarity, an increase in urine osmolarity and a decrease in diuresis support the diagnosis (65).

Radiological examinations: In newborns, bone age is assessed with knee radiography. Epiphyseal dysgenesis should also to be investigated. Brain and hypophysis imaging should be done in infants suspected of having hypopituitarism. The degree of severity of the hypopituitarism will be proportional to the extent of the neuro-radiological abnormalities (18). Pituitary gland height neurohypophysis brightness or ectopia, an undescended posterior lobe, infundibulum morphology, absence of corpus callosum and of septum pellucidum, optic nerve and chiasma, holoprosencephaly, schizencephaly, cerebellar hypoplasia, absence of fornix and presence of Chiari malformation should be assessed with imaging (66). Lack of neurohypophysis brightness supports the diagnosis in cases of central diabetes insipidus. Data on pituitary gland height in newborns are presented in Table 6 (67,68,69,70).

Genetic studies: Genetic studies should be targeted depending on family history, physical examination and laboratory and radiological findings (13,15).

Treatment Approaches and Follow-up

Cases diagnosed with neonatal hypopituitarism should be followed-up by a multidisciplinary team. Suitable hormonal treatments, providing follow-up baring in mind that some hormone deficiencies may develop slowly, ocular and neurodevelopmental follow-up in syndromic cases, acquisition of genetic data and establishing a good relationship with the family are important during follow-up.

Treatment of central hypothyroidism is started with 6-8 microgram/kg/day L-thyroxine. The aim is to keep the free T4 level in the upper part of the normal range. After starting treatment dose insufficiency is monitored by measuring free T4 and overdose by free T3 concentrations (71). It is important to know the cortisol level before thyroxine replacement. This is because cortisol clearance increases and cortisol deficiency occurs when thyroxine is given to infants with low cortisol level, especially in preterm cases (72). In cases of cortisol deficiency oral hydrocortisone should be started first and thyroid replacement should be initiated subsequently. In preterm infants, daily cortisol production is reported to be 7.28 mg/m^2/day on the fifth day and 6 mg/m^2/day in the second week (73). Oral cortisol should be higher than daily production and should be given by dividing into three doses of 12-18 mg/m^2/day. In case of stress, the dose should be increased two- to threefold. Diabetes insipidus may develop with hydrocortisone treatment and the infant should be observed closely (33). In infants with cholestasis at initiation of treatment oral thyroxine and hydrocortisone should be administered at high dose due to absorption deficiency and it should be kept in mind that the doses should be decreased after cholestasis resolves (74).

Testosterone injection, dihydrotestosterone gel application or recombinant human gonadotropin subcutaneous infusion treatments can be initiated between the ages of 1-6 months in boys in whom HH is diagnosed (30,75,76). Acceptable results were obtained by giving 25 mg depot testosterone intramuscularly, every three weeks over a period of three months (77).

Intranasal or peroral desmopressin should be used in cases of central diabetes insipidus. The maximum plasma

Table 6. Pituitary gland height values in the neonatal period (mm)

Researcher	Number of cases and age	Pituitary gland height in mm (mean ± standard deviation)
Kitamura et al (67)	88 (0-122 days)	3.9 ± 0.7
Dietrich et al (68)	17 (0.1-1.5 weeks)	4.12 ± 1.13
	17 (1.7-6.0 weeks)	3.94 ± 0.6
Argyropoulou et al (69)	12 (0-12 months)	3.5 ± 0.5
Sari et al (70)	14 (0-12 months)	Girls 3.81 ± 0.68
	13 (0-12 months)	Boys 3.91 ± 0.75

concentration was observed after 40-55 minutes with intranasal or oral use and the half-life is nearly 3.5 hours. Urine output starts to decrease after 1-2 hours and the effect lasts from six to 18 hours (78). It is recommended to start treatment with a low dose and titrate according to the response. The intranasal form should be started with a dose of 0.05-0.1 micrograms and should also be titrated (79). Oral tablets are dissolved in 3-5 mL of water and given by dividing the daily dose of 5 micrograms/kg into two (80,81). When the treatment is started, the daily liquid intake should be lowered to maintain fluid quantities which will prevent hyponatremia (65,80).

GH treatment can be started in the neonatal period. However. treatment is often started after the neonatal period since diagnosis is generally delayed. GH treatment can contribute to hypoglycemia recovery (16).

Conclusion

Neonatal period is different from other periods of life. Assessment of hypothalamus-hypophysis axis different from the other stages of life. The hormonal deficiencies particularly in this period being asymptomatic make the interpretation of pathologic conditions difficult. Therefore, efforts have been made to shed light on the diagnosis and the therapeutic approach specific to this period.

Ethic

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Selim Kurtoğlu, Nihal Hatipoğlu, Ahmet Özdemir, Data Collection and Processing: Ahmet Özdemir, Analysis and Interpretation: Nihal Hatipoğlu, Literature Research: Selim Kurtoğlu, Writing: Selim Kurtoğlu, Nihal Hatipoğlu, Ahmet Özdemir.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Alatzoglou KS, Dattani MT. Genetic forms of hypopituitarism and their manifestation in the neonatal period. Early Hum Dev 2009;85:705-712. Epub 2009 Sep 16
2. Massa G, Vanderschueren-Lodeweyckx M, Van Vliet G, Craen M, deZegher F, Eggermont E. Hypothalamo-pituitary dysfunction in congenital toxoplasmosis. Eur J Pediatr 1989;148:742-744.
3. Daaboul JJ, Kartchner W, Jones KL. Neonatal hypoglycemia caused by hypopituitarism in infants with congenital syphilis. J Pediatr 1993;123:983-985.
4. Chang AK, Sopher AB, Pat Gallagher M, Khandji AG, Oberfield SE. Congenital pituitary gland abnormalities--a possible association with maternal hyperglycemia: two case reports. Clin Pediatr (Phila) 2011;50:662-665. Epub 2011 Feb 21
5. Indolfi G, Bèrczes R, Pelliccioli I, Bosisio M, Agostinis C, Resti M, Zambelli M, Lucianetti A, Colledan M, D'Antiga L Neonatal haemochromatosis with revesible pituitary involvement. Transpl Int 2014;27:76-79. Epub 2014 May 10
6. Saranac L, Bjelakovic B, Djordjevic D, Novak M, Stankovic T. Hypopituitarism occurring in neonatal sepsis. J Pediatr Endocrinol Metab 2012;25:847-848.
7. Akin MA, Kurtoğlu S, Sarici D, Akin L, Hatipoğlu N, Korkmaz L, Güneş T, Oztürk MA, Akçakuş M. Endocrine abnormalities of patients with cleft lip and/or cleft palate during neonatal period. Turk J Med Sci 2014;44:696-702.
8. Geffner ME. Hypopituitarism in childhood. Cancer Control 2002;9:212-222.
9. Bar C, Zadro C, Diene G, Oliver I, Pienkowski C, Jouret B, Cartault A, Ajaltouni Z, Salles JP, Sevely A, Tauber M, Edouard T. Pituitary stalk interruption syndrome from infancy to adulthood: Clinical, hormonal, and radiological assessment according to the initial presentation. PLoS One 2015;10:e0142354.
10. Voutetakis A, Sertedaki A, Dacou-Voutetakis C. Pituitary stalk interruption syndrome: cause, clinical manifestations, diagnosis, and management. Curr Opin Pediatr 2016;28:545-550.
11. Castinetti F, Reynaud R, Saveanu A, Jullien N, Quentien MH, Rochette C, Barlier A, Enjalbert A, Brue T. MECHANISMS IN ENDOCRINOLOGY: An update in the genetic aetiologies of combined pituitary hormone deficiency. Eur J Endocrinol 2016;174:239-247. Epub 2016 Jan 5
12. Kelberman D, Dattani MT. Hypopituitarism oddities: congenital causes. Horm Res 2007;68:138-144. Epub 2007 Dec 10
13. Kelberman D, Dattani MT. Hypothalamic and pituitary development: novelinsights into the aetiology. Eur J Endocrinol 2007;157(Suppl 1):3-14.
14. Arslan D, Patiroğlu T, Kendirci M, Kurtoğlu S. X-linked agammaglobulinemia and isolated growth hormone deficiency. Turk J Pediatr 1998;40:609-612.
15. Davis SW, Castinetti F, Carvalho LR, Ellsworth BS, Potok MA, Lyons RH, Brinkmeier ML, Raetzman LT, Carninci P, Mortensen AH, Hayashizaki Y, Arnhold IJ, Mendonça BB, Brue T, Camper SA. Molecular mechanisms of pituitary organogenesis: In search of novel regulatory genes. Mol Cell Endocrinol 2010;323:4-19. Epub 2009 Dec 16
16. Cavarzere P, Biban P, Gaudino R, Perlini S, Sartore L, Chini L, Silvagni D, Antoniazzi F. Diagnostic pitfalls in the assessment of congenital hypopituitarism. J Endocrinol Invest 2014;37:1201-1209. Epub 2014 Aug 1
17. Mehta A, Hindmarsh PC, Stanhope RG, Turton JP, Cole TJ, Preece MA, Dattani MT. The role of growth hormone in determining birth size and early postnatal growth, using congenital growth hormone deficiency (GHD) as a model. Clin Endocrinol(Oxf) 2005;63:223-231.
18. Arrigo T, Wasniewska M, De Luca F, Valenzise M, Lombardo F, Vivenza D, Vaccaro T, Coradi E, Biason-Lauber A. Congenital adenohypophysis aplasia: clinical features and analysis of the transcriptional factors for embryonic pituitary development. J Endocrinol Invest 2006;29:208-213.
19. Ahmad T, Borchert M, Geffner M. Optic nerve hypoplasia and hypopituitarism. Pediatr Endocrinol Rev 2008;5:772-777.
20. Mehta A, Hindmarsh PC, Mehta H, Turton JP, Russell-Eggitt I, Taylor D, Chong WK, Dattani MT. Congenital hypopituitarism: clinical, molecular and neuroradiological correlates. Clin Endocrinol (Oxf) 2009;71:376-382. Epub 2009 Mar 6
21. Mehta A, Hindmarsh PC, Dattani MT. An update on the biochemical diagnosis of congenital ACTH insufficiency. Clin Endocrinol (Oxf) 2005;62:307-314.

22. Gonc EN, Kandemir N, Sen Y, Yordam N. Hyponatremia can be a presenting finding of multiple pituitary hormone deficiency in children: report of a case and review of literature. Clin Pediatr 2005;44:623-628.

23. Gönç EN, Kandemir N, Andiran N, Ozön A, Yordam N. Cholestatic hepatitis as a result of severe cortisol deficiency in early infancy: report of two cases and review of literature. Turk J Pediatr 2006;48:376-379.

24. McQueen MC, Copeland KC. Congenital hypopituitarism. With free water intolerance and lack of thymic involution. Early recognition of clinical presentation. Clin Pediatr (Phila) 1989;28:579-580.

25. Filges I, Bischof-Renner A, Röthlisberger B, Potthoff C, Glanzmann R, Günthard J, Schneider J, Huber AR, Zumsteg U, Miny P, Szinnai G. Panhypopituitarism presenting as life-threatening heart failure caused by an inherited microdeletionin 1q25 including LHX4. Pediatrics 2012;129:529-534. Epub 2012 Jan 9

26. Sheehan AG, Martin SR, Stephure D, Scott RB. Neonatal cholestasis, hypoglycemia, and congenital hypopituitarism. J Pediatr Gastroenterol Nutr 1992;14:426-430.

27. Binder G, Martin DD, Kanther I, Schwarze CP, Ranke MB. The course of neonatal cholestasis in congenital combined pituitary hormone deficiency. J Pediatr Endocrinol Metab 2007;20:695-702.

28. Karnsakul W, Sawathiparnich P, Nimkarn S, Likitmaskul S, Santiprabhob J, Aanpreung P. Anterior pituitary hormone effects on hepatic functions in infants with congenital hypopituitarism. Ann Hepatol 2007;6:97-103.

29. Braslavsky D, Grinspon RP, Ballerini MG, Bedecarra's P, Loreti N, Bastida G, Ropelato MG, Keselman A, Campo S, Rey RA, Bergada I. Hypogonadotropic Hypogonadism in infants with Congenital Hypopituitarism: A Challenge to diagnose at an Early Stage. Horm Res Paediatr 2015;84:289-297. Epub 2015 Sep 11

30. Hatipoglu N, Kurtoglu S. Micropenis: etiology, diagnosis and treatment approaches. J Clin Res Pediatr Endocrinol 2013;5:217-223.

31. Herman TE, Siegel MJ. Congenital adenohypophysis aplasia. J Perinatol 2008;28:448-450.

32. Scommegna S, Galeazzi D, Picone S, Farinelli E, Agostino R, Bozzao A, Boscherini B, Cianfarani S. Neonatal identification of pituitary aplasia: a life-saving diagnosis. Review of five cases. Horm Res 2004;62:10-16. Epub 2004 Apr 1

33. Olilvy-Suart A, Midgley P. Practical Neonatal Endocrinology. Cambridge: Cambridge University Press 2006;83-93.

34. de Weerth C, Zijl RH, Buitelaar JK. Development of cortisol circadian rhythm in infancy. Early Hum Dev 2003;73:39-52.

35. Padidela R, Kapoor RR, Moyo Y, Gilbert C, Flanagan SE, Ellard S, Hussain K. Focal congenital hyperinsulinism in a patient with septo-optic dysplasia. Nat Rev Endocrinol 2010;6:646-650. Epub 2010 Sep 14

36. Karlsson R, Kallio J, Irjala K, Ekblad S, Toppari J, Kero P. Adrenocorticotropin and corticotropin-releasing hormone tests in preterm infants. J Clin Endocrinol Metab 2000;85:4592-4595.

37. van Tijn DA, de Vijlder JJ, Vulsma T. Role of corticotropin-releasing hormone testing in assessment of hypothalamic-pituitary-adrenal axis function in infants with congenital central hypothyroidism. J Clin Endocrinol Metab 2008;93:3794-4803. Epub 2008 Jul 22

38. Braslavsky D, Keselman A, Galoppo M, Lezama C, Chiesa A, Galoppo C, Bergadá I. Neonatal cholestasis in congenital pituitary hormone deficiency and isolated hypocortisolism: characterization of liver dysfunction and follow-up. Arq Bras Endocrinol Metabol 2011;55:622-627.

39. Filippi L, Pezzati M, Cecchi A, Serafini L, Poggi C, Dani C, Tronchin M, Seminara S. Dopamine infusion and anterior pituitary gland function in very low birth weight infants. Biol Neonate 2006;89:274-280. Epub 2006 Feb 23

40. van Tijn DA, de Vijlder JJ, Verbeeten B Jr, Verkerk PH, Vulsma T. Neonatal detection of congenital hypothyroidism of central origin. J Clin Endocrinol Metab 2005;90:3350-3359. Epub 2005 Mar 22

41. Mehta A, Hindmarsh PC, Stanhope RG, Brain CE, Preece MA, Dattani MT. Is the thyrotropin-releasing hormone test necessary in the diagnosis of central hypothyroidism in children. J Clin Endocrinol Metab 2003;88:5696-5703.

42. Kurtoğlu S, Baştuğ O. Mini puberty and its interpretation. Turk Pediatri Ars 2014;49:186-191.

43. Grinspon RP, Bedecarrás P, Ballerini MG, Iñiguez G, Rocha A, Mantovani Rodrigues Resende EA, Brito VN, Milani C, Figueroa Gacitúa V, Chiesa A, Keselman A, Gottlieb S, Borges MF, Ropelato MG, Picard JY, Codner E, Rey RA; LAREP Group. Early onset of primary hypogonadism revealed by serum anti-Müllerian hormonedetermination during infancy and childhood in trisomy 21. Int J Androl 2011;34:487-498. Epub 2011 Aug 10

44. Bergadá I, Milani C, Bedecarrás P, Andreone L, Ropelato MG, Gottlieb S, Bergadá C, Campo S, Rey RA. Time course of the serum gonadotropin surge, inhibins, and anti-Müllerian hormone in normal newborn males during the first month of life. J Clin Endocrinol Metab 2006;91:4092-4098. Epub 2006 Jul 18

45. Bergadá I, Bergadá C, Campo S. Role of inhibins in childhood and puberty. J Pediatr Endocrinol Metab 2001;14:343-353.

46. Andersson AM, Toppari J, Haavisto AM, Petersen JH, Simell T, Simell O, Skakkebaek NE. Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. J Clin Endocrinol Metab 1998;83:675-681.

47. Resende EA, Lara BH, Reis JD, Ferreira BP, Pereira GA, Borges MF. Assessment of basal and gonadotropin-releasing hormone-stimulated gonadotropins by immunochemiluminometric and immunofluorometric assays in normal children. J Clin Endocrinol Metab 2007;92:1424-1429. Epub 2007 Feb 6.

48. van Tijn DA, Schroor EJ, Delemarre-van de Waal HA, de Vijlder JJ, Vulsma T. Early assessment of hypothalamic-pituitary-gonadal function in patients with congenital hypothyroidism of central origin. J Clin Endocrinol Metab 2007;92:104-109. Epub 2006 Oct 17

49. Segal TY, Mehta A, Anazodo A, Hindmarsh PC, Dattani MT. Role of gonadotropin-releasing hormone and human chorionic gonadotropin stimulation tests in differentiating patients with hypogonadotropic hypogonadism from those with constitutional delay of growth and puberty. J Clin Endocrinol Metab 2009;94:780-785. Epub 2008 Nov 18

50. Bin-Abbas B, Conte FA, Grumbach MM, Kaplan SL. Congenital hypogonadotropic hypogonadism and micropenis: effect of testosterone treatment on adult penile size why sex reversal is not indicated. J Pediatr 1999;134:579-583.

51. Ogilvy-Stuart AL. Growth hormone deficiency (GHD) from birth to 2 years of age: diagnostic specifics of GHD during the early phase of life. Horm Res 2003;60:2-9.

52. Ogilvy-Stuart AL, Hands SJ, Adcock CJ, Holly JM, Matthews DR, Mohamed-Ali V, Yudkin JS, Wilkinson AR, Dunger DB. Insulin, insulin-like growth factor I(IGF-I), IGF-binding protein-1, growth hormone, and feeding in the newborn. J Clin Endocrinol Metab 1998;83:3550-35542.

53. Kurtoğlu S, Kondolot M, Mazicioğlu MM, Hatipoğlu N, Akin MA, Akyildiz B. Growth hormone, insulin like growth factor-1, and insulin-like growth factor-binding protein-3 levels in the neonatal period: a preliminary study. J Pediatr Endocrinol Metab 2010;23:885-889.

54. Binder G, Weidenkeller M, Blumenstock G, Langkamp M, Weber K, Franz AR. Rational approach to the diagnosis of severe growth hormone deficiency in the newborn. J Clin Endocrinol Metab 2010;95:2219-2226. Epub 2010 Mar 23

55. Binder G, Hettmann S, Weber K, Kohlmüller D, Schweizer R. Analysis of the GH content within archived dried blood spots of newborn screening cards from children diagnosed with growth hormone deficiency after the neonatal period. Growth Horm IGF Res 2011;21:314-317. Epub 2011 Sep 8

56. Hawkes CP, Grimberg A. Measuring growth hormone and insulin-like growth factor-I in infants: what is normal? Pediatr Endocrinol Rev 2013;11:126-146.

57. Hussain K, Hindmarsh P, Aynsley-Green A. Spontaneous hypoglycemia in childhoodis accompanied by paradoxically low serum growth hormone and appropriate cortisolcounterregulatory hormonal responses. J Clin Endocrinol Metab 2003;88:3715-3723.

58. Crofton PM, Midgley PC. Cortisol and growth hormone responses to spontaneous hypoglycaemia in infants and children. Arch Dis Child 2004;89:472-478.

59. Chung ST, Chi CH, Haymond MW, Jeha GS. Infantile Growth Hormone Deficiency and X- Linked Adrenal Hypoplasia Congenita. Jacobs J Pediatr 2015;1. Epub 2015 May 4

60. Aitkenhead H, Heales SJ. Establishment of paediatric age-related reference intervals for serum prolactin to aid in the diagnosis of neurometabolic conditions affecting dopamine metabolism. Ann Clin Biochem 2013;50:156-158. Epub 2013 Feb 12

61. Ben-Jonathan N, Hnasko R. Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 2001;22:724-763.

62. Cheetham T, Baylis PH. Diabetes insipidus in children: pathophysiology, diagnosis and management. Paediatr Drugs 2002;4:785-796.

63. Saborio P, Tipton GA, Chan JC. Diabetes insipidus. Pediatr Rev 2000;21:122-129.

64. Wang LC, Cohen ME, Duffner PK. Etiologies of central diabetes insipidus in children. Pediatr Neurol 1994;11:273-277.

65. Ferlin ML, Sales DS, Celini FP, Martinelli Junior CE. Central diabetes insipidus: alert for dehydration in very low birth weight infants during the neonatal period. A case report. Sao Paulo Med J 2015;133:60-63. Epub 2014 Sep 26

66. Garel C, Léger J. Contribution of magnetic resonance imaging in non-tumoral hypopituitarism in children. Horm Res 2007;67:194-202. Epub 2006 Dec 6

67. Kitamura E, Miki Y, Kawai M, Itoh H, Yura S, Mori N, Sugimura K, Togashi K. T1signal intensity and height of the anterior pituitary in neonates: correlation with postnatal time. AJNR Am J Neuroradiol 2008;29:1257-1260. Epub 2008 Apr 16

68. Dietrich RB, Lis LE, Greensite FS, Pitt D. Normal MR appearance of the pituitary gland in the first 2 years of life. AJNR Am J Neuroradiol 1995;16:1413-1419.

69. Argyropoulou M, Perignon F, Brunelle F, Brauner R, Rappaport R. Height ofnormal pituitary gland as a function of age evaluated by magnetic resonanceimaging in children. Pediatr Radiol 1991;21:247-249.

70. Sari S, Sari E, Akgun V, Ozcan E, Ince S, Saldir M, Babacan O, Acikel C, Basbozkurt G, Ozenc S, Yesilkaya S, Kilic C, Kara K, Vurucu S, Kocaoglu M,Yesilkaya E. Measures of pituitary gland and stalk: from neonate to adolescence. J Pediatr Endocrinol Metab 2014;27:1071-1076.

71. Persani L. Clinical review: Central hypothyroidism: pathogenic, diagnostic, and therapeutic challenges. J Clin Endocrinol Metab 2012;97:3068-3078. Epub 2012 Jul 31

72. Yagasaki H, Kobayashi K, Nemoto A, Naito A, Sugita K, Ohyama K. Late-onset circulatory dysfunction after thyroid hormone treatment in an extremely low birth weight infant. J Pediatr Endocrinol Metab 2010;23:153-158.

73. Heckmann M, Hartmann MF, Kampschulte B, Gack H, Bödeker RH, Gortner L, Wudy SA. Assessing cortisol production in preterm infants: do not dispose of the nappies. Pediatr Res 2005;57:412-418. Epub 2005 Jan 5.

74. Higuchi A, Hasegawa Y. Dose Adjustments of Hydrocortisone and L-thyroxine in Hypopituitarism Associated with Cholestasis. Clin Pediatr Endocrinol 2006;15:93-96. Epub 2006 Aug 2

75. Bouvattier C, Maione L, Bouligand J, Dodé C, Guiochon-Mantel A, Young J. Neonatal gonadotropin therapy in male congenital hypogonadotropic hypogonadism. Nat Rev Endocrinol 2011;8:172-182.

76. Main KM, Schmidt IM, Toppari J, Skakkebaek NE. Early postnatal treatment of hypogonadotropic hypogonadism with recombinant human FSH and LH. Eur J Endocrinol 2002;146:75-79.

77. Guthrie RD, Smith DW, Graham CB. Testosterone treatment for micropenis during early childhood. J Pediatr 1973;83:247-252.

78. Di Iorgi N, Napoli F, Allegri AE, Olivieri I, Bertelli E, Gallizia A, Rossi A, Maghnie M. Diabetes insipidus--diagnosis and management. Horm Res Paediatr 2012;77:69-84. Epub 2012 Mar 16

79. Van der Kaay DC, Van Heel WJ, Dudink J, van den Akker EL. Transient diabetes insipidus in a preterm neonate and the challenge of desmopressin dosing. J Pediatr Endocrinol Metab 2014;27:769-771.

80. Korkmaz HA, Demir K, Kılıç FK, Terek D, Arslanoğlu S, Dizdarer C, Ozkan B. Management of central diabetes insipidus with oral desmopressin lyophilisate in infants. J Pediatr Endocrinol Metab 2014;27:923-927.

81. Stick SM, Betts PR. Oral desmopressin in neonatal diabetes insipidus. Arch Dis Child 1987;62:1177-1778.

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0117

J Clin Res Pediatr Endocrinol 2019;11(1):13-23

Neonatal Screening for Congenital Adrenal Hyperplasia in Turkey: A Pilot Study with 38,935 Infants

Tülay Güran[1], Başak Tezel[2], Fatih Gürbüz[3], Beray Selver Eklioğlu[4], Nihal Hatipoğlu[5], Cengiz Kara[6], Enver Şimşek[7], Filiz Mine Çizmecioğlu[8], Alev Ozon[9], Firdevs Baş[10], Murat Aydın[6], Feyza Darendeliler[10]

[1]Marmara University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, İstanbul, Turkey
[2]Turkish Directorate of Public Health, Ankara, Turkey
[3]Çukurova University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Adana, Turkey
[4]Necmettin Erbakan University Meram Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Konya, Turkey
[5]Erciyes University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Kayseri, Turkey
[6]Ondokuz Mayıs University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Samsun, Turkey
[7]Osmangazi University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Eskişehir, Turkey
[8]Kocaeli University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Kocaeli, Turkey
[9]Hacettepe University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Ankara, Turkey
[10]İstanbul University İstanbul Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, İstanbul, Turkey

What is already known on this topic?

Classical congenital adrenal hyperplasia (CAH) occurs in 1:13,000 to 1:15,000 live births. 21-hydroxylase enzyme deficiency (21-OHD) occurs in 90 to 95% of all cases of CAH. CAH is a potentially life-threatening condition that requires accurate diagnosis and urgent treatment with glucocorticoid and mineralocorticoid replacement. Neonatal screening for CAH is effective in detecting the salt-wasting form and thereby reducing mortality.

What this study adds?

The estimated incidence of classical 21-hydroxylase enzyme deficiency (21-OHD) congenital adrenal hyperplasia (CAH) in the screened population in Turkey was 1:7,787. The incidence of CAH due to classical 21-OHD is higher in Turkey in comparison to previous reports in the literature. Thus, it may be worthwhile to add CAH to the newborn screening panel in Turkey.

Abstract

Objective: Congenital adrenal hyperplasia (CAH) is the most common form of primary adrenal insufficiency in children. Neonatal screening for CAH is effective in detecting the salt-wasting (SW) form and in reducing mortality. In this study, our aim was to estimate the incidence of CAH in Turkey and to assess the characteristics and efficacy of the adopted newborn CAH screening strategy.

Methods: A pilot newborn CAH screening study was carried out under the authority of the Turkish Directorate of Public Health. Newborn babies of ≥32 gestational weeks and ≥1500 gr birth weight from four cities, born between March 27-September 15, 2017 were included in the study. Screening protocol included one sample two-tier testing. In the first step, 17α-hydroxyprogesterone (17-OHP) was measured by fluoroimmunoassay in dried blood spots (DBS) obtained at 3-5 days of life. The cases with positive initial screening were tested by steroid profiling in DBS using a liquid chromatography-tandem mass spectrometry method to measure 17-OHP, 21-deoxycortisol (21-S), cortisol (F), 11-deoxycortisol and androstenedione as a second-tier test. The babies with a steroid ratio (21-S + 17-OHP)/F of ≥0.5 were referred to pediatric endocrinology clinics for diagnostic assessment.

Results: 38,935 infants were tested, 2265 (5.82%) required second-tier testing and 212 (0.54%) were referred for clinical assessment, six of whom were diagnosed with CAH (four males, two females). Four cases were identified as SW 21-hydroxylase deficiency (21-OHD) (two males, two females). One male baby had simple virilizing 21-OHD and one male baby had 11-OHD CAH. The incidence of classical 21-OHD in the screened population was 1:7,787.

Address for Correspondence: Tülay Güran MD, Marmara University Faculty of Medicine, Department of Paediatric Endocrinology and Diabetes, Istanbul, Turkey
Phone: +90 216 625 45 45 **E-mail:** tulayguran@yahoo.com **ORCID ID:** orcid.org/0000-0003-2658-6866

Conflict of interest: None declared
Received: 15.05.2018
Accepted: 10.08.2018

Conclusion: The incidence of CAH due to classical 21-OHD is higher in Turkey compared to previous reports. We, therefore, suggest that CAH be added to the newborn screening panel in Turkey. The use of steroid profiling as a second-tier test was found to improve the efficacy of the screening and reduce the number of false-positives.
Keywords: Newborn screening, congenital adrenal hyperplasia, second-tier, steroid profiling

Introduction

Congenital adrenal hyperplasias (CAH) arise from biallelic gene defects encoding the enzymes and cofactor proteins involved in cortisol (F) biosynthesis. The most common enzyme deficiency that accounts for more than 90% of all cases with CAH is 21-hydroxylase deficiency (21-OHD). 21-OHD is classified into three subtypes according to clinical severity: classical salt wasting (SW), classical simple virilizing (SV) and nonclassical CAH (NCCAH; mild or late onset) (1). Data from nearly 6.5 million newborn screenings (NBS) worldwide indicate that classical CAH occurs in 1:13,000 to 1:15,000 live births (2).

CAH is the most common cause of primary adrenal insufficiency in childhood and is a potentially life-threatening condition that requires accurate diagnosis and urgent treatment with glucocorticoid and mineralocorticoid replacement. Symptoms and signs may easily be overlooked, particularly in male infants who do not have genital ambiguity. Because of delayed or missed diagnosis in affected male infants (and some very virilized female infants), in 2002 the Joint Lawson Wilkins Paediatric Endocrine Society/European Society for Pediatric Endocrinology Working Group recommended biochemical screening for CAH in the newborn period (3,4). The majority of states in the United States (US) and more than 50 countries are currently performing NBS for CAH (5). Infant screening programs have markedly decreased the time to diagnosis, theoretically decreasing morbidity (6,7). Based on proven importance, a pilot NBS programme for CAH was initiated by the Turkish Directorate of Public Health (TDPH) on March 27, 2017 in four Turkish cities. We have evaluated the data collected from this pilot study to describe the incidence of CAH in Turkey. We have also described the cases with CAH identified by this pilot study in detail. Additionally, we assessed the results of the pilot study in detail with regard to the characteristics and efficacy of the adopted NBS strategy to determine if any modifications to the strategy would enhance screening performance.

Methods

The pilot screening programme for CAH was carried out between March 27 and July 15, 2017 by the TDPH, in four Turkish cities (Adana, Kayseri, Konya and Samsun). According to the programme, dried blood spots (DBS) were obtained using filter paper ("Guthrie" cards) between the 3rd and 5th day of life or as soon as possible after 48 hours of age, by heel prick. The samples were obtained simultaneously with the ongoing nationwide NBS program for congenital hypothyroidism, phenylketonuria, biotinidase deficiency and cystic fibrosis. The CAH screening algorithm was developed in consultation with an expert scientific committee, consisting of paediatric endocrinologists from several universities in Turkey (Figure 1). Newborn babies ≥32 gestational weeks (gw) and ≥1500 gr birth weight from the four cities where the pilot study was conducted were included.

Initial CAH screening was based on the measurement of 17α-hydroxyprogesterone (17-OHP) in DBS on filter paper by fluoroimmunoassay (FIA) (Labsystems Diagnostics, Finland). Cut-off values for 17-OHP were based primarily on gestational age and birth weight. 17-OHP values of 10 ng/mL and 15 ng/mL have been used as cut-off points for newborn babies ≥36 gw and/or ≥2500 gr birth weight and for newborn babies between 32-36 gw and/or 1500-2500 gr birth weight, respectively (8,9,10). If the 17-OHP level was above the cut-off level in the first-tier test using immunoassay, the filter paper was directly analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for a steroid profiling assay for simultaneous analysis of 17-OHP, 21-deoxycortisol (21-S), F, androstenedione (4AS) and 11-deoxycortisol (11-S). Normal values for babies of 32-36 gw and/or 1500-2500 gr were; 17-OHP: <8 ng/mL, 21-S: <1.5 ng/mL, F: >50 ng/mL, 4AS: <4.5 ng/mL. Normal ranges for babies ≥36 weeks and/or ≥2500 gr were; 17-OHP: <1.5 ng/mL, 21-S: <1.5 ng/mL, F: > 50 ng/mL, 4AS: <4.5 ng/mL. Although all of the steroids were evaluated for each baby; a (21-S + 17-OHP)/F ratio of ≥0.5 was considered as the main criterion for referral (Figure 1) (11,12,13).

Reagent, Instruments, and Analytical Conditions for Liquid Chromatography-tandem Mass Spectrometry

The steroid standards for F, 17-OHP, 21-S, 11-S, 4AS and deuterated steroid standards for d4-F, d8-17-OHP, d3-testosterone were purchased from Sigma-Aldrich (MO, USA). Acetonitrile (ACN), methanol, ethanol, isopropyl alcohol

(IPA), formic acid and LC-MS grade water were purchased from Merck (Darmstadt, Germany).

Detection and measurement were performed on a QTRAP® 5500 tandem MS equipped with an Sciex Exion AC LC system (AB Sciex, Concord, Ontario, Canada) that was operated using an electrospray ionization source in positive and multiple reactions monitoring mode. The column used was Phenomenex Kinetex C18, 100 mm Å ~ 2.1 mm, 2.7 µ (Phenomenex, Torrance, CA, USA) that was maintained at 50 °C. The mobile phase gradient conditions consisted of water (A) (containing 0.1 % v/v Formic Acid in Water) and ACN (B) (containing 0.1 % v/v Formic Acid in ACN). The flow rate was 0.35 mL/min and the final injection volume of each sample was 20 µL. All sample extracts were maintained in the autosampler at 4 °C while awaiting injection. The ionization source conditions were as follows: curtain gas: 25 psi; ion spray voltage: 5500V; temperature: 500 °C; nebulizer gas (GS1): 50 psi and heater gas (GS2): 50 psi. The optimized precursor and product ion pairs, collision energy and retention times for the analytes and internal standards are listed in Table 1.

Sample Preparation

In order to obtain calibrators and control DBS, blood from a healthy donor was washed four times with saline to remove all plasma. The washed cells were then combined with steroid-free serum in proportions that resulted in a hematocrit of 0.50. A mix of unlabeled steroid hormones stock solutions of 1 mg/mL in ethanol was diluted in steroid-free serum to obtain four points for calibration and two points for control. F concentrations were 0, 4.12, 37.0, 333.33 nmol/L in calibrators and 12.3 and 111.1 nmol/L in controls. All other analyte concentrations were 0, 2.1, 18.5, 166.7 nmol/L in calibrators and 6.2 and 55.6 nmol/L in controls. Internal standard stock solutions of 1 mg/mL were prepared in ethanol for all deuterium-labeled steroid hormones and diluted to 40 mmol/L in IPA/ACN.

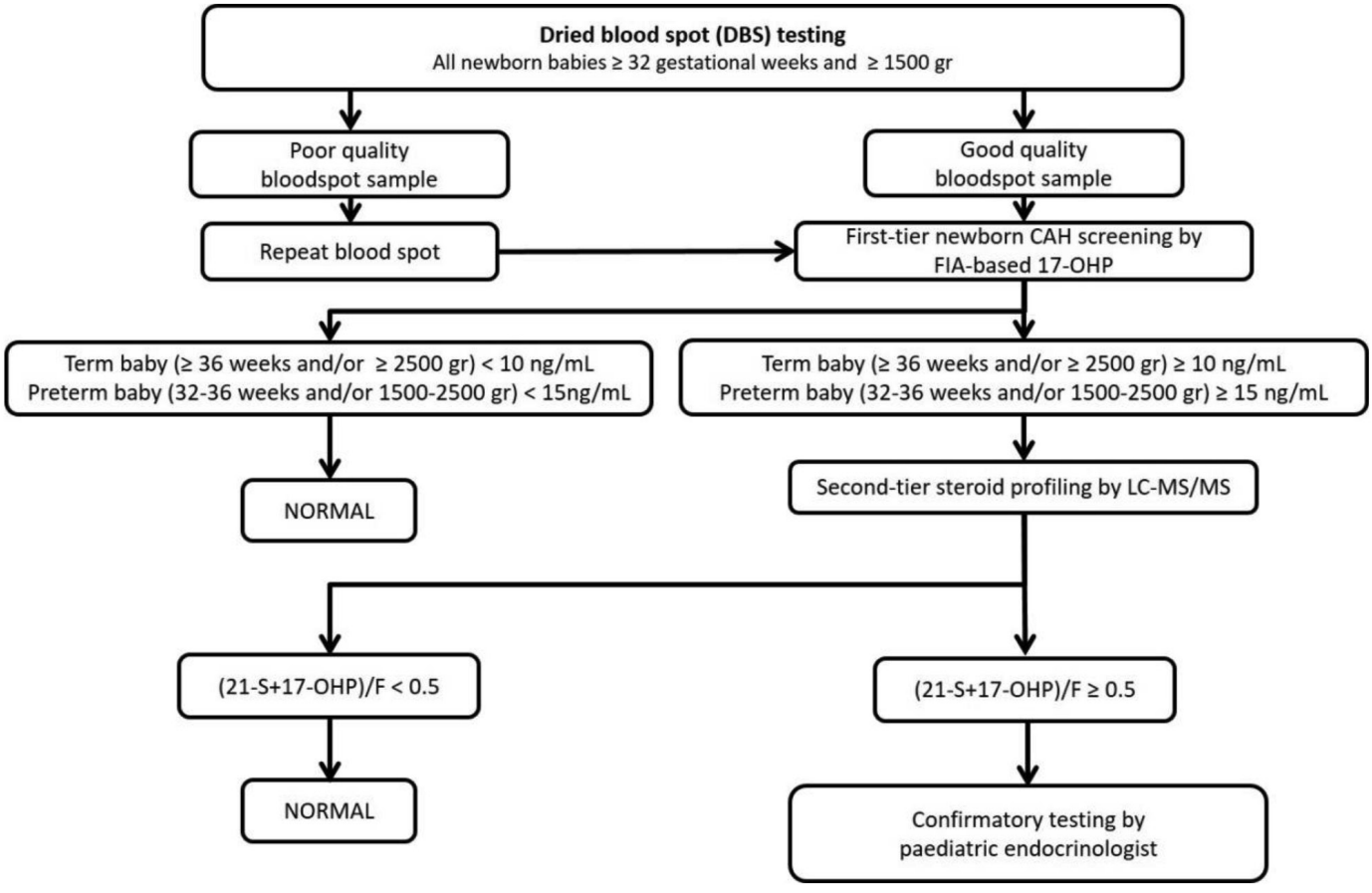


Figure 1. Flowchart for pilot neonatal congenital adrenal hyperplasia screening initiated by the Turkish Directorate of Public Health

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

The blood spots, each 4x3 mm in diameter were punched out of each DBS calibrator, control and sample using a manual puncher into a tube and 500 uL of internal standard mix was added. The tubes were mixed for 60 min by an orbital shaker. Supernatant was transferred to a 96-well plate and evaporated at 50 °C by vacuum centrifuge. 50 uL of a methanol/water mixture was added to reconstitute the dry residues and 20 uL injected by limited insert vials.

Ethics

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

Statistical Analysis

Statistical evaluation was performed using GraphPad Prism® V5.0 software (GraphPad Software Inc., San Diego, California, USA). The results for each steroid are reported as mean, standard deviation (SD) or as median in the text. We performed a *t-test* for the comparison of the means of two independent samples. Values were considered statistically significant when p value was less than 0.05.

Results

The total number of newborns that underwent CAH screening was 38,935. Of those babies, 33,967 (87.2%) were ≥36 gw and ≥2500 gr birth weight. There were 3,022 babies (7.8%) between 1500-2500 gr birthweight and 3,684 babies (9.5%) born between 32-36 gw. 1,744 (4.5%) babies were born between 32-36 gw and had a birthweight of 1500-2500 gr.

Results of first-tier 17-OHP measurement using DBS of the normal newborn population (those without CAH) are summarized in Table 2. We have presented 99.8 and 99.5% of 17-OHP for healthy babies to define healthy cut-off values with a greater sensitivity (14).

2,265 (5.8%) babies had second-tier testing by LC-MS/MS steroid profiling using the same DBS. During screening the babies born between 32-36 gw and/or of 1500-2500 gr birthweight were more likely to fail to pass first-tier and a much higher proportion in these categories required second-tier testing in comparison to those with a birthweight of ≥2500 gr and/or a gestational age ≥36 weeks (Table 3).

Two hundred and twelve babies who failed to pass second-tier testing were referred to paediatric endocrinology clinics for further evaluation, which corresponds to an overall recall rate of 0.54%.

Table 4 shows the distribution of second-tier testing values of babies referred for further analysis. The results are summarized with respect to gestational age and birth weight. The highest proportion of the babies referred to clinics had a (21-S + 17-OHP)/F ratio between 0.5-1.

The babies referred to paediatric endocrinology clinics were evaluated by medical history and physical examination for CAH symptoms and signs. Serum electrolytes were measured and in most of the babies 17-OHP testing was repeated, mainly by LC-MS/MS or immunoassay. Based on this evaluation, further biochemical assessments including synacthen test, ACTH, renin and detailed plasma steroid measurements by LC-MS/MS were undertaken when necessary and only for the cases suggestive of CAH. Genetic testing was performed only if the diagnosis of CAH was established by clinical and biochemical findings. Molecular analysis of the *CYP21A2* gene was performed at the diagnostic molecular genetic laboratories of university hospitals of the four enrolled cities. The *CYP21A2* gene was screened first for the detection of the eight most common

Table 1. Multiple reaction monitoring functions and settings for detecting steroids by liquid chromatography-tandem mass spectrometry

	Precursor (m/z)	Dwell time (msec)	Product (m/z)	Collision energy (eV)	Retention time (min)
17-hydroxyprogesterone	331	100	109	43	3.8
d8-17-hydroxyprogesterone	339	100	112	44	3.8
Cortisol	363	100	121	30	2.8
d4-cortisol	367	100	121	30	2.8
21-deoxycortisol	347	100	97	30	3.1
d3-testosterone	292	100	97	30	3.6
Androstenedione	287	100	109	30	3.3
11-deoxycortisol	347	100	97	27	3.3

mutations [p.P30L, IVS2-13C>G (IVS-2), p.I172N, exon 6 mutation cluster (p.I236N, p.V237E, p.M239K), p.V281L, p.Q318X, p.R356W, 8-bp-deletion]. Subsequent testing for large deletion and conversion by MLPA or allele specific semi-quantitative PCR/enzyme restriction method and sequencing when needed was performed when indicated.

Consequently, six babies were diagnosed with CAH (four males, two females). Four cases were diagnosed with classical SW 21-OHD (two males, two females), one male baby had SV 21-OHD and one male baby had 11-OHD CAH. None of these babies was premature nor had low birth weight. Diagnosis of CAH was verified by molecular analysis of *CYP21A2* and *CYP11B1* genes in five of the cases (Table 5). The identified mutations in our patients were among the previously known and common mutations analyzed by Sanger sequencing and heterozygosity of parents was confirmed for the identified mutations. There was no report of a case with SW 21-OHD missed during the period of screening in the screening area.

The estimated incidence of classical 21-OHD CAH in the screened population was 1:7,787. In 206 of 38,935 infants there was a false positive recall rate of 0.52%.

Table 2. Fluoroimmunoassay based 17-hydroxyprogesterone values of the screened population by birth weight and gestational age

17-OHP (ng/mL) [nmol/L] (n)	1500-2500 gr (3,022)	≥2500 gr (35,907)	32-36 gw (3,684)	≥36 gw (35,245)	32-36 gw + 1500-2500 gr (1,744)	≥36 gw + ≥2500 gr (33,967)
Mean (SD)	**8.29 (8.68)** [25.08 (26.2)]	**4.07 (2.75)** [12.3 (8.3)]	**8.60 (8.27)** [26.02 (25.0)]	**3.96 (2.53)** [11.9 (7.6)]	**10.80 (10.11)** [32.6 (30.5)]	**3.92 (2.43)** [11.8 (7.3)]
Minimum-maximum	0.10-137.30 [0.3-415]	0.05-56.63 [0.15-171]	0.11-137.30 [0.33-415]	0.05-57.66 [0.15-174]	0.11-137.30 [0.33-415]	0.05-56.63 [0.15-171]
Median	5.33 [16.1]	3.53 [10.6]	5.92 [17.9]	3.48 [10.5]	7.36 [22.2]	3.47 [10.5]
IQR (25-75%)	3.40-9.96 [10.2-30.1]	2.49-4.89 [7.5-14.7]	3.84-10.52 [11.6-31.8]	2.47-4.80 [7.4-14.5]	4.58-13.72 [13.8-41.5]	2.47-4.77 [7.4-14.2]
99.5%	50.80 [17.5]	18.05 [54.6]	49.99 [151]	16.71 [50.5]	58.27 [176]	15.97 [48.1]
99.8%	63.64 [191]	23.48 [71]	59.95 [181]	21.38 [64]	77.21 [233]	20.21 [61]

SI units are given in brackets.

FIA: fluoroimmunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, 17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol, 4AS: androstenedione, 11-S: 11-deoxycortisol, SD: standard deviation, gw: gestational weeks, IQR: median (17-OHP conversion factor from ng/mL to nmol/L: multiply by 3.02)

Table 3. Rate of second-tier testing among babies based on birth weight and gestational weeks

	1500-2500 gr	≥2500 gr	32-36 gw	≥36 gw	32-36 gw + 1500-2500 gr	≥36 gw + ≥2500 gr
Number of babies	3,022	35,907	3,684	35,245	1,744	33,967
Second-tier testing (number; %)	(722; 24)	(1,543; 4)	(973; 26)	(1,292; 4)	(607; 34)	(1,117; 3)

gw: gestational weeks

Table 4. Distribution of babies based on (21-deoxycortisol + 17-hydroxyprogesterone)/cortisol ratio adjusted for gestational age and birth weight

(21-S + 17-OHP)/F ratio	1500-2500 gr	≥2500 gr	32-36 gw	≥36 gw	32-36 gw + 1500-2500 gr	≥36 gw + ≥2500 gr
0.5-1.0	54	107	68	93	45	84
1.0-2.0	19	20	22	17	18	16
2.0-5.0	8	1	9	0	8	0
>5.0	1	2	2	1	1	1
Total (n)	82	130	101	111	72	101

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

Table 5. Clinical characteristics and laboratory details of the patients with congenital adrenal hyperplasia diagnosed through newborn screening

Case no	Karyotype	Birth weight (gr)/ gestational week	17-OHP by FIA (ng/mL)	Second-tier testing by LC-MS/MS (ng/mL)		Day of treatment initiation	Blood biochemistry at diagnosis		Diagnosis	Molecular defect
1	46, XX	3290/38	137.3	17-OHP	263.34	10th day	Na (mEq/L)	132	21-OHD (SW)	*CYP21A2* cluster E6 (c.707T > A, c.710T > A, c.716 T > A) homozygous
				21-S	40.65		K (mEq/L)	6.1		
				F	29.95		17-OHP (ng/mL)	12.5		
				(21-S + 17-OHP)/F	10.14					
				4AS	90.44					
				11-S	7.17					
2	46, XX	3139/38	137.3	17-OHP	262.59	28th day	Na (mEq/L)	113	21-OHD (SW)	ND
				21-S	36.52		K (mEq/L)	6.5		
				F	9.21		17-OHP (ng/mL)	> 128		
				(21-S + 17-OHP)/F	32.47					
				4AS	23.10					
				11-S	0.83					
3	46, XY	2900/38	96	17-OHP	908.45	21st day	Na (mEq/L)	137	21-OHD (SW)	*CYP21A2* IVS2-13C > G (c.293-13C > G) homozygous and *CYP21A2* p.Q319X (c.955C > T) homozygous
				21-S	0.49		K (mEq/L)	6.6		
				F	41.27		17-OHP (ng/mL)	> 20		
				(21-S + 17-OHP)/F	22.02					
				4AS	346.9					
				11-S	27.84					
4	46, XY	3200/39	96	17-OHP	58.73	30th day	Na (mEq/L)	NA	21-OHD (SW)	*CYP21A2* IVS2-13C > G (c.293-13C > G) and p.R357W (c.1069C > T) Compound heterozygous
				21-S	0.02		K (mEq/L)	NA		
				F	12.69		17-OHP (ng/mL)	> 20		
				(21-S + 17-OHP)/F	4.62					
				4AS	6.83					
				11-S	3.84					
5	46, XY	2950/39	44.29	17-OHP	19.66	19th day	Na (mEq/L)	134	21-OHD (SV)	*CYP21A2* p.I172N (c.518 T > A) homozygous
				21-S	0.05		K (mEq/L)	5.7		
				F	14.99		17-OHP (ng/mL)	47.2		
				(21-S + 17-OHP)/F	1.31					
				4AS	1.53					
				11-S	0.62					
6	46, XY	3250/36	16.33	17-OHP	4.82	71th day	Na (mEq/L)	137	11-OHD	*CYP11B1* p.Asn394ArgfsX37 (c.1180_1181insGA) and p.Phe487Cys (c.1460T > G) Compound heterozygous
				21-S	0.002		K (mEq/L)	5.3		
				F	31.31		17-OHP (ng/mL)	NA		
				(21-S + 17-OHP)/F	0.15					
				4AS	52.33					
				11-S	113.83					

NBS: newborn screening, FIA: fluoroimmunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, 17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol, 4AS: androstenedione, 11-S: 11-deoxycortisol, 21-OHD:21-hydroxylase deficiency, 11-OHD: 11β-hydroxylase deficiency, SW: salt wasting, SV: simple virilising, NA: not available, ND: not done.
Conversion factors to SI units: 17-OHP, ng/mL x 3.02 → nmol/L; 4AS, ng/mL x 3.49 → nmol/L; F, ng/mL x 27.5 → nmol/L; 11-S, ng/mL x 2.88 → nmol/L

None of the recalled babies with false-positive results had any clinical signs or symptoms suggestive of CAH. The mean ± SD duration from birth to clinical evaluation of abnormal screening test results of false-positive cases was 25.8 ± 6.4 days. We have compared first-tier 17-OHP and (21-S + 17-OHP)/F values of false-positive recalled babies and babies with 21-OHD. Both of these parameters were significantly higher in babies with 21-OHD compared to term babies and ≥2500 gr birthweight (n = 101) with false-positive screening results (Table 6).

Discussion

The purpose of this prospective pilot study was to estimate the incidence of CAH in Turkey and to assess the characteristics and efficacy of the adopted NBS strategy determine if any modifications to the strategy would enhance screening performance. Newborns were screened for CAH in parallel with the normal Turkish National Newborn Screening Programme in four cities during a six-months time period. Data analysis revealed an estimate of the incidence of classical 21-OHD CAH in the screened population as 1:7,787. Data were analysed to reassess the strategy for the upcoming extended NBS for CAH in Turkey.

NBS for CAH is universal in the US (15) and many other developed countries (5,6). The incidence of classical CAH is approximately 1:13,000 to 1:15,000 1:14,000 to 1:18,000 in most populations (2,5). However, it is reported to be more prevalent in populations with high rates of consanguinity. The data from NBS for CAH in the United Arab Emirates and Saudi Arabia revealed an incidence of 1:9,030 and 1:7,908, respectively for classical CAH (16,17). Our data demonstrated that incidence of classical CAH in Turkey is similar to that in the Gulf Arab region. This is most probably due to the high rate of consanguinity (overall rate of consanguinity is 22% in Turkey, increasing to 34% in the South East Anatolia region) (18). Therefore, one may expect an increase in the incidence of homozygous biallelic mutations in our population in comparison to compound heterozygotes for two or more different mutant *CYP21A2* alleles. This is indeed the case, three of the five patients identified in the current study were homozygous carriers of biallelic mutations causing classical 21-OHD. Together with the high carrier rate for classical CAH in the general population, which is ~2%, we expect to have a relatively higher incidence of classical CAH in Turkey, which we calculated to be 1:7,787. This is a finding which supports the incorporation of CAH in the core programme of NBS in Turkey.

Table 6. Comparison of first-tier 17-hydroxyprogesterone levels and second-tier (21-deoxycortisol + 17-hydroxyprogesterone)/cortisol ratios between the 206 false-positive healthy recalled infants and 5 infants with 21-hydroxylase deficiency

	False-positive healthy recalled babies					21-OHD CAH babies	
(n)	**1500-2500 gr (82)**	**≥2500 gr (130)**	**32-36 gw (101)**	**≥36 gw (111)**	**32-36 gw + 1500-2500 gr (72)**	**≥36 gw + ≥2500 gr (101)**	**≥36 gw + ≥2500 gr (5)**	**p value***
First-tier 17-OHP (ng/mL) [nmol/L]								
Mean ± SD	29.52 ± 18.68 [89.3 ± 56.5]	15.11 ± 6.52 [45.7 ± 19.7]	28.00 ± 17.73 [84.7 ± 53.6]	14.02 ± 4.69 [42.4 ± 14.1]	31.09 ± 19.33 [94 ± 58.4]	13.61 ± 4.42 [41.1 ± 13.3]	302.6 ± 357 [915 ± 1080]	**< 0.0001**
Median (IQR)	24.9 (18-35) [75 (54-105)]	12.8 (10.3-17) [39 (31-51.5)]	24.2 (16-33.5) [73 (49-101)]	12.5 (10.3-16) [38 (31-48)]	27 (18.5-35) 82 (56-106)	12.3 (10-15.3) [37 (30-46)]	262 (39-586) [793 (118-1773)]	
99.5%	117.8 [356]	39.8 [120.4]	112.8 [341]	28.9 [87.4]	120 [363]	27 [81.7]		
99.8%	129 [390]	45 [136]	127.5 [386]	29.8 [90.1]	130.3 [394]	27.5 [83.2]		
(21-S + 17-OHP)/F ratio								
Mean ± SD	1.38 ± 2.92	1.01 ± 1.81	1.49 ± 3.24	0.85 ± 0.68	1.47 ± 3.11	0.85 ± 0.70	14.1 ± 12.94	
Median (IQR)	1.38 (0.7-1.1)	0.7 (0.6-0.9)	0.8 (0.65-1.2)	0.66 (0.6-0.85)	0.9 (0.7-1.15)	0.65 (0.6-0.86)	10.1 (3-27.2)	**< 0.0001**
99.5%	17.9	11.6	23.3	4.2	19	4.5		
99.8%	23.17	16.64	25.35	5.89	23.61	5.99		

17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol, 4AS: androstenedione, 11-S: 11-deoxycortisol, 21-OHD: 21-hydroxylase deficiency, IQR: median
*p values indicate the comparison of the parameters in babies with 21-OHD with the term and ≥2500 gr birthweight babies (n = 101) with false-positive second-tier screening results.
SI units are given in brackets.

Screening markedly reduces the time to diagnosis of infants with CAH and will have an impact in reducing serious morbidity and mortality (19,20,21,22). A retrospective analysis of neonatal DBS in the Czech Republic and Austria identified three genotype-proven cases of classical CAH among 242 samples from cases of sudden infant death that were not screened for CAH (23). Previous studies have reported a death rate of ~10% in infants with SW CAH without screening (24), but recent estimates from developed countries are lower, 0-4% (25). We had no mortality due to unrecognized classical CAH among the screened cohort and only one of the cases had severe hyponatremia at the time of diagnosis. However, the fact that there was no mortality so far, does not ensure that the screening strategy is efficient to prevent delay in the diagnosis of CAH cases in the long run is not adding any safety for the screening program in the long run since it is well known that the crisis may occur at one week of age or even earlier. Furthermore, the initiation of hydrocortisone treatment in our study ranged between 10 to 30 days of life in four cases with SW 21-OHD and the mean ± SD duration from birth to clinical evaluation of abnormal screening test results of false positive cases was 25.8 ± 6.4 days. In this regard, our pilot study should be criticised for delayed recall of positive screening results. This delayed recall can partially be explained by our single sample second-tier screening approach. A potential disadvantage of single sample second-tier testing or a second specimen programme is that the infant would have been symptomatic by the time of second-tier testing or a second specimen was collected and tested. Therefore, a significantly high 17-OHP value in the first-tier should alarm the clinician for a suspicion of CAH and neonates with elevated 17-OHP levels should be recalled directly. Awaiting a second analysis, even on the same sample may only delay the recall until after the time when the child develops detrimental salt crisis. Furthermore, in cases with a markedly elevated first 17-OHP result, a second tier does not necessarily add important information. Indeed, our first-tier 17-OHP results of 21-OHD CAH babies were very significantly higher than that found in 101 recalled term babies with false positive screening results (302.6 ± 357 ng/mL vs 13.61 ± 4.42 ng/mL, $p < 0.0001$) and in 33967 health term babies with normal first-tier results (3.92 ± 2.43).

