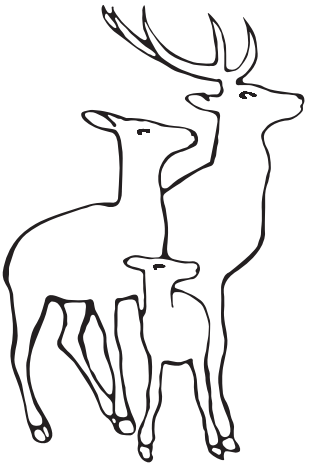
ISSN 0041-4301
Online ISSN 2791-6421
www.turkishjournalpediatrics.org

THE TURKISH JOURNAL OF PEDIATRICS

volume 65
issue 5
September-October 2023



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Volume 65 ▪ Issue 5
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ISSN: 0041-4301
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ISSN 0041-4301 Online ISSN 2791-6421
www.turkishjournalpediatrics.org

Cilt: 65 Sayı: 5, Eylül-Ekim 2023

YAYIN SAHİBİ
Yayımlayan Kurumlar Adına
Elif Nursel ÖZMERT

SORUMLU YAZI İŞLERİ MÜDÜRÜ
Enver HASANOĞLU

YAYIMLAYAN
Türkiye Milli Pediatri Derneği
Hacettepe Üniversitesi Çocuk Sağlığı Enstitüsü
Uluslararası Çocuk Merkezi

EDİTÖR ADRESİ
The Turkish Journal of Pediatrics
P.K. 36, Samanpazarı 06240 Ankara, Türkiye
Faks: +90 (312) 305 22 64
E-posta: editorial@turkishjournalpediatrics.org

YAYIN İDARE MERKEZİ
The Turkish Journal of Pediatrics Editör Ofisi
Hacettepe Üniversitesi
İhsan Doğramacı Çocuk Hastanesi
06100 Ankara
Tel : +90 (312) 305 26 76
Faks: +90 (312) 305 22 64

YAYININ TÜRÜ
Uluslararası hakemli dergi

YAYIN SIKLIĞI VE DİLİ
İki aylık ▪ İngilizce

BASIM YERİ
Meteksan Matbaacılık ve Teknik Sanayi A.Ş.
Beytepe No: 3, 06530 Bilkent, Ankara, Türkiye
Tel: +90 (312) 266 44 10 (Pbx)

BASIM TARİHİ: XX.XX.2023

YAYINCILIK HİZMETLERİ
Akdema Bilişim Yayıncılık ve Danışmanlık Tic. Ltd. Şti.
Kızılay Mah. Gazi Mustafa Kemal Bulvarı No: 23/8 06420
Çankaya/Ankara, Türkiye
Tel: +90 (533) 166 80 80 ▪ Web: www.akdema.com

ISSN 0041-4301 Online ISSN 2791-6421
www.turkishjournalpediatrics.org

Vol: 65 Issue: 5, September-October 2023

PRODUCTION MANAGER
Owner on behalf of the Publishers
Elif Nursel ÖZMERT

ADMINISTRATOR
Enver HASANOĞLU

PUBLISHED BY
Turkish National Pediatric Society
Hacettepe University Institute of Child Health
The International Children's Center

EDITORIAL OFFICE
The Turkish Journal of Pediatrics
P.K. 36, Samanpazarı 06240 Ankara, Türkiye
Fax: +90 (312) 305 22 64
E-mail: editorial@turkishjournalpediatrics.org

SUBSCRIPTION ADDRESS
The Turkish Journal of Pediatrics Editorial Office
Hacettepe University
İhsan Doğramacı Children's Hospital
06100 Ankara
Tel : +90 (312) 305 26 76
Fax: +90 (312) 305 22 64

PUBLICATION TYPE
International peer-reviewed journal

PUBLICATION FREQUENCY AND LANGUAGE
Bi-monthly ▪ English

PRINTED BY
Meteksan Matbaacılık ve Teknik Sanayi A.Ş.
Beytepe No: 3, 06530 Bilkent, Ankara, Türkiye
Tel: +90 (312) 266 44 10 (Pbx)

PRINT DATE: XX.XX.2023

PUBLISHING SERVICES
Akdema Informatics, Publishing, and Consultancy Trade LLC
Kızılay Mah. Gazi Mustafa Kemal Bulvarı No: 23/8 06420
Çankaya/Ankara, Türkiye
Tel: +90 (533) 166 80 80 ▪ Web: www.akdema.com

THE TURKISH JOURNAL OF PEDIATRICS

The Turkish Journal of Pediatrics is the joint publication of the Turkish National Pediatric Society, Hacettepe University Institute of Child Health and The International Children's Center. The journal has been published since 1958 and has been available online since 2002. The Turkish Journal of Pediatrics is published 6 times a year. The journal does not have article processing charges or article submission charges.

The Turkish Journal of Pediatrics is a multidisciplinary, peer reviewed, open access journal that seeks to publish research to advance the field of Pediatrics. The Journal publishes original articles, case reports, review of the literature, short communications, clinicopathological exercises and letters to the editor in the field of pediatrics. Articles published in this journal are evaluated in an independent and unbiased, double blinded peer-reviewed fashion by an advisory committee.

This publication is indexed in BIOSIS Previews, CABI Abstracts (Helminthological Abstracts, Nutrition Abstracts and Reviews Series A, Protozoological Abstracts, Review of Medical and Veterinary Entomology), EMBASE/Excerpta Medica, EBSCOhost (Medline with Full Text), IBIDS (International Bibliographic Information on Dietary Supplements), ProQuest (Medline, Professional ProQuest Central, ProQuest Health and Medical Complete, ProQuest Medical Library, ProQuest Pharma Collection), Web of Science - Science Citation Index (SCI) Expanded, Turkiye Citation Index and ULAKBİM TR-Index.

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The Turkish Journal of Pediatrics 2023; 65: 709-718
https://doi.org/10.24953/turkjped.2023.223

Review

Can early life interventions prevent food allergies?

Aysegül Ertuğrul[1], Özge Soyer[2]

[1]Department of Pediatric Allergy, Dr Sami Ulus Maternity and Children Research and Training Hospital, Ankara; [2]Department of Pediatric Allergy, Hacettepe University Faculty of Medicine, Ankara, Türkiye.

ABSTRACT

Background. The incidence of food allergies is increasing all over the world. Prevention strategies intend to reduce food sensitization risk and subsequent allergies. In this review, we will discuss the recent data concerning different geographic regions for the prevention of food allergies in children.

Methods. This review provides recommendations for the prevention of food allergies based on the recent data available in the PUBMED database in English (up to December 2022).

Results. The best strategy to prevent food allergies is regarded as the early introduction of allergenic foods to an infant's diet. A healthy and diverse diet is recommended for infants and their mothers, in accordance with the family's eating habits and regional food culture, rather than avoiding certain foods or using supplements. Avoiding common food allergens in the maternal diet during pregnancy and/or breastfeeding is not recommended. Exclusive breastfeeding is generally recommended for all mothers for at least 6 months. There is no specific association between exclusive breastfeeding and the primary prevention of any specific food allergy. Where a breastmilk substitute is needed, the best alternative should be chosen according to the infant's nutritional needs. There is no substantial evidence to support the use of hydrolyzed or soy formula in infancy against food allergies or sensitization.

Conclusions. Feeding patterns in infancy play an important role in the risk of developing food allergies. Existing strategies to prevent allergies are relatively ineffective and further research is needed to figure out strategies for food allergy prevention, particularly in high-risk infants.

Key words: allergy, child, food allergy, prevention.

One of the main causes of chronic illness in children is food allergy (FA), a complex immunological disorder that appears to have become more prevalent over the past 20 years in many different nations.[1] A multifactorial interaction of genetic factors and nutritional-environmental exposures is blamed for the underlying reason.[1] The content and diversity of the human microbiome of the gut and skin have been negatively impacted by inappropriate antibiotic usage, altered diets, non-vaginal deliveries, ultra-sanitary lifestyles, and less time spent outdoors.[2,3] These factors also had significant effects on the maturation of the immune system, which in turn affected the emergence of allergy illnesses like FA.[2,3] The most significant modifiable risk factors for FA development early in childhood include skin barrier dysfunction and delayed introduction of allergic solid foods.[4,5] As the dual allergen exposure hypothesis suggests, exposure to solid foods through the skin can lead to allergic sensitization, while oral exposure to solid foods induces tolerance.[4] Early oral exposure to allergens produces T-cell deviation toward Th1 and Treg subtypes, whereas early cutaneous exposure to allergens causes T-cell deviation toward a Th2 type and subsequent FA.[5] When the skin's protective barrier is compromised, allergens and microorganisms penetrate the skin and compromise the immune system. The formation of IgE and the allergic cascade then begin. Skin barrier dysfunction is made worse

✉ Ayşegül Ertuğrul
aysegull.ertugrul@gmail.com

Received 23rd March 2023, revised 15th June 2023, accepted 14th July 2023.

by immunological dysfunction.[5] Primary oral exposure of antigens allowing for presentation via a healthy mucosal immune system, rather than through an impaired skin epidermal barrier, is crucial to prevent FA.[4,5] Years of research have been conducted to determine whether changes in a mother's or an infant's diet can affect the risk of FA. These studies have searched for dietary patterns during infancy, pregnancy, and breastfeeding, avoiding certain allergenic foods, when to introduce certain foods, and dietary supplements.[2,5]

Since 2002, documents on the prevention of FA have been reported globally.[4] The European Academy of Allergy and Clinical Immunology (EAACI) Guidelines[6], Japanese Society of Pediatric Allergy and Clinical Immunology (JSPACI)[7], Chinese Expert Consensus[8], Indian Society of Pediatric Gastroenterology, Hepatology and Nutrition (ISPGHAN)[9], American Academy of Allergy, Asthma, and Immunology (AAAAI)[10], American College of Allergy, Asthma, and Immunology (ACAAI)[10]; Canadian Society for Allergy and Clinical Immunology (CSACI)[10], Australasian Society of Clinical Immunology and Allergy (ASCIA)[11], and the Malaysia Allergy Prevention (MAP)[12]; are the ones that have been updated in the last two years among these guidelines. These recommendations include prevention strategies to stop the onset of allergy symptoms or their progression, and risk groups have been defined to specify the recommendations.[4] A child with a family history of allergies is considered at high risk for allergic disease, according to numerous previous international papers.[10,13] Halken et al.[6] defined an increased risk of FA due to having a condition associated with FA such as eczema or asthma or having close relatives with a history of any allergy. AAAAI, ACAAI and CSACI proposed a risk gradient for the development of FA among infants.[10] The bottom of the pyramid represents a standard risk for infants in the general population. The ascending gradient of risk assessment for the development of FA lines up with a family history of atopy, mild to moderate eczema, and other food allergies and peaks with severe eczema as the highest risk for the development of FA.[10] A summary of current guidelines for the prevention of food allergies is provided in Fig. 1.

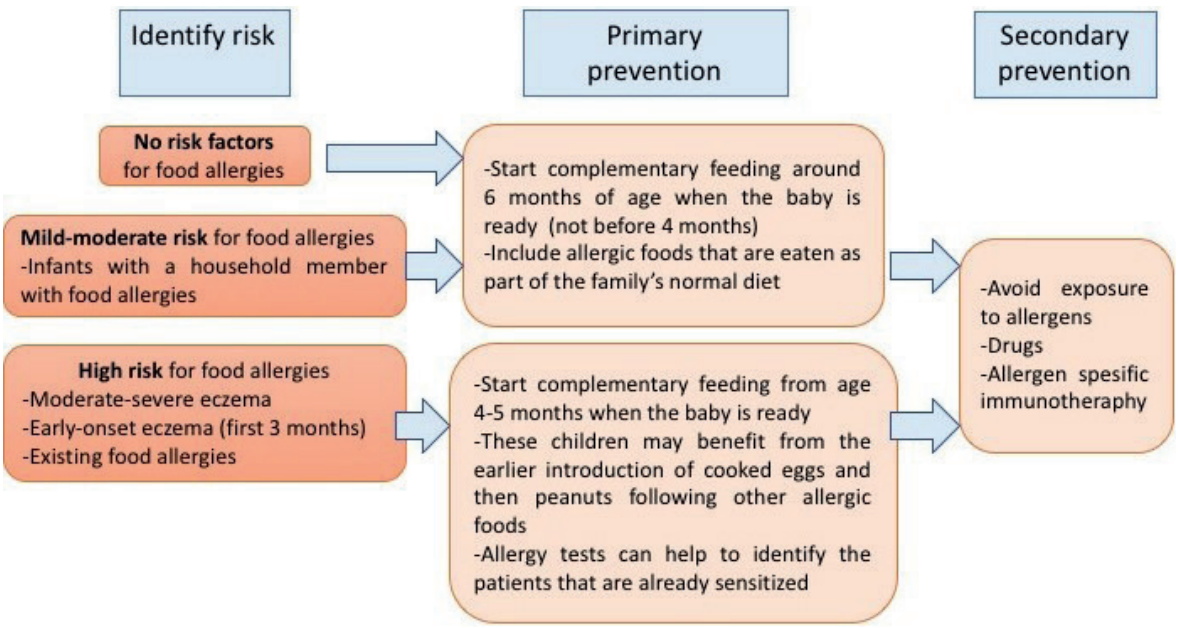

Fig. 1. Summary of current international guidelines for the prevention of food allergies.
Primary prevention refers to inhibiting the development of clinical disease before it occurs.[38]
Secondary prevention refers to the prevention of symptoms in those who have food allergies.[38]

Maternal diet in pregnancy and lactation

According to the World Health Organization (WHO), infants should be breastfed exclusively for the first six months of life and for the next two years to ensure their health.[14] Although most of the international food allergy guidelines recommend breastfeeding, there is no consistent evidence of allergy prevention through breastfeeding.[4,6,10,15-18] The EAACI guideline makes no recommendations for or against using breastfeeding to avoid FAs, but the guideline emphasizes that breastfeeding has many benefits for infants and mothers and supports WHO's global breastfeeding advice.[6] The American Academy of Pediatrics (AAP) and Japanese Pediatric Guideline for Food Allergy (JPGFA) reported that no implications can be made about the effect of breastfeeding on preventing or delaying any specific FAs.[7,19] In a systematic review and meta-analysis it was reported that breastfeeding of any duration does not seem to be an effective way to prevent FA, but that there is some weak evidence for the prevention of the development of AD in infants.[20]

Uncertainty exists about the effect of maternal diet during pregnancy and lactation on a child's immune development and risk of allergic diseases.[4] Some earlier studies and guidelines recommended avoiding highly allergenic foods, but these were not supported by later studies.[4,21] There is no evidence that maternal avoidance of known food allergens during pregnancy reduces the risk of their children developing allergic diseases.[4] Studies investigating diet indices on allergy outcomes have also failed to prove a consistent eating pattern based on the Healthy Eating Index, Dietary Inflammatory Index, or Mediterranean diet, during pregnancy and subsequent allergy prevention in the infant.[22] According to a review that considered 11 randomized controlled trials and 32 cohort studies including over 40 000 children, eating patterns including vegetable oils, margarine, nuts, and fast food are associated with a higher risk of developing atopy.[21] The authors also stated that Mediterranean dietary patterns, diets rich in fruits and vegetables, fish and vitamin D-containing foods are dietary patterns that are more likely to be associated with a lower risk of allergic diseases.[21] In a systematic review, Garcia et al.[20] indicated that a mother's diet during pregnancy and lactation might influence her child's risk of occurrence of the atopic disease. The authors found that supplementation with probiotics during late pregnancy and lactation may diminish the risk of eczema, and fish oil supplementation may reduce the risk of sensitization to food allergens.[20] The World Allergy Organization (WAO) guideline panel does not have a recommendation about prebiotic supplementation in pregnancy or during breastfeeding due to a lack of evidence.[23] Similarly, the EAACI task force does not have a recommendation for or against the use of vitamin supplements, fish oil, prebiotics, probiotics or symbiotics during pregnancy or lactation.[6] As a consequence, mothers should consume a healthy diet during pregnancy or breastfeeding and should neither reduce nor increase the intake of potentially allergenic foods to prevent the development of FA.[4]

Complementary foods

Infant feeding habits change according to local eating habits and food culture.[18] The introduction of allergenic foods into an infant's diet and the prevention of FA is not part of the WHO recommendations.[24] Early reports about preventing FA stated that eliminating allergenic foods or delaying the introduction of allergenic foods might reduce sensitization by preventing the transition of allergens through the increased permeability of the immature infant gut.[4] However, numerous subsequent studies demonstrated no benefits of allergen avoidance.[25]

The 2015 randomized trial Learning Early About Peanut Allergy on a sizable cohort of high-risk infants showed that early peanut introduction dramatically reduced the likelihood of peanut allergy in high-risk infants.[26] Early and frequent consumption of peanuts lowered the relative risk of developing a peanut allergy

by 81% at the age of five years.[26] According to a meta-analysis of 5 studies, consuming eggs between the ages of 4-6 months was found to be associated with a lower risk of developing an egg allergy than introducing eggs later in life.[27] Early introduction of fish into an infant's diet was found to reduce fish sensitization with very low certainty evidence.[27] There is no consistent evidence that the early introduction of cow's milk (CM) reduces the risk of CM allergy.[27] In 2016, a randomized trial involving breastfed infants evaluated whether the early introduction of six allergenic foods (CM, hen's egg, peanut, sesame, codfish, and wheat) in the diet would protect against the development of FA. In this trial, lower relative risks of peanut allergy and egg allergy were observed in the early-introduction group than in the standard-introduction group.[28] After these studies, updates were made to the guidelines; allergen avoidance gave way to the early consumption of certain solid foods.[5]

Recently, some authors have suggested introducing common allergenic solid foods at 4 to 6 months of age[6,19,28] indeed, some of them recommend not delaying the introduction of allergenic foods.[7] Asia Pacific Association of Pediatric Allergy, Respirology & Immunology (APAPARI) consensus statement's recommendations for infant feeding and the introduction of allergenic foods in infants differ according to the risk status of the infant.[18] APAPARI indicates that healthy infants should be introduced to complementary foods at 6 months of age however, the introduction of allergenic foods in healthy infants with a family history of atopy (at-risk infants) and in high-risk infants with severe eczema should not be delayed.[18] Regardless of risk status, the EAACI 2020 guideline on the prevention of the development of FA in children recommends introducing allergenic foods (cooked eggs and peanuts) to the infant's diet between the ages of 4 and 6 months as a part of complementary feeding.[6] Australian guidelines and ASCIA recommend introducing allergic foods within the first year of life.[11,15] AAP indicates that there is no evidence that delaying the introduction of allergenic foods, including peanuts, eggs, and fish, beyond 4 to 6 months, prevents allergic disease.[19] ACAAI, AAAI, and CSACI recommend that all infants irrespective of risk should be given cooked eggs and peanuts at around the age of 6 months, but not before 4 months of life, at home when the infant is developmentally ready.[10] Before allergen introduction, screening is not required, but the decision to screen is optional.[10] A medical assessment may be advisable in infants with severe AD and/or FA, before introducing common food allergens into the infant's diet.[29] In conclusion, it is difficult to make a standardized recommendation on when to introduce complementary foods and when to defer the introduction of solids for all infants. Recent guidelines in the USA and Europe advising early peanut introduction for high-risk infants with severe eczema or egg allergy have been published as a result of the high prevalence of peanut allergy in Western societies.[18,26] Due to the comparatively low prevalence of peanut allergy in most of Asia compared to the West, Asian recommendations diverge from US and European recommendations, which are primarily focused on preventing peanut allergy.[18] Early peanut introduction should be encouraged in nations with a high frequency of peanut allergy.[18,26] In Asian countries, egg allergy is more prevalent; therefore, the early introduction of a cooked egg is considered in infants with severe AD.[18]

How to begin complementary feeding is a controversial issue. It is advised to introduce new foods singly and a few days apart so that the responsible food can be identified in the event of an allergic reaction.[30] Current ASCIA[11] and BSACI[17] guidelines recommend introducing one new common allergen alone, but updated European[6] and the US/Canadian[10] guidelines have now removed this recommendation. The AAP indicated that it is safe to introduce multiple foods at once.[31] The order in which foods should be introduced varies according to studies. The recent US/Canadian consensus guideline stated that there

is no set order for these foods to be started.[10] The infant's developmental stages, nutritional needs, and cultural dietary practices should be taken into account for the introduction of complementary foods (eg, grains, fruits, or vegetables first followed by a cooked egg and then a peanut).[10] The family's needs and the local food culture should be taken into account when making recommendations about other common allergens like tree nuts and shellfish.[10] Ongoing exposure is needed to maintain tolerance; an introduction without ongoing consumption may be counterproductive.[30]

According to the EAACI position paper, high diet diversity in the first year of life was significantly associated with reduced food allergy risk up to 6 years of age.[32] The authors recommend that infants in any risk category for an allergic disease should have a diverse diet.[32] It has been proposed that a varied diet during infancy can affect allergy outcomes by exposing the gut microbiota to a variety of foods, increasing the intake of fiber, prebiotics, and omega-3 fatty acids, and promoting the development of immunological tolerance.[32] The introduction of solid foods during the weaning phase and greater dietary variety may increase the gut microbiome's diversity, positively impact its structure and function, and indirectly influence the development of tolerance through the microbiome.[10,32] A more diverse diet may also lead to exposure to different food antigens that impact the development of immune tolerance.[10,32] Consequently, diet diversity in the first year of life is associated with reduced food allergy outcomes and is recommended by allergen prevention strategies.[6,10]

A recent study has shown that commercial baby foods are low in common food allergen content.[33] If the infant's diet is heavily reliant on commercial infant food products, there would be a risk of limited exposure to allergens.[33] This low food allergen content may be disadvantageous for infants fed mostly with commercial infant foods because they are at risk of consuming insufficient amounts of the major food allergens on a regular basis during infancy.[33] Decreased levels of food variety can disrupt nutrient variety and a balanced diet.[33]

Based on recent data, consumption of solid foods (including allergenic foods) is recommended during the first year of life, according to the infant's neuro-developmental abilities and familial or cultural habits.[29] The use of home-prepared foods including the major food allergens according to regional food culture and considering the infant's readiness seems best.[29]

Formulas

Current evidence do not support that partially hydrolyzed formula (pHF) or extensively hydrolyzed formula (eHF) prevents FA in children, even in infants at high risk of allergy, but discrepancies among guidelines exist.[4] In a recent review of the Cochrane database, HF was compared with a CM-based formula for the prevention of allergic disease in infants who were not able to exclusively breastfeed however, no evidence was found for the suggestion of the use of HF.[34] According to the consensus approach of AAAAI, ACAAI and CSACI, the use of HF in infancy has not been shown to have any superiority in preventing FA or food sensitization.[10] The BSACI 2017[17] guideline doesn't recommend a hypoallergenic formula for the prevention of FA in children however the Japanese guideline does not have a recommendation. If possible, the BSACI guideline indicates that infants should be breastfed while starting complementary foods.[17] If breastmilk is unavailable, a CM-based formula should be used rather than a hypoallergenic formula, unless the infant has been diagnosed with CM allergy.[17] Irregular consumption of CM-based formula has been shown to enhance the risk of CM allergy, regularity appears to be crucial to promoting ongoing tolerance, with a few ingestions per week.[35] For this reason, continuous consumption of CM-based formula (as little as 10 mL daily) is crucial as not to lose tolerance.[35] The EAACI Task Force indicates that breastfed infants should not be given CM-based formula in the first week of life to prevent CM allergy.[6] No suggestion has been made for

or against using pHF or eHF to prevent CM allergy.[6] In case of not being able to breastfeed, the choice is up to families, including HF.[6] The EAACI Task Force recommends against utilization the of soy protein formula in the first 6 months of life to prevent FA.[6]

Dietary supplements

There is no common consensus for the utilization of dietary supplements globally.[4] In studies to date, no substantial evidence indicates that dietary supplementation diminishes the risk of any allergic findings in children.[4] In the EAACI 2020 update guideline, no suggestion is made for or against the use of vitamin supplements, fish oil, prebiotics, probiotics or symbiotics in infancy to prevent FA in infants and young children.[6] The 2020 ASCIA guideline[11] does not recommend their use however, the WAO guideline suggests the use of prebiotic supplements in not-exclusively breastfed infants but does not suggest their usage in exclusively breastfed infants, based on the very low certainty of the evidence.[23] The researchers concluded that prebiotic supplementation in infants reduces the risk of developing recurrent wheezing and possibly also the development of FA.[23] They also emphasized that prebiotic-containing formulas should not be used instead of breast milk.[23]

Consumption of omega-3 fatty acids and fibres/prebiotics may be significant, but the optimal dosage and the target group expected to benefit the most are not obvious yet.[29] There is no sufficient evidence to advise vitamin D, omega 3, or pre-or-probiotic supplements to prevent FA in infants. A diverse range of food ingestion in infancy may enhance the intake of nutrients and positively influence the gut microbiome composition and subsequently FA.[4,29]

Others

Atopic dermatitis is accepted as the initial step of allergic diseases. The development of AD often represents the beginning of the "atopic march", and usually proceeds with the development of FA, allergic asthma and allergic rhinitis.[2] It is not clear whether AD is the main pathology or the earliest finding of the underlying condition. Many studies showed a causal role for the skin barrier and cutaneous sensitization in the development of FA.[2] Although reported studies point to a shred of strong evidence for improving the skin to prevent AD and thus FA, two preventative emollient trials "Barrier Enhancement for Eczema Prevention (BEEP)[36]" and "Preventing Atopic Dermatitis and ALLergies in Children (PreventADALL)[37]" found no decrease in AD or FA. Brough et al.[2] suggested that improving the skin's integrity may prevent epicutaneous sensitization or give the possibility of gaining oral tolerance through early food consumption. It is expected that maintaining skin integrity and preventing AD would positively impact the prevention of allergic diseases, especially FAs.[2] Despite discrepancies, AD seems to be a significant risk factor for FA and may have an important role in FA prevention.[2]

Th1-stimulating infant vaccines are expected to act to prime the immune response away from a Th2-driven allergic phenotype.[6] The EAACI Task Force suggests against using the Bacillus Calmette-Guérin (BCG) vaccination to prevent FA in infants and young children.[6] No recommendation has been made for or against using emollients as skin barriers or using preventive oral immunotherapy to prevent FA in infants and young children.[6]

Conclusion

- Infant feeding recommendations alter according to regional public health priorities (Table I).
- Eliminating allergenic foods from the diet during pregnancy or breastfeeding to prevent sensitization is not suggested.
- Infants in any risk group for allergic disease and their mothers should consume a healthy and diverse diet, similar to what is ordinary for the family.