Another reason for relatively late recall in our screening programme may be the thrice-weekly postal service of samples from hospitals to screening laboratory and the different location of laboratories for FIA and LC-MS/MS. However, efforts to reduce the recall time are ongoing for the upcoming extended CAH NBS in Turkey by performing two steps of screening in a single central laboratory so that second-tier testing can be performed on the same day that a positive first screening result is obtained. Moreover, the filter paper samples will be collected from hospitals every day and sent to the screening laboratory by regular daily postal service. Nevertheless, since three of five cases with classical 21-OHD diagnosed through our pilot screening were males, and thus without ambiguous genitalia, it is safe to say that the diagnosis and treatment would have been delayed much more without screening.

Another weakness of single sample second-tier screening is the high false negative rate compared to second sample testing, particularly to diagnose the classical CAH cases with a delayed rise in 17-OHP levels. The Minnesota program has the longest experience in using a single sample two-tier screening algorithm with steroid profiling by LC-MS/MS as the second tier in specimens that exceeded the first-tier 17-OHP cut-off (26). If the second tier test results were negative, further follow-up of the child was considered unnecessary. They have evaluated their 11 years of experience on screening and came to the conclusion that the overall false negative rate doubled with their two-tiered algorithm. This is highlighted by the finding that seven missed cases were not tested by LC-MS/MS because their first-tier 17-OHP values were within range, and four more were missed by the second tier testing after initial abnormal screening values (26). Our single sample, two-tier screening may still have a similar risk to misidentify some CAH cases with delayed rise in 17-OHP. Therefore, physicians should have a high level of suspicion in patients presenting with signs and symptoms of CAH even if they have (false) negative screening results. In view of the high false negative results associated with a single NBS two-tier approach, some programs have opted to collect and screen a second specimen as an alternative means of improving the results of CAH screening. However, this may further confuse the screening results. For example, the Colorado screening program collects the sample in the first two days of life, which may be the main reason for their high false negative rates. Therefore, this program has routinely obtained a second specimen, 1-2 weeks after birth for repeat screening, which was reported to further complicate the screening, due to longer time before recall (27).

We have also questioned our high recall rate during NBS for CAH in comparison to previous studies (6). Recall rate was reported between 0.002-1.2%, generally <0.5%, in many developed countries with long established screening programs for CAH (6). Furthermore, such low recall rates are reported in the course of single tier DBS screening. We could achieve a recall rate of 0.54%, in the face of a higher cost adopting single DBS-two-tier

screening approach. This can be explained by the lower cut-off values we used for the first step 17-OHP FIA measurements as well as second step (21-S + 17-OHP)/F ratio to increase the sensitivity of this pilot study. The lower cut-off values have the advantage of increasing screening sensitivity with a markedly increased risk of higher false positive rate, higher cost and higher likelihood of unnecessary treatment of NCCAH cases, and even of non-CAH healthy babies. Further analysis of our data suggests that 99.8th percentile of FIA based 17-OHP levels in our healthy population (excluding the babies with SW 21-OHD) is 50 ng/mL for 1500-2500 gr and 32-36 gw babies and is 20 ng/mL for ≥2500 gr and ≥36 gw babies. When these 17-OHP levels were used as cut-off values in the first-tier we would have expected 253 babies failing to pass, which corresponds to only 11 % of population undergoing the second-tier test. Likewise, 161 of 212 babies (75%) had a (21-S + 17-OHP)/F ratio of < 1 in the second-tier testing while this ratio ranges between 4.6-32.4 in classical SW 21-OHD cases. Even in the single case with SV 21-OHD, the measurement of (21-S + 17-OHP)/F ratio was 1.31. Therefore, if we had used 1.0 as the cut-off for (21-S + 17-OHP)/F ratio, the recall rate would decrease by 75%. This observation is similar to that of Janzen et al (11) who analyzed the (21-S + 17-OHP)/F ratio in around 8000 retrospective and prospective DBS samples in order to compare healthy newborns (including preterms) with 66 CAH cases. None of the cases with CAH had a (21-S + 17-OHP)/F ratio < 1. Analysis of data from the current study helped us to reassess and modify the screening strategy for the upcoming extended NBS screening for CAH in Turkey. It emerges that the above-mentioned cut-off values may contribute to designing a less labour intensive and more efficient screening strategy for CAH, and also with a better cost-benefit profile.

Immunoassays of DBS for 17-OHP are the most widely used and least costly initial screening methods for CAH. However, poor antibody specificity in addition to abundant cross-reacting hormones in the newborn circulation as well as the necessity of variation in the cut-offs with respect to gestational ages (28) and/or birthweight (29), limit their use in the detection of CAH. Furthermore, stress due to prematurity or critical illness generally increases adrenal F and 17-OHP secretion, which further hamper interpretation of screening results for CAH. Therefore, using 17-OHP as the sole marker may increase the recall rate as well as likelihood of false positive healthy infants who may be started on glucocorticoids unnecessarily. Employing the LC-MS/MS based steroid panel appears as an effective second-tier screen that would better separate false positives, avoid false negatives and potentially save a great deal in unnecessary health care expenditure, which subsequently will relieve much of the stress and work burden experienced by health professionals and parents with quite confusing immunoassay results (11,13,30,31,32). We adopted a modified LC-MS/MS protocol developed by Janzen et al (11) that utilized a ratio of the sum of 17-OHP and 21-S levels, divided by the F level as a second-tier screening test. This protocol was reported to identify all affected children with no false positives for a positive predictive value of 100% (11). Particularly 21-S, which is produced by 11β-hydroxylation of 17-OHP, is not expected to be secreted in large amounts even in preterm infants, and thus elevated levels are highly specific for 21-OHD (33,34).

It is encouraging that simultaneous measurement of 17-OHP, 21-S and F by LC-MS/MS increased the positive predictive value of our CAH screening 9-fold over that for 17-OHP FIA alone. This pilot study also measured 11-S in addition to 17-OHP, 21-S, F and 4AS, which is specifically diagnostic for 11-hydroxylase deficiency. Hence, with our tandem MS method, it was possible to detect a male newborn with (later genotype-proven) classical 11-OHD in addition to cases with 21-OHD. To our knowledge, this is the first patient with 11-OHD identified directly during NBS for CAH. Therefore, the method of steroid profiling has a potential to distinguish other rare forms of classical CAH, beyond 21-OHD, more efficiently. Even though the hormones measured in LC-MS/MS based panels are not specifically diagnostic for the rare forms of CAH such as 11-hydroxylase or 3β-hydroxysteroid dehydrogenase type 2 deficiency, perturbations in simultaneous steroid measurements would provide preliminary information suggesting the need for further evaluation (35,36). In fact, these apparently "rare" forms of classic CAH are far more common in the Middle East and in Turkey, due to the high rate of consanguinity (37,38).

Study Limitations

The prevelance of 21-OHD was calculated among approximately 40.000 babies screened in 4 pilot cities. This figure may change and may need to be recalculated after an extended newborn screening is completed.

Conclusion

In conclusion, this pilot study suggests that incidence of CAH in Turkey may be higher than previously reported figures. Hence, it may be recommended that CAH due to 21-OHD be included in

the screening panel for the Turkish NBS program. Employing the current LC-MS/MS based steroid panel as second-tier testing may be expected to reduce the time to diagnosis of infants with 21-OHD CAH. It may also enable detection of rare forms of classical CAH. On the other hand, further efforts are needed in our CAH screening programme for earlier clinical recall of babies with positive NBS tests, which is critically important to both prevent salt loss and to shorten the period of unclear sex in classical CAH cases. Prospective analyses of screening strategy, cut-off values and results would help to increase the sensitivity and reduce the false positive rate of screening. Such measures will subsequently serve to alleviate the medical, psychological and economic burden of CAH and its associated health problems.

Ethics

Ethics Committee Approval: The study was carried out with the written permission of the Scientific Committee of the Turkish Directorate of Public Health.

Informed Consent: The parents were informed about NBS. Heel-prick blood samples were collected from live-born babies after written consents from the parents were obtained.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Medical Practices: Fatih Gürbüz, Beray Selver Eklioğlu, Nihal Hatipoğlu, Cengiz Kara, Enver Şimşek, Filiz Mine Çizmecioğlu, Concept: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Design: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Data Collection or Processing: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Analysis or Interpretation: Tülay Güran, Başak Tezel, Alev Ozon, Feyza Darendeliler, Literature Search: Tülay Güran, Başak Tezel, Alev Ozon, Firdevs Baş, Feyza Darendeliler, Writing: Tülay Güran.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Hannah-Shmouni F, Chen W, Merke DP. Genetics of Congenital Adrenal Hyperplasia. Endocrinol Metab Clin North Am 2017;46:435-458. Epub 2017 Mar 1
2. Pang SY, Wallace MA, Hofman L, Thuline HC, Dorche C, Lyon IC, Dobbins RH, Kling S, Fujieda K, Suwa S. Worldwide experience in newborn screening for classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Pediatrics 1988;81:866-874.
3. Clayton PE, Miller WL, Oberfield SE, Ritzén EM, Sippell WG, Speiser PW; ESPE/ LWPES CAH Working Group. Consensus statement on 21-hydroxylase deficiency from the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society Horm Res 2002;58:188-195.
4. Joint LWPES/ESPE CAH Working Group. Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. J Clin Endocrinol Metab 2002;87:4048-4053.
5. Therrell BL, Padilla CD, Loeber JG, Kneisser I, Saadallah A, Borrajo GJ, Adams J. Current status of newborn screening worldwide: 2015. Semin Perinatol 2015;39:171-187.
6. Gidlöf S, Falhammar H, Thilén A, von Döbeln U, Ritzén M, Wedell A, Nordenström A. One hundred years of congenital adrenal hyperplasia in Sweden: a retrospective, population-based cohort study. Lancet Diabetes Endocrinol 2013;1:35-42. Epub 2013 Feb 26
7. Dumic K, Krnic N, Skrabic V, Stipancic G, Cvijovic K, Kusec V, Stingl K. Classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency in croatia between 1995 and 2006. Horm Res 2009;72:310-314. Epub 2009 Oct 19
8. Working Group on Neonatal Screening of the European Society for Paediatric Endocrinology. Procedure for neonatal screening for congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Horm Res 2001;55:201-205.
9. Cavarzere P, Camilot M, Teofoli F, Tatò L. Neonatal screening for congenital adrenal hyperplasia in North-Eastern Italy: a report three years into the program. Horm Res 2005;63:180-186. Epub 2005 Apr 7
10. Lee JE, Moon Y, Lee MH, Jun YH, Oh KI, Choi JW. Corrected 17-alpha-hydroxyprogesterone values adjusted by a scoring system for screening congenital adrenal hyperplasia in premature infants. Ann Clin Lab Sci 2008;38:235-240.
11. Janzen N, Peter M, Sander S, Steuerwald U, Terhardt M, Holtkamp U, Sander J. Newborn screening for congenital adrenal hyperplasia: additional steroid profile using liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 2007;92:2581-2589. Epub 2007 Apr 24
12. Kim B, Lee MN, Park HD, Kim JW, Chang YS, Park WS, Lee SY. Dried blood spot testing for seven steroids using liquid chromatography-tandem mass spectrometry with reference interval determination in the Korean population. Ann Lab Med 2015;35:578-585.
13. Boelen A, Ruiter AF, Claahsen-van der Grinten HL, Endert E, Ackermans MT. Determination of a steroid profile in heel prick blood using LC-MS/MS. Bioanalysis 2016;8:375-384. Epub 2016 Feb 19
14. Hayashi G, Faure C, Brondi MF, Vallejos C, Soares D, Oliveira E, Brito VN, Mendonca BB, Bachega TA. Weight-adjusted neonatal 17-OH-progesterone cutoff levels improve the efficiency of newborn screening for congenital adrenal hyperplasia. Arq Bras Endocrinol Metabol 2011;55:632-637.
15. National Newborn Screening and Global Resource Center. 2006. National Newborn Screening System [Online]. National Newborn Screening & Global Resource Center. Available: http://genes-r-us.uthscsa.edu/newborn_reports [Accessed August 28 2017].
16. Al Hosani H, Salah M, Osman HM, Farag HM, El-Assiouty L, Saade D, Hertecant J. Expanding the comprehensive national neonatal screening programme in the United Arab Emirates from 1995 to 2011. East Mediterr Health J 2014;20:17-23.
17. Alfadhel M, Al Othaim A, Al Saif S, Al Mutairi F, Alsayed M, Rahbeeni Z, Alzaidan H, Alowain M, Al-Hassnan Z, Saeedi M, Aljohery S, Alasmari A, Faqeih E, Alwakeel M, AlMashary M, Almohameed S, Alzahrani M, Migdad A, Al-Dirbashi OY, Rashed M, Alamoudi M, Jacob M, Alahaidib L, El-Badaoui F, Saadallah A, Alsulaiman A, Eyaid W, Al-Odaib A. Expanded Newborn Screening Program in Saudi Arabia: Incidence of screened disorders. J Paediatr Child Health 2017;53:585-591. Epub 2017 Mar 24
18. Koc I. Prevalence and sociodemographic correlates of consanguineous marriages in Turkey. J Biosoc Sci 2008;40:137-148.

19. Balsamo A, Cacciari E, Piazzi S, Cassio A, Bozza D, Pirazzoli P, Zappulla F. Congenital adrenal hyperplasia: neonatal mass screening compared with clinical diagnosis only in the Emilia-Romagna region of Italy, 1980-1995. Pediatrics 1996;98:362-367.

20. Brosnan PG, Brosnan CA, Kemp SF, Domek DB, Jelley DH, Blackett PR, Riley WJ. Effect of newborn screening for congenital adrenal hyperplasia. Arch Pediatr Adolesc Med 1999;153:1272-1278.

21. Therrell BL Jr, Berenbaum SA, Manter-Kapanke V, Simmank J, Korman K, Prentice L, Gonzalez J, Gunn S. Results of screening 1.9 million Texas newborns for 21-hydroxylase-deficient congenital adrenal hyperplasia. Pediatrics 1998;101:583-590.

22. Thil'en A, Nordenström A, Hagenfeldt L, von Döbeln U, Guthenberg C, Larsson A. Benefits of neonatal screening for congenital adrenal hyperplasia (21-hydroxylase deficiency) in Sweden. Pediatrics 1998;101:11.

23. Strnadová KA, Votava F, Lebl J, Mühl A, Item C, Bodamer OA, Torresani T, Bouska I, Waldhauser F, Sperl W. Prevalence of congenital adrenal hyperplasia among sudden infant death in the Czech Republic and Austria. Eur J Pediatr 2007;166:1-4. Epub 2006 Sep 22

24. Watson MS, Mann MY, Lloyd-Puryear MA, Rinaldo P, Howell RR. Newborn screening: toward a uniform screening panel and system. Genet Med 2006;8(Suppl 1):1-252.

25. Grosse SD, Van Vliet G. How many deaths can be prevented by newborn screening for congenital adrenal hyperplasia? Horm Res 2007;67:284-291. Epub 2007 Jan 3

26. Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. Pediatrics 2012;130:1261-1268. Epub 2012 Oct 15

27. Chan CL, McFann K, Taylor L, Wright D, Zeitler PS, Barker JM. Congenital adrenal hyperplasia and the second newborn screen. J Pediatr 2013;163:109-113. Epub 2013 Feb 12

28. Gruneiro-Papendieck L, Prieto L, Chiesa A, Bengolea S, Bossi G, Bergada C. Neonatal screening program for congenital adrenal hyperplasia: adjustments to the recall protocol. Horm Res 2001;55:271-277.

29. Allen DB, Hoffman GL, Fitzpatrick P, Laessig R, Maby S, Slyper A. Improved precision of newborn screening for congenital adrenal hyperplasia using weight-adjusted criteria for 17-hydroxyprogesterone levels. J Pediatr 1997;130:128-133.

30. Seo JY, Park HD, Kim JW, Oh HJ, Yang JS, Chang YS, Park WS, Lee SY. Steroid profiling for congenital adrenal hyperplasia by tandem mass spectrometry as a second-tier test reduces follow-up burdens in a tertiary care hospital: a retrospective and prospective evaluation. J Perinat Med 2014;42:121-127.

31. Lacey JM, Minutti CZ, Magera MJ, Tauscher AL, Casetta B, McCann M, Lymp J, Hahn SH, Rinaldo P, Matern D. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry. Clin Chem 2004;50:621-625. Epub 2003 Dec 4

32. Kim B, Lee MN, Park HD, Kim JW, Chang YS, Park WS, Lee SY. Dried blood spot testing for seven steroids using liquid chromatography-tandem mass spectrometry with reference interval determination in the Korean population. Ann Lab Med 2015;35:578-585.

33. Fiet J, Villette JM, Galons H, Boudou P, Burthier JM, Hardy N, Soliman H, Julien R, Vexiau P, Gourmelen M, Kuttenn F. The application of a new highlysensitive radioimmunoassay for plasma 21-deoxycortisol to the detection of steroid-21-hydroxylase deficiency. Ann Clin Biochem 1994;31:56-64.

34. Cristoni S, Cuccato D, Sciannamblo M, Bernardi LR, Biunno I, Gerthoux P, Russo G, Weber G, Mora S. Analysis of 21-deoxycortisol, a marker of congenital adrenal hyperplasia, in blood by atmospheric pressure chemical ionization and electrospray ionization using multiple reaction monitoring. Rapid Commun Mass Spectrom 2004;18:77-82.

35. Krone N, Grötzinger J, Holterhus PM, Sippell WG, Schwarz HP, Riepe FG. Congenital adrenal hyperplasia due to 11-hydroxylase deficiency--insights from two novel CYP11B1 mutations (p.M92X, p.R453Q). Horm Res 2009;72:281-286. Epub 2009 Oct 19

36. Araújo VG, Oliveira RS, Gameleira KP, Cruz CB, Lofrano-Porto A. 3β-hydroxysteroid dehydrogenase type II deficiency on newborn screening test. Arq Bras Endocrinol Metabol 2014;58:650-655.

37. Khattab A, Haider S, Kumar A, Dhawan S, Alam D, Romero R, Burns J, Li D, Estatico J, Rahi S, Fatima S, Alzahrani A, Hafez M, Musa N, Razzghy Azar M, Khaloul N, Gribaa M, Saad A, Charfeddine IB, Bilharinho de Mendonça B, Belgorosky A, Dumic K, Dumic M, Aisenberg J, Kandemir N, Alikasifoglu A, Ozon A, Gonc N, Cheng T, Kuhnle-Krahl U, Cappa M, Holterhus PM, Nour MA, Pacaud D, Holtzman A, Li S, Zaidi M, Yuen T, New MI. Clinical, genetic, and structural basis of congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. Proc Natl Acad Sci U S A 2017;114:1933-1940. Epub 2017 Feb 22

38. Kandemir N, Yilmaz DY, Gonc EN, Ozon A, Alikasifoglu A, Dursun A, Ozgul RK. Novel and prevalent CYP11B1 gene mutations in Turkish patients with 11-β hydroxylase deficiency. J Steroid Biochem Mol Biol 2017;165:57-63. Epub 2016 Mar 5

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0185

J Clin Res Pediatr Endocrinol 2019;11(1):24-33

Androgen Insensitivity Syndrome: Clinical Phenotype and Molecular Analysis in a Single Tertiary Center Cohort

Maria Sol Touzon*[1,2], Natalia Perez Garrido*[1], Roxana Marino[1], Pablo Ramirez[1], Mariana Costanzo[1], Gabriela Guercio[1,2], Esperanza Berensztein[1], Marco A. Rivarola[1,2], Alicia Belgorosky[1,2]

[1]Hospital de Pediatria Garrahan, Endocrinology Service, Buenos Aires, Argentina

[2]National Scientific and Technical Research Council (CONICET), Endocrinology Service, Buenos Aires, Argentina

**These two authors contributed equally to this work.*

What is already known on this topic?

Androgen insensitivity syndromes (AIS) the most frequent known monogenic cause of 46,XY disorder of sexual differentiation. Mutations of variable severity in androgen receptor gene are associated with a wide phenotypic spectrum, ranging from complete AIS to a partial form or a mild form.

What this study adds?

Characterization of the clinical phenotype, long term follow up, in particular gender identityand the contribution of the *AR* gene to the molecular cause of 46,XY disorder of sexual differentiation in a single tertiary pediatric center of Buenos Aires, Argentina are reported. Nine novel *AR* mutations are described

Abstract

Objective: The aim of this study was the molecular characterization of the *AR* gene as the cause of 46,XY disorder in our population.
Methods: We studied 41, non related, 46,XY disorder of sexual differentiation index cases, having characteristics consistent with androgen insensivity syndrome (AIS). Genomic DNA was isolated from peripheral blood leukocytes of all patients and 25 family members from 17 non-related families.
Results: The *AR* gene analysis revealed an abnormal sequence in 58.5% of the index patients. All of the complete AIS (CAIS) cases were genetically confirmed, while in the partial form (PAIS) a mutation in *AR* was detected in only 13 (43.3%). Molecular studies revealed other affected or carrier relatives in 87% of the index cases. The *AR* mutations were found spread along the whole coding sequence, with a higher prevalence in the ligand binding domain. Nine out of 23 (39%) *AR* mutations were novel. In 17% of patients with detected *AR* mutations, somatic mosaicism was detected in leucocyte DNA. In our cohort, long-term follow up gender dysphoria, raised as male or female, was not found. Finally, in suspected PAIS, the identification of *AR* mutation occurred significantly less than in CAIS patients.
Conclusion: Improved knowledge of the components of the *AR* complex and signaling network might contribute to long term outcome and genetic counseling in AIS patients.
Keywords: 46,XY disorders of sex development, androgen insensitivity syndrome, androgen receptor gene mutations, mosaicism, clinical phenotype

Introduction

The endogenous androgens, testosterone (T) and dihydrotestosterone (DHT), exert their effects via a single intracellular receptor protein, the androgen receptor (*AR*) (1). *AR*-mediated androgen action is essential for normal primary male sexual development before birth and for normal secondary male sexual development around puberty, whereas in females, androgens also participate in sexual development around puberty and in adult

Address for Correspondence: Alicia Belgorosky MD, Hospital de Pediatria Garrahan, Endocrinology Service, Buenos Aires, Argentina
Phone: +541143080034 **E-mail:** abelgo12345@gmail.com **ORCID ID:** orcid.org/0000-0002-4234-400X

Conflict of interest: None declared
Received: 24.07.2018
Accepted: 20.09.2018

female sexual function (2). The *AR* gene is located on the X-chromosome inthe Xq11–12 region and encodes a protein with a molecular mass of approximately 110 kDa. The gene consists of eight coding exons (I to VIII) (3). The *AR* is a transcription factor that belongs to the nuclear receptor subfamily 3, group C, member 4. The protein consists of 920 amino acids that, like other nuclear receptors, is composed of an N-terminal domain (NTD), located on exon 1, a DNA-binding domain (DBD), located on exons 2 and 3 containing two zinc fingers, a hinge region connecting the ligand-binding domain (LBD) to the DBD and a C-terminal LBD, located on exons 4-8 (4).

Androgen insensitivity syndrome (AIS; OMIM 300068) is the most frequent known monogenic cause of 46,XY disorders of sex development (DSD) and is an X-linked recessive condition. Mutations of variable severity in the *AR* gene are associated with a wide phenotypic spectrum, ranging from complete AIS (CAIS) to a partial form (PAIS) or a mild form (MAIS). Patients who present with CAIS exhibit female external genitalia, testes located in the inguinal or abdominal area, and complete breast development with sparse to absent axillary and pubic hair. Patients with PAIS present with a predominantly male phenotype with hypospadias or a predominantly female phenotype with cliteromegaly and/or posterior labial fusion, ambiguous genitalia and variable degrees of gynecomastia at puberty. Patients with MAIS present with normal external male genitalia associated with infertility (5).

The aim of this study was to characterize the clinical phenotype and the contribution of the *AR* gene to the molecular cause of 46,XY DSD in our population.

Methods

We studied 41 unrelated 46,XY DSD patients with clinical and hormonal characteristics consistent with AIS. CAIS was suspected in 11 of the patients and PAIS in 30. All patients presented with female or ambiguous external genitalia, adequate T production without evidence of steroidogenic blockade and no Müllerian structures evident on abdominal ultrasound. Patients with hormonal determinations previous to gonadal biopsy or gonadectomy presented no biochemical evidence of gonadal dysgenesis and had normal male follicle-stimulating hormone (FSH) levels. In these individuals, the *AR* gene was the first candidate for molecular analysis.

Informed consent for the genetic study was obtained from all of the patients or their parents guardians after full explanation of the purpose and nature of all procedures. The study was approved by the Independent Ethics Committee "Prof. Dr. J. P. Garrahan Pediatric Hospital" (reference number: 971).

Hormonal Assays

Serum luteinising hormone (LH) and FSH levels were determined by the IMX systems (Abbott Laboratories, Abbott Park, IL); assay sensitivity was 0.3 IU/L for LH and 0.2 IU/L for FSH; interassay coefficient of variation ranged from 3.1-8.7% for LH and from 3.8-12% for FSH. Serum anti-Müllerian hormone (AMH) levels were determined by ELISA; assay sensitivity was 0.5 pmol/L and assay limit of quantification 1.2 pmol/L serum T was determined by a DPC Immulite® Assay System (Diagnostic Products, Los Angeles, CA); assay sensitivity was 0.17 nmol/L; interassay coefficient of variation ranged from 7.4 to 13%.

AR Gene Mutation Analysis

Genomic DNA was isolated from peripheral blood leukocytes of all patients (41 index cases) and 25 family members from 17 families according to standard procedures. There were seven families in which family members were not available for molecular studies. The entire coding region (exons 1-8) and splice sites in flanking intronic regions of *AR* gene were polymerase chain reaction (PCR) amplified and sequenced by automated analysers (6).

After PCR, the products were assessed by electrophoresis on a 1% agarose gel stained with ethidium bromide and showed a single band with expected size. The PCR products were purified (Qia Quick PCR Purification Kit, Qiagen, Buenos Aires, Argentina) and sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Buenos Aires, Argentina) on an ABI PRISM® 3130 Genetic Analyzer capillary DNA sequencer (Applied Biosystems, Buenos Aires, Argentina). The primers used for sequencing were the same as those used for PCR. Previously reported intronic mutations were also analysed [Human Gene Mutation Database (HGMD), www.hgmd.cf.ac.uk/]. The nucleotide sequences obtained were compared with those from Genebank accession number: NG_009014.2. Nucleotide changes were reconfirmed in each sample DNA by antisense sequence and resequencing after a new PCR product was produced from the original DNA extract.

In silico Protein Analysis

Nonsense and frameshift mutations which implicate a premature stop codon and a truncated protein were considered deleterious.

The sequence homology-based tool, [Sorting Intolerant from Tolerant (SIFT); http://sift.jcvi.org/], version 2.0.6,

the structure-based tool PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (http://www.mutationtaster.org/) were used to predict the pathogenicity of the previously undescribed missense variants using default settings. To evaluate the implication of a novel synonymous mutation, we used The Berkeley Drosophila Genome Project (http://www.fruitfly.org/) as a splice site prediction program.

The SIFT algorithm predicts the functional importance of the substitutions based on the alignment of orthologous and/or paralogous protein sequences. The PolyPhen-2 algorithm predicts the functional effects of amino acid changes by considering conservation, physicochemical differences and the proximity of the substitution to the predicted functional domains. Unlike SIFT or PolyPhen which handle only single amino acid substitutions, MutationTaster works on the DNA level and allows insertions and deletions up to 12 base pairs.

The original sequence of the protein was obtained from the Ensembl and UniProt/Swiss-Prot databases.

Statistical Analysis

This study describes the genotype and clinical phenotype of patients with AIS. A statistical analysis was not necessary.

Results

In our study *AR* gene analysis revealed an abnormal sequence in 24 individuals (58.5% of total index patients). All of the CAIS cases (n = 11) were genetically confirmed, while in PAIS (n = 30) a mutation in *AR* was detected in only 13 patients (43.3%).

Family studies were performed in 25 family members from 17 families. The molecular studies and affected family members are shown in Table 1. Molecular studies revealed other affected or carrier relatives in 87% of the index cases.

Table 1. Clinical phenotype, social sex and molecular studies

Patient	Clinical form	Social sex	Mutation	Location		Type of AIS	Family studies
				Protein domain	Exon		
1	CAIS	F	[a]p.Trp399Valfs*95 c.1197_1213del	NTD	1	Hereditary	Mother/Sistercarriers
2	CAIS	F	p.Leu822Pro c.2464T > C (27)	LBD	7	Hereditary	Mother/Sistercarriers
3	CAIS	F	p.Arg832* c.2494C > T (28)	LBD	7	*De novo*	Notdetected (mother)
4	CAIS	F	p.Pro767Ser c.2299C > T (26)	LBD	5	Hereditary	Mothercarrier
5	PAIS	M	p.[Cys602 = /Cys602Phe] c.[= /1805G > T] (29)	DBD	3	*De novo*, mosaic	Not detected (mother)
6	PAIS	F	[a]p.[Glu804 = /Glu804*] c.[= /2410G > T]	LBD	6	*De novo*, mosaic	Not detected (mother)
7	CAIS	F	p.Ile899Phe c.2695A > T (30)	LBD	8	-	Not done
8	PAIS	F	[a]p.[His730 = /His730Glnfs*38] c.[= /2188_2194dupACTTACA]	LBD	5	Mosaic	Not done
9	CAIS	F	p.Met750Val c.2248A > G (31)	LBD	5	-	Not done
10	PAIS	M	p.Arg608Gln c.823G > A (32)	DBD	3	Hereditary	Mother/aunt/ Grandmother carriers Cousin affected

Table 1. Continued

Patient	Clinical form	Social sex	Mutation	Location		Type of AIS	Family studies
				Protein domain	Exon		
11	CAIS	F	p.Val890Met c.2668G > A (33)	LBD	8	Hereditary	Mother carrier
12	PAIS	F	p.Asp691del c.2071_2073del (34)	LBD	4	Hereditary	Mother carrier/ Sister affected
13	CAIS	F	[a]p.Phe726Cys c.2176T > G	LBD	5	Hereditary	Mother carrier
14	CAIS	F	p.Arg856Cys c.2566C > T (35)	LBD	7	-	Not done
15	CAIS	F	[a]p.Gln658Argfs*3 c.1972_1973del	LBD	4	Hereditary	1 Sister affected/ 1 Sister carrier
16	PAIS	M	[a]p.[Gln98 = /Gln98Hisfs*8] c.[= /292_311del]	NTD	1	Mosaic	Not done
17	CAIS	F	ex2-ex8del c.1617-1768_2608-2763del (36)	DBD-LBD	2-8	-	Not done
18	PAIS	M	p.Arg841Cys c.2521C > T (35)	LBD	7	Hereditary	Mother carrier
19	PAIS	M	p.Arg841Cys c.2521C > T (35)	LBD	7	Hereditary	Mother carrier
20	PAIS	M	p.(Ser889 =) c.2667C > T (37)	LBD	8	-	Not done
21	PAIS	M	p.Ala597Thr c.1789G > A (38)	DBD	3	Hereditary	Mother carrier
22	PAIS	M	[a]p.Asn611Ile c.1832A > T	DBD	3	Hereditary	Mother carrier
23	PAIS	M	[a]p.Glu707Asp c.2121A > C	LBD	4	Hereditary	Mother carrier
24	PAIS	M	[a]p.His886Tyr c.2656C > T	LBD	8	Hereditary	Mother carrier

[a]novel mutation. CAIS: complete androgen insensitivity syndrome, PAIS: partial androgen insensitivity syndrome, F: female, M: male, NTD: N-terminal domain, DBD: DNA-binding domain, LBD: ligand-binding domain, AIS: androgen insensitivity syndromes

De novo AR mutations were found in three (P3, P5 and P6) out of 13 mothers analyzed. In two non-related index cases (P12 A and P15), two 46,XY affected siblings raised as female were detected. Interestingly, even though in P12A PAIS was established, normal external female genitalia, in the affected sister, was observed (P12 B). As shown in Table 1, 23 *AR* mutations were detected. The *AR* mutations were found spread along the whole coding sequence, with a higher prevalence in LBD: 8.3% were located in NTD; 16.6% in the DBD; 70.8% in the LBD and 4.3% were gross deletions (7).

Nine out of 23 (39%; P1, P6, P8, P13, P15, P16, P22, P23 and P24) *AR* mutations were novel. Two novel mutations were located in the NTD domain (P1 and P16). They

were both out of frame deletions that ultimately created a nonsense stop codon and premature truncation of the protein. The others, located in the LBD, were:four missense mutations, a nonsense mutation together with a 2bp deletion and a duplication of 7bp that produce a frameshift with a premature stop codon. Three patients (P6, P8 and P16) harboured somatic mosaicisms: a nonsense mutation, a 7bp duplication and a 20bp deletion which result in a truncating frameshift mutation. One missense mutation was located in the DBD. All novel mutations were predicted to be pernicious by all *in silico* tools.

In four individuals (P5, P6, P8 and P16), 17% of AR-mutated gene patients, somatic mosaicism of mutant and wild type alleles was detected in DNA derived from blood leukocytes.

Of the 17 individuals without a defect in the AR, two patients were finally diagnosed (and genetically confirmed) with 5-alpha reductase deficiency. In the others, diagnosis remains unknown.

The clinical phenotype and follow-up of the genetically confirmed patients is shown in Supplemental Table 1. Interestingly, during follow-up, no gender dysphoria,

Supplemental Table 1. Clinical phenotype at diagnosis and long term follow-up

Patient	CA at diagnosis (years)	Clinical form	Phenotype	Gonadal position	EMS at diagnosis (PAIS)	Social sex	Follow-up: puberty, gender dysphoria	Hormonal profile
1	4	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Female gender identity.	
2	5.7	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Lost to follow-up.	
3		CAIS	Female genitalia, inguinal hernia	Inguinal		F		
4	6.7	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Female gender identity.	
5	1.7	PAIS	Penoscrotal hypospadias	Scrotal	9	M	Pubertal onset at 9.6 years, gynecomastia, male gender identity.	Gonadotropins, T and AMH levels according to male reference range.
6	0.5 months	PAIS	Micropenis, penoscrotal hypospadias	Scrotal	6	M	Spontaneous pubertal onset, pubertal development not available. Gender identity not available.	
7	0.3 months	PAIS	Micropenis, penoscrotal hypospadias	Inguinal/scrotal	5.5	F	Female gender identity. Speech delay.	Gonadotropins, T, and AMH levels according to male reference range in the neonatal period.
8	0.8 months	PAIS	Micropenis, penoscrotal hypospadias	Scrotal	6	M	Lost to follow-up	
9	9.8	PAIS	Female genitalia, clítoris hypertrophy	NA	NA	F	Female gender identity, hypoplastic upper vagina requiring sigmoidal vaginal replacement.	Gonadotropins, T, and AMH levels according to male reference range in the neonatal period.
10	4 months	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Female gender identity.	

Supplemental Table 1. Continued

Patient	CA at diagnosis (years)	Clinical form	Phenotype	Gonadal position	EMS at diagnosis (PAIS)	Social sex	Follow-up: puberty, gender dysphoria	Hormonal profile
11	9.8	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Female gender identity. Wolf-Parkinson White syndrome.	Gonadotropins levels according to male reference range.
12	1.5 months	PAIS	Penoscrotal hypospadias	Scrotal	9	M	Precocious pubertal development (onset at 7 years), mild gynecomastia, male gender identity.	Gonadotropins, T and AMH levels in according male reference range.
13	2 months	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Female gender identity.	Gonadotropins, T and AMH levels according to male neonatal reference range.
14	7.3	PAIS	Female genitalia, complete fusion of the labia majora	Inguinal	5	F	Female gender identity.	Gonadotropins, T and AMH levels according to male reference range.
15	1.8	PAIS	Female genitalia, posterior fusion of the labia majora, inguinal hernia	Inguinal	2	F	Female gender identity. Obesity.	Gonadotropins and T levels according to male reference range.
16	14.6	CAIS	Female genitalia	Abdominal		F	Spontaneous telarche. Pubertal development difficult to evaluate because antecedents of BMT for neuroblastoma. Neurosensorial hypoacusia and developmental delay.	T levels according to male reference range.
17	7	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Peripheral precocious puberty secondary to unilateral gonadal tumor (Sertoli cell + sexual cords)	
18	1.3	PAIS	Micropenis, penoscrotal hypospadias	Scrotal	6	M	Spontaneous pubertal development, severe gynecomastia. Gender identity not available.	Gonadotropins and T levels according to male reference range.
19	17	CAIS	Female genitalia, inguinal hernia	Inguinal		F	Spontaneous pubertal development, female gender identity. Postpubertal gonadectomy.	Gonadotropins and T levels according to male reference range.
20	0.7 months	PAIS	Penoscrotal hypospadias	Scrotal	9	M	Spontaneous pubertal development, severe gynecomastia, male gender identity.	Increased neonatal gonadotropins, and T levels for male reference range.

Supplemental Table 1. Continued

Patient	CA at diagnosis (years)	Clinical form	Phenotype	Gonadal position	EMS at diagnosis (PAIS)	Social sex	Follow-up: puberty, gender dysphoria	Hormonal profile
21	7 months	PAIS	Micropenis, penoscrotal hypospadias	Scrotal	6	M	Gender identity and follow-up not yet evaluatable (toddler).	Gonadotropins T and AMH levels according to male reference range.
22	0.5 months	PAIS	Micropenis, penoscrotal hypospadias, inguinal hernia	Inguinal	5	M	Gender identity and follow-up not yet evaluatable (toddler).	Gonadotropins T and AMH levels according to male reference range.
23	2 months	PAIS	Micropenis, penoscrotal hypospadias	Scrotal	6	M	Gender identity and follow-up not yet evaluatable (toddler).	Gonadotropins T and AMH levels according to male reference range.
24	0.3 months	PAIS	Penoscrotal hypospadias, unilateral cryptorchidism	Inguinal/scrotal	8.5	M	Gender identity and follow-up not yet evaluatable (toddler).	Gonadotropins and T levels according to male reference range. Increased AMH levels.

CA: chronologycal age, EMS: external masculinization score (39), CAIS: complete androgen insensitivity syndrome, PAIS: partial androgen insensitivity syndrome, F: female, M: male, NA: not available, BMT: bone marrow transplant, T: testosterone, AMH: anti-Müllerian hormone.

Male reference gonadotropin levels (MU/mL). 0-3 months: follicle-stimulating hormone (FSH): 2.43 ± 1.67, luteinising hormone (LH): 2.52 ± 1.74; 3-12 months: FSH: 1.35 ± 0.81, LH: 1.21 ± 1.65; 12-24 months: FSH: 0.90 ± 0.59, LH: 0.15 ± 0.17; > 24 months: FSH: 1.10 ± 0.82, LH: 0.13 ± 0.32; 9-12 years: FSH: 2.26 ± 0.96 (MU/mL), LH: 0.78 ± 0.99.

Male reference testosterone levels (ng/mL): 1-5 months < 0.05-1.77; 6-11 months ≤0.07; 1-5 years ≤0.25; 6-9 years ≤0.30; 10-11 years 0.05-0.50; 12-14 years 0.10-5.72; 15-17 years 2.20-8.00.

Male reference AMH levels (pmol/L): 0-14 days: 250-1000; 15 days-3 years 400-2400; > 3 years prepubertal Tanner 1 300-1400; > 3 years pubertal Tanner 2 70-1000; > 3 years pubertal Tanner 3 30-400; > 3 years pubertal Tanner 4/5 30-180.

including those PAIS patients assigned male or female sex, were observed. Unfortunately, in toddler patients, gender identity could not be evaluated. According to previous reports, very low frequency of gonadal tumors was found. Only in P17 was a Sertoli cell tumor detected (8).

Discussion

We describe a series of unrelated patients affected by different degrees of AIS. *AR* gene mutations are the main cause of 46,XY DSD. To date, the *AR* gene mutations database (http://www.mcgill.ca/androgendb/) has reported more than 800 different *AR* mutations from patients with AIS.

In all CAIS cases, *AR* mutations responsible for the phenotype were identified. However, similar to other cohorts, in PAIS phenotype cases, *AR* mutations were identified in only 38%. Overall, in our series of 41 index patients, the *AR* gene proved to be abnormal in 58.5%, confirming the diagnosis. Similarly, Boehmer et al (9) and Audi et al (2) report a frequency of detection of 44-65% which is in line with our results. In contrast de Silva et al (10) and Akcay et al (11) describe cohorts with 15-18% of genetically confirmed AIS. In these studies, the significantly lower percentage of *AR* mutation detection could be due to the presence of overlaps in the clinical presentation of the patients, such as 5-α reductase deficiency or the fact that patients with a T biosynthetic defect were also included. Therefore, it has been proposed that even though AR is essential for virilization, other components of the AR complex and signaling network are required for complete masculinization. It has been suggested that in non-detected cases androgen resistance might be secondary to mutations in the 5'UTR, or other regulatory regions. Moreover, several necessary AR cofactor(s) should also be taken into consideration. Several cofactors, such as coactivators steroid receptor coactivator 1 (SRC1), transcriptional mediators/intermediary factor 2, SRC3 and corepressors nuclear receptor-interacting

protein 1, nuclear receptor subfamily 0 group B member 1, are actively involved in the regulation of *AR*-mediated transcription, and might play an important role in AIS etiopathogenesis (12,13,14,15). Interestingly, in order to confirm androgen resistance, Hornig et al (16) developed a DHT-dependent transcriptional induction of the androgen-regulated *APOD (apolipoprotein D)* gene in cultured genital fibroblasts (*APOD*-assay). However, the usefulness of this *APOD assay* needs to be confirmed in a large cohort.

Mutations in the *AR* gene are distributed throughout the sequence with a preponderance (70.8%) located in the LBD (17). In our cohort, nine novel AR mutations were found, expanding the mutational spectrum of 46, XY DSD. In three of these novel mutations, located in the LBD, a truncated, significantly reducedor inactive protein was predicted due to a premature stop codon, secondary to gene deletion (P15), gene duplication (P8) or nonsense mutation (P6). The p.Phe726Cys missense mutation located in the LBD was also detected. Interestingly the study of Quigley et al (18) demonstrated by functional assays that a missense mutation in the same position (p.Phe726Leu),caused the disruption of the N/C terminal interaction of the mutated protein. Hence it might be reasonable to suppose that the novel missense mutation found in our cohort might also affect the transactivation activity of the AR, impairing the binding of the ligand to its LBD. The remaining novel mutations, two gene deletions (P1 and P16) located in the NTD domain, result in a truncated protein due to a premature stop codon.

A lack of correlation between genotype and clinical phenotype has previously been reported (19). Interestingly, in siblings of family 12, harbouring p.Asp691del mutation, a clinical variability was evident. A CAIS phenotype was observed in one case, while in the other a PAIS phenotype was observed. Petroli et al's (20) study showed, in N/C terminal interaction assays, different profiles of the mutant AR protein in response to DHT stimulation, explaining the phenotypic diversity observed in PAIS cases.

Somatic mosaicism has been reported. Interestingly, even though the patients carried severe *AR* mutations, PAIS clinical phenotype was reported. In these affected patients the *de novo* mutation occurred after the zygote stage and probably very early, during the first few cell divisions. Thus, different proportions of cells containing mutant or wild-type protein are present in various tissues of the same individual explaining the mild phenotype. Similarly, in four patients of our cohort (P5, P6, P8 and P16) a severe mutation was detected but presenting with a PAIS phenotype. It is noteworthy that detection of somatic mosaicism in AR has a great impact for patients with AIS because further virilization is possible after birth and this is an important consideration for genetic counseling (21).

No gender dysphoria was observed in our cohort, even though systematic assessment was not available in all cases.

In contrast to previous reports, in this cohort, AMH serum concentrations during the neonatal period were within the normal male reference range in the only two PAIS cases in whom it was assessed (22,23). *AMH* gene expression in Sertoli cells is inhibited via the AR receptor pathway (24). The lack of *AR* expression in Sertoli cells during mini puberty could explain our findings, suggesting that other as yet unidentified factors might be involved in the regulation of AMH synthesis (25).

In agreement with previous reports, normal gonadotropin levels were the most frequent finding (26).

Study Limitations

Even though all *in silico* tools predicted the novel mutations to be damaging for protein structure and function, functional assays should be performed to confirm pathogenicity.

Conclusion

In summary, we report a series of 41 46,XY DSD index patients in whom AR was the candidate gene. Molecular diagnosis is useful for genetic counseling of the families. However, similar to other series, the percentage of suspected casesin whom an AR mutation was found wasonly around 60%.

Emerging technological advances might contribute to an increase in the accuracy of determining the etiology in suspected AIS cases.

Acknowledgements

The authors want to express their thanks to Marta Ciaccio, Monica Warman, Elisa Vaiani, Silvia Gil, Isabel Di Palma, Veronica Forclaz, Maria del Carmen Malosetti, Carmen Riu and Valeria de Dona for their help with the clinical data.

Ethics

Ethics Committee Approval: The study was approved by the Independent Ethics Committee "Prof. Dr. J. P. Garrahan Pediatric Hospital" (reference number: 971).

Informed Consent: Informed consent for the genetic study was obtained from all of the patients or their parents guardians after full explanation of the purpose and nature of all procedures.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: Maria Sol Touzon, Natalia Perez Garrido, Gabriela Guercio, Mariana Costanzo, Roxana Marino, Marco A. Rivarola, Alicia Belgorosky, Data Collection and Analysis: Sol Touzon, Gabriela Guercio, Mariana Costanzo, Roxana Marino, Pablo Ramirez, Esperanza Berensztein, Natalia Perez Garrido, Writing: Maria Sol Touzon, Natalia Perez Garrido.

Financial Disclosure: Supported by grants from the National Scientific and Technical Research Council (CONICET), Argentina and Fondo para la Investigación Científica y Tecnológica (FONCYT), Argentina.

References

1. GalaniA, Kitsiou-Tzeli S, Sofokleous C, Kanavakis E, Kalpini-Mavrou A. Androgen insensitivity syndrome: clinical features and molecular defects. Hormones (Athens) 2008;7:217-229.
2. Audi L, Fernández-Cancio M, Carrascosa A, Andaluz P, Torán N, Piró C, Vilaró E, Vicens-Calvet E, Gussinyé M, Albisu MA, Yeste D, Clemente M, Hernández de la Calle I, Del Campo M, Vendrell T, Blanco A, Martínez-Mora J, Granada ML, Salinas I, Forn J, Calaf J, Angerri O, Martínez-Sopena MJ, Del Valle J, García E, Gracia-Bouthelier R, Lapunzina P, Mayayo E, Labarta JI, Lledó G, Sánchez Del Pozo J, Arroyo J, Pérez-Aytes A, Beneyto M, Segura A, Borrás V, Gabau E, Caimarí M, Rodríguez A, Martínez-Aedo MJ, Carrera M, Castaño L, Andrade M, Bermúdez de la Vega JA; Grupo de Apoyo al Síndrome de Insensibilidad a los Andrógenos (GrApSIA). Novel (60%) and recurrent (40%) androgen receptor gene mutations in a series of 59 patients with a 46,XY disorder of sex development. J Clin Endocrinol Metab 2010;95:1876-1888. Epub 2010 Feb 11
3. Brinkmann AO. Molecular basis of androgen insensitivity. Mol Cell Endocrinol 2001;179:105-109.
4. Tadokoro-Cuccaro R, Hughes IA. Androgen insensitivity syndrome. Curr Opin Endocrinol Diabetes Obes 2014;21:499-503.
5. Gottlieb B, Trifiro MA. Androgen Insensitivity Syndrome. GeneReviews®. University of Washington, Seattle, 1999.
6. Chu J, Zhang R, Zhao Z, Zou W, Han Y, Qi Q, Zhang H, Wang JC, Tao S, Liu X, Luo Z. Male Fertility Is Compatible with an Arg(840) Cys substitution in the AR in a Large Chinese Family affected with Divergent Phenotypes of AR insensitivity Syndrome. J Clin Endocrinol Metab 2002;87:347-351.
7. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The Androgen Receptor Gene Mutations Database: 2012 update. Hum Mutat 2012;33:887-894. Epub 2012 Mar 13
8. Cools M, Wolffenbuttel KP, Hersmus R, Mendonca BB, Kaprová J, Drop SLS, Stoop H, Gillis AJM, Oosterhuis JW, Costa EMF, Domenice S, Nishi MY, WunschL, Quigley CA, T'Sjoen G, Looijenga LHJ. Malignant testicular germ cell tumors in postpubertal individuals with androgen insensitivity: prevalence, pathology and relevance of single nucleotide polymorphism-based susceptibility profiling. Hum Reprod 2017;32:2561-2573.
9. Boehmer AL, Brinkmann O, Brüggenwirth H, van Assendelft C, Otten BJ, Verleun-Mooijman MC, Niermeijer MF, Brunner HG, Rouwé CW, Waelkens JJ, Oostdijk W, Kleijer WJ, van der Kwast TH, de Vroede MA, Drop SL. Genotype versus phenotype in families with androgen insensitivity syndrome. J Clin Endocrinol Metab 2001;86:4151-4160.
10. de Silva KS, Sirisena ND, Wijenayaka HK, Cooray JG, Jayasekara RW, Dissanayake VH. Androgen insensitivity syndrome in a cohort of Sri Lankan children with 46, XY disorders of sex development (46, XY DSD). Ceylon Med J 2015;60:139-142
11. Akcay T, Fernandez-Cancio M, Turan S, Güran T, Audi L, Bereket A. AR and *SRD5A2* gene mutations in a series of 51 Turkish 46,XY DSD children with a clinical diagnosis of androgen insensitivity. Andrology 2014;2:572-578. Epub 2014 Apr 16
12. Heemers HV, Tindall DJ. Androgen Receptor (AR) Coregulators: A Diversity of Functions Converging on and Regulating the AR Transcriptional Complex. Endocr Rev 2007;28:778-808. Epub 2007 Oct 16
13. Van de Wijngaart D, Dubbink H, VanRoyen M, Trapman J, Jenster G. Androgen receptor coregulators: Recruitment via the coactivator binding groove. Mol Cell Endocrinol 2012;352:57-69. Epub 2011 Aug 17
14. Känsäkoski J, Jääskeläinen J, Jääskeläinen T, Tommiska J, Saarinen L, Lehtonen R, Hautaniemi S, Frilander MJ, Palvimo JJ, Toppari J, Raivio T. Complete androgen insensitivity syndrome caused by a deep intronic pseudoexon-activating mutation in the androgen receptor gene. Sci Rep 2016;9;6:32819.
15. Hornig NC, de Beaufort C, Denzer F, Cools M, Wabitsch M, Ukat M, Kulle AE, Schweikert HU, Werner R, Hiort O, Audi L, Siebert R, Ammerpohl O, Holterhus PM. A Recurrent Germline Mutation in the 5'UTR of the Androgen Receptor Cause Complete Androgen Insensitivity by Activating Aberrant uORF Translation. PLoS One 2016;11:0154158.
16. Hornig NC, Ukat M, Schweikert HU, Hiort O, Werner R, Drop SL, Cools M, Hughes IA, Audi L, Ahmed SF, Demiri J, Rodens P, Worch L, Wehner G, Kulle AE, Dunstheimer D, Müller-Roßberg E, Reinehr T, Hadidi AT, Eckstein AK, van der Horst C, Seif C, Siebert R, Ammerpohl O, Holterhus PM. Identification of an AR-mutation negative class of androgen insensitivity by determining endogenous AR-activity. J Clin Endocrinol Metab 2016;101:4468-4477. Epub 2016 Sep 1
17. Hughes IA, Werner R, Bunch T, Hiort O. Androgen insensitivity syndrome. Semin Reprod Med 2012;30:432-442. Epub 2012 Oct 8
18. Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, Forest MG, Chatelain P, Ritzén EM, French FS, Wilson EM. Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH(2)- and carboxyl-terminal interaction. Mech Ageing Dev 2004;125:683-695.
19. Williams textbook of Endocrinology 13th ed. Testicular disorders. Philadelphia, PA. Elsevier Saunders, 2016.
20. Petroli RJ, Hiort O, Struve D, Gesing JK, Soardi FC, Spínola-Castro AM, Melo K, Prado Arnhold IJ, Maciel-Guerra AT, Guerra-Junior G, Werner R, de Mello MP. Functional Impact of Novel Androgen Receptor Mutations on the Clinical Manifestation of Androgen Insensitivity Syndrome. Sex Dev 2017;11:238-247. Epub 2017 Dec 14
21. Köhler B, Lumbroso S, Leger J, Audran F, Grau ES, Kurtz F, Pinto G, Salerno M, Semitcheva T, Czernichow P, Sultan C. Androgen insensitivity syndrome: somatic mosaicism of the androgen receptor in seven families and consequences for sex assignment and genetic counseling. J Clin Endocrinol Metab 2005;90:106-111. Epub 2004 Nov 2
22. Rey R, Mebarki F, Forest MG, Mowszowicz I, Cate RL, Morel Y, Chaussain JL, Josso N. Anti-müllerian hormone in children with androgen insensitivity. J Clin Endocrinol Metab 1994;79:960-964.
23. Rey RA, Belville C, Nihoul-Fékété C, Michel-Calemard L, Forest MG, Lahlou N, Jaubert F, Mowszowicz I, David M, Saka N, Bouvattier C, Bertrand AM, Lecointre C, Soskin S, Cabrol S, Crosnier H, Léger J, Lortat-Jacob S, Nicolino M, Rabl W, Toledo SP, Baş F, Gompel A, Czernichow P, Josso N. Evaluation of gonadal function in 107 intersex patients by means of serum antimüllerian hormone measurement. J Clin Endocrinol Metab 1999;84:627-631.

24. Boukari K, Meduri G, Brailly-Tabard S, Guibourdenche J, Ciampi ML, Massin N, Martinerie L, Picard JY, Rey R, Lombès M, Young J. Lack of androgen receptor expression in Sertoli cells accounts for the absence of anti-Mullerian hormone repression during early human testis development. J Clin Endocrinol Metab 2009;94:1818-1825. Epub 2009 Mar 10

25. Berensztein EB, Baquedano MS, Gonzalez CR, Saraco NI, Rodriguez J, Ponzio R, Rivarola MA, Belgorosky A. Expression of aromatase, estrogen receptor alpha and beta, androgen receptor, and cytochrome P-450scc in the human early prepubertal testis. Pediatr Res 2006;60:740-744. Epub 2006 Oct 25

26. Ahmed SF, Cheng A, Hughes IA. Assessment of the gonadotrophin-gonadal axis in androgen insensitivity syndrome. Arch Dis Child 1999;80:324-329.

27. Akella RR. Mutational analysis of androgen receptor gene in two families with androgen insensitivity Indian. Indian J Endocrinol Metab 2017;21:520-523.

28. Audi L, Fernández-Cancio M, Carrascosa A, Andaluz P, Torán N, Piró C, Vilaró E, Vicens-Calvet E, Gussinyé M, Albisu MA, Yeste D, Clemente M, Hernández de la Calle I, Del Campo M, Vendrell T, Blanco A, Martínez-Mora J, Granada ML, Salinas I, Forn J, Calaf J, Angerri O, Martínez-Sopena MJ, Del Valle J, García E, Gracia-Bouthelier R, Lapunzina P, Mayayo E, Labarta JI, Lledó G, Sánchez Del Pozo J, Arroyo J, Pérez-Aytes A, Beneyto M, Segura A, Borrás V, Gabau E, Caimarí M, Rodríguez A, Martínez-Aedo MJ, Carrera M, Castaño L, Andrade M, Bermúdez de la Vega JA; Grupo de Apoyo al Síndrome de Insensibilidad a los Andrógenos (GrApSIA). Novel (60%) and Recurrent (40%) Androgen Receptor Gene Mutations in a Series of 59 Patientswith a 46,XY Disorder of Sex Development. J Clin Endocrinol Metab 2010;95:1876-1888. Epub 2010 Feb 11

29. Baldazzi L, Baroncini C, Pirazzoli P, Balsamo A, Capelli M, Marchetti G, Bernardi F, Cacciari E. Two mutations causing complete androgen insensitivity: a frame-shift in the steroid binding domain and a Cys-->Phe substitution in the second zinc finger of the androgen receptor. Hum Mol Genet 1994;3:1169-1170.

30. Melo KF, Mendonca BB, Billerbeck AE, Costa EM, Inácio M, Silva FA, Leal AM, Latronico AC, Arnhold IJ. Clinical, Hormonal, Behavioral, and Genetic Characteristics of Androgen Insensitivity Syndrome in a Brazilian Cohort: Five Novel Mutations in the Androgen Receptor Gene. J Clin Endocrinol Metab 2003;88:3241-3250.

31. Bouvattier C, Carel JC, Lecointre C, David A, Sultan C, Bertrand AM, Morel Y, Chaussain JL. Postnatal changes of T, LH, and FSH in 46, XY infants with mutations in the AR gene. J Clin Endocrinol Metab 2002;87:29-32.

32. Weidemann W, Linck B, Haupt H, Mentrup B, Romalo G, Stockklauser K, Brinkmann AO, Schweikert HU, Spindler KD. Clinical and biochemical investigations and molecular analysis of subjects with mutations in the androgen receptor gene. Clin Endocrinol (Oxf) 1996;45:733-739.

33. Pinsky L, Trifiro M, Kaufman M, Beitel LK, Mhatre A, Kazemi-Esfarjani P, Sabbaghian N, Lumbroso R, Alvarado C, Vasiliou M. Androgen resistance due to mutation of the androgen receptor. Clin Invest Med 1992;15:456-472.

34. Schwartz M, Skovby F, Mueller J, Nielsen O, Skakkebaek NE. Partial androgen insensitivity (PAIS) in a large eskimo kindred caused by a del D690 mutation in the androgen receptor *(AR)* gene. Horm Res 1994;41:117.

35. McPhaul MJ, Marcelli M, Zoppi S, Wilson CM, Griffin JE, Wilson JD. Mutations in the ligand-binding domain of the androgen receptor gene cluster in two regions of the gene. J Clin Invest 1992;90:2097-2101.

36. Jakubiczka S, Nedel S, Werder EA, Schleiermacher E, Theile U, Wolff G, Wieacker P. Mutations of the androgen receptor gene in patients with complete androgen insensitivity. Hum Mutat 1997;9:57-61.

37. Hellwinkel OJ, Holterhus PM, Struve D, Marschke C, Homburg N, Hiort O. A unique exonic splicing mutation in the human androgen receptor gene indicates a physiologic relevance of regular androgen receptor transcript variants. J Clin Endocrinol Metab 2001;86:2569-2575.

38. Gast A, Neuschmid-Kaspar F, Klocker H, Cato AC. A single amino acid exchange abolishes dimerization of the androgen receptor and causes Reifenstein syndrome. Mol Cell Endocrinol 1995;111:93-98.

39. Ahmed SF, Cheng A, Dovey L, Hawkins JR, Martin H, Rowland J, Shimura N, Tait AD, Hughes IA. Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. J Clin Endocrinol Metab 2000;85:658-665.

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0121

J Clin Res Pediatr Endocrinol 2019;11(1):34-40

Genetic and Clinical Characteristics of Patients with Vitamin D Dependent Rickets Type 1A

Fatma Dursun[1], Gamze Özgürhan[2], Heves Kırmızıbekmez[1], Ece Keskin[3], Bülent Hacıhamdioğlu[4]

[1]Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
[2]Süleymaniye Maternity and Children's Training and Research Hospital, Clinic of Paediatrics, İstanbul, Turkey
[3]Süleymaniye Maternity and Children's Training and Research Hospital, Clinic of Medical Genetic, İstanbul, Turkey
[4]İstinye University Faculty of Medicine, Department of Pediatric Endocrinology, İstanbul, Turkey

What is already known on this topic?

Although vitamin D dependent rickets type 1A (VDDR1A) is a rare disease, it is relatively more common in Turkey. Thus far intron-1 mutations have only been reported from Turkey. Intron-1 mutations have been reported to be associated with milder clinical findings. Clinical and laboratory findings can overlap with other types of rickets. Serum 1,25-dihydroxyvitamin D levels are usually reported to be low in cases of VDDR1A.

What this study adds?

Patients with intron-1 mutations can present with clinical findings of variable severity. We also found that the concentrations of 1,25-dihydroxyvitamin D levels may be within inappropriately normal ranges in genetically proven vitamin D dependent rickets type 1A and lead to diagnostic confusion.

Abstract

Objective: Vitamin D dependent rickets type 1A (VDDR1A) is an autosomal recessive disorder caused by mutations in the 1α-hydroxylase gene (*CYB27B1*). As it may be confused with nutritional rickets and hypophosphatemic rickets, genetic analysis is important for making a correct diagnosis.

Methods: We analysed genomic DNA from 11 patients from eight different Turkish families. The patients were recruited for our studies if they presented with a diagnosis of VDDR.

Results: The mean ± standard deviation age at diagnosis was 13.1 ± 7.4 months. Seven patients had mild hypocalcemia at presentation while four patients had normal calcium concentrations. All patients underwent *CYP27B1* gene analysis. The most prevalent mutation was the c.195 + 2T > G splice donor site mutation, affecting five out of 11 patients with VDDR1A. Two patients from the fourth family were compound heterozygous for c.195 + 2T > G and c.195 + 2 T > A in intron-1. Two patients, from different families, were homozygous for a previously reported duplication mutation in exon 8 (1319_1325dupCCCACCC, Phe443Profs*24). One patient had a homozygous splice site mutation in intron 7 (c.1215 + 2 T > A) and one patient had a homozygous mutation in exon 9 (c.1474 C > T).

Conclusion: Intron-1 mutation was the most common mutation, as previously reported. All patients carrying that mutation were from same city of origin suggesting a "founder" or a "common ancestor" effect. VDDR1A should definitely be considered when a patient with signs of rickets has a normal 25-OHD level or when there is unresponsiveness to vitamin D treatment.

Keywords: Vitamin D, vitamin D dependent rickets, *CYP27B1* gene, 1α hydroxylase

Address for Correspondence: Fatma Dursun MD, Ümraniye Training and Research Hospital, Clinic of Pediatric Endocrinology, İstanbul, Turkey
Phone: +90 505 267 14 03 **E-mail:** fatmadursun54@yahoo.com **ORCID ID:** orcid.org/0000-0003-4880-8175

Conflict of interest: None declared
Received: 07.05.2018
Accepted: 03.10.2018

Introduction

Vitamin D (calciferol) comprises two biologically inactive, fat-soluble pro-hormones. The first is ergocalciferol (vitamin D2), derived from ergosterol after ultraviolet (UV) light exposure and the second is cholecalciferol (vitamin D3), derived from animal tissues and 7-dehydrocholesterol, formed in human skin by the action of UV rays in sunlight (1). Both forms need a two-step hydroxylation at the 25th and 1st carbons for activation. The first step occurs in the liver, where vitamin D is hydroxylated to 25-hydroxyvitamin D (25-OHD) by hepatic 25-hydroxylase. The second step occurs mainly in the kidney, where 25-OHD is further hydroxylated by the mitochondrial vitamin D 1α-hydroxylase to the biologically active hormone 1,25-dihydroxyvitamin D (1,25-OH_2D), which binds to its nuclear receptor and exerts its biological activities (1,2,3). The biologically active 1,25-OH_2D plays a central role in calcium homeostasis and bone metabolism and also has a significant influence on cell proliferation and differentiation of a variety of tissues (1,3,4). The renal synthesis of 1,25-OH_2D from its precursor 25-OHD is a rate-limiting step and is tightly regulated by exisiting serum concentrations of 1,25-OH_2D, parathyroid hormone (PTH), fibroblast growth factor-23 (FGF-23), calcium and phosphate concentrations, with renal 1α-hydroxylase being stimulated by PTH, hypophosphatemia, or hypocalcaemia and inhibited by FGF-23 (4).

Four rare genetic errors of vitamin D metabolism that can cause rickets have been described. The first one involves 1α-hydroxylase deficiency, which is also described as vitamin D dependent rickets type 1A (VDDR1A). A selective mutation in *CYP2R1* gene, which leads to 25-hydroxylase deficiency, is called type 1B (VDDR1B). This second type involves a defective vitamin D receptor (VDR), resulting in vitamin D resistant rickets (VDRR), also known as VDDR type 2A (VDDR2A). VDDR2B is an unusual form of rickets due to abnormal expression of a hormone response element-binding protein that interferes with normal function of VDR (5,6,7,8).

VDDR1A is an autosomal recessive disorder caused by mutations in the 25-OHD 1α-hydroxylase gene (*CYB27B1*). *CYB27B1* is composed of nine exons and is approximately 5 Mb in size. The gene has been mapped to the chromosomal region 12q14.1 (9,10,11,12). Clinically, VDDR1A is characterized by hypotonia, muscle weakness, inability to walk, growth failure and radiographic findings of rickets. Typical laboratory findings are hypocalcaemia, elevated serum levels of alkaline phosphatase (ALP) and of PTH with low or normal levels of 1,25-OH_2D despite normal or increased concentrations of 25-OHD (9,13). Patients with VDDR1 may present with aminoaciduria and hyperchloremic acidosis (3).

To date, over 100 patients with 78 mutations have been identified in the *CYP27B1* gene in patients from multiple ethnic groups. These mutations span all exons of the gene and mostly include missense and nonsense changes, along with splice site changes, insertions, deletions and duplications [Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk/ac/index.php] (14). Mutations in *CYP27B1* lead to a loss of 1α-hydroxylase activity and require treatment with calcitriol to normalize the clinical and laboratory abnormalities (15).

In the present study, we report 11 patients with VDDR1A from eight unrelated Turkish families. The most prevalent mutation was the c.195 + 2T > G splice donor site mutation, affecting five out of 11 patients with VDDR1A. Clinical findings of patients were examined in detail and genotype-phenotype correlations were evaluated.

Methods

We analyzed genomic DNA in 11 patients from eight different Turkish families. In five of these families, the parents were consanguineous. The study was approved by the University of Health Sciences Ümraniye Training and Research Hospital Clinical Research Ethical Committee (approved number: 19/01/2018-2926). Informed consent was obtained from patients and/or families.

Eleven patients had the clinical findings of rickets including X-bain deformity or bowed leg, chest rosary, Harrison's groove, frontal bossing, widening of the wrist, growth retardation, hypotonia and inability to walk together with hypocalcaemic seizures. The patients also had biochemical features suggestive of rickets such as hypophosphatemia, hypo- or normocalcemia, elevated PTH and ALP, normal or high 25-OHD levels and low or normal 1,25-OH_2D levels. Wrist and knee radiographs of all patients demonstrated widened epiphyses and metaphyseal cupping and fraying. Differentiation of nutritional rickets and VDDR1A was made by normal/high 25-OHD levels, low/inappropriately normal 1,25-OH_2 D levels and improvement in the clinical, biochemical and radiological findings of rickets after replacement with calcitriol. All patients received calcitriol and patients with hypocalcaemia received calcium replacement. Calcitriol was started at a dose of 1-1.5 mcg/day, twice daily. Subsequently the calcitriol dose was titrated according to the results of biochemical analyses. The aims of the treatment were to achieve normocalcemia, to maintain PTH levels within normal limits and to avoid hypercalciuria.

Targeted Second Generation Sequence Analysis

DNA was isolated from a 200 microlitre peripheral blood sample using QIAamp DNA Blood Mini QIAcube Kit and QIAcube device (QIAGEN, Hilden, Germany). Then, the exons of the *CYP27B1* gene were amplified for targeted sequencing. Amplification was controlled with agarose gel electrophoresis technique. Sequencing was carried out using Illumina MiSeq NGS System (Illumina Inc., San Diego, CA, USA) and the Miseq Reagent Kit V3 (600 cycles) from the same manufacturer. The readings were aligned with human genome 19 genomic sequence and compared.

Sanger Sequencing

10 mL venous blood sample was taken from each patient into EDTA tubes. DNA isolation was performed using the QIAamp DNA Mini QIAcube Kit from the peripheral blood. The Primer design included *CYP27B1* gene exons and close introns (Table 1). The products of polymerase chain reaction (PCR) [94 °C-5 min (95 °C-30 sec - 60 °C-30 sec - 72 °C 30 sec) x 34, 72 °C-5 min] with the primers, also shown in Table 1, were checked on a 2% agarose gel. After the amplification of correct gene regions, purification of PCR products was made by maintenance for 15 minutes at 37 °C (enzyme activation temperature) and 15 minutes at 80 °C (enzyme inactivation temperature) in the thermal cycler using ExoSAP enzyme. After purification, the primer and the cleaned template DNA were added to the PCR solution, using "The Big Dye Ready Reaction Mix Sequencing Kit" (Applied Biosystems® Big Dye®, Foster City, Calif., USA) and the PCR reaction was started. The purification process was repeated after the PCR sequencing for the removal of uncoupled dideoxynucleotide triphosphates in the solution. Sanger sequencing of the purified samples was performed on the ABI 3130 XL (Applied Biosystems® 3130 Genetic Analyzers, Foster City, Calif., USA) capillary sequencing device. The obtained data were analysed by Applied Biosystems SeqScape® Software (Calif., USA) analysis program.

Table 1. List of primers used for polymerase chain reaction amplification of the nine coding exons of *CYP27B1* gene

Primer name	Primer sequence
*CYP27B1*_1F:	GTCATCACCTCACCCAAAGG
*CYP27B1*_1R:	TCTGACGCTGTCAAAACCAG
*CYP27B1*_2F:	GAAGCTCCCTATTCCCAAGC
*CYP27B1*_2R:	CATGCCCCCAGATTGATAGT
*CYP27B1*_3-4F:	CTCCTTCACTGCAGCCAGTC
*CYP27B1*_3-4R:	GTGGGTAGAAGGCACGTGAA
*CYP27B1*_5F:	GCATTTGGTAAGGCACAGGT
*CYP27B1*_5R:	CATAATGGATCCCCTGCAAC
*CYP27B1*_6-7F:	CCATAATCTGCACCCTCTGC
*CYP27B1*_6-7R:	GGGCCCAAGATAGTGAGGA
*CYP27B1*_8F:	TCTTCATGCCTGCCCTATTC
*CYP27B1*_8R:	CAGGGGAAAGAGCTCACAAC
*CYP27B1*_9F:	CACCCAATCATTGACCATTC
*CYP27B1*_9R:	CATACTTCACACATTGGTCAGG

Data Analysis

Sequenced data were analyzed with the Genomize Variant Analysis Program (NHLBI GO Exome Sequencing Project, Seattle, USA) and Integrative Genomics Viewer (1000 Genomes Project, Calif., USA). The homozygote or compound heterozygote variants in the databases such as National Center for Biotechnology Information, HGMD, and Clinvar were primarily selected for data filtering. The effects of mutations on protein structures were tested with various *in silico* prediction tools, particularly Mutation Taster (16), PolyPhen-2 (17), and Sorting Tolerant From Intolerant (18).

Statistical Analysis

Statistical analysis was performed using IBM SPSS 21.0 for windows statistical software (IBM Inc., Chicago, Ill., USA). The data were presented as mean ± standard deviation (SD) (ranges).

Results

Among patients diagnosed with VDRR1A, six were males and five were females, from eight families. Clinical presentation and laboratory findings of the patients are summarized in Table 2. The mean age at diagnosis was 13.1 ± 7.4 months. Seven patients had mild hypocalcemia at presentation while four patients had normal calcium levels. Five of eight families had consanguineous marriages. The two families that were not consanguineous were from the same city.

All patients had clinical and laboratory features of rickets at the time of diagnosis. All patients had low levels of phosphorus with quite high levels of PTH and ALP levels (see Table 2). Five patients had fairly high levels of 25-OHD due to being formerly diagnosed with nutritional rickets and treated with vitamin D. Levels of 1,25-OH_2D, on the other hand, were normal in three patients. One patient was previously followed for hypophosphatemic rickets and treated with calcitriol and phosphate. When he was diagnosed with VDDR1A, he had elevated PTH levels and typical radiological findings of rickets (Figure 1).

After the definitive diagnosis of VDDR1A all patients received calcitriol treatment. The duration of treatment with calcitriol ranged between six months and seven years.

Biochemical improvement with treatment occurred within a period ranging between four and 12 months.

All patients underwent *CYP27B1* gene analysis (Table 3). The most prevalent mutation was the c.195 + 2T > G splice donor site homozygous mutation, affecting five out of 11 patients with VDDR1A. Two patients from family-4 had a compound heterozygous mutation for c.195 + 2T > G and c.195 + 2 T > A in intron-1. Two patients from different families had homozygous duplication mutation in exon 8 (1319_1325dupCCCACCC, Phe443Profs*24), which has been previously reported (Figure 2). A homozygous c.1215 + 2T > A mutation in the splice donor site of intron-7 was found in one patient and one patient was found to have a homozygous mutation in exon 9 (c.1474 C > T).

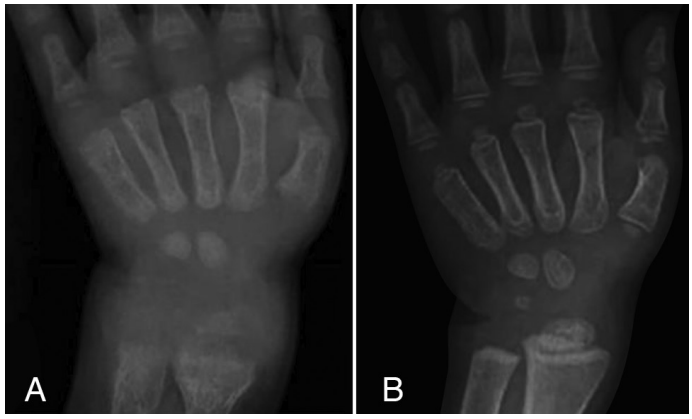


Figure 1. X-rays of this patient before (A) and at the 6th month of calcitriol treatment (B)

(A) Abnormal cupping, widening and fraying of the metaphyses consistent with rickets. (B) Recovery of cupping and fraying, and a provisional calcification zone suggesting healing rickets

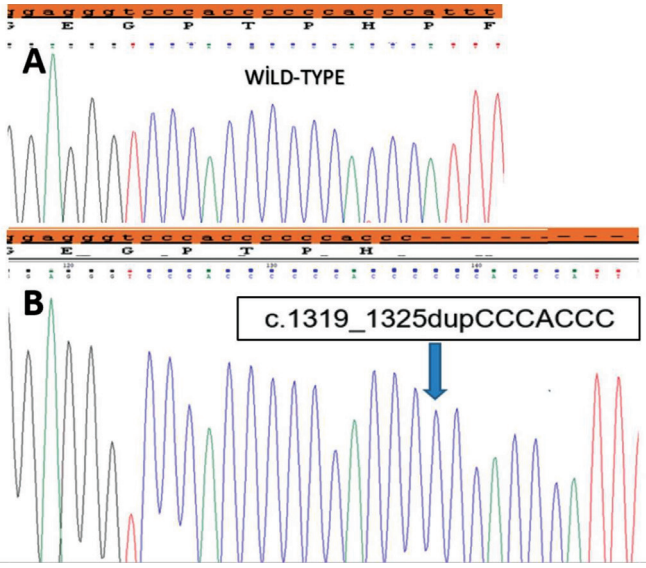


Figure 2. A) Wild type sequence of exon 8 in *CYP27B1* gene. B) Sequencing analysis of the *CYP27B1* gene exon 8 showing the homozygous mutation (1319_1325dupCCCACCC, Phe443Profs*24)

Table 2. Clinical and laboratory findings of 11 patients with vitamin D dependent rickets type 1A from 8 families

Subjects	Age (months)	Presenting symptoms	Height SD	Ca mg/dL NR: 9-11.5	P mg/dL NR: 4-6.5	PTH pg/mL NR: 11-67	ALP IU/L NR < 455	25-OHD ng/mL NR > 20	1,25-OH_2D pg/mL NR: 20-153
1.1	18	Bowed legs, growth retardation	-2	8.0	2.3	441	2120	26	15
1.2	36	Bowed legs, growth retardation	-2.5	7.1	3.1	784	3100	64	10
1.3	18	Bowed legs, growth retardation	-2.1	6.2	2.8	980	1940	120	12
2	9	O-bine deformity, failure to thrive	-3.1	7.8	3.0	625	1445	45	6
3	14	Hypotonia	-4.7	8.4	2.2	972	3111	194	38
4.1	6	History of VDDR sibling	0.28	8.0	2.9	546	1325	50	11
4.2	7	Inability to work	-1.06	8.9	2.1	423	1400	132	45
5	14	Growth retardation	-2.2	5.91	3.4	925	2531	41	< 1.3
6	11	Hypocalcaemic seizure	-0.55	6.7	3.6	467	651	89	15.4
7	24	Inability to walk	-2.1	8.7	2.3	1397	4479	18.7	21.5
8	24	Elevated ALP, inability to walk	-3.1	8.2	2	571	1001	135	5.5

SD: standard deviation, Ca: calcium, P: phosphate, PTH: parathyroid hormone, ALP: alkaline phosphatase, NR: normal range, VDDR: vitamin D dependent rickets, 25-OHD: 25-hydroxylase, 1,25-OH_2D: 1,25-dihydroxyvitamin D.

The patients are indicated with their respective family number and their number in that family. Patients 1.1, 1.2, 1.3. and 4.1 and 4.2 were siblings.

Table 3. Characteristics of the mutations detected in 11 patients with vitamin D dependent rickets type 1A from eight families

Family number	Exon/intron	DNA description	Zygosity	CF	MO/PO
1.1	Intron-1	c.195 + 2 T > G	HM	Yes	Batman/Batman
1.2	Intron-1	c.195 + 2 T > G	HM	Yes	Batman/Batman
1.3	Intron-1	c.195 + 2 T > G	HM	Yes	Batman/Batman
2	Intron-1	c.195 + 2 T > G	HM	No	Batman/Batman
3	Intron-1	c.195 + 2 T > G	HM	No	Batman/Batman
4.1	Intron-1	c.195 + 2 T > G / c.1215 + 2 T > A	CHT	No	Batman/Bitlis
4.2	Intron-1	c.195 + 2 T > G / c.1215 + 2 T > A	CHT	No	Batman/Bitlis
5	Exon 8	p.Phe443Profs*24 (c.1319_1325dupCCCACCC)	HM	Yes	Erzurum/Erzurum
6	Intron 7	c.1215 + 2T > A	HM	Yes	Mardin/Mardin
7	Exon 9	c.1474C > T p.R492W	HM	Yes	Mersin/Mersin
8	Exon 8	p.Phe443Profs*24 (c.1319_1325dupCCCACCC)	HM	Yes	Elazığ/Elazığ

CF: consanguineous family, HM: homozygyous, CHT: compound heterozygosity, MO: maternal origin, PO: paternal origin.

Locations of origins: Batman, Bitlis, Mardin: South-eastern Anatolia, Erzurum, Elazığ: Eastern Anatolia, Mersin: Mediterranean Region

Discussion

In the present study, we report the clinical, biochemical and genetic analysis of 11 patients with VDDR1A. We identified five previously reported mutations. The most prevalent mutation was the c.195 + 2T > G splice donor site mutation. Five patients from two different families had this mutation as homozygous and two patients from the same family had hemizygous inheritance as a part of compound heterozygous mutation. Durmaz et al (19) reported this mutation for the first time in a Turkish patient. Currently the c.195 + 2T > G homozygous mutation in intron-1 is present in a total of 20 patients including the patients described herein, all reported from Turkey (4,9,19). These patients were homozygous for the previously described splice donor site mutation c.195 + 2T > G, where a thiamine is substituted for a guanine in the second nucleotide of intron-1. Since this mutation is common in Turkish patients and has not been reported in other ethnic groups, it may be unique, representing a 'founder' or "common ancestor" effect, given the high rates of consanguinity. Although it has not been reported in other publications, all patients in the study by Tahir et al (9) were living in Diyarbakır or neighbouring provinces; all of our patients carrying that mutation were from Batman, which is geographically very close to Diyarbakır.

While Tahir et al (9) reported that patients with intron-1 mutation had a milder clinical presentation, Demir et al (4) reported that the most severe form of the disease occurred in a patient with intron-1 mutation thus the phenotype may be variable and a larger evidence base would be necessary to determine the genotype/phenotype relationship more clearly. We could not identify any relationship between genotype and phenotype although our series adds to the existing evidence. All patients in the literature who had an intron-1 mutation had delayed walking and bowed legs at admission. While four of our patients were also affected thus, another patient presented with hypotonia. Although 4 of 5 patients with intron-1 mutation had a height below -2 SD, patients with other mutations also had short stature.

We had only one patient presenting at the age of 11 months with a hypocalcaemic convulsion. Hypocalcaemic convulsion has also been reported rarely by other studies from Turkey. Tahir et al (9) reported hypocalcaemic convulsion in five of 22 patients; Demir et al (4) in 4 of 8 patients; and Durmaz et al (19) in two of seven patients. Kim et al (20) reported that 4 of their 10 patients presented with hypocalcaemic convulsion. Edouard et al (21) reported that the admission symptom was hypocalcaemic convulsion in 4 of 21 pediatric patients. Since these patients had blood calcium levels that were in the lower limit of the normal range, hypocalcemic convulsion was not frequently encountered.

The clinical presentations of patients with VDDR1A could lead to a misdiagnosis of nutritional rickets or hypophosphatemic rickets, which can be differentiated from hypophosphatemic rickets by a high PTH level and from nutritional rickets by a normal 25-OHD level. The hypophosphatemia in VDDR1A is a result of elevated PTH and renal excretion of phosphate. The clinical and laboratory features of VDDR1A are very similar to nutritional rickets although the differential diagnosis can be made by a low or inappropriately normal 1, 25-OH_2D level and unresponsiveness to vitamin D treatment. In our study, six patients had also had long-term therapy

with vitamin D because of an initial diagnosis of nutritional rickets and they had extremely high 25-OHD levels. Four patients had normal calcium levels and one of them had been followed with hypophosphatemic rickets. There are a few patients with normal 1,25-OH_2D levels diagnosed with VDDR1A in the literature (4,8). In fact, the expected 1,25-OH_2D levels in 1α-hydroxylase deficiency are low and inappropriately normal 1,25-OH_2D levels also indicate that the enzyme activity is insufficient. Recently, Nishikawa et al (22) reported that liver mitochondrial *CYP27A1* can catalyse 1α-hydroxylation of 25-OHD. A small increase in serum 1,25-OH_2D concentration has been observed in *CYP27B1* knockout mice after being given high dietary vitamin D, suggesting a conversion from 25-OHD to 1,25-OH_2D by a non-*CYP27B1* enzyme. Three of eleven patients in our study had normal 1,25-OH_2D levels and there was a history of high dose vitamin D intake in two of these three patients. In these patients, conversion from 25-OHD to 1,25-OH_2D by a non-*CYP27B1* enzyme may have contributed to the normal 1,25-OH_2D level.

Maternal 1,25-OH_2D does not cross the fetoplacental barrier (21,23). 1,25-OH_2D increases 2-3 fold in the first weeks of pregnancy when maternal 25-OHD crosses the placental barrier. The rise in circulating 1,25-OH_2D concentrations in the mother facilitates optimal *in utero* bone development by attaining a positive calcium balance (24). Edouard et al (21) reported that, unlike patients with severe vitamin D deficiency who can present within the first six months of life, none of the VDDR1A patients were symptomatic before the age of six months. Indeed, the infant who was diagnosed with VDDR1A at the age of one month had a low serum 1,25-OH_2D and a positive *CYP27B1* sequencing result but did not have any clinical or radiological signs of rickets (21). This indicates that 1,25-OH_2D is not critical for mineral ion homeostasis and growth plate mineralization in the first months of life owing to *in utero* positive calcium balance in these patients. All patients in this study group were aged 6-months or older at admission.

Generally, a good response to treatment with alfacalcidol or calcitriol (10-400 ng/kg/day) is expected in cases with VDDR1A (4,21). Calcitriol dose was tailored based on biochemical and clinical findings. Edouard et al (21) indicated short and long-term outcomes of calcitriol treatment in their patients. They started calcitriol treatment at a dose of 1.0 μg/day, given in two doses of 0.5 μg. Treatment with calcitriol resulted in the normalization of biochemical parameters within three months. The aims of the treatment were to achieve normocalcemia, to maintain PTH levels within normal limits and to avoid hypercalciuria. Our patients had not reached their final height and their treatment durations ranged between six months and seven years. Improvement of biochemical parameters occurred somewhat later than previously reported at between four and 12 months.

Study Limitations

The main limitation of our study is the relatively small number of patients.

Conclusion

Although VDDR1A is a rare disease, it is more common in Turkey where autosomal recessive disorders are common. In this study, we evaluated the genetic and clinical features of 11 patients with the diagnosis of VDDR1A. Intron-1 mutation was the most common mutation, as in the previous studies, and all patients carrying this mutation were from the same city of origin, suggesting a "founder" or a "common ancestor" effect. As it may be confused with nutritional rickets and hypophosphatemic rickets, genetic analysis is important for making a correct diagnosis. VDDR1A should be considered when a patient with signs of rickets has a normal 25-OHD level or when there is unresponsiveness to vitamin D treatment. We should emphasize that concentrations of 1,25-OH_2D levels can be within normal ranges in patients with VDDR1A and this may lead to diagnostic confusion.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Ümraniye Training and Research Hospital Clinical Research Ethical Committee (approved number: 19/01/2018- 2926).

Informed Consent: Informed consent was obtained from patients and/or families.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Fatma Dursun, Bülent Hacıhamdioğlu, Concept: Fatma Dursun, Heves Kırmızıbekmez, Ece Keskin, Design: Fatma Dursun, Bülent Hacıhamdioğlu, Heves Kırmızıbekmez, Ece Keskin, Data Collection or Processing: Fatma Dursun, Bülent Hacıhamdioğlu, Heves Kırmızıbekmez, Ece Keskin, Gamze Özgürhan, Analysis or Interpretation: Fatma Dursun, Bülent Hacıhamdioğlu, Heves Kırmızıbekmez, Ece Keskin, Gamze Özgürhan, Literature Search: Fatma Dursun, Heves Kırmızıbekmez, Ece Keskin, Gamze Özgürhan, Bülent Hacıhamdioğlu, Writing: Fatma Dursun, Heves Kırmızıbekmez, Ece Keskin, Bülent Hacıhamdioğlu.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266-281.
2. Alzahrani AS, Zou M, Baitei EY, Alsshaikh OM, Al-Rijjal RA, Meyer BF, Shi Y. A novel G102E mutation of CYP27B1 in a large family with vitamin D-dependent rickets type 1. J Clin Endocrinol Metab 2010;95:4176-4183. Epub 2010 Jun 9
3. Miller WL, Portale AA. Vitamin D biosynthesis and vitamin D 1αlpha-hydroxylase deficiency. Endocr Dev 2003;6:156-174.
4. Demir K, Kattan WE, Zou M, Durmaz E, BinEssa H, Nalbantoğlu Ö, Al-Rijjal RA, Meyer B, Özkan B, Shi Y. Novel CYB27B1 Gene Mutations in Patients with Vitamin D-Dependent Rickets Type 1A. PloS One 2015;10:e0131376.
5. Malloy PJ, Feldman D. Genetic disorders and defects in vitamin D action. Rheum Dis Clin North Am 2012;38:93-106. Epub 2012 Apr 12
6. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russel DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci 2004;101:7711-7715. Epub 2004 May 5
7. Babiker AM, Al Gadi I, Al-Jurayyan NA, Al Nemri AM, Al Haboob AA, Al Boukai AA, Al Zahrani A, Habib HA. A novel pathogenic mutation of the CYP27B1 gene in a patient with vitamin D-dependent rickets type 1: a case report. BMC Res Notes 2014;7:783.
8. Acar S, Demir K, Shi Y. Genetic causes of rickets. J Clin Res Pediatr Endocrinol 2017;9(Suppl 2):88-105. Epub 2017 Dec 27
9. Tahir S, Demirbilek H, Ozbek MN, Baran RT Tanrıverdi S, Hussain K. Genotype and Phenotype Characteristics in 22 Patients with Vitamin D-Dependent Rickets Type I. Horm Res Paediatr 2016;85:309-317. Epub 2016 Mar 17.
10. Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA. Cloning of human 25-hydroxyvitamin D- 1 alpha-hydroxylase and mutations causing vitamin D-dependent rickets type I. Mol Endocrinol 1997;11:1961-1970.
11. St-arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH. The 25-hydroxyvitamin D1-αlpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res 1997;12:1552-1559.
12. Monkowa T, Yoshida T, Wakino S, Anazawa H, Deluca HF, Suda T, Hayashi M, Saruta T. Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D3 1alpha-hydroxylase. Biochem Biophys Res Commun 1997;239:527-533.
13. Kim CJ. Vitamin D dependent rickets type I. Korean J Pediatr 2011;54:51-54. Epub 2011 Feb 28
14. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, Abeysinghe S, Krawczak M, Cooper DN. Human Gene Mutation Database (HGMD): 2003 update. Hum Mut 2003;21:577-581.
15. Shaw NJ. Vitamin D deficiency rickets. Endocr Dev 2003;6:93-104.
16. Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. Mutation Taster evaluates disease-causing potential of sequence alterations. Nat Methods 2010;7:575-576.
17. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248-249.
18. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073-1081. Epub 2009 Jun 25
19. Durmaz E, Zou M, Al-Rijjal RA, Bircan I, Akcurin S, Meyer B, Shi Y. Clinical and genetic analysis of patients with vitamin D-dependent rickets type 1A. Clin Endocrinol (Oxf) 2012;77:363-369.
20. Kim JC, Kaplan LE, Perward F, Huang N, Sharma A, Choi Y, Miller WL, Portale AA. Vitamin D 1alpha-hydroxylase gene mutations in patients with 1alpha-hydroxylase deficiency. J Clin Endocrinol Metab 2007;92:3177-3182. Epub 2007 May 8
21. Edouard T, Alos N, Chabot G, Roughley P, Glorieux FH, Rauch F. Short- and long-term outcome of patients with pseudo-vitamin D deficiency rickets treated with calcitriol. J Clin Endocrinol Metab 2011;96:82-89. Epub 2010 Oct 6
22. Nishikawa M, Yasuda K, Takamatsu M, Abe K, Nakagawa K, Tsugawa N, Hirota Y, Tanaka K, Yamashita S, Ikushiro S, Suda T, Okano T, Sakaki T. Generation of 1,25-dihydroxyvitamin D3 in cyp27b1knockout mice by treatment with 25-hydroxyvitamin D3 rescued their rachitic phenotypes. J Steroid Biochem Mol Biol 2019;185:71-79. Epub 2018 Jul 18
23. Kovacs CS. Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies. Am J Clin Nutr 2008;88:520-528.
24. Karras SN, Wagner CL, Castracane VD. Understanding vitamin D metabolism in pregnancy: from physiology to pathophysiology and clinical outcomes. Metabolism 2018;86:112-123. Epub 2017 Oct 21

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0149

J Clin Res Pediatr Endocrinol 2019;11(1):41-45

Birth Size in Neonates with Congenital Adrenal Hyperplasia due to 21-hydroxylase Deficiency

Helmuth G. Dörr, Theresa Penger, Andrea Albrecht, Michaela Marx, Thomas M. K. Völkl

University Hospital of Erlangen, Department of Paediatrics, Division of Paediatrics Endocrinology, Erlangen, Germany

What is already known on this topic?

Prenatal weight gain and birth weight are partially influenced by androgen action. Some reports in the literature mention higher birth weight and length in congenital adrenal hyperplasia newborns. However data on this topic are inconsistent.

What this study adds?

We studied birth size in term newborns with classic congenital adrenal hyperplasia who were followed in our hospital. Our data support the assumption that prenatal hyperandrogenism has no effect on fetal growth.

Abstract

Objective: Classic congenital adrenal hyperplasia (CAH) secondary to 21-hydroxylase deficiency is characterized by increased prenatal adrenal androgen secretion. There are a small number of reports in the literature showing higher birth weight and length in CAH newborns.

Methods: We analyzed birth weight and length data of 116 German newborns (48 boys, 68 girls) with classic CAH who were born during the period from 1990 to 2017. All children have been followed or are currently treated as outpatients in our clinic. All children were born at term. The mothers were healthy and their pregnancies were uneventful. The diagnosis of CAH was confirmed by molecular analyses of the *CYP21A2* gene. Birth data were calculated as standard deviation (SD) scores according to German reference values.

Results: Weight and length in male CAH newborns (mean ± SD) (3601 ± 576 g; 52.4 ± 2.85 cm) were significantly higher than in female CAH newborns (3347 ± 442 g; 51.2 ± 2.55 cm), but male-female differences in the CAH cohort were lost when the data were converted into SD scores. The birth sizes of the CAH newborns did not differ from the reference group. The birth sizes also did not differ between the different CAH genotypes. Maternal age, mode of delivery and maternal parity had no influence on birth size.

Conclusion: Our data show that prenatal hyperandrogenism does not affect fetal growth.

Keywords: Term newborn, congenital adrenal hyperplasia, 21-hydroxylase deficiency, genotype

Introduction

Classical congenital adrenal hyperplasia (CAH) is the most common form of inherited disorders of cortisol biosynthesis in the adrenal cortex. It is due to mutations in the active gene *CYP21A2* causing varying degrees of impairment of 21-hydroxylase deficiency (21-OHD) activity. Classical CAH with 21-OHD occurs in two forms, namely, a three-times more frequent form with salt wasting (cortisol + aldosterone deficiency) and a simple virilizing form without aldosterone deficiency (1,2,3). Both forms are characterized by increased adrenal androgen secretion which prenatally causes virilization of the external female genitalia and postnatally, in both sexes if untreated, results in pseudoprecocious puberty, accelerated growth and bone maturation.

Since term-born male newborns are heavier than females, it has been speculated that prenatal weight gain and birth weight are also at least partially influenced by androgen action (4). In contrast, there are also data showing that birth size is not explained by the effects of prenatal androgen exposure (5).

Address for Correspondence: Helmuth G. Dörr MD, University Hospital of Erlangen, Department of Paediatrics, Division of Paediatrics Endocrinology, Erlangen, Germany
Phone: +49(0)91318533732 **E-mail:** helmuth-guenther.doerr@uk-erlangen.de
ORCID ID: orcid.org/0000-0002-4838-6737

Conflict of interest: None declared
Received: 06.06.2018
Accepted: 03.09.2018

Some reports in the literature show higher birth weight and length in CAH newborns. According to data from Finland, both girls and boys with classical CAH are significantly longer at birth than healthy newborns of the same ethnic origin (6). Italian authors confirmed these results and speculated that birth size of newborns with classical CAH correlates with the severity of the phenotype (7). In another study from the UK and Sweden, no differences between birth weight standard deviation (SD) scores (SDS) in CAH girls and boys compared with national references and no correlation to the severity of the gene mutation were found (5). Thus the literature contains inconsistent data on this topic.

The objective of our study was to analyse birth weight and length of children with classical CAH who were treated in the outpatient department of our hospital. The severity of the CAH phenotype was determined by molecular genetic classification of the common mutations (8,9,10,11). We attempted to adjust for most factors that might additionally affect birth weight and length.

Methods

Birth weight and length data of 116 German newborns (48 boys, 68 girls) with classical CAH who were born during the period from 1990 to 2017 were analyzed. All children have been followed or are currently treated as outpatients in our endocrinology clinic. All children were born at term (gestational age: 38 to 41 weeks) either by spontaneous vaginal delivery (n = 95) or by caesarean section (n = 21). The mothers (age: 20 to 42 years) were healthy primipara (n = 77) or multipara (n = 39), and the pregnancies were uneventful. Data on maternal body mass index at delivery were not available.

The diagnosis of CAH was confirmed by molecular analyses of the *CYP21A2* gene. Molecular genetic classification of the severity of CAH was performed according to Krone et al (8) as follows. The genotype 'Null' included patients with biallelic mutations that resulted in completely inactive enzymes (e.g. gene deletions), genotype A included patients with homozygous I2G or heterozygous I2G in trans with a null mutation, and B patients with homozygous p.I173N mutation or heterozygous p.I173N mutation in trans with a mutation from group 'Null' or group A. Genotype 'Null' was found in 43 children, genotype A in 51 children, and 22 children were identified with genotype B.

Birth weight (g) and length (cm) data were obtained from the patient records ("Vorsorgeheft"). As birth weight we used the weight measured at birth and for length we used the data obtained between three and seven days after birth. The length measurement at that age is part of the clinical examination of the newborn, usually before discharge from the hospital and the value obtained is more reliable than the length measured at birth. Birth data were calculated as SDS according to German reference values as follows (12): SDS = (patient's measured value-mean value for age- and sex-matched normal subjects) ÷ SD of the values for age- and sex-matched normal subjects. The German reference data used are based on the perinatal data of 2.3 million singleton newborns from 1995-2000 (12). We defined all neonates with a birth weight and length of < -2 SDS as small for gestational age (SGA) and with the same parameters, those with measurements of > 2 SDS as large for gestational age (LGA).

The study design (retrospective analysis of the data) was approved by the Ethical Committee of our Hospital without an approval number. Informed consent has been obtained from the parents after full explanation of the purpose and nature of all procedures used.

Statistical Analysis

Statistical analysis was performed using SPSS, Version 21 (IBM Inc., Chicago, Ill., USA). Data are expressed as mean ± SD and median. Kruskal Wallis test was used to compare values between different genetic groups. Student t-test for unpaired samples was used to compare weight and length values between status of maternal parity and mode of delivery.

Results

Birth size (weight and length) in term newborns with classic CAH are shown in Tables 1 and 2. We found no statistically

Table 1. Birth size in term male and female newborns with classical congenital adrenal hyperplasia. P values refer to comparison between the genders

	All (n = 116)	Male (n = 48)	Female (n = 68)
Birth weight (g)	3452 ± 515 (3410)	3601 ± 576** (3510)	3347 ± 442** (3355)
Birth weight (SDS)	-0.07 ± 1.12 (0.19)	0.06 ± 1.26 (-0.08)	-0.17 ± 1.02 (-0.24)
Birth length (cm)	51.7 ± 2.73 (52.0)	52.4 ± 2.85* (52.1)	51.2 ± 2.55* (50.9)
Birth length (SDS)	-0.07 ± 1.15 (-0.21)	0.05 ± 1.20 (-0.20)	-0.16 ± 1.12 (-0.24)

The data are expressed as mean ± SD (median); *p < 0.02; **p < 0.01, SDS: standard deviation scores

Table 2. Birth size in newborns with classical congenital adrenal hyperplasia according to genotype

	Genotype		
	Null (n = 43)	**A (n = 51)**	**B (n = 22)**
Birth weight (g)	3397 ± 491 (3410)	3474 ± 466 (3410)	3511 ± 665 (3420)
Birth weight (SDS)	-0.19 ± 1.04 (-0.22)	-0.01 ± 1.04 (-0.22)	-0.03 ± 1.44 (-0.22)
Birth length (cm)	51.4 ± 2.68 (51.2)	51.9 ± 2.45 (51.7)	51.2 ± 3.44 (51.2)
Birth length (SDS)	-0.19 ± 1.13 (-0.39)	0.04 ± 1.05 (-0.11)	-0.10 ± 1.42 (-0.40)

SDS: standard deviation scores, values are shown as mean ± SD (median)

significant difference between the birth size of the CAH newborns compared with the reference group. In terms of birth weight, three children of the CAH cohort were classified as SGA and six as LGA newborns. Mean birth size (weight in grams and length in centimeters) in male CAH newborns was statistically significantly higher than in females (weight: $p < 0.01$; length $p < 0.02$). However, when comparing the data after SDS conversion, neither weight-SDS nor length-SDS values were significantly different between the two sexes. Birth size was also not different between the different CAH genotypes. We analyzed the data also according to genotype and sex and found no difference. Moreover, maternal age, mode of delivery and maternal parity had no influence on birth size.

Discussion

Androgen action on birth size is implicated by the fact that healthy male newborns are longer and weigh more than female newborns (13). Children with classical CAG and 21-OHD (CAH) have increased adrenal androgen secretion that prenatally causes virilization of the external female genitalia. Additionally, there are some reports in the literature showing that prenatal hyperandrogenism also affects birth size in CAH newborns.

In 1971, a study from Canada compared the birth weights of CAH newborns with their unaffected siblings and normal newborns and found that only females with CAH were heavier than the female controls and female siblings (14). Jaaskelainen and Voutilainen (6) from Finland reported that both girls and boys with classic CAH are significantly longer at birth than healthy newborns of the same ethnic origin. The authors did not make any distinction between the different clinical forms of classic CAH.

In addition Italian authors reported that the mean birth length in both boys and girls with classical CAH was significantly greater than the mean birth length in healthy Italian children and speculated that the birth data correlated with the severity of the phenotype (7). In contrast data from the UK and Sweden showed no differences between birth weight SDS in CAH girls and boys in relation to the national references and no correlation to the severity of the gene mutation (5). In a study from Munich, mean birth "height" SDS data of 51 newborns with classical CAH, diagnosed by newborn screening, was found to be slightly above average (15). Chalmers et al (16) identified 105 CAH newborns over a long period of 50 years and found no difference in birth weight from the standard population median and also no sex difference in favour of heavier males. They speculated that these differences were ameliorated because of increased levels of prenatal androgens experienced by the female infants.

Furthermore, in a large retrospective observational cohort study from France, heavier male than female CAH newborns, but overall normal mean birth weight and birth length were reported (17). Our data confirm this sex-related difference. In our analysis, male CAH newborns had statistically significant higher birth weight (g) and length (cm) values than females. When the data were transformed to SDS values, the male newborns still had slightly higher values than the females, but the difference was not statistically significant. Birth sizes of our cohort were not different from the German reference population. The severity of CAH, maternal age, parity and mode of delivery had no influence on birth size. In terms of birth weight, three children in our cohort were classified as SGA and six as LGA newborns.

It is not surprising that the results in the literature are inconsistent. Most reports on birth size in CAH newborns do not provide data on the course of pregnancy, maternal age, maternal parity status, or mode of delivery. The somatic classification of neonates is primarily based on birth weight. Birth weight is affected by a multitude of different factors such as socio-economic status, maternal age and concomitant diseases of the mother in pregnancy, as well as placental, fetal and environmental conditions (18,19,20,21). Additionally, the correct interpretation of birth sizes is complicated by the methodological heterogeneity and limitations of birth size charts available worldwide. In addition secular trends in birth size over the last 25 year period might play a role. Consequently, there are numerous

factors that limit the interpretation of birth size in CAH newborns.

Study Limitations

There were some limitations to our study. The sample size is too small to exclude a type 2 statistical error. The study is retrospective. The data analyzed cover a period from 1990 to 2017. It was not possible to exclude all the different factors which might affect birth size of newborns with CAH.

Conclusions

In general data on birth size of newborns with CAH secondary to 21-OHD are scarce. We tried to clarify existing conflicting published data on this topic. Our data support the assumption that prenatal hyperandrogenism has no effect on fetal growth.

Acknowledgements

Some of the birth data used (newborns from our hospital born between 1969 and 2008) have been the subject of a doctoral thesis.

Ethics

Ethics Committee Approval: The study was approved by the Local Ethics Committee of the Department of Pediatrics of Erlangen.

Informed Consent: Consent has been obtained from the parents after full explanation of the purpose and nature of all procedures used.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Helmuth G. Dörr, Thomas M. K. Völkl, Design: Helmuth G. Dörr, Thomas M. K. Völkl, Data Collection or Processing: Theresa Penger, Andrea Albrecht, Michaela Marx, Analysis or Interpretation: Helmuth G. Dörr, Theresa Penger, Andrea Albrecht, Michaela Marx, Thomas M. K. Völkl, Literature Search: Helmuth G. Dörr, Michaela Marx, Writing: Helmuth G. Dörr.

Financial Disclosure: This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

1. El-Maouche D, Arlt W, Merke DP. Congenital adrenal hyperplasia. Lancet 2017;390:2194-2210. Epub 2017 May 30
2. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, Meyer-Bahlburg HF, Miller WL, Montori VM, Oberfield SE, Ritzen M, White PC; Endocrine Society. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2010;95:4133-4160.
3. Auchus RJ. The Classic and Nonclassic Congenital Adrenal Hyperplasias. Endocr Pract 2015;21:383-389. Epub 2014 Dec 22
4. de Zegher F, Francois I, Boehmer AL, Saggese G, Müller J, Hiort O, Sultan C, Clayton P, Brauner R, Cacciari E, Ibáñez L, Van Vliet G, Tiulpakov A, Saka N, Ritzén M, Sippell WG. Androgens and fetal growth. Horm Res 1998;50:243-244.
5. Miles HL, Gidlöf S, Nordenström A, Ong KK, Hughes IA. The role of androgens in fetal growth: observational study in two genetic models of disordered androgen signalling. Arch Dis Child Fetal Neonatal Ed 2010;95:435-438.
6. Jaaskelainen J, Voutilainen R. Growth of patients with 21-hydroxylase deficiency: an analysis of the factors influencing adult height. Pediatr Res 1997;41:30-33.
7. Balsamo A, Wasniewska M, Di Pasquale G, Salzano G, Baronio F, Bombaci S, De Luca F. Birth length and weight in congenital adrenal hyperplasia according to the different phenotypes. Eur J Pediatr 2006;165:380-383. Epub 2006 Mar 7
8. Krone N, Roscher AA, Schwarz HP, Braun A. Comprehensive analytical strategy for mutation screening in 21-hydroxylase deficiency. Clin Chem 1998;44:2075-2082.
9. Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, Lesser M, New MI, White PC. Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Clin Invest 1992;90:584-595.
10. Wedell A, Thilén A, Ritzén EM, Stengler B, Luthman H. Mutational spectrum of the steroid 21-hydroxylase gene in Sweden: implications for genetic diagnosis and association with disease manifestation. J Clin Endocrinol Metab 1994;78:1145-1152.
11. Krone N, Braun A, Roscher AA, Knorr D, Schwarz HP. Predicting phenotype in steroid 21-hydroxylase deficiency? Comprehensive genotyping in 155 unrelated, well defined patients from southern Germany. J Clin Endocrinol Metab 2000;85:1059-1065.
12. Voigt M, Fusch C, Olbertz D, Hartmann K, Rochow N, Renken C, Schneider KTM. Analyse des Neugeborenenkollektivs der Bundesrepublik Deutschland. 12. Mitteilung. Vorstellung engmaschiger Perzentilwerte (-kurven) für die Körpermaße Neugeborener. Geburtsh Frauenheilk 2006;66:956-970.
13. Fields DA, Krishnan S, Wisniewski AB. Sex differences in body composition early in life. Gend Med 2009;6:369-375.
14. Qazi QH, Thompson MW. Birthweight in congenital virilizing adrenal hyperplasia. Arch Dis Child 1971;46:350-352.
15. Bonfig W, Schmidt H, Schwarz HP. Growth patterns in the first three years of life in children with classical congenital adrenal hyperplasia diagnosed by newborn screening and treated with low doses of hydrocortisone. Horm Res Paediatr 2011;75:32-37. Epub 2010 Aug 12
16. Chalmers LJ, Doherty P, Migeon CJ, Copeland KC, Bright BC, Wisniewski AB. Normal sex differences in prenatal growth and abnormal prenatal growth retardation associated with 46,XY disorders of sex development are absent in newborns with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Biol Sex Differ 2011;2:5.
17. Bretones P, Riche B, Pichot E, David M, Roy P, Tardy V, Kassai B, Gaillard S, Bernoux D, Morel Y, Chatelain P, Nicolino M, Cornu C; French Collaborative CAH Growth Study Group. Growth curves for congenital adrenal hyperplasia from a national retrospective cohort. J Pediatr Endocrinol Metab 2016;29:1379-1388.
18. Voigt M, Jorch G, Briese V, Kwoll G, Borchardt U, Straube S. The combined effect of maternal body mass index and smoking status

on perinatal outcomes - an analysis of the german perinatal survey. Z Geburtshilfe Neonatol 2011;215:23-28. Epub 2011 Feb 23

19. Voigt M, Heineck G, Hesse V. The relationship between maternal characteristics, birth weight and pre-term delivery: evidence from Germany at the end of the 20th century. Econ Hum Biol 2004;2:265-280.
20. Spada E, Chiossi G, Coscia A, Monari F, Facchinetti F. Effect of maternal age, height, BMI and ethnicity on birth weight: an Italian multicenter study. J Perinat Med 2018;46:1016-1021.
21. Valero De Bernabé J, Soriano T, Albaladejo R, Juarranz M, Calle ME, Martínez D, Domínguez-Rojas V. Risk factors for low birth weight: a review. Eur J Obstet Gynecol Reprod Biol 2004;116:3-15.

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0046

J Clin Res Pediatr Endocrinol 2019;11(1):46-54

Evaluation of Renal Function in Obese Children and Adolescents Using Serum Cystatin C Levels, Estimated Glomerular Filtration Rate Formulae and Proteinuria: Which is most Useful?

Dilşah Önerli Salman[1], Zeynep Şıklar[2], Eda Nisa Çullas İlarslan[2], Z. Birsin Özçakar[3], Pınar Kocaay[2], Merih Berberoğlu[2]

[1]Ankara University Faculty of Medicine, Department of Pediatrics, Ankara, Turkey
[2]Ankara University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey
[3]Ankara University Faculty of Medicine, Department of Pediatric Nephrology, Ankara, Turkey

What is already known on this topic?

The effects of obesity and metabolic syndrome on kidney function in the child and adolescent age groups have not been adequately examined. There is insufficient data concerning the degree of impairment of renal function and its clinical significance. There is also no consensus on the parameters that assess renal function most reliably.

What this study adds?

Cystatin C could be used as a biomarker which detects impaired renal function at an earlier stage than creatinine (Cr) in obese children, especially those with metabolic syndrome (MetS). Cr based formulae detect hyperfiltration as the first change in renal function. Decreasing estimated glomerular filtration rate (GFR), seen with cystatin C-based formulae, in MetS patients, may represent the early stages of renal damage. Using fat free mass or body cell mass for estimated GFR formulae in obese children appears to provide no additional information.

Abstract

Objective: There is a growing interest in the relationship between obesity and renal damage. The effect of obesity on renal function in children and adolescents has not been adequately investigated. In addition, there is no complete consensus on the reliability of various renal function parameters. The primary goal of this study was to evaluate renal function in obese children and adolescents using glomerular filtration rate (GFR), cystatin C, and creatinine (Cr)-derived formulas. We also compared classical GFR measurement methods with methods based on bioimpedance analysis-derived body cell mass (BCM).

Methods: We enrolled 108 obese and 46 healthy subjects aged 6-18 years. Serum cystatin C, serum Cr, 24-hour proteinuria, Cr clearance, and GFR were evaluated in both groups. Estimated GFR was measured with Cr-based, cystatin C-based, combined (cystatin C and Cr) and BCM-based formulae. Both actual and fat-free mass body surface areas (BSA) were used when required. Metabolic parameters (blood glucose, insulin, and lipids) were analyzed in the obese subjects. International Diabetes Federation criteria were used to identify metabolic syndrome (MetS).

Results: We did not detect statistically significant differences between the obese and control groups for mean Cr ($p = 0.658$) and mean cystatin C ($p = 0.126$). Mean cystatin C levels of MetS patients were significantly higher than those of non-MetS obese participants ($p < 0.001$). Cr-based GFR measurements, BCM-based measurements and a combined Cr and cystatin C measurement showed a statistically significant increase in the GFR of obese subjects compared to controls ($p = 0.002$ and $p < 0.001$). This increase was negatively correlated with duration of obesity. Estimations based on actual or fat-free mass BSA did not differ either. Only the Filler equation showed a statistically significant decrease in eGFR in MetS patients. There were no statistically significant differences between the obese and control groups for proteinuria ($p = 0.994$) and fat-free mass proteinuria ($p = 0.476$).

Conclusion: We conclude that cystatin C could be used as an earlier biomarker than Cr in the detection of impaired renal function in obese children, especially those with MetS. Cr-based formulae reveal hyperfiltration as the first change in renal function. Decreasing eGFR seen in MetS patients with cystatin C-based formulae, but not Cr-based formulae, may represent the early stages of renal damage. Using fat-free mass or BCM for eGFR formulae in obese children seems to provide no additional information.

Keywords: Obesity, glomerular filtration rate, body cell mass, cystatin C

Address for Correspondence: Dilşah Önerli Salman MD, Ankara University Faculty of Medicine, Department of Pediatrics, Ankara, Turkey
Phone: +90 532 344 94 42 **E-mail:** dr_dilsah@hotmail.com **ORCID ID:** orcid.org/0000-0002-0267-4696

Conflict of interest: None declared
Received: 29.05.2018
Accepted: 17.08.2018

Introduction

Obesity is a serious health problem that adversely affects whole-body systems, particularly the cardiovascular and endocrine systems (1). Among the adverse effects of obesity, kidney problems have recently begun to attract more attention. Increased obesity-related glomerulopathy has become apparent in the last 20 years as the role of obesity in the onset and progression of adult kidney disease has been better understood (2). In the last 30 years, with increased obesity prevalence, a significant increased prevalence of chronic kidney disease (CKD) and end-stage renal failure has been observed (2). Vivante et al (3) found that overweight and obesity were serious risk factors for end-stage renal failure in their 30-year survey of 1.2 million adolescents. Obesity was also found to be associated with negative effects on the allograft and reduced allograft survival in patients undergoing renal transplantation (4).