Table I. Summary of current recommendations about infant feeding, maternal diet and dietary supplements for food allergy prevention.

Guideline	Complementary foods /formulas / dietary supplements in infants	Maternal diet in pregnancy, during lactation
WHO, 2003[24]	- Suggest CF to be introduced no earlier than 6 mo of age, with no specific allergen advice	-
APAPARI, 2018[18]	-Healthy infants; Introduce CF at 6 mo of age	-
	-At-risk infants (healthy infants with a family history of atopy or non-severe eczema); No delay in the introduction of allergenic foods (including egg, cow's milk, peanut, soy, wheat, and shellfish).	
	-High-risk infants with severe eczema; Introduction of all allergenic foods should not be delayed and aggressive control of eczema	
BSACI, 2018[17]	- Suggest CF to be introduced at around 6 mo	-
	-Introduce cooked egg/peanut before 1 year (high-risk infants from 4 mo) and continue as part of a usual diet	
AAP, 2019[19]	-Healthy infants; peanut should be introduced together with other solid foods, in accordance with family preferences	-
	- Infants with mild-to-moderate eczema; should be introduced to peanut at around 6 mo, in accordance with family preferences	
	-High-risk infants (presence of severe eczema and/or egg allergy); should be introduced to peanut at 4–6 mo.	
Japanese guidelines, 2020[7]	-Healthy infants; peanut should be introduced together with other solid foods, in accordance with family preferences	-Food elimination is not recommended
	- Infants with mild-to-moderate eczema; should be introduced to peanut at around 6 mo, in accordance with family preferences	- Insufficient evidence supporting the use of probiotics
	-High-risk infants (presence of severe eczema and/or egg allergy); should be introduced to peanut at 4–6 mo.	
ASCIA, 2020[11]	- Suggest CF to be introduced at around 6 mo	- Food elimination is not recommended
	-Cooked egg in the first year (high-risk infants from 8 months)	
	- Wheat, fish, peanut, and other nuts before 12 months	
	- There is no consistent evidence to support a protective role for partially or extensively hydrolyzed formulas	
EAACI, 2021[6]	-Suggest introducing well-cooked egg and peanut from 4 to 6 mo	-Advise against restricting consumption of potential food allergens
	- Suggest avoidance of cow's milk-based formula for breastfed infants in the first week of life	
	- Advise against using soy protein formula in the first 6 months to prevent cow's milk allergy	
	No recommendation for or against for the use of vitamin supplements, fish oil, prebiotics, probiotics or symbiotic	
American and Canadian Consensus, 2021[10]	-Introduce peanut/cooked egg-containing products to all infants, irrespective of their relative risk of developing food allergy, at around 6 months of life, not before 4 months of life.	- Food elimination is not recommended
	-Do not delay the introduction of other potentially allergenic CF	-No recommendation to support any particular food or supplement
	-Infants should be fed a diverse diet	
	-Recommends against the routine usage of any hydrolyzed formula	
	- Use of supplements has no clear role	

*AAP: American Academy of Pediatrics, APAPARI: Asia Pacific Association of Pediatric Allergy, Respirology & Immunology, BSACI: British Society of Clinical Immunology and Allergy, EEACI: European Academy of Allergy and Clinical Immunology, WHO: World Health Organization, CF: complementary foods

- For optimal health of the infant exclusive breastfeeding is strongly suggested.
- Early introduction of allergenic foods (in the first year of life) into an infant's diet and increased diet diversity is currently the most promising prevention strategies for FA. However, there is no trial-based comparative data regarding the quantity or the frequency of allergen intake that is clearly associated with tolerance.
- Hydrolyzed (partially or extensively) infant formula or soy protein formula for the prevention of allergic disease is not recommended. Where a breastmilk substitute is needed, the best alternative should be chosen according to the infant's nutritional needs.
- Protecting skin integrity with the use of appropriate emollient care against proinflammatory conditions may prevent the development of AD and subsequently sensitization to foods.
- Th1-stimulating infant vaccines such as BCG vaccination to prevent FA are not suggested.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: ÖS, AE; data collection: ÖS, AE; analysis and interpretation of results: ÖS, AE; draft manuscript preparation: ÖS, AE. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 719-738
https://doi.org/10.24953/turkjped.2022.765

Review

Safety and efficacy of pharmacological approaches available for multisystem inflammatory syndrome in children (MIS-C): a systematic review

Yasothaa Velusamy[1], Govinathan Vivekanandan[2], Muhammad Hibatullah Romli[3], Aissvarya Shankar[1], Thilakavathy Karuppiah[1], Putri Yubbu[2]

[1]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; [2]Department of Pediatrics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia; [3]Department of Nursing and Rehabilitation, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia.

ABSTRACT

Background. To describe the existing pharmacological managements for Multisystem Inflammatory Syndrome in Children (MIS-C) in a systematic way, to identify the available pharmacological managements in MIS-C, evaluate its safety and efficacy and identify the best treatment procedures for practice recommendation.

Methods. A systematic search using six databases was conducted on August 18, 2021, updated in January 26th 2023. Terminologies that were used in this search are children, MIS-C/PIMS and SARS-CoV-2. A PRISMA flow diagram was used to report the study selection process. Quality analysis was done based on NOS and GRADE tools. Data synthesis was conducted by extracting the information on drugs used, efficacy and side effects.

Results. From the 32 articles included, a total of 2331 children with MIS-C were studied. The main pharmacological approaches were immunomodulatory therapy, i.e., intravenous immunoglobulin (IVIG) (77.3%), steroids (60.5%), and a combination of IVIG and steroids (41.3%). IVIG and steroids were found to be potentially effective and safe treatments for MIS-C. Combination of IVIG and steroids was found favorable in severe cases with higher recovery rate. Refractory treatments include second dose of initial treatment and biological response modifier drugs like anakinra, tocilizumab, infliximab. A small number of studies investigating supportive treatment consisted of vasoactive, inotropic and anticoagulation. The mortality rate was 1.28% and only three studies reported side effects from the treatment. Evidence of outcome from GRADE were mostly at moderate, low and very low levels.

Conclusions. This review provides preliminary evidence to support the current standard treatment practices in managing MIS-C pharmacologically. However, comprehensive investigation is required using clinical trials to provide stronger outcome evidence.

Key words: Multisystem Inflammatory Syndrome in Children (MIS-C), Paediatric Inflammatory, Multisystem Syndrome (PIMS), pharmacological treatment, systematic review.

Coronavirus Disease 2019 (COVID-19) became a global pandemic in early 2020.[1] Shortly, a newly recognized syndrome in children that causes severe multisystem inflammation and has clinical presentation like Kawasaki disease (KD) and toxic shock syndrome were reported.[2,3] This was then identified as a new disease associated with COVID-19 known as Multisystem Inflammatory Syndrome in Children (MIS-C). Alternately named pediatric inflammatory multisystem syndrome temporally associated with SARS- CoV-2 (PIMS-TS).

World Health Organization (WHO) criteria for MIS-C comprises, patients are less than 19 years old with fever (more than three days),

✉ Putri Yubbu
drputri@upm.edu.my

Received 17th August 2022, revised 1st March 2023, accepted 1st May 2023.

inflammatory laboratory evidence, clinically severe inflammation that needs hospitalization, no other credible diagnoses and positive result for the SARS-CoV-2 infection.[4] The overlapping features between MIS-C and KD suggest that they may share a similar immunopathogenesis explaining their responsiveness to similar treatments.[3]

Unlike KD, cardiogenic or vasoplegic shock are prominent features in MIS-C, with majority of cases requiring hemodynamic support and intensive care admission. Nonetheless, survival rates are high and a mortality rate of 1-9% alone, has been reported.[5,6] However, there is concern on the harm and risk of MIS-C if treatment is not properly given. Moreover, there is a need to identify which treatment effectively reduces pharmaceutical waste resources and cost.

Currently, there are several suggestions on MIS-C treatments, pharmacological approaches, interventions and managements.[4,7,8] Yet no randomized trial is has been conducted to support the pharmacological approach for MIS-C. There are several reviews available pertaining to the topic.[1,9,10] However, they only provide an overall summary and narratively focus on the suggested treatment, as well as the epidemiological perspective. There is a limited understanding on the evidence level of available pharmacological treatment for MIS-C on its safety, efficacy and side effects.

This systematic review aims to describe the existing pharmacological managements for MIS-C in a systematic way, to identify the available pharmacological treatment approaches in MIS-C, evaluate its safety and efficacy and identify the best treatment procedure.

Methods

This systematic review was registered in International Platform of Registered Systematic Review and Meta-analysis (INPLASY202220052). The review question was developed using the patient/population, intervention, comparison and outcomes (PICO) model and determined as "What is the available pharmacological treatment for MIS-C and its level of evidence on the safety and efficacy?"

Searching and selection

Systematic search was done on six databases (Academic Search Complete, CINAHL, Cochrane Clinical Answers, Cochrane Database of Systematic Reviews, MEDLINE and Scopus). Manual searching was done by reviewing the reference list of included studies, other reviews and articles known by the authors. Search terms used were children MIS-C, PIMS and SARS-CoV-2. Inclusion criteria were studies reporting pharmacological treatment for MIS-C, being an original study, and involving patients age of 19 years and below. The exclusion criteria were non-English, no full-text available, grey literature (e.g., conference abstract, guideline), non-research article (e.g., editorial), and review articles. No restriction was imposed on study design. The search was initially done on August 18, 2021, and updated in January 26th 2023. Screening and selection were conducted independently involving at least 2 authors (YV, SA and GV) compared for pre-consensus agreement, disagreements were resolved by discussion. Other authors (MHR, PY and TK) validated the process through review. The process is reported using the PRISMA diagram.

Quality and Methodology Assessment

Methodological quality was assessed using the Newcastle-Ottawa Scale (NOS). NOS has eight items with multiple choices and scores. Each quality item choice in NOS is given a star with the top-quality research receiving up to nine scores. Among the eight items, any one of it can be given up to two stars. The score interpreted as good (7-9), fair (2-6) and poor (≤1). Grading of Recommendations Assessment, Development, and Evaluation (GRADE) was also used in this review to analyse different clinical management treatments and to assess the certainty in evidence and strength of recommendations in health care.

Data extraction and analysis

Essential information from the included article has been extracted to a matrix table on study design, setting, main, refractory and supportive pharmacological treatment, outcome, side effects and assessed quality. Main treatments are defined as first treatments given and considered as the best treatment protocol. Refractory treatment used for refractory disease is considered when there is persistence fever or worsening of inflammatory markers and significant end organ involvement despite receiving initial treatment.[8] Supportive treatments are categorized as treatments used to control or prevent complications and side effects. Quality assessments were reported explicitly and overall limitations were identified. Narrative synthesis employed each pharmacological category on its evidence of efficacy and safety. Procedural aspects such as dosage, follow-up, side effects and precautionary measures were also synthesized.

Results

The systematic search resulted in a total of 2393 documents, and 32 studies being included with pre-consensus agreement of 88.3% (eDiagram in the supplementary information available online). Most studies have good and fair methodological quality (Mean: 6.5 Range: 5-8) according to NOS and the majority is at moderate, low, or very low evidence level according to GRADE (Table I). For methodological aspects most of the studies have issues on the accurate follow-up data. Most studies (71%) reported no follow-up data. Study design and lack of comprehensive reporting of treatment recommendations contributes to the poor GRADE level. Almost 75% of the studies were from developed and Western countries with a total of 2331 patients excluding a study[11] that surveyed the management protocols from 40 different centres in the United States. All studies included were conducted from March 2020 till January 2023. A detailed report on the included articles is presented in Table II.

Pharmacological Approaches

Table II shows the treatments used and the outcome for each of the included studies while the summarization of the treatments is reported in Table III.

• *Main treatment*

Intravenous immunoglobulin (IVIG) was the predominant treatment option used in 1839 of all patients (77.3%) in all studies in any combination as an anti-inflammatory measure with a dose of 2 g/kg or divided into two doses of 1g/kg. The overall evidence level supporting this was 4% good[12], 28% moderate[6,13-19], 36% low[11,20-29] and 32% very low.[30-41] IVIG alone was used in 902 patients (37.9%). Following that, 1439 patients (60.5%) received steroids in any combination as the main anti-inflammatory treatment. Out of these four hundred and four (16.9%) patients received steroids alone. A combination of IVIG and steroids were given to 43.1% of the total patients (72 % of the studies), which were preferred in severe cases. Methylprednisolone was the most common type of steroid used, which was administered with either low or high dose depending on the severity (1-4 mg/kg to 10-30 mg/kg). The evidence supporting the use of methylprednisolone was at good[12], moderate[6,14,17-19], low[22-24,27] and very low levels.[31,34,36] Another anti-inflammatory measure that was used in combination with IVIG was high dose aspirin (30-80 mg/kg/day) in 1.1% of the total patients.[15,19,24,35]

• *Refractory treatment*

A secondary infusion of the main treatment is given to patients who were unresponsive to the initial therapy. Patients were given a second dosage of IVIG as reported by 43% of the total studies.[6,11,12,15,17,21,27,32,34-37] Patients unresponsive to methylprednisolone were given a secondary infusion with similar or increased dosage in 29% of the studies.[12,17,18,22,27,32,34,35] Another immunomodulatory therapy that has been used for refractory disease is anakinra, an Interleukin-1 inhibitor which was given to

Table I. Quality analysis summary of the included studies using NOS and GRADE tools.

Article	Newcastle-Ottawa Scale (NOS)									GRADE		
	1	2	3	4	5	6	7	8	Total Score	Initial Quality	Upgrade/ Downgrade	Overall Quality
Ouldali et al. (2021)	a	a	a	a	a	a	b	d	6	C	↑↑	A
Cattalini et al. (2021)	a	a	a	a	a	a	a	b	8	C	↑	B
Emeksiz et al. (2021)	a	a	b	a	b	b	a	c	8	C	↑	B
Pouletty et al. (2020)	b	a	a	a	a	b	a	b	8	D	↑↑	B
Jonat et al. (2020)	a	a	a	a	a	a	b	b	7	C	↑	B
Ramcharan et al. (2020)	b	a	a	a	a	a	b	b	7	C	↑	B
Son et al. (2021)	a	a	a	a	a	a	b	d	6	C	↑	B
Vukomanovic et al. (2021)	b	a	a	a	a	a	b	c	6	C	↑	B
Lee et al. (2020)	a	a	c	b	a	a	a	d	5	C	↑	B
Balagurunathan et al. (2021)	a	a	a	a	a	b	a	b	8	C	-	C
Feldstein et al. (2020)	a	a	a	a	b	b	b	d	7	C	-	C
Savas Sen et al. (2021)	b	b	a	a	b	b	a	c	7	C	-	C
Borgi et al. (2021)	a	a	b	a	a	a	a	d	7	C	-	C
Kurz and Gombala (2021)	a	a	b	a	a	a	a	d	7	D	↑	C
Capone et al. (2020)	a	b	a	a	a	a	a	a	7	D	↑	C
McArdle et al. (2021)	b	a	b	a	b	a	b	c	7	C	-	C
Dove et al. (2020)	b	a	a	a	a	a	b	d	6	C	-	C
Angurana et al. (2021)	a	b	a	a	a	b	a	d	6	C	-	C
Kaushik et al. (2020)	a	a	a	a	a	c	b	d	5	C	-	C
Jain et al. (2020)	a	a	a	a	b	b	b	d	7	C	↓	D
Maskari et al. (2021)	a	a	b	a	a	b	a	d	7	D	-	D
Toubiana et al. (2020)	a	a	b	a	a	a	b	d	6	C	↓	D
Shobhavat et al. (2020)	a	a	b	a	a	b	b	d	6	D	-	D
Garcia-Dominguez et al. (2020)	a	b	b	a	a	a	a	d	6	D	-	D
Dhanalakshmi et al. (2020)	a	b	b	a	a	b	a	d	6	D	-	D
Cattaneo et al. (2021)	a	b	b	a	a	b	b	b	6	C	↓	D
Belhadjer et al. (2021)	a	b	b	a	a	a	b	d	5	D	-	D
Davies et al. (2021)	a	a	a	a	a	c	b	d	5	C	↓	D
Mehra et al. (2021)	a	a	a	a	a	a	b	a	8	C	-	C
Sethy et al. (2021)	a	a	a	a	b	a	b	d	5	C	-	C
Mane et al. (2022)	a	a	a	a	b	a	a	a	7	C	-	C
Ouldali et al. (2022)	a	a	a	a	b	a	b	a	7	D	-	D

(NOS: Selection- 1) Representativeness of the exposed cohort, 2) Selection of the non-exposed cohort, 3) Ascertainment of exposure, 4) Demonstration that outcome of interest was not present at start of study; Comparability- 5) Comparability of cohorts on the basis of the design or analysis and; Outcome- 6) Assessment of outcome, 7) Was follow-up long enough for outcomes to occur, 8) Adequacy of follow up of cohorts. GRADE (Grading of Recommendations Assessment, Development and Evaluation) quality: B- Moderate, C- Low, D- Very Low)

patients who were refractory to IVIG and steroids in 189 of the total patients (8.9%). A study reported the use of tocilizumab for patients unresponsive to anakinra.[14] Tocilizumab (IL-6 inhibitor), infliximab (TNF inhibitors) and rituximab were used in 92 patients (4.3%).

• ***Supportive treatment***

Vasoactive drugs (4.5%) such as epinephrine, norepinephrine and vasopressin were used as a supportive treatment modality in vasogenic shock cases while inotropic drugs (3.3%) such as milrinone and dobutamine were used in cardiogenic shock cases, reported in 16% of all the studies.[13,14,27,28,31,33,35] Anticoagulation (12.2%) was used in cases of severe left ventricular dysfunction, coronary artery aneurysm (CAA) or evidence of thrombosis complication with elevated d-dimer and fibrinogen levels. Besides, antiplatelet were administered to 5.3% of patients, while broad-spectrum antibiotics were taken by 6.8% of the patients. Antiviral and antimalarial treatments were used in 17 patients (0.6%) and 3 patients (0.1%), respectively.

Efficacy of treatment

The vast majority of children recovered under proper diagnosis and early treatment. The main outcomes focused on in this review are recovery reported on resolution in fever and other clinical manifestations, improved biochemical and cardiac parameters, as well as echocardiographic improvements of ventricular functions and resolution of coronary artery involvement. Other outcomes such median length of hospitalization/PICU stay were also reported in Table II. However, only 19 studies (59%) fully or partially reported data on recovery.[11,13-15,17-19,22-28,31,32,36-38]

Twenty-nine percent of the studies reported follow-up data either with good improvement in clinical findings and resolution of echocardiogram or partial resolution or with persistent heart abnormalities, although some of the follow-up data was incomplete. Fever, immunomodulatory markers, echocardiographic measures and other clinical signs were resolved on day 8 after treatment.[15] Vukomanovic et al.[18] reported that patients treated with corticosteroids had faster normalization of fever (afebrile on day 1) compared to patients treated with IVIG (afebrile on day 4) while Lee et al.[19] reported that patients treated with immunomodulators had fever that resolved after a median of 4 days. IVIG was found to be effective as first-line immunomodulator in treating MIS-C with inflammatory process improvements[19,33] followed by steroids (methylprednisolone) which is the most efficient in cases with shock or coronary artery aneurysm.[25]

However, a combination of these IVIG and steroids are best suggested to be efficient in severely ill cases.[6,12-16,19,20,22-24,26,27,30,32,35,37,38] This strategy is associated with quick fever resolution, low rate of treatment failure, reduced need for refractory treatment and rapid recovery of myocardial dysfunction compared to using IVIG or steroids alone.[27] Anakinra has therapeutic benefits with its efficacy in treating systemic inflammation and overall safety profile is evident.[6,12-15,19,21,22,25,28,30,37,38] Tocilizumab has shown encouraging benefits in patients with refractory disease.[15] Tocilizumab plays an important role as an IL-6 receptor inhibitor and efficiently mediates the cytokine storm and myocardial injury in patients with high IL-6 levels.[28] Infliximab was recommended as an alternative if IVIG was unavailable due to its efficacy in IVIG-resistant cases.[16]

There were not many studies focusing on the details or efficacy of supportive pharmacological treatment. Kaushik et al reported that the use of these vasopressors were associated with improvements by day 4 to 5 in most patients admitted.[28] Capone et al had found that low-molecular weight heparin (LMWH) is efficient in decreasing elevated levels of D-dimer or fibrinogen.[25]

Table II. General characteristics, primary and secondary treatment, outcome and side effects.

Author (year)	Population (Country)	Study type	Sample size	Treatment		Treatment	Outcome		
				Main	Refractory	Supportive	Recovery	Follow-up	Side effects
1. Ouldali et al. (2021)	Children (median age 8.6 years) (French)	Retrospective cohort	106	IVIG (2g/kg) IVIG + MP	IVIG second infusion IVIG + MP Anakinra Tocilizumab	Vasoactive or inotropic support (62% in IVIG+MP) and 32% in IVIG alone	IVIG + MP lower risk of treatment failure, OR [0.25(CI 0.09-0.7), P = 0.008) compared to IVIG alone Echo: Acute left ventricular dysfunction after initial therapy- 16/72(22.2%) with IVIG, 2/34(5.8%) with IVIG + MP. Median length of PICU stay- IVIG group is 6 days; IVIG + MP is 4 days. Mortality: None	No CVS complication in short term follow up	NR
2. Cattalini et al. (2021)	Children hospitalized with Kawasaki disease-like multi-inflammatory syndrome (Italy).	Observational Retrospective	53	IVIG GC IVIG + GC	Tocilizumab Anakinra	HCQ Antiviral Antibiotics Vasoactive Anticoagulation (Heparins) Low dose aspirin (ASA)	Good treatment response to GC and IVIG. Echo: Coronary involvement resolution. Mortality: None	Attended: NR 8 – persistent heart ultrasonography abnormalities	NR
3. Emeksiz et al. (2021)	Patients with severe MIS-C (Ankara, Turkey).	Observational, descriptive, retrospective	27	IVIG (1–2 g/kg) MP (30mg/kg/day) IVIG + MP	Anakinra Tocilizumab given to patients unresponsive to anakinra	Vasoactive drugs (epinephrine & norepinephrine) Anticoagulant (enoxaparin)	Improvement in clinical findings with immunomodulatory therapy. Median length of hospitalization is 15 days. Echo: 25/27 – complete recovery of ventricular function Mortality: 2	Attended: NR 8/27-clear stabilization of hemodynamics.	NR
4. Pouletty et al. (2020)	Children (median age 10 y/o) (Paris, France)	Multicen ter cohort, case series	16	IVIG Steroids IVIG + steroids High dose aspirin	IVIG second infusion Anakinra Tocilizumab	HCQ Low dose aspirin	All clinical signs resolved on day 8 after treatment. Mortality: None	Attended: 9/16 7 to 15 days: 9/9-asymptomatic. negative inflammatory biomarkers; 7/16 – normal heart ultrasounds; 2/16 – mild persistent cardiac dysfunction	Second infusion of IVIG-hemolytic anemia

Table II. Continued.

5. Jonat et al. (2020)	Pediatric patients (<21 y/o) (New York City)	Observational	54	IVIG GC IVIG + GC	Anakinra Infliximab	-	Clinical improvement of symptom resolution & improvement of shock with steroids. Median length of hospitalization is 4 days. Mortality: None	NR	NR
6. Ramcharan et al. (2020)	Children with PIMS-TS (United Kingdom)	Observational	15	IVIG (2 g/kg) MP	IVIG second infusion and/or three-day course of MP (In response to the first line IVIG)	Antibiotics Low dose aspirin	All have clinical improvement. Echo: 2/15- mild impairment LVEF at discharge; 3/15- abnormal ECG at discharge. Mortality: None	Attended: 12/15 12/15 - stable clinical and echocardiogram findings.	NR
7. Son et al. (2021)	Children (≤ 21 y/o) (United States)	Retrospe ctive cross- sectional	596	IVIG (2 g/kg) GC IVIG + GC	IVIG second infusion (2g/kg) Anakinra Infliximab Tocilizumab	-	Patients treated with IVIG + GC had a lower risk of receiving adjunctive treatment than IVIG alone. Echo: IVIG + GC was associated with a lower risk of cardiovascular dysfunction than IVIG alone. Mortality: 2	NR	NR
8. Vukoman ovic et al. (2021)	Children (average 13.2 ± 3.8 y/o) (Belgrade, Serbia).	Retrospe ctive cohort	22	IVIG GC (MP & dexamethasone)	GC: IVMP given to patients unresponsive to IVIG	-	Patients treated with GC had a faster normalization of fever than patients treated with IVIG (Day 1vs Day 4) Patients treated with CS had a rapid decline in proinflammatory parameters in the blood than patients treated with IVIG. 9/22 - Treatment failure (CS: 2, IVIG: 7). Mortality: None	NR	NR
9. Lee et al. (2020)	Children (1 month to 17 y/o) (Boston, United States).	Retrospective	28	IVIG (2 g/kg) MP (1–4 mg/kg/day) IVIG + MP High dose aspirin	Anakinra (5-13mg/kg/day)	Low-dose aspirin (3–6 mg/kg/day) enoxaparin Remdesivir HCQ	28/28- Clinical Improvement of inflammatory markers. Median length of hospitalization is 8 days. Echo: 3/6- Normalization of coronary vessel size after treatment (all 6 received IVIG, 4 received steroids) Mortality: None	NR	NR

Table II. Continued.

10. Balagurunathan et al. (2021)	Children (≤ 16 y/o) (South India).	Retrospective and prospective observational	21	IVIG Steroids IVIG + Steroids	-	Low dose aspirin Antibiotic Anticoagulant Antiepileptics	All with clinical improvement Median length of hospital stay: 6 days l Mortality: None	Attended: 16/21 16/16 - had 'no clinical concerns. 12/16 –echo partial or complete resolution from previous abnormalities.	NR
11. Feldstein et al. (2020)	Children (≤ 18 y/o) (United State)	Prospective, retrospective surveillance	186	IVIG GC	IVIG second infusion Tocilizumab Anakinra	Vasoactive support Anticoagulation	Majority recovered. 28% still hospitalized. Median length of Hospitalization: 7 days. Mortality: 4	NR	NR
12. Savas Sen et al. (2021)	Children (5.6 to 11.7 y/o) (Turkey).	Retrospective	45	IVIG (2 g/kg) IVIG + MP	MP given when Unresponsive to IVIG. Anakinra was used due to lack of adequate clinical response to previous IVIG and MP.	Anticoagulant (LMWH) Low dose aspirin (ASA)	I32/45 - recovered with IVIG alone. 45/45- had a favorable course. Mortality: None	NR	NR
13. Borgi et al. (2021)	Children (≤ 15 y/o) (South India).	Retrospective	8	IVIG (1 dose of 2 g/kg) + MP (10 mg/kg/day)	-	Low dose aspirin Dobutamine, Milrinone, Levosimendan Norepinephrine) Anticoagulant (LMWH) Antibiotic	All recovered with median length of PICU stay is 5.5 days. Echo: 8/8 - Complete recovery of left ventricular function was observed at a median delay of 4 days. Mortality: None	NR	NR
14. Kurz and Gombala (2021)	Children (2-18 y/o) (Vienna, Austria).	Case series	8	IVIG (2 g/kg) + high dose of MP (20–30 mg/kg) High dose aspirin (ASA, 30 mg/kg)	-	Antibiotics Anticoagulant (LMWH)	Rapid clinical improvement. Median length of hospitalization is 13 days. Echo: 8/8- function recovered Mortality: None	NR	NR

Table II. Continued.

15. Capone et al. (2020)	Children (United States)	Case series	33	IVIG CS	Anakinra Tocilizumab Infliximab	enoxaparin Aspirin	33/33- Rapid clinical improvement. Median length of hospitalization is 4 days. Echo: 14 had normal cardiac function, 10 had lower than normal cardiac function & 9 still have mild cardiac dysfunction at discharge. Mortality: None	Attended: 33/33 25/35 - complete recovery of left ventricle	NR
16. McArdle et al. (2021)	Patients with suspected MIS-C (a total of 32 countries).	Observat ional cohort	614	IVIG IVIG + GC GC	-	-	Decreased disease severity in patients treated with IVIG+GC (54/208) compared to patients treated with GC alone (20/99). Reduction of frequency of organ failure with GC benefits alone. Echo: NR Mortality: 12	NR	Drug complication occurred in 16/411patients who received GC in any combination and 9/508 patients who received IVIG in any combination- GC: Hyperglycemia-7 patients. Hypertension-7 patients. IVIG: Rash & lip swelling- 1 Anakinra: Superficial cutaneous infection- 1 Anticoagulation: Bleeding- 2
17. Dove et al. (2020)	Patients with MIS- C protocols (United States).	Cross-sectional survey	40 cent ers	IVIG (2 g/kg) CS	IVIG second infusion for cases that were refractory to the first dose. Tocilizumab Infliximab	Low dose aspirin Warfarin Remdesivir Antiplatelet Clopidogrel	Echo: Many patients have recovery of left ventricular systolic function at the time of discharge. Mortality: None	NR	NR

Table II. Continued.

18. Angurana et al. (2021)	Children (median age [IQR] age 7 months [5-10] (North India)	Retrospective	40	IVIG (2 g/kg) – 100% received IVIG. And combination with MP in 85% (10 -30 mg/kg/day)	IVIG second infusion given for non-improvement after first dose IVIG (2.5%) In patient received MP 10mg/kg/day, the MP dose was increased to 20 or 30 mg/kg/day. If there was no improvement in next 24-48 hours	Vasoactive 29/40 (72.5%) Low dose aspirin (3mg/kg/day) given in 80% Anticoagulant (LMWH) in 7.5% cases	Clinical improvements after treatment at discharge. Echo: Improvement in myocardial dysfunction but 6/40(15%) still have residual myocardial dysfunction at discharge Mortality: 2(6.2%)	NR	NR
19. Kaushik et al. (2020)	Children (median age 10 y/o) (New York City)	Retrospective, Cohort	33	IVIG IVIG + steroids Steroids	Anakinra Tocilizumab	Norepinephrine dopamine enoxaparin Antibiotics Remdesivir	Most patients showed improvements by day 4-5 with vasopressors. Median length of hospitalization is 7.8 days. Echo: Recovery of ventricular function & normalization of myocardial dysfunction. Mortality: 1	NR	NR
20. Jain et al. (2020)	Children with MIS- C with COVID-19 (Mumbai, India)	Cohort	23	IVIG Steroids IVIG + steroids	Anakinra Infliximab	Inotropic	Clinical improvements after treatment at discharge. Echo: 34.8% with LV dysfunction but no report on repeat echo Mortality: 1	NR	NR
21. Maskari et al. (2021)	Children (≤ 21 y/o) (Oman).	Case series	6	IVIG MP	Tocilizumab	-	Good clinical response within 24-48 hours. Range of hospital stay4-12 days Mortality: None	NR	NR
22. Toubiana et al. (2020)	Children (≤ 18 y/o) (Paris, France)	Prospective, observational	21	IVIG (2 g/kg) IVIG +steroids (2-10 mg/kg/day)	IVIG second infusion IVIG + steroids	Low dose aspirin (3-5 mg/kg/day) Vasoactive Inotropic Antibiotic	21/21- Rapid resolution of symptoms after treatment with IVIG. Median length of hospitalization is 8 days. Echo: NR Mortality: None	NR	NR
23. Shobhavat et al. (2020)	Children (West India).	Cohort	21	IVIG Steroids	Tocilizumab	Anticoagulant (LMWH) Aspirin	18/21 recovered with median length of PICU stay is 5 days. Echo: NR Mortality: 3	NR	NR

Table II. Continued.

24. Garcia-Dominguez et al. (2020)	Children with MIS- C associated a SARS- CoV-2 infection (Mexico).	Case series	4	IVIG (2 g/kg) MP (10 mg/kg)	Second infusion of IVIG and MP	epinephrine norepinephrine Antibiotics	4/4 – Responded adequately to IVIG, steroids, & vasopressors. 4/4 - Discharged without complications. Mortality: None	NR	2 patients developed acute kidney injury with recovery of kidney function 72 hours after IVIG & MP treatment.
25. Dhanalakshmi et al. (2020)	Children who met the case definition of PIMS- TS (Chennai, India).	Case series	19	IVIG (2 g/kg) Steroids IVIG + steroids	IVIG second infusion High dose steroids Tocilizumab	Antibiotics Aspirin	All recovered with median length of hospitalization is 6 days. Echo: NR Mortality: None	NR	NR
26. Cattaneo et al. (2021)	Children (≤ 18 y/o) (Mayotte Island, France).	Retrospective, descriptive	11	IVIG (2 g/kg) MP (2 mg/kg/day) Aspirin (ASA, 40–60 mg/kg/day)	IVIG second infusion (2 g/kg)	Inotropic Vasoactive Antibiotics Low-dose aspirin (5 mg/kg/day)	Median length of hospitalization is 8 days. Echo: 11/11 - Complete recovery of left ventricular function. Mortality: None	Attended: 8/11 8/8 – no cardiac abnormalities detected	NR
27. Belhadjer et al. (2021)	Children admitted to PICU (12 hospitals in France and 1 hospital in Switzerlan d).	Retrospective case series	35	IVIG IVIG + steroids	Repeated IVIG for persistent fever 48 hours after the first infusion; Anakinra	Inotropic Anticoagulant (Heparin)	28/35- Favorable clinical evolution. Median length of hospitalization is 10 days Echo: 7/35- Still in hospital/ with residual left ventricular dysfunction. Mortality: None	Attended: 28/35 25/35 -complete recovery of left ventricle (5/35: mild to moderate residual left ventricular systolic dysfunction)	NR
28. Davies et al. (2021)	Children (≤ 18 y/o) (United Kingdom)	Observational	78	IVIG Steroids IVIG + steroids	Anakinra Tocilizumab Infliximab Rituximab	Remdesivir	Median length of hospitalization is 5 days. Echo: NR Mortality: None	NR	NR
29. Sethy et al. (2021)	Children (Mean age of 9.09 years) (India)	Retrospective Observational	21	IVIG (2g/kg) MP (1-10 mg/kg/day) Steroids+ IVIG	NIL	Inotropic support (10/2147.6%) LMWH (10/21,47.6%)	17/2, 81% recovered Echo: 2 patients with myocardial dysfunction on discharge Mortality: 2	NR	NR
30. Mehra et al. (2021)	Children (< 18 y/o) (Delhi, India)	Retrospective, cohort Median age 7 (IQR: -10year)	120	IVIG Steroids No IVIG/ Steroids:	Non received tocilizumab or anakinra	Vasoactive Aspirin Enoxaparin/heparin, Remdesivir	96.6% survival outcome Mortality: 4	NR	NR

Table II. Continued.

31. Mane et al. (2022) (India)	Children with MIS-C	Case Series 2 month-9 year	7	IVIG MP		Inotropic Enoxaparin	5/7 patients improved with IVIG+/MP. 1/7 recovered without immunomodulator. Median hospital day: 7 days Mortality :1	NR
32. Ouldali et al. (2022) (French)	Children Age:12-17 years	Prospective national population-based surveillance following Covid-19 Vaccination	12	IVIG (2g/kg) + MP MP alone (3)	1 received MP(10mg/kg/day)	Antiplatelet dose (3-5 mg/kg) per	All recovered fully with median hospital length of stay 7 days IQR (7,9)	NR

ASA: acetylsalicylic acid, BNP: brain natriuretic peptide, CS: corticosteroids, ECG: electrocardiogram, Echo: echocardiography, GC: glucocorticoids, HCQ: hydroxychloroquine HSP: Henoch- Schoenlein purpura, IM: inflammatory markers, IVIG: intravenous immunoglobulin, IVMP: intravenous methylprednisolone, KD: Kawasaki disease, LMWH: low molecular weight heparin, LVEF: left ventricular ejection fraction, MIS-C: multisystem inflammatory syndrome in children, MP: methylprednisolone, NR: not reported, PICU: Paediatric Intensive Care Unit, PIMS-TS: paediatric inflammatory multisystem syndrome, WHO: World Health Organization.

Safety and Side Effects

Several precautions need to be taken to ensure the treatments are safe to be utilised. Consultation with specialists is crucial in cases of giving drug combinations with higher dosage as it is less safe. Ramcharan et al.[17] described the use of milrinone in patients with vasogenic shock as the drug is known to produce a counterproductive effect of peripheral vasodilation.

Only three studies reported side effects from the pharmacological treatments given (Table II). Hemolytic anemia was observed in one patient who received a repeated dose of IVIG.[15] Drug complications occurred in 16 patients who received glucocorticoids and 9 patients who received IVIG in any combination.[26] One patient developed acute kidney injury following IVIG and methylprednisolone treatment.[34]

The reported mortality was 30 out of 2331 patients (1.28%). Due to the severity of SARS-CoV-2-associated MIS-C, it is critical to diagnose and treat it as soon as possible.[14] Angurana et al.[27] reported death in their cohort by highlighting the importance of early recognition and timely diagnosis. Another study reported the death of MIS-C patients with shock and possibly due to late admission to hospital.[33]

Discussion

MIS-C is rare with serious inflammatory complications that manifests late following SARS-CoV-2 infection (SARS-CoV-2). This has been postulated as post-infectious immune dysregulation characterized by a hyperinflammatory cytokine storm and macrophage activation in genetically predisposed children. Since the novel coronavirus was identified in late 2019, various COVID-19 variants had been reported, i.e., Ancestral type, Beta, Delta, and the latest Omicron. However, a study comparing the clinical phenotype of MIS-C in children across four distinct variant-driven waves reported that regardless of variant, MIS-C remains a severe disease with a stable clinical presentation.[42]

Auspiciously, compared to Alpha wave, MIS-C was less common in the waves driven by Delta and Omicron.[43] In addition, Zambrano et al. reported that the introduction of Covid-19 mRNA vaccine in children and adolescents is protective against MIS-C.[44]

The pharmacological approaches of MIS-C were extrapolated from KD treatment as they may share some immunopathogenesis, however, both demonstrated distinct epidemiology, clinical manifestations, and laboratory markers. Compared to KD, cardiogenic or vasoplegic shock are prominent features in MIS-C, with 60-75% of cases requiring hemodynamic support that may be responsible for the mortality.[45] Main treatments consist of immunomodulatory therapy aimed at decreasing tissue inflammation that aid in tissue recovery and supportive management of acute life-threatening complications and prevent long-term sequelae like CAA.[9] The pharmacological approach of MIS-C has been strongly influenced by recommended KD management in which IVIG and aspirin are the standard first-line treatments; whereas corticosteroids and/or biological response modifier drugs (BRMDs) are indicated in a case of IVIG refractory or high-risk cardiac complication. However, MIS-C has a refractory nature due to a greater degree of inflammations with more severe multiorgan involvement such that most children require corticosteroids as part of main treatments.[46] On the other hand, a few studies reported that treatment with steroids alone is a plausible initial treatment for MIS-C.[26,38] A progressive approach for MIS-C management with immunomodulatory therapy is indicated based on the severity of clinical manifestation or MIS-C spectrum. The findings from this review are in line with published guidelines.[8,47] We have demonstrated that immunomodulatory therapy effectively treats MIS-C with a low risk of side effects. However, the available data was limited to nonrandomized studies with evidence using GRADE that was mostly at low and moderate levels.

Main Immunomodulatory Therapy

• *Intravenous Immunoglobulin (IVIG)*

IVIG is a blood product composed of purified serum immunoglobulin G protein, which serves as an immunomodulator of both innate and adaptive immunity.[48] IVIG can be substituted from pro-inflammatory to anti-inflammatory due to its cytotoxic properties towards neutrophils and eosinophils.[49] In cellular immunity, IVIG inhibits the activation of monocytes and macrophages. It also triggers the release of anti-inflammatory cytokines from innate cells that give rise to a reduction in macrophage response towards interferon.[49]

IVIG is suggested to be used as the first-line of pharmacological treatment for MIS-C.[33] We demonstrated that 38.2% of the study population received IVIG alone. However, in several studies, MIS-C patients did not respond to IVIG treatment.[12,18,50] Early addition of glucocorticoids to IVIG therapy resulted in fever resolution and significant decrease in inflammatory markers[18], whereas other studies found that a combination of IVIG and glucocorticoids caused lower risk of fever recurrence, cardiovascular dysfunction, and the need for adjunct treatment.[6,12] In addition, other studies reported that early administration of IVIG and glucocorticoid in MIS-C is related with a decrease in ICU admissions and length of hospital stay.[12,16] Simon et al. suggested that MIS-C patients who are at high risk of developing immunoglobulin resistance should be given a combination of IVIG and steroids, which appears to lower the incidence of coronary anomalies and the duration of fever.[51]

Therefore, early intensified therapy with combination of IVIG and steroids are beneficial in more severely ill patients and who are at high risk of developing immunoglobulin resistance. A recent study on the trends of MIS-C treatment for patients who required ICU admission in the United States, demonstrated increased practice of using combination of IVIG and steroids from 43% in April 2020 to 76.1% in Jun 2021 and the

proportion of patients who received IVIG alone decreasing from 22% to only 6.5%.[52]

In refractory cases associated with unresolved fever or symptoms, a treatment protocol of secondary infusion of IVIG within 2 to 3 days has been suggested.[9] In this review, 43% of the total studies used a second infusion of IVIG in refractory cases.[6,11,12,15,17,21,27,32,34-37] The need for higher doses of IVIG is related to body size and age of the patients which may increase the risk of volume overload, particularly for cases with underlying myocardial dysfunction.[47] Therefore, it is suggested that fluid status and ventricular function should be assessed prior to IVIG administration to avoid complications of fluid overload.

Furthermore, high doses of IVIG can also be associated with hemolytic anemia, however, only one patient in a study by Pouletty et al.[15] reported this side effect in our review. According to the clinical guidance of American College of Rheumatology (ACR)[8], MIS-C patients with refractory disease are not recommended a second administration of IVIG. Alternatively, glucocorticoids with low-moderate doses may be considered. Despite that, a recent study on treatment trends of MIS-C in the United States showed that IVIG is the most utilized treatments, administered to 85.6% of patients and about 18.1% received a second dose of IVIG.[52]

- ***Glucocorticoids***

Glucocorticoids usage has a tendency to decrease the development rate of CAA in patients with classical KD with increased risk of resistance to IVIG.[47] Molecules associated with inflammation such as cytokines, metabolites and chemokines are hindered by glucocorticoids.[53] Mechanisms of glucocorticoids are mainly moderated via classic glucocorticoid receptors. Glucocorticoids have anti-inflammatory effects that are said to result from transrepression- a vital negative regulatory mechanism.[54] The rapid action of glucocorticoids enables the reduction in hyperinflammatory response, inhibits vasodilation and increases permeability via inhibition of IL-1α and IL-1β.[55]

Vukomanovic et al.[18] investagated the infusion of corticosteroids as a first-line treatment for MIS-C patients with cardiovascular involvement. In comparison to IVIG-treated patients, glucocorticoids were linked with faster normalisation of fever, laboratory parameters, cardiac function and shorter ICU stays. Based on a study, there was also no evidence of delayed recovery from organ failure in individuals who received glucocorticoids alone as their first therapy.[26] In this systematic review, almost 17.3% of patients received steroids alone.[6,11,13-21,25,26,28,30,31,33-36,38] Some children with shock who required numerous inotropes and/or vasopressors reacted best to large doses of intravenous glucocorticoids (10–30 mg/kg/day).