Despite this growing interest the effects of obesity and metabolic syndrome (MetS) in children and adolescents on renal function have not been sufficiently investigated. In addition, there is no consensus on the reliability of renal function parameters and which of these best represents "true" renal function (4). Glomerular filtration rate (GFR) is one of the most important parameters used to determine renal function and can be calculated with different formulae. GFR is generally calculated using body surface area (BSA)-based formulae. However, these calculations may give incorrect results, particularly for obese children, due to a higher BSA than in normal-weight children. Cystatin C is a biomarker recommended for use in GFR calculations because it is easily glomerular-filtered, has a low molecular weight and is not dependent on muscular mass (5,6). Studies have indicated that cystatin C-derived formulae provide more accurate results than conventional GFR calculation methods (6). However, both conventional GFR formulae and cystatin C-derived formulae may be affected by the amount of adipose tissue, so that calculation of GFR based on non-adipose tissue is considered a more accurate method (7). Proteinuria, one of the best predictors of renal damage, is another parameter that should be considered in renal function evaluation (8).

The primary goal of the present study was to extensively evaluate renal function in obese children and adolescents, using serum cystatin C concentration, cystatin C- and creatinine (Cr)-based eGFR and measures of proteinuria. We also investigated the relationship between these parameters and MetS components and obesity duration. Furthermore, we compared classical GFR measurement methods with those based on bioimpedance analysis-derived body cell mass (BCM) and fat-free mass BSA.

Methods

Study Design

This prospective, observational study was conducted between January 2014 and January 2015 at the Pediatric Endocrinology Outpatient Clinic of Ankara University Faculty of Medicine. All participants or parents gave informed consent prior to participation. Institutional Ethics Committee Approval was obtained (Ankara University Ethics Committee, decision dated: 23 September 2013, no: 14-540-15, for the study entitled "Control of renal function in obese children and adolescents and relation with MetS components"). Project support was obtained from the Association of Pediatric Endocrinology and Diabetes.

Patient Enrollment

We enrolled consecutive patients aged 6-18 years with a body mass index (BMI) $>95^{th}$ percentile. We excluded patients with comorbidities (diabetes mellitus, congenital heart disease and chronic systemic disorders) and those who were receiving systemic drugs at the time of presentation. Normal-weight (BMI $<85^{th}$ percentile) healthy subjects constituted the control group.

Measurements and Outcomes

The demographic data (age, gender and duration of obesity) and physiological measurements [weight, height, height standard deviation score (SDS), BMI, blood pressure, and pubertal stage] of the participants were recorded. Laboratory evaluations were performed, including fasting plasma glucose, blood Cr, blood total cholesterol, blood low-density lipoprotein cholesterol (LDL-C), blood high-density lipoprotein cholesterol (HDL-C), blood triglycerides, and 24-hour urine protein and urine Cr levels by using automated Roche® Moduler (Germany). Fasting plasma insulin measured by radioimmunoassay. Cystatin-C was measured by nephelometric immunoassayby using BNII® Nephelometer (Siemens, Germany). The homeostatic model assessment-insulin resistance (HOMA-IR) of each patient was calculated. HOMA-IR levels of >2.22 in prepubertal girls, >2.67 in prepubertal boys, >3.82 in pubertal girls and >5.22 in pubertal boys were accepted as demonstrating insulin resistance (9). The body-fat mass of each participant was measured with a bioimpedance analyzer (Tanita® BC 418) to compare GFR and cystatin C levels with BCM and Cr clearance (CrCl). Both BSA (for CrCl) and fat-free mass BSA (adopted using total fat-free mass in GFR formulae as body weight) were analyzed. BCM was calculated as intracellular fluid divided by 0.70 (7). We identified MetS patients, 10-18 years old, based on the International Diabetes Federation (IDF) MetS criteria

(10). The IDF criteria define MetS as central obesity (waist circumference > 90th percentile) combined with any two of the following: dyslipidemia (triglycerides > 150 mg/dL), reduced HDL-C (< 40 mg/dL), increased blood pressure (systolic > 130 mmHg or diastolic > 85 mmHg), increased fasting plasma glucose (> 100 mg/dL), or previously diagnosed type 2 diabetes.

For the GFR measurements, we used four groups of formulae: Cr-based, cystatin C-based, combined (Cr- and cystatin C-based), and BCM-based (Table 1) (11,12,13,14,15,16,17,18). We used only Cr-based formulas for GFR measurements with fat-free cell mass.

For evaluation of proteinuria, 24-hour urine samples were collected. Protein excretion of 100 mg/m^2/day indicated a nephritic status, while > 1 g/m^2/day indicated a nephrotic status (19). Fat-free mass adjusted proteinuria was also calculated.

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS version 20.0 for Windows; SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± SD or median (minimum-maximum), and nominal variables were expressed as numbers (%) in the descriptive analyses. Percentage comparisons of groups were performed using the chi-square test and multivariate logistic regression analyses were performed for statistically significant variables. Sperman's rho correlation was used. Normality of data was tested using the Kolmogorov-Smirnov test. Normally distributed variables were compared using the t test, and non-normally distributed variables were compared using the Mann-Whitney U test. For all statistical analyses, $p < 0.05$ was considered significant.

Results

Clinical Characteristics of Participants

A total of 154 children and adolescents were enrolled in the study. Of these, 108 consituted the obese group and 46 made up the control group. The age and gender distributions of the two groups were similar although there was a higher proportion of subjects in puberty compared with the control group (see Table 2). Unsurprisingly weight ($p < 0.001$), height ($p < 0.001$), BMI ($p < 0.001$), BMI SDS ($p < 0.001$), height SDS ($p < 0.001$) and waist circumference ($p < 0.001$) were greater in the obese group than in the control group (Table 2).

Table 1. Formulae used for glomerular filtration rate calculations (7,11,12,13,14,15,16,17,18)

Creatinine-based formulas
Creatinine clearance = [urine creatinine (mg/dL) x urine volume (mL) x 1.73] / [serum creatinine (mg/dL) x 1440 x m² (BSA)] (mL/minute/1.73 m²) (11)
BSA = 0.007184 x height (cm)$^{0.7152}$ x weight (kg)$^{0.425}$ (12)
Fat-free mass creatinine clearance: [urine creatinine (mg/dL) x urine volume (mL) x 1.73] / [serum creatinine (mg/dL) x 1440 x m² (fat-free mass BSA)] (mL / minute / 1.73 m²) (11)
BSA = 0.007184 x height (cm)$^{0.715}$ x fat-free mass (kg)$^{0.425}$ (12)
Bedside Schwartz et al (13): 0.413 x height (cm) / serum creatinine (mg/dL)
Cystatin C-based formulas
Filler and Lepage (14) formula: 91.62 x (1/cystatin C)^{1,123}
Cystatin C = mg/L
Zappitelli et al (15) formula: 75.94 / cystatin C$^{1.17}$ (renal transplant patients x 1.2)
Cystatin C = mg/L
Creatinine- and cystatin c-based formulas
Bouvet et al's (16) formula: 38.4 x (serum creatinine)$^{-0.35}$ x (cystatin C)$^{-0.56}$ x [weight (kg)]$^{0.30}$ x (age)$^{0.40}$ mL/minute
Serum creatinine: mg/dL; cystatin C: mg/L
Donadio et al's (17) formula: 0.426 x [weight (kg) / cystatin C]$^{0.39}$ x [height (cm) x BSA / serum creatinine]$^{0.64}$
Serum creatinine: mg/dL; cystatin C: mg/L
Body cell mass formulas
Andersen's (7) formula: 10.2 x (BCM / cystatin C)$^{0.40}$ x (height x BSA/serum creatinine)$^{0.65}$
GFR: mL/minute; serum creatinine: mmol/L; serum creatinine: mg/dL x 88.4
BCM (kg) = intracellular fluid / 0.7
Donadio et al's (18) formula: (BCM x 2.231 / serum creatinine) - 2.73
BCM (kg) = intracellular fluid / 0.7

BSA: body surface area, BCM: body cell mass, GFR: glomerular filtration rate

Laboratory analysis of all cases are given in Table 3. Based on the IDF criteria, MetS was identified in 14.8% of the obese participants and in none of the control participants.

Creatinine and Cystatin C Results

Serum Cr and cystatin C levels were compared to evaluate renal function. There were no statistically significant differences in mean levels of Cr and cystatin C between the all obese ($p = 0.658$) and control groups ($p = 0.126$) (Table 4). The mean concentration of cystatin C in the obese children with MetS was significantly higher than in the controls and the non-MetS obese participants ($p < 0.01$).

We performed Spearman's correlation and a regression analysis to evaluate the factors affecting cystatin C and Cr levels. There was a positive correlation between cystatin C

Table 2. Clinical characteristics of all subjects and controls

	Obese group (n = 108)	Control group (n = 46)	p
Male n (%) Female n (%)	47 (43.5) 61 (56.5)	21 (45.7) 25 (54.3)	0.860
Pubertal/prepubertal (N)	88/20	25/21	**0.002**
Age (years) mean ± SD (range)	**13.2 ± 2.7** (6.1-18)	**12.9 ± 3.6** (7.5-17.6)	0.209
Height (cm) mean ± SD (range)	**156.6 ± 12.6** (116.7-187.3)	**144.3 ± 17.1** (117.5-176)	**< 0.001**
Height SDS mean ± SD (range)	**0.48 ± 0.98** (-1.76-3.18)	**-0.38 ± 0.92** (-2.25-1.53)	**< 0.001**
Body weight (kg) mean ± SD (range)	**71.1 ± 19.3** (26.5-124.6)	**38.7 ± 14.4** (19-63)	**< 0.001**
BMI (kg/m²) mean ± SD (range)	**28.3 ± 4.5** (19.4-42.3)	**17.8 ± 3.3** (12.4-24.8)	**< 0.001**
RBMI Mean ± SD (range)	**142.9 ± 18.3** (115.5-217.8)	**92.8 ± 11.4** (68.6-119)	**< 0.001**
BMI SDS Mean ± SD (range)	**2.2 ± 0.63** (1.1-3.8)	**-0.57 ± 1.11** (-3.48-1.18)	**< 0.001**
Waist circumference (cm) mean ± SD (range)	**87.7 ± 10.7** (65-120)	**58.9 ± 8** (48-82)	**< 0.001**

SD: standard deviation, SDS: standard deviation score, BMI: body mass index, RBMI: relative body mass index

Table 3. Laboratory characteristics of all cases

	Obese cases (n = 108) Mean ± SD (range)	Controls (n = 46) Mean ± SD (range)	p
Fasting blood glucose (mg/dL)	84.8 ± 8.0 (57.0-102.0)	78.3 ± 8.1 (50.0-94.0)	< 0.001
Fasting insulin (mIU/mL)	17.4 ± 7.1 (3.0-41.9)	7.7 ± 3.6 (1.5-17.1)	< 0.001
Total cholesterol (mg/dL)	167.3 ± 36.7 (85.0-277)	154.2 ± 25.6 (117-211)	0.032
LDL-cholesterol (mg/dL)	102.1 ± 30.9 (45.0-208.0)	87.8 ± 24.5 (45.0-145.0)	0.007
HDL-cholesterol (mg/dL)	44.7 ± 10.3 (26.0-76.0)	49.2 ± 10.1 (30.0-75.0)	0.007
VLDL-cholesterol (mg/dL)	21 ± 11.4 (5.0-52.0)	17.1 ± 7.2 (3.0-35.0)	0.140
Triglyceride (mg/dL)	104 ± 56.6 (26.0-262.0)	85.3 ± 36.2 (17.0-177.0)	0.156

SD: standard deviation, LDL: low-density lipoprotein, HDL: high-density lipoprotein, VLDL: very-low-density lipoprotein

and total cholesterol (r = 0.275, p = 0.001), LDL-C (r = 0.277, p < 0.001), triglycerides (r = 0.318, p < 0.001) and fasting insulin (r = 0.255, p = 0.001). There was an inverse correlation with HDL-C (r = – 0.219, p = 0.006). There was no significant correlation between Cr and total cholesterol (r = –0.085, p = 0.296), LDL-C (r = –0.098, p = 0.225), HDL-C (r = 0.091, p = 0.260), triglycerides (r = 0.11, p = 0.889), fasting plasma glucose (r = 0.016, p = 0.840), and fasting insulin (r = 0.133, p = 0.101).

Renal Function Evaluation Based on Glomerular Filtration Rate Formulae

GFR results in the obese patients were calculated with CrCl, fat-free mass CrCl, Bedside Schwartz et al (13), Andersen (7), Donadio et al (18), and Donadio et al (17) formulae. The results were significantly higher in the obese group than in the control group. In the obese group without MetS, the GFR results calculated with the CrCl and Bedside Schwartz formulas were significantly higher than those in the control group (p < 0.05). The GFR values calculated with the cystatin C-derived Filler formula and the cystatin C and serum Cr-derived Bouvet formula were lower in the MetS-diagnosed obese patients than in the non-MetS obese patients and the controls (p < 0.05). In both the MetS obese group and the non-MetS obese group, GFR levels calculated with fat-free mass CrCl, and the formulae of Andersen (7), Donadio et al's (18), and Donadio et al's (17) formula were higher than those of the control group (see Table 5). Correlation of the duration obesity and the changes of GFR were analysed. As the duration of obesity increased, GFR calculated with both the Donadio et al's (17,18) formulae were increased, but GFRs calculated with Filler (p = 0.008), Bouvet (p = 0.020), and Bedside Schwartz (p = 0.038) showed a decrease.

Renal Function Evaluation Based on Proteinuria

There were no statistically significant differences between the obese and control groups for proteinuria (p = 0.994) and fat-free mass proteinuria (p = 0.476) (Table 6). There were also no statistically significant differences between the MetS obese, non-MetS obese and control groups with regard to proteinuria and fat-free mass proteinuria results. Nephritic-range proteinuria was detected in 12 non-MetS obese participants (11.1%) and in six control-group participants (12%). Nephrotic-range proteinuria was not detected in any of the participants.

Discussion

Obesity has a deleterious effect on renal function, so the capability to determine exact renal function is more important in obese patients than in those of normal weight. One of the most useful parameters of renal function is eGFR. Accurate calculation of GFR has a vital role in the accurate identification of kidney disease, drug-dose calculations, CKD management and prognosis (20). There are several models for GFR measurements, but none is accepted as the gold standard for GFR calculation (7). There is a potential risk that Cr-based formulae may yield GFR results that are even lower in obese patients than in normal-weight individuals. Since CrCl is subject to variability due to a number of causes including acute and chronic disease, it is reported that this method is not very sensitive for GFR (21). However, up to 80% of clinical laboratories use CrCl as the main method for determining GFR (20). It is accepted that serum cystatin C gives more accurate GFR results because it is less affected by muscle mass and diet than Cr based methods (22). Roos et al (23) compared 24 cystatin C and Cr studies involving a total of 2,007 participants. They found that at a 95% confidence interval and according to the Moses-Littenberg linear regression model, cystatin C was more interoceptive for indicating renal dysfunction compared to Cr [cystatin C: 3.99 (3.41-4.57) versus Cr: 2.79 (2.12-3.4)] (23).

There is an increasing number of studies investigating eGFR and Cistatin-C measurements in children. Miliku et al (24) compared the relationship between body composition and eGFR calculated from both Cr and cystatin-C concentrations. They found that, eGFR was influenced by BMI and BSA. Moreover, eGFR calculated on the basis of Cr concentrations,

Table 4. Serum creatinine and cystatin C levels

	All obese patients (n = 108) Mean ± SD (range)	Non-MetS obese patients (n = 92) Mean ± SD (range)	MetS obese patients (n = 16) Mean ± SD (range)	Controls (n = 46) Mean ± SD (range)	p1	p2
Creatinine (mg/dL)	0.5 ± 0.11 (0.28-0.88)	0.5 ± 0.11 (0.28-0.88)	0.54 ± 0.15 (0.35-0.79)	0.52 ± 0.15 (0.23-0.91)	0.658	0.649
Cystatin C (mg/L)	0.69 ± 0.12 (0.35-1.08)	0.67 ± 0.11 (0.35-0.93)	0.8 ± 0.12 (0.66-1.08)	0.66 ± 0.1 (0.5-0.93)	p = 0.126	< 0.001

SD: standard deviation, MetS: metabolic syndrome.

p1: All obese vs control group, p2: Non MetS obese patients vs MetS obese patients

was also influenced by lean mass percentage and fat mass percentage of the patients. This study was limited to six year-old healthy children. In another study, Correia-Costa et al (25) evaluated 163 normal and 150 overweight/obese children, between eight and nine years of age. They compared eGFR, CrCl, Cr and cystatin-C levels of the patients. Results showed that, overweight/obese children had lower eGFR values using several formulae except when using CrCl and the Schwartz formula.

In the present study, kidney function of obese participants was assessed with Cr-based, cystatin C-based, combined Cr and cystatin C and BCM-based GFR formulae and with proteinuria levels. We calculated the BCM and fat-free mass of obese participants from BSA-based GFR measurement techniques based on the hypothesis that the increased BSA of these participants may lead to inaccurate results. In a new model, Andersen (7) found that both the BCM and the weight models are reliable methods for

Table 5. Comparison of glomerular filtration rate measurement methods in obese subjects with and without metabolic syndrome and the control group

	GFR measurement method	Controls (n = 46)	All obese patients (n = 108)	Obese without MetS (n = 92) Mean ± SD	Obese with MetS (n = 16) Mean ± SD	p1	p2
Creatinine-based formulas	Creatinine clearance (mL/min/1.73 m²) (11)	125.3 ± 38.1 (74-212)	171.4 ± 82.5 (51-473)	173.2 ± 83.4* (65-473)	161.3 ± 78.9* (51-330)	**< 0.001**	**0.001**
	Fat-free mass creatinine clearance (mL/min/1.73 m²) (11)	147 ± 45.8 (88-250)	215.5 ± 102.3 (65-578)	218.2 ± 104* (81-578)	200 ± 93.4* (65-410)	**< 0.001**	**< 0.001**
	Bedside Schwartz et al (13)	118.2 ± 26.9 (77-194)	131.5 ± 25.9 (76-225)	132 ± 25.1 (76-225)	129.1 ± 31.2 (87-180.5)	**0.004**	**0.012**
Cystatin C-based formulas	Filler and Lepage (14)	143.7 ± 20.4 (101.5-186)	139 ± 26.1 (87.9-264.8)	141.5 ± 29.2 (101.5-264.8)	118.5 ± 15.9 (87.9-141.9)	0.116	**< 0.001**
	Zappitelli et al (15)	121.2 ± 21.5 (82.6-166.9)	123.2 ± 27.5 (69.4-259.3)	123.9 ± 29 (69.4-259.3)	119.2 ± 16.8 (94.4-1149.6)	0.862	0.894
Creatinine and cystatin C combined formulas	Bouvet et al (16)	124 ± 17 (88.1-159)	121.7 ± 21.6 (79-224.8)	124.4 ± 21.3 (90.7-224.8)	106 ± 16 (79-141.3)	0.223	**0.002**
	Donadio et al (17)	89 ± 29.1 (46.5-161)	147 ± 34.8 (70.3-256.8)	148.3 ± 36.4 (70.3-256.8)	145.6 ± 24.4 (82.1-172.7)	**< 0.001**	**< 0.001**
BCM-based formulas	Andersen (7)	119 ± 35.7 (61-191)	183.3 ± 43.1 (78.2-323.8)	183.6 ± 44.9 (78.2-323.8)	181.5 ± 31.2 (103.4-212)	**< 0.001**	**< 0.001**
	Donadio et al (18)	156 ± 51.6 (79.1-278)	242.1 ± 77.8 (88.5-557.1)	237.7 ± 77.5 (88.5-557.1)	267.2 ± 77.6 (120.4-391)	**< 0.001**	**< 0.001**

SD: standard deviation, MetS: metabolic syndrome, BSA: body surface area, BCM: body cell mass, GFR: glomerular filtration rate.

*Findings: mean ± standard deviation (range). p1 indicates statistical comparison between obese group and control group, p2 indicates statistical comparison between MetS obese and non-MetS obese groups.

*BCM and GFR estimations calculated using fat-free mass BSA were similar to actual BSA-based GFR estimations.

Table 6. Proteinuria in obese cases with or without metabolic syndrome

	All obese cases n (%)	MetS obese n (%)	Non-MetS obese n (%)	Control group n (%)
Proteinuria at nephritic level (4-40 mg/m²/h)	12 (11)	0 (0)	12 (13)	6 (12)
Proteinuria at nephrotic level (> 40 mg/m²/h)	0 (0)	0 (0)	0 (0)	0 (0)

p value: 0.886.

MetS: metabolic syndrome

estimating GFR in children, with a higher accuracy than the currently recommended Schwartz model. To the best of our knowledge, our study is the first to use the Andersen method. We did not find any differences between using the BCM model and CrCl methods. However, we obtained similar results using fat-free cell mass for GFR calculations with Cr-based formulae. We obtained higher GFR values in the obese group compared to the control group using calculations with combined Cr and cystatin C (Donadio et al. (17)) and all BCM or Cr-based formulae. We consider that the increased GFR with Cr-based formulae found in this study support the hyperfiltration and renal-function effects in obese participants. However, there was no difference between GFR rates using the cystatin C-based formula of Filler and Lepage (14) or Zappitelli et al (15) We believe that this is due to similar cystatin C levels between the obese and control groups.

We detected higher cystatin C levels in the MetS obese group compared to the non-MetS obese group, as an indicator of renal damage. Cystatin C is recommended as an interoceptive biomarker indicating kidney function when Cr levels are not yet affected, such as during the early stages of kidney damage and with mildly decreased GFR (7). Cystatin C is less affected by muscle mass and diet compared to Cr and so should be used instead of Cr for more accurate GFR measurements (22). Research by Marwyne et al (26) showed that cystatin C gave more accurate results compared to Cr in abnormal GFR measurements when compared to 99mTc-diethylenetriamine pentaacetic acid ($r = 0.526$, $p = 0.001$).

Some pediatric researchers have made comparisons between cystatin C and Cr when predicting renal damage. In five of 12 studies done using receiver operating characteristic analyses, it was confirmed that cystatin C was significantly more sensitive than Cr, but another five studies did not find any statistically significant difference between the biomarkers. In the remaining two studies, statistical comparisons were not performed. One study reported cystatin C to be significantly better than Cr while Cr was not superior to cystatin C in any of these 12 studies (7). Our results showed that elevated serum cystatin C is an earlier biomarker than elevated serum Cr in the detection of impaired renal function in obese children. Furthermore, in cystatin C-based formulae, a steady decline in GFR parallel to the duration of obesity may be noted, which may be an indication that functional damage was superceeded by structural damage over time. Based on these results, we conclude that Cr-based formulae may not reflect real renal function, because of a tendency to give inaccurate higher GFRs, particularly during the early stages of renal damage in obese patients.

With regard to GFR estimations using Cr- or cystatin C-based formulae, the question of whether decreased GFR in obese children may be overlooked with increased cystatin C concentrations, when using these formulae, arises. We believe that since cystatin C concentration increases with renal function impairment, it can be useful when GFR begins to decrease.

Dyslipidemia is a metabolic parameter that indicates increased risk of renal failure. As a result of reabsorption of fatty acids and cholesterol from tubular epithelial cells, tubulointerstitial inflammation may stimulate foam cell formation and tissue damage. At the same time, dyslipidemia may damage mesangial cells and glomerular capillary endothelial cells, such as podocytes. Both hypercholesterolemia and hypertriglyceridemia may lead to podocyte damage. Accumulation of lipoproteins in the glomerular mesangium may stimulate matrix production and glomerulosclerosis (8). This hypothesis led to the idea of investigating the effect of dyslipidemia in the etiology of CKD. In a study by Servais et al (27) on 925 dyslipidemic patients, cystatin C values were significantly higher in patients with MetS than in patients without (0.86 ± 0.23 vs 0.79 ± 0.20 mg/L, p 0.0001) and were correlated with dyslipidemia ($p < 0.001$). In our study, in accordance with the literature, cystatin C values were found to be significantly higher in patients with MetS; the Spearman analysis showed positive correlation between cystatin C and total cholesterol, triglycerides and LDL-C, but a negative correlation with HDL-C. This result suggests that cystatin C is more accurate than Cr as a biomarker for detecting the negative effects of dyslipidemia on renal function in obese children.

When we assessed our results in terms of proteinuria, we found no statistically significant difference between the obese and control groups. In addition, we found no statistically significant difference between the control and obese groups with and without MetS. Proteinuria and microalbuminuria are accepted as indicators, as well as risk factors, for chronic renal failure (28). BMI is the second most common factor after proteinuria in increased risk of end-stage renal failure. Obesity-related renal disease involves a wide spectrum of disorders, from excretion of urinary albumin to proteinuria and/or decreased GFR. The adverse effects of fat accumulation on kidney hemodynamics and obesity-related glomerulopathy are two important possible mechanisms for this. Hemodynamic changes cause inflammation, oxidative stress, apoptosis and finally, the development of renal scarring (29). The absence of significant differences between our study groups in terms of proteinuria indicates that no apparent structural renal damage had begun in our participants

during the study period. Studies examining the relationship between renal protein loss and MetS have reported that increased albuminuria and proteinuria or the presence of microalbuminuria are risk factors for MetS (8). However, the accepted conclusion in the current literature is that protein loss does not increase the risk of MetS, unlike chronic renal failure development (30). We believe that proteinuria is not useful for indicating renal function impairment in obese pediatric and adolescent patients.

Study Limitations

Measurement of inulin clearence, which is a valuable tool for GFR estimation, was not performed in our study groups.

Conclusion

Serum cystatin C can be used as an earlier biomarker than Cr-based GFR estimations in the detection of impaired renal function in obese children, especially those with MetS. In a comparison of GFR measurement formulae, we found that Cr-based formulae may give normal or higher GFR results, particularly during the early stages of renal dysfunction in obese children. In addition cystatin C may be a more sensitive biomarker, when compared to Cr-based GFR estimations, for detecting dyslipidemia-mediated renal impairment in obese children. Proteinuria is not an appropriate early biomarker for indicating damaged renal function. It also appears that there is no need to use fat-free mass or BCM for determining eGFR in obese children.

Ethics

Ethics Committee Approval: Institutional Ethics Committee Approval was obtained (Ankara University Ethics Committee, decision dated: 23 September 2013, no: 14-540-15, for the study entitled "Control of renal function in obese children and adolescents and relation with MetS components").

Informed Consent: All participants or parents gave informed consent prior to participation.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Dilşah Önerli Salman, Concept: Zeynep Şıklar, Design: Zeynep Şıklar, Merih Berberoğlu, Data Collection or Processing: Dilşah Önerli Salman, Eda Nisa Çullas İlarslan, Z. Birsin Özçakar, Pınar Kocaay, Analysis or Interpretation: Zeynep Şıklar, Merih Berberoğlu, Dilşah Önerli Salman, Z. Birsin Özçakar, Literature Search: Zeynep Şıklar, Dilşah Önerli Salman, Writing: Dilşah Önerli Salman, Zeynep Şıklar.

Financial Disclosure: The authors declared that this study received from the Association of Pediatric Endocrinology and Diabetes financial support.

References

1. No authors listed. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894. https://www.ncbi.nlm.nih.gov/pubmed/11234459
2. Gunta SS, Mak RH. Is obesity a risk factor for chronic kidney disease in children? Pediatr Nephrol 2013;28:1949-1956. Epub 2012 Nov 14
3. Vivante A, Golan E, Tzur D, Leiba A, Tirosh A, Skorecki K, Calderon-Margalit R. Body mass index in 1.2 million adolescents and risk for end-stage renal disease. Arch Intern Med 2012;172:1644-1650.
4. Espinoza R, Gracida C, Cancino J, Ibarra A. Effect of Obese Living Donors on the Outcome and Metabolic Features in Recipients of Kidney Transplantation. Transplant Proc 2006;38:888-889.
5. Samyn M, Cheeseman P, Bevis L, Taylor R, Samaroo B, Buxton-Thomas M, Heaton N, Rela M, Mieli-Vergani G, Dhawan A. Cystatin C, an easy and reliable marker for assessment of renal dysfunction in children with liver disease and after liver transplantation. Liver Transpl 2005;11:344-349.
6. Simonsen O, Grubb A, Thysell H. The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate. Scand J Clin Lab Invest 1985;45:97-101.
7. Andersen TB. Estimating renal function in children: a new GFR-model based on serum cystatin C and body cell mass. Dan Med J 2012;59:4486.
8. Wickman C, Kramer H. Obesity and Kidney Disease: Potential Mechanisms. Semin Nephrol 2013;33:14-22.
9. Kurtoğlu S, Hatipoğlu N, Mazıcıoğlu M, Kendirici M, Keskin M, Kondolot M. Insulin resistance in obese children and adolescents: HOMA-IR cut-off levels in the prepubertal and pubertal periods. J Clin Res Pediatr Endocrinol 2010;2:100-106. Epub 2010 Aug 2
10. International Diabetes Federation. The IDF consensus worldwide definition of the metabolic syndrome [Internet]. The IDF consensus worldwide definition of the metabolic syndrome. 2006;1–7. Available from: http://www.idf.org/webdata/docs/MetS_def_update2006.pdf
11. Stevens LA, Coresh J, Greene T, Levey AS. Assessing kidney function--measured and estimated glomerular filtration rate. N Engl J Med 2006;354:2473-2483.
12. Du Bois D, Du Bois EF. Clinical calorimetry: tenth paper a formula to estimate the approximate surface area if height and weight be known. Arch Intern Med 1916;17:863-871.
13. Schwartz GJ, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, Furth SL. New equations to estimate GFR in children with CKD. J Am Soc Nephrol 2009;20:629-637. Epub 2009 Jan 21
14. Filler G, Lepage N. Should the Schwartz formula for estimation of GFR be replaced by cystatin C formula? Pediatr Nephrol 2003;18:981-985. Epub 2003 Aug 13
15. Zappitelli M, Parvex P, Joseph L, Paradis G, Grey V, Lau S, Bell L. Derivation and validation of cystatin C-based prediction equations for GFR in children. Am J Kidney Dis 2006;48:221-230.
16. Bouvet Y, Bouissou F, Coulais Y, Séronie-Vivien S, Tafani M, Decramer S, Chatelut E. GFR is better estimated by considering both serum cystatin C and creatinine levels. Pediatr Nephrol 2006;21:1299-1306. Epub 2006 Jun 22
17. Donadio C, Lucchesi A, Tramonti G, Bianchi C. Creatinine clearance can be predicted from plasma creatinine and body composition

analysis by means of electrical bioimpedance. Ren Fail 1998;20:285-293.

18. Donadio C, Consani C, Ardini M, Caprio F, Grassi G, Lucchesi A. Prediction of glomerular filtration rate from body cell mass and plasma creatinine. Curr Drug Discov Technol 2004;1:221-228.
19. Utsch B, Klaus G. Urinalysis in children and adolescents. Dtsch Arztebl Int 2014;111:617-625.
20. Inker L, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van Lente F, Zhang YL, Coresh J, Levey AS; CKD-EPI Investigators. Estimating glomerular filtration rate from serum creatinine and cystatin C. N Engl J Med 2012;367:20-29.
21. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J; CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-612.
22. Tangri N, Stevens LA, Schmid CH, Zhang YL, Beck GJ, Greene T, Coresh J, Levey AS. Changes in dietary protein intake has no effect on serum cystatin C levels independent of the glomerular filtration rate. Kidney Int 2011;79:471-477. Epub 2010 Oct 27
23. Roos JF, Doust J, Tett SE, Kirkpatrick CM. Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children-A meta-analysis. Clin Biochem 2007;40:383-391. Epub 2007 Jan 5
24. Miliku K, Bakker H, Dorresteijn EM, Cransberg K, Franco OH, Felix JF, Jaddoe VW. Childhood Estimates of Glomerular Filtration Rate Based on Creatinine and Cystatin C: Importance of Body Composition. Am J Nephrol 2017;45:320-326. Epub 2017 Mar 1
25. Correia-Costa L, Afonso AC, Schaefer F, Guimarães JT, Bustorff M, Guerra A, Barros H, Azevedo A. Decreased renal function in overweight and obese prepubertal children. Pediatr Res 2015;78:436-444. Epub 2015 Jul 7
26. Marwyne MN, Loo CY, Halim AG, Norella K, Sulaiman T, Zaleha MI. Estimation of Glomerular Filtration Rate using Serum Cystatin C in Overweight and Obese Subjects. Med J Malaysia 2011;66:313-317.
27. Servais A, Giral P, Bernard M, Bruckert E, Deray G, Isnard Bagnis C. Is serum cystatin-C a reliable marker for metabolic syndrome? Am J Med 2008;121:426-432.
28. Thomas G, Sehgal AR, Kashyap SR, Srinivas TR, Kirwan JP, Navaneethan SD. Metabolic syndrome and kidney disease: a systematic review and meta-analysis. Clin J Am Soc Nephrol 2011;6:2364-2373. Epub 2011 Aug 18
29. Redon J, Lurbe E. The kidney in obesity. Curr Hypertens Rep 2015;17:555.
30. Prasad GV. Metabolic syndrome and chronic kidney disease: Current status and future directions. World J Nephrol 2014;3:210-219.

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0195

J Clin Res Pediatr Endocrinol 2019;11(1):55-60

SLC30A8 Gene rs13266634 C/T Polymorphism in Children with Type 1 Diabetes in Tamil Nadu, India

Ramasamy Thirunavukkarasu[1], Arthur Joseph Asirvatham[2], Ayyappan Chitra[3], Mariakuttikan Jayalakshmi[1]

[1]Madurai Kamaraj University, School of Biological Sciences, Department of Immunology, Madurai, India
[2]Government Rajaji Hospital, Clinic of Diabetology, Madurai, India
[3]Government Rajaji Hospital, Institute of Child Health and Research Centre, Madurai, India

What is already known on this topic?

SLC30A8 rs13266634 C/T polymorphism in type 1 diabetes (T1D) patients from four different populations was previously reported. This gene polymorphism is associated with T1D in the German population, but not in Danish, Japanese and British populations.

What this study adds?

To our knowledge, this is the first family-based report addressing *SLC30A8* gene polymorphism in South Indian patients. The present study and the meta-analysis show that the rs13266634 C/T polymorphism is not associated with type 1 diabetes in this population.

Abstract

Objective: Zinc transporter 8 (ZnT8) is a multi-transmembrane protein situated in the insulin secretory granule of the islets of β-cells and is identified as a novel auto-antigen in type 1 diabetes (T1D). The gene coding for ZnT8, solute carrier family 30 member 8 (*SLC30A8*) is located on chromosome 8q24.11. This study aimed to identify the association of *SLC30A8* rs13266634 C/T gene polymorphism with T1D in a sample of T1D children in Tamil Nadu, India.

Methods: The family based study was conducted in 121 T1D patients and 214 of their family members as controls. The *SLC30A8* gene rs13266634 C/T polymorphism was evaluated by polymerase chain reaction-restriction fragment length polymorphism.

Results: No significant differences were observed in either allele (odds ratio: 0.92; confidence interval: 0.33-2.58; p = 0.88) and genotype (CC: p = 0.74; CT: p = 0.82; TT: p = 0.80) frequencies of rs13266634 C/T between T1D patients and controls. Transmission disequilibrium test has identified over-transmission of mutant T allele from parents to affected children (T: U = 9:7) without statistical significance. Meta-analysis on the overall effects of rs13266634 C allele frequency was not different (p = 0.10 and $P_{heterogeneity}$ = 0.99) in T1D patients as compared to the controls.

Conclusion: The present study along with the meta-analysis does not show any substantial association of the rs13266634 C/T polymorphism with T1D development in this population.

Keywords: Type 1 diabetes, auto-antigen, polymorphisms, zinc transporter 8 autoantibody, meta-analysis

Introduction

Type 1 diabetes (T1D) is a complex, multifactorial disease caused by the selective destruction of insulin-producing pancreatic β-cells (1,2). The autoimmune destruction of pancreatic β-cells by pathogenic T cells predominately targets a number of well-known β-cell auto-antigens (3). Islet cell auto-antigens identified in T1D are Zinc transporter 8 (ZnT8), glutamic acid decarboxylase 65, tyrosine phosphatase-related molecules-2 and insulin (4). ZnT8 is a multi-transmembrane protein, belonging to the family of zinc transporters, having a role in the transport of zinc ions generated from the cytoplasm to the insulin vesicles and plays a major role in insulin maturation (5). During the process of insulin biosynthesis and secretion, frequent exocytosis of glucose stimulated insulin secretion

Address for Correspondence: Mariakuttikan Jayalakshmi PhD, Madurai Kamaraj University, School of Biological Sciences, Department of Immunology, Madurai, India
E-mail: jayalakshmim@genomicsmku.org - jayalakshmimk11@yahoo.com
ORCID ID: orcid.org/0000-0002-7035-3755

Conflict of interest: None declared
Received: 28.07.2018
Accepted: 07.09.2018

increase the chance of ZnT8 expression on the β-cell surface (6), which further causes more ZnT8 antigen to be exposed. Once ZnT8 is exposed, it can trigger or exacerbate the production of ZnT8 autoantibodies in genetically susceptible individuals (7). Previous studies have reported autoantibodies to ZnT8 to be highly prevalent among new-onset T1D children and have suggested that they could be a marker for disease risk (8,9,10,11). The cation efflux transporter ZnT8 may influence the development of ZnT8 immunogenicity and the phenotypic features of T1D. The solute carrier family 30 member 8 (*SLC30A8*) gene, located in chromosome 8q24.11, encodes for the ZnT8 auto-antigen and comprises 369 amino acids (12,13). Notably, aa268-369 of the cytoplasmic domain of ZnT8, especially ZnT8-325R and ZnT8-325W, is the dominant epitope in T1D. A common non-synonymous single-nucleotide polymorphism (SNP) of *SLC30A8* rs13266634 (C/T polymorphism) encodes either arginine (R) by the C allele or tryptophan (W) by the T allele at aa325 of ZnT8 (14) suggesting that rs13266634 SNP might be critical for humoral autoimmunity in T1D (11,15). Thus, the present study is based on the evidence that *SLC30A8* gene polymorphism is involved in T1D development. The objective of this study was to investigate the association between rs13266634 C/T gene polymorphism and T1D among the children of Tamil Nadu and to apply these results in a meta-analysis to reveal the association between the *SLC30A8* risk allele and T1D for comparison in different ethnic groups.

Methods

Subjects

The study subjects comprised 121 T1D patients from the Department of Diabetology, Government Rajaji Hospital in Madurai, Tamil Nadu, India, along with 214 their first degree relatives (120 parents and 94 siblings) as controls. All patients were evaluated by clinical history and routine laboratory tests. The patients met the revised criteria of the American Diabetes Association (ADA) for the screening of T1D (16). Genomic DNA was extracted from 5 mL of peripheral blood sample by salting out method (17).

Ethic board consent for the study was approved by the Institutional Ethics Committees of Govt. Rajaji Hospital (Ref. No. 23339/E4/3/10) and Madurai Kamaraj University (MKU/IRB/11/11) and consented in writing by the participants.

Genotype Analysis

Subjects were genotyped for rs13266634 C/T polymorphism of *SLC30A8* gene by polymerase chain reaction (PCR)-restriction fragment length polymorphism (18,19). The region surrounding the polymorphism was amplified with the following primers: Forward, 5'-GGACAGAAAGAGTTCCCATAGCG-3'; Reverse, 5'-ATAGCAGCATGTTTGAAGGTGGC-3'. PCR was performed at 95 °C for 5 minutes, followed by 40 cycles at 94 °C for 40 seconds and 69 °C for 45 seconds. A final extension step was carried out at 72 °C for 5 minutes. The PCR products were digested using enzyme MSp1 (Thermo Scientific, USA) incubated at 37 °C for 4 hours and visualized on 2% agarose gel. In the wild-type genotype (CC) the fragments obtained were of 234 and 195 bp. In the heterozygote genotype (CT), three fragments were detected of 429, 234 and 195 bp. Only one fragment of 429 bp was identified in the homozygote genotype (TT).

Meta-analysis

An extensive literature search was done to examine the association between T1D and *SLC30A8* gene. The original data were collected from the following electronic databases: PubMed, Elsevier, Science Direct, Web of Science and Google Scholar with key words "Zinc transporter protein member 8, ZnT8, *SLC30A8* gene polymorphism, *SLC30A8* or *SLC30A8* variant, combined with autoimmunity, autoimmune diabetes, T1D mellitus". All searches were done independently by more than two research investigators. The following inclusion criteria were applied: 1) studies should be case-controlled; and 2) all patients should meet the diagnostic criteria for T1D according to the ADA. Studies were excluded if they did not report on genotype frequency or if they had insufficient data.

Statistical Analysis

The obtained clinical data were subjected to Student t-test and χ^2 test after segregating the data based on age, number and sex of the subjects. Odds ratio (OR) and their p-values were calculated by logistic regression, which was performed using STATA 14v software (STATA Corporation, College road, TX, USA). In addition, the transmission/disequilibrium test (TDT) was employed to detect preferential transmission from heterozygous parents to affected offspring (20). The TDT analysis was done by Haploview 4.2v. software (Broad Institute, Cambridge, MA, USA). The level of significance was set at $p < 0.05$. Heterogeneity evaluation was performed by the Cochran's Q-test (21) and $p < 0.10$ was considered statistically significant. If not significant, OR and 95% confident interval (CI) was estimated by fixed effect model (22), otherwise the random effect model was used (23). Heterogeneity of the data was quantified using the I^2 test (24). I^2 value of 25%, 50% and 75% were nominally considered low, moderate and high estimates, respectively. Funnel plot and Egger's linear regression test was used

for the analysis of publication bias (25). Meta-analysis was performed with Rev Man 5.0v. software (RevMan 5.0, The Cochrane Collaboration, Oxford, UK).

Results

The demographic details of the T1D subjects and controls are given in Table 1. There was no significant differences observed in allele (OR = 0.92; CI = 0.33-2.58; p = 0.88) and genotype (CC: OR = 0.92; CI = 0.58-1.47; p = 0.74; CT: OR = 1.05; CI = 0.64-1.71; p = 0.82; TT: OR = 1.13; CI = 0.42-3.00; p = 0.80) frequencies of rs13266634 C/T between T1D patients and controls, respectively (Table 2). Upon analysis of 30 parent-offspring trios (one affected child and both parents) of the study cohort, TDT analysis identified over-transmission of mutant T allele of rs13266634 C/T polymorphism from parents to affected children (T: U = 9:7; MAF = 0.194; $\chi^2 = 0.25$; p = 0.61) without statistical significance.

Meta-analysis of the data via literature survey was able to retrieve 18 studies. Of these, nine were excluded after screening the abstracts, review and irrelevant subject matter. Three studies did not provide comprehensive information. Two studies were not considered as they provided insufficient genotype frequencies. The remaining four studies (14,26,27,28) associated with rs13266634 C/T polymorphism in the *SLC30A8* gene of T1D, which met the required criteria, were included in the present meta-analysis. Along with the present study, a total of five eligible studies with a total of 10,376 T1D patients and 10,027 control subjects were included in the meta-analysis.

Characteristics of the said studies and the distribution of rs13266634 C/T genotypes and alleles in T1D patients and controls are given in Table 3. Overall effects of rs13266634 C allele frequency in T1D patients (OR = 0.97; CI = 0.92-1.01; p = 0.10) based on pooled analysis were not different from the controls (Table 4). There was no evidence of virtual asymmetry ($\chi^2 = 0.29$; $I^2 = 0\%$; $P_{heterogeneity} = 0.99$) which indicated that no publication bias crept in the meta-analysis (Figure 1).

In the Forest plot the area of squares, horizontal lines and diamond shows the weight of specific study, confidence intervals and the summary of fixed-effects OR, respectively (Table 4).

In the Funnel plot the open circle represents various studies considered for this plot correlation (Figure 1). No evidence of publication bias was found.

Table 1. Demographic details of the type 1 diabetes patients and controls

Details	Type 1 diabetes patients		Controls
	Male	Female	
No. of Subjects (n)	70	51	214
Age (year)	15.8 ± 10.2	22.8 ± 10.0	32.2 ± 15.1
Age at diagnosis (year)	15.5 ± 8.4	14.8 ± 7.9	
TDDM (year)	9.5 ± 5.8	8.0 ± 5.6	

TDDM: time duration of diabetes mellitus

Table 2. *SLC30A8* rs13266634C/T genotypes and allele frequencies in type 1 diabetes patients and healthy controls

rs13266634C/T	T1D patients (n = 121)	Controls (n = 214)	OR (95% CI)	p-value
Genotype				
CC (RR)	77 (63.6%)	140 (65.4%)	0.92 (0.58-1.47)	0.74
CT (WW)	37 (30.6%)	63 (29.4%)	1.05 (0.64-1.71)	0.82
TT (WW)	7 (5.8%)	11 (5.2%)	1.13 (0.42-3.00)	0.8
CC + CT (RR + RW)[1]	114 (72.2%)	203 (73.3%)	0.88 (0.33-2.33)	0.8
CT + TT (RW + WW)[2]	44 (27.8%)	74 (26.7%)	1.08 (0.67-1.72)	0.74
Allele				
C (R)	191 (78.9%)	343 (80.1%)	0.92 (0.33- 2.58)	0.88
T (W)	51 (21.1%)	85 (19.9%)		-

OR: odds ratio, CI: confidence interval, n: number in sample, T1D: type 1 diabetes

[1]Dominant model (CC + CT vs TT)

[2]Recessive model (CT + TT vs CC)

Discussion

ZnT8 is highly expressed in the pancreatic islet β-cells and recognized as one of the four major auto-antigens in T1D patients. It has been observed that autoantibodies are generated against ZnT8 prior to the onset of disease. It is known that rs13266634 C/T SNP is responsible for the autoimmune response to ZnT8 (12). The rs13266634 C/T plays a susceptibility role in the presence of impaired, autoimmunity-mediated β-cell dysfunction which leads to T1D development (13). Studies of the role of rs13266634 C/T polymorphism in T1D among a global population are scanty. This work appears to be the first family based TDT analysis on rs13266634 SNP with its allele transmission from parents to offspring. As for TDT results, the present study documents over-transmission of mutant T allele of rs13266634 in T1D. In a case control scenario, the present study indicates that there is a lack of association of rs13266634 C/T polymorphism to T1D. A few earlier studies also lent support to this contention in the Danish, Japanese and British populations (14,26,28). However, a German study indicates a higher occurrence of the C allele and CC genotype of rs13266634 C/T polymorphism in early onset of T1D patients compared to controls (27). A recent study revealed that an adjacent locus of rs2466293 in the *SLC30A8* gene seems to predispose to

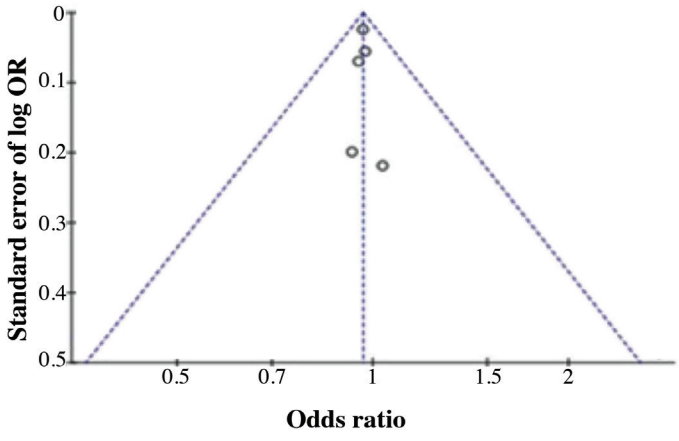


Figure 1. Begg's funnel plot of *SLC30A8* rs13266634 C/T with type 1 diabetes patients included in this meta-analysis

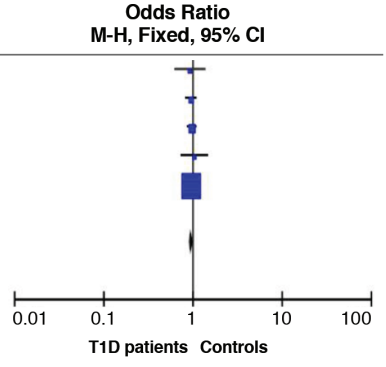
Table 3. Distribution of *SLC30A8* genotype and allele among type 1 diabetes patients and controls included in the meta-analysis

Study	Arms	C	T	CC	CT	TT	Population	Method
Brorsson et al (26)	Cases (n = 1530)	2100	960	736	628	166	Danish	Sequencing & Taqman
	Controls (n = 1478)	2045	911	725	595	158		
Kawasaki et al (14)	Cases (n = 171)	198	144	63	72	36	Japanese	PCR-RFLP
	Controls (n = 114)	130	98					
Gohlke et al (27)	Cases (n = 874)	1193	555	400	393	81	German	MOLDI-TOF-MS
	Controls (n = 1021)	1416	626	493	430	98		
Raj et al (28)	Cases (n = 7680)	10481	4879				British	Taq-man PCR
	Controls (n = 7200)	9937	4463					
Present study	Cases (n = 121)	191	51	77	37	7	South Indian	PCR-RFLP
	Controls (n = 214)	343	85	140	63	11		

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism

Table 4. Forest plot depicting the association of *SLC30A8* rs13266634 C-allele in type 1 diabetes

Study or Subgroup	T1D patients Events	T1D patients Total	Controls Events	Controls Total	Weight	Odds Ratio M-H, Fixed, 95% CI	Year
Present study	191	242	343	428	1.2%	0.93 (0.63, 1.37)	
Gohlke et al (27)	1193	1748	1416	2042	9.3%	0.95 (0.83, 1.09)	2008
Brorsson et al (26)	2100	3060	2045	2956	14.7%	0.97 (0.87, 1.09)	2008
Kawasaki et al (14)	198	342	130	228	1.5%	1.04 (0.74, 1.45)	2008
Raj et al (28)	10481	15360	9937	14400	73.3%	0.96 (0.92, 1.01)	2009
Total (95% CI)		**20752**		**20054**	**100.0%**	**0.97 (0.93, 1.01)**	
Total events	14163		13871				
Heterogeneity: Chi²=0.29, df=4 (p=0.99); I²=0%							
Test for overall effect: Z=1.64 (p=0.10)							



the risk of T1D in individuals of non-European descent (29).

Until now, several publications have investigated the correlation of rs13266634 C/T polymorphisms with T1D (14,26,27,28). However, the results remain inconclusive. In order to reach a more concrete opinion of this contentious matter, a meta-analysis was performed with expanded sample size, aiming to explore the relationship of polymorphism at rs13266634 C/T of the *SLC30A8* gene with susceptibility to T1D. However the result of the meta-analysis indicated that the C allele conferred no risk in the development of T1D. Nevertheless, we should point out that one of the previous meta-analyses on T2D revealed that the rs13266634 C/T polymorphism is significantly associated with impaired glucose tolerance (30).

Study Limitations

The study is limited by a relatively small number of subjects. Varied studies from different ethnicities with large sample size are required to conclusively confirm the role of rs13266634 C/T polymorphism in T1D.

Conclusion

This result demonstrates that the allele, genotype, genetic models and allele transmission of rs13266634 C/T polymorphism are not strongly associated with T1D in the children of a Tamil Nadu population. The meta-analysis also indicates that the rs13266634 C/T polymorphism was not associated with T1D.

Acknowledgements

This work was supported by the grants of the Tamil Nadu State Council for Science and Technology, Govt. of Tamil Nadu. India.

Ethics

Ethics Committee Approval: Ethic board consent for the study was approved by the Institutional Ethics Committees of Govt. Rajaji Hospital (Ref. No. 23339/E4/3/10) and Madurai Kamaraj University (MKU/IRB/11/11).

Informed Consent: All parents were informed about the purpose of the study, and a signed consent for study participation was obtained.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Mariakuttikan Jayalakshmi, Design: Mariakuttikan Jayalakshmi, Ramasamy Thirunavukkarasu, Data Collection or Processing: Ramasamy Thirunavukkarasu, Arthur Joseph Asirvatham, Ayyappan Chitra, Analysis or Interpretation: Ramasamy Thirunavukkarasu, Mariakuttikan Jayalakshmi, Literature Research: Ramasamy Thirunavukkarasu, Writing: Ramasamy Thirunavukkarasu, Mariakuttikan Jayalakshmi.

Financial Disclosure: This study was supported by the grants of the Tamil Nadu State Council for Science and Technology (ref. No.TNSCST/S&T Proj/ MS/ VR/2011-2012 by Government of Tamil Nadu and UGC - NRCBS, CAS, DST-PURSE, and UGC - Meritorious fellowship by Government of India.

References

1. Gillespie KM. Type 1 diabetes: pathogenesis and prevention. CMAJ 2006;2:165-170.
2. Pihoker C, Gilliam LK, Hampe CS, Lernmark A. Autoantibodies in diabetes. Diabetes 2005;54(Suppl 2):52-61.
3. Lieberman SM, DiLorenzo TP. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens 2003;62:359-377.
4. Arvan P, Pietropaolo M, Ostrov D, Rhodes CJ. Islet autoantigens: Structure, function, localization, and regulation. Cold Spring Harb Perspect Med 2012;2.
5. Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M. Overview of mammalian zinc transporters. Cell Mol Life Sci 2004;61:49-68.
6. Chimienti F, Devergnas S, Favier A, Seve M. Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 2004;53:2330-2337.
7. Yi B, Huang G, Zhou Z. Different role of zinc transporter 8 between type 1 diabetes mellitus and type 2 diabetes mellitus. J Diabetes Investig 2016;7:459-465. Epub 2016 Jan 9.
8. Vaziri-Sani F, Oak S, Radtke J, Lernmark K, Lynch K, Agardh CD, Cilio CM, Lethagen AL, Ortqvist E, Landin-Olsson M, Törn C, Hampe CS. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity 2010;43:598-606. Epub 2010 Mar 19
9. Yang L, Luo S, Huang G, Peng J, Li X, Yan X, Lin J, Wenzlau JM, Davidson HW, Hutton JC, Zhou Z. The diagnostic value of zinc transporter 8 autoantibody (ZnT8A) for type 1 diabetes in Chinese. Diabetes Metab Res Rev 2010;26:579-584.
10. Yi B, Huang G, Zhou ZG. Current and Future Clinical Applications of Zinc Transporter-8 in Type 1 Diabetes Mellitus. Chin Med J (Engl) 2015;128:2387-2394.
11. Elmaoğulları S, Uçaktürk SA, Elbeg Ş, Döğer E, Tayfun M, Gürbüz F, Bideci A. Prevalence of ZnT8 antibody in Turkish children and adolescents with new onset type 1 diabetes. J Clin Res Pediatr Endocrinol 2018;10:108-112. Epub 2017 Sep 25
12. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007;104:17040-17045. Epub 2007 Oct 17
13. Wenzlau JM, Liu Y, Yu L, Moua O, Fowler KT, Rangasamy S, Walters J, Eisenbarth GS, Davidson HW, Hutton JC. A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes. Diabetes 2008;57:2693-2697. Epub 2008 Jun 30

14. Kawasaki E, Uga M, Nakamura K, Kuriya G, Satoh T, Fujishima K, Ozaki M, Abiru N, Yamasaki H, Wenzlau JM, Davidson HW, Hutton JC, Eguchi K. Association between anti-ZnT8 autoantibody specificities and SLC30A8 Arg325Trp variant in Japanese patients with type 1 diabetes. Diabetologia 2008;51:2299-2302. Epub 2008 Oct 11
15. Wenzlau JM, Moua O, Liu Y, Eisenbarth GS, Hutton JC, Davidson HW. Identification of a major humoral epitope in Slc30A8 (ZnT8). Ann N Y Acad Sci 2008;1150:252-255.
16. American Diabetes Association. Standards of Medical Care in Diabetes - 2010. Diabetes Care 2010;33(Suppl 1):11-61.
17. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.
18. Feghih H, Khatami SR, Azarpira N, Foroughmand AM. SLC30A8 gene polymorphism (rs13266634 C/T) and type 2 diabetes mellitus in south Iranian population. Mol Biol Rep 2014;41:2709-2715. Epub 2014 Jan 22
19. Huang Q, Yin JY, Dai XP, Wu J, Chen X, Deng CS, Yu M, Gong ZC, Zhou HH, Liu ZQ. Association analysis of SLC30A8 rs13266634 and rs16889462 polymorphisms with type 2 diabetes mellitus and repaglinide response in Chinese patients. Eur J Clin Pharmacol 2010;66:1207-1215. Epub 2010 Aug 31
20. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet 1993;52:506-516.
21. Cochran WG. The combination of estimates from different experiments. Biometrics 1954;10:101-129.
22. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 1959;22:719-748.
23. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986;7:177-188.
24. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002;21:1539-1558.
25. Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ 1997;315:629-634.
26. Brorsson C, Bergholdt R, Sjögren M, Eising S, Sorensen KM, Hougaard DM, Orho-Melander M, Groop L, Pociot F. A non-synonymous variant in SLC30A8 is not associated with type 1 diabetes in the Danish population. Mol Genet Metab 2008;94:386-388. Epub 2008 Apr 8
27. Gohlke H, Ferrari U, Koczwara K, Bonifacio E, Illig T, Ziegler AG. SLC30A8 (ZnT8) polymorphism is associated with young age at type 1 diabetes onset. Rev Diabet Stud 2008;5:25-27. Epub 2008 May 10
28. Raj SM, Howson JM, Walker NM, Cooper JD, Smyth DJ, Field SF, Stevens, Todd JA. No association of multiple type 2 diabetes loci with type 1 diabetes. Diabetologia 2009;52:2109-2116. Epub 2009 May 20
29. Gomes KF, Semzezem C, Batista R, Fukui RT, Santos AS, Correia MR, Passos-Bueno MR, Silva ME. Importance of Zinc Transporter 8 Autoantibody in the Diagnosis of Type 1 Diabetes in Latin Americans. Sci Rep 2017;16:7:207.
30. Xu K, Zha M, Wu X, Yu Z, Yu R, Xu X, Chen H, Yang T. Association between rs13266634 C/T polymorphisms of solute carrier family 30 member 8 (SLC30A8) and type 2 diabetes, impaired glucose tolerance, type 1 diabetes--a meta-analysis. Diabetes Res Clin Pract 2011;91:195-202. Epub 2010 Dec 4

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0153

J Clin Res Pediatr Endocrinol 2019;11(1):61-69

Epicardial Fat Thickness in Children with Classic Congenital Adrenal Hyperplasia

Kotb Abbass Metwalley[1], Hekma Saad Farghaly[1], Abdelrahman Abdelhamid[2]

[1]Assiut University Faculty of Medicine, Department of Pediatrics, Assiut, Egypt
[2]South Valley University Qena Faculty of Medicine, Department of Clinical Pathology, Qena, Egypt

What is already known on this topic?