Intravenous glucocorticoids with high doses have been used safely and successfully in MIS-C, KD and shock patients.[16,56,57] Given these findings, treatment with just steroids is plausible to be considered as an initial treatment for MIS-C. Nevertheless, rather than following predefined protocols, having a personalized treatment for each patient is equally crucial, coordinated by a multidisciplinary team.[9]

According to ACR guidance[8], low to moderated dose of glucocorticoids (1-2 mg/kg/day) should be added early to IVIG treatment in MIS-C cases that require hospitalization and high dose glucocorticoids (10-30 mg/kg/day) in refractory cases. High dose may be considered as an emergency and immediate treatment for budget-constrained regions as their access to IVIG is limited. Many lower and middle-income countries have issues with adequate and proper medication supplies, therefore, there is a need for randomized control trials to study if steroids or IVIG alone or combination groups are more effective than others.[26,38] More evidence to assist the use of cheaper anti-inflammatory groups such as glucocorticoids is needed. In this systematic review, commonly reported side

effects from corticosteroids were hypertension and hyperglycemia in a very small number of patients. Only one patient developed acute kidney injury from using the combination of IVIG and steroids.[34]

• ***Aspirin***

Salicylate is the active ingredient of aspirin which is responsible for the anti-inflammatory activity.[58] It is a member of a large family of pleiotropic and short-lived mediators that are produced by the cell membrane's arachidonic acid moiety and have biological effects on a variety of cell types, including platelets and endothelial cells. By suppressing cyclooxygenase enzymes, lipid mediators are synthesized such as thromboxane, prostacyclin, and prostaglandin; which also possesses anti-inflammatory, antipyretic, and antiplatelet activities.[59] High dose aspirin has been used as a treatment option for anti-inflammatory effects in KD and Rheumatic carditis. However, an anti-inflammatory dose of aspirin is not part of ACR recommendations in MIS-C management. It has been received by only 1.1% of the total patients in our review, mostly in the initial phase of MIS-C where treatment depends strongly on the KD treatment protocol.

• ***Biological Response Modifier Drugs (BRMDs)***

BRMDs are a novel class of therapeutic treatments that include recombinant human monoclonal antibodies or receptor antagonists and have been utilized to treat a number of autoimmune disorders.[60] MIS-C can lead to immune-mediated multiorgan damage[61], hence, immunomodulatory therapy is recommended such as anakinra, tocilizumab and infliximab as recommended by the ACR in refractory treatments.[8,47] Patients with severe inflammation and refractory disease are recommended to consult rheumatologist and/or immunologist when considering immunomodulatory beyond required dosage with anakinra.[16] However, there is very limited evidence to suggest that anakinra is more preferable compared to other biological immunomodulatory agents such as infliximab in effectively treating MIS-C.[16,62] Anakinra is more preferable than glucocorticoids in patients with sickle cell anemia because glucocorticoids are reported to have a tendency of complicating vaso-occlusive pain crises.[19] Compared to ACR, the United Kingdom's PIMS-TS National Consensus Management Study Group recommend the use of a second dose of IVIG with preference of infliximab over anakinra for refractory cases of KD-like phenotype presentation.[63] Currently, there is ongoing clinical trials to investigate the effectiveness of infliximab, methylprednisolone, anakinra, and tocilizumab in MIS-C patients.[64]

Supportive Therapy

Therapeutic anticoagulants that were used in the studies were LMWH, enoxaparin and warfarin (12.1% of the patients). It should be heavily considered for patients with evidence of thrombosis ,giant CAA or ventricular dysfunction.[45] As recommended by the ACR, anticoagulants should be given to high-risk patients, however, patients' risk for bleeding should be taken into consideration.[8] A study by Abrams et al. on trends of MIS-C treatment in US, about 88% of 2000 patients were treated with anticoagulant, the majority received enoxaparin (86.8%) followed by heparin (18.9%), rivaroxaban(3.2%) and apixaban(1%).[52]

Low-dose aspirin is recommended for MIS-C to reduce the risk of thrombosis.[65] Patients who fit the criteria for KD, have coronary artery abnormalities, or who have additional thrombosis risk factors may be considered for antiplatelet treatment.[45] According to ACR, aspirin with a low dose of 3-5 mg/kg/day not exceeding 81 mg/day is recommended to be utilised in MIS-C patients and continued up till platelet counts and coronary artery return to normal.[8] However, only 5.4% of the patients in this review received this treatment. The low result could be explained by lack of reporting or detailed information. The current trend of MIS-C treatment in the US, demonstrated that 73.7% of 4901 patients with suspected MIS-C received an antiplatelet dose of aspirin.[52]

Table III. Summary of pharmacological treatments identified and its usage.

Treatment	Usage, n/2331 (%)	Studies used, n/32 (%)
Total IVIG [6,11-37]	1958 (84%)	32 (100%)
IVIG alone [6,11-22,25-37]	902 (38.7%)	267 (84%)
Total Steroids [6,11-37]	1439 (61.7%)	32 (100%)
Steroids alone [6,11,13-21,25,26,28-30,32-35,37]	404 (17.3%)	27 (84%)
IVIG + Steroids [6,12-16,19,20,22-24,26,27,29,31,34,36,37]	982 (42.1%)	23 (72%)
High dose Aspirin (Anti-inflammatory) [15,19,24,35]	26 (1.1%)	4 (13%)
Anakinra [6,12-15,19,21,22,25, 28,29,36,37]	189 (8.1%)	13 (41%)
Tocilizumab, Infliximab, Rituximab [6,11,12,14,15,21,25,28-30,32,34,37]	92 (3.9%)	13 (41%)
Anticoagulation (LMWH, enoxaparin, warfarin, heparin, etc.) [11,13,14,19-25,27,28,32,36]	257 (11%)	14 (44%)
Low dose Aspirin (Antiplatelet) [11,15,17,19,22,23,27,31,35]	126 (5.4%)	9 (28%)
Unspecified Aspirin [13,20,25,32,34]	86 (3.7%)	5 (16%)
Vasoactive drugs (epinephrine, norepinephrine, vasopressin, etc.) [13,14,27,28,31,33,35]	105 (4.5%)	7 (22%)
Inotropic drugs (milrinone, dobutamine) [23,29,31,35,36]	78 (3.3%)	5 (16%)
Antibiotics [13,17,20,23,24,28,31,33-35]	162 (6.9%)	10 (31%)
Antiviral drugs (remdesivir) [11,13,19,28,37]	17 (0.7%)	5 (16%)
Antimalarial drugs (hydroxychloroquine) [13,15,19]	3 (0.1)	3 (6%)

Inotropic agents such as dobutamine and milrinone are efficient in improving echocardiographic measures of patients with severe left ventricular dysfunction. A patient with severe cardiac dysfunction can require more than one inotropic drug.[23] Milrinone is an inotropic agent and a vasoactive drug which aids in increasing cardiac output by vasodilation and its inotropic effect. However, milrinone is known to produce a counterproductive effect of peripheral vasodilation as a side effect.[17]

Antibiotics are found to be efficient in cases of bacterial infections. In cases of patients with shock, antibiotic administration plays an important role and cannot be delayed.[51] The role of antiviral efficacy such as remdesivir is currently not evident.[61] These treatments do not contain any evidence that can be used to treat MIS-C.[11,13,19,20,38]

This current review can serve as preliminary evidence in supporting the major pharmacological treatment for MIS-C. However, the findings should be accepted cautiously as evidence available is limited due to methodological design. The reliance on observational studies and pharmacological approach is most likely limited to moderate and severe cases with limited long term follow up. The absence of high-quality studies such as large-scale prospective longitudinal studies or randomized controlled trials, warrant for more studies to be conducted to provide more confidence on the medication efficacy and safety. Another limitation of this current review is on the inability to conduct meta-analysis due to insufficient data availability and designs of the included studies. However, this review is still valuable in giving pharmacological treatment suggestions for MIS-C, especially with the discussion on its safety, potential side effects, and current guidelines or trends in pharmacological management.

In conclusion, IVIG or steroids alone, or combination of the two were found to be effective and relatively safe as the main MIS-C treatment. In severe refractory conditions, second infusion of the main treatment and biological response

modifier drugs should be considered in a stepwise approach. However, majority of included studies were at moderate, low, or very low evidence levels according to GRADE. Therefore, comprehensive investigation using randomized clinical trials are required to provide stronger outcome evidence.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: PY, YV and MHR; data collection: YV, GV, PY and SA; analysis and interpretation of results: MHR, PY, YV and KT. Author; draft manuscript preparation: YV, GV, PY, MHR and KT. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

Supplementary information

Online supplementary information is available at http://www.turkishjournalpediatrics.org/uploads/turkjped.2022.765.S1.pdf

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The Turkish Journal of Pediatrics 2023; 65: 739-747
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Original Article

The effect of the COVID-19 pandemic on long-term treatment compliance and disease control in children with persistent asthma

Sevilay Özerden Özcan[1], Sinem Polat Terece[2], Gamze Yalçın[1], Hacer İlbilge Ertoy Karagöl[2], Arzu Bakırtaş[2]

[1]Department of Pediatrics, Gazi University School of Medicine, Ankara; [2]Department of Pediatric Allergy, Gazi University School of Medicine, Ankara, Türkiye.

ABSTRACT

Background. No long-term data exists on asthma treatment compliances (ATC), exacerbations (AE), and control (AC) during the COVID-19 pandemic in children. This study aimed to evaluate ATC, AE, AC and the related factors among children with persistent asthma (PA) within the first year of the pandemic

Methods. Children aged 6-18 years with PA who were under regular inhaled corticosteroid treatment for at least a year prior to the first COVID-19 case in Türkiye were included. Data on AE and AC were collected from medical files. Factors affecting ATC and AC as well as COVID-19 history were assessed by means of a questionnaire.

Results. The study included 247 cases. COVID-19 was detected in 14.5% of them. In the first year of the pandemic, ATC decreased to 56.7% and the most common reason was the absence of asthma symptoms. There was a significant improvement in AC ($p<0.001$). The number of upper respiratory tract infections (URTI) and AE were significantly decreased during the first year of the pandemic ($p<0.001$). COVID-19 infection, smoking in the household, school attendance, a family member working outside the home, house dust mite sensitization or allergic rhinitis had no significant effect on AC ($p>0.05$). Regression analysis determined that children who did not have any URTI had 2.4 times better AC compared to those who had ($p= 0.02$; %95 CI: 1.1-5.4).

Conclusions. Although ATC decreased significantly in the long-term in the first year of the pandemic, significant improvement was observed in AE and AC compared to the previous year, which was related only to not having URTI.

Key words: asthma, children, compliance, COVID-19, exacerbation, treatment.

The COVID-19 epidemic was declared as a pandemic on March 11, 2020[1]. The COVID-19 infection has produced milder illness in children than in adults.[2-4] During the pandemic, chronic lung disease, diabetes, and cardiovascular diseases were considered comorbidities that represented a risk in terms of COVID-19.[5] However, studies have not as yet pointed to an increased risk for COVID-19 among individuals with asthma.[2,6-8] In fact, improvements in pediatric AC during the pandemic have been reported in cross-sectional studies.[9-11] Almost all of the studies have indicated that the main reason for this was the decline in the incidence of upper respiratory tract infections (URTI) and reduced air pollution.[9,12-14] On the other hand, asthma treatment compliance (ATC) has a significant impact on asthma control (AC).[15-17] Generally, ATC among children was reported between 30-70% prior to the pandemic.[18-20] There is no study that assesses the long-term ATC in children with asthma during the COVID-19 pandemic. During the pandemic there were a

✉ Arzu Bakırtaş
arzubakirtas@gmail.com

Received 13th February 2023, revised 2nd May 2023, accepted 25th July 2023.

limited number of studies in which ATC and its effect on AC were investigated.[21] The few studies that explore ATC either compare the period of quarantine with the previous period[21] or provide a one-year extrapolation based on a short interval.[10] Most studies, however, are short-term accounts[22,23] based on doctor's statements[9], or studies that have not queried the patient's history of COVID-19 infection.[10,21,23] In addition, there are a few long-term studies about asthma exacerbations (AE) and AC among children during the pandemic.[24,25] Therefore, we aimed to compare the first year of the pandemic with the previous year in terms of its effects on long-term ATC, AE, and AC among 6-18 year old children with persistent asthma (PA) who exhibited high levels of ATC before the pandemic.

Materials and Methods

Study sample

Children between 6-18 years of age who were being followed up with a confirmed diagnosis of persistent asthma and had been regularly taking inhaled corticosteroids (ICS) for at least one year prior to the first recorded case of the COVID-19 infection in Türkiye in March 2020 were invited to participate in the study. Cases with a diagnosis of asthma that were being followed up for less than one year prior to the pandemic, and those whose compliance was poor were excluded from the study since they were not receiving ICS treatment regularly.

Questionnaire

A questionnaire of 26 items (ATC: 8 questions, COVID-19 history: 11 questions, factors that may affect AC other than COVID-19 infection during pandemic: 7 questions) was administered to families and children consenting to participate at designated appointment times (Supplement). The meetings were held separately, face-to-face, and in keeping with COVID-19 safety precautions.

Definitions

Definitive COVID-19 infection: The child with asthma is symptomatic and has tested positive on the SARS-CoV-2 PCR test.

Possible COVID-19 infection: The child with asthma is symptomatic but the SARS-CoV-2 PCR test has not been administered by the filiation team because at least one person in the same household has a diagnosis of COVID-19 with a PCR test at the same time.

Compliance: The participants who had stopped taking ICS treatment after March 2020 without consulting to the department were defined as *noncompliant,* while those who continued their treatment as advised were defined as *compliant*.

Asthma exacerbations: AE were grouped according to the medical files using a written AE plan at home, need for systemic corticosteroids use, emergency department application or hospitalization.

Asthma control: AC was evaluated categorically as recommended by the GINA 2020 asthma diagnosis and treatment guidelines and defined as well-, partially-controlled and uncontrolled.[26]

In the study, AC and AE were evaluated from the medical records of the participants. The first year of the COVID-19 pandemic (March 2020-March 2021) was compared to the year prior to the pandemic (February 2019-February 2020) in terms of compliance with asthma treatment and AE. Evaluation of AC was made twice–at the last visit in 2019 and at the end of the first year of the pandemic. History of COVID-19 infection, ATC, and the factors that may affect AC during COVID-19 pandemic were evaluated by the questionnaire.

Ethics committee approval

Ethical approval was obtained from Gazi University Ethics Committee (Date: 31.05.2021/ No:498). All participants gave informed consent for the study.

Statistical analysis

The data of the study were analyzed with the SPSS (Statistical Package for Social Sciences) for Windows 22.0 program (SPSS Inc, Chicago, IL) package program. The Kolmogorov-Smirnov Test was used to assess normal distribution. The McNemar and chi-square tests were used in the comparison of categorical variables. In the multivariate analysis, significant parameters in univariate analysis were subjected to logistic regression analysis. The Hosmer-Lemeshow test was used to determine the goodness of fit of the model. A p value of less than 0.05 was accepted as statistically significant.

Results

Screening

There were 486 children with persistent asthma between 6-18 years of age who were on regular ICS treatment and attending their check-up visits regularly for more than a year. The final analysis included 247 participants after exclusion of the cases that did not give consent, those who stopped taking ICS treatment before the pandemic and could not be contacted (Fig. 1). The demographic details of the participants are presented in Table I. Forty-eight percent of the participants were taking a high dose of ICS. Of those taking high doses, 97.5% were between the ages 6-11 years old.

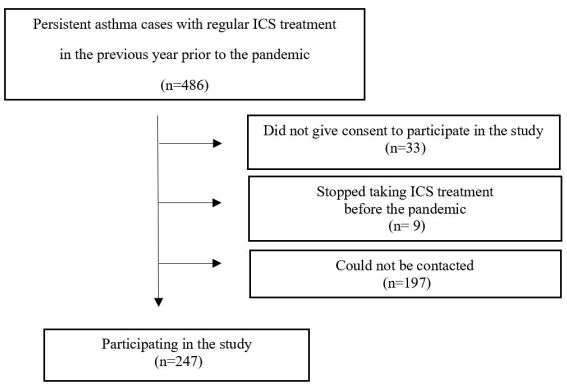

Fig. 1. Study group.

Long term compliance with asthma treatment during the COVID-19 pandemic

In the first year of the pandemic, 107 patients stopped taking ICS treatment (43.3%). Of these individuals, 8 had recurrence of asthma symptoms upon stopping their treatment (7.4%), and 7 of them (87.5%) started taking their medications again but none were hospitalized for asthma.

Table I. Demographics.

	n (%)
Age, year	
6-11	167 (67.6)
≥12	80 (32.4)
Male	156 (63.2)
Current age, months	121 (75-234)
Age at asthma diagnosis, months	66 (8-176)
Aeroallergen sensitivity	107 (43.3)
Pollen	78 (72.8)
Dust mites	56 (52.3)
Animal epithelia	49 (45.7)
Mold	18 (16.8)
Allergic comorbidity	79 (31.9)
Allergic rhinoconjunctivitis	29 (36.7)
Atopic dermatitis	11 (13.9)
Food allergy	3 (3.7)
Asthma controller treatment	247 (100)
ICS	132 (53.4)
ICS- LABA (Fixed dose combination treatment)	95 (38.5)
ICS plus LTRA	11 (4.5)
ICS- LABA (Fixed dose combination treatment) plus LTRA	9 (3.6)
Daily ICS doses, by age	
Age 6-11 years	167 (67.6)
Low	21 (12.5)
Moderate	29 (17.5)
High	117 (70)
Age ≥12 years	80 (32.4)
Low	64 (80.0)
Moderate	13 (16.2)
High	3 (3.8)

Continuous variables are shown as median (min-max). ICS: Inhaled corticosteroid; LABA: Long-acting bronchodilator inhaler; LTRA: Leukotriene receptor antagonists.

The discontinuation of ICS treatment, which began shortly after the onset of the pandemic, persisted throughout the study period. During this first year of the pandemic, the highest and lowest termination rates of ICS treatment were seen in April 2020 (n: 23, 21.4%) and November 2021 (n:2, 1.8%), respectively (Fig. 2). The absence of asthma symptoms was the most commonly cited reason for discontinuing ICS treatment (n: 94, 92.5%).

Asthma control during the COVID-19 pandemic

A comparison of the AC of the participants prior to the COVID-19 pandemic and at their last checkup at the end of the first year of the pandemic is presented in Table II. Asthma control did not change in 59.2%, improved in 35.2% and worsened in 5.6% of the cases, respectively compared to the last control before the pandemic. There was a significant improvement overall in AC compared to the period prior to the pandemic (p<0.001) (Table II).

Factors that may impact AC during the COVID-19 pandemic

COVID-19 infection

Seventy-five cases had a household member with a definite history of COVID-19 infection (30.4%). There were 21 children with definite and 15 with probable COVID-19 infection (8.5% and 6.1%, respectively). Three of them applied to the hospital due to COVID-19 symptoms (fever, myalgia, diarrhea, etc) but not for asthma symptoms. COVID-19 infection did not have any impact on AC (p= 0.52).

Asthma exacerbations

Table II presents a comparison of AE before and during the COVID-19 pandemic. The percentage of cases with AE decreased from 30.8 to 10.5% at the end of the first year of pandemic compared to the previous year (p<0.001). There were significant differences in terms of exacerbations requiring systemic corticosteroid (SCS) use and application to the emergency department (p<0.001 for both) but not for those that required hospitalization (p>0.05).

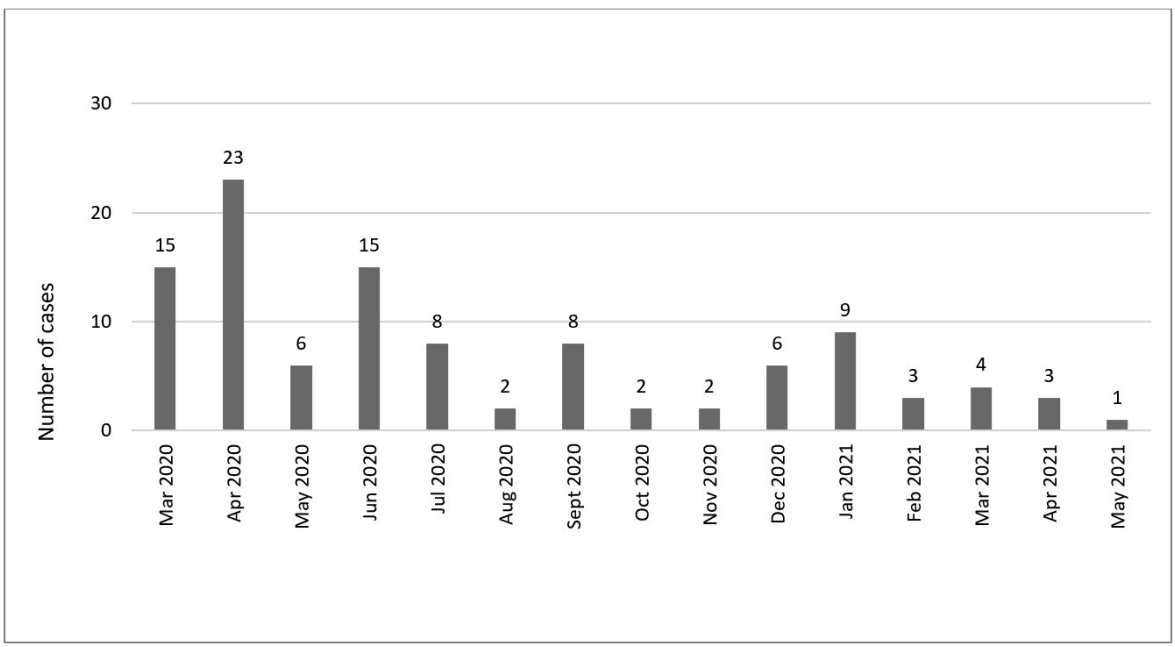

Fig. 2. Distribution of patients that stopped taking ICS treatment by months.

Table II. Comparison of asthma control and exacerbations before and after the first year of COVID-19 pandemic.

	In the year before pandemic n (%)	In the first year of pandemic n (%)	p
Asthma control			
Well-controlled	146 (59.1)	216 (87.4)	<0.001
Partially controlled	85 (34.5)	30 (12.1)	<0.001
Uncontrolled	16 (6.4)	1 (0.5)	<0.001
Asthma exacerbations			
Systemic corticosteroid use	55 (72.3)	7 (26.9)	<0.001
Emergency department admission	53 (69.7)	9 (34.6)	<0.001
Hospitalization	5 (6.5)	2 (7.6)	0.453

Other factors

Only 21.4% of persistent asthmatic children whose AC worsened stopped taking their daily ICS treatment. Distribution of treatment non-compliance was similar between AC groups and it did not affect the overall AC during the pandemic (p= 0.17). In the univariate analysis, it was found that pollen sensitization (p= 0.04) had an effect on AC during the pandemic, whereas house dust mite sensitization (p= 0.07), any aeroallergen sensitization (p= 0.09), and comorbid allergic rhinitis (p= 0.21) did not have a significant impact.

Data about remaining factors that may affect AC are shown in Table III. During the pandemic the number of URTI decreased in 79.7% of the cases and the median number of URTI was found to be significantly lower compared to the year before the pandemic (p<0.001). Smoking in the household, attendance at school and the presence of someone employed outside the home did not impact AC during the COVID-19 pandemic (Table III). There were 172 participants who have a sibling (69.6%) and 105 of them had continued daycare (61%) for the periods permitted by the Ministry of Health during the pandemic. Presence of a sibling who goes to daycare during the pandemic did not affect AC (p= 0.115).

A logistic regression analysis was performed to evaluate the independent effect of URTI, AE requiring SCS use or emergency department admission and pollen sensitization on AC, all of which showed statistically significant impacts in the univariate analyses. Only URTI was found to have a significant impact on AC throughout the pandemic. Children who did not get URTI had 2.4 times better AC compared to children that experienced URTI during the pandemic (p= 0.02; %95 CI:1.1-5.4).

Discussion

Although many studies exist on asthmatic children during the COVID-19 pandemic, none of them have examined the long-term effects of COVID-19 exclusively on children with persistent asthma.[8,13,21,25,26] Therefore,

Table III. Other factors that impact asthma control during the COVID-19 pandemic.