There is an increased risk for cardiac abnormalities in children with congenital adrenal hyperplasia. Epicardial fat thickness is an emerging cardio-metabolic risk factor and has been shown to be related to atherosclerosis.

What this study adds?

To our knowledge, this is the first study assessing epicardial fat thickness (EFT) in children with congenital adrenal hyperplasia (CAH). EFT is higher in children with CAH than in healthy children and correlated with carotid intima media thickness, left ventricular mass and mitral deceleration time. EFT may be used as a possible marker of early atherosclerosis and myocardial function in children with CAH.

Abstract

Objective: Epicardial fat thickness (EFT) is an emerging cardio-metabolic risk factor and has been shown to be related to atherosclerosis. EFT has not been studied in the context of CAH. This study aimed to evaluate EFT in children with CAH and its relation to carotid artery intima-media thickness (CA-IMT) and left ventricular (LV) functions.

Methods: Thirty-six children with classical CAH were compared with 36 healthy controls. All patients had confirmed CAH and were receiving steroid substitution therapy. Patients and controls underwent anthropometric evaluation, measurement of fasting lipids, glucose, insulin, homeostasis model assessment for insulin resistance (HOMA-IR). LV functions and EFT were assessed using conventional echocardiography. Duplex ultrasonography was used to measure CA-IMT.

Results: Compared to controls, patients had greater EFT ($p = 0.001$), CA-IMT ($p = 0.01$), LV mass index (LVMI) ($p = 0.001$) and prolonged mitral deceleration time (DcT) ($p = 0.01$). CAH patients also had significantly worse HOMA-IR ($p = 0.001$) than controls. Abnormalities were worse in uncontrolled CAH on treatment. Multivariate analysis in CAH subjects showed EFT correlated positively with waist circumference odds ratio (OR) [OR = 1.9; 95% confidence interval (CI): 1.07-1.14; $p = 0.01$], 17-hydroxyprogesterone [OR = 1.6; 95% CI: 1.33-2.89; $p = 0.05$], testosterone concentration (OR = 1.7; 95% CI: 1.55-2.13; $p = 0.01$), LVMI (OR = 1.14; 95% Cl: 1.08-1.13; $p = 0.01$), mitral DcT (OR = 2.25; 95% CI: 1.15-2.05; $p = 0.01$) and CA-IMT (OR = 1.6; 95% CI: 1.15-2.05; $p = 0.01$).

Conclusion: EFT is elevated in children with classical CAH, particularly in those with poor control, and is correlated with CA-IMT, LV mass and mitral DcT. Measurement of EFT in CAH children may help to identify those at high risk of developing LV dysfunction and subclinical atherosclerosis.

Keywords: Diastolic function, echocardiography, epicardial fat thickness, left ventricular function, left ventricular mass index, congenital adrenal hyperplasia, carotid intima media thickness, mitral deceleration time

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive condition resulting from mutations in enzymes required for adrenal steroid synthesis (1). Defects in the enzyme 21-hydroxylase, leading to enzyme deficiency, are responsible for approximately 95% of cases (2). CAH is commonly divided into the severe classical and the milder nonclassical form. Classical CAH is generally subdivided, depending on the extent of enzymatic impairment, into the salt-wasting (SW) form, presenting with both cortisol

Address for Correspondence: Kotb Abbass Metwalley MD, Assiut University Faculty of Medicine, Department of Pediatrics, Assiut, Egypt
Phone: +0020882368373 **E-mail:** kotb72@gmail.com **ORCID ID:** orcid.org/0000-0003-4763-488X

Conflict of interest: None declared
Received: 07.06.2018
Accepted: 03.10.2018

and aldosterone deficiency and the simple virilizing (SV) form, characterized by an isolated cortisol deficiency. Both conditions are associated with androgen excess resulting in virilization of female external genitalia (3). Researchers have long thought that patients with 21-hydroxylase deficiency are at increased risk for cardiovascular diseases due to the resulting high plasma levels of androgens and/or the harmful effects of glucocorticoid and mineralocorticoid treatment (4). 21-hydroxylase deficiency may also have detrimental effects on vascular structures as well as ventricular systolic and diastolic function (5). Obesity, hypertension, dyslipidaemia and insulin resistance (IR) have been found to be associated with both CAH itself and the treatment strategies (6).

Few studies have utilized carotid artery intima-media thickness (CA-IMT) to assess vascular structural changes in children with CAH (4,5,7). A hindrance to the wider use of CA-IMT measurements in the pediatric population is the lack of standardization of CA-IMT values in this age group (8). Epicardial fat thickness (EFT) is a layer of adipose tissue surrounding the heart and coronary vessels which can be measured by ultrasound, a simple, noninvasive procedure (9). EFT is a reliable and sensitive marker of cardiovascular risk and has become an emerging target for therapeutic and medical interventions (10). We are not aware of any published data on EFT in children with CAH.

The aim of this study was to evaluate the EFT measurement and its relation to CA-IMT and left ventricular function in a cohort of children with classical CAH.

Methods

Patients and Methods

This cross-sectional, controlled study included 36 children (11 males and 25 females; mean ± standard deviation age = 13.7 ± 2.4 years) with a confirmed diagnosis of classic CAH (4). Diagnosis was made based on clinical signs and biochemical assessment [elevated adrenocorticotropic hormone (ACTH), 17-hydroxyprogesterone (17-OHP), androstenedione and testosterone, in addition to low cortisol]. SW was diagnosed in patients with frank hyponatraemia and hyperkalaemia accompanied by low plasma aldosterone and elevated rennin concentrations (11). Patients were included if they were on glucocorticoid therapy for a minimum of five years. They were recruited during the period between January and December 2017 from the Pediatric Endocrinology Unit of Assiut University Children Hospital, Assiut, Egypt. Patients who had chest deformities, chronic lung disease, poor echo window, pericardial and/or pleural effusion on transthoracic echocardiography were excluded. Thirty six healthy children (7 males and 27 females) matched for age, gender, pubertal status and socioeconomic status were recruited as control subjects from the General Pediatric Outpatient Clinic of the same hospital. None of the controls were hypertensive and none were smokers, on any medication, or had a chronic illness. Controls were attending the outpatient clinic either because of minor illness or accompanying their sick siblings. All patients had classical CAH with 21-hydroxylase deficiency (SW n = 30; SV n = 6) and were receiving glucocorticoid substitution therapy with hydrocortisone (HC) (n = 30) or prednisone (PR) (n = 6). PR dose was converted to HC using the conversion assumption that 20 mg of HC is equivalent to 5 mg of PR (12). SW patients were also on 9-alphafludrocortisone therapy at a dose of 50-100 ug/m^2/day. The adequacy of steroid therapy was monitored periodically during follow-up visits every 3-6 months by clinical parameters, such as signs of androgen excess, growth curves, bone age and hormonal assay (13). Patients were divided into two groups according to the degree of control on medical treatment, that is those patients with acceptable disease control and children with poor disease control, based on the previously mentioned data (14).

The study protocol was approved by the Local Ethics Committee of Assiut University Children Hospital, Assiut, Egypt (approval number: 312/2017) and also by the Ethics Committee of the Faculty of Medicine, in accordance to the Declaration of Helsinki. Written informed consent was obtained from the parents of all participants.

All patients and controls were subjected to a full medical history-taking as well as a thorough clinical examination. Demographic and clinical data included age, gender, duration of treatment, type and dose of steroids, blood pressure (BP), height, weight and body mass index (BMI). Systolic BP (SBP) and diastolic BP (DBP) were measured in all subjects in the right arm with a standard sphygmomanometer (Exacta) by the same operator. Height and weight were measured using a wall-mounted stadiometer (seca 213 l) and a calibrated weight scale (Uline Industrial Platform Scales), with the child wearing underwear only. BMI was calculated using the following formula: BMI = weight (kg)/height (m)^2. BMI was expressed as standard deviation scores (SDS) using the Egyptian Growth Reference Data (15). Waist circumference was measured at the midpoint between the lower edge of the ribs in the midaxillary line and the top of the iliac crest by the same clinician. Waist-to-height ratio was then calculated as an index of visceral adiposity. Pubertal status was assessed according to Tanner staging (16). A radiograph of the left hand was used to determine BA using the Greulich-Pyle method (17). This assessment was made in a blinded fashion by a single pediatric endocrinologist. BA

was defined as advanced when greater than the subject's chronological age by one year or more (18).

Blood samples were drawn after an overnight fast for at least 12 hours at 8.00-10.00 a.m. before the first dose of steroids for assessment of serum levels of total cholesterol (TC), triglycerides (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), glucose and insulin. Serum TG and TC were assessed by quantitative enzymatic colorimetric technique (Bio Merieux-Diagnostic Chemicals Ltd., Charlottetown, CA, USA). Serum high-density lipoproteins (HDL) were measured by the phosphotungstate precipitation method (Biomerieux kit, Marcy L'etoile, Craponne, France). LDL cholesterol was calculated by Friedewald's formula: (TC) - (HDL-C) - 1/5 (TG) (19). IR was calculated using the homeostasis model assessment for IR (HOMA-IR) equation formula:

HOMA-IR = Fasting insulin (uU/mL) multiplied by fasting glucose (mmol/L) divided by 22.5.

A cut-off level of 2.7 was used for diagnosing IR as previously described (20). ACTH, plasma 17-OHP, serum cortisol, androstenedione and testosterone were also measured with commercially available RIA kits (Siemens Healthcare Diagnostics Inc., Los Angeles, CA, USA). The plasma level of high sensitivity C-reactive protein (hsCRP) was measured using the hsCRP enzyme immunoassay test (ELISA) kit for quantitative determination of the CRP concentration in human serum (catalog no. E29-056; Immunospec Corp., Canoga Park, CA, USA).

Echocardiographic Examination

All echocardiographic examinations were performed according to the recommendations of the American Society of Echocardiography (21). A Philips Envisor Ultrasound System with a S4-2 Broadband Sector (Philips Medical Systems, Inc., Netherlands) was used. Measurements were performed using the machine's incorporated analysis package. An M-mode echocardiography was obtained at the left sternal border. Left ventricular (LV) dimension, LV fractional shortening (FS) and LV ejection fraction (EF) were measured. LV mass index (LVMI) was measured using a LVMI calculator. LV diastolic function was evaluated by mitral inflow velocities obtained in the apical four-chamber view. Mitral filling was assessed with the peak velocity of the transmitral early filling wave (E) and the peak velocity of atrial late filling (A) and the ratio of both (E/A) was calculated. The interval from the early peak velocity to the zero intercept of the extrapolated deceleration time (DcT) slope (early filling mitral DcT) was measured. The interval between the end of the LV outflow velocity and the onset of mitral inflow [isovolumic relaxation time (IVRT)] obtained by pulsed-wave Doppler with the cursor placed in the LV out-flow near the anterior leaflet of the mitral valve, was measured from the end of the LV ejection to the onset of the mitral inflow.

Epicardial Fat Thickness Measurement

A two-dimensional (2D) echocardiogram, using a standardised procedure, was performed with the patient in the left lateral decubitus position. EFT thickness was measured by an experienced pediatric echocardiologist, who was blinded to the subjects' clinical and demographic data, using the procedure validated by Iacobellis et al (9). EFT was identified as the echolucent region between the external wall of the myocardium and the visceral layer of the pericardium (Figure 1). This thickness was measured perpendicularly on the free wall of the right ventricle at the end of systole over three cardiac cycles, using a parasternal long and a parasternal short axis. The average value of the three cardiac cycles from each echocardiographic view was used for the statistical analysis.

Carotid Intima Media Thickness Measurement

All participants underwent an ultrasound scan to measure CA-IMT. The studies were performed in the morning between 7:30 and 9:30 a.m. after the children had fasted overnight. All ultrasound scans were performed by an experienced vascular operator who was unaware of the subject's clinical details. Examination of CA-IMT was manually performed using a color duplex flow imaging system (Acuson 128 XP; Acuson Corporation, Mountain View, CA, USA). The examinations were performed while the patients were in a supine position, with their necks slightly extended and their heads turned 450 away from the examination side. From both sides of the head, three images were obtained from the distal common carotid

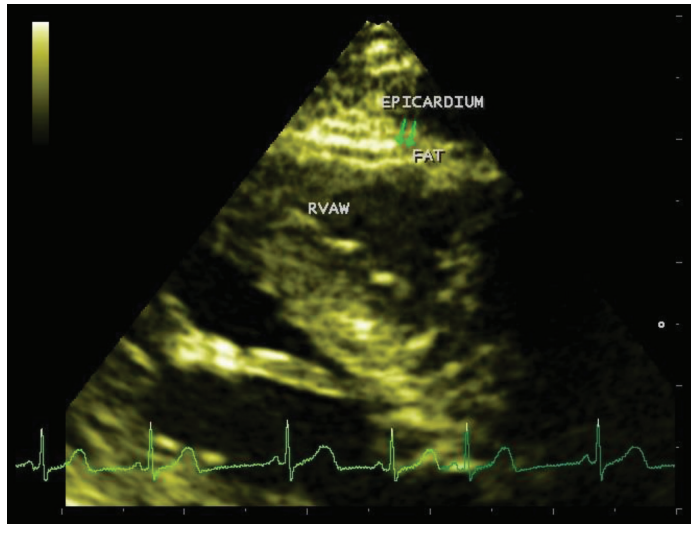


Figure 1. Echocardiographic imaging of the epicardial fat

artery, 1-2 cm proximal to the carotid bulb at end diastole. These images were then stored for later offline analyses. All studies were done according to a predetermined, standardized scanning protocol for the right and left carotid arteries (22). All measurements were performed in all participants by the same pediatric cardiologist who was blinded to the clinical and treatment status of the study participants. Reliability of echocardiographic measurements of CA-IMT and EFT were assessed by intra-observer correlation coefficient in all subjects.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) for Windows, version 16.0 (SPSS Inc, Chicago, IL, USA). Data were expressed as means + standard deviation. Comparisons of quantitative variables between the study groups were made using the paired Student t-test. Correlations between EFT and demographic, clinical, and laboratory variables were assessed using Pearson test. Multiple logistic regression analysis was used to determine the factors that were significantly associated with high EFT. The odds ratios, 95% confidence intervals and significances were calculated. For all tests, values of $p < 0.05$ were considered statistically significant.

Results

Demographic and anthropometric data of the patients and controls are shown in Table 1. Bone age in the patient group was advanced by an average of two years compared with chronological age. Compared with healthy controls, children with CAH exhibited increased visceral adiposity, as suggested by higher values of BMI SDS, waist circumference, hip circumference and waist to height ratio. Moreover, CAH children had higher SBP and DBP, although all children had blood pressures within the normal ranges.

Laboratory data of the patients and controls are shown in Table 2. Concentrations of TC, TG, LDL-C, fasting blood glucose, fasting insulin, hsCRP, 17-OHP, androstenedione and testosterone were significantly higher while concentration of HDL-C was significantly lower in CAH patients compared to control subjects. HOMA-IR values were also significantly higher in the patients compared with controls.

The results of echocardiographic EFT and CA-IMT examinations are shown in Table 3. There were no significant differences in EF and FS values between patients and control subjects. However, compared to control subjects, patients had higher LVMI value, indicating myocardial hypertrophy, and lower E/A ratio, higher IVRT values and prolonged mitral DcT, indicating impaired diastolic function and increased CA-IMT and higher EFT. Intra-observer agreement on CA-IMT and EFT measurements were excellent. Intra-observer correlation coefficient was 0.94 and 0.95, respectively, indicating excellent reproducibility of these measures. Compared to patients who were well controlled (n = 16), patients who were uncontrolled (n = 20) were older, had advanced bone ages and had higher levels of 17-OHP, testosterone and hsCRP. In addition poorly controlled patients had higher values of LVMI, mitral DcT, EFT and CA-IMT (see Table 4).

EFT thickness showed a statistically significant positive correlation with BMI, waist circumference, SBP, DBP, HOMA-IR, hsCRP, 17-OHP, testosterone, LVMI, mitral DcT and CA-IMT (see Table 5).

Multivariate analysis in children with CAH revealed that EFT was significantly correlated with waist circumference, 17-OHP, HOMA-IR, testosterone, mitral DcT, CA-IMT and LVMI (see Table 6).

Table 1. Demographic, anthropometric and clinical data of the congenital adrenal hyperplasia patients compared with controls

	CAH patients (n = 36)	Controls (n = 36)	p value
Age, years	13.7 ± 2.4	13.6 ± 2.5	NS
SDS-BMI	1.02 ± 0.92	-0.24 ± 1.5	0.01
Waist circumference, cm	83 ± 13	72 ± 14	0.01
Hip circumference, cm	86 ± 9	78 ± 12	0.05
Waist to height ratio	0.55 ± 0.08	0.47 ± 0.07	0.001
SBP (mmHg)	119.76 ± 8.11	106.35 ± 7.47	0.001
DBP (mmHg)	74.70 ± 5.23	65.43 ± 4.91	0.001
Bone age, years	15.3 ± 2.3	12.2 ± 1.2	0.001

Data are expressed as mean ± standard deviation.

SDS-BMI: standard deviation scores of body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, CAH: congenital adrenal hyperplasia, NS: non-significant

Discussion

This study demonstrates that a) children with classical CAH may have subclinical LV hypertrophy, diastolic dysfunction and subclinical atherosclerosis; b) EFT was higher in patients with CAH than in the healthy controls; c) EFT is correlated to carotid intima media thickness, LV mass and mitral DcT suggesting that EFT may be used as an additional marker of endothelial and myocardial dysfunction in children with CAH. The classical cardiovascular risk factors in children with CAH, namely obesity, hypertension, dyslipidemia, steroid treatment and others have been extensively discussed elsewhere (4,5,6). To our knowledge, this study is the first to demonstrate that EFT was significantly increased in children with classical CAH compared with control children ($p < 0.001$). In addition, we showed that EFT was correlated positively with CA-IMT. The multiple linear regression analysis showed that the CA-IMT was the variable that most influenced EFT. EFT was reported to be increased in children with a positive family history of type 2 DM and has been suggested as a risk factor for early atherosclerosis (23). A meta-analysis showed that EFT may be an effective marker for the prediction of coronary heart disease (24). Epicardial fat is thought to play a pivotal role in the pathogenesis of coronary artery disease (CAD)

Table 2. Laboratory data of the congenital adrenal hyperplasia patients compared with controls

	CAH cases (n = 36)	Controls (n = 36)	p value
Total cholesterol (mg/dL)	173.65 ± 43.34	142.22 ± 18.14	0.01
Triglycerides (mg/dL)	138.22 ± 34.23	104.23 ± 12.21	0.01
LDL-C (mg/dL)	113.55 ± 65.21	73.66 ± 13.32	0.001
HDL-C (mg/dL)	44.12 ± 7.8	53.9 ± 7.8	0.01
Fasting blood glucose (mg/dL)	92.4 ± 15.8	81.6 ± 12.9	0.01
Fasting insulin (lU/mL)	15.2 ± 6.2	7.6 ± 2.8	0.001
HOMA-IR	3.21 ± 1.2	1.8 ± 0.8	0.001
hsCRP (mg/L)	329 ± 20.5	154.9 ± 16.8	0.001
ACTH (pg/mL)	102.5 ± 12.7	26.3 ± 2.2	0.001
17-OHP (nmol/L)	184.2 ± 54.9	1.75 ± 0.95	0.001
Testosterone (ng/dL)	544.3 ± 195.7	181.6 ± 62.5	0.001
Androstenedione (ng/dL)	182 ± 15.2	89.3 ± 17.2	0.001

Data are expressed as mean ± standard deviation.

LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, ACTH: adrenocorticotropin hormone, 17-OHP: 17-hydroxyprogesterone, hsCRP: high-sensitivity C-reactive protein, CAH: congenital adrenal hyperplasia

Table 3. The echocardiographic, carotid artery intima-media thickness and epicardial fat thickness findings in the congenital adrenal hyperplasia patients compared to controls

	CAH children (n = 36)	Controls (n = 36)	p value
LVEDD (mm)	43.7 ± 2.2	37.9 ± 3.8	0.001
LVESD (mm)	23.5 ± 2.1	22.2 ± 2.8	NS
EF (%)	67.5 ± 6.6	66.3 ± 4.1	NS
FS (%)	41.2 ± 3.1	49.9 ± 4.2	NS
IVSWT (mm)	9.36 ± 1.20	4.33 ± 1.32	0.001
LVPWT (mm)	7.54 ± 0.43	4.17 ± 0.45	0.001
LVMI (gm/m^2)	59.71 ± 7.24	42.29 ± 5.75	0.001
E/A ratio	1.28 ± 0.12	1.64 ± 0.22	0.01
IVRT (ms)	76.2 ± 6.3	46.5 ± 5.1	0.001
Mitral DcT (ms)	189.5 ± 16.2	129.4 ± 15.3	0.001
CA-IMT; mm	0.52 ± 0.20	0.43 ± 0.02	0.001
EFT (mm)	6.95 ± 0.81	4.01 ± 0.52	0.001

Data are expressed as mean ± standard deviation.

LVEDD: left ventricular end-diastolic diameter, LVESD: left ventricular end-systolic diameter, EF: ejection fraction, FS: fractional shortening, IVSWT: interventricular septal wall thickness, LVPWT: left ventricular posterior wall thickness, LVMI: left ventricular mass index, E/A: ratio of mitral E-to mitral A-wave peak velocity, IVRT: isovolumic relaxation time, DcT: deceleration time, EFT: epicardial fat thickness, NS: non-significant, CA-IMT: carotid artery intima-media thickness

as it releases a wide range of biologically active molecules that modulate vascular smooth-muscle contraction (25). The paracrine effects of these molecules might be attributable to their location being close to the adventitia and extravascular bed (26). Gastaldelli and Basta (27) reported the existence of a link between epicardial fat and hypertension, atherosclerosis and coronary heart disease. Several studies have emphasized the link between EFT and the severity of CAD (28). Epicardial fat has an important role in the inflammatory process within the atherosclerotic plaque (29). Furthermore, it has been shown that epicardial fat products induce increased cell surface expression of adhesion molecules, enhance adhesion of monocytes to coronary artery endothelial cells, and facilitate migration of adherent monocytes (30).

Echocardiographic EFT measurements, provide some advantages when used to assess the cardiometabolic risk. They are objective, quantified, non-invasive, low cost, have routine applicability, avoid exposure to radiation and have a potential for monitoring therapeutic effects. They may also be used as a simple marker for identification of CAH patients with higher cardiovascular risk who may need further cardiac evaluation (31).

In the present study, children with CAH had echocardiographic changes indicating the presence of LV hypertrophy, as indicated by increased LVMI. Moreover, our study showed significant positive correlations between EFT and LVMI that remained significant after regression analysis, which suggested a detrimental effect of EFT excess on the myocardium of patients with CAH. This is in agreement with Corradi et al (32) who reported that EFT levels have an important role in LV hypertrophy. Some mechanisms may be suggested to explain this correlation. It could be assumed that the increased visceral fat directly effects LV output and stroke volume to perfuse the increased body mass. Additionally, the biochemical properties of visceral adipose tissue, such as increased IR, high free fatty acids (FFA) levels, and adrenergic activity, could contribute to LV hypertrophy (33).

In the present study, children with CAH had echocardiographic changes indicating presence of diastolic dysfunction (as evidenced by reduced E/A ratio and prolonged IVRT and mitral DcT). Regression analysis revealed that EFT in CAH patients correlates with mitral DcT. This is in agreement with the study of Van der Meer (34) which showed that myocardial fat has progressive and harmful effects on LV diastolic function. Diastolic dysfunction has been considered as one of the first echocardiographic abnormalities to appear in patients with atherosclerotic cardiovascular disease with a high rate of release of FFA (35), which encounter no physical barrier or fascia before reaching the cardiomyocytes (36). Therefore, the myocardium receives a double dose of FFA from both the epicardial fat and the systemic circulation. Epicardial fat is a source of several bioactive molecules that might directly influence the myocardium (37). In metabolic and cardiovascular disease states, these fat tissues expand, becoming hypoxic and dysfunctional and recruiting phagocytic cells which would lead to a reduction in the production of protective cytokines and, eventually, impaired cardiac function (38,39).

BMI and waist circumference are widely accepted measures of generalized adiposity. However they are poor indicators for visceral obesity. It is well known that visceral adipose tissue

Table 4. The demographic, laboratory and echocardiographic characteristics of patients, according to the degree of control on medical treatment

	Uncontrolled patients (n = 20)	Controlled patients (n = 16)	p value
Age (years)	16.2 ± 0.8	13.1 ± 2.5	0.01
Bone age (years)	17.1 ± 1.2	14.3 ± 2.2	0.01
HOMA-IR	4.42 ± 1.9	2.7 ± 1.9	0.001
hsCRP (mg/L)	452 ± 33.1	216.4 ± 14.6	0.001
17-OHP (nmol/L)	188.3 ± 32.5	8.21 ± 1.3	0.001
Testosterone (ng/dL)	498.8 ± 191.2	34.5 ± 12.7	0.001
LVMI (gm/m^2)	44.4 ± 6.5	32.4 ± 7.2	0.01
EFT (mm)	8.95 ± 1.21	6.66 ± 1.76	0.001
DcT (milliseconds)	187.0 ± 23.0	120.0 ± 25.0	0.01
CA-IMT (mm)	0.54 ± 0.30	0.43 ± 0.02	0.05

Data are expressed as mean ± standard deviation.

HOMA-IR: homeostasis model assessment for insulin resistance, EFT: epicardial fat thickness, 17-OHP: 17-hydroxyprogesterone, hsCRP: high-sensitivity C-reactive protein, LVMI: left ventricular mass index, DcT: deceleration time, CA-IMT: carotid artery intima-media thickness

Table 5. The correlation between epicardial fat thickness and anthropometric, laboratory and echocardiographic data in children with congenital adrenal hyperplasia

Parameter	(r and p values)
Age (years)	+0.639**
SDS-BMI	0.155
Waist circumference (cm)	+0.569**
Waist to height ratio	+0.657*
SBP (mmHg)	+0.432**
DBP (mmHg)	+0.361**
HOMA-IR	+0.562**
hsCRP (mg/L)	+0.389**
Total cholesterol (mg/dL)	0.062
Triglycerides (mg/dL)	0.155
HDL-C (mg/dL)	-0.658*
17-OHP (nmol/L)	+0.743**
Testosterone (ng/dL)	+0.659**
LVMI (gm/m^2)	+0.301*
Mitral DcT (ms)	+0.39**
CA-IMT (mm)	+0.415**

*Indicates $p < 0.05$.

**Indicates $p < 0.01$.

Statistical significance is indicated by asterisks after the correlation coefficient.

SDS-BMI: standard deviation scores of body mass index, LVMI: left ventricular mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, hsCRP: high-sensitivity C-reactive protein, HOMA-IR: the homeostasis model assessment of insulin resistance, HDL-C: high density lipoprotein cholesterol, EFT: Epicardial fat thickness, mitral DcT: mitral deceleration time, CA-IMT: carotid intima media thickness

Table 6. Multivariate correlation coefficients between epicardial fat thickness and various confounding variables in children with congenital adrenal hyperplasia

Confounding variables	Odds ratio	95% CI
Waist circumference (cm)	1.9**	1.45-2.4
17-OHP (nmol/L)	1.6*	1.33-2.89
Testosterone (ng/dL)	1.7**	1.55-2.13
HOMA-IR	1.3*	1.04-1.34
LVMI (gm/m^2)	1.1**	1.08-1.13
Mitral DcT (ms)	1.4**	1.15-2.05
CA-IMT (mm)	2.7**	1.16-1.57

Statistical significance is indicated by asterisks after the odds ratio.

*Indicates $p < 0.05$.

**Indicates $p < 0.01$.

17-OHP: 17-hydroxyprogesterone, EFT: epicardial fat thickness, HOMA-IR: the homeostasis model assessment of insulin resistance, LVMI: left ventricular mass index, mitral DcT: mitral deceleration time, CA-IMT: carotid intima media thickness, CI: confidence interval

accumulation is associated with subclinical atherosclerosis and increased cardiovascular mortality and morbidity. In this study, we found a very good correlation between EFT and waist circumference by multiple regression analysis in children with CAH. However we did not find a significant correlation between BMI-SDS and EFT. These findings suggest that waist circumference is a better anthropometric cardiovascular risk predictor and support the evidence that EFT is related to visceral fat, rather than total adiposity (40). Mavri et al (41) suggested that CA-IMT regression may also be achieved by weight reduction programs. Altin et al (42) suggested that laparoscopic sleeve gastrectomy induced weight loss results in regression of CA-IMT and EFT. Marked adipose mass reduction is associated with dramatic changes in circulating adipokine levels, with leptin reduction and adiponectin increase, thereby leading to a reduced leptin/adiponectin ratio. Of note, such a ratio was found to be directly correlated with CA-IMT in male subjects (43).

Testosterone concentrations were significantly higher in our subjects with CAH compared to controls, particularly in children who were poorly controlled on medical treatment. In addition, testosterone correlated positively and significantly with EFT. Colgecen et al (44) reported that subjects in advanced stages of androgenetic alopecia had higher echocardiographically measured EFT than controls. Moreover, Cakir et al (45) reported a strong positive correlation between testosterone concentrations and EFT in patients with polycystic ovarian disease. This finding suggests that androgen excess may be responsible for the increased EFT in patients with CAH. Physicians treating these patients should be aware that amelioration of androgen excess in patients with CAH should also be considered as a way to prevent cardiovascular symptoms and not only as a tool to improve hyperandrogenic symptoms.

In the present study, children with CAH had higher HOMA-IR than controls. EFT correlated positively and significantly with HOMA-IR by multiple regression analysis. This finding is in agreement with Manco et al (46) who reported that epicardial fat is a significant marker of increased insulin resistance. These observations suggest that epicardial fat is a tissue with high IR (47). EFT is associated with high lipolytic activity, probably because of the reduced antilipolytic effect of insulin in this tissue and an increased expression of B-adrenergic receptors, especially B-3 receptors. Stimulation by these receptors activates lipolysis and increases the release of FFAs which are able to promote blood pressure increase through different pathways, including adrenergic stimulation, increased oxidative stress, endothelial dysfunction and vascular cell growth (48).

Study Limitations

We recognize that this study has some limitations such us; Due to the difficulties in enrolling and studying CAH children, the sample was relatively small in size and included patients with a wide age range. Due to the cross-sectional design, it is difficult to generalize the results to the general population. CA-IMT measurements were performed manually. We were not able to confirm EFT using the standard magnetic resonance imaging methods. However, echocardiographic calculation of epicardial fat has been reported to show good reliability when compared with magnetic resonance epicardial fat measurements (49). Epicardial adipose tissue has a three-dimensional distribution. Therefore 2D echocardiography may not accurately reflect the total amount of epicardial adiposity.

Conclusions

EFT is increased in children with classic CAH, particularly in those with poor control and is correlated with CA-IMT, LV mass and mitral DcT. Measurement of EFT by echocardiography in CAH children may help to identify those at high risk of developing LV dysfunction and subclinical atherosclerosis. Future prospective and multicenter studies are required to confirm our results.

Ethics

Ethics Committee Approval: The study protocol was approved by the Local Ethics Committee of Assiut University Children Hospital, Assiut, Egypt (approval number: 312/2017).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Kotb Abbass Mewalley, Hekma Saad Farghaly, Concept: Kotb Abbass Mewalley, Hekma Saad Farghaly, Design: Kotb Abbass Mewalley, Hekma Saad Farghaly, Data Collection or Processing: Kotb Abbass Mewalley, Hekma Saad Farghaly, Analysis or Interpretation: Kotb Abbass Mewalley, Hekma Saad Farghaly, Abdelrahman Abdelhamid, Literature Search: Kotb Abbass Mewalley, Hekma Saad Farghaly, Writing: Kotb Abbass Mewalley, Hekma Saad Farghaly.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, Meyer-Bahlburg HF, Miller WL, Montori VM, Oberfield SE, Ritzen M, White PC; Endocrine Society. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2010;95:4133-4160.
2. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev 2000;21:245-291.
3. Speiser PW, White PC. Congenital adrenal hyperplasia. N Engl J Med 2003;349:776-788.
4. Amr NH, Ahmed AY, Ibrahim YA. Carotid intima media thickness and other cardiovascular risk factors in children with congenital adrenal hyperplasia. J Endocrinol Invest 2014;37:1001-1008. Epub 2014 Aug 12
5. Marra AM, Improda N, Capalbo D, Salzano A, Arcopinto M, De Paulis A, Alessio M, Lenzi A, Isidori AM, Cittadini A, Salerno M. Cardiovascular abnormalities and impaired exercise performance in adolescents with congenital adrenal hyperplasia. J Clin Endocrinol Metab 2015;100:644-652. Epub 2014 Nov 18
6. Mooij CF, Kroese JM, Claahsen-van der Grinten HL, Tack CJ, Hermus AR. Unfavourable trends in cardiovascular and metabolic risk in paediatric and adult patients with congenital adrenal hyperplasia? Clin Endocrinol(Oxf) 2010;73:137-146. Epub 2009 Aug 29
7. Wasniewska M, Balsamo A, Valenzise M, Manganaro A, Faggioli G, Bombaci S, Conti V, Ferri M, Aversa T, Cicognani A, De Luca F. Increased large artery intima media thickness in adolescents with either classical or non-classical congenital adrenal hyperplasia. J Endocrinol Investig 2013;36:12-15. Epub 2011 Dec 21
8. Fusaro MF, Zanini JL, Silva IN. Increased carotid intima-media thickness in Brazilian adolescents with type 1 diabetes mellitus. Diabetol Metab Syndr 2016;8:74.
9. Iacobellis G, Assael F, Ribaudo MC, Zappaterreno A, Alessi G, Di Mario U, Leonetti F. Epicardial fat from echocardiography: a new method for visceral adipose tissue prediction. Obes Res 2003;11:304-310.
10. Bachar GN, Dicker D, Kornowski R, Atar E. Epicardial adipose tissue as a predictor of coronary artery disease in asymptomatic subjects. Am J Cardiol 2012;110:534-538. Epub 2012 May 10
11. Merke DP, Bornstein SR. Congenital adrenal hyperplasia. Lancet 2005;365:2125-2136.
12. Labarta JI, Bello E, Ruiz-Echarri M, Rueda C, Martul P, Mayayo E, Ferrández Longás A. Childhood-onset congenital adrenal hyperplasia: long-term outcome and optimization of therapy. J Pediatr Endocrinol Metab 2004;17(Suppl 3):411-422.
13. Joint LWPES/ESPE CAH Working Group. Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. J Clin Endocrinol Metab 2002;87:4048-4053.
14. Finkielstain GP, Kim MS, Sinaii N, Nishitani M, Van Ryzin C, Hill SC, Reynolds JC, Hanna RM, Merke DP. Clinical characteristics of a cohort of 244 patients with congenital adrenal hyperplasia. J Clin Endocrinol Metab 2012;97:4429-4438. Epub 2012 Sep 18
15. Diabetes Endocrine Metabolism Pediatric Unit, Cairo University Children's Hospital. Egyptian growth curves 2002. [http://dempuegypt.blogspot.com].
16. Tanner JM, Whitehouse RH. A note on the bone age at which patients with true isolated growth hormone deficiency enter puberty. J Clin Endocrinol Metab 1975;41:788-790.
17. Greulich WW, Pyle SI, Stanford CA. Radiographic Atlas of Skeletal Development of the Hand and Wrist. Calif Med 1959;91:53.

18. Bretones P, Riche B, Pichot E, David M, Roy P, Tardy V, Kassai B, Gaillard S, Bernoux D, Morel Y, Chatelain P, Nicolino M, Cornu C; French Collaborative CAH Growth Study Group. Growth curves for congenital adrenal hyperplasia from a national retrospective cohort. J Pediatr Endocrinol Metab 2016;29:1379-1388.

19. Chen Y, Zhang X, Pan B, Jin X, Yao H, Chen B, Zou Y, Ge J, Chen H. A modified formula for calculating low-density lipoprotein cholesterol values. Lipids Health Dis 2010;9:52.

20. Gutch M, Kumar S, Razi SM, Gupta KK, Gupta A. Assessment of insulin sensitivity/resistance. Indian J Endocrinol Metab 2015;19:160-164.

21. Park MK. Pediatric cardiology for practitioners. Noninvasive techniques (Chap. 6), 5th ed. An imprint of Elsevier, Part2: Special tools in evaluation of cardiac patients Elsevier, Mosby, 2008.

22. Järvisalo MJ, Jartti L, Näntö-Salonen K, Irjala K, Rönnemaa T, Hartiala JJ, Celermajer DS, Raitakari OT. Increased aortic intima-media thickness: a marker of preclinical atherosclerosis in high-risk children. Circulation 2001;104:2943-2947.

23. Mahfouz RA, Alzaiat A, Yousry A. Relationship of epicardial fat thickness with endothelial and cardiac functions in children with family history of type 2 diabetes mellitus. Echocardiography 2015;32:28-33. Epub 2014 Mar 25

24. Baker AR, Silva NF, Quinn DW, Harte AL, Pagano D, Bonser RS, Kumar S, McTernan PG. Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease. Cardiovasc Diabetol 2006;5:1.

25. Bachar GN, Dicker D, Kornowski R, Atar E. Epicardial adipose tissue as a predictor of coronary artery disease in asymptomatic subjects. Am J Cardiol 2012;110:534-538. Epub 2012 May 10

26. Sengul C, Cevik C, Ozveren O, Duman D, Eroglu E, Oduncu V, Tanboga HI, Can MM, Akgun T, Dindar I. Epicardial fat thickness is associated with non-dipper blood pressure pattern in patients with essential hypertension. Clin Exp Hypertens 2012;34:165-170. Epub 2011 Oct 18

27. Gastaldelli A, Basta G. Ectopic fat and cardiovascular disease: what is the link? Nutr Metab Cardiovasc Dis 2010;20:481-490. Epub 2010 Jul 24

28. Nakanishi R, Rajani R, Cheng VY, Gransar H, Nakazato R, Shmilovich H, Otaki Y, Hayes SW, Thomson LE, Friedman JD, Slomka PJ, Berman DS, Dey D. Increase in epicardial fat volume is associated with greater coronary artery calcification progression in subjects at intermediate risk by coronary calcium score: a serial study using non-contrast cardiac CT. Atherosclerosis 2011;218:363-368. Epub 2011 Jul 23

29. Şengül C, Özveren O. Epicardial adipose tissue: a review of physiology, pathophysiology, and clinical applications. Anadolu Kardiyol Derg 2013;13:261-265. Epub 2013 Feb 6

30. Cengel A. Epicardial adipose tissue, metabolic syndrome, inflammation, and cardiovascular risk. Turk Kardiyol Dern Ars 2012;40:696-698.

31. Iacobellis G, Willens HJ. Echocardiographic epicardial fat: a review of research and clinical applications. J Am Soc Echocardiogr 2009;22:1311-1319.

32. Corradi D, Maestri R, Callegari S, Pastori P, Goldoni M, Luong TV, Bordi C. The ventricular epicardial fat is related to the myocardial mass in normal, ischemic and hypertrophic hearts. Cardiovasc Pathol 2004;13:313-316.

33. Iacobellis G, Ribaudo MC, Zappaterreno A, Iannucci CV, Leonetti F. Relation between epicardial adipose tissue and left ventricular mass. Am J Cardiol 2004;94:1084-1087.

34. van der Meer RW, Rijzewijk LJ, Diamant M, Hammer S, Schär M, Bax JJ, Smit JW, Romijn JA, de Roos A, Lamb HJ. The ageing male heart: myocardial triglyceride content as independent predictor of diastolic function. Eur Heart J 2008;29:1516-1522. Epub 2008 May 19

35. Harada M, Tabako S. Carotid atherosclerosis is associated with left ventricular diastolic function. J Echocardiogr 2016;14:120-129. Epub 2016 Jun 30

36. Iacobellis G, Corradi D, Sharma AM. Epicardial adipose tissue: anatomic, biomolecular and clinical relationships with the heart. Clin Pract Cardiovasc Med 2005;2:536-543.

37. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA, Heagerty AM. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. Circulation 2009;119:1661-1670. Epub 2009 Mar 16

38. Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, Meda P, Chizzolini C, Meier CA. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? Arterioscler Thromb Vasc Biol 2005;25:2594-2599. Epub 2005 Sep 29

39. Xu Y, Cheng X, Hong K, Huang C, Wan L. How to interpret epicardial adipose tissue as a cause of coronary artery disease: a meta-analysis. Coron Artery Dis 2012;23:227-233.

40. Janssen I, Heymsfield SB, Allison DB, Kotler DP, Ross R. Body mass index and waist circumference independently contribute to the prediction of nonabdominal, abdominal subcutaneous, and visceral fat. Am J Clin Nutr 2002;75:683-688.

41. Mavri A, Stegnar M, Sentocnik JT, Videcnik V. Impact of weight reduction on early carotid atherosclerosis in obese premenopausal women. Obes Res 2001;9:511-516.

42. Altin C, Erol V, Aydin E, Yilmaz M, Tekindal MA, Sade LE, Gulay H, Muderrisoglu H. Impact of weight loss on epicardial fat and carotid intima media thickness after laparoscopic sleeve gastrectomy: A prospective study. Nutr Metab Cardiovasc Dis 2018;28:501-509. Epub 2018 Feb 9

43. Norata GD, Raselli S, Grigore L, Garlaschelli K, Dozio E, Magni P, Catapano AL. Leptin: adiponectin ratio is an independent predictor of intima media thickness of the common carotid artery. Stroke 2007;38:2844-2846. Epub 2007 Sep 6

44. Colgecen E, Ede H, Erkoc MF, Akyuz Y, Erbay AR. The Relation of Androgenetic Alopecia Severity with Epicardial Fat Thickness. Ann Dermatol 2016;28:205-209. Epub 2016 Mar 31

45. Cakir E, Doğan M, Topaloglu O, Ozbek M, Cakal E, Vural MG, Yeter E, Delibasi T. Subclinical atherosclerosis and hyperandrogenemia are independent risk factors for increased epicardial fat thickness in patients with PCOS and idiopathic hirsutism. Atherosclerosis 2013;226:291-295. Epub 2012 Nov 15

46. Manco M, Morandi A, Marigliano M, Rigotti F, Manfredi R, Maffeis C. Epicardial fat, abdominal adiposity and insulin resistance in obese pre-pubertal and early pubertal children. Atherosclerosis 2013;226:490-495. Epub 2012 Dec 3

47. Marchington JM, Pond CM. Site specific properties of pericardial and epicardial adipose tissue: the effects of insulin and high-fat feeding on lipogenesis and the incorporation of fatty acids in vivo. Int J Obes 1990;14:1013-1022.

48. Fortier M, Wang SP, Mauriège P, Semache M, Mfuma L, Li H, Levy E, Richard D, Mitchell GA. Hormone-sensitive lipase-independent adipocyte lipolysis during β-adrenergic stimulation, fasting, and dietary fat loading. Am J Physiol Endocrinol Metab 2004;287:282-288.

49. Iacobellis G, Assael F, Ribaudo MC, Zappaterreno A, Alessi G, Di Mario U, Leonetti F. Epicardial fat from echocardiography: a new method for visceral adipose tissue prediction. Obes Res 2003;11:304-310.

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0017

J Clin Res Pediatr Endocrinol 2019;11(1):70-75

Effect of Telehealth System on Glycemic Control in Children and Adolescents with Type 1 Diabetes

Esra Döğer, Rukiye Bozbulut, A. Şebnem Soysal Acar, Şebnem Ercan, Aylin Kılınç Uğurlu, Emine Demet Akbaş, Aysun Bideci, Orhun Çamurdan, Peyami Cinaz

Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey

What is already known on this topic?

In the management of diabetic patients, diabetes education and communication with the diabetes team is as important as medical treatment. Telehealth systems facilitate communication with the diabetes team.

What this study adds?

The use of telehealth systems in Turkish children and adolescents with type 1 diabetes mellitus was shown to improve glycemic control.

Abstract

Objective: A close diabetes team-patient relationship is required for establishing satisfactory metabolic control. The purpose of this study was to investigate the effect of a telehealth system on diabetes control.

Methods: The study was carried out between June 2015 and January 2016 at the Gazi University Faculty of Medicine, Pediatric Endocrinology Department. The telehealth system was developed by the diabetes team. The demographic characteristics, frequency of use and hemoglobin A1c (HbA1c) changes of type 1 diabetic (T1DM) patients using this communication network were analysed.

Results: Eighty two patients [43 (52.4%) females, mean (± standard deviation) age 10.89 ± 4 years] used the telehealth system. Fourteen (17.1%) of the cases were on pump therapy and 59 (72.0%) were counting carbohydrates. The individuals with diabetes or their families preferred WhatsApp communication. Whatsapp provided a means for instant messaging in most instances (57.3%), contact with diabetes education nurse (32.9%) and consultation with the diabetes team about insulin doses and blood glucose regulation (42.7%). HbA1c values after six months were significantly lower in patients/parents calling frequently ($p < 0.001$) compared with HbA1c values recorded at the beginning of the study.

Conclusion: Increase in frequency of counselling by the diabetes team led to improved blood glucose control in T1DM patients. A telehealth system is useful for early detection of the need for changes in treatment and for intervention. It also promoted better self care.

Keywords: Type 1 diabetes, telehealth, diabetes team, HbA1c

Introduction

Diabetes management is clinically demanding (1). Insulin treatment, healthy eating appropriate for the diabetic state and physical activity are important in diabetes management. Diabetic patients need to learn how to keep these factors in balance (2). A close health professional-patient relationship, individualized care and education are essential for attaining good glycemic control in diabetic patients (3). Educational interventions in children and adolescents with diabetes are known to enhance glycemic control and psychosocial health (4).

Diabetes education should involve both the diabetic child and his/her family and should be provided by a team of professionals including the physician, nurse, dietitian and psychologist (5). To maintain their blood glucose at normal levels and thus prevent complications of diabetes, these patients should be able to contact the diabetes team frequently and easily and be able to access health services

Address for Correspondence: Rukiye Bozbulut MD, Gazi University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey
E-mail: dyt_rukiye@hotmail.com **ORCID ID:** orcid.org/0000-0003-4317-9226

Conflict of interest: None declared
Received: 07.02.2018
Accepted: 17.07.2018

at all times (2,3). However, reasons such as failure to increase the number of health professionals in line with the increase in the number of patients with diabetes has led to decreased quality of service for providing a full care package, including the educational interventions necessary for the self-management of patients (6,7). Inadequacies in the training of the medical team may be another factor contributing to poorer levels of service offered in diabetic care (8). Moreover, diabetic children residing in rural areas are faced with greater difficulty in accessing healthcare (2,6). Providing health information using newer communication technologies will help to overcome some of the difficulties cited above. This type of rapid communication provides greater flexibility in time and means for contacting the healthcare team, thus decreasing the number of patients presenting to outpatients. It will also lead to a decrease in the patient education tasks required from physicians and eventually to a decrease in the need for hospital care and lower financial costs (9,10,11,12).

The use of information technology and providing online education are recommended in undergraduate and postgraduate educational programs for all health workers, including continuing education programs (2). Ever more widespread use of the internet enables its greater use in the field of education (13). Web-based education should also be provided because it provides an accessible and permanent record of information for patients (14). Web-based applications also enable fast and effective communication with health professionals. Internet communication programs also serve rural patients, allowing them to contact health professionals from their home environment, thus decreasing the time and expenses spent in transportation (6,15,16). Another positive feature of these programs is that the patient can receive feedback and suggestions in line with his/her requirements very rapidly. Moreover, it has been reported that communications between the diabetes team and diabetic patients by phone and/or text messaging (SMS) using cellular phones increased compliance to treatment and enhanced glucose control (17,18,19).

The purpose of this study was to assess the effects of counselling services offered to diabetics by the diabetes team via communication networks (internet, phone) on diabetes control.

Methods

The study was conducted between June 2015 and January 2016 at the Gazi University Faculty of Medicine Pediatric Endocrinology Department, Ankara. The telehealth system was developed in-house by the diabetes team. The demographic characteristics, frequency of use and hemoglobin A1c (HbA1c) changes over six months of use using this communication network were recorded for type 1 diabetic (T1DM) patients.

The telehealth system used in the study was developed by a pediatric diabetes team which included a nurse, dietician, psychologist and physician. Counselling was conducted via communication networks including the internet and smart phones. Counselling hours were scheduled for 11:30-14:30 and 21:00-24:00 hours. Oral communications were recorded for later analysis.

The purpose of the study was explained to each participant and written informed consent was obtained. The study procedures were in accordance with the Declaration of Helsinki. Prior to the study, families consented to statistical analysis and publishing of the data with the exception of any confidential communications. The study protocol was approved by Gazi University Ethics Committee (with the approval number: 583.10.09.2018).

The analyses were based on variables such as call frequency, duration of the diabetes, use of infusion pump or carbohydrate counting. We obtained the mean from frequency distribution of the patients who reached us and we grouped them according to it. Patients/parents calling daily, 5-6 times a week, 1-2 weekly or once every 15 days were identified as the frequent caller group, while those calling once a month, every two months or once every three months were identified as the infrequent caller group.

Demographic characteristics and patient history information were collected from patient files. HbA1c levels at baseline and at follow-up were evaluated.

Statistical Analysis

Statistical analyses were carried out by using the Statistical Package for Social Sciences (SPSS version 21.0, IBM Inc., Chicago, Ill., USA). The conformity to normal distribution of numerical data (age and HbA1c values) was tested with the Shapiro-Wilk test.

In the comparison of the numerical data of dependent paired groups (HbA1c values changes after six months for both frequent and rare call groups), the paired t-test was used for data with normal distribution.

Patients were grouped according to HbA1c levels after six months ($<7.5\%$, 5-9% and $>9\%$ group), rare and frequent call groups on HbA1c levels after six months.

To examine the effects on HbA1c levels after six months of the call frequency groups, cross-reference tables were formed and evaluation was made with the chi-square test.

For categorical data such as demographic and communication features, tables were formed and values were presented as number of cases and percentage. For all the statistical calculations a value of $p < 0.05$ was accepted as statistically significant.

Results

The study was conducted on diabetic children aged between two and 18 years (mean ± standard deviation = 10.89 ± 4.00) consisting of 43 girls (52.4%) and 39 boys (47.6%) of comparable ages.

Eleven (13.4%) of the participants were newly diagnosed diabetics (< 1 year), 36 (43.9%) were patients with diabetes for 1-3 years, 19 (32.3%) for 4-6 years, and 16 (19.5%) had been diagnosed ≥7 years earlier. In the study group, 14 (17.1%) were using pumps while 59 (72.0%) counted carbohydrates. Of the participants 78 (95.1%) attended their check-up visits regularly and 11 (13.4%) had suffered from episodes of ketoacidosis after diagnosis.

Parental data are given in Table 1.

In Table 2, the communication network used by the children or by other family members contacting the diabetes team, frequency of counselling, the most consulted diabetes team members and the purpose of the call are shown.

There was a preference for using WhatsApp (57.3%) which is an instant messaging service, to contact the diabetes education nurse (32.9%) or consult with the diabetes team most frequently (42.7%) on their insulin dose and glucose regulation. Frequency of use of WhatsApp (28.0%) was higher than the other means of communication.

In Table 3, HbA1c levels were compared between the call frequency groups (frequent versus infrequent callers, defined above). It was found that HbA1c values at baseline compared with after six months were lower in patients calling frequently ($p < 0.001$).

Table 4 shows that HbA1c levels of the diabetic patients consulting with the diabetes team by using the telehealth method decreased significantly after six months. It was also observed that 32/36 (89%) of individuals with diabetes

Table 1. Demographic data of the parents

	Mother	Father
Mean (± SD) age (years)	36.1 ± 5.9	40 ± 6.5
Education n (%)		
Primary school	24 (29.3)	14 (17.1)
Secondary school	10 (12.2)	7 (8.5)
High school	30 (36.6)	36 (43.9)
University	16 (19.5)	22 (26.8)
Postgraduate education	2 (2.4)	3 (3.7)
Working status n (%)		
Working	27 (32.9)	82 (100)
Housewife	55 (67.1)	-

SD: standard deviation

Table 2. Communication features

Communication network used	n	%
WhatsApp	47	57.3
Phone	24	29.3
Short message service	11	13.4
People who contacted the diabetes team		
Mother	53	64.6
Patient	20	24.4
Father	7	8.5
Sister/brother	1	1.2
Teacher	1	1.2
Frequency of counselling		
Every day	23	28.0
2-3 times a week	22	26.8
Every 15 days	16	19.5
Once a month	13	15.9
Once every 3 months	8	9.8
Most consulted member of the diabetes team		
Diabetes education nurse	27	32.9
Nurse + physician	18	22.0
Nurse + diabetes dietician	14	17.1
Physician + nurse + diabetes dietician	14	17.1
Nurse + psychologist	6	7.3
Nurse + diabetes dietician + psychologist	2	2.4
Counselling topics		
Insulin dose and blood glucose regulation	35	42.7
Carbohydrate count	24	29.3
Hyperglycemia and hypoglycemia	14	17.1
Spreading in pump		
Technical problems relating to instruments		
Doses and diet on special occasions		
Insulin doses in case of fever, diarrhea and vomiting	9	10.1

Table 3. Hemoglobin A1c values by frequency of calling

Frequency of telehealth use	Initial HbA1c (mean ± SD)	HbA1c after 6 months (mean ± SD)	p
Rarely (n = 34)	9.10% ± 1.26	9.28% ± 1.25	0.172
Frequently (n = 48)	8.30% ± 1.16	7.45% ± 0.87	< 0.001

Paired T test was used for comparison, HbA1c: hemoglobin A1c, SD: standard deviation

Table 4. Comparison of call frequency groups (rare vs. frequent) on hemoglobin A1c levels after six months

	HbA1c			p
	< 7.5%	7.5-9%	> 9%	
Frequent calls (n)	32	10	6	< 0.001
Rare calls (n)	4	10	20	

Chi-square test was used for comparison, HbA1c: hemoglobin A1c

whose HbA1c value was lower than 7.5% consulted frequently while only 6/26 (23%) of patients whose HbA1c value was > 9% consulted frequently.

Discussion

The use of communication technologies as a means of providing healthcare services has increased the ability of patients living far from health centres to obtain information and advice and removed many of the barriers for other patients trying to access healthcare services (20). The telehealth system enables the use of information storage and delivery in the form of audio, image, speech or video through a range of communication technologies such as landline, satellite connection and cellular phone. This system provides duplex audiovisual communication between health professionals and patient (21).

In this study, communication between pediatric T1DM patients, their families and the diabetes therapy team via WhatsApp, phone and SMS was investigated. The results indicate that the diabetes team was contacted most frequently via WhatsApp, an intermessaging and file transfer/sending program which uses the internet (57.3%) and phone (23.2%) (Table 2). Whatsapp is used in numerous fields including in the field of education. "We conjecture that the reason why the children and their families prefer WhatsApp is that they can easily communicate with the diabetes team via SMS, a voice mail system and audio and video calls using wifi connectivity. An additional and important benefit is that they can immediately share photos showing their blood glucose values. It was found that most frequently (64.6%) it is the mothers of diabetic children who communicate with the diabetes team. The second most frequent contact was directly from the diabetic patient (Table 2).

An American Diabetes Association (2014) report, emphasizes that the acute and chronic complications of diabetes can be prevented or delayed in diabetics who receive education on self-management and who are being supported regularly (22). Diabetes-oriented education increases the quality of life and glycemic control is also improved to an optimal level in T1DM children and adolescents (23). However, it is also important that this education be continuous and that it is reinforced at frequent intervals to be effective in terms of glycemic control (24).

In recent years, the telehealth system was introduced for use in the treatment of chronic diseases such as diabetes. This system is both flexible and cost effective. The patients can benefit from one-to-one education sessions, offering social support and information on self-management (25). In this study, a decline in HbA1c values was observed as the frequency of consulting sessions with the diabetes team increased. While the initial mean HbA1c value of frequent callers was $8.30 \pm 1.16\%$, their mean HbA1c decreased to $7.45 \pm 0.87\%$. In contrast, while the initial HbA1c mean value of the rare callers group was $9.10 \pm 1.26\%$, after six months their mean HbA1c levels increased to $9.28 \pm 1.25\%$. In the study carried out by Thompson et al (17), T1DM were contacted by phone for 15 minutes over the course of three weeks and additionally when necessary and insulin dose adjustments were made by the diabetes nurse. They reported significant reduction in HbA1c values of the patients at the end of six months. Our results are consistent with this. Xu et al (26) reported that the telehealth system they applied to individuals with T1DM reduced the mean HbA1c and glucose variability, and that the specific diabetes care provided by this method was associated with time and cost savings and compliance with diabetes treatment. In a study reported by Kwon et al (3), in which diabetic patients were able to contact the diabetes team using SMS and the internet, a significant decrease in HbA1c values was reported after three months. Likewise, in the study carried out by Bin-Abbas et al (19) daily information messages regarding the procedures related to diabetes care, weekly interactive messages and optional multimedia messages were sent to diabetic patients. Significant decreases in postprandial glucose and HbA1c levels, in the frequency of hypoglycemic episodes and in blood glucose levels were observed.

It has been reported that web-based telehealth services improve patient satisfaction, patient compliance to diabetes treatment and clinical results (19,27). Telehealth systems which include individualized evaluations, supervision and skills development by feedback are reported to be more effective in improvement of glycemic control. In this present study, it was found that the most frequently consulted therapy team member was the diabetes nurse and the most frequently issue consulted about was insulin dose and blood glucose regulation (42.7%). Questions concerning carbohydrate counting

(29.3%) and appropriate actions to be taken in the case of hyperglycemia and hypoglycemia (17.1%) were next most frequent. Questions about methods of insulin injection, technical problems with instruments, such as pump failure, message failure, battery running out, set change or blockage in set, adjustments in insulin dose on special days, for example birthdays, weddings or picnics and in special cases such as intercurrent illness were the least often consulted topics (10.1%; see Table 2). We hypothesise that the diabetes team making suggestions, providing information and giving online support to the patients over six months provided greater continuity of communication with the patients than previously and led to improved self-esteem. It was also noted that this continuity in communication ensured that the changes in the patient's status were detected earlier than was previously happening and appropriate interventions were more rapid. It was observed that 88.9% of 36 individuals with diabetes whose HbA1c value after six months was below 7.5% had called frequently while 76.9% of those whose HbA1c value was above 9.5% had called rarely.

Study Limitations

The short duration of the study could be construed as a limitation although significant differences were detected and have been detected in shorter duration studies than this one (3). The limited number of cases constitute a definite limitation of the study. A final limitation was that the our telehealth system did not belong to our institution and was not accessible 24 hours a day.

Conclusion

Increased frequency of consulting with the diabetes team improved the control of blood glucose levels of T1DM patients. Guidelines published in recent years emphasize individualized treatment and a multidisciplinary approach in diabetes management. A telehealth system developed by the diabetes team was a useful system in early detection of problems and facilitated rapid intervention, thus enhancing the self-care.

In general we suggest that there is a need to establish an institutional system to provide a regular, effective tele-education service. The system should allow the whole team to access and monitor the patient's entries simultaneously for a synchronized intervention.

Acknowledgements

We thank all patients and their parents for participating in this study.

Ethics

Ethics Committee Approval: The study was approved by the Gazi University Ethics Committee (the approval number: 583.10.09.2018).

Informed Consent: Consent form was filled out by the parents of all children and adolescents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: Esra Döğer, Aylin Kılınç Uğurlu, Emine Demet Akbaş, Aysun Bideci, Orhun Çamurdan, Peyami Cinaz, Concept: Esra Döğer, Şebnem Ercan, Rukiye Bozbulut, A. Şebnem Soysal Acar, Design: Esra Döğer, Rukiye Bozbulut, A. Şebnem Soysal Acar, Data Collection or Processing: Şebnem Ercan, Rukiye Bozbulut, A. Şebnem Soysal Acar, Analysis and Interpretation: A. Şebnem Soysal Acar, Rukiye Bozbulut, Esra Döğer, Literature Search: Rukiye Bozbulut, Esra Döğer, Emine Demet Akbaş, Aylin Kılınç Uğurlu, Writing: Rukiye Bozbulut, Esra Döğer, A. Şebnem Soysal Acar, Emine Demet Akbaş.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Nylander C, Tindberg Y, Haas J, Swenne I, Torbjörnsdotter T, Akesson K, Örtqvist E, Gustafsson J, Fernell E. Self-and parent-reported executive problems in adolescents with type 1 diabetes are associated with poor metabolic control and low physical activity. Pediatr Diabetes 2018;19:98-105. Epub 2017 Mar 20
2. Ayatollahi H, Hasannezhad M, Fard HS, Haghighi MK. Type 1 diabetes self-management: developing a web-based telemedicine application. Health Inf Manag 2016;45:16-26.
3. Kwon HS, Cho JH, Kim HS, Lee JH, Song BR, Oh JA, Han JH, Kim HS, Cha BY, Lee KW, Son HY, Kang SK, Lee WC, Yoon KH. Development of web-based diabetic patient management system using short message service (SMS). Diabetes Res Clin Pract 2004;66(Suppl 1):133-137.
4. Lange K, Swift P, Pankowska E, Danne T. Diabetes education in children and adolescents. Pediatr Diabetes 2014;15(Suppl 20):77-85.
5. Silverstein J, Klingensmith G, Copeland K, Plotnick L, Kaufman F, Laffel L, Clark N; American Diabetes Association. Care of children and adolescents with type 1 diabetes: a statement of the American Diabetes Association. Diabetes Care 2005;28:186-212.
6. Azar M, Gabbay R. Web-based management of diabetes through glucose uploads: Has the time come for telemedicine? Diabetes Res Clin Pract 2009;83:9-17. Epub 2008 Dec 3
7. Kim HS. A randomized controlled trial of a nurse short-message service by cellular phone for people with diabetes. Int J Nurs Stud 2007;44:687-692. Epub 2006 Apr 17
8. Tao D, Or CK. Effects of self-management health information technology on glycaemic control for patients with diabetes: a meta-analysis of randomized controlled trials. J Telemed Telecare 2013;19:133-143. Epub 2013 May 23

9. Heidgerken AD, Lewin AB, Geffken GR, Gelfandw KM, Storch EA, Malasanos T. Online diabetes education: design and evaluationwith prospective diabetes camp counsellors. J Telemed Telecare 2005;11:93-96.

10. Newton KT, Ashley A. Pilot study of a web-based intervention for adolescents with type 1 diabetes. J Telemed Telecare 2013;19:443-449. Epub 2013 Nov 6

11. Whittemore R, Jaser SS, Jeon S, Liberti L, Delamater A, Murphy K, Faulkner MS, Grey M. An Internet Coping Skills Training Program for Youth With Type 1 Diabetes: six-month outcomes. Nurs Res 2012;61:395-404.

12. Esmatjes E, Jansà M, Roca D, Pérez-Ferre N, del Valle L, Martínez-Hervás S, Ruiz de Adana M, Linares F, Batanero R, Vázquez F, Gomis R, de Solà-Morales O; Telemed-Diabetes Group. The Efficiency of Telemedicine to Optimize Metabolic Control in Patients with Type 1 Diabetes Mellitus: Telemed Study. Diabetes Technol Ther 2014;16:435-441. Epub 2014 Feb 14

13. Dougherty JP, Lipman TH, Hyams S, Montgomery KA. Telemedicine for Adolescents With Type 1 Diabetes. West J Nurs Res 2014;36:1199-1221. Epub 2014 Apr 1

14. Nguyen HQ, Carrieri-Kohlman CV, Rankin SH, Slaughter R, Stulbarg MS. Internet-based patient education and support interventions: A review of evaluation studies and directions for future research. Comput Biol Med 2004;34:95-112.

15. Kyngas H. Patient education: perspective of adolescents with a chronic disease. J Clin Nurs 2003;12:744-751.

16. Mcmahon GT, Gomes HE, Hohne SH, Ming-Jye Hu T, Levine BA, Conlin PR. Web-Based Care Management in Patients With Poorly Controlled Diabetes. Diabetes Care 2005;28:1624-1629.

17. Thompson DM, Kozak SE, Sheps S. Insulin adjustment by a diabetes nurse educator improves glucose control in insulin-requiring diabetic patients: a randomized trial. CMAJ 1999;161:959-962.

18. Baumer JH, Hunt LP, Shield JP. Audit of diabetes care by caseload. Arch Dis Child 1997;77:102-107.

19. Bin-Abbas B, Jabbari M, Al-FaresA, El-Dali A, Al-Orifi F. Effect of mobile phone short text messages on glycaemic control in children with type 1 diabetes. J Telemed Telecare 2014;20:153-156. Epub 2014 Mar 18

20. Öz F. Sağlık Alanında Temel Kavramlar. Hemşirelik. 2. Baskı, Ankara, Mattek Matbaacılık, 2010;44-48.

21. Miller EA. Solving the disjuncture between research and practice: Telehealth trends in the 21st century. Health Policy 2007;82:133-141. Epub 2006 Oct 13

22. American Diabates Association. Standards of medical care in diabetes-2014. Diabetes Care 2014;37(Suppl 1):14-80.

23. Abolfotouh MA, Kamal MM, El-Bourgy MD, Mohamed SG. Quality of life and glycemic control in adolescents with type 1 diabetes and the impact of an education intervention. Int J Gen Med 2001;4:141-152.

24. International Society for Pediatric and Adolescence Diabetes (ISPAD). (2011). Global IDF/Ispad Guideline For Diabetes in Childhood and Adolescence: Diabetes in Adolecent. 6th. Edition. Date accessed: 19.06.2014. Available from: https://cdn.ymaws.com/www.ispad.org/resource/resmgr/ISPAD_News/idf-ispad_diabetes_in_childh.pdf

25. Franc S, Daoudi A, Mounier S, Boucherie B, Dardari D, Laroye H, Neraud B, Requeda E, Canipel L, Charpentier G. Telemedicine and diabetes: achievements and prospects. Diabetes Metab 2011;37:463-476. Epub 2011 Sep 1

26. Xu T, Pujara S, Sutton S, Rhee M. Telemedicine in the Management of Type 1 Diabetes. Prev Chronic Dis. 2018;15:E13.

27. Pérez-Ferre N, Galindo M, Fernández MD, Velasco V, de la Cruz MJ, Martín P, del Valle L, Calle-Pascual AL. A Telemedicine system based on Internet and short message service as a new approach in the follow-up of patients with gestational diabetes. Diabetes Res Clin Pract 2010;87:15-17. Epub 2009 Dec 30

ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2018.2018.0108

J Clin Res Pediatr Endocrinol 2019;11(1):76-81

Remarkable Increase in the Prevalence of Overweight and Obesity Among School Age Children in Antalya, Turkey, Between 2003 and 2015

Gamze Çelmeli[1], Yusuf Çürek[1], Zümrüt Arslan Gülten[2], Mehmet Yardımsever[3], Mustafa Koyun[2], Sema Akçurin[4], İffet Bircan[4]

[1]University of Health Sciences Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Turkey
[2]Akdeniz University Faculty of Medicine, Department of Pediatrics, Antalya, Turkey
[3]Akdeniz University Faculty of Medicine, Department of Biostatistics and Medical Informatics, Antalya, Turkey
[4]Akdeniz University Faculty of Medicine, Department of Pediatric Endocrinology, Antalya, Turkey

What is already known on this topic?

The increase in prevalence of obesity and overweight among children and adolescents is a major global public health problem in both developed and developing countries.

What this study adds?

This is a 12-year interval study referring to our previous study conducted in 2003. Our findings suggest that the prevalence of OW and OB in school-age children living in the same geographical region of Turkey has increased around three-fold.

Abstract

Objective: Childhood obesity (OB) is an acknowledged global problem with increasing prevalence reported around the world. We conducted this study with the aim of determining the local trend in OB and overweight (OW) prevalence in the last decade and to observe the alteration of OB and OW prevalence by age group. An additional aim was to construct new age- and gender-specific body mass index (BMI) reference percentile charts for Turkish children living in the city center of Antalya.

Methods: This cross-sectional study included 1687 school aged children. International Obesity Task Force guidelines were used to determine the OB and OW prevalence. OW was defined as a BMI between 85th and 95th percentile, and OB > 95th percentile. The data were compared with a previous study carried out in the same region in 2003. The least mean square method was used to construct the BMI reference percentile charts.

Results: The prevalence rates for OB and OW were 9.8% and 23.2%, respectively, with a combined OW/OB rate of 33%. OB prevalence was higher in boys than girls ($p < 0.05$). The prevalence of combined OW/OB was highest at age 9-10 years. The prevalence of OB has increased 2.9 times during twelve years in this location.

Conclusion: Comparing the current findings with rates of OW and OB in the previous decade, childhood OB in Antalya has reached alarming levels. Urgent measures integrated into the national education system should be taken to prevent OB. In addition more surveillance studies should be planned to show the future trend of OB prevalence nationally.

Keywords: Obesity, prevalence, school age children, Turkey

Introduction

During the last few decades, the number of obese (OB) and overweight (OW) children and adolescents has significantly increased in both developed and developing countries. This change poses a major public health threat, globally (1).

From 1980 to 2013, the prevalence of combined OW/OB among children and adolescents in developed countries has risen from 16.9% to 23.8% in boys and from 16.2% to 22.6% in girls. In developing countries, the prevalence at these ages has also increased from 8.1% in 1980 to 12.9% in 2013 among boys, and 8.4% to 13.4% among girls (2). In

Address for Correspondence: Gamze Çelmeli MD, University of Health Sciences Antalya Training and Research Hospital, Clinic of Pediatric Endocrinology, Antalya, Turkey
Phone: +90 242 249 44 00 **E-mail:** gcelmeli@hotmail.com **ORCID ID:** orcid.org/0000-0002-6768-6700

Conflict of interest: None declared
Received: 12.04.2018
Accepted: 20.09.2018

2016, it was estimated that 50 million girls and 74 million boys worldwide were OB (3).

Although there are many reports from different regions of Turkey, there is no nationwide systematic study investigating OB trends in Turkish children. Alper et al (4) reported, in a meta-analysis of 58 publications from Turkey, an increase in prevalence of OB from 0.7% in 1990-1995 to 7.1% in 2011-2015 (1.2% to 6.8% for girls, 0.3% to 7.4% for boys). Bereket and Atay (5) reported that OW and OB prevalence was higher in the western regions of Turkey where the population generally has a higher socioeconomic status (6).

The primary aim of this study was to determine the prevalence of OW and OB among school aged children in Antalya, Turkey, and to compare our data with those of a similar study conducted in 2003 in the same region (7). The data from this study will also enable the creation of age- and sex-specific body mass index (BMI) reference percentile charts and BMI curves for Turkish children living in the city center of Antalya.

Methods

Data collection for this cross-sectional study was carried out in March-April 2015. The study included children from 58 out of the 124 schools located in the Muratpaşa district of Antalya city, a district with a relatively high socio-economic level population. From a total of 61092 school children, 1687 healthy children (873 boys and 814 girls) aged between 6-14 were selected for the study using a population-based, stratified, cluster-sampling method.

Written permits for the study were obtained from Antalya Provincial Directorate of Health and Antalya Province National Education Directorate. Informed consent was obtained from all students and their parents. The study was approved by the Ethics Committee of Akdeniz University (decision no: 108, date: 25.02.2015).

The ages of all participants were calculated from the day of data collection according to their date of birth to calculate decimal age. Decimal ages were grouped in years, for example; 6 years (6-6.99 years). Weight was measured with light clothes and without shoes, using a digital portable scale and was rounded up to the nearest 100 g. Height was measured with the subjects standing in the Frankfurt plane, using a laser rangefinder (BOSCH, Leinfelden-Echterdingen, Germany) calibrated with Harpenden stadiometer, sensitive to the nearest 0.1 cm. BMI was calculated as weight/ height² (kg/m²). Age and gender specific International Obesity Task Force references as defined by Cole et al (8) were used to determine the prevalence of OW and OB. OW was defined as BMI between 85th and 95th percentile and OB as BMI above the 95th percentile. To be able to perform comparisons with the current study, the data of 1775 children aged 6-14 years were selected from a previous study with adjustments for age and sex (7).

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences version 22 software (IBM Inc., Chicago, Ill., USA). Differences between categorical variables were tested by the Pearson and Fisher chi-square tests, while BMI values were compared with the z-test.

We used the LMS method to construct age and gender specific BMI reference percentile charts and BMI curves for Turkish children living in the city center of Antalya (9). In this method, L represents the skewness, M represents the median and S represents the coefficient of variation of the data. The BMI centile curves were smoothed by using the distance-weighted least squares procedure.

Results

The overall prevalence of combined OW/OB in the current study was 33 %, while the prevalence figures for OB and OW were 9.8% and 23.2%, respectively. There was no significant difference between boys and girls for OW prevalence. However, OB prevalence was higher in boys (11.3%) than in girls (8.1%) ($p < 0.05$; see Table 1). The prevalence of combined OW/OB was also higher in boys (35.2 %) than in girls (30.6 %). The distribution of OB and

Table 1. Frequency of overweight and obese children of ages 6-14 years in Antalya in 2015

Gender	Overweight n (%)	Obese n (%)	OW + OB n (%)	Non OW/OB n	Total n
Boys	209 (23.9)*	99 (11.3)**	308 (35.2)**	565	873
Girls	183 (22.5)*	66 (8.1)**	249 (30.6)**	565	814
Total	392 (23.2)	165 (9.8)	557 (33)	1130	1687

*$p > 0.05$, **$p < 0.05$ comparing boys with girls.

OW: overweight, OB: obese

OW prevalence for all children according to age group is shown on Figure 1. We observed that the prevalence of combined OW/OB increased rapidly from seven years to nine years of age ($p < 0.05$), formed a plateau between the ages of nine and 10 years and then decreased from the age of 10 onwards. The prevalence of OB and OW by age group is depicted in Figure 2 for each gender separately. The prevalence of combined OW/OB was found to increase with age between six and 10 years among girls ($p < 0.05$), while a rapid increase in prevalence was found from seven to nine years in boys ($p < 0.05$). The peak prevalence of combined OW/OB was at 10 years of age in girls (38.8%) and at nine years in boys (47.1%). The prevalence of OB alone was not statistically significant when age groups were compared.

Mean BMI (± standard deviation) values and the cutoff points of BMI, obtained by using the LMS method for OW and OB (85th and 95th percentiles) by age group are shown in Table 2. The BMI centile curves were also generated using the LMS method (data not shown).

The prevalence of combined OW/OB was found to increase up to 1.8-fold (from 18% to 33%) from 2003 to 2015, while OB prevalence showed a 2.9-fold increase during the same period (3.4% to 9.8%) (Table 3).

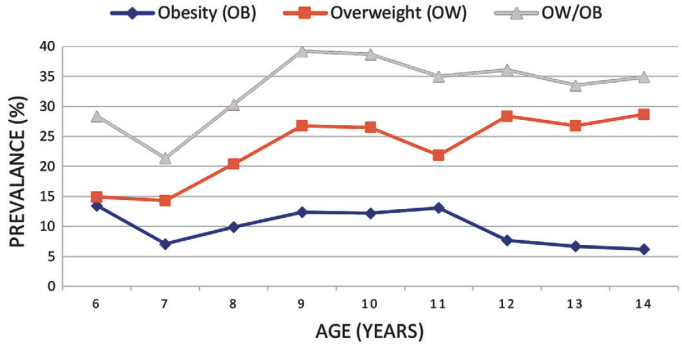


Figure 1. The prevalence of obesity and overweight combining both genders in by age group, in Antalya, Turkey

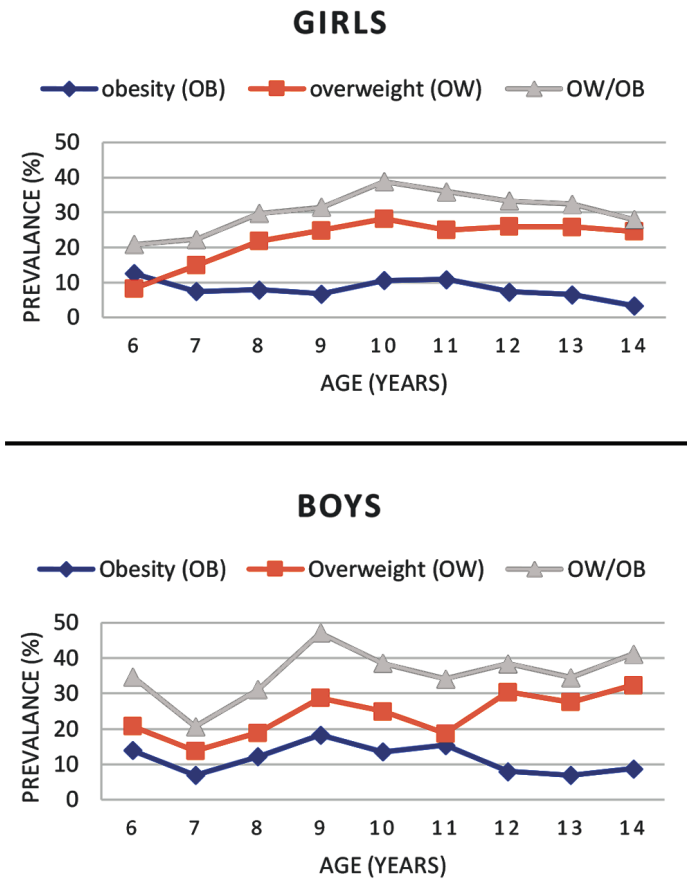


Figure 2. The prevalence of obesity and overweight among girls and boys by age group in Antalya, Turkey

Table 2. Body mass index percentiles of children aged 6-14 years, in Antalya, Turkey

Age	Girls				Boys			
	n	BMI (mean ± SD)	Overweight (85th perc.)	Obese (95th perc.)	n	BMI (mean ± SD)	Overweight (85th perc.)	Obese (95th perc.)
6	71	17±2.7	18.9	19.8	72	17.1±2.9	19.3	20.5
7	94	17.2±2.4	19.4	20.5	116	17.7±2.5	20.4	21.8
8	101	17.6±3.2	20.3	21.8	90	18.3±3.4	21.4	23.1
9	105	18.2±3.0	21.4	23.3	104	18.9±4.0	22.4	24.3
10	85	19.0±3.7	22.6	24.9	104	19.5±4.0	23.2	25.4
11	92	19.8±4.2	23.8	26.3	91	20.1±4.1	24.0	26.4
12	96	20.7±3.9	24.8	27.3	112	20.7±3.7	24.7	27.2
13	108	21.6±3.8	25.4	27.6	116	21.1±3.6	25.2	27.8
14	61	22.3±3.9	25.4	27.2	68	21.5±4.1	25.6	28.2

SD: standard deviation, BMI: body mass index

Table 3. Obesity and overweight figures for 6-14 years old school children in Antalya in 2003 and 2015

	2003*	2015	p
Number	1775	1687	-
Age (years)	6-14	6-14	-
Gender (girls/boys)	867/908	814/873	0.05
OB (%)	3.4	9.8	< 0.001
OW (%)	14.6	23.2	< 0.001
OB/OW (%)	18	33	< 0.001

*Reference 7.

OW: overweight, OB: obese

Discussion

This study has merit because it is one of the most recent studies in Turkey investigating OW and OB prevalence and its trend among school children residing in the same geographical region by age and sex. This study was performed as a sequel to our previous cross-sectional study, conducted in 2003. The comparison shows that the prevalence of combined OW/OB has increased nearly twofold (from 18% in 2003 to 33% in 2015) and the prevalence of OB alone has increased nearly threefold (from 3.4% to 9.8%) during the course of 12 years (2003-2015) in Antalya, Turkey. Although there are several reports which have shown a plateau or a decreasing trend of childhood OW and OB in recent years from some countries including the United Kingdom (10), Ireland (3,11), France (12), Sweden (13), Italy (14), Germany (15), Australia (16) and the United States (17) the general prevalence trend for OW and OB is increasing among children and adolescents in both developed and developing countries, as is the case in Turkey (2,4,18). Alper et al (4) showed in a meta-analysis that the overall prevalence of OB in Turkey is 7.3% among school aged children (6.8 % in girls, 7.4 % in boys). However, the prevalence of OW and OB in our study appears to be much higher than in other regions of Turkey (4,5,19,20). A possible cause of this difference may be the fact that the current study is one of the latest in the literature and reflects the upward trend in Turkish OB. A further possible reason may be that the study was conducted in a region with high socioeconomic status. The ratios we report are very high compared to the literature and even higher than those of developed countries (2).

In our 2003 study, we reported that there was no difference in OB rates between girls and boys, while OW prevalence was higher in girls. This situation has changed. OB has become significantly more common in boys (11.3%) than girls (8.1%) ($p < 0.05$) and that OW prevalence is similar in both sexes (23.9% in boys, 22.5% in girls). Alper et al (4) also reported that the prevalence of OB increased markedly from 1.2% to 6.8% in girls and from 0.3% to 7.4% in boys over a longer period in Turkey; between 1990-1995 and 2011-2015. The trend of increase in boys was also higher than girls in this study which suggests that, in recent years, boys have become more likely to be OB than girls on a national scale. It is not known why the prevalence of OB in boys increases faster than girls. A study from the Netherlands also showed a noticeable increase in the OW and OB prevalence among Turkish children living in the Netherlands although children of Moroccan, Surinamese, South Asian and Dutch descent showed no similar trends. This finding was more pronounced among Turkish boys than Turkish girls with only a mild increase in OB prevalence in the girls from 1999 through 2007 (21).

When analyzed according to age groups, we observed that the prevalence of combined OW/OB increased rapidly the mid-childhood years and appeared to plateau in late childhood. While the prevalence of OB gradually decreased after age 11, the prevalence of OW did not. Koca et al (22) reported that the prevalence of OB in children under 11 years of age was higher than that of older children in Isparta, a city located in the south-west of Turkey in line with our results. We found that girls reach the highest prevalence of combined OW/OB at 10 years of age while boys arrive at peak prevalence a year earlier. The distributions of combined OW/OB prevalence and OB prevalence alone by age are compatible with global data in girls (2). In the meta-analysis of American data by Wang and Beydoun (18) the highest prevalence of OB in childhood is between the ages of 6-11 years for girls and boys. In another study conducted in the Netherlands among subjects aged 0-21 years, the prevalence of OB and OW was shown to peak between 4-7 years of age (23).

All these studies indicate that the prevalence of OB in children shows its peak during the primary school years. As the highest prevalence of OB appears to occur in the primary school years, targetted preventative and education programmes should be considered for children and parents during these ages and shoud probably be implemented at even earlier ages. In March 2016 the Turkish Ministry of National Education and the Ministry of Health published a joint statement including the list of foods suitable or unsuitable for sale in school canteens. This was a small but positive step towards increasing awareness among children and their parents. However, surveillance and monitoring of trends in the prevalence of OW and OB are required to determine whether such actions are beneficial and to plan future actions.

The LMS method, which depends on the BMI calculation, is generally used to define OB and OW in childhood (8). The

age- and sex-specific BMI reference percentile charts derived from our data had higher cutoffs than those of the study conducted by Turkkahraman et al (7) in 2003 and other studies conducted in different regions of Turkey (İstanbul in 2002 and Kayseri in 2008) (19,20). Since the etiology of childhood OB is multifactorial, it is difficult to explain the underlying cause of these differences in BMI cutoff values.

Study Limitations and Strengths

There are some limitations to this study. Firstly, as this was a sequel to a previous study and since the studies were conducted 12 years apart, it is not possible to show the fluctuation in the prevalence of OW and OB during these years. Secondly, this was a cross-sectional study performed in a relatively limited area of a single city and thus cannot reflect the characteristics of the whole Turkish population. There are also some important strengths to our study. These include the fact that both studies were performed by the same pediatric endocrinology team. Furthermore, all measurements in the study were performed by experienced health personnel, which increases the reliability of results despite inter-observer variation. Another important strength of the study was the reliable comparison of OW and OB prevalence and their trends via two studies with very similar characteristics and two sets of data which were adjusted for age and sex.

Conclusion

The results of our study demonstrate a striking increase in the prevalence of OW and OB in the city center of Antalya, Turkey in line with data from other pediatric populations. If this trend is replicated nationally then there is a pressing need for both regional and national OB prevention strategies. The effectiveness of these interventions should be measured by on-going surveillance studies and the OB prevalence trend among children should be closely monitored.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Akdeniz University (decision no: 108, date: 25.02.2015).

Informed Consent: Informed consent was obtained from all students and their parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Gamze Çelmeli, Yusuf Çürek, Zümrüt Arslan Gülten, Concept: Zümrüt Arslan Gülten, Mehmet Yardımsever, Mustafa Koyun, İffet Bircan, Design: Mehmet Yardımsever, Mustafa Koyun, Sema Akçurin, İffet Bircan, Data Collection or Processing: Gamze Çelmeli, Yusuf Çürek, Zümrüt Arslan Gülten, Analysis or Interpretation: Gamze Çelmeli, Mehmet Yardımsever, Sema Akçurin, Literature Search: Gamze Çelmeli, Yusuf Çürek, Zümrüt Arslan Gülten, Writing: Gamze Çelmeli, Mustafa Koyun, Sema Akçurin, İffet Bircan.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Wang Y, Lobstein T. Worldwide trends in childhood overweight and obesity. Int J Pediatr Obes 2006;1:11-25.
2. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, Abraham JP, Abu-Rmeileh NM, Achoki T, AlBuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, Ammar W, Anwari P, Banerjee A, Barquera S, Basu S, Bennett DA, Bhutta Z, Blore J, Cabral N, Nonato IC, Chang JC, Chowdhury R, Courville KJ, Criqui MH, Cundiff DK, Dabhadkar KC, Dandona L, Davis A, Dayama A, Dharmaratne SD, Ding EL, Durrani AM, Esteghamati A, Farzadfar F, Fay DF, Feigin VL, Flaxman A, Forouzanfar MH, Goto A, Green MA, Gupta R, Hafezi-Nejad N, Hankey GJ, Harewood HC, Havmoeller R, Hay S, Hernandez L, Husseini A, Idrisov BT, Ikeda N, Islami F, Jahangir E, Jassal SK, Jee SH, Jeffreys M, Jonas JB, Kabagambe EK, Khalifa SE, Kengne AP, Khader YS, Khang YH, Kim D, Kimokoti RW, Kinge JM, Kokubo Y, Kosen S, Kwan G, Lai T, Leinsalu M, Li Y, Liang X, Liu S, Logroscino G, Lotufo PA, Lu Y, Ma J, Mainoo NK, Mensah GA, Merriman TR, Mokdad AH, Moschandreas J, Naghavi M, Naheed A, Nand D, Narayan KM, Nelson EL, Neuhouser ML, Nisar MI, Ohkubo T, Oti SO, Pedroza A, Prabhakaran D, Roy N, Sampson U, Seo H, Sepanlou SG, Shibuya K, Shiri R, Shiue I, Singh GM, Singh JA, Skirbekk V, Stapelberg NJ, Sturua L, Sykes BL, Tobias M, Tran BX, Trasande L, Toyoshima H, van de Vijver S, Vasankari TJ, Veerman JL, Velasquez-Melendez G, Vlassov VV, Vollset SE, Vos T, Wang C, Wang X, Weiderpass E, Werdecker A, Wright JL, Yang YC, Yatsuya H, Yoon J, Yoon SJ, Zhao Y, Zhou M, Zhu S, Lopez AD, Murray CJ, Gakidou E. Global regional and national prevalence of overweight and obesity in children and adults during 1980-2013: A systematic analysis for the Global Burden of Disease Study 2013. Lancet 2014;384:766-781. Epub 2014 May 29
3. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. Lancet 2017;390:2627-2642. Epub 2017 Oct 10
4. Alper Z, Ercan İ, Uncu Y. A Meta-Analysis and the Evaluation of Trends in Obesity Prevalence among Children and Adolescents aged 5-19 in Turkey: 1990 through 2015. J Clin Res Pediatr Endocrinol 2018;10:59-67. Epub 2017 Sep 13
5. Bereket A, Atay Z. Current status of childhood obesity and its associated morbidities in Turkey. J Clin Res Pediatr Endocrinol 2012;4:1-7.
6. Sağlam H, Tarım Ö. Prevalence and correlates of obesity in schoolchildren from the city of Bursa, Turkey. J Clin Res Pediatr Endocrinol 2008;1:80-88. Epub 2008 Nov 4
7. Turkkahraman D, Bircan I, Tosun O, Saka O. Prevalence and risk factors of obesity in school children in Antalya, Turkey. Saudi Med J 2006;27:1028-1033.
8. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000;320:1240-1243.

9. Cole TJ. The LMS method for constructing normalized growth standards. Eur J Clin Nutr 1990;44:45-60.
10. Stamatakis E, Wardle J, Cole TJ. Childhood obesity and overweight prevalence trends in England: evidence for growing socioeconomic disparities. Int J Obes (Lond) 2010;34:41-47. Epub 2009 Nov 3
11. Keane E, Kearney PM, Perry IJ, Kelleher CC, Harrington JM. Trends and prevalence of overweight and obesity in primary school aged children in the Republic of Ireland from 2002-2012: a systematic review. BMC Public Health 2014;14:974.
12. Péneau S, Salanave B, Maillard-Teyssier L, Rolland-Cachera MF, Vergnaud AC, Méjean C, Czernichow S, Vol S, Tichet J, Castetbon K, Hercberg S. Prevalence of overweight in 6- to 15-year-old children in central/western France from 1996 to 2006: trends toward stabilization. Int J Obes (Lond) 2009;33:401-407. Epub 2009 Feb 24
13. Lissner L, Sohlström A, Sundblom E, Sjöberg A. Trends in overweight and obesity in Swedish schoolchildren 1999-2005: has the epidemic reached a plateau? Obes Rev 2010;11:553-559. Epub 2009 Dec 16
14. Lazzeri G, Panatto D, Pammolli A, Azzolini E, Simi R, Meoni V, Giacchi MV, Amicizia D, Gasparini R. Trends in overweight and obesity prevalence in Tuscan schoolchildren (2002-2012). Public Health Nutr 2015;18:3078-3085. Epub 2015 May 29
15. Keß A, Spielau U, Beger C, Gausche R, Vogel M, Lipek T, Körner A, Pfäffle R, Kiess W. Further stabilization and even decrease in the prevalence rates of overweight and obesity in German children and adolescents from 2005 to 2015: a cross-sectional and trend analysis. Public Health Nutr 2017;20:3075-3083. Epub 2017 Sep 21
16. Olds TS, Tomkinson GR, Ferrar KE, Maher CA. Trends in the prevalence of childhood overweight and obesity in Australia between 1985 and 2008. Int J Obes (Lond) 2010;34:57-66. Epub 2009 Oct 13
17. Kamali A, Hameed H, Shih M, Simon P. Turning the Curve on Obesity Prevalence Among Fifth Graders in the Los Angeles Unified School District, 2001-2013. Prev Chronic Dis 2017;14:16.
18. Wang Y, Beydoun MA. The obesity epidemic in the United States--gender, age, socioeconomic, racial/ethnic, and geographic characteristics: a systematic review and meta-regression analysis. Epidemiol Rev 2007;29:6-28. Epub 2007 May 17
19. Bundak R, Furman A, Gunoz H, Darendeliler F, Bas F, Neyzi O. Body mass index references for Turkish children. Acta Paediatr 2006;95:194-198.
20. Senol V, Unalan D, Bayat M, Mazicioglu MM, Ozturk A, Kurtoglu S. Change in reference body mass index percentiles and deviation in overweight and obesity over 3 years in Turkish children and adolescents. J Pediatr Endocrinol Metab 2014;27:1121-1129.
21. de Wilde JA, van Dommelen P, Middelkoop BJ, Verkerk PH. Trends in overweight and obesity prevalence in Dutch, Turkish, Moroccan and Surinamese South Asian children in the Netherlands. Arch Dis Child 2009;94:795-800. Epub 2009 Jun 24
22. Koca T, Dereci S, Pirgon Ö, Akçam M. Evaluation of the Change in the Prevalence of Overweight and Obesity in Schoolchildren in South-west Turkey from 2005 to 2014. Iran J Public Health 2018;47:33-39.
23. Fredriks AM, Van Buuren S, Sing RA, Wit JM, Verloove-Vanhorick SP. Alarming prevalences of overweight and obesity for children of Turkish, Moroccan and Dutch origin in The Netherlands according to international standards. Acta Pædiatrica 2005;94:496-498.

CASE REPORT

DOI: 10.4274/jcrpe.galenos.2018.2018.0077

J Clin Res Pediatr Endocrinol 2019;11(1):82-87

Congenital Hyperinsulinism and Evolution to Sulfonylurea-responsive Diabetes Later in Life due to a Novel Homozygous p.L171F *ABCC8* Mutation

Emregül Işık[1], Hüseyin Demirbilek[2], Jayne A. Houghton[3], Sian Ellard[3], Sarah E. Flanagan[3], Khalid Hussain[4]

[1]Gaziantep Children's Hospital, Clinics of Paediatric Endocrinology, Gaziantep, Turkey
[2]Hacettepe University Faculty of Medicine, Department of Paediatric Endocrinology, Ankara, Turkey
[3]University of Exeter Medical School, Institute of Biomedical and Clinical Science, Exeter, United Kingdom
[4]Sidra Medical and Research Center, Clinic of Paediatric Medicine, Doha, Qatar

What is already known on this topic?

Homozygous *ABCC8* mutations cause severe, persistent, diffuse hyperinsulinemic hypoglycaemia (HH) which is usually diazoxide unresponsive and requires surgical therapy. In medically managed patients with congenital hyperinsulinism, disease symptoms become milder over time. HH in the neonatal period, and subsequent diabetes, have been reported in heterozygous mutations of *HNF4A* and *HNF1A* as well as heterozygous *ABCC8* mutations.

What this study adds?

We describe the first homozygous *ABCC8* mutation with hyperinsulinemic hypoglycaemia (HH) in the neonatal period and its evolution to complete insulin deficient, sulphonylurea responsive diabetes mellitus. Findings from this present work, which show a broad clinical spectrum from asymptomatic to mild symptomatic hypoglycemia and severe hypoglycemia as well as insulin deficient diabetes mellitus in family members with identical mutation confirm the phenotypical variations in *ABCC8* mutations. This present case report emphasizes the need for long-term follow up of patients with HH in the neonatal period due to *ABCC8* mutations, particularly in those who have received medical therapy for risk of developing diabetes in later life.

Abstract

Congenital hyperinsulinism (CHI) is the most common cause of persistent hypoglycemia in infants and children. Recessive inactivating mutations in the *ABCC8* and *KCNJ11* genes account for approximately 50% of all CHI cases. Hyperinsulinaemic hypoglycaemia in infancy and diabetes in later life have been reported in patients with *HNF1A*, *HNF4A* and *ABCC8* mutations. Herein, we present a child who was diagnosed with CHI at birth, then developed diabetes mellitus at the age of nine years due to a novel homozygous missense, p.L171F (c.511C>T) mutation in exon 4 of *ABCC8*. The parents and one sibling were heterozygous carriers, whilst a younger sibling who had transient neonatal hypoglycemia was homozygous for the mutation. The mother and (maternal) uncle, who was also heterozygous for the mutation, developed diabetes within their third decade of life. The preliminary results of sulphonylurea (SU) treatment was suggestive of SU responsiveness. Patients with homozygous *ABCC8* mutations can present with CHI in the newborn period, the hyperinsulinism can show variability in terms of clinical severity and age at presentation and can cause diabetes later in life. Patients with homozygous *ABCC8* mutations who are managed medically should be followed long-term as they may be at increased risk of developing diabetes after many years.

Keywords: Congenital hyperinsulinism, MODY, *ABCC8* mutation, children

Address for Correspondence: Hüseyin Demirbilek MD, Hacettepe University Faculty of Medicine, Department of Paediatric Endocrinology, Ankara, Turkey
Phone: +90 312 305 11 24 **E-mail:** dr_huseyin@hotmail.com **ORCID ID:** orcid.org/0000-0001-6374-5884

Conflict of interest: None declared
Received: 02.03.2018
Accepted: 08.05.2018

Introduction

Adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels play an essential role in the regulation of insulin secretion from the pancreatic beta-cell; the key mechanism maintaining the blood glucose level in a narrow range of 3.5-5.5 mmol/L (1,2,3). K_{ATP} channels are open at low glucose levels (1). Increased metabolism, resulting in an increased ATP/adenosine diphosphate ratio, leads to closure of the K_{ATP} channel, depolarisation of the beta cell membrane and subsequent calcium influx through voltage-gated calcium channels. This in turn leads to insulin secretion via the exocytosis of secretory granules (2,3). Dysfunction of the K_{ATP} channel can cause either congenital hyperinsulinism (CHI) or diabetes (neonatal or adult onset) (1,4,5,6,7,8,9). CHI occurs when K_{ATP} channels are absent on the cell membrane or when they remain closed despite low glucose levels. In contrast, diabetes occurs if K_{ATP} channels remain open despite high blood glucose concentrations and increased metabolism in the beta cell (1,4). Recessive inactivating mutations of the K_{ATP} channel genes (*ABCC8* and *KCNJ11*) are the most common cause of severe, diazoxide unresponsive, diffuse CHI which usually requires pancreatectomy (1,10). Patients with dominant mutations of K_{ATP} channel genes, *ABCC8* and *KCNJ11*, may cause variable phenotype ranging from asymptomatic macrosomia, mild diazoxide responsive CHI to severe persistent hyperinsulinaemic hypoglycaemia (HH) as well as diabetes mellitus in later life (7,8,9,11).

CHI within the neonatal period and evolution to diabetes later in life has been reported in individuals with heterozygous inactivating mutations in the hepatocyte nuclear factor 4A and 1A genes (*HNF4A* and *HNF1A*) (12,13,14) and dominant mutations in *ABCC8* genes in a very limited number of cases (1,7,11,13,15,16,17,18,19,20,21).

To the best of our knowledge, CHI due to homozygous *ABCC8* mutations and evolution to complete insulin deficient-diabetes later in life has not been reported. Herein, we present a patient with a novel, homozygous *ABCC8* mutation who was diagnosed with CHI in the neonatal period and developed diabetes at the age of nine years.

Case Report

A nine year-old Turkish boy (VI.2 in Figure 1) presented with abdominal pain and fever. He was diagnosed with perforated appendicitis and was referred to the endocrine clinic for coexisting hyperglycaemia (blood glucose level was 27.75 mmol/L). A detailed family history revealed the presence of diabetes in multiple members of the maternal family (see details on the pedigree and footnotes). Specifically, the patient's mother was on insulin therapy for diabetes mellitus which had been diagnosed during the first trimester of pregnancy, when she was 24 years of age. A maternal uncle was also affected. There was also a history of neonatal hypoglycaemia of varying duration and severity affecting two of the patient's siblings.

The patient was the first living child of the family and was born with a birth weight of 3750 grams (+6.6 SD) via caesarian section at a gestation age of 29 weeks. Parents were distantly related. He presented with a hypoglycaemic episode at postnatal day one (blood glucose was 1.33

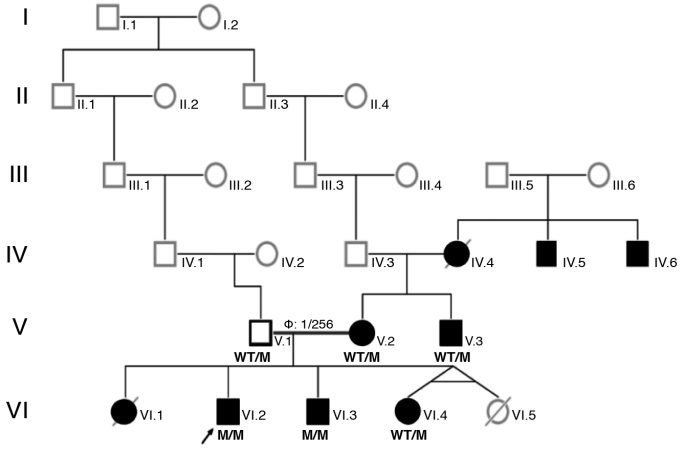


Figure 1. Pedigree of the family. The members developed either hypoglycaemia, diabetes or both are indicated as affected and shown with black-filled boxes. IV.4: Insulin dependent diabetes since 35 years-old, developed diabetic nephropathy (chronic renal failure) (reportedly), IV.5: Had insulin dependent diabetes and diabetic nephropathy (reportedly), IV.6: Diabetes and bilateral visual loss was reported, V.1: Father, 41 years old, apparently healthy with normal glucose and HbA1c (5.6%) levels, V.2: Mother 37 years old, developed insulin dependent diabetes during pregnancy and has been on insulin treatment since 24 years old, changing the treatment to SU therapy is in progress (see the section of the case report concerning sulphonylurea treatment), V.3: 40 years old, had insulin dependent diabetes mellitus since 32 years-old, VI.1: Born at term, macrosomic birth weight (4750 gram; 2.8 SDS), hypoglycaemia was detected during the neonatal period, died at 1-month during hospitalization with unknown etiology, VI.2: Index patient, VI.3: 9.5 years-old, born at term, birth weight was 3750 gram (0.4 SDS), had transient hypoglycemia during the neonatal period, latest blood glucose and HbA1c levels were normal, VI.4: 6.5 years old, born at seven months of gestation from a twin pregnancy, birth weight was 1250 gram (1.05 SD), HbA1c is normal

M: mutated, WT: wild type, SDS: standard deviation score, SU: sulphonylurea, HbA1c: haemoglobin A1c

mmol/L and simultaneous insulin level was 22.7 µIU/mL, C-peptide 5.42 ng/mL (0.9-7.1). A diagnosis of HH was considered and diazoxide was commenced. The patient developed pulmonary edema, which was considered likely to be a complication of treatment with diazoxide. Diazoxide was subsequently stopped and octreotide therapy was introduced. Hypoglycaemia remitted at the age of three months and the child remained free of hypoglycaemic episodes until nine years of age, when he was admitted to our hospital.

On admisson, the child was lethargic and had pale and grayish colour skin. His height was 140 cm [0.7 standard deviation score (SDS)], weight was 35 kg (0.8 SDS), and body mass index (BMI) 17.8 (0.7 SDS). Respiratory rate was 20 breaths/minute, heart rate was 72 beats/minute and blood pressure was 90/60 mmHg. There was abdominal distention, rigidity and rebound tenderness on physical examination. The patient underwent emergency appendectomy. During the post-operative period hyperglycaemia persisted and subcutaneous insulin therapy was introduced. Laboratory investigations revealed a blood glucose of 13.2 mmol/L with a simultaneous insulin of 8.82 µIU/mL (2.6-25), C-peptide: 1.28 ng/mL (0.9-7.1). Glycosylated haemoglobin A1c (HbA1c) was 9.1% (76 mmol/L). Islet cell, anti-insulin and anti-glutamic acid decarboxylase antibodies were negative. Over the following two months the insulin requirement gradually decreased until insulin treatment could be completely withdrawn. During the follow-up, HbA1c remained within the range of the high normal limits (6.2% to 6.4%) with dietary intervention and lifestyle changes. At the age of 11.5 years HbA1c was shown to be again significantly raised at 9.6% (81 mmol/L). At this time his weight was 46 kg (0.9 SDS), height was 152 cm (0.8 SDS) and BMI was 19.9 (0.9 SDS). The patient and family refused recommencement of insulin therapy. Subsequently, HbA1c increased to 11.4% (101 mmol/l) at the age of 12 years when an oral glucose tolerance test suggested insulin deficient-diabetes mellitus (Table 1).

Table 1. Oral glucose tolerance test results of index case at age 12 years

	Glucose (mmol/L)	Insulin (µIU/mL)	C-peptide (ng/mL)
0'	16.5	9.05	1.68
30'	21.8	10.09	1.94
60'	25.2	9.66	1.93
90'	28.2	7.76	1.92
120'	25	7.62	1.88
Normal lab. values	(3.5-5.5)	(2.6-25)	(0.9-7.1)

Genetic Testing

Genomic DNA was extracted from peripheral leukocytes using standard procedures and the coding regions and intron/exon boundaries of the *ABCC8*, *KCNJ11*, *HNF4A* and *HADH* genes were amplified by polymerase chain reaction (primers available on request). Amplicons were sequenced using the BigDye Terminator Cycle Sequencing Kit V.3.1 (Applied Biosystems, Warrington, UK) according to manufacturer's instruction and reactions were analysed on an ABI 3730 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the reference sequences (NM_001287174.1, NM_000525.3, NM_175914.4 and NM_005327.4) using Mutation Surveyor v5.0.1 software (SoftGenetics, State College, Pennsylvania, USA). The variant was classified using the American College of Medical Genetic and Genomics/Association for Molecular Pathology guidelines (22).

A written informed consent was obtained from the patients and/or their legal guardians.

Results

The index patient (VI.2, see Figure 1) was found to be homozygous for a novel missense c.511C>T (p.L171F) variant in exon 4 of *ABCC8* (Figure 2). The p.L171F variant affects a highly conserved amino acid and *in silico* analysis predicted the variant to be disease-causing (Alamut Visual V2.10 Software, Rouen, France). Mutation testing showed that the variant co-segregated with diabetes and hypoglycemia within the family, with an incomplete penetrance of heterozygous carriers (Figure 1).

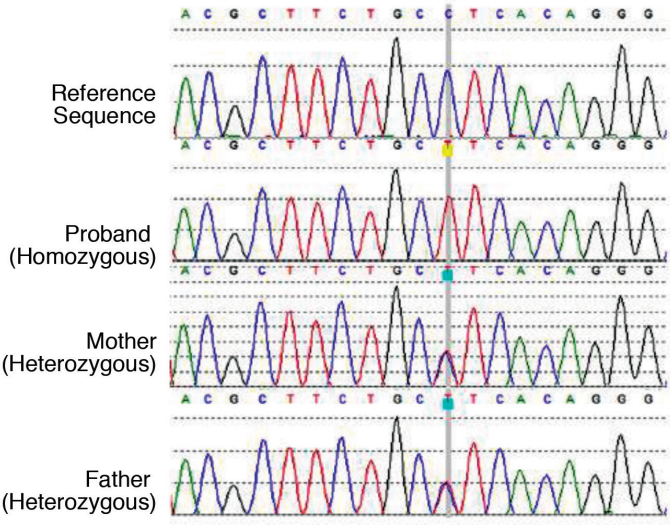


Figure 2. Electropherograms of the reference, index case and parents for c.511C>T (p.L171F) mutation

Treatment and Follow-up

Following detection of the *ABCC8* mutation, a trial of sulphonylurea (SU) treatment was commenced in the index case and his mother, who had been on insulin therapy for 13 years in an outpatient setting (Table 2). The mother's daily insulin dose requirement was reduced by approximately 50% from the baseline at the first week of the SU therapy with improved blood glucose measurements. The index case also responded to SU therapy and even developed one hypoglycaemic episode following SU therapy. Although the SU doses were adjusted accordingly, the family avoided giving the glibenclamide regularly due to the severe hypoglycaemic episodes which had not been observed while he was on insulin therapy or during fasting.

Discussion

Herein, we present a patient with a novel homozygous *ABCC8* mutation who was diagnosed with HH in the neonatal period and diabetes at the age of nine years. Hyperglycemia was first recognized during acute appendicitis which suggested stress-induced hyperglycemia. However, the patient had persistent hyperglycemia which required insulin therapy, a history of HH and relatives with autoantibody-negative diabetes. These findings were suggestive of monogenic diabetes, which was confirmed by molecular genetics analysis.

Homozygous K_{ATP} channel gene mutations are the most common cause of severe, diazoxide unresponsive HH which often requires pancreatectomy (3,13). However, clinical heterogeneity is observed in patients with dominant *ABCC8* mutations (9). Kapoor et al (23) reported a marked clinical heterogenity in siblings with identical mutations in *ABCC8*, ranging from asymptomatic hypoglycaemia to macrosomia, transient HH or severe HH and development of diabetes mellitus later in life. Besides, a heterogeneous nature is observed regarding severity and response to medical treatment and age of onset of symptoms (23).

Variations in the severity of HH and clinical course have also been reported in a mother and her daughter with a heterozygous *E1506K* mutation in *ABCC8* (20). In this report the child had severe symptoms and hypoglycaemic convulsions at age three months while the mother had subtle symptoms of hypoglycaemia followed by gestational diabetes which persisted after delivery.

Similarly, a marked clinical heterogenity was also observed in this present family. While one of the homozygous siblings (VI.2, index case) had prolonged HH and required medical therapy, the other sibling with an identical homozygous mutation (VI.3) suffered from transient hypoglycemia in the first week of life which remitted within three months. Diabetes was observed in the heterozygous mother and a maternal uncle, but the father (V.1) who was also heterozygous for the *ABCC8* mutation, had normal fasting plasma glucose and HbA1c levels. Unfortunately, the family refused performance of oral glucose tolerance test in the individuals who carried the mutation, but had not yet developed fasting hyperglycaemia or elevated HbA1c. We could also not perform genetic analysis in other relatives who also had diabetes with renal and ocular complications. We, therefore, were not able to confirm whether the diabetes in these additional family members was due to the *ABCC8* mutation.

Indeed, the clinical course for patients with *ABCC8* mutations is also substantially variable (1,7,13,15,16,17,18,19,20). Clinical features include hypoglycaemia within the neonatal period which remits over time, coexistence of hypoglycaemia and post-fed hyperglycaemic episodes, impaired fasting glucose or impaired glucose tolerance in response to a glucose load and in a few cases the development of diabetes (1,7,11,13,15,16,17,18,19,20). Regarding the type of mutation reported in *ABCC8* which caused neonatal HH and diabetes later in life, only a few cases were reported to have a homozygous mutation (18), whilst the majority

Table 2. Sulphonlyurea treatment trial results in the index case and his heterozygous carrier mother with diabetes mellitus

	Index case	Mother
Baseline fasting glucose (mmol/L)	14.2	16.6
Baseline fasting C-peptide (ng/mL)	1.13	1.04
Fasting C-peptide (ng/mL) post-SU therapy	2.12	NA
HbA1c	12.1% (109 mmol/L)	10.4% (90 mmol/L)
Glibenclamide dose	3.75 mg/day	10 mg/day
Change in insulin dose (%)	NA	-50
Evidence for SU responsivenes	Developed severe hypoglycaemic episodes after SU administration	Decrease in insulin dose requirement and improved glycaemic control with SU therapy

SU: sulphonylurea, HbA1c: hemoglobin A1c, Na: not applicable

had heterozygous or compound heterozygous mutations (7,11,15,17,19,20).

Biallelic (either homozygous or compound heterozygous) *ABCC8* mutations usually cause severe, diazoxide unresponsive HH which often requires surgical management (3). Therefore, the number of medically managed cases, particularly those with long-term follow-up, is very small. This limits our understanding of the underlying mechanisms and experience in the management of cases who develop hyperglycemia later in life. The data from the reported cases and experimental studies suggest that the key mechanisms are dysregulated insulin secretion, impaired first phase insulin secretion, delayed insulin response and β-cell apoptosis mediated via enhanced β-cell depolarisation, resulting in increased calcium ion entry into the cell (7,8,9, 13,15,16,17,18,19,20,24,25,26).

Taking into account the previously reported cases, our patient is the only case with a homozygous *ABCC8* mutation who presented with CHI (confirmed by clinical and biochemical evidence and mutation analysis) within the neonatal period which evolved into complete insulin deficient diabetes later in life. Therefore, this family provides novel insights into the clinical heterogeneity of CHI and later onset diabetes in patients with homozygous *ABCC8* mutations.

Neonatal diabetes due to a dominant activating mutation of a K_{ATP} channel gene (*KCNJ11* or *ABCC8*) is usually sulphonylurea responsive (27,28). We also performed a trial of SU therapy in the index case and his heterozygous mother who had insulin dependent diabetes. Preliminary results suggested a favorable SU responsiveness. Since SU drugs work by binding to the SUR1 subunit of the K_{ATP} channel, a positive response to SU therapy suggested that the presence of a homozygous mutation may not completely abolish the channel function.