	In the year before pandemic n (%)	In the first year of pandemic n (%)	p
Number of URTI	3 (0-10)	0 (0-5)	<0.001
Smoking in the household	119 (48.1)	124 (50.2)	0.568
School attendence	247 (100)	167 (67.6)	n.a.
Family member working outside home	247 (100)	224 (90.7)	n.a.

Continuous variables have been expressed in medians (min-max). URTI: upper respiratory tract infections.
n.a.: non attributable.

this study examined ATC, AC, and AE in persistent asthmatic children aged 6-18 years old who had previously demonstrated good ATC before the onset of the COVID-19 pandemic for a one year period. At the end of the first year of the pandemic, almost half of the cases (43.3%) had stopped taking their ICS treatment. Despite the significant decline in ATC during the first year of the pandemic, there were significant improvements in AC and a decrease in AE that required the use of SCS and emergency department admissions, compared to the previous year. Infection with COVID-19 did not impact AC or the risk of AE in the study population. Moreover, the number of URTI decreased by almost 80% compared to the previous year. The absence of URTI was identified as the only factor that influenced AC in the study.

Long term compliance with asthma treatment during COVID-19 pandemic

While studies on childhood AC during the pandemic have provided data on AE, many fail to evaluate long-term adherence to asthma treatment.[9,10,21,23,25] In published studies, ATC has generally been evaluated for short periods of time ranging from 3 to 6 months at the beginning of the pandemic.[10,12,14,21,23] It was usually reported to increase or improve in studies evaluating the beginning of pandemic for shorter periods such as 3 months[10,21] although the contrary also exists.[12,14] On the other hand, another study comparing the first 6 months of the pandemic to the same periods two years ago, reported a decrease in the refills of asthma medications.[23] We found that long term ATC over a year during the pandemic is low even in children with persistent asthma. The initial rise in ATC during the pandemic, followed by a subsequent decrease in later stages in other studies, can be attributed to asthma being initially categorized as a high-risk chronic illness in relation to COVID-19. In our study, parents mostly stopped ICS treatment for their children in the initial months of the pandemic. The most common reason for non-compliance was reported as their children having no asthma symptoms. Anxiety related to the pandemic was a distant second reason. We think that the greater decrease in ATC at the beginning of the pandemic may be related to families' concerns about the potential COVID-19 risk associated with their children's inhaler corticosteroid treatment.

Asthma control

In our study, we assessed AC categorically and observed a significant improvement in more than one third of our patients. Specifically, we found that the number of well-controlled asthma had increased, while that of partially controlled and uncontrolled asthma had decreased over the first year of the pandemic compared to the previous year. This finding supports previous short-term studies that have shown improvements in asthma control at the beginning of the pandemic.[10-12,14,22] The improvement during the first wave of the COVID-19 pandemic, was explained by reduced exposure to asthma triggers such as URTI[10,12,14,22], outdoor pollution[14], and increased treatment adherence[10] in these studies. We investigated many factors that may affect AC such as ATC, COVID-19 infection, AE, number of URTI, allergen sensitizations, comorbid allergic rhinitis, smoking in the household, school attendance, sibling that attends a nursery, and family members working outside the home in this study. Despite a decreasing ATC during the first year of the pandemic, worsening of asthma control was observed in only a small proportion of our patients, around 5.6%. However, treatment non-compliance even in children with persistent asthma did not have an impact on AC during the first year of the pandemic. This is certainly an unexpected finding, but it appears that all the precautions taken during the pandemic helped mitigate the impact of treatment noncompliance on AC.

The second factor investigated that may impact AC was COVID-19 infection. Almost 15% of the participants in our study were infected with COVID-19, but none of them experienced any

worsening of AC, nor any significant AE related to COVID-19 or severe COVID-19 infection. Consistent with our study findings, a study evaluating 1205 children with mild asthma for COVID-19 infection revealed that none of the 16 cases that tested positive for COVID-19 exhibited a decline in AC.[22] Although the exact reason is unknown, COVID-19 infection seems not be an important trigger of asthma symptoms as human rhinoviruses. Additionally, guidelines have not shown an increased risk for severe COVID-19 in individuals with well-controlled asthma.[27] Despite all of our patients having persistent asthma, the majority of them were either well- or partially-controlled with very few cases of uncontrolled asthma before the pandemic, which may help to explain our findings.

Another important factor that may impact AC is AE. Both short-term and long-term studies have demonstrated a decrease in asthma-related emergency department visits and hospitalizations in relation to AE.[10,13,21,23,24] It is believed that this reduction was partly due to a decrease in widespread viral triggers of asthma, such as the human rhinovirus. Our study also found a decrease in viral URTI during the COVID-19 pandemic compared to the previous year. This reduction was associated with a decrease in AE that required the use of SCS and admission to the emergency department. However, no significant difference was observed in the number of AEs requiring hospitalization in the first year of the pandemic compared to the year before. We believe this may be due to the already low rates of hospitalization in the pre-pandemic period.

Considering aeroallergen sensitization and related comorbid atopic disease AR, only pollen sensitization but not house dust mite (HDM) sensitization or AR was found to be significantly different in children whose AC was worsened. These findings are consistent with those of a short-term study in which the researches did not show any detrimental effect of HDM allergy in AC.[14] This may be due to increased hygienic measures also implemented in many houses during the pandemic which might have also decreased HDM load. There could be a connection between pollen allergies and the increase in people spending more time outdoors when quarantine restrictions were lifted, as well as the frequent ventilation of homes during quarantine periods.

Presence of a sibling who goes to daycare, school attendance, and family members working outside the home, smoking in the household during the pandemic were other factors investigated considering their impact on AC but they did not influence AC. Last but not least is the number of URTI during the first year of the pandemic which was found to significantly decrease with respect to the pre-pandemic period. The reduction may have been influenced by several factors such as the absence of in-person education, children staying at home, social distancing, increased use of masks, and hand washing. A logistic regression of factors that impacted AC in the univariate analysis revealed that only the number of URTI had an independent effect on AC. Children who did not experience URTI had 2.4 times better AC compared to those who did.

The strengths of our study include the fact that all study participants had persistent asthma and were fully and regularly compliant with ICS treatment prior to the pandemic. To the best of our knowledge, this is the first study in Türkiye to conduct a long-term comparison between the year prior to the pandemic and the first year of the pandemic, with regards to ATC, AE and AC among children with asthma, and the factors influencing these parameters. The weaknesses of the study include the limited number of cases with worsened AC during the first year of the pandemic and reliance on data sources from medical records and self-reported questionnaires.

In conclusion, this study is important because it is the first to investigate the impact of the COVID-19 pandemic on long-term ATC, AC, and AE. Despite a significant decline in treatment compliance, the reduction in asthma

exacerbations, emergency room visits, and use of SCS during the first year of the pandemic compared to the year prior is a positive outcome. Moreover, it is noteworthy that these factors were associated with a decrease in the number of URTI experienced. Asthma control was not affected in children with persistent asthma who were infected with COVID-19.

Ethical approval

Ethical approval was obtained from Gazi University Ethics Committee. (Date: 31.05.2021/ No:498). All procedures performed were in accordance with the ethical standards and with the 1964 Helsinki Declaration and its later amendments. All participants gave informed consent for the study.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AB, SOO, SPT, GY, HIEK; data collection: SOO, GY; analysis and interpretation of results: SOO, AB; draft manuscript preparation: SOO, AB. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 748-757
https://doi.org/10.24953/turkjped.2023.275

Original Article

Evaluation of the relationship between neonatal serum asprosin levels and anthropometric measurements in newborns of mothers with and without gestational diabetes mellitus

Emine Esin Yalınbaş[1], Raziye Akcılar[2]

[1]*Department of Neonatology, Eskişehir City Hospital, Eskişehir;* [2]*Department of Physiology, Kütahya Health Sciences University Faculty of Medicine, Kütahya, Türkiye.*

ABSTRACT

Background. Asprosin is a newly identified adipokine that is expressed in the placenta. Its production is increased in women with gestational diabetes mellitus (GDM), and it is a factor related to insulin resistance. This study aimed to determine whether neonatal serum asprosin levels are associated with anthropometric characteristics of newborns born to mothers with and without GDM.

Methods. This study included 51 newborns of mothers with GDM (insulin-treated or diet-treated) and 55 control newborns with their mothers. In newborns, anthropometric parameters were measured, and the concentrations of asprosin were detected by ELISA. Maternal blood glucose levels, body weight, and length were measured and body mass index (BMI) was calculated.

Results. Serum asprosin levels were significantly higher and linked to a higher risk in the newborns of mothers with GDM compared with those of the control newborns (170.3 [132.6] vs. 91.4 [68.7] ng/mL, $p < 0.001$). Serum asprosin levels were negatively correlated with blood glucose concentrations ($r = -0.282$, $p = 0.045$) in the newborns of mothers with GDM and significantly positively correlated with birth weight ($r = 0.315$, $p = 0.019$) in the control newborns. Newborn serum asprosin levels were positively correlated with the glucose levels ($r = 0.264$, $p = 0.006$) of all mothers. In addition, newborns born to an insulin-treated mother with GDM had significantly higher birth weight and length than newborns born to a diet-treated mother with GDM (3262.9 vs. 3137 g, $p = 0.032$, and 49.7 vs. 49.2 cm, $p = 0.05$). Although asprosin levels were higher in newborns of mothers treated with insulin, these differences were not statistically significant. Mothers with GDM had high blood glucose levels ($p = 0.032$).

Conclusions. Serum levels of asprosin are increased and negatively correlated with glucose concentrations in newborns of mothers with GDM. Asprosin could be used as an early biomarker in newborns of GDM mothers.

Key words: asprosin, newborn, gestational diabetes mellitus.

The asymptomatic condition known as gestational diabetes mellitus (GDM) is defined by carbohydrate intolerance that transforms into diabetes during pregnancy despite normal glucose metabolism prior to pregnancy.[1] There are many short- and long-term consequences for the mother and fetus when maternal diabetes occurs during pregnancy. GDM has a number of causes, including the occurrence of insulin resistance as a result of the anti-insulin actions of placental hormones and the increase in maternal adipose tissue during pregnancy.[2] Babies born to mothers with GDM are more likely to acquire type II diabetes later in life and to be overweight or obese at an early age.[3]

Two exons (exons 65 and 66) of the fibrillin-1 gene (*FBN1*) encode the newly identified,

✉ Raziye Akcılar
raziye.akcilar@ksbu.edu.tr

Received 24th April 2023, revised 3rd June 2023, 10th July 2023, 3rd August 2023, 21st August 2023, accepted 23rd August 2023.

glucogenic adipokine asprosin, which is produced and released by white adipose tissue during fasting. Asprosin plays a complex role in the central nervous system (CNS), peripheral tissues, and organs.[4] Through a multitude of signaling channels, asprosin significantly influences things like hunger, insulin resistance, glucose metabolism, and cell death.[5] Asprosin is crucial in the treatment of metabolic disorders such as insulin resistance, type 2 diabetes, and polycystic ovary syndrome.[6,7] Asprosin concentrations are considerably greater in type 2 diabetic women, and asprosin and insulin resistance are positively correlated in polycystic ovarian syndrome patients.[8] Patients with glucose dysregulation have significantly higher asprosin concentrations, which are linked to a number of clinical indicators of lipid and glucose metabolic disorders.[9] Zhong et al.[10] found that the placenta expresses asprosin and that GDM pregnant women have higher levels. Baykus et al.[11] reported that infants with intrauterine growth restriction had the lowest asprosin concentrations while pre-eclampsia, gestational diabetes, and fetal macrosomia were associated with higher salivary and blood asprosin levels. Although there are studies on maternal and neonatal asprosin levels, including gestational diabetes, pre-eclampsia, and fetal growth retardation[11,12], the underlying mechanisms for its formation are not fully known. Therefore, the goal of this study was to investigate the relationship between neonatal serum asprosin levels and anthropometric features in babies delivered in Turkish to women with and without gestational diabetes.

Material and Methods

Study population

This prospective study was conducted in the Neonatology Unit of Kütahya Health Sciences University from December 2020 to December 2021. The study was approved by the Local Ethics Committee of Kütahya Health Sciences University (date: November 25, 2020; no. 2020-07/04). Parental consent was obtained before blood samples were taken. The mothers were diagnosed with gestational diabetes by an oral glucose tolerance test (OGTT) performed between 24 and 28 gestational weeks, and their 51 newborns of mothers with gestational diabetes (30 diet-treated vs. 21 insulin-treated) were included in the study. The mothers, in whom the OGTT results were normal, and their 55 newborns (37–41 weeks of gestation, both groups) were accepted into the control group. Maternal blood glucose levels, body weight and length were measured and body mass index (BMI) was calculated by dividing weight by the square of height (kg/m^2).

Gestational diabetes mellitus was diagnosed according to the American Diabetes Association's suggested criteria (fasting blood sugar of ≥ 92 < 126 mg/dL at the time of the first examination and the presence of at least one abnormal result (fasting ≥92 mg/dL, 1st hour ≥180 mg/dL, 2nd hour ≥153 mg/dL) in a 75 g oral glucose tolerance test after 24–28 weeks).[13] It was noted that glucose monitoring, diet, and exercise were recommended to pregnant women diagnosed as having GDM, and insulin therapy was initiated by consulting endocrinology for pregnant women whose targeted glucose values could not be determined.

After birth, the gestational age at delivery, and weight, length, and head circumference of the newborns were recorded and ponderal index (PI) was calculated using the formula: PI = [weight (g) x 100] ÷ [length (cm)]^3. The body weight of newborns was measured using electronic scales sensitive to 5 g. Length measurements were taken (head part fixed, foot part movable) using a length measurement board.

Blood samples were taken from newborns of mothers with and without GDM to measure glucose levels. Blood glucose assessments of newborns were made according to the American Academy of Pediatrics (AAP) criteria.[14] Since the newborns of mothers with GDM are at risk, blood glucose measurement was repeated 30 minutes after early feeding. If the blood glucose levels of newborns born to mothers with GDM

were normal at 12 hours of screening in large for gestational age (LGA) babies and at 24 hours in small for gestational age (SGA) babies, the screening was terminated.

Blood samples obtained from newborns to measure asprosin levels were placed in non-heparinized tubes and promptly centrifuged at 1,000 g for 10 min. The serum samples were then kept at −20 °C until analysis. Asprosin measurement in serum was performed with the Human Asprosin ELISA measurement kit (Bioassay Technology Laboratory, Catalogue no: E4095Hu, Shanghai, China). Absorbance reading was done on a Chromate 4300 brand ELISA Reader device (Awareness Technology, Inc., Palm City, USA). Asprosin test results were given as ng/mL, with an assay range of 0.5 to 100 ng/mL and a sensitivity of 0.23 ng/mL.

Babies of pregnant mothers with similar BMI values were included in the study. Mothers with pregestational diabetes or a history of chronic disease and infants with maternal clinical conditions such as parathyroid, a bone, kidney, or gastrointestinal disorder or with congenital anomalies were excluded.

Statistical analysis

All analyses were performed using SPSS version 20.0 software (SPSS Inc., Chicago, USA). The Kolmogorov–Smirnov test was performed to check the normality of distributions. The independent samples t test was used for normally distributed variables such as birth weight, body weight, length, and BMI, and are presented as mean ± standard deviation (SD). The Mann–Whitney U test was used for non-normally distributed variables such as gestational age, birth length, head circumference, PI, glucose, and asprosin, and are presented as median and interquartile range. Categorical data like sex are presented as number and percentage using chi-square ($\chi 2$) test. To evaluate the variables related with mothers and their newborns were analyzed using binary and multivariate logistic regression analysis to evaluate the odds ratios (OR) and 95% confidence intervals (CIs). Hosmer-Lemeshow test was used to check the model fitness. Pearson's correlation was used to correlate serum asprosin with birth weight, body weight, length, BMI. Spearman's correlation was used to correlate serum asprosin levels with gestational age, birth length, head circumference, PI, and glucose. Linear regression analysis was used to evaluate the relationship between neonatal serum asprosin and other variables. P values ≤ 0.05 were considered statistically significant.

Results

The newborn and mother characteristics are summarized in Table I. In all, 106 newborns were consecutively recruited, including 51 newborns of mothers with GDM and 55 newborns of non-GDM mothers (control), and matched for gestational age [37.0 (36.0–38.0) vs. 38.0 (37.0–39.0) weeks; $p < 0.001$] and sex (28 males/23 females vs. 26/29; $p = 0.432$). There was no significant difference in birth weight, birth length, BMI, head circumference, PI, or blood glucose levels between the newborn groups. Factors associated with mothers and their newborns were determined by logistic regression analysis and the results are shown in Table II. Serum asprosin levels were significantly higher in the newborns of mothers with GDM than in the control ($p < 0.001$), and the OR was approximately 1.08 (95% CI 1.04–1.12, $p < 0.001$). In newborns of mothers with non-GDM there was increased gestational age compared to the newborns of mothers with GDM ($p < 0.001$), and OR value was 0.61 (95% CI 0.46–0.82, $p = 0.001$). In addition, mothers with GDM had a significantly higher glucose concentration compared to non-GDM mothers [90.0 (81.0–103.0) vs. 98.0 (88.0–110.0) mg/dl; $p = 0.032$], and the OR was 1.02 (95% CI 1.00–1.04, $p = 0.041$) as shown in Table I and Table II.

In our study, the GDM group was divided into two subgroups including those treated with diet (n=30) and insulin (n=21). As depicted in Table III, neonates born to an insulin-treated mother with GDM had a significantly higher

Table I. Demographic and clinical characteristics of mothers and their newborns.

	Control newborns (n = 55)	Newborns of mothers with GDM (n = 51)	*p*
Male / Female (n)	26 / 29	28 / 23	0.432
Gestational age (weeks, range)	38 (37 - 39)	37 (36 - 38)	< 0.001*
Birth weight (g)	3194 ± 321	3189 ± 567	0.958
Birth length (cm)	50 (49 - 51)	49 (48 - 50)	0.245
BMI (kg/m^2)	12.9 ± 0.95	12.9 ± 1.69	0.797
Head circumference (cm)	34.1 (34.0 - 35.0)	34.5 (34.0 - 35.0)	0.868
PI (kg/m^3)	2.60 (2.50 - 2.70)	2.55 (2.40 - 2.86)	0.766
Glucose (mg/dL)	78.0 (64.0 - 89.0)	71.0 (62.0 - 87.0)	0.171
Asprosin (ng/mL)	91.4 (68.7 - 114.3)	170.3 (132.6 – 236.9)	< 0.001*
	Control mothers (n = 55)	Mothers with GDM (n = 51)	*p*
Glucose (mg/dL)	90.0 (81.0 - 103.0)	98.0 (88.0 - 110.0)	0.032*
Body weight (kg)	77.2 ± 13.7	76.9 ± 12.0	0.769
Length (cm)	161.8 ± 6.8	160.5 ± 5.5	0.105
BMI (kg/m^2)	29.4 ± 4.5	29.8 ± 4.3	0.872
Delivery method			
Vaginal (n, %)	25 (59.5)	17 (40.5)	0.202
Cesarean (n, %)	30 (46.9)	34 (53.1)	
Treatment Diet/Insulin (n, %)	-	30 (58.8) / 21 (41.2)	-

Birth weight, body weight, length, BMI were described as mean ± standard deviation and determined by independent Student t-test. Other parameters were described as median and interquartile range and determined by Mann-Whitney U test. Proportion n (%) was determined by chi-square (χ2).
*p ≤ 0.05 is considered signifcant. BMI: body mass index, GDM: gestational diabetes mellitus, PI: ponderal index.

Table II. Logistic regression analysis determining the factors associated with mothers and their newborns.

Newborns	Univariate logistic regression		Multivariate logistic regression	
	OR (95% CI)	p	OR (95% CI)	p
Male/Female (n)	0.73 (0.34 – 1.58)	0.433	-	-
Gestational age (weeks)	0.61 (0.46 – 0.82)	0.001*	0.59 (0.30 – 1.14)	0.120
Birth weight (g)	1.00 (0.99 – 1.00)	0.957	-	-
Birth length (cm)	0.91 (0.73 – 1.14)	0.432	-	-
BMI (kg/m^2)	1.03 (0.78 – 1.37)	0.795	-	-
Head circumference (cm)	0.94 (0.64 – 1.38)	0.775	-	-
PI (kg/m^3)	1.78 (0.42 – 7.62)	0.431	-	-
Glucose (mg/dL)	0.98 (0.96 – 1.00)	0.096	1.01 (0.97 – 1.05)	0.498
Asprosin (ng/mL)	1.08 (1.04 – 1.12)	<0.001*	1.08 (1.048 – 1.13)	<0.001*
Mothers	Univariate logistic regression		Multivariate logistic regression	
	OR (95% CI)	p		
Glucose (mg/dL)	1.02 (1.00 – 1.04)	0.041*	1.01 (0.98 – 1.05)	0.377
Body weight (kg)	0.99 (0.96 – 1.02)	0.890	-	-
Length (cm)	0.96 (0.90 – 1.02)	0.273	-	-
BMI (kg/m^2)	1.02 (0.93 – 1.11)	0.654	-	-
Method of Delivery				
Vaginal (n, %)	1.67 (0.75 – 3.66)	0.204	-	-
Cesarean (n, %)				
Treatment Diet/Insulin (n, %)	-	-	-	-

Logistic regression analysis was performed to evaluate the odds ratios (OR) and 95% confidence intervals (CIs).
*p ≤ 0.05 is considered signifcant. BMI: body mass index, GDM: gestational diabetes mellitus, PI: ponderal index.

birth weight and birth length compared with neonates born to a diet-treated mother (p = 0.032, p ≤ 0.05, respectively). Although the newborn asprosin levels tended to be higher in the insulin group, these differences were not statistically significant (p = 0.108) (Table III). In addition, glucose levels were greater in the insulin-treated GDM mothers compared to the diet-treated mothers (p ≤ 0.05).

Correlation analyzes between anthropometric variables and serum asprosin concentrations in the newborns and mothers are shown in Table IV. For all newborn groups, serum asprosin levels correlated negatively with gestational age (r = −0.272; p = 0.005) and glucose concentration (r = −0.267; p = 0.006). In addition, a positive correlation between serum levels of asprosin, birth weight (r = 0.187; p = 0.05), BMI (r = 0.247; p = 0.011), and the mother's glucose concentration (r = 0.264; p = 0.006) was found in all groups. There was a significant positive correlation between asprosin, birth weight, and BMI in the newborns of the non-GDM mothers (r = 0.315; p = 0.019 and r = 0.291; p = 0.031, respectively). In newborns of mothers without GDM, both gestational age and birth weight were high, and there was a positive correlation with each other (r = 0.415, p < 0.001). In the newborns of mothers with GDM, serum asprosin levels correlated negatively with blood glucose (r = −0.282; p = 0.045) and positively with BMI (r = −0.336; p = 0.016). There was no relationship between serum asprosin levels and birth length, head circumference or PI in newborns (Table IV). The BMI of mothers with GDM was negatively correlated with newborn glucose levels (r = −0.307; p = 0.02), while birth weight (r = 0.416; p = 0.002) and birth length (r = 0.365; p = 0.008) were positively correlated. After multivariate linear regression analysis, neonatal serum asprosin levels continued to be negatively related to gestational age and glucose level of newborns and positively related to maternal glucose level in the all group (Table IV).

Informed consent was obtained from all individual participants' legal guardians included in the study.

Discussion

In this study, we investigated the relationship between blood asprosin concentrations and anthropometric characteristics in newborns born to women with gestational diabetes in the Turkish population. Asprosin, an orexigenic hormone that increases hepatic glucose production, is a possible therapeutic target for the treatment of both obesity and diabetes.[4,15] Patients with impaired glucose regulation have significantly higher asprosin levels, which are correlated with several clinical markers of lipid and glucose metabolic disorders.[16] Asprosin, a

Table III. Clinical outcomes of newborns of mothers with GDM treated with diet and insulin.