In conclusion, we present the novel missense c.511C>T (p.L171F) *ABCC8* mutation causing neonatal HH and SU-responsive diabetes mellitus later in life. There are, however, some limitations in interpreting the phenotype-genotype relationships observed in this family. Firstly, we were not able to analyse the mutation status of other family members with diabetes mellitus. Secondly, although clinical evidences and bioinformatic tools confirmed the pathogenicity of the novel mutation, functional analyses have not been undertaken to assess the role of the variant *in vitro*. These results highlight the need for the long-term follow up of a larger series of CHI patients with homozygous *ABCC8* mutations who have been managed medically. In addition further evaluation of these variants, including functional analysis, to better understand the underlying molecular mechanism and phenotype-genotype relationships should be performed.

Ethics

Informed Consent: A written informed consent was obtained from the patients and/or their legal guardians.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Concept: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Sian Ellard, Sarah E. Flanagan, Khalid Hussain, Design: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Sian Ellard, Sarah E. Flanagan, Khalid Hussain, Data Collection or Processing: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Sian Ellard, Sarah E. Flanagan, Analysis or Interpretation: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Sian Ellard, Sarah E. Flanagan, Khalid Hussain, Literature Search: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Writing: Emregül Işık, Hüseyin Demirbilek, Jayne A. Houghton, Sian Ellard, Sarah E. Flanagan, Khalid Hussain.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Bennett K, James C, Hussain K. Pancreatic beta-cell KATP channels: Hypoglycaemia and hyperglycaemia. Rev Endocr Metab Disord 2010;11:157-163.
2. Demirbilek H, Rahman SA, Buyukyilmaz GG, Hussain K. Diagnosis and treatment of hyperinsulinaemic hypoglycaemia and its implications for paediatric endocrinology. Int J Pediatr Endocrinol 2017;2017:9. Epub 2017 Aug 29
3. Shah P, Rahman SA, Demirbilek H, Guemes M, Hussain K. Hyperinsulinaemic hypoglycaemia in children and adults. Lancet Diabetes Endocrinol 2017;5:729-742. Epub 2016 Dec 1
4. Clark R, Proks P. ATP-sensitive potassium channels in health and disease. Adv Exp Med Biol 2010;654:165-192.
5. Bowman P, Flanagan SE, Edghill EL, Damhuis A, Shepherd MH, Paisey R, Hattersley AT, Ellard S. Heterozygous ABCC8 mutations are a cause of MODY. Diabetologia 2012;55:123-127. Epub 2011 Oct 12
6. Hartemann-Heurtier A, Simon A, Bellanne-Chantelot C, Reynaud R, Cave H, Polak M, Vaxillaire M, Grimaldi A. Mutations in the ABCC8 gene can cause autoantibody-negative insulin-dependent diabetes. Diabetes Metab 2009;35:233-235. Epub 2009 Apr 1
7. Huopio H, Otonkoski T, Vauhkonen I, Reimann F, Ashcroft FM, Laakso M. A new subtype of autosomal dominant diabetes attributable to a mutation in the gene for sulfonylurea receptor 1. Lancet 2003;361:301-307.
8. Huopio H, Reimann F, Ashfield R, Komulainen J, Lenko HL, Rahier J, Vauhkonen I, Kere J, Laakso M, Ashcroft F, Otonkoski T. Dominantly inherited hyperinsulinism caused by a mutation in the sulfonylurea receptor type 1. J Clin Invest 2000;106:897-906.

9. Kapoor RR, Flanagan SE, James CT, McKiernan J, Thomas AM, Harmer SC, Shield JP, Tinker A, Ellard S, Hussain K. Hyperinsulinaemic hypoglycaemia and diabetes mellitus due to dominant ABCC8/KCNJ11 mutations. Diabetologia 2011;54:2575-2583. Epub 2011 Jun 15
10. Kapoor RR, Flanagan SE, Arya VB, Shield JP, Ellard S, Hussain K. Clinical and molecular characterisation of 300 patients with congenital hyperinsulinism. Eur J Endocrinol 2013;168:557-564.
11. Ocal G, Flanagan SE, Hacihamdioglu B, Berberoglu M, Siklar Z, Ellard S, Savas Erdeve S, Okulu E, Akin IM, Atasay B, Arsan S, Yagmurlu A. Clinical characteristics of recessive and dominant congenital hyperinsulinism due to mutation(s) in the ABCC8/KCNJ11 genes encoding the ATP-sensitive potasium channel in the pancreatic beta cell. J Pediatr Endocrinol Metab 2011;24:1019-1023.
12. Pearson ER, Boj SF, Steele AM, Barrett T, Stals K, Shield JP, Ellard S, Ferrer J, Hattersley AT. Macrosomia and hyperinsulinaemic hypoglycaemia in patients with heterozygous mutations in the HNF4A gene. PLoS Med 2007;4:118.
13. Rahman SA, Nessa A, Hussain K. Molecular mechanisms of congenital hyperinsulinism. J Mol Endocrinol 2015;54:119-129. Epub 2015 Mar 2
14. Stanescu DE, Hughes N, Kaplan B, Stanley CA, De Leon DD. Novel presentations of congenital hyperinsulinism due to mutations in the MODY genes: HNF1A and HNF4A. J Clin Endocrinol Metab 2012;97:2026-2030. Epub 2012 Jul 16
15. Abdulhadi-Atwan M, Bushman J, Tornovsky-Babaey S, Perry A, Abu-Libdeh A, Glaser B, Shyng SL, Zangen DH. Novel De Novo Mutation in Sulfonylurea Receptor 1 Presenting as Hyperinsulinism in Infancy Followed by Overt Diabetes in Early Adolescence. Diabetes 2008;57:1935-1940. Epub 2008 Apr 4
16. Bin-Abbas BS, Al-Ashwal AA. Diabetes in a nonpancreatectomized child with nesidioblastosis. Diabetes Care 2004;27:626-627.
17. Gussinyer M, Clemente M, Cebrian R, Yeste D, Albisu M, Carrascosa A. Glucose intolerance and diabetes are observed in the long-term follow-up of nonpancreatectomized patients with persistent hyperinsulinemic hypoglycemia of infancy due to mutations in the ABCC8 gene. Diabetes Care 2008;31:1257-1259. Epub 2008 Mar 13
18. Harel S, Cohen AS, Hussain K, Flanagan SE, Schlade-Bartusiak K, Patel M, Courtade J, Li JB, Van Karnebeek C, Kurata H, Ellard S, Chanoine JP, Gibson WT. Alternating hypoglycemia and hyperglycemia in a toddler with a homozygous p.R1419H ABCC8 mutation: an unusual clinical picture. J Pediatr Endocrinol Metab 2015;28:345-351.
19. Saito-Hakoda A, Yorifuji T, Kanno J, Kure S, Fujiwara I. Nateglinide is Effective for Diabetes Mellitus with Reactive Hypoglycemia in a Child with a Compound Heterozygous ABCC8 Mutation. Clin Pediatr Endocrinol 2012;21:45-52. Epub 2012 Jul 25
20. Vieira TC, Bergamin CS, Gurgel LC, Moises RS. Hyperinsulinemic hypoglycemia evolving to gestational diabetes and diabetes mellitus in a family carrying the inactivating ABCC8 E1506K mutation. Pediatr Diabetes 2010;11:505-508.
21. Haghverdizadeh P, Sadat Haerian M, Haghverdizadeh P, Sadat Haerian B. ABCC8 genetic variants and risk of diabetes mellitus. Gene 2014;545:198-204. Epub 2014 Apr 21
22. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-424. Epub 2015 Mar 5
23. Kapoor RR, Flanagan SE, Ellard S, Hussain K. Congenital hyperinsulinism: marked clinical heterogeneity in siblings with identical mutations in the ABCC8 gene. Clin Endocrinol (Oxf) 2012;76:312-313.
24. Miki T, Tashiro F, Iwanaga T, Nagashima K, Yoshitomi H, Aihara H, Nitta Y, Gonoi T, Inagaki N, Miyazaki J, Seino S. Abnormalities of pancreatic islets by targeted expression of a dominant-negative KATP channel. Proc Natl Acad Sci U S A 1997;94:11969-11973.
25. Nichols CG, Koster JC, Remedi MS. Beta-cell hyperexcitability: from hyperinsulinism to diabetes. Diabetes Obes Metab 2007;(9 Suppl 2):81-88.
26. Remedi MS, Rocheleau JV, Tong A, Patton BL, McDaniel ML, Piston DW, Koster JC, Nichols CG. Hyperinsulinism in mice with heterozygous loss of K(ATP) channels. Diabetologia 2006;49:2368-2378. Epub 2006 Aug 19.
27. Pearson ER, Flechtner I, Njolstad PR, Malecki MT, Flanagan SE, Larkin B, Ashcroft FM, Klimes I, Codner E, Iotova V, Slingerland AS, Shield J, Robert JJ, Holst JJ, Clark PM, Ellard S, Sovik O, Polak M, Hattersley AT; Neonatal Diabetes International Collaborative Group. Switching from insulin to oral sulfonylureas in patients with diabetes due to Kir6.2 mutations. N Engl J Med 2006;355:467-477.
28. Rafiq M, Flanagan SE, Patch AM, Shields BM, Ellard S, Hattersley AT; Neonatal Diabetes International Collaborative Group. Effective treatment with oral sulfonylureas in patients with diabetes due to sulfonylurea receptor 1 (SUR1) mutations. Diabetes Care 2008;31:204-209. Epub 2007 Nov 19

CASE REPORT

DOI: 10.4274/jcrpe.galenos.2018.2018.0005

J Clin Res Pediatr Endocrinol 2019;11(1):88-93

Inherited Deletion of 1q, Hyperparathyroidism and Signs of Y-chromosomal Influence in a Patient with Turner Syndrome

Alejandro F. Siller[1]*, Alex Shimony[1]*, Marwan Shinawi[1], Ina Amarillo[2], Louis P. Dehner[2], Katherine Semenkovich[1], Ana María Arbeláez[1]

[1]Washington University Faculty of Medicine, Department of Pediatrics, Washington, USA

[2]Washington University Faculty of Medicine, Department of Pathology and Immunology, Washington, USA

**Dr. Siller and Alex Shimony contributed equally to this article.*

What is already known on this topic?

Turner syndrome (TS) is caused by partial or complete absence of a second sex chromosome resulting in phenotypes involving multiple organ systems. Endocrine problems typically manifest as short stature, gonadal failure and hypothyroidism. Hyperparathyroidism, though reported in a small number of TS cases, is not considered a typical feature.

What this study adds?

This case highlights the importance of genetic testing beyond karyotype in patients with atypical clinical features of Turner syndrome (TS), particularly in those with hyperparathyroidism (HPT). The findings also led us to postulate a potential molecular mechanism for HPT in prior TS cases.

Abstract

We report a detailed phenotypic, cytogenetic and molecular characterization of a patient prenatally diagnosed with Turner syndrome (TS). In addition to having typical TS clinical characteristics including webbed neck, high arched palate and coarctation of the aorta, the patient had features less frequently seen in TS. These included recurrent parathyroid adenomas, growth along the 75th-90th centiles on the TS height curve despite minimal treatment with growth hormone, behavioral problems and evidence of gonadal dysgenesis with testicular-like structures, such as seminiferous tubules lined by Sertoli cells and a contiguous nodule of Leydig cells. While fluorescence *in situ* hybridization (FISH) failed to detect Y-chromosome material in gonadal tissue or blood samples, chromosomal microarray analysis (CMA) confirmed X monosomy and a 4.69 Mb copy number loss on 1q31.2q31.3 (bp 192,715,814 to 197,401,180). This region contains the *CDC73* gene which has been associated with hyperparathyroidism-jaw tumor syndrome, features of which include recurrent, functional parathyroid adenomas and behavioral issues. This case illustrates how atypical features in a TS patient, such as robust growth and recurrent parathyroid adenomas, may suggest an underlying molecular etiology that should be explored by additional genetic diagnostic modalities. It is therefore appropriate in such cases to conduct further genetic testing, such as CMA and FISH, to explore other diagnostic possibilities and possibly prevent further complications.

Keywords: Turner syndrome, genetic testing, hyperparathyroidism, inherited 1q deletion, signs of Y-chromosomal influence

Introduction

Turner syndrome (TS) is a disorder caused by partial or complete absence of a second sex chromosome and results in a wide variety of phenotypes with an estimated incidence of 1/2000-1/3000 live births (1,2,3). Certain features, such as short stature and primary ovarian failure, are present in virtually all cases. Coarctation of the aorta, bicuspid aortic valve, renal anomalies, autoimmune thyroid disease and hearing loss are also frequently present in patients with TS. While rare, hyperparathyroidism (HPT) has been reported in a small number of TS patients (4,5,6,7,8). Further genetic testing in these cases was not performed, thus the etiology of these concurrent morbidities is unknown. Other reported

Address for Correspondence: Ana Maria Arbeláez MD, Washington University Faculty of Medicine, Department of Pediatrics, Washington, USA
Phone: +3144546051 **E-mail:** arbelaez_a@kids.wustl.edu **ORCID ID:** orcid.org/0000-0001-6977-9786

Conflict of interest: None declared
Received: 03.01.2018
Accepted: 08.05.2018

atypical clinical features have been suggestive of other clinically relevant genomic abnormalities (9,10).

Herein, we present a case of TS with HPT who was found to have a 1q deletion. Patients with 1q deletions have been reported to have growth retardation, psychomotor delay and genital, cardiac and facial anomalies as well extremity anomalies (11). Additionally, *de novo* deletions in this region have been associated with developmental delay, agenesis of the corpus callosum and cerebellar hypoplasia (12). Moreover, this region includes genes that have been associated with recurrent, functional parathyroid adenomas as well as behavioral issues. While HPT has been shown to be associated with both 1q deletions (13) and TS separately, to our knowledge HPT has not been documented in an individual patient with both a 1q deletion and TS. The findings in our patient suggest a possible genetic cause, beyond the missing sex chromosome, of other TS patients exhibiting these types of atypical clinical features and highlights the importance of a multidisciplinary approach and genetic testing, beyond karyotyping, in atypical TS cases.

Case Report

The proband was a 20-year-old woman with classical features of TS, including webbed neck, widely-spaced nipples, a high-arched palate, a bicuspid aortic valve, coarctation of the aorta (surgically repaired at one year of age) and a 45,X karyotype on an antenatal amniocentesis. Other comorbidities included bipolar disorder, dyscalculia, bilateral kidney malrotation, steatohepatitis and an episode of hemorrhagic gastritis of unclear etiology. At age 11, she was found to have an elevated plasma calcium level of 12.1 mg/dL [reference range (RR): 8.5-10.3 mg/dL], an intact parathyroid hormone (PTH) level of 369 pg/mL (RR: 14-72 pg/mL), a plasma phosphorus level of 1.7 mg/dL (RR: 3.0-6.0 mg/dL) and a urinary calcium to urinary creatinine ratio of 0.19. Technetium-99m (Tc-99m) sestamibi scan revealed an enlarged right superior parathyroid gland. She underwent resection of the enlarged parathyroid and surgical pathology showed a right superior parathyroid adenoma measuring 1.1x1.0x1.6 cm and weighing 1.07 grammes. Intraoperative sampling of the right internal jugular vein showed a drop in PTH from 815 to 42 pg/mL following resection. Five months post-surgery, she developed abdominal pain and emesis and was found to have a left distal ureteral calculus, left hydronephrosis and bilateral nephrocalcinosis and bilateral nephrolithiasis, leading to a ureteroscopy with stone extraction. At that time her urinary calcium to urinary creatinine ratio was 0.12. Post-stone extraction, she remained normocalcemic until age 16, when she was found to have an elevated plasma calcium level of 11.4 mg/dL, elevated intact PTH level of 108 pg/mL and a plasma phosphorus level of 3.8 mg/dL. Neck ultrasound showed a solid, hypoechoic nodule posterior to the midportion of the right thyroid measuring 9x6x4 mm with detectable internal vascularity on Doppler, consistent with a second enlarged parathyroid. The Tc-99m sestamibi scan did not show an area of increased activity, but given ultrasound findings and biochemical results she had a second parathyroidectomy, yielding a 0.136 gramme, hypercellular parathyroid and a decrease of the intraoperative PTH from 136 to 28 pg/mL. She has been normocalcemic since.

The patient grew along the 75th-90th percentiles of the TS height-for-age growth chart (14) since birth. Her final height prediction, given her parental heights, was 171 cm. Growth hormone therapy (0.35 mg/kg/week) was initiated at seven years of age. However, her family felt this treatment led to agitation and overactivity and was therefore discontinued after less than one year of therapy. It was never restarted and she continued to grow along the 90th percentile for TS, achieving an adult height of 150 cm, consistent with roughly the 1st percentile of the CDC growth chart for girls without TS (Figure 1) (14).

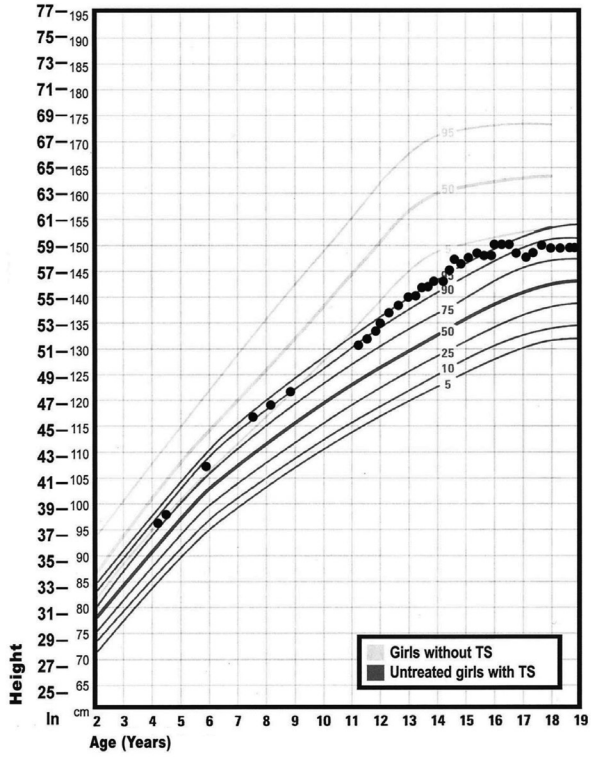


Figure 1. Proband's growth points on growth chart for children with Turners syndrome (Adapted from Frías JL, Davenport ML; Committee on Genetics and Section on Endocrinology. Health supervision for children with Turner syndrome. Pediatrics 2003;111:692-702)

TS: Turners syndrome

J Clin Res Pediatr Endocrinol
2019;11(1):88-93

The proband required special education classes for learning disabilities, especially in mathematics which is typical of girls with TS, and was also diagnosed with attention-deficit/hyperactivity disorder. Last audiogram at age 20, revealed mild left ear hearing loss at 4-8 kHz and mild right ear conductive hearing loss from 250-8 kHz. The proband can do most of her daily life activities without any help.

Verbal informed consent was obtained from the patient and the family.

She had documented primary ovarian failure at age 14 with elevated gonadotropins (luteinizing hormone: 18.9 IU/L and follicle-stimulating hormone: 99.8 IU/L) levels. Gradual estrogen replacement therapy with conjugated estrogen was started at that time and she experienced menarche a year later. She then began combined oral contraceptive therapy (OCT), but developed severe mood-related symptoms and extreme distress from breakthrough bleeding that required treatment with multiple mood stabilizing medications (Prozac, Zyprexa, Lithium, Seroquel). The progesterone in her OCT was felt to be the primary trigger for this exacerbation in her mood symptoms. Thus, she elected to undergo a hysterectomy with bilateral salpingo-oophorectomy (BSO) at age 18 in order to resume estrogen-only therapy. Following hysterectomy/BSO, the patient was continued on estrogen-only replacement with improvement of mood disturbance. The pathology showed a diminutive uterus weighing 33 grammes. The bilateral adnexa had fallopian tubes and fibrous streak gonads. In addition, the right-side streak gonad (Figure 2a) was accompanied microscopically by ovarian-like stroma, dysgenetic testicular-like structures and an apparent vas deferens. The right gonad showed the presence of a fibroepithelial structure with the features of an epididymis (Figure 2b) and a second nodule composed of Sertoli-like tubules with an adjacent focus of Leydig cells (Figure 2c). Inhibin immunostain confirmed the presence of Leydig cells (Figure 2d). The patient had never had any physical examination findings suggestive of virilization.

Since the patient presented with atypical features of TS, including HPT, an unusual growth pattern, behavioral abnormalities and the presence of gonadal dysgenesis with Sertoli-only tubules, endocrinology recommended that the genetics team become involved. Thus both a chromosomal microarray analysis (CMA) of the proband's peripheral blood and a fluorescence *in-situ* hybridization (FISH) analysis of the peripheral blood and of the testis-like structures in the streak

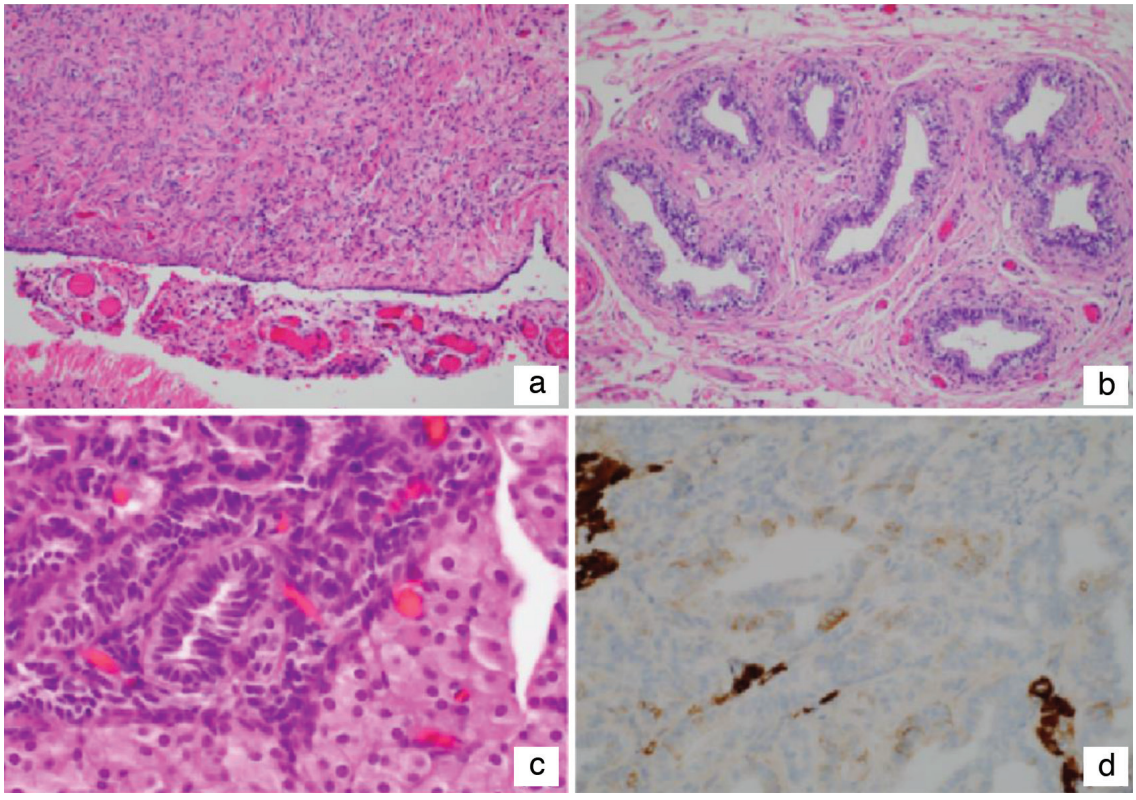


Figure 2. Histopathological findings of gonadal tissue. (a) The right (shown) and left gonads show the typical fibrous, ovarian-like stromal features of the streak gonad. (b) This circumscribed nodule is composed of epithelial-lined tubules surrounded by fibrous stroma with a resemblance to the epididymis. (c) The tubular structures resembling Sertoli tubules in the absence of germ cells are adjacent to a nodule of Leydig cells with abundant pale eosinophilic cytoplasm. (d) Inhibin immunostain shows pale staining of the tubules and intense reactivity in the Leydig cells

gonad tissue were performed. The Affymetrix CytoScan HD (www.affymetrix.com) was utilized to interrogate the genomic DNA for copy number variants (CNVs) and regions of homozygosity (ROH). The array was designed with 2.6 million copy number markers, including 1.9 million non-polymorphic probes, selected for their linear response to copy number and genomic position. The average intragenic marker spacing is equivalent to 1 probe per 880 base pairs. A genomic imbalance is reported when deletions are greater than 200 kb and duplications are greater than 500 kb, unless they represent a region clearly associated with benign copy number polymorphism in multiple independent studies. ROH are reported when they are greater than 10 Mb. The genomic linear positions are given relative to GRCh37/hg19 (UCSC Genome Browser) (15). Copy number analysis was done using the Affymetrix Chromosome Analysis Suite (version 3.0.0.42 r8004). The CMA of the proband revealed two CNV: a loss of the entire chromosome X (~155 Mb) indicative of monosomy X and a 4.69 Mb copy number loss on 1q31.2q31.3 (bp 192,715,814 to 197,401,180) (Figure 3).

Interphase and metaphase FISH analyses on peripheral blood lymphocytes, obtained from the patient and her parents, were performed using standard cytogenetic methods, to confirm the 1q deletion in the proband, which was also found to be maternally inherited. The RP11-78E12 BAC clone and CEP 1 FISH probes (Empire Genomics LLC, Williamsburg NY) were used to detect the 1q deletion and centromere 1 (control) regions, respectively.

Interphase *SRY*/Y FISH was also performed on paraffin-embedded tissue obtained from the testicular-like structures in the dysgenic right gonad with locus-specific Vysis commercial FISH probes localizing to centromere X (CEPX; DXZ1; Xp11.1-q11.1 Alpha satellite DNA; Spectrum Green) and sex-determining region Y (SRY; Yp11.31-p11.32; Spectrum Aqua) and Yq12 Satellite III DNA locus (DYZ1; Spectrum Orange (Abbott Molecular, Des Plaines, IL). The testicular-like structures showed a single X signal pattern. None of the nuclei showed the presence of *SRY* or Yq-specific signals such as *DYZ1*. The tubular structures were weakly positive for *WT-1*, but *SALL4* was non-reactive indicating an absence of germ cells in the tubules (not shown).

Discussion

Awareness of the typical features of TS is important in order to diagnose this disorder as early as possible and treat any

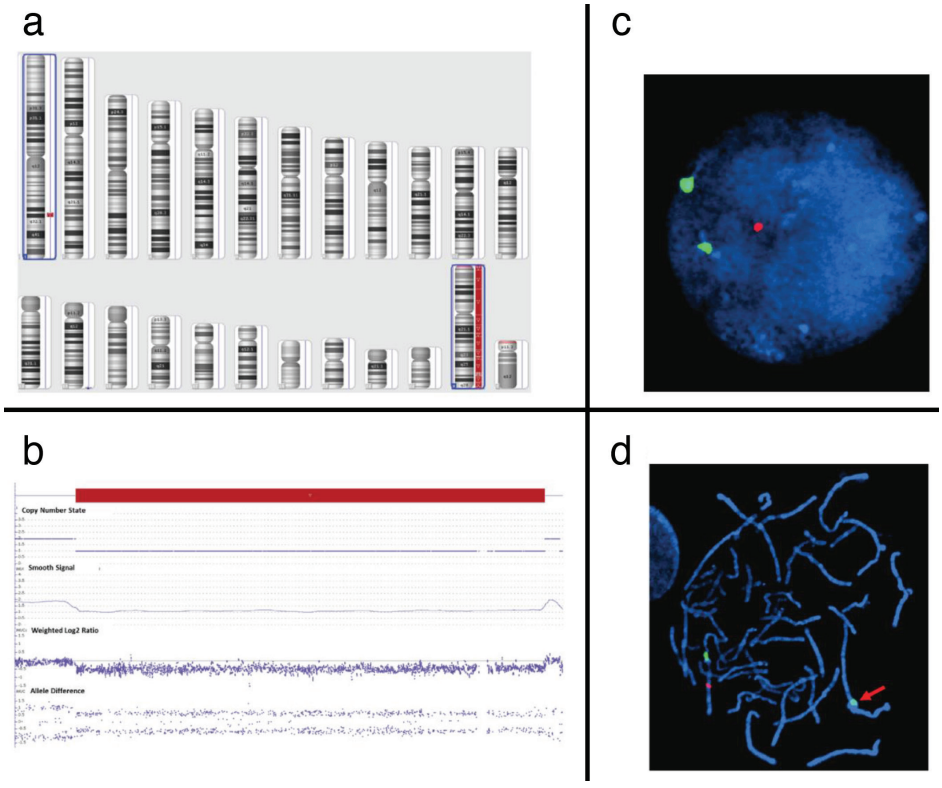


Figure 3. Proband CMA: (a) Karyoview showing concurrent copy number losses (blue boxes) on chromosomes X (monosomy X) and 1 (1q31.2q31.3; ~4.69 Mb, 4,113 markers/probes, arr[hg19] 1q31.2q31.3(192715814-197401180)x1 mat). (b) Detailed view of the 1q31.2q31.3 deletion (CN = 1, smooth signal = 1, weighted log2 ration = -0.5, allele difference = + 0.5, -0.5). Post-CMA familial FISH: (c) Interphase and (d) metaphase proband and maternal cells revealed one copy number loss

CMA: chromosomal microarray analysis, FISH: fluorescence in situ hybridization

associated comorbidity. There are studies showing that the recognition of TS is occurring earlier, with serial surveys in Belgium suggesting that the age at diagnosis, especially TS with a 45,X genotype, declined from 11.2 years of age in 1991 to 6.6 years of age in 2003 (16). Moreover, it is crucial for clinicians to be aware of atypical aspects of a TS patient's presentation and to pursue further genetic testing if necessary. In addition multidisciplinary involvement in such cases will help to mitigate the risk posed by these atypical manifestations.

Primary HPT has been reported in a small number of TS cases (4), but to our knowledge, there have been no studies investigating the etiology of primary HPT in patients with TS. HPT-jaw tumor (JT) syndrome, which is associated with recurrent functional parathyroid adenomas as well as behavioral issues, has been shown to be associated with a large-scale 1q31 deletion, specifically the *CDC73* gene (13). CMA in our patient revealed a maternally inherited 4.69 Mb deletion at 1q31.2q31.3. Interestingly, the deleted interval in this patient includes 16 OMIM genes, including *CDC73* gene, also known as *HPRT2*. Loss of function mutations in *HPRT2* have been associated with HPT, parathyroid adenoma, parathyroid carcinoma or HPT-JT (17). *HPRT2*, which encodes parafibromin, is a regulator of gene expression through its association with the RNA polymerase II subunit *POLR2A* and with a histone methyltransferase complex (18). In eight previously documented cases of 1q deletions encompassing the 1q31.2q31.3 region, most presented with growth retardation, psychomotor retardation, and lip/palate anomaly phenotypes. In our patient, her bipolar disorder, behavioral abnormalities, learning disabilities and atypical aspects might be associated with inherited deletion of 1q (11). The patient's parental FISH revealed that the proband's mother is a carrier for the same 1q31.2-31.3 deletion, but she has no clinical or laboratory evidence of HPT. This may be due to incomplete penetrance or phenotypic variability. However, she will warrant surveillance for calcium and parathyroid abnormalities in the future.

There are currently no consensus guidelines for monitoring calcium and/or PTH levels in patients with *CDC73* related conditions. One report suggests screening for HPT in HPT-JT with serum calcium and PTH levels every 6-12 months (19), similar to predisposing syndromes for parathyroid tumors, such as multiple endocrine neoplasia syndrome type 1 (20).

Relative height stature has been reported in TS patients with Y chromosome material or with karyotypes other than 45,X (21). A wide phenotypic variability in mixed gonadal dysgenesis has been previously described, with unilateral testicular structures, due in part to isodicentric Y(p) (idicY(p)) mosaicism or the presence of *SRY* in early gonadal ontogenesis of Sertoli cells (22). We report the phenotypic and cytogenetic characterization of an apparently female patient, with mixed gonadal dysgenesis who unexpectedly was found to have a histologically male gonad with Sertoli cells. However, despite histopathological evidence of Sertoli cells in our proband, FISH analyses of peripheral blood and gonadal tissue did not demonstrate evidence of *SRY* material or idicY(p) mosaicism, suggesting that additional factors may play a role in gonadal determination and differentiation, such as the timing of the mitotic loss of the Y material during gonadal ontogenesis and the proportion of *SRY* positive pre-Sertoli cells in the gonad. TS patients with presence of *SRY* or Y-chromosome material have a 7-33% risk of developing gonadoblastoma (23,24,25,26). Therefore, given the pathologic findings in the right gonad, the risk of gonadoblastoma in this patient was not insignificant (regardless of whether or not we were able to find evidence of Y-chromosome material) and it was addressed with surgery. It is important to note, however, that it can be difficult to locate Y-chromosome material even in those with clear evidence of developmental influence of *SRY*, as seen in the proband in this report. Further research is needed to determine the risk of gonadoblastoma in patients with testicular tissue but with negative *SRY* on peripheral blood and gonadal tissue.

While classical cases of TS with 45,X karyotype and typical features of neonatal pedal edema, short stature, ovarian failure and cardiac comorbidities are frequently diagnosed earlier by discerning physicians, atypical features warrant investigation beyond the conventional karyotype study. CMA analysis continues to improve the detection of additional chromosomal aberrations in patients with TS and facilitates genotype-phenotype correlation. Such clinically significant information can lead to additional interventions, such as gonadectomy to eliminate the risk of gonadoblastoma in the presence of cryptic Y-chromosome material or establish surveillance for other complications. In this case, the proband's height, recurrent parathyroid adenoma, behavioral problems and learning disabilities warranted CMA, which led to the discovery of a 1q31.2q31.3 deletion. The male-differentiated structures in one streak gonad suggested the influence of SRY during gonad development, which ultimately led to gonadectomy. Since this may in fact be the first documented case of TS, HPT and a large 1q deletion, it should prompt further evaluation of patients exhibiting phenotypes that may not be attributed simply to TS. Therefore, it is crucial for clinicians to be aware of the typical TS phenotype and pursue careful examination and further genetic diagnostic investigation in cases with unusual phenotypic features or less common co-morbidities. This will facilitate better understanding of the underlying molecular etiologies and genotype-phenotype correlation in atypical cases of TS. Such clinically significant information can lead

to additional interventions and better patient surveillance to prevent complications.

Ethics

Informed Consent: Verbal Informed consent was obtained from the parents and the patient.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Marwan Shinawi, Ana María Arbeláez, Concept: Marwan Shinawi, Ana María Arbeláez, Design: Ana María Arbeláez, Data Collection or Processing: Louis P. Dehner, Ina Amarillo, Ana María Arbeláez, Analysis or Interpretation: Marwan Shinawi, Ina Amarillo, Louis P. Dehner, Ana María Arbeláez, Literature Search: Alejandro F. Siller, Alex Shimony, Writing: Alejandro F. Siller, Alex Shimony, Marwan Shinawi, Ina Amarillo Louis P. Dehner, Katherine Semenkovich, Ana María Arbeláez.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Gravholt CH. Epidemiological, endocrine and metabolic features in Turner syndrome. Eur J Endocrinol 2004;151:657-687.
2. Gonzalez L, Witchel SF. The Patient with Turner Syndrome: Puberty and Medical Management Concerns. Fertility Sterility 2012;98:780-786. Epub 2012 Aug 9
3. Davenport ML. Approach to the patient with Turner syndrome. J Clin Endocrinol Metab 2010;95:1487-1495.
4. Park J, Kim YM, Choi JH, Lee BH, Yoon JH, Jeong WY, Yoo HW. Turner syndrome with primary hyperparathyroidism. Ann Pediatr Endocrinol Metab 2013;18:85-89. Epub 2013 Jun 30
5. Shirzad N, Tehrani MR, Soltani A. A rare association of hyperparathyroidism and Turner's syndrome--a case report. Endokrynologia Polska 2008;59:232-234.
6. Paul TV, Dinakar J, Thomas N, Mathews SS, Shanthly N, Nair A. A case of Turner syndrome with hyperparathyroidism in an adult. Ear Nose Throat J 2008;87:110-112.
7. Kishida M, Otsuka F, Mimura Y, Ogura T, Makino H. A lethal complication, acute necrotizing pancreatitis, of Turner's Syndrome with primary hyperparathyroidism. Endocr J 2003;50:835-836.
8. Chen JF, Chen JY, Huang HS. [Primary hyperparathyroidism with parathyroid adenocarcinoma in a patient with Turner's syndrome complicated with thyrotoxicosis]. Changgeng Yi Xue Za Zhi 1993;16:133-139.
9. Peabody J, Martin M, DeMaria L, Florentino J, Paculdo D, Paul M, Vanzo R, Wassman ER, Burgon T. Clinical Utility of a Comprehensive, Whole Genome CMA Testing Platform in Pediatrics: A Prospective Randomized Controlled Trial of Simulated Patients in Physician Practices. PLoS One 2016;11:e0169064.
10. Sdano MR, Vanzo RJ, Martin MM, Baldwin EE, South ST, Rope AF, Allen WP, Kearney H. Clinical utility of chromosomal microarray analysis of DNA from buccal cells: detection of mosaicism in three patients. J Genet Couns 2014;23:922-927. Epub 2014 Aug 15
11. Hu P, Wang Y, Meng LL, Qin L, Ma DY, Yi L, Xu ZF. 1q25.2-q31.3 Deletion in a female with mental retardation, clinodactyly, minor facial anomalies but no growth retardation. Mol Cytogenet 2013;6:30.
12. Sajan SA, Fernandez L, Nieh SE, Rider E, Bukshpun P, Wakahiro M, Christian SL, Riviere JB, Sullivan CT, Sudi J, Herriges MJ, Paciorkowski AR, Barkovich AJ, Glessner JT, Millen KJ, Hakonarson H, Dobyns WB, Sherr EH. Both rare and de novo copy number variants are prevalent in agenesis of the corpus callosum but not in cerebellar hypoplasia or polymicrogyria. PLoS Genet 2013;9:e1003823. Epub 2013 Oct 3
13. Rubinstein JC, Majumdar SK, Laskin W, Lazaga F, Prasad ML, Carling T, Khan SA. Hyperparathyroidism-Jaw Tumor Syndrome Associated With Large-Scale 1q31 Deletion. J Endocr Soc 2017;1:926-930.
14. Frías JL, Davenport ML, Committee on Genetics, and the Section on Endocrinology. Health Supervision for Children with Turner Syndrome. Pediatrics 2003;111:692-702.
15. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at UCSC. Genome Res 2002;12:996-1006.
16. Massa G, Verlinde F, De Schepper J, Thomas M, Bourguignon JP, Craen M, de Zegher F, Francois I, Du Caju M, Maes M, Heinrichs C; Belgian Study Group for Paediatric Endocrinology. Trends in age at diagnosis of Turner syndrome. Arch Dis Child 2005;90:267-268.
17. Frank-Raue K, Leidig-Bruckner G, Lorenz A, Rondot S, Haag C, Schulze E, Buchler M, Raue F. [Hereditary variants of primary hyperparathyroidism--MEN1, MEN2, HPT-JT, FHH, FIHPT]. Dtsch Med Wochenschr 2011;136:1889-1894. Epub 2011 Sep 13
18. Rozenblatt-Rosen O, Hughes CM, Nannepaga SJ, Shanmugam KS, Copeland TD, Guszczynski T, Resau JH, Meyerson M. The parafibromin tumor suppressor protein is part of a human Paf1 complex. Mol Cell Biol 2005;25:612-620.
19. Bradley KJ, Hobbs MR, Buley ID, Carpten JD, Cavaco BM, Fares JE, Laidler P, Manek S, Robbins CM, Salti IS, Thompson NW, Jackson CE, Thakker RV. Uterine tumours are a phenotypic manifestation of the hyperparathyroidism-jaw tumour syndrome. J Intern Med 2005;257:18-26.
20. Thakker RV, Newey PJ, Walls GV, Bilezikian J, Dralle H, Ebeling PR, Melmed S, Sakurai A, Tonelli F, Brandi ML; Endocrine Society. Clinical practice guidelines for multiple endocrine neoplasia type 1 (MEN1). J Clin Endocrinol Metab 2012;97:2990-3011. Epub 2012 Jun 20
21. Gunther DF, Eugster E, Zagar AJ, Bryant CG, Davenport ML, Quigley CA. Ascertainment bias in Turner syndrome: new insights from girls who were diagnosed incidentally in prenatal life. Pediatrics 2004;114:640-644.
22. Shinawi M, Cain MP, Vanderbrink BA, Grignon DJ, Mensing D, Cooper ML, Bader P, Cheung SW. Mixed gonadal dysgenesis in a child with isodicentric Y chromosome: Does the relative proportion of the 45,X line really matter? Am J Med Genet A 2010;152:1832-1837.
23. Gravholt CH, Fedder J, Naeraa RW, Müller J. Occurrence of gonadoblastoma in females with Turner syndrome and Y chromosome material: a population study. J Clin Endocrinol Metab 2000;85:3199-3202.
24. Schoemaker MJ, Swerdlow AJ, Higgins CD, Wright AF, Jacobs PA; UK Clinical Cytogenetics Group. Cancer incidence in women with Turner syndrome in Great Britain: a national cohort study. Lancet Oncol 2008;9:239-246. Epub 2008 Feb 20
25. Canto P, Kofman-Alfaro S, Jimenez AL, Soderlund D, Barron C, Reyes E, Mendez JP, Zenteno JC. Gonadoblastoma in Turner syndrome patients with nonmosaic 45,X karyotype and Y chromosome sequences. Cancer Genet Cytogenet 2004;150:70-72.
26. Coleman JF, MacLennan GT. Gonadoblastoma. J Urol 2006;175:2300.

CASE REPORT

DOI: 10.4274/jcrpe.galenos.2018.2018.0134

J Clin Res Pediatr Endocrinol 2019;11(1):94-99

Hyperphosphatemic Familial Tumoral Calcinosis in Two Siblings with a Novel Mutation in *GALNT3* Gene: Experience from Southern Turkey

Rabia Miray Kışla Ekinci[1], Fatih Gürbüz[2], Sibel Balcı[1], Atıl Bişgin[3], Mehmet Taştan[2], Bilgin Yüksel[2], Mustafa Yılmaz[1]

[1]Çukurova University Faculty of Medicine, Department of Pediatric Rheumatology, Adana, Turkey
[2]Çukurova University Faculty of Medicine, Department of Pediatric Endocrinology, Adana, Turkey
[3]Çukurova University Faculty of Medicine, Department of Medical Genetics, Adana, Turkey

What is already known on this topic?

Mutations in the *FGF23*, *KL* and *GALNT3* genes cause hyperphosphatemic familial tumoral calcinosis (HFTC), which is a rare disorder. Patients with HFTC commonly present with hyperphosphatemia and tumor-like soft tissue calcifications. The main management strategy for HFTC is pain control and phosphate depletion.

What this study adds?

We describe two siblings with hyperphosphatemic familial tumoral calcinosis due to a novel homozygote *GALNT3* mutation and add to the scarce literature. We wish to emphasize that physicians should also consider this rare condition in the differential diagnosis of calcinosis.

Abstract

Inactivating autosomal recessive mutations in fibroblast growth factor 23 *(FGF23)*, *klotho (KL)* and *polypeptide N-acetylgalactosaminotransferase 3 (GALNT3)* genes lead to a rare disorder, hyperphosphatemic familial tumoral calcinosis (HFTC). Patients with HFTC present with hyperphosphatemia and tumor like soft tissue calcifications. Although 78% of patients develop their first symptoms between the ages of 2-13 years, diagnosis is usually delayed until adulthood. Some individuals with the same genetic defect develop a condition named hyperphosphatemic hyperostosis syndrome. Herein we report two siblings suffering from periarticular, warm, hard and tender subcutaneous masses. Subcutaneous calcifications were present on X-ray and biopsy results were consistent with calcinosis in both patients. Laboratory results showed marked hyperphosphatemia and elevated renal tubular phosphate reabsorption rates, normal renal function tests and normal serum 25-hydroxyvitamin D levels. Thus, we suspected HFTC and performed next generation sequencing for the *GALNT3* gene, reported as the most frequent cause. A novel homozygote P85Rfs*6 (c.254_255delCT) mutation in *GALNT3* was identified in both siblings. Our report adds two new patients to the literature about this rare genetic disease and suggests that small deletions in the *GALNT3* gene may be related with HFTC phenotype.
Keywords: *GALNT3*, hyperphosphatemia, tumoral calcinosis

Introduction

Hyperphosphatemic familial tumoral calcinosis (HFTC) is a very rare disorder of phosphate homeostasis resulting from decreased fibroblast growth factor 23 (FGF23) synthesis or activity (1). *FGF23* gene encodes this protein which inhibits the sodium phosphate cotransporter in proximal renal tubules and 25-hydroxyvitamin D 1-α-hydroxylase expression, by its co-receptor *klotho (KL)*. The *polypeptide N-acetylgalactosaminotransferase 3 (GALNT3)* gene codes the enzyme known variously as UDP-N-acetyl-alpha-D galactosamine or polypeptide N-acetylgalactosaminyltransferase-3 (GalNAc-T3), which protects intact *FGF23* from catabolism and inactivation by

Address for Correspondence: Rabia Miray Kışla Ekinci MD, Çukurova University Faculty of Medicine, Department of Pediatric Rheumatology, Adana, Turkey
Phone: +90 322 458 68 68 **E-mail:** mir_kisla@hotmail.com **ORCID ID:** orcid.org/0000-0001-6234-822X

Conflict of interest: None declared
Received: 21.05.2018
Accepted: 17.07.2018

posttranslational glycosylation (2). Inactivating autosomal recessive mutations in *FGF23*, *KL* or the *GALNT3* genes lead to increased renal tubular phosphate reabsorption and, usually, elevated 1,25-dihydroxyvitamin D_3 (1,25-OH_2D_3), promoting gastrointestinal absorption of calcium and phosphorus (1,3,4).

Patients with HFTC usually present with hyperphosphatemia and tumor-like soft tissue calcifications. Although 78% of patients develop their first symptoms between two and 13 years of age, diagnosis is usually delayed until adulthood. Some individuals with the same genetic defect develop hyperphosphatemic hyperostosis syndrome (HHS), a condition which was formerly described as a distinct entity (5). Here we report childhood onset HFTC in two siblings with a novel homozygote *GALNT3* mutation.

Case Reports

Case 1

A previously healthy 10 year-old female patient presented with complaints of pain and swelling in her left elbow. Due to the limitation of movement of the elbow, surgery was performed in another medical center at the age of eight years. Excisional biopsy revealed well-circumscribed subcutaneous tissue including widespread dystrophic calcification and multinuclear giant cells. She was referred to us upon recurrence of bilateral calcinosis in her elbows and in her right upper thigh.

The patient was the offspring of a first-degree cousin marriage. Her past medical history revealed no myositis, skin lesions or renal disease. Physical examination revealed calcinous masses of approximately 3 cm-6 cm diameters in the left elbow, the right elbow and in the right upper thigh (Figure 1). The masses were warm, hard and tender. Laboratory results showed marked hyperphosphatemia, normal serum creatinine, 25-hydroxyvitamin D and parathormone levels and an elevated ratio of tubular maximum reabsorption of phosphorus/glomerular filtration rate (TmP/GFR), consistent with HFTC (Table 1). Direct radiographs demonstrated radio-opaque soft tissue masses around the elbows bilaterally and right upper femur diaphysis (Figure 2). Bone mineral density Z-score was 0.7. Dental and ophthalmological examination showed no involvement. Milimetric calcified plaques were present inside the right lower eyelid. A novel homozygote P85Rfs*6 (c.254_255delCT) mutation in exon 1 of the *GALNT3* gene was detected by next generation sequencing (NGS). *In silico* analyses was performed with Mutation Taster, which confirmed that the mutation led to frameshift and a premature stop codon. Both parents were heterozygous carriers for the same mutation.

Case 2

This nine year-old female patient was simultaneously referred to our department with her older sister, Case 1. She had developed similar but milder complaints over the preceding two years, including swelling of the left elbow which required surgery due to joint contracture and bilateral recurrence in her elbows thereafter. Direct radiographs demonstrated radio-opaque soft tissue masses around both elbows (Figure 2). Dental and ophthalmological examination showed no involvement. Hyperphosphatemia, elevated

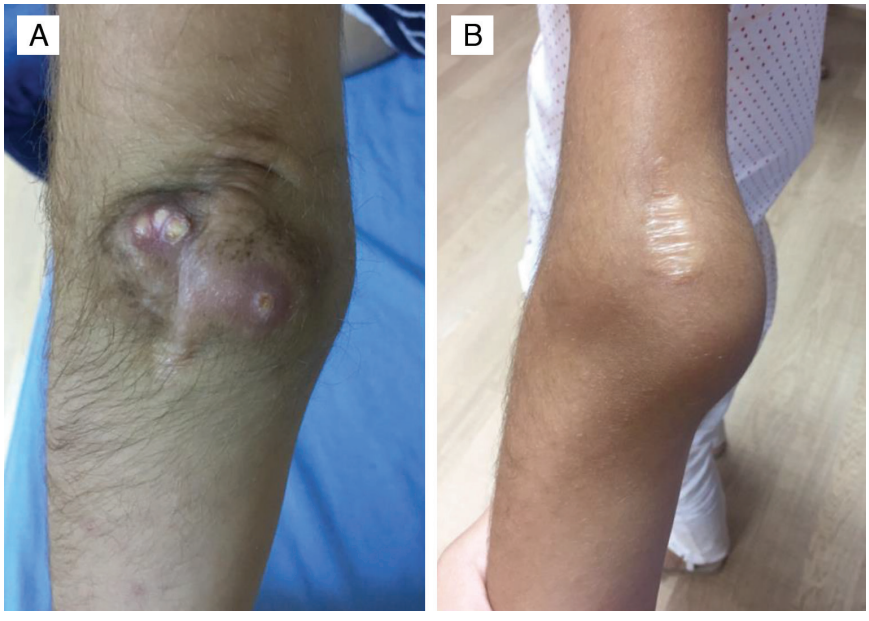


Figure 1. A) Calcinosis in the left elbow of Case 1. B) Subcutaneous mass around the left elbow of Case 2

Table 1. Clinical characteristics, laboratory and genetic results of the two patients with hypertrophic familial tumoral calcinosis

Parameters	Case 1	Case 2
Gender (M/F)	F	F
Age at calcinosis onset (years)	6	7
Age at HFTC diagnosis (years)	10	9
Dental involvement	No	No
Eyelid calcifications	Yes	No
Arterial calcifications on echocardiography	No	No
Serum phosphorus (normal: 3.7-5.6 mg/dL)	7.6	9.25
Serum calcium (normal: 9-11 mg/dL)	9.5	11
Serum creatinine (normal: 0.3-0.7 mg/dL)	0.3	0.4
Alkaline phosphatase (normal: 50-160 U/L)	97	100
Parathormone (normal: 10-65 pg/mL)	26	19.3
25-hydroxyvitamin D (normal: 20-100 ng/mL)	39.3	41.8
Erythrocyte sedimentation rate (normal: 0-20 mm/h)	31	13
C-reactive protein (normal: <0.5 mg/dL)	1.4	0.9
Leukocyte count (normal: 4000-10000/mm^3)	9300	6380
Renal tubular reabsorption of phosphate (normal: $>85\%$)	97.8	96.9
TmP/GFR ratio (normal: 2.9-6.5 mg/dL)	7.34	7.73
Bone mineral density Z-score	0.7	0.0
GALNT3 gene variant	P85Rfs*6 homozygote	P85Rfs*6 homozygote

HFTC: hypertrophic familial tumoral calcinosis, TmP/GFR: tubular maximum reabsorption of phosphorus/glomerular filtration rate, *GALNT3*: polypeptide N-acetylgalactosaminyltransferase 3, M: male, F: female

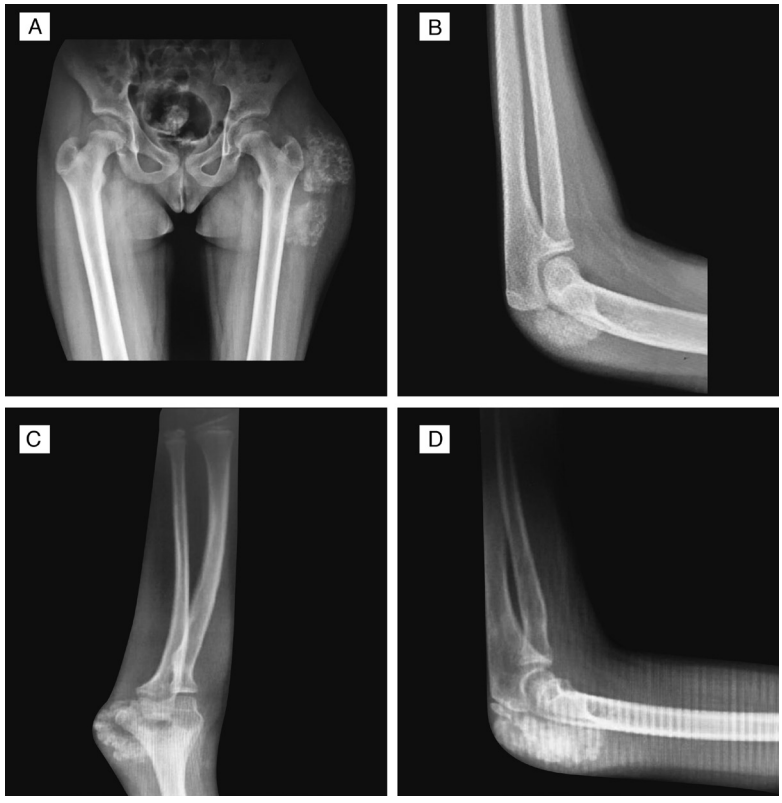


Figure 2. Radiographic findings in our two patients. Two giant radio-opaque soft tissue masses around the left femur neck of Case 1 in anteroposterior view (A). Lateral view of the left elbow of Case 1, revealing subcutanous calcifications (B). Anteroposterior view of upper extremities shows a radio-opaque mass around the elbow joint of Case 2 (C). A subcutaneous calcified mass behind the olecranon is identified in a lateral view of the left elbow (D)

TmP/GFR ratio, family history, biopsy result and presence of the same homozygote P85Rfs*6 (c.254_255delCT) mutation in *GALNT3* gene confirmed the diagnosis of HFTC.

Figure 3 shows the pedigree and NGS results of our patients.

A written informed consent for this report was obtained from the parents of the patients.

Discussion

Tumoral calcinosis (TC) is a condition in which calcium crystals accumulate in soft tissues, particularly in periarticular regions. HFTC is the autosomal recessive inherited form of TC with hyperphosphatemia and normal renal function. Differential diagnosis includes chronic renal failure, hypervitaminosis D, primary hyperparathyroidism or connective tissue diseases with particular emphasis on dermatomyositis and scleroderma. HFTC is very rare, approximately 75 cases have been genetically described worldwide to the best of our knowledge and almost all of the information is based on case reports (5,6,7,8,9,10). Homozygote mutations in the *GALNT3*, *FGF23* and *KL* genes were found in patients with the HHS phenotype. HHS is characterized by painful diaphyseal hyperostosis and may overlap with the TC phenotype in some cases. One study speculated that nonsense and missense *GALNT3* mutations are associated with TC and HHS phenotypes, respectively (11). Indeed, the majority of reported *GALNT3* mutations are missense or nonsense, and only five distinct small deletions were identified in HFTC patients, according to The Human Gene Mutation Database. Small deletions were reported to cause only the TC phenotype, as was also true for our patients (8,12,13).

Besides subcutaneous calcifications, patients often present with dental abnormalities and occasionally anemia, low-grade fever, regional lymphadenopathy, splenomegaly, amyloidosis, chronic recurrent osteomyelitis and eyelid calcifications (14,15,16). Vascular calcifications may rarely occur and can cause significant morbidity (17). Some HFTC patients develop hyperphosphatemia several years after the onset of dental abnormalities and calcinosis (11). Eyelid calcification was present in one of our patients. However, other clinical traits had not yet developed at presentation.

The management of HFTC mainly targets pain control and phosphate depletion. Surgery is not recommended, due to recurrences, until the calcinosis causes restricted joint movement. A phosphate restricted diet and phosphate binders are the mainstays of the medical treatment (18). A calcium-free phosphate binder, Sevelamer alters the intestinal absorption of phosphorus. Although Sevelamer

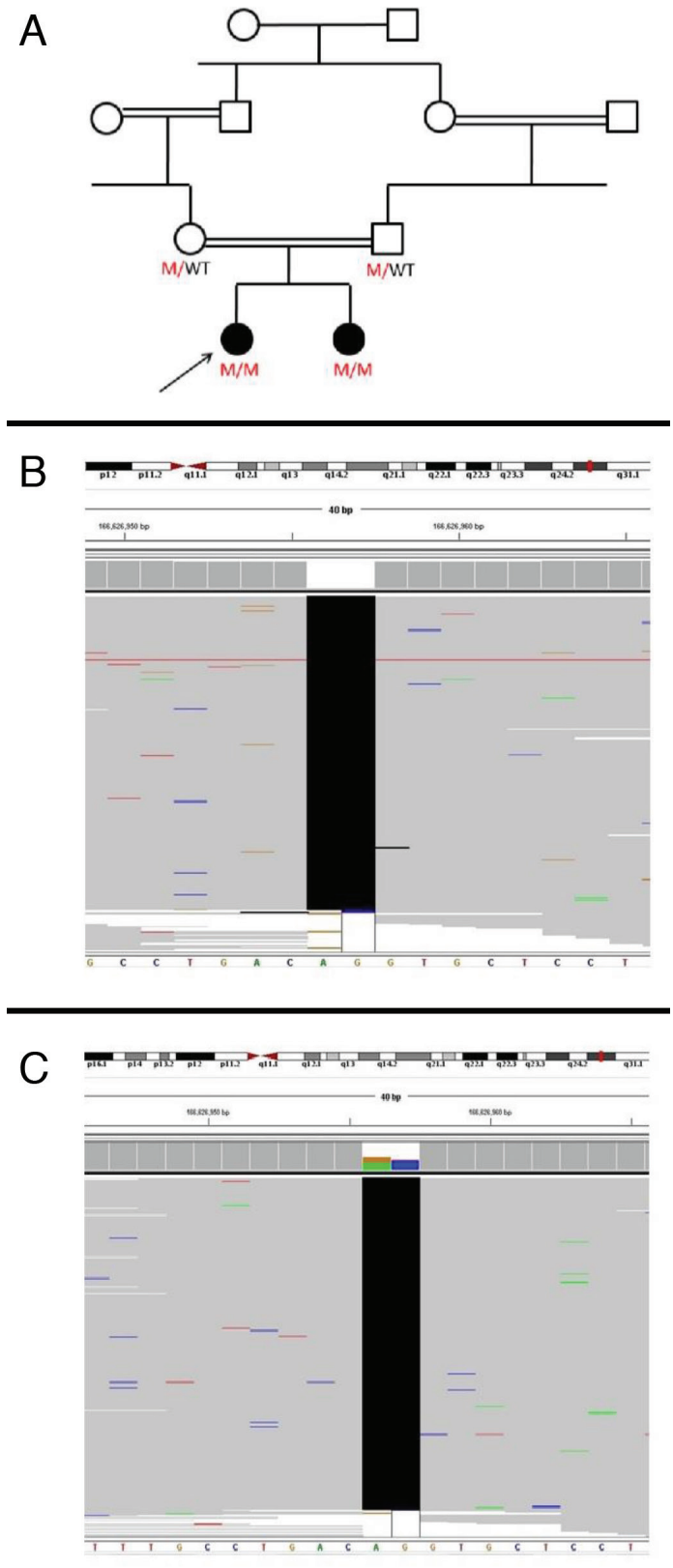


Figure 3. Genetic pedigree of the hyperphosphatemic familial tumoral calcinosis patients is presented in A. Next generation sequence view of the variant identified in the two cases are shown in B (Case 1) and C (Case 2)

and dietary phosphate restriction is reported to lead to complete or partial recovery of the calcinosis, recurrences have been reported due possibly to self-discontinuation or ineffectiveness of the drug in a significant proportion of patients. Other agents, including acetazolamide, probenocid and topical sodium thiosulfate, have been reported to be beneficial with variable outcomes (8,19,20,21).

The limitation of our study was the unavailability of serum 1,25-OH_2D_3 level determinations in both our patients. However, the patients had other clinical and laboratory results consistent with HFTC diagnosis. We believe that elevated serum 1,25-OH_2D_3 levels are only supportive in the diagnosis of HFTC.

In conclusion, we report two siblings with a novel homozygote *GALNT3* mutation representing an HFTC phenotype. HFTC is a rare cause of tenderness and pain around the joints in children and should be kept in mind in the differential diagnosis of arthritis. Our report adds two new patients to the information on a rare genetic disease and we wish to highlight the need for attention to this rare disorder. We speculate that small deletions in *GALNT3* gene may be related with HFTC phenotype. However, this speculation can be confirmed only with genotype-phenotype correlation studies including long-term outcomes of more patients in the future.

Ethics

Informed Consent: A written informed consent for this report was obtained from the parents of the patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Rabia Miray Kışla Ekinci, Sibel Balcı, Fatih Gürbüz, Concept: Rabia Miray Kışla Ekinci, Mustafa Yılmaz, Bilgin Yüksel, Design: Mustafa Yılmaz, Bilgin Yüksel, Data Collection or Processing: Atıl Bişgin, Fatih Gürbüz, Analysis or Interpretation: Sibel Balcı, Mehmet Taştan, Atıl Bişgin, Literature Search: Mehmet Taştan, Rabia Miray Kışla Ekinci, Writing: Rabia Miray Kışla Ekinci, Mustafa Yılmaz.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Benet-Pages A, Orlik P, Strom TM, Lorenz-Depiereux B. An FGF23 missense mutation causes familial tumoral calcinosis with hyperphosphatemia. Hum Mol Genet 2005;14:385-390. Epub 2004 Dec 8
2. Kato K, Jeanneau C, Tarp MA, Benet-Pagès A, Lorenz-Depiereux B, Bennett EP, Mandel U, Strom TM, Clausen H. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. J Biol Chem 2006;281:18370-18377. Epub 2006 Apr 25
3. Topaz O, Shurman DL, Bergman R, Indelman M, Ratajczak P, Mizrachi M, Khamaysi Z, Behar D, Petronius D, Friedman V, Zelikovic I, Raimer S, Metzker A, Richard G, Sprecher E. Mutations in GALNT3, encoding a protein involved in O-linked glycosylation, cause familial tumoral calcinosis. Nat Genet 2004;36:579-581. Epub 2004 May 9
4. Ichikawa S, Imel EA, Kreiter ML, Yu X, Mackenzie DS, Sorenson AH, Goetz R, Mohammadi M, White KE, Econs MJ. A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. J Musculoskelet Neuronal Interact 2007;7:318-319.
5. Rafaelsen S, Johansson S, Ræder H, Bjerknes R. Long-term clinical outcome and phenotypic variability in hyperphosphatemic familial tumoral calcinosis and hyperphosphatemic hyperostosis syndrome caused by a novel GALNT3 mutation; case report and review of the literature. BMC Genet 2014;15:98.
6. Keskar VS, Imel EA, Kulkarni M, Mane S, Jamale TE, Econs MJ, Hase NK. The Case | Ectopic calcifications in a child. Kidney Int 2015;87:1079-1081.
7. Shah A, Miller CJ, Nast CC, Adams MD, Truitt B, Tayek JA, Tong L, Mehtani P, Monteon F, Sedor JR, Clinkenbeard EL, White K, Mehrotra R, LaPage J, Dickson P, Adler SG, Iyengar SK. Severe vascular calcification and tumoral calcinosis in a family with hyperphosphatemia: a fibroblast growth factor 23 mutation identified by exome sequencing. Nephrol Dial Transplant 2014;29:2235-2243.
8. Ramnitz MS, Gourh P, Goldbach-Mansky R, Wodajo F, Ichikawa S, Econs MJ, White KE, Molinolo A, Chen MY, Heller T, Del Rivero J, Seo-Mayer P, Arabshahi B, Jackson MB, Hatab S, McCarthy E, Guthrie LC, Brillante BA, Gafni RI, Collins MT. Phenotypic and Genotypic Characterization and Treatment of a Cohort With Familial Tumoral Calcinosis/Hyperostosis-Hyperphosphatemia Syndrome. J Bone Miner Res 2016;31:1845-1854. Epub 2016 Sep 20
9. Shawar SM, Ramadan AR, Ali BR, Alghamdi MA, John A, Hudaib FM. FGF23-S129F mutant bypasses ER/Golgi to the circulation of hyperphosphatemic familial tumoral calcinosis patients. Bone 2016;93:187-195. Epub 2015 Nov 24
10. Masi L, Beltrami G, Ottanelli S, Franceschelli F, Gozzini A, Zonefrati R, Galli G, Ciuffi S, Mavilia C, Giusti F, Marcucci G, Cioppi F, Colli E, Fossi C, Franchi A, Casentini C, Capanna R, Brandi ML. Human Preosteoblastic Cell Culture from a Patient with Severe Tumoral Calcinosis-Hyperphosphatemia Due to a New GALNT3 Gene Mutation: Study of In Vitro Mineralization. Calcif Tissue Int 2015;96:438-452. Epub 2015 Apr 23
11. Dumitrescu CE, Kelly MH, Khosravi A, Hart TC, Brahim J, White KE, Farrow EG, Nathan MH, Murphey MD, Collins MT. A case of familial tumoral calcinosis/hyperostosis-hyperphosphatemia syndrome due to a compound heterozygous mutation in GALNT3 demonstrating new phenotypic features. Osteoporos Int 2009;20:1273-1278. Epub 2008 Nov 4
12. Garringer HJ, Fisher C, Larsson TE, Davis SI, Koller DL, Cullen MJ, Draman MS, Conlon N, Jain A, Fedarko NS, Dasgupta B, White KE. The role of mutant UDP-N-acetyl-alpha-D-galactosamine-polypeptide N-acetylgalactosaminyltransferase 3 in regulating serum intact fibroblast growth factor 23 and matrix extracellular phosphoglycoprotein in heritable tumoral calcinosis. J Clin Endocrinol Metab 2006;91:4037-4042. Epub 2006 Jul 25
13. Ichikawa S, Baujat G, Seyahi A, Garoufali AG, Imel EA, Padgett LR, Austin AM, Sorenson AH, Pejin Z, Topouchian V, Quartier P, Cormier-Daire V, Dechaux M, Malandrinou FCh, Singhellakis PN, Le Merrer M, Econs MJ. Clinical variability of familial tumoral calcinosis caused by novel GALNT3 mutations. Am J Med Genet A 2010;152:896-903.
14. Chefetz I, Heller R, Galli-Tsinopoulou A, Richard G, Wollnik B, Indelman M, Koerber F, Topaz O, Bergman R, Sprecher E, Schoenau E. A novel

homozygous missense mutation in FGF23 causes Familial Tumoral Calcinosis associated with disseminated visceral calcification. Hum Genet 2005;118:261-266. Epub 2005 Nov 15

15. Demellawy DE, Chang N, de Nanassy J, Nasr A. GALNT3 gene mutation-associated chronic recurrent multifocal osteomyelitis and familial hyperphosphatemic familial tumoral calcinosis. Scand J Rheumatol 2015;44:170-172. Epub 2014 Oct 29
16. Ichikawa S, Imel EA, Sorenson AH, Severe R, Knudson P, Harris GJ, Shaker JL, Econs MJ. Tumoral Calcinosis Presenting with Eyelid Calcifications due to Novel Missense Mutations in the Glycosyl Transferase Domain of the GALNT3 Gene. J Clin Endocrinol Metab 2006;91:4472-4475. Epub 2006 Aug 29
17. Chefetz I, Heller R, Galli-Tsinopoulou A, Richard G, Wollnik B, Indelman M, Koerber F, Topaz O, Bergman R, Sprecher E, Schoenau E. A novel homozygous missense mutation in FGF23 causes familial tumoral calcinosis associated with disseminated visceral calcification. Hum Genet 2005;118:261-266. Epub 2005 Nov 15
18. Claramunt-Taberner D, Bertholet-Thomas A, Carlier MC, Dijoud F, Chotel F, Silve C, Bacchetta J. Hyperphosphatemic tumoral calcinosis caused by FGF23 compound heterozygous mutations: what are the therapeutic options for a better control of phosphatemia? Pediatr Nephrol 2018;33:1263-1267. Epub 2018 Mar 28
19. Finer G, Price HE, Shore RM, White KE, Langman CB. Hyperphosphatemic familial tumoral calcinosis: Response to acetazolamide and postulated mechanisms. Am J Med Genet Part A 2014;164:1545-1549. Epub 2014 Mar 25
20. Balachandran K, Kamalanathan S, Sahoo JP, Das AK, Halanaik D. Differential response of idiopathic sporadic tumoral calcinosis to bisphosphonates. Indian J Endocr Metab 2014;18:521-525.
21. Jost J, Bahans C, Courbebaisse M, Tran TA, Linglart A, Benistan K, Lienhardt A, Mutar H, Pfender E, Ratsimbazafy V, Guigonis V. Topical Sodium Thiosulfate: A Treatment for Calcifications in Hyperphosphatemic Familial Tumoral Calcinosis? J Clin Endocrinol Metab 2016;101:2810-2815. Epub 2016 May 10

CASE REPORT

DOI: 10.4274/jcrpe.galenos.2018.2018.0110

J Clin Res Pediatr Endocrinol 2019;11(1):100-103

Severe Neonatal Cholestasis as an Early Presentation of McCune-Albright Syndrome

Nicole Coles[1], Ian Comeau[2], Tatiana Munoz[3], Jennifer Harrington[1], Roberto Mendoza-Londono[3], Andreas Schulze[3], Sari Kives[4], Binita M. Kamath[5], Jill Hamilton[1]

[1]University of Toronto, Hospital for Sick Children, Clinic of Endocrinology, Toronto, Canada
[2]Montreal Children's Hospital, Clinic of Adolescent Medicine and Paediatric Gynaecology, Montreal, Canada
[3]University of Toronto, Hospital for Sick Children, Clinic of Clinical and Metabolic Genetics, Toronto, Canada
[4]University of Toronto, Hospital for Sick Children, Clinic of Paediatric Gynaecology, Toronto, Canada
[5]University of Toronto, Hospital for Sick Children, Clinic of Gastroenterology, Hepatology and Nutrition, Toronto, Canada

What is already known on this topic?

Hepatic disease is a rare but described feature of McCune-Albright syndrome (MAS). Previous descriptions of cholestasis in MAS have detailed a benign phenotype with gradual improvement and resolution over time.

What this study adds?

This case demonstrates a presentation of severe cholestasis in McCune-Albright syndrome with significant associated morbidity, ultimately leading to liver transplantation.

Abstract

McCune-Albright syndrome (MAS) is a rare genetic disorder characterized by café-au-lait macules, polyostotic fibrous dysplasia and multiple endocrinopathies. Liver involvement, although described, is a rare complication. We review the case of a child with MAS whose initial presentation was characterized by severe neonatal cholestasis. The case demonstrates a severe phenotype of persistent cholestasis in MAS requiring liver transplantation. This phenotype has been previously considered to be a more benign feature. This case highlights the importance of consideration of MAS as an uncommon but important cause of neonatal cholestasis. Early diagnosis may allow for prompt recognition and treatment of other endocrinopathies.

Keywords: McCune-Albright syndrome, neonatal cholestasis, precocious puberty

Introduction

Classically, McCune-Albright syndrome (MAS) is characterized by café-au-lait macules, polyostotic fibrous dysplasia and multiple endocrinopathies, including precocious puberty. The condition presents with different tissue involvement depending on the location of the postzygotic somatic mutations, resulting in wide phenotypic variability. Understanding the diverse manifestations of this rare diagnosis is important as it is associated with potentially significant complications including short stature, fractures, facial deformity and hearing and vision loss.

Case Report

The index patient is a female infant born following an uncomplicated pregnancy. She was delivered at 40 weeks gestation with a birth weight of 2020 g and presented at two weeks of life with significant neonatal cholestasis. She had persistent jaundice and mild hepatomegaly with laboratory investigations showing evidence of conjugated hyperbilirubinemia [peak conjugated bilirubin 193 micromol/L, (normal range: 0-2 micromol/L)] and marked elevation of liver enzymes [peak aspartate aminotransferase (AST): 3633 U/L, (normal range: 0-110 U/L), peak alanine

Address for Correspondence: Jill Hamilton MD, University of Toronto, Hospital for Sick Children, Clinic of Endocrinology, Toronto, Canada
Phone: +4168135115 **E-mail:** Jill.hamilton@sickkids.ca **ORCID ID:** orcid.org/0000-0002-1958-2800

Conflict of interest: None declared
Received: 02.05.2018
Accepted: 01.07.2018

aminotransferase (ALT): 2120 U/L, (normal range: 0-60 U/L) and peak gamma-glutamyl transferase: 106, (normal range: 0-45 U/L)]. Further investigations revealed normal coagulation parameters [international normalized ratio: 0.9 (normal range: 0.6-1.6), partial thromboplastin time 38s (normal range: 25-44s)] and albumin levels [41g/L (normal range: 26-41 g/L)]. The patient underwent a comprehensive panel of investigations to determine the cause of the hepatobiliary dysfunction, but no clear diagnosis was identified. She had a normal TORCH screen, hepatitis B and C serologies, alpha-1-anti-trypsin level, galactosemia screen, metabolic and mitochondrial studies, urine bile acids and negative genetic testing for Niemann-Pick type C, Alagille syndrome and progressive familial intra-hepatic cholestasis. Imaging of the liver revealed mild hepatomegaly with non-specific mild heterogeneity and no evidence of biliary obstruction. A liver biopsy was performed at one month of age and showed significant liver damage with cytoplasmic and canalicular cholestasis, broad areas of resolving hepatocellular necrosis, giant cell transformation and abundant extramedullary hematopoiesis.

She experienced ongoing severe cholestasis with secondary complications, namely significant fat malabsorption and failure to thrive requiring placement of an enterostomy tube. The patient experienced episodes of recurrent respiratory infections and sepsis. She also sustained a non-traumatic femur fracture, which was thought to be related to metabolic bone disease in the setting of profound cholestasis and poor nutrition. As a result of these progressive complications, she was listed for liver transplantation and underwent a living related donor transplant at the age of 10 months. Histologic examination of the liver explant showed severe cholestatic liver disease with intrahepatic cholestasis, focal bile cannalicular plugs, mild to moderate focal peri-portal and sinusoidal fibrosis, yielding no definitive cause for the liver disease.

At 14 months of age, she presented with a five-day history of vaginal bleeding. She was otherwise well, with no history of fevers, discharge or abdominal pain. Physical examination was significant for sexual precocity including Tanner stage 2 breasts and pubic hair with an estrogenized vulva. Dermatological examination revealed multiple ragged-edge café-au-lait macules in the lumbosacral area and bilaterally on her legs.

Laboratory investigations revealed undetectable follicle-stimulating hormone and luteinizing hormone (<0.1/<0.1 IU/L), an elevated estradiol [168 pmol/L, (normal range <60 pmol/L)], a suppressed thyroid-stimulating hormone [<0.01 mIU/L, (normal range: 0.73-4.09 mIU/L)] and elevated free T4 [20.1 pmol/L, (normal range: 10-17.6 pmol/L)]. Cortisol level was normal [214 nmol/L, (normal range: 14-458 nmol/L)]. An initial bone age assessment after the episode of vaginal bleeding, revealed a slight advancement (by 4 to 10 months). However, follow up radiography performed at 21 months of age revealed an advancement of 21 months, with a bone age of 42 months. Skeletal survey revealed evidence of lesions in the femur and humerus suggestive of polyostotic fibrous dysplasia (Figure 1). A routine abdominal ultrasound showed an incidental finding of a bi-lobed left adnexal mass suggestive of an ovarian cyst (Figure 2). Assessment of the renal tubular absorption rate of phosphate did not reveal evidence of FGF23 mediated phosphate wasting. However, upon retrospective chart review, it was noted that she had had hypophosphatemia requiring phosphate supplements for several months prior to her transplant.

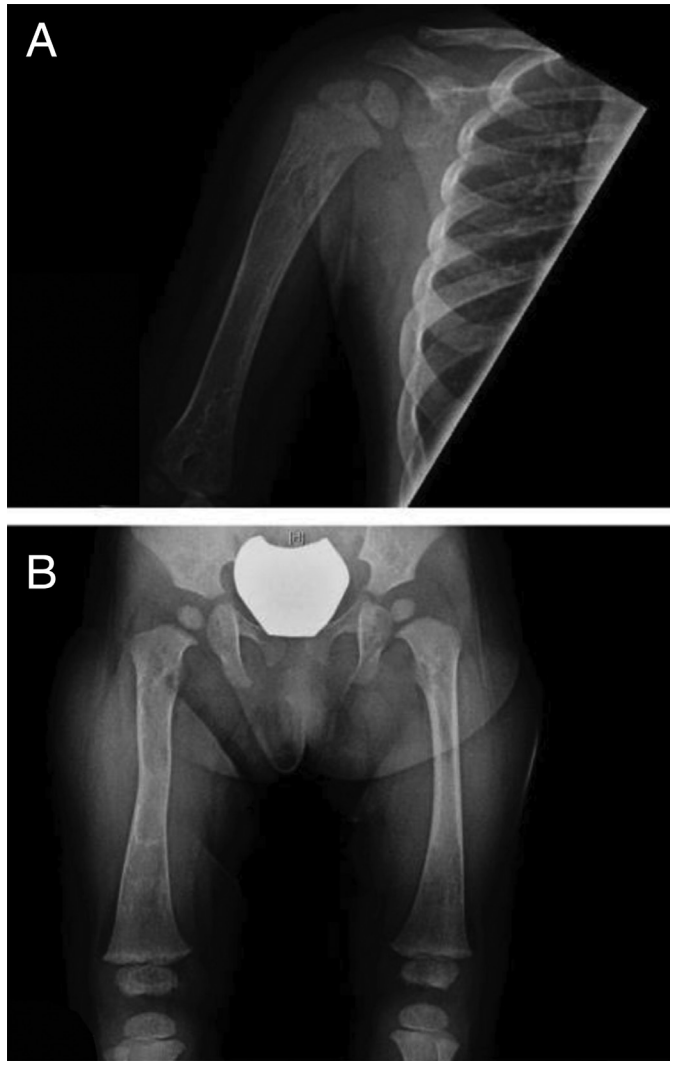


Figure 1. Skeletal survey demonstrating findings suggestive of polyostotic fibrous dysplasia. A) Ill-defined foci of linear sclerosis and lucency in the right proximal humerus. B) Ill-defined lucency with associated sclerosis in the left proximal femoral shaft

Written informed consent was obtained from the parents of the patient.

Final Diagnosis

Given the constellation of findings of gonadotropin independent precocious puberty, mild hyperthyroidism, café-au-lait macules and the appearance of polyostotic fibrous dysplasia, a clinical diagnosis of MAS was made. The question of whether the previous hepatobiliary dysfunction was an early pathologic manifestation of MAS was raised. G-protein coupled receptor mutation analysis was completed on native liver tissue obtained at transplant which identified one of the typical pathogenic variants (c.602G>A.p.R201H) in exon 8 of the *GNAS1 (adenylate cyclase stimulatory G protein)* gene that has been associated with the MAS phenotype (1,2,3).

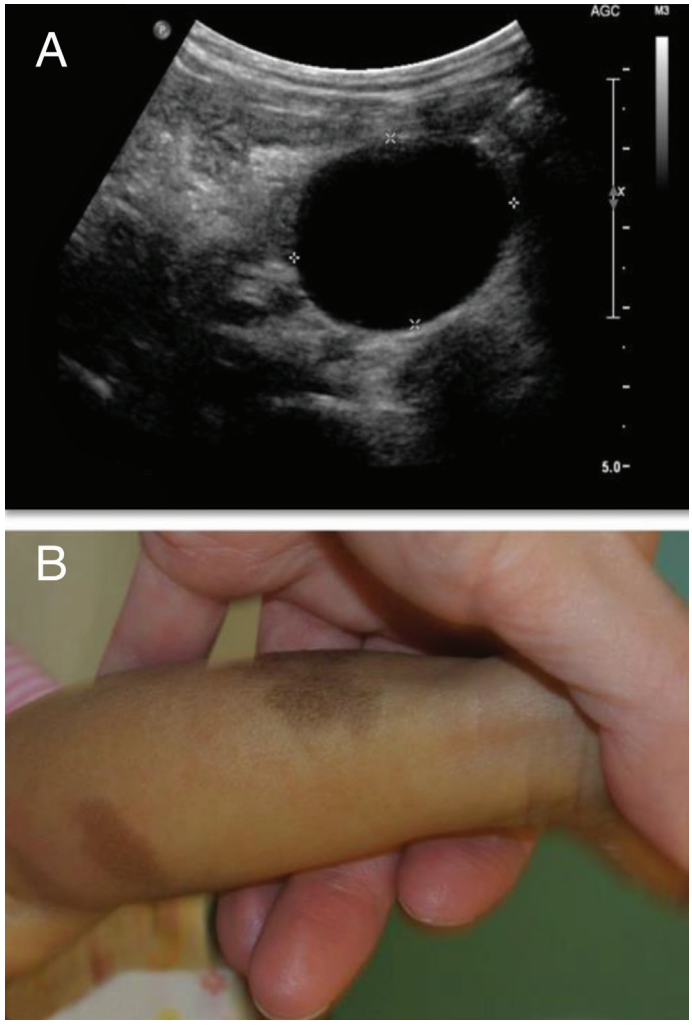


Figure 2. Clinical features suggestive of McCune-Albright syndrome. A) Abdominal ultrasound demonstrating large cystic structure in left ovary. B) Café-au-lait macules present bilaterally on legs and lumbosacral area

Hospital Course

Over the next six months, the patient continued to develop recurrent episodes of vaginal bleeding with evidence of an advancing bone age. She was initiated on a trial of an aromatase inhibitor for management of precocious puberty. She sustained recurrent fractures of her femur and received a course of bisphosphonate therapy. She has required intermittent treatment with methimazole for thyroid over-activity. She is receiving ongoing clinical surveillance of bone lesions and screening for other comorbidities of MAS.

Discussion

MAS is caused by activating somatic mutations within the *G protein α stimulatory subunit (GNAS)* gene (1). These mutations occur in the early post-zygotic period and the clinical presentation of patients will vary depending on the unique pattern of affected cells. Hepatic involvement has been described in some of the earliest case reports as an uncommon but early manifestation of MAS (1). One case series reported 16 patients with MAS and evidence of liver disease between 1937 and 1993 (2). Lumbroso et al (3) presented a case series of 113 patients with MAS, six of whom had evidence of cholestasis. Specific G-protein mutations have been identified in two case reports of patients with cholestasis and MAS (4,5). To our knowledge, our case is the first presentation of severe cholestasis with significant associated morbidity, ultimately leading to liver transplantation (6).

The underlying mechanism by which the constitutive activity of the G protein leads to cholestasis is unclear, however it has been suggested to play a role in bile metabolism (4). G protein coupled receptors play an important role in regulating intracellular signaling pathways in biliary epithelial cells. Interruption of normal signal transduction could impair cellular function, affecting bile formation and secretion by the cholangiocyte (4,7). Normalization of the liver function tests and complete resolution of the cholestasis does not always occur. However, existing descriptions of the natural history of the cholestasis suggest a benign course in most patients, with slow improvement and stabilization over time (4). In previously published case reports, where biochemical data are available, the liver enzymes are typically only mildly elevated (four to five-fold). The profound abnormalities seen in our patient (peak AST 33-fold elevation and peak ALT 35-fold elevation) are in marked contrast to these previous reports. With somatic mosaicism, the severity of the hepatic phenotype likely varies according to the number of cells affected by the mutation. This may account for the spectrum of liver disease described in the literature. Clinical presentation in our patient was affected

by a number of secondary complications including failure to thrive, recurrent infections and fractures. Interestingly, following liver transplantation, many of these comorbidities resolved or improved significantly.

Hepatobiliary lesions and hepatic adenomas have been identified in adult patients with MAS, further supporting the concept of persistent liver involvement as a pathologic feature of the syndrome (8). Less commonly, other gastrointestinal manifestations of MAS have been described, including intestinal polyps, pancreatitis and intra-ductal papillary mucinous neoplasms (4,8,9). Importantly, the authors advocate for consideration of radiographic screening of patients with MAS given the risk of malignancy associated with pancreatic intraductal papillary mucinous neoplasms, hepatic adenomas and choledochal cysts. This may be particularly relevant for patients with identified gastrointestinal manifestations.

The reported patient was started on letrozole for treatment of precocious puberty. Aromatase inhibitors have been associated with mild liver abnormalities among women taking it as an adjuvant treatment for breast cancer and rarely it has been associated with more significant hepatoxicity (10). Longitudinal follow up of a pediatric cohort of patients with MAS treated with letrozole for precocious puberty did not report on any hepatic side effects (11). However, it is unclear if any patients in this series had pre-existing hepatic disease. Given this patient's history of liver transplantation, continued surveillance of liver function will be performed during her treatment course. This adverse reaction should also be considered for other patients with MAS and potential hepatic involvement.

The case reported here exhibits a severe phenotype of persistent cholestasis in MAS which has been previously considered to be a more benign feature. While this is a rare finding, we propose that MAS should be considered in the differential diagnosis of unexplained cholestasis. Occurring in the neonatal period, the presence of cholestatic liver disease may provide an early clue to the underlying diagnosis of MAS. In turn, prompt diagnosis may allow for screening, recognition and treatment of other endocrinopathies and bone disease.

Ethics

Informed Consent: Written consent was obtained from the patient to report this case.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nicole Coles, Ian Comeau, Tatiana Munoz, Jennifer Harrington, Sari Kives, Andreas Schulze, Concept: Jennifer Harrington, Design: Jennifer Harrington, Nicole Coles, Ian Comeau, Data Collection or Processing: Nicole Coles, Tatiana Munoz, Ian Comeau, Analysis or Interpretation: Nicole Coles, Ian Comeau, Tatiana Munoz, Roberto Mendoza-Londono, Jennifer Harrington, Sari Kives, Andreas Schulze, Literature Search: Nicole Coles, Ian Comeau, Binita M. Kamath, Jill Hamilton, Writing: Nicole Coles, Ian Comeau, Tatiana Munoz, Roberto Mendoza-Londono, Jennifer Harrington, Sari Kives, Andreas Schulze.

Financial Disclosure: Nicole Coles, Ian Comeau, Tatiana Munoz, Jennifer Harrington, Sari Kives and Andreas Schulze have nothing to declare. Jill Hamilton is supported by the Mead Johnson Chair in Nutritional Science at the University of Toronto. Binita Kamath is a consultant for Retrophin.

References

1. McCune D, Bruch H. Osteodystrophia fibrosa: report of a case in which the condition was combined with precocious puberty, pathologic pigmentation of the skin and hyperthyroidism, with a review of the literature. Am J Dis Child 1937;54:806-848.
2. Shenker A, Weinstein LS, Moran A, Pescovitz OH, Charest NJ, Boney CM, Van Wyk JJ, Merino MJ, Feuillan PP, Spiegel AM. Severe endocrine and nonendocrine manifestations of the McCune-Albright syndrome associated with activating mutations of stimulatory G protein GS. J Pediatr 1993;123:509-518.
3. Lumbroso S, Paris F, Sultan C; European Collaborative Study. Activating Gsalpha mutations: analysis of 113 patients with signs of McCune-Albright syndrome--a European Collaborative Study. J Clin Endocrinol Metab 2004;89:2107-2113.
4. Silva ES, Lumbroso S, Medina M, Gillerot Y, Sultan C, Sokal EM. Demonstration of McCune-Albright mutations in the liver of children with high gammaGT progressive cholestasis. J Hepatol 2000;32:154-158.
5. Schwindinger WF, Levine MA. McCune-Albright syndrome. Trends Endocrinol Metab 1993;4:238-242.
6. Boyce A, Collins M. Fibrous Dysplasia/McCune-Albright Syndrome: Gene Reviews. Gene Reviews. Seattle, University of Washington, 2015.
7. Leite M, Andrade V, Nathanson M. Signaling Pathways in Liver Diseases. In: Dufour JF, Clavien PA, Trautwein C, Graf R (eds). Berlin, Heidelberg: Springer Berlin Heidelberg, 2005.
8. Gaujoux S, Salenave S, Ronot M, Rangheard AS, Cros J, Belghiti J, Sauvanet A, Ruszniewski P, Chanson P. Hepatobiliary and Pancreatic neoplasms in patients with McCune-Albright syndrome. J Clin Endocrinol Metab 2014;99:97-101. Epub 2013 Dec 20
9. Zacharin M, Bajpai A, Chow CW, Catto-Smith A, Stratakis C, Wong MW, Scott R. Gastrointestinal polyps in McCune Albright syndrome. J Med Genet 2011;48:458-461. Epub 2011 Feb 28
10. Gharia B, Seegobin K, Maharaj S, Marji N, Deutch A, Zuberi L. Letrozole-induced hepatitis with autoimmune features: a rare adverse drug reaction with review of the relevant literature. Oxford Med Case Reports 2017;2017.
11. Estrada A, Boyce AM, Brillante BA, Guthrie LC, Gafni RI, Collins MT. Long-term outcomes of letrozole treatment for precocious puberty in girls with McCune-Albright syndrome. Eur J Endocrinol 2016;175:477-483. Epub 2016 Aug 25

BRIEF REPORT

DOI: 10.4274/jcrpe.galenos.2018.2018.0162

J Clin Res Pediatr Endocrinol 2019;11(1):104-108

Extreme Premature Small for Gestational Age Infants Have Appropriate Catch-up Growth at Term Equivalence Compared with Extreme Premature Appropriate for Gestational Age Infants

Sze May Ng[1,2], Donatella Pintus[2], Mark A. Turner[1]

[1]University of Liverpool, Institute of Translational Medicine, Department of Women's and Children's Health, Liverpool, UK
[2]Southport and Ormskirk Hospital NHS Trust, Department of Paediatrics, Southport, UK

What is already known on this topic?

Small for gestational age (SGA) infants are at risk of impaired postnatal growth. Majority of SGA infants born at term will have catch-up growth by 2 years of age but there are limited studies on extreme premature SGA infants.

What this study adds?

Extremely premature small for gestational age infants born less than 28 week's gestation have appropriate catch-up growth by the time they reach term equivalence suggesting that postnatal nutrition and care are important determinants of catch-up growth in these infants.

Abstract

Recent studies have shown that small for gestational age (SGA) term infants undergo catch-up growth during infancy but there is limited studies on early growth outcomes of extreme premature SGA infants. The aim of this study was to compare factors associated during birth in extremely premature infants less than 28 weeks' gestation who were born SGA (< 10^{th} percentile for gestational age) with those who were born appropriate-for-gestational age (AGA) (10^{th}-89^{th} percentile) and to determine whether there was catch-up growth at term equivalence. One hundred fifty-three extreme premature infants (89 males) born below 28 weeks' gestation were prospectively recruited. All infants had auxological measurements undertaken and prospective data on pregnancy, maternal factors, perinatal and postnatal data obtained. SGA infants at birth had significantly higher Clinical Risk Index for Babies scores and mortality, lower birth weight, smaller head circumference, smaller mid arm circumference and shorter leg length at time of birth compared with AGA infants. However, at term equivalence, weight and leg length of were not significant between AGA and SGA infants born at extreme prematurity. Our study shows that extreme premature SGA infants have appropriate catch-up growth by the time they reach term equivalence suggesting that postnatal nutrition and care are important determinants of catch-up growth in SGA infants.

Keywords: Small for gestational age, prematurity, growth

Introduction

Small for gestational age (SGA) is a term most commonly used to indicate infants born with a weight below the 10^{th} percentile for their gestational age. Infants born SGA are at increased risk of perinatal morbidity, persistent short stature and metabolic alterations in later life (1). Infants born SGA with low birth length who do not achieve catch-up growth by the age of two years have a 7-fold higher relative risk of short stature than children born at normal size (2) and this risk is further increased by extreme prematurity (3). While the vast majority of SGA infants do show catch-up growth by 2 years of age, one in 10 does not (4,5). SGA infants who fail to catch-up and do not reach their target height range, remain short throughout childhood (6,7) and approximately 10% of SGA children will continue to further fall below the 3^{rd} percentile of height into adulthood (8,9). Intrauterine and neonatal growth failure of very low birth weight SGA and premature infants may also have long-term implications for adult health with risk of future adverse metabolic outcomes (8,10,11).

Address for Correspondence: Sze May Ng MD, University of Liverpool, Institute of Translational Medicine, Department of Women's and Children's Health, Liverpool, UK
Phone: +01695656163 **E-mail:** may.ng@nhs.net **ORCID ID:** orcid.org/0000-0002-3449-0541

Conflict of interest: None declared
Received: 25.06.2018
Accepted: 27.08.2018

Accordingly, the aim of this study was compare factors associated during birth in extremely premature infants who were born SGA (< 10th percentile for gestational age) with those who were born appropriate-for-gestational age (AGA) (10th-89th percentile) and to determine whether there was catch-up growth at term equivalence, defined in our study at 36 weeks corrected gestational age (CGA).

Methods

In our study 153 extreme premature infants (89 males) born less than 28 weeks' gestation were prospectively recruited from the TIPIT study and the infants were randomised according to trial protocol (12,13). The following were excluded: infants born to mothers with known thyroid disease or on anti-thyroid medications or amiodarone, and infants with major congenital or chromosomal abnormalities. This study is part of a post-hoc analysis to look at comparisons between babies born SGA versus AGA carried out at the Neonatal Intensive Care Unit of Liverpool Women's Hospital, UK. All infants had auxological measurements undertaken and prospective data on pregnancy, maternal factors, perinatal and postnatal data obtained. Ultrasound assessment of the thyroid gland at 36 weeks' CGA were also undertaken. Auxological measurements and data of SGA infants were compared with AGA infants to compare outcome measures at term equivalence.

Auxology Measurements

a) Mini-knemometry

The mini knemometer was used to measure the length of the lower leg of the infants and to determine short term growth in SGA and AGA premature babies. This device has been validated as an accurate device for the detection of small growth spurts that in neonates occur within days (14). The mini knemometer measures the distance from the bottom of the heel to the top of the knee in neonates with the knee flexed at an approx. 90 degrees angle (Figure 1). In order to avoid measurement bias and to develop a standardised method for measuring the infants leg length using this device, a 4-week learning period was required to provide reliable results. Lower leg length measurements of 6 preterm infants were taken using the mini knemometer followed by repeated measurements within a day period for each infant. The acquired mean technical error (mean standard deviation of 5 sequential readings) in all 6 studied infants was 0.15 mm (Table 1).

b) Mid arm circumference (MAC)

The MAC for each infant was measured using a normal non-stretch measuring tape and measurements were rounded to the nearest 0.1 cm. The measuring point called the mid-point of the upper arm was located in the mid upper arm half way between the tip of the shoulder blade and tip of the elbow. All infants were undressed during these measurements.

c) Head circumference

The occipitofrontal circumference for each infant was measured using a normal non-stretch measuring tape and measurements were rounded to the nearest 0.1 cm.

Statistical Analyses

Distributions of continuous outcomes were checked. P values were calculated using a t-test or Mann-Whitney U test as appropriate. Comparisons of SGA and AGA infants

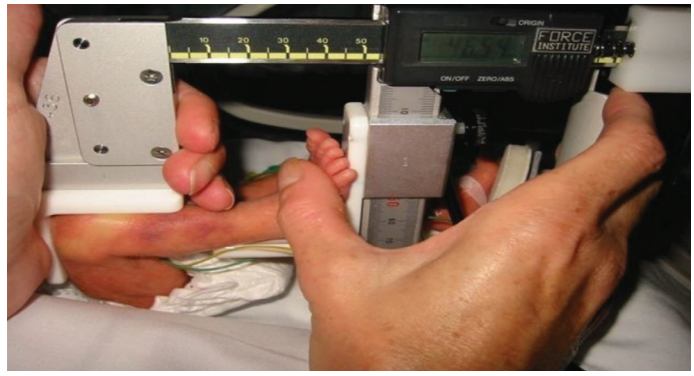


Figure 1. Knemometry measurement

Table 1. Reliability of mini-knemometry

	1st	2nd	3rd	4th	5th	SD
Case 1	45.62	45.67	45.10	45.29	45.6	0.23
Case 2	50.12	50.12	50.14	50.23	50.16	0.04
Case 3	53.45	53.44	53.45	53.41	53.44	0.14
Case 4	39.89	39.88	39.90	39.73	39.56	0.16
Case 5	51.11	51.1	51.12	51.11	51.13	0.13
Case 6	42.56	42.56	42.55	42.59	42.61	0.14
Mean SD						0.15

SD: standard deviation

were made using univariate analyses. Statistical Package for the Social Sciences 21.0 was used in the data analysis. The study was approved by North West Research Ethics Committee (reference number 07/MRE08/37).

Consent

The study was approved by North West Research Ethics Committee (reference number: 07/MRE08/37) and by the Medicines for Human Regulatory Agency. The parents of each potentially eligible baby were informed of the study's objectives and overall requirements after birth when the baby had achieved respiratory and haemodynamic stability. The Investigator explained the study fully to the patient's parent(s)/guardian(s) using the patient information leaflet. The parent/guardian was then given at least 12 hours to consider the study. If a parent/guardian was willing for the patient to participate in the study written informed consent was obtained.

Results

One hundred fifty-three infants were recruited to the study. The median gestational age at birth was 26.2 ± 1.0 and mean birth weight was 893.8 ± 178.1 grams. This study found that SGA infants at birth had significantly higher Clinical Risk Index for Babies scores and mortality, lower birth weight, smaller head circumference, smaller MAC and shorter leg length at time of birth compared with AGA infants. However, at term equivalence 36 weeks' gestation, weight and leg length of SGA and AGA infants were not significant (Table 2).

Discussion

SGA is a sign of growth failure due to maternal factors such as poor nutrition, chronic disease and infections (5,15), as well as potential environmental toxins (e.g. smoking and alcohol consumption) (16) and paternal factors including diabetes may also contribute to being born SGA (17). The aetiology of most SGA births remains unknown; however, several factors involving the foetus and placenta have been evaluated (15,18). Among these causes, lack of nutritional supply to the foetus is believed to be the primary cause of reduced foetal growth (19). In a Swedish population-based study, highest rates of mortality were observed in extremely premature SGA infants (15). This was in concordance with the results shown in our study.

General postnatal growth pattern can be divided into three phases: infancy, childhood, and puberty and failure of growth in any of these phases can reduce growth potential and eventually cause adult short stature (19). Studies have shown that SGA born neonates may experience a compensatory growth spurt or catch-up growth in infancy and childhood, however the timing of this growth spurt is not well described (20). Catch-up growth of infants born SGA mainly occurs from 6 months to 2 years and approximately 85% of SGA children will have catch-up by two years of age (21), however, babies born prematurely who are SGA may take around four years to achieve catch-up growth (22). The majority of infants born SGA show catch-up growth during the first few months of life followed by a normal pattern of development (19,23). In our study, SGA infants showed appropriate catch-up growth by the time they reached 36 weeks' CGA suggesting that early postnatal nutrition and care were important determinants of catch-up growth in SGA infants.

The thyroid hormones are essential for normal growth and development of the foetus and deficiency of thyroid hormones perinatally can impairs growth of the infant and compromises its adaptation to extrauterine life (24). Our results have not found any significant difference in either the maternal or infant thyroid status.

It is generally recognised that children born SGA have a significantly higher risk of short stature in adulthood than children born AGA at normal size, with a possible explanation that the programming of the endocrine axes occurs during critical phases of foetal development affected by intrauterine growth retardation (6). Accurate gestational dating and measurements of birth weight and length are important in identifying SGA infants. We recommend that comprehensive pregnancy, maternal, perinatal, and immediate postnatal data be obtained to help confirm diagnosis although often the cause of SGA remains unclear.

Limitations of the Study

Limitations of this study is the small sample size of this cohort of extremely premature infants born less than 28 weeks' gestation and the lack of data on maternal size or maternal glycaemic indices which could be potential confounding factors. Due to their extremes of viability, we were unable to obtain blood sampling for growth hormone factors and body size measurement of these infants were not feasible. The authors believe that the mini knemometer is a practical and novel method used to measure the length of the lower leg of the infants and to determine short term growth these extremely premature babies. The authors also acknowledge that many of extremely premature infants is unlikely to have appropriate catch-up growth by the time they reach term equivalence and factors associated with growth will require further research in a larger population group, which would be best performed as a multi-centered study.

Table 2. Comparative variables of small for gestational age and appropriate-for-gestational age infants

	AGA (N = 97)	SGA (N = 56)	Difference (95% CI) AGA-SGA	p value
Gestation (weeks)	25.86 (1.01)	25.80 (1.78)	0.06 (-0.38, 0.51)	0.78
Birth weight (kg)	0.81 (0.17)	0.70 (0.21)	0.09 (0.02, 0.15)	**0.01**
Gender (male)	55 (57%)	34 (60%)	-0.04 (-0.19, 0.12)	0.61β
CRIB score	5.05 (3.56)	6.41 (4.12)	-1.35 (-2.61, -0.11)	**0.03**
Infant baseline FT4 (pmol/L)	10.86 (5.1)	12.32 (5.1)	-1.45 (-4.12, 1.21)	0.28
Maternal age (years)	26.03 (7.0)	28.62 (6.90)	-0.99 (-3.51, 1.52)	0.43
Maternal history of smoking during pregnancy	20 (21.0%)	16 (29.0%)	-0.08 (-0.23, 0.058)	0.24β
Maternal history of alcohol during pregnancy	7 (7.2%)	3 (5.4%)	0.02 (-0.08, 0.098)	0.65β
Maternal FT4 (pmol/L)	16.57 (2.63)	15.52 (3.0)	1.04 (-0.66, 2.16)	0.06
Premature rupture of membrane	34 (35.1%)	20 (35.7%)	-0.01 (0.16, 0.15)	0.97β
Evidence of maternal pyrexia > 38 °C in the week before birth or raised maternal CRP in the week before birth	31 (32.0%)	9 (16.1%)	0.16 (0.02, 0.29)	**0.03**β
Caesarian section delivery	63 (64.97%)	38 (67.8%)	-0.03 (-0.18, 0.13)	0.60β
Antenatal steroids given	94 (96.9%)	51 (91.1%)		0.12β
Head circumference at screening (cm)	23.81 (1.68)	22.95 (1.90)	0.85 (0.22, 1.49)	**0.001**
Head circumference at 36 weeks (cm)	30.77 (1.960)	29.9 (2.14)	0.86 (0.06, 1.66)	0.51
Leg length at screening (mm)	40.19 (6.10)	39.70 (8.01)	2.95 (0.37, 5.53)	**0.03**
Leg length at 36 weeks (mm)	63.54 (7.3)	62.80 (10.0)	0.74 (-2.69, 4.16)	0.67
Mid arm circumference at screening (mm)	5.26 (0.55)	5.04 (0.60)	0.22 (0.008, 0.43)	**0.04**
Mid arm circumference at 36 weeks (mm)	8.23 (1.12)	8.22 (1.12)	0.39 (-0.06, 0.84)	0.09
Weight at screening (kg)	0.81 (0.17)	0.70 (0.21)	0.09 (0.02, 0.15)	**0.01**
Weight at 36 weeks (kg)	1.99 (0.40)	1.84 (0.43)	0.15 (-0.008, 0.16)	0.06
Subarachnoid space (mm)	0.20 (0.05)	0.19 (0.07)	0.01 (-0.01, 0.03)	0.57
Thyroid volume	0.57 (0.18)	0.58 (0.16)	-0.01 (-0.08, 0.06)	0.70
Mortality	14 (14%)	16 (28%)	-0.18 (-0.34, -0.037)	0.01β
Duration of mechanical ventilation (days)	Median 19.0 IQR (13.38)	Median 22.0 IQR (14.42)	-6.0 (0.00, 13.0)	0.50#
Number with CLD	57 (58.8%)	24 (42.9%)	0.16 (-0.01, 0.32)	0.06β
Oxygen levels required at 36 weeks (L/min) for CLD	Median 0.2 IQR (0.05, 1.5)	Median 0.5 IQR (0.08, 1.8)	0.00 (-0.23, 0.20)	0.86#

Data expressed as mean (SD) for continuous parametric outcomes, Student t-test; x (%) for categorical outcomes, βchi-square; median and IQR for non parametric outcomes; #Mann-Whitney U-Wilcoxon test.

SGA: small for gestational age, AGA: appropriate-for-gestational age, CLD: chronic lung disease, CRIB: Clinical Risk Index for Babies, CRP: C-reactive protein, IQR: interquartile ranges, SD: standard deviation

Conclusions

The mechanism of catch-up growth in premature SGA infants remains unclear (11). Our study shows that extremely premature SGA infants have appropriate catch-up growth by the time they reach term equivalence suggesting that postnatal nutrition and care are important determinants of catch-up growth in SGA infants. We recommend that early surveillance in a growth clinic for those without catch-up is advisable and neurodevelopment evaluation and interventions are warranted in at-risk children who fail to catch-up.

Ethics

Informed Consent: The study was approved by North West Research Ethics Committee (reference number 07/MRE08/37) and by the Medicines for Human Regulatory Agency (MHRA). Informed consent was obtained for all patients/parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sze May Ng, Mark A. Turner, Concept: Sze May Ng, Mark A. Turner, Design: Sze May Ng,

Mark A. Turner, Data Collection or Processing: Sze May Ng, Donatella Pintus, Mark A. Turner, Analysis or Interpretation: Sze May Ng, Literature Search: Sze May Ng, Donatella Pintus, Writing: Sze May Ng, Donatella Pintus, Mark A. Turner.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Clayton PE, Cianfarani S, Czernichow P, Johannsson G, Rapaport R, Rogol A. Management of the child born small for gestational age through to adulthood: a consensus statement of the International Societies of Pediatric Endocrinology and the Growth Hormone Research Society. J Clin Endocrinol Metab 2007;92:804-810. Epub 2007 Jan 2
2. Tenovuo A, Kero P, Piekkala P, Korvenranta H, Sillanpaa M, Erkkola R. Growth of 519 small for gestational age infants during the first two years of life. Acta Paediatr Scand 1987;76:636-646.
3. Farooqi A, Hagglof B, Sedin G, Gothefors L, Serenius F. Growth in 10- to 12-year-old children born at 23 to 25 weeks' gestation in the 1990s: a Swedish national prospective follow-up study. Pediatrics 2006;118:1452-1465.
4. Saenger P, Czernichow P, Hughes I, Reiter EO. Small for gestational age: short stature and beyond. Endocr Rev 2007;28:219-251. Epub 2007 Feb 23
5. Lee PA, Chernausek SD, Hokken-Koelega AC, Czernichow P; International Small for Gestational Age Advisory Board. International Small for Gestational Age Advisory Board consensus development conference statement: management of short children born small for gestational age, April 24-October 1, 2001. Pediatrics 2003;111:1253-1261.
6. Albertsson-Wikland K, Boguszewski M, Karlberg J. Children born small-for-gestational age: postnatal growth and hormonal status. Horm Res 1998;49(Suppl 2):7-13.
7. Hediger ML, Overpeck MD, McGlynn A, Kuczmarski RJ, Maurer KR, Davis WW. Growth and fatness at three to six years of age of children born small- or large-for-gestational age. Pediatrics 1999;104:33.
8. Hack M, Schluchter M, Cartar L, Rahman M, Cuttler L, Borawski E. Growth of very low birth weight infants to age 20 years. Pediatrics 2003;112:30-38.
9. Argente J, Mehls O, Barrios V. Growth and body composition in very young SGA children. Pediatr Nephrol 2010;25:679-685. Epub 2010 Jan 27
10. Cutfield WS, Regan FA, Jackson WE, Jefferies CA, Robinson EM, Harris M, Hofman PL. The endocrine consequences for very low birth weight premature infants. Growth Horm IGF Res 2004;14(Suppl A):130-135.
11. Darendeliler F, Bas F, Bundak R, Coban A, Sancakli O, Eryilmaz SK, Kucukemre B, Disci R, Gokcay G, Aki S, Ince Z, Eskiyurt N. Insulin resistance and body composition in preterm born children during prepubertal ages. Clin Endocrinol (Oxf) 2008;68:773-779. Epub 2007 Nov 2
12. Ng SM, Turner MA, Gamble C, Didi M, Victor S, Weindling AM. TIPIT: A randomised controlled trial of thyroxine in preterm infants under 28 weeks' gestation. Trials 2008;9:17.
13. Ng SM, Turner MA, Gamble C, Didi M, Victor S, Manning D, Settle P, Gupta R, Newland P, Weindling AM. An explanatory randomised placebo controlled trial of levothyroxine supplementation for babies born < 28 weeks' gestation: results of the TIPIT trial. Trials 2013;14:211.
14. Kaempf DE, Pflüger MS, Thiele AM, Linderkamp O. Validation of a newly developed mini-knemometer for premature infants. Ann Hum Biol 1999;26:259-266.
15. Clausson B, Cnattingius S, Axelsson O. Preterm and term births of small for gestational age infants: a population-based study of risk factors among nulliparous women. Br J Obstet Gynaecol 1998;105:1011-1017.
16. Baba S, Wikstrom AK, Stephansson O, Cnattingius S. Changes in snuff and smoking habits in Swedish pregnant women and risk for small for gestational age births. BJOG 2013;120:456-462. Epub 2012 Nov 27
17. Gluckman PD, Harding JE. The physiology and pathophysiology of intrauterine growth retardation. Horm Res 1997;48(Suppl 1):11-16.
18. Pollack RN, Divon MY. Intrauterine growth retardation: definition, classification, and etiology. Clin Obstet Gynecol 1992;35:99-107.
19. Botero D, Lifshitz F. Intrauterine growth retardation and long-term effects on growth. Curr Opin Pediatr 1999;11:340-347.
20. Beger C, Merker A, Mumm R, Gausche R. Growth prediction of small for gestational age infants within the first weeks after birth. Anthropol Anz 2018;74:377-382. Epub 2018 Feb 20
21. Karlberg JP, Albertsson-Wikland K, Kwan EY, Lam BC, Low LC. The timing of early postnatal catch-up growth in normal, full-term infants born short for gestational age. Horm Res 1997;48(Suppl 1):17-24.
22. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. BMJ 2000;320:967-971.
23. Leger J, Limoni C, Collin D, Czernichow P. Prediction factors in the determination of final height in subjects born small for gestational age. Pediatr Res 1998;43:808-812.
24. Forhead AJ, Fowden AL. Thyroid hormones in fetal growth and prepartum maturation. J Endocrinol 2014;221:87-103. Epub 2014 Mar 19.

DOI: 10.4274/jcrpe.galenos.2019.e001

J Clin Res Pediatr Endocrinol 2019;11(1):109

DOI: 10.4274/jcrpe.3238

Keskin M, Muratoğlu Şahin N, Kurnaz E, Bayramoğlu E, Savaş Erdeve Ş, Aycan Z, Çetinkaya S. A Rare Cause of Short Stature: 3M Syndrome in a Patient with Novel Mutation in *OBSL1* Gene. J Clin Res Pediatr Endocrinol 2017;9:91-94. Epub 2016 Oct 31

These mistakes have made by author inadvertently. The errors correction in the article has been demonstrated in the following list. "p.T45Nfs*40" word in page 91, Abstract section, line 8 has been corrected as "p.T425Nfs*40" and "p.T45Nfs*40" in Page 92, Case Report Section paragraph 2, line 12 has been corrected as "p.T425Nfs*40".

Location	Error	Correction
Page 91, Abstract section, line 8	p.T45Nfs*40	p.T425Nfs*40
Page 92, Case Report section paragraph 2, line 12	p.T45Nfs*40	p.T425Nfs*40

JCRPE
www.jcrpe.org
Journal of Clinical Research in Pediatric Endocrinology

Affirmation of Originality, Assignment of Copyright and Authorship Confirmation

Fax this form to: Feyza Darendeliler M.D.
Editor-in-Chief **Manuscript No.:**
The Journal of Clinical Research in Pediatric Endcorinology
Fax: +90 212 621 99 27

The author(s) hereby affirms that the manuscript entitled:

.....

.....

.....

is original, that all statement asserted as facts are based on author(s) careful investigation and research for accuracy, that the, manuscript does not, in whole or part, infringe any copyright, that it has not been published in total or in part and is not being submitted or considered for publication in total or in part elsewhere.

In signing this form, each author acknowledge that he/she participated in the work in a substantive way and is prepared to take public responsibility for the work.

In signing this form, each author further affirms that he or she has read and understands the "Ethical Guidelines for Publication of Research".

The author(s), in consideration of the acceptance of the above work for publication, does, hereby assign and transfer to the Journal of Clinical Research in Pediatric Endocrionolgy all of the rights and interest in and the copyright of the above titled work in its current form and in any form subsequently revised for publication ond/or electronic dissemination.

All authors must sign in the order in which each is listed in the authorship.

Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____
Date _____ Name (print) _____ Signature _____