Newborns	Diet (n=30)	Insulin (n=21)	p
Male / Female (n)	17 / 13	11 / 10	0.493
Gestational age (weeks)	37 (36 - 38)	37 (36 - 38)	0.410
Birth weight (g)	3137 ± 473	3263 ± 686	0.032*
Birth length (cm)	49.2 ± 1.7	49.7 ± 2.3	0.05*
BMI (kg/m^2)	12.9 ± 1.51	13.0 ± 1.95	0.703
Head circumference (cm)	34.8 (34-35)	34 (33 - 35)	0.260
PI (kg/m^3)	2.64 ± 0.32	2.61 ± 0.34	0.604
Glucose (mg/dL)	73.8 ± 17.1	73.2 ± 18.5	0.511
Asprosin (ng/mL)	161.4 (131.4 – 227.5)	194.8 (149.2 - 252.9)	0.108

Birth weight, birth length, BMI, PI, glucose were described as mean ± standard deviation and determined by independent Student t-test. Other parameters were described as median and interquartile range and determined by Mann-Whitney U test. Proportion n (%) was determined by chi-square (χ2).
*p ≤ 0.05 is considered signifcant. BMI: body mass index, GDM: gestational diabetes mellitus, PI: ponderal index.

Table IV. Correlation between neonatal serum asprosin concentrations and various parameters in mothers and their newborns.

Newborns	Control newborns (n = 55)				Newborns of mothers with GDM (n = 51)				All newborns (n = 106)			
	Univariate correlation analysis		Multivariate regression analysis		Univariate correlation analysis		Multivariate regression analysis		Univariate correlation analysis		Multivariate regression analysis	
	r	p	β	p	r	p	β	p	r	p	β	p
Gestational age (weeks)	0.095	0.488	-	-	−0.151	0.290	-	-	−0.272	0.005*	-0.369	<0.001*
Birth weight (g)	0.315	0.019*	0.226	0.302	0.257	0.069	-	-	0.187	0.05*	0.079	0.687
Birth length (cm)	0.154	0.261	-	-	−0.045	0.756	-	-	−0.036	0.713	-	-
BMI (kg/m^2)	0.291	0.031*	0.111	0.612	0.336	0.016*	0.294	0.036*	0.247	0.011*	0.239	0.203
Head circumference (cm)	0.173	0.207	-	-	−0.011	0.938	-	-	0.050	0.611	-	-
PI (kg/m^3)	0.143	0.297	-	-	0.255	0.071	-	-	0.135	0.166	-	-
Glucose (mg/dL)	−0.223	0.101	-	-	−0.282	0.045*	-0.201	0.146	−0.267	0.006*	-0.267	0.003*
Mothers	Control mothers (n = 55)				Mothers with GDM (n = 51)				All mothers (n = 106)			
	Univariate correlation analysis		Multivariate regression analysis		Univariate correlation analysis		Multivariate regression analysis		Univariate correlation analysis		Multivariate regression analysis	
	r	*p*	β	*p*	r	*p*	β	*p*	r	*p*	β	*p*
Glucose (mg/dL)	0.083	0.547	-	-	0.210	0.138	-	-	0.264	0.006*	0.193	0.047*
Body weight (kg)	−0.084	0.543	-	-	0.030	0.834	-	-	-0.018	0.851	-	-
Length (cm)	−0.163	0.233	-	-	0.148	0.300	-	-	−0.062	0.530	-	-
BMI (kg/m^2)	0.007	0.957	-	-	-0.036	0.803	-	-	0.018	0.857	-	-

Pearson correlation analysis was used for normally distributed variables as birth weight, body weight, length, BMI. Spearman correlation analysis was used for skewness distribution as gestational age, birth length, head circumference, PI, and glucose. Linear regression analysis was used to evaluate the relationship between serum asprosin and other variables. *$p \leq 0.05$ is considered signifcant. BMI: body mass index, GDM: gestational diabetes mellitus, PI: ponderal index.

novel factor associated with insulin resistance, may play a role in the development of GDM.[10] There is currently little information available about asprosin levels in pregnant women with GDM and their newborns. The findings of Baykus et al.[11] indicated that venous and arterial cord blood asprosin levels in newborns of GDM pregnant women were higher. Birth weights of infants and asprosin levels in arterial and venous cord blood were positively correlated in women with gestational diabetes. Zhong et al.[10] demonstrated that asprosin levels were elevated in the umbilical cord of newborns from GDM mothers; neonatal cord asprosin levels showed a positive correlation with birth weight. In this study, serum concentrations of asprosin were significantly positively correlated with birth weighs and BMI, and negatively correlated with gestational age and blood glucose levels in all newborn groups. Serum concentrations of asprosin were also significantly positively correlated with birth weight and BMI in newborns of non-GDM mothers. In addition, levels of serum asprosin were significantly higher in newborns of mothers with GDM and were correlated negatively with blood glucose concentration and positively with BMI. In newborns born to mothers with GDM, increased serum asprosin levels may be an important risk factor.

Baykus et al.[11] and Zhong et al.[10] demonstrated that asprosin levels are elevated in the plasma of pregnant women with GDM. Further, asprosin levels are positively associated with age in pregnant women but not with maternal BMI.[10] In our study, mothers with GDM had high blood glucose levels. We were unable to look at maternal serum insulin, hemoglobin A1c (HbA1c), and asprosin levels in this study. Therefore, we could not examine the relationship between maternal blood sugar and asprosin level. But, there was a positive correlation between the asprosin levels of newborns and maternal blood glucose levels. The reason for the increase in serum asprosin levels in newborns of mothers with GDM may be due to the increase in hepatic glucose production due to changes in maternal insulin sensitivity during pregnancy.

GDM is a developing health problem worldwide affecting up to 14% of pregnancies according to the diagnostic criteria and demographics analyzed.[13-17] Pre-pregnancy maternal obesity, excessive weight gain during pregnancy and gestational diabetes are the main causes of pathological pregnancy conditions. A newborn's energy metabolism becomes compromised due to insulin resistance, high blood glucose, and hormonal changes.[18] In our study, gestational age was significantly lower among newborns of mothers with GDM than among newborns of non-GDM mothers. There was no significant difference in birth weights, birth length, BMI, head circumference, or PI between the newborn groups. Other studies have not shown differences in birth measurements between neonates exposed to GDM and those not.[19,20] According to a study by Bayoumi et al.[21], there is no statistically significant difference between babies born to healthy non-diabetic women and babies born to women with GDM in terms of length or head circumference. Contrarily, Baptiste-Roberts et al.[22] demonstrated that mothers with GDM delivered infants with higher birth weight than mothers without diabetes. In addition, Sletner et al.[23] found that compared to offspring of non-GDM mothers, offspring of GDM mothers were smaller in the middle of pregnancy but developed more rapidly until delivery. This disparity may be attributable to the diverse ethnic backgrounds of the participants.

GDM is linked to complications in both mothers and newborns.[24] Neonatal hypoglycaemia is one such complication; it generally develops in the first 24 hours after birth as newborns go through their metabolic transition during the first few days of life.[25,26] Rarely, prolonged or recurrent hypoglycemia can have serious and long-lasting neurological health consequences, and babies with GDM mothers are especially at risk.[27] Neonatal hypoglycaemia and congenital anomalies are more prevalent in infants born to women with GDM.[28,29] Among neonates whose

mothers had GDM, neonatal hypoglycemia and hyperbilirubinemia are more common.[28] The likelihood of neonatal hypoglycemia in diabetic mothers is the consequence of a combination of risk factors, including maternal blood glucose levels, maternal treatment, and birth weight, according to studies.[30-33] In our study, although the blood glucose levels of babies born to mothers with GDM were lower than those of non-diabetic mothers, the difference was not significant. In addition, babies of mothers with GDM treated with insulin or diet showed no difference in blood glucose levels. The nonsignificance of these results may be due to the low number of pregnant women included in the study.

In this study, glucose levels were higher in insulin-treated mothers with GDM compared with diet-treated mothers with GDM. Compared to neonates born to diet-treated mothers, neonates born to insulin-treated mothers with GDM had considerably higher birth weight and birth length. In research by Koning et al.[34] newborns in the insulin group had lower birth weights and gestational ages at birth than those in the diet group. We found that although not statistically significant, serum asprosin levels tended to be higher in neonates born to insulin-treated mothers with GDM. Placental asprosin levels are correlated with maternal insulin levels and rise after starting insulin therapy in GDM patients, according to Hoffman et al.[35] There are no literature data showing serum asprosin levels in neonates born to insulin-treated mothers with GDM.

The current study has the following limitations: 1) asprosin levels were only tested in the neonates' peripheral blood; 2) asprosin levels in the peripheral blood of the mothers were not analyzed; and 3) the number of newborns was relatively low because this was a single-center clinical study involving the Turkish population. The relationship between anthropometric measurements and asprosin in neonates during GDM as well as the causes for increased asprosin release are unknown. It is advised that our findings be replicated in larger, more ethnically diverse populations in order to support the validity of our conclusions.

In the newborns of mothers with GDM, asprosin concentration was considerably higher and had a negative correlation with blood glucose levels. Higher serum asprosin levels may raise the risk of short- and long-term negative health outcomes, such as neonatal and obstetric problems during delivery and obesity and diabetes later in life for these babies. Therefore, elevated asprosin levels may be an important risk factor for newborns born to mothers with GDM, independent of anthropometric measurements.

Acknowledgement

The authors thank all the parents for their cooperation and participation.

Ethical approval

This study was started following the decision of the Local Ethics Committee of Kütahya Health Sciences University (date: November 25, 2020, and no. 2020- 07/04). Informed consent was obtained from all individual participants' legal guardians included in the study.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EEY, RA; data collection: EEY; analysis and interpretation of results: RA, EEY; draft manuscript preparation: EEY, RA. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 758-768
https://doi.org/10.24953/turkjped.2023.389

Original Article

Evaluation of toll-like receptors 2 and 4 polymorphism and intestinal microbiota in children with food allergies

Mehmet Kılıç[1], Elif Beyazıt[2], Ebru Etem Önalan[3], Tuğçe Kaymaz[3], Erdal Taşkın[4]

[1]Division of Allergy and Immunology, Department of Pediatrics, Fırat University Faculty of Medicine, Elazığ; [2]Department of Pediatrics, Fırat University Faculty of Medicine, Elazığ; [3]Department of Medical Biology, Fırat University Faculty of Medicine, Elazığ; [4]Division of Neonatology, Department of Pediatrics, Elazığ, Türkiye.

ABSTRACT

Background. Mutual regulation between immune system and gut microbiota is achieved through several mechanisms including the engagement of toll-like receptors (TLRs) which is expressed on numerous cell types.

In this study we aimed to explore the association between food allergies and TLR gene polymorphisms in association with gut microbiota.

Methods. Toll-like receptors polymorphism frequencies and some bacteria in the gut microbiota in 130 infants aged 1-24 months with egg and/or milk allergy in a prospective cohort were compared with 110 non-food allergic controls. Four candidate polymorphisms (*TLR2* rs1898830/rs5743708 and *TLR4* rs4986790/rs4986791) were genotyped by allelic discrimination polymerase chain rection (PCR) method. Gut microbiota analysis was achieved by using high-throughput sequencing.

Results. The *TLR4* rs4986790 (Asp299Gly) single nucleotide polymorphism (SNP) major/minor allele frequency was 0.788/0.212 in food allergy patients and 0.719/0.280 in controls (p=0.017). There was a statistically significant difference between groups in terms of genotype frequencies (AA, AG, GG). Gut microbiota analysis revealed increased Firmicutes phylum in stool of the patients with food allergy. Except for *TLR4* rs4986791 (Thr399lle) allele, the other TLR polymorphisms were not associated with food allergies in children. When the bacteria in the intestinal microbiota and *TLR2* and *TLR4* gene polymorphisms were compared; we determined a statistically significant increase in *Bifidobacterium* concentration in the intestinal microbiota in *TLR4* rs4986791 CT heterozygous genotype (p=0.004).

Conclusions. This study demonstrated a partial role of *TLR4* gene polymorphism and gut microbiota in the development of food allergies. Future work in this area will be required to clarify the roles of different microbial strains that modulate gut microbiota composition and function in conjunction with TLR transcription pathways.

Key words: food allergy, Toll-like receptor 2, Toll-like receptor 4, genetic polymorphism, gut microbiota, children.

Food allergies are immune reactions against food proteins and may cause life-threatening severe systemic reactions. The prevalence of food allergies is 6-8% in children up to three years of age, whereas this rate decreases to 2% over seven years of age.[1] The incidence of food allergies has increased in the last 10-20 years, and is considered to be related to numerous risk factors.[2] The factors that directly play roles in food sensitivity development are generally divided into two groups: host factors and antigenic features. Numerous host factors such as genetic predisposition, the age at the time of exposure to the antigen, a high socioeconomic status, urban living, breastfeeding, formula feeding, exposure to domestic animals, time of transition to complementary foods, the mother's diet, disruption of the intestinal barrier, and quantitative changes of the intestinal microbiota have been defined.[3]

✉ Mehmet Kılıç
drmkilic@gmail.com

Received 23rd May 2023, revised 13th July 2023, accepted 20th August 2023.

The human gut microbiota involves hundreds of different phylogenetic species classified into six primary microbial phyla: Firmicutes, Bacterioidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Euryarchaeota. The first four primary phyla constitute 98% of the intestinal microbiota.[4] The microbiota's taxonomic combination also changes with age. For example, prior to maturing an adult-like microbiota dominated by Firmicutes and Bacteroidetes, the neonatal intestinal microbiota is initially dominated by Proteobacteria such as *Escherichia* and *Shigella*, and then by Actinobacteria such as *Bifidobacterium*. Such maturing is reflected in the ratio between *Enterobacteriaceae* (Proteobacteria and Actinobacteria) and *Bacterioidaceae* (Firmicutes and Bacteroidetes). The relative ratio of *Enterobacteriaceae* decreases, and the rate of *Bacteroidaceae* increases with age. Microbial colonization developing in the human gut during infancy plays a role in the maturation and epigenetic regulation of the immune system.[5] The factors affecting early-life intestinal microbial colonization and combination have been shown to impact the development of atopic disorders, including food allergies. Impairment of the original microbiota combination (dysbiosis) has been found to be associated with food allergy development.[6] Dysbiosis developing in children with food allergies might cause immune imbalances among effector T cells and regulatory T cells (Treg) cells. Microbiota deficiency has been found to be associated with Th2 development and IgE production against food allergens in germ-free mice.[7] On the other hand, the high concentration of some microorganisms in the intestinal microbiota may lead the immune system towards a T helper (Th) 1 phenotype that is protective against atopy rather than the Th2 cell phenotype associated with atopy in infancy/childhood.[5,8]

Toll-like receptors (TLRs) play essential roles in responses against microbial agents, inflammatory pathways, and the regulation of innate immune responses.[9] The innate immune system can control the adaptive immune system's developing response against food proteins. Dendritic cells and TLRs play essential roles in this task.[10] TLRs induce the development/differentiation of Th1, Th2, Th17, and $CD4^+$ Tregs. TLRs regulate the development and functions of Tregs by mediated signals and alter the development of atopic disorders. TLRs flawed expressions or genetic changes can contribute to imbalanced Th1 or Th2 immunity levels.[11] Besides, it was reported that TLRs could affect the intestinal microbiota's development and combination.[9] In some studies on mice have demonstrated that the Treg cells, stimulated by *TLR2* activation, were necessary for successful intestinal colonization.[12,13] In addition, it was reported in animal studies that the *TLR2* and *TLR4* gene expression levels changed due to alterations in the intestinal microbiota's combination.[9,14] Therefore, TLR gene polymorphisms (particularly *TLR2* and *TLR4*) and, accordingly, the TLR system's impaired signaling mechanisms have been reported to have associations with the risk of allergy development.[9] On the other hand, TLR4-related signals induced by intestinal flora were found to inhibit the development of reactions against food antigens. In recent years, several simple nucleotide polymorphisms (SNPs) have been described in the *TLR4* gene (*TLR4* rs4986790, *TLR4* rs4986791) and *TLR2* gene (*TLR2* rs1898830, *TLR2* rs5743708).

This study aimed to investigate the associations between food allergies, intestinal microbiota combination, the genetic polymorphisms of rs5743708 and rs1898830 in the *TLR2* gene and rs4986791 and rs4986790 in the *TLR4* gene.

Methods

Study design

A total of 130 children, aged between 1 and 24 months, followed up with the diagnosis of food allergy in our hospital's Pediatric Allergy and Immunology Department were included in the study. A total of 110 children with

similar age characteristics who had applied to the healthy child follow-up clinic of our hospital constituted the control group. In the healthy pediatric outpatient clinic; children were vaccinated, age-appropriate nutrition education was provided, and their growth and development were monitored. Children with chronic pulmonary and other respiratory system disorders, gastrointestinal system diseases such as inflammatory intestinal disorders, renal/urologic, hepatic, cardiovascular, metabolic, and neurologic diseases, children with cystic fibrosis, bone diseases, and history of immune deficiencies, children under continuous antibiotic prophylaxis, and those with the history of antibiotic use within the last six months were excluded from the study.

Food allergy was diagnosed by obtaining the patient's medical history, the skin prick test (SPT) with foods, the child food panel (fx5), food sIgE measurement, and oral provocation test with foods. Commercial allergen solutions were utilized for SPTs with foods (Allergopharma, Reinbek, Germany). Moreover, SPTs for milk and egg white were performed with commercial allergen solutions, and a prick-to-prick test was used with fresh food. Commercial allergen solutions were used for aeroallergens and food in children aged two years (Allergopharma, Reinbek, Germany). Patients with >95% predictive values in the enduration diameter of skin prick tests for milk or egg or sIgE levels and who had a medical history of anaphylaxis due to these foods within the last six months did not undergo a provocation test.[15] Diagnoses of these cases were directly considered food allergies; they went on an elimination diet and were included in the study. Besides, provocation tests were performed in patients with <95% predictive value for these foods. The diagnosis was considered a food allergy in patients who developed a reaction during the test, and they were included in the study. The Ethics Committee of Fırat University approved the study (date June 21, 2018, and decision # 11/04). Moreover, the participants' families were informed about the study, and their written consent was obtained.

Sample collection

In addition to routine tests of the cases in the patient and control groups, one ml of blood was obtained for genotyping. In addition, fresh stool (100 mg) was obtained for microbiota analysis; the samples were placed in sterile, sealed, covered stool sample containers within 0-2 hours and kept at -80°C until the time of analysis.

Molecular genetic analysis

The Real-time Polymerase Chain Reaction (RT-PCR) method was used to analyze rs5743708 and rs1898830 polymorphisms in the *TLR2* gene and rs4986791 and rs4986790 polymorphisms in the *TLR4* gene. Blood was thawed to room temperature before isolating Deoxyribonucleic acid (DNA). The Genomic DNA isolation WIZARD Genomic DNA Purification Kit (Catalog # A1125, Promega, MA, USA) was used for isolation. The samples' DNA concentrations were measured in a nanodrop (Maestrogen, Maestro NanoDrop, USA) device, and the concentrations were adjusted to 1-10 ng. The PCR reaction was prepared by adding 2.5 µl of DNA sample, 0.5 µl of polymorphism-specific Tag-Man probe, 2.5 µl of Taqman genotyping master mix (Catalog# 4371355), and 2.5 µl of ddH_2O to each well of a 96-well plate. The plate was placed in the ABI 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA) device. The conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (92°C for 15 s), annealing, and extension in one step (60°C for 1 min). After the Polymerase Chain Reaction, the homozygous mutant, heterozygous, and homozygous normal genotypes were determined using the device's software system and according to Allele1 / Allele2 differentiation.[16]

DNA extraction from stool

One hundred mg of stool sample was weighed, 3 ml of 0.9% saline was added to the sample, and vortexed. The supernatant was taken into

a new tube following centrifugation at 3.000 rpm for 2 minutes. It was centrifuged at 1.000 rpm for one minute in this new tube, and the supernatant was discarded following the centrifugation process. Two ml of phosphate-buffered saline (PBS) was added to the pellet that remained at the bottom of the tube and then vortexed. The supernatant was discarded following centrifugation at 10.000 rpm for two minutes., As previously described by Kumar et al.[17], bacterial 16S rRNA amplification was performed following the isolation of bacterial DNA in the obtained pellet samples. Table I shows the studied microbiota-specific bacteria and used primers. Microbiota analysis was performed by breaking down the formed amplicons according to their hypervariable regions.

Statistical analysis

Statistical Package for Social Sciences (SPSS) v.22 software was used for statistical analysis. The quantitative variables with a normal distribution were expressed as the mean ± the mean's standard error, and those not having a normal distribution as the median and interquartile range. The Kruskal Wallis test was used to compare groups, whereas the Mann Whitney-U test was used for paired-group comparisons. Fisher's exact chi-square and Pearson's chi-square tests were used for comparing qualitative data. The results of the analyzes were assessed within the 95% confidence interval, and $p<0.05$ was considered statistically significant.

Results

Of 130 children with food allergies, 89 (68.5%) were male, and 41 (31.5%) were female, whereas 61 (55.5%) were male and 49 (44.5%) were female in the control group. The mean age was 8.1 ± 5.9 months in the patient group and 8.8 ± 4.6 months in the control group. No significant difference was found between the groups regarding age ($p=0.847$). In addition, a comparison of the groups for type of delivery, gestational age, and birth weight revealed no statistically significant differences. The clinical and demographic data of the food allergy and control groups are presented in Table II.

No significant differences were determined between the patient and control groups regarding genotype allele distributions for rs5743708 and rs1898830 SNPs in the *TLR2* gene and rs4986791 SNP in the *TLR4* gene ($p>0.05$). For *TLR2* rs5743708, Ma/Mi AF was 0.884/0.116 in the patient group and 0.911/0.09 in the control group. For *TLR2* rs1898830, Ma/Mi AF was calculated as 0.60/0.40 in the patient group and 0.546/0.453 in the control group. For the *TLR4* gene's rs4986790 SNP (A/G), the Ma/Mi AF allele frequencies were determined as 0.788/0.212 and 0.719/0.280 in the patient and control groups, respectively. In addition, it was determined that the minor allele frequency of the *TLR4* gene's rs4986790 polymorphism was 0.07 in the patient group and 0.04 in the control group. The incidences of *TLR4* rs4986790 polymorphism heterozygote genotype (AG) and G allele significantly increased in the patient group ($p=0.017$ and $p=0.017$, respectively) (Table III).

Table I. The bacteria studied and primers used in stool samples in QRT-PCR.

Bacteria (16S rRNA specific)	Forward primer	Reverse primer
Bacteroides	5′-GGCGACCGGCGCACGGG-3′	5′-GRCCTTCCTCTCAGAACCC-3′
Bifidobacterium	5′-CCTGGTAGTCCACGCCGTAA -3′	5′-CAGGCGGGATGCTTAACG-3′
Firmicutes	5′-TGAAACTYAAAGGAATTGACG-3′	5′-ACCATGCACCACCTGTC-3′
Fusobacterium	5′-TGAAACTYAAAGGAATTGACG-3′	5′-ACCATGCACCACCTGTC-3′
Actinobacteria	5′-TACGGCCGCAAGGCTA-3′	5′-TCRTCCCCACCTTCCTCCG-3′
Lactobacillus	5′-AGCAGTAGGGAATCTTCCA-3′	5′-CATGGAGTTCCACTGTCCTC-3′
Universal	5′-AAACTCAAAKGAATTGACGG-3′	5′-CTCACRRCACGAGCTGAC-3′

Table II. Comparison of clinical and demographic data for food allergy and control groups.

Features	Patient (n=130)	Control (n=110)	p value
Gender, n (%)†			0.38
Boy	89 (68.5)	61 (55.4)	
Girl	41 (31.5)	49 (44.6)	
Age (months), (mean ± SD)†	8.1±5.9	8.8±4.6	0.84
Type of birth, n (%)			0.63
Cesarean section	78 (60)	68 (61.8)	
Normal delivery	52(40)	42 (38.2)	
Gestational age (weeks), n (%)			0.40
≥38 weeks	82 (63.1)	76 (69.0)	
<38 weeks	48 (36.9)	34 (31.0)	
Birth weight (grams), n (%)			0.31
≥3500	33 (25.4)	19 (17.3)	
3000-3500	45 (34.6)	44 (40)	
<3000	52 (40.0)	47 (42.7)	
Feeding style, n (%)			0.21
Breast milk	64 (49.2)	49 (44.5)	
Breast milk + formula	22 (16.9)	15 (13.6)	
Breast milk + formula + cow's milk	21 (16.2)	19 (17.2)	
Breast milk + cow's milk	20 (15.4)	17 (15.4)	
Formula	3 (2.3)	10 (9.3)	
Antibiotic use, n (%)			0.54
Yes	73 (56.2)	66 (60)	
No	57 (43.8)	44 (40)	

†Data were presented as mean ± standard deviation (SD) and percentage.

No significant correlations of patients' characteristics participating in the study, such as gender, admission complaints, allergy types, feeding characteristics, and history of antibiotic use, with polymorphisms of *TLR2* rs5743708, *TLR2* rs1898830 and *TLR4* rs4986791 were determined (p>0.05). On the other hand, a significant relationship was present between the *TLR4* rs4986790 gene polymorphism and the type of food allergy, and the *TLR4* rs4986790 variant significantly increased in the group with isolated milk allergy compared to the control group (p=0.025). This difference was determined to be more significant between the group with isolated milk allergy and the group with multiple food allergies (p=0.036) (Table IV).

When the relationships of the patients' SPTs with the *TLR2* and *TLR4* gene polymorphisms were investigated, it was determined that in the patients with SPT positivity for milk only, the *TLR4* rs4986790 and *TLR4* rs4986791 heterozygote genotypes were significantly increased compared to the other SPT positivities (p=0.02, and p=0.003, respectively). However, no significant correlations of skin prick tests and oral provocation tests with the other analyzed polymorphisms were determined (p>0.05) (Table IV).

Microbiota analysis revealed that the partial concentration of Firmicutes increased 4.14-fold in the patient group compared to the control group, and this increase was statistically significant (p<0.001). On the other hand, even though Actinobacteria were 2.31-fold higher in the patient group than in the control group, this increase was not statistically significant (p>0.05) (Table V). In addition, there was a positive

Table III. Genotype and allele frequencies of TLR2 and TLR4 gene polymorphisms in patients with food allergies and the healthy control group.

TLR	SNP	Major/ Minor allele	Patient group (n=130)		Control group (n=110)		Minör OR (%95 CI)	p value for minor allele	OR (95% CI) for genotype	p value for genotype
			Major/ Minor allele frequency	Genotype number (n)	Major/ Minor allele frequency	Genotype number (n)				
TLR2	Rs5743708 (Arg753Gln)	A/G	0.88/ 0.12	127/3/0	0.91/ 0.09	108/2/0	0,79 (0.13-4.75)	0.578	0.78 (0.13-4.78)	0.578
	Rs1898830	A/G	0.60/ 0.40	43/59/ 28	0.55/ 0.45	40/48/ 22	0.91 (0.63-1.30)	0.643	0.91 (0.49-1.70)	0,860
TLR4	Rs4986791 (Thr399Ile)	C/T	0.68/ 0.32	122/8/0	0.62/ 0.38	102/8/0	1.18 (0.44-3.22)	0.463	1.10 (0.43-3.23)	0,463
	Rs4986790 (Asp299Gly)	A/G	0.93/ 0.07	111/19/0	0.96/ 0.04	102/8/0	0.35 (0.14-0.81)	**0.017***	0,34 (0.13-0.88)	**0.017***

CI: confidence interval, OR: odds ratio, SNP: single nucleotide polymorphism, TLR: toll like receptor.

correlation between egg-specific IgE level and *Bacteroides* concentration in the gut microbiota in the patient group (p=0.009, r=0.270).

When quantitative values of bacteria within the gut microbiota were compared to the *TLR2* and *TLR4* gene polymorphisms, it was determined that the concentration of *Bifidobacterium* significantly increased in the *TLR4* rs4986791 CT heterozygote genotype (p=0.004) (Table VI).

Discussion

In our study, we found that the concentration of Firmicutes bacteria increased in children with food allergies compared to healthy children. There was a positive correlation between egg-specific IgE level and Bacteroides concentration in the gut microbiota in the patient group. Also, the incidences of *TLR4* rs4986790 polymorphism heterozygote genotype (A/G) significantly increased in children with food allergies. In addition, the *TLR4* rs4986790 variant significantly increased in the group with isolated milk allergy compared to the control group.

Information about the roles of gut microbiota in the development and course of food allergies has been progressively increasing day by day.[18,19] Evidence from studies shows that dysbiosis starts long before the onset of food allergy symptoms and signs. However, a specific type of microorganism responsible for food allergies has not yet been clearly isolated.[5,19] Numerous studies evaluating the relationship between food allergy development and the content of gut microbiota have been published in the literature. In some of these studies comparing children with eczema and developing a food allergy with healthy children, the concentrations of *Streptococcaceae, Lachnospiraceae, Leuconostocaceae, Ruminococcaceae, Clostridium*, Clostridia, *Enterococcus, Lactobacillus, Staphylococcus, Faecalibacterium* (Firmicutes phylum), *Escherichia, Shigella, Enterobacter* (Proteobacteria phylum), Firmicutes, and Bacteroidetes microorganisms have been

Table IV. The relationships of *TLR4* gene polymorphism with patients' demographic, clinical, and laboratory characteristics.

Features	The frequency and distribution for *TLR4* genotype n (%)							
	Rs4986790 (Asp299Gly)				Rs4986791(Thr399lle)			
	AA	AG	GG	p value	CC	CT	TT	p value
Gender								0.23
Boy	76 (58.5)	13 (10)	0	0.98	82 (63.24)	7 (5.3)	0	
Girl	35 (26.9)	6 (4.6)	0		40 (30.7)	1 (0.76)	0	
Complaints								0.36
Eczema	77 (59.2)	10 (7.6)	0		82 (63)	5 (3.8)	0	
Vomiting	11 (8.6)	2 (1.5)	0		13 (10)	0	0	
Cough	7 (5.6)	2 (1.5)	0	0.32	7 (5.3)	2 (1.5)	0	
Infantile asthma	6 (4.6)	2 (1.5)	0		8 (6.44)	0	0	
Other	10 (7.69)	3 (2.30)	0		12 (9.2)	1 (0.76)	0	
Allergy type								0.47
Isolated egg allergy	53 (40.7)	9(6.9)	0		59 (45.3)	3 (2.3)	0	
Isolated milk allergy	22 (16.9)	9(6.9)	0	0.025	26 (20)	5 (3.8)	0	
Multiple food allergy	35 (26.9)	1(0.76)	0		36 (27.6)	0	0	
Type of birth								0.26
Cesarean section	67 (51.5)	12 (9.2)	0	0.87	72 (55.46)	7 (5.38)	0	
Normal delivery	44 (33.8)	7 (5.5)	0		50 (38.4)	1 (0.76)	0	
Gestational age (weeks), n (%)								0.79
≥38 weeks	71 (54.6)	11 (8.4)	0	0.81	78 (60.07)	4 (3.07)	0	
<38 weeks	40 (30.85)	8 (6.15)	0		44 (33.79)	4 (3.07)	0	
Birth weight (grams), n (%)								0.18
≥3500	29 (22.3)	3 (2.3)	0		33 (25.3)	0	0	
3000-3500	41 (31.6)	5 (3.8)	0	0.45	43 (33.26)	2 (1.53)	0	
<3000	41 (31.6)	11 (8.4)	0		46 (35.3)	6 (4.61)	0	
Feeding style, n (%)								0.97
Breast milk	54 (41.5)	9 (6.9)	0		60 (46.1)	4 (3.07)	0	
Breast milk + formula	20 (15.3)	3 (2.3)	0		21 (16.1)	1 (0.76)	0	
Breast milk + formula + cow's milk	15 (11.64)	6 (4.6)	0	0.34	19 (14.6)	2 (1.53)	0	
Breast milk + cow's milk	19 (14.6)	1 (0.76)	0		19 (14.6)	1 (0.76)	0	
Formula	3 (2.4)	0	0		3 (2.48)	0	0	

Table V. Gut microbiota for patients with food allergies, expressed as partial fold increase in comparison to the control group

Microbiota	Partial fold increase in patients with food allergies	p value
Bacteroides	1.11	0.788
Bifidobacterium	0.64	0.171
Firmicutes	4.14	**<0.001***
Fusobacteria	0.99	0.626
Actinobacteria	2.31	0.204
Lactobacillus	1.36	0.985

Table VI. The relationships between gut microbiota and *TLR2*/*TLR4* gene polymorphisms in the patient group with food allergies.

Microbiota	*TLR2*						
	Rs1898830				Rs5743708 (Arg753Gln)		
	Partial fold increase				Partial fold increase		
	AA	AG	GG	p value	AA	AG	p value
Bacteroides	1.04	1.17	1	0.973	1.69	1	0.389
Bifidobacterium	2.87	1.19	1	0.140	1.15	1	0.169
Firmicutes	1.73	1.68	1	0.499	1.10	1	0.937
Fusobacteria	0.20	0.57	1	0.344	2.64	1	0.676
Actinobacteria	0.71	1.40	1	0.871	0.15	1	0.431
Lactobacillus	0.46	0.43	1	0.696	0.12	1	0.335
Microbiota	*TLR4*						
	Rs4986790 (Asp299Gly)			Rs4986791 (Thr399lle)			
	Partial fold increase			Partial fold increase			
	AA	AG	p value	CC	CT	p value	
Bacteroides	1.76	1	0.459	0.25	1	0.229	
Bifidobacterium	1.98	1	0.318	0.05	1	0.004	
Firmicutes	1.02	1	0.961	2.42	1	0.276	
Fusobacteria	2.48	1	0.197	1.70	1	0.781	
Actinobacteria	2.78	1	0.265	0.83	1	0.926	
Lactobacillus	1.76	1	0.459	0.25	1	0.229	

determined to increase.[8,20-24] However, in some other studies comparing children with eczema to healthy children, the concentrations of bacteria such as *Streptococcaceae*, *Oscillibacter*, *Lactococcus*, *Dorea* (Firmicutes phylum); *Enterobacter*, *Citrobacter* (Proteobacteria phylum), *Bifidobacterium* (Actinobacteria phylum), and *Bacteroides* (Bacteroidetes phylum) have been found to decrease in intestinal microbiota of children with eczema.[8,23,25] On the other hand, there are also studies in the literature showing that a statistical relationship was not present between food allergies and gut microbiota.[26,27] Our case-control study analyzed bacteria within the intestinal microbiota at three phylum and three genus levels. We determined that Firmicutes bacteria were increased in children with food allergies compared to the control group. Nylund et al.[25] reported higher levels of Firmicutes species such as *Clostridium* and lower levels of *Bacteroides* species within the gut microbiota in 6-month and 18-month old infants with atopic eczema than in healthy infants. The study of Ling et al.[24] showed that the amounts of *Bacteroides*, Proteobacteria, and Actinobacteria decreased, whereas Firmicutes filum significantly increased in the gut microbiota of children with food allergies. Firmicutes, one of the anaerobic bacteria species, play roles in stimulating the immune system and the inflammatory response. Furthermore, our study determined that *Bifidobacterium* and *Fusobacteria* microorganisms were relatively low, and Actinobacteria and *Lactobacillus* bacteria were relatively high in children with food allergies compared to the control group. However, no statistically significant difference was present between the groups.

In children with food allergies, the intestinal microbiota's composition might be specific to an isolated food allergen. Fazlollahi et al.[22], in their case-control study, reported that *Lachnospiraceae*, *Streptococcaceae*, and *Leuconostocaceae* species significantly increased in amount in the intestinal microbiota of

children with an egg allergy. Berni Canani et al.[23] determined increased concentrations of *Lachnospiraceae* and *Ruminococcaceae* in the gut microbiota of infants with cow's milk allergy. On the other hand, the same study reported a decreased level of Streptococcaceae within the intestinal microbiota. In our study, there was a positive correlation between egg-specific IgE level and *Bacteroides* concentration in the gut microbiota in the patient group. Obtaining different results in microbiome studies in children with food allergies; we think that it may be due to factors such as geographical and racial differences, the age of the child at the time of collection of stool samples, differences in collection and analysis of stool samples, and heterogeneity of food allergies.

The roles of *TLR2* and *TLR4* polymorphisms in food allergies have not yet been clearly investigated. Conducted studies reported that impaired signaling of *TLR2* and *TLR4* in mice caused allergic sensitivity to food proteins.[28,29] Bashir et al.[28] determined that the absence of *TLR4* expression in mice was associated with sensitivity development to food allergy. In a *TLR4* receptor-negative mouse model, intragastric administration of food allergens triggered the allergen-specific IgE release and a high histamine discharge. Moreover, it was shown that when sufficient time was provided for the flora's regeneration, antigen-specific IgE response and allergic symptoms decreased in these mice. Berin et al.[29] evaluated the relationship between TLR4 signal and food allergy, intestinal microbiota. The authors showed that TLR4 could negatively or positively affect allergic reactions according to individual genetic differences and the food antigens' nature and types. Conversely, Galli et al.[30] reported that there was no association between cow's milk allergy and *TLR2*/*TLR4* polymorphisms in children with food allergies. Our study determined that the AG heterozygote genotype and allele prevalence for the *TLR4* gene's rs4986790 SNP of the patient group were significantly increased. Moreover, there were significant relationships of the *TLR4* rs4986790 gene polymorphism in the food allergy group with the number and type of foods diagnosed as food allergy with the oral provocation test. Besides, our study found that, when compared to the other food allergy positivities, the heterozygote genotypes of the *TLR4* rs4986790 and *TLR4* rs4986791 gene polymorphisms were significantly increased in the patients with only milk-positivity in the skin prick test. When *TLR2* and *TLR4* gene polymorphisms were compared regarding quantitative ratios of bacteria in the intestinal microbiota, we determined that only the *Bifidobacterium* ratio significantly increased in the *TLR4* rs4986791 heterozygote variant genotype compared to the wild genotype. In the light of these results, we suggest that the *TLR2* and *TLR4* polymorphisms may lead to a predisposition to food allergy development by affecting TLR expressions and functions. We think that the probable mechanism for the *TLR4* rs4986790 polymorphism's contribution to the pathophysiology of food allergies is stimulating the pro-inflammatory process. Ferwerda et al.[31] demonstrated that the production of TNF-alpha, a potent pro-inflammatory cytokine, increased after stimulating with LPS in mice with the *TLR4* rs4986790 SNP.

Our study has various limitations. First, there was a relatively small number of cases in the study and control groups in our study. In addition, there was a small number of bacteria investigated in the intestinal microbiota and half of the analyzed bacteria were at the phylum level. The studies revealed that performing the analysis at the genus and species level was more critical in food-allergy-related microbiota analysis.[19,32] Secondly, the cases in the study group constituted a heterogeneous group. Some children had allergies to more than one food. The gut microbiota composition and TLR polymorphisms might be specific to an isolated food allergen. Different microorganism species in the gut microbiota might be associated with each food allergy's development.[19] For this reason, investigating and comparing microbial compositions in children with different food allergies continues to be a field deserving

further study. Thirdly, because our study was cross-sectional, we underline that definitive causality cannot be retrieved from the results of our study.

In conclusion, we believe that our study provided some data about potential relationships between food allergies and the bacteria within the intestinal microbiota, TLR2 and TLR4 receptors. When children with food allergies were compared to healthy children, we determined that the concentration of *Firmicutes* bacteria increased, and the A/G genotype of the *TLR4* rs4986790 polymorphism was statistically significantly increased. Because the intestinal microbiota has the potential to be modulated, it provides new research fields to develop innovative strategies for the prevention and treatment of food allergies. Furthermore, studies investigating the composition and functions of the intestinal microbiota and disclosing their relationships with TLR might also enable the evolution of novel treatment models.

Acknowledgements

This study was supported by Scientific Research Projects Coordination Unit of Fırat University, Grant/Award Number: TF.18.37.

Ethical approval

The Ethics Committee of Fırat University approved the study (date June 21, 2018, and decision # 11/04). Moreover, the participants' families were informed about the study, and their written consent was obtained.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: MK, EB; data collection: EB, TK; analysis and interpretation of results: MK, EB, ET, EEO; draft manuscript preparation: MK, EB, ET. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 769-777
https://doi.org/10.24953/turkjped.2022.169

Original Article

Risk factors for and incidence of hospital-acquired infections after cardiac surgery in children with congenital heart disease: a single center experience

Nimet Cındık[1], Mahmut Gökdemir[1], Mehmet Çelik[2], Asım Çağrı Günaydın[2]

[1]Department of Pediatric Cardiology, Konya Practice and Research Center, Başkent University, Konya; [2]Department of Cardiovascular Surgery, Konya Practice and Research Center, Başkent University, Konya, Türkiye.

ABSTRACT

Background. The epidemiology of hospital-acquired infections (HAIs) has been less well studied in critically ill children in pediatric cardiothoracic intensive care units. This study aimed to investigate independent risk factors for and incidence of HAIs after cardiac surgery in children with congenital heart disease (CHD).

Methods. Our study included 574 patients who underwent congenital heart surgery and were followed up in the cardiothoracic intensive care unit between September 2016 and December 2020. All patients were divided into four groups according to age: 0-1 months, 1-6 months, 6-12 months, and 1-18 years, and into two subgroups according to HAI development.

Results. The patients' median age and weight at surgery were 3.28 (interquartile range [IQR]): 0.43-8.1) months and 4.34 (IQR: 4.34-6.69) kg, respectively. HAIs and infection-related deaths were observed in 223 and 21 patients, respectively. Age at surgery, weight at surgery, concomitant syndromes and immunodeficiency status, presence of cyanotic heart disease, intubation, and use of antibiotics during hospitalization were statistically significant between the two groups with and without infection ($p<0.05$). In logistic regression analysis, surgical weight <5 kg (odds ratio [OR]: 2.55; 95% confidence interval [CI]: 1.56-4.17; $p<0.001$), preoperative mechanical ventilation (OR: 2.0; 95% CI: 1.26-3.12; $p=0.003$), complexity of cardiac surgery according to the risk-adjusted congenital heart surgery classification score 3 (OR: 3.13; 95% CI: 1.24-7.92; $p=0.016$), presence of an concomitant syndrome (OR: 1.56; 95% CI: 1.02-2.88; $p=0.040$), age (OR: 1.01; 95% CI: 1.01-1.04; $p=0.044$) were independent risk factors for HAIs after cardiac surgery in children with CHD.

Conclusions. In this study, younger age, presence of an associated syndrome, preoperative mechanical ventilation, and weight less than 5 kg were found to be independent risk factors for HAI after cardiac surgery in children with CHD.

Key words: congenital heart diseases, pediatric cardiac surgery, hospital-acquired infection.

Hospital-acquired infections (HAIs) are a well-defined problem in pediatric and neonatal intensive care units (ICUs). Individual HAI rates are higher in developing countries than in developed countries.[1] However, the epidemiology of HAIs in critically ill children in pediatric cardiothoracic ICUs has been less well studied.[1,2] More than 50% of patients treated in ICUs are affected by HAIs due to severe congenital heart disease (CHD), an immature immune system, and multiple invasive procedures.[3-5] HAIs impair the clinical outcome and may lead to mortality, morbidity, increased treatment costs, and prolonged hospital stay in pediatric patients undergoing cardiac surgery.[3,6] The incidence and severity of HAIs are higher in children after cardiac surgery. This is because impaired nutrition, pulmonary congestion, some forms of CHD with genetic abnormalities, or syndromes leading to some degree of immunosuppression increase susceptibility

✉ Nimet Cındık
nmtcindik@yahoo.com

Received 11th February 2022, revised 2nd May 2023, 21st June 2023, 5th July 2023, accepted 10th July 2023.

to infection.[1,3,4] In developing countries, the severity of HAIs is underestimated, mainly due to the lack of an effective surveillance system that requires expertise and resources.[7]

After cardiac surgery, HAIs in patients with CHD are influenced by several factors. Younger age, longer preoperative hospital stay, longer duration of surgery, open chest, congenital malformations, and postoperative complications are associated with HAIs in pediatric patients undergoing cardiac surgery.[1,6,8] In addition, pediatric cardiothoracic ICUs should identify their risk factors. It is important to identify the risk factors for each unit and take appropriate precautions. Therefore, identifying risk factors for infections in these patients would be beneficial. This study aimed to investigate the incidence of HAIs and their independent risk factors after cardiac surgery with or without cardiopulmonary bypass (CPB) in children with CHD based on age.

Material and Methods

This retrospective cohort study included children with CHD who underwent cardiac surgery and were treated in pediatric cardiovascular ICUs between September 2016 and December 2020. Data were collected from a digital database and immediately entered into detail. This retrospective observational study was approved by the Research Ethics Committee of Başkent University Hospital, and the need for individual informed consent was waived. Patients who underwent surgery and were transferred on the day of surgery, those who died within 24 hours of surgery, and preterm infants after ductal ligation were excluded from this study. All patients were divided into four groups according to age: 0-1 months, 1-6 months, 6-12 months, and 1-18 years, and into two subgroups according to HAI development. Patients were clinically examined daily for signs of HAIs until hospital discharge. Patients were evaluated for signs of infection by determining their daily blood count, C-reactive protein, and procalcitonin. Urine analysis and cultures (blood, urine, tracheal aspirate) were obtained from patients with signs of or suspected HAIs. The Centers for Disease Control and Prevention (CDC) criteria were used as standard definitions for HAIs.[9] Infections occurring 48-72 hours after hospitalization were considered HAIs. Culture specimens were collected only when clinical signs indicated HAIs. Postoperative infections were categorized as hospital-acquired bloodstream or catheter-associated bloodstream infections or sepsis, ventilator-associated pneumonia (VAP), urinary tract or catheter-associated urinary tract infections, and surgical site or wound infections. According to the criteria adopted at the International Sepsis Conference in 2001, the deaths of patients diagnosed with sepsis were considered infection-related deaths.[10] In patients with suspected immunodeficiency, a complete blood count and immunoglobulin levels were analyzed. Lymphocyte subset analysis by flow cytometric examination was performed in patients with lymphopenia and hypogammaglobulinemia to establish the diagnosis of immunodeficiency.[11] The degree of hypothermia was classified as mild (32 °C to 34 °C), moderate (26 °C to 31°C), severe (20 °C to 25 °C) hypothermia. For preoperative antibiotic prophylaxis, cefazolin sodium was administered to some patients, whereas ampicillin-gentamicin, vancomycin, and meropenem were administered to other patients. Antibiotic prophylaxis was administered for a maximum of 3 days, even in patients whose chest tube remained in place for >3 days. Antibiotic prophylaxis was discontinued in most patients on the day of chest tube removal. Culture samples were obtained and analyzed according to standard methods when clinical signs of infection appeared after surgery.

Age and weight at surgery, sex, type of open/closed heart surgery, duration of CPB, complexity of cardiac surgery according to the risk-adjusted classification for congenital heart surgery (RACHS-1)[12], preoperative mechanical ventilation and antibiotic use, concomitant syndromes, immune status, hypothermia classification, and length of hospital stay were considered independent variables for analysis.

Statistical analysis

Variables are presented as means ± standard deviations for continuous variables with normal distribution, medians with interquartile range (IQR) (25th-75th percentiles) for continuous data with abnormal distribution, or numbers and percentages for categorical variables. T-tests or Mann-Whitney U tests for continuous variables and chi-squared or Fisher's exact tests for categorical data were used for comparison between groups. Spearman or Pearson tests were performed for the correlations. Point biserial correlation coefficient was used to determine the relation between numerical variables and dichotomous variables. Multi-variate analysis was performed using logistic regression analysis to determine the independent risk factors for HAIs. All variables with a p-value <0.20 in univariate analysis were included in the multi-variate analysis. Hosmer– Lemeshow goodness-of-fit statistics was used to assess model fit. Statistical significance was set at a p value <0.05. Data input and statistical analyses were performed using IBM SPSS statistical software (version 25.0; IBM Corp. 25.0, Armonk, NY, United States)

Results

The study population was comprised of 574 patients (259 girls and 315 boys). Their median age and weight at surgery were 3.28 (IQR: 0.43-8.1) months and 4.34 (IQR: 3.3–6.69) kg, respectively. Complete correction was performed in 417 (72.6%) patients, and palliative surgery (pulmonary banding, Blalock–Taussig shunt, central shunt, bidirectional cavopulmonary anastomosis) was performed in 157 (27.4%) patients. In total, 72 (12.5%) and 502 (87.5%) patients had univentricular cardiac and biventricular physiologies, respectively. A total of 252 (43.9%) patients had cyanotic heart disease. Cardiac surgery was performed in 457 (79.6%) patients with CPB and in 117 (20.4%) patients without CPB. Patients were classified according to the RACHS-1 classification: RACHS-1 score 1, 53 (9.2%); RACHS-1 score 2, 192 (33.4%); RACHS-1 score 3, 211 (36.8%); RACHS-1 score 4, 110 (19.2%); and RACHS-1 score 6, 8 (1.4%). In total, 146 (25.4%) patients were administered alprostadil before cardiac surgery (121 with cyanotic heart disease and 25 with acyanotic heart disease). A total of 145 (25.3%) patients required mechanical ventilation preoperatively. Moreover, 148 (25.7%) patients (including 14 patients with DiGeorge syndrome; 70 with Down syndrome; one with Turner syndrome; five with trisomy 18, one with 9-12 chromosomal translocation; one with Alagille syndrome; one with Robinow syndrome; one with vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies, and limb abnormalities anomaly; two with Williams syndrome and 52 with unclassified syndromes) were syndromic. In total, 42 (7.3%) patients were diagnosed with immunodeficiency. Postoperative infections and HAI-related deaths were observed in 223 (38.9%) and 21 (9.4%) patients, respectively. Age, CPB duration, duration of aortic cross-clamping, RACHS-1 classification, preoperative mechanical ventilation, preoperative antibiotic use, presence of associated syndrome, presence of immunodeficiency, and length of hospital stay differed between patients with and without infection. HAI-related deaths were observed in 21 (52.4% in <1 month, 42.9% between 1 and 6 months, and 4.76% in >1 year) patients. The demographic characteristics of patients with and without infection are shown in Table I and Table II. Preoperatively, 149 (26%) patients received antibiotic therapy (prophylaxis or treatment).

There was a moderate correlation between the RACHS-1 classification score and CPB duration (r=0.42, p<0.001). In addition, according to point-biserial correlation coefficient, the incidence of infections was found to increase with increasing aortic cross-clamping and CPB duration and RACHS-1classification score and with decreasing weight and age of patients at the time of surgery (Table III).

In our study, 223 (38.9%) patients had HAIs, including hospital-acquired bloodstream or

Table I. Demographic features of the group with and without HAIs (*significant *p* values).

	HAIs (+)		HAIs (-)		*p*
	n	%	n	%	
Patient (female/male)	223 (95/128)	38.9	351 (164/187)	61.1	0.35
Preoperative use of antibiotics	86	38.6	63	17.9	<0.001
Preoperative mechanical ventilation	87	39.0	58	16.5	<0.001
Presence of immune deficiency	26	11.6	16	4.6	0.003
Cyanotic heart disease	122	54.7	130	37.0	<0.001
Absent of thymus	12	5.4	18	5.1	0.71
Alprostadil adminastered	82	36.8	64	18.2	<0.001
Presence of open chest	15	6.7	27	7.7	0.74
Presence of accompanied syndrome	70	31.4	78	22.2	0.019*
Presence of umbilical line	39	17.5	39	11.1	0.033*
Weight at surgery <5 kg	179	80.3	163	46.4	<0.001* *
Type of surgical procedure (Complete correction/palliative)	141/82	63.2	276/75	78.6	<0.001*
Physiology of univentricular heart	34	15.2	38	10.8	0.123
Presence of CPB	165	73.9	292	83.2	0.011*
Ductus dependent heart disease	88	39.4	64	18.2	<0.001*
Classification of hypothermia					0.025*
Normal	67	30.0	80	22.8	
Mild	66	29.6	129	36.8	
Moderate	47	21.0	94	26.8	
Deep	43	19.3	48	13.6	
Classification of age group					<0.001*
<1 month	103	46.2	92	26.2	
1-6 months	85	38.1	116	33.0	
6- 12 months	20	9.0	59	16.8	
>1year	15	6.7	84	23.9	
Classification of RACHS-1					<0.001*
score 1	7	3.1	46	13.1	
score 2	58	26.0	134	38.2	
score 3	107	48.0	104	29.6	
score 4	48	21.5	62	17.7	
score 6	3	1.35	5	0.9	

CPB= cardiopulmonary bypass, HAIs=hospital-acquired infections, n: number of patients, RACHS-1= complexity of cardiac surgery according to the risk-adjusted classification for congenital heart surgery.

catheter-associated bloodstream infections or sepsis in 171 (76.6%), VAP in 12 (5.4%), surgical site or wound infections in 39 (17.4%), and urinary tract or catheter-associated urinary tract infections in one (0.4%) patient. A positive culture was observed in 99 (44.4%) patients. The isolated pathogens are listed in Table IV.

Logistic regression analysis identified surgical weight of <5 kg, preoperative mechanical ventilation, RACHS-1 classification score of 3, presence of a syndrome, and young age as independent risk factors for the development of infection (Table V).

Table II. Demographic features of the group with and without HAIs. Data are expressed as median (25th– 75th percentile).

	HAIs (+) n: 223	HAIs (-) n: 351	*p*
Length of stay in the hospital (day)	35(20-60)	23(7.6-40.5)	<0.001
Duration of CPB (minute)	112 (93-142)	103 (88-145)	<0.001
Duration of aortic clamping (minute)	44 (32-72)	38 (26-68.5)	<0.001

CPB=cardiopulmonary bypass, HAIs=hospital-acquired infections, n: number of patients.

Table III. Evaluation of variables affecting the development of infection with point-biserial correlation coefficient.

	r_{pb}	*p*
Weight (kg)	-0.334	<0.001
Classification of age group	-0.283	<0.001
CPB time, minute	0.265	<0.001
The length of the clamped aorta, minute	0.188	<0.001
Length of stay in the hospital, days	0.557	<0.001
RACHS-1 classification	0.190	<0.001

CPB= cardiopulmonary bypass, RACHS-1= complexity of cardiac surgery according to the risk-adjusted classification for congenital heart surgery, r_{pb}= point-biserial correlation coefficient.

Table IV. Pathogens isolated from the cultures.

	n (%)
Klebsiella pneumoniae	20 (20.2)
Coagulase (-) staphylococci	18 (18.1)
Enterobacter aerogenes	17 (17.1)
Escherichia coli	11 (11.1)
Enterococcus species	9 (9.09)
Staphylococcus aureus	9 (9.09)
Streptococcus species	5 (5.05)
Pseudomonas aeruginosa	3 (3.03)
Acinetobacter species	2 (2.02)
Klebsiella oxytoca	2 (2.02)
Serratia marcescens	1 (1.01)
Morganella morganii	1 (1.01)
Candida non-albicans	1 (1.01)

n: number of patients.

Table V. Independent risk factors for developing HAIs.

Risk factor	Odds ratio (95% confidence interval)	*p*
<5 kg	2.55 (1.56-4.17)	<0.001
Presence of syndrome	1.56 (1.02-2.88)	0.040
Age (months)	1.01 (1.01-1.04)	0.044
Preoperative mechanical ventilation	2.0 (1.26-3.12)	0.003
RACHS-1 score 3	3.13 (1.24-7.92)	0.016

RACHS-1= complexity of cardiac surgery according to the risk-adjusted classification for congenital heart surgery, HAIs=hospital-acquired infections.

Discussion

HAIs are a major cause of morbidity, mortality, and prolonged hospitalization in pediatric cardiac surgery units.[1,6,8] In these patients, incomplete maturation of the immune system, immunosuppression from CPB, multiple invasive procedures, deep hypothermia, and delayed complete enteral nutrition increase the propensity for HAIs, which also increase the length of hospital stay and antibiotic use.[3,4] Therefore, it is important to identify the risk factors in children undergoing cardiac surgery and take the necessary preventive measures to reduce the risk of HAIs. Each cardiology and cardiothoracic ICU should identify the risk factors in children specific to its department and

take the necessary precautions. Accordingly, we studied the risk factors for HAI development in our department. In our study, it was found that the percentage of HAI was 38.9%, which is similar to the percentage in developing countries but higher than in developed countries owing to a smaller age distribution, complex cardiac anomalies, the use of preoperative antibiotics, and preoperative intubation. In a study conducted in developing countries, Sen et al.[13] reported that the incidence of HAIs was 6.9% in their study, which included 28 regions from 17 developing countries. Most patients in their study were aged >1 year (54.1%). Only 6.2% of the patients were aged <1 month. For RACHS-1 classification, 18.6%, 49.8%, and 23% of the patients had RACHS-1 classification scores of 1, 2, and 3, respectively. In contrast, in our study, 82.8% of the patients were aged <1 year, 34% were aged <1 month, and 57.4% had an RACHS-1 score of ≥3. In addition, most patients with severe complex heart disease were followed up in the ICU of an outside center and were referred to our hospital for surgery. As a result of these findings, the fact that our patients were younger and had more complex heart disease increased the incidence of HAIs. The age of the patients was one of the most important factors in our study. The incidence rates of HAIs were 46.2% in patients aged <1 month, 38.1% in those aged between 1 and 6 months, 9% in those aged between 6 and 12 months, and 6.7% in those aged >1 year. The incidence rate of HAIs increased with decreasing age and was higher in children aged <6 months, especially those aged <1 month, than other age groups. Therefore, age is considered one of the most important causes of increased susceptibility to infections.[3,8,14] The lack of trained personnel or new employees is also a major problem for the development of HAIs in developing countries, such as ours.

The incidence rates of HAIs in our study were 3.1% at RACHS-1 score 1, 26% at score 2, 48% at score 3, 21.5% at score 4, and 1.3% at score 6. The incidence rate of infection increases with the increasing complexity of cardiac surgery. According to the RACHS-1 classification, surgery itself is an important risk factor.[1,4,15,16] In our study, cardiac complexity was an independent risk factor for HAIs, and the risk of HAIs increased with increasing complexity; specifically, a threefold increase in this risk was observed with a RACHS-1 score of 3. Yu et al.[8] found similar results in a multicenter study in which increased cardiac complexity was an independent risk factor for HAIs. In addition, CPB duration was longer in the HAI group, which is consistent with the results of other studies.[8,14,16] A possible reason for this can be the risk of bacterial contamination, which increases with longer duration. In addition, it can also be hypothesized that the increased incidence of systemic inflammatory syndrome due to the impaired immune response and prolonged time contributes to an increased risk of HAIs.

There is an increased tendency for infection in immunodeficiency-concomitant syndromes.[17] In some syndromes, such as Down syndrome, the tendency for infection is higher with concomitant immunodeficiency. As expected, having a syndrome was found to be an independent risk factor in these patients owing to immunodeficiencies and developmental disorders.

VAP was observed in 12 (5.4%) patients. It was the second most common form of HAIs after bloodstream infections. In pediatric patients, the VAP incidence rate in the pediatric ICU setting ranged from 3 to 10% in mechanically ventilated children.[18] Preoperative mechanical ventilation was a risk factor in patients who were preoperatively intubated because of their unstable general condition or severe heart failure. Preoperative mechanical ventilation, cardiac surgery, and CPB increased the incidence of VAP and subsequent pulmonary infections. Moreover, CPB and cardiac surgery impair endogenous defense in children. The origin and pathogenesis of VAP remain unclear, although it is likely that VAP is the result of microaspirations rather than bloodstream-associated infiltration of the lungs. Bacterial invasion of the lungs may be directly facilitated

by the endotracheal tube during separation from the ventilatory circuit, as most bacteria found in the endotracheal aspirates of patients with VAP are also found in the nasal and oropharyngeal passages, and even in gastric secretions.[19] The increased incidence of VAP in children after cardiac surgery may be due to several factors, including young age, lower body weight, heart failure, failure to thrive, and poor general condition. The current literature highlights the importance of strategies to prevent VAP, which contributes to prolonged hospital stays, increased costs, mortality, and morbidity.[3,19,20]

The incidence rate of HAIs increased with decreasing patient weight. Weight was an independent risk factor in our study, and an inverse relationship was found between weight and infection. Diet plays an important role in the immune system[21], and low protein status may increase the risk of infection due to low antibody production.[22] An optimal nutritional status is also important for the regulation of inflammatory and oxidative stress processes, all of which are related to the immune system.[23]

Children undergoing heart surgery are placed in a special type of intensive care unit. Many of these patients are infants and neonates who require CPB and invasive procedures. Therefore, pediatric patients undergoing cardiac surgery may have a significantly higher risk of infection. Central lines and invasive monitoring are common in postoperative children with cardiac disease, who are often infants or neonates requiring complex and high-risk cardiac surgery.[24] In our study, a bloodstream or catheter-related bloodstream infection or sepsis was detected in 76.6% of the patients. The combination of younger age and high-risk surgical category in our cohort may have increased this risk. The most common infectious agent in our study was gram-negative bacteria, which also occurred in infections after cardiac surgery.[7,25] The increased prevalence of gram-negative organisms can also be attributed to the current practice of using prophylactic antibiotics that cover mainly Gram-positive organisms in the postoperative period.[26]

As expected, the length of hospital stay was longer in our group of infected patients than in the group without infection. This result is consistent with the results of previous studies and suggests that as the length of hospital stay increases, the tendency to become infected also increases, which directly affects the length of hospital stay. Thus, HAIs are an important factor in prolonging the length of hospital stay.[2,6,24]

Although advancements in surgical experience and intensive care conditions over the years have led to earlier surgery for complex and critically ill patients, the incidence of postoperative infections remains high and multifactorial. Culture positivity is low owing to the high proportion of antibiotics started before surgery. Prevention strategies, such as optimization of surgical procedures, early nutrition, infection control measures, and experienced staff, can reduce the risk of postoperative infection. Although this study has limitations, such as its retrospective, single-center design and small sample size we believe it is important in identifying risk factors for HAI specific to pediatric cardiovascular ICUs. However, further studies are required to identify the contributing factors and prevention strategies. The results of this single-center study cannot be generalized, and a large prospective multicenter study is required to confirm these findings.

Ethical approval

This study was approved by Başkent University Institutional Review Board (Project no: KA21/234).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: NC, MG; data collection: NC, MG, MC, ACG; analysis and interpretation of results: NC, MG; draft manuscript preparation: NC. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 778-788
https://doi.org/10.24953/turkjped.2023.233

Original Article

Sociodemographic and social barriers to early detection of autism

Müslüm Kul[1], Pelin Dağ[1], Berhan Akdağ[2], Mahmut Zabit Kara[3]

[1]*Department of Child and Adolescent Psychiatry, Mersin City Training and Research Hospital, Mersin;* [2]*Department of Child and Adolescent Psychiatry, Silifke State Hospital, Mersin;* [3]*Department of Child and Adolescent Psychiatry, University of Health Sciences, Antalya Training and Research Hospital, Antalya, Türkiye.*

ABSTRACT

Background. The increasing incidence of autism spectrum disorder (ASD) is a common finding of many studies. Early diagnosis and appropriate treatment approaches for ASD can provide favourable clinical outcomes. This study aimed to determine the factors affecting the age at diagnosis, in children with ASD.

Methods. Two hundred and two cases diagnosed with ASD were included in the study, according to the DSM-5 diagnostic criteria, at the Mersin City Training and Research Hospital Child and Adolescent Psychiatry outpatient clinics, between April 2021 and August 2022. Clinical features and sociodemographic data that may be related to early diagnosis were investigated.

Results. The mean age at diagnosis was 36.76 ± 15.30 months. In 71.3% of cases parents were the first to suspect that children were developmentally different. In 38.1% of the cases, at least one of the parents denied the symptoms and evaluated their child's development as age-appropriate. It was found that 32.7% of the cases evaluated by pediatricians and 32.5% of cases evaluated by family physicians, were referred to child psychiatry examination. The present study revealed that higher educational level of the father and the middle-high socioeconomic status, were associated with early diagnosis. There was also a positive correlation between paternal age and age at diagnosis.

Conclusions. The age at diagnosis is below the target level for early diagnosis. Studies should focus on increasing awareness of health professionals and parents about ASD.

Key words: autism, early diagnosis, sociodemographic factors, awareness.

Autism spectrum disorder (ASD) refers to a group of differences that typically appear in early childhood and are characterized by repetitive and restricted patterns in behaviours, interests and activities, as well as difficulties with social interaction and communication.[1] It's reported that the incidence of ASD is increasing all over the world both within society and in the health community, mainly as a result of an increase in awareness of the disorder, increase in the number of diagnostic centers and changes in diagnostic practices, although the increasing paternal age and other factors have not yet been adequately clarified.[2,3] According to the Center for Disease Control and Prevention (CDC), the prevalence of ASD was reported to be 1/160, 1/68, and 1/59 in 2000, 2014, and 2018, respectively, in the United States, and a striking increase in the incidence of ASD has been revealed.[4] This increase in incidence rates shows the high probability of all healthcare professionals, especially physicians, of encountering children with ASD.

The most important issue emphasized in the literature in terms of the prognosis of the disorder is the early diagnosis and the initiation of appropriate educational approaches, without delay.[5-7] The main goals of special education practices that are used effectively in the

✉ Mahmut Zabit Kara
mahmutz.kara@yahoo.com

Received 28th March 2023, revised 25th April 2023, 12th June 2023, 27th July 2023, accepted 28th July 2023.

treatment of ASD, are to reduce core symptoms and increase adaptation skills, in order to enable the individual to reach the upper limit of their own potential. Results from extensive clinical experience show that families begin to notice ASD symptoms after the age of one.

However, in studies conducted in Türkiye, it's known that the age at diagnosis is still far behind the target. The inability to reach early intervention due to delayed diagnosis has a direct negative impact on the clinical severity of the disorder. Although there is no fully accepted time frame for early diagnosis in the literature, 3 years of age is accepted as the age limit for early diagnosis in studies investigating various factors related to early diagnosis and the clinical importance of early intervention.[8-10]

A number of studies have been conducted for early diagnosis, both in Türkiye and in other developed countries, in the hope that the age of diagnosis may be reduced to the lowest level possible.[11,12] In addition to family-related social variables, quality and access to health care are also important for age at diagnosis. Families in our country are evaluated by various health personnel, primarily by pediatricians and family physicians, until they apply to Child and Adolescent Psychiatry Clinics where they are diagnosed with ASD. The rising incidence of the disorder and the ease of access to health services both increase the possibility for an encounter between these patients and health professionals. However, the most significant reason the targeted age for early diagnosis has not yet been reached is the lack of sufficient awareness regarding the issue. Consequently, increasing awareness in healthcare professionals who are likely to encounter children with ASD, will assist to achieve this goal.

In this study, it was aimed to investigate the age of diagnosis and the variables thought to be effective in the diagnosis process. Another aim of the study was to contribute to the early diagnosis of the disorder by raising the awareness among healthcare professionals, who are especially likely to encounter toddlers and preschool children.

Material and Methods

This cross-sectional study was conducted in Mersin City Training and Research Hospital Child and Adolescent Psychiatry Unit, between April 2021 and August 2022. Cases diagnosed with ASD for the first time according to the DSM-5 diagnostic criteria were included in the study. Those who had previously been diagnosed with ASD in another center, those with neurometabolic disease and genetic syndromes, were excluded from the study. The diagnosis of ASD was made after two or more consecutive clinical interviews, and the patients were re-evaluated by a second child and adolescent psychiatrist to confirm the diagnosis.

Sociodemographic and clinic information forms were completed, where the age and gender of the child, concomitant diseases, birth history, family's complaints, family structure, age and educational status of the parents, and previous health care institution applications of the family were collected. The Hollingshead-Redich Scale was used to determine the socioeconomic status of the family, whereby parents were divided into three groups as low, middle, and high. The health facility applications of the patients in the last six months were controlled via electronic information systems, with the approval of the family.

Statistical analyses were performed using the SPSS Statistics for Windows, version 26.0. Variables were tested for normal distribution using the Kolmogorov-Smirnov test. The demographic variables were expressed as mean ± standard deviation, number (n), and percentage (%). The relationship between variables was assessed using Spearman correlation analysis. The Pearson chi-square test or Fisher's exact test were used to compare categorical variables. A p value of less than 0.05 indicated a statistically significant difference.

The approval of the Clinical Research and Ethics Committee of the Mersin City Training and Research Hospital were obtained for the study (03.03.2021/194). Written consents were obtained after detailed information was given about the subject and purpose of the study.

Results

Two hundred and two cases diagnosed with ASD were included in the study; the sociodemographic data of the participants are summarized in Table I. The mean age at diagnosis was 36.76 ± 15.30 months. Of the participants, 165 (81.7%) were male; the clinical characteristics of the cases are summarized in Table II. While mothers came to the interview alone in 114 cases (56.4%), mothers and fathers came together in 88 cases (42.6%). During the diagnostic interviews, the most common reason for referral was speech delay. The complaints of the families at the time of application and their frequency are provided in Table III.

The cases were divided into two groups, as those with an early diagnosis and those with late diagnosis, the cut-off was selected as 36 months. Determination of this cut-off point was based on studies in which the age of early diagnosis was defined and it was also aimed to distribute the number of patients into the two groups in a similar fashion: 117 of the cases (57.9%) were included in the early diagnosis group, while 85 cases (42.1%) were included in the late diagnosis group.

The results of our research reveal that the educational status of the father is associated with early diagnosis. The children of fathers with a secondary education level and below, were diagnosed later than the children of fathers with a higher education level. In addition, it was found that cases in which at least one of their parents denied their symptoms, were diagnosed later than those whose parents did not deny their symptoms. Again, children whose symptoms were noticed for the first time by third parties other than their parents, were also diagnosed late. Early diagnosis was significantly higher in the children of families with a medium-high socioeconomic level, compared to those with a low socioeconomic level. There was no relationship between the gender of the child and the educational status of the mother and early diagnosis. It was determined that variables such as epilepsy, preterm birth, presence of regression in the history and family structure, were not associated with age at diagnosis. Being evaluated by a pediatrician or family doctor in the last six months was also not associated with early diagnosis (Table IV).

Table I. Sociodemographic characteristics of children (n=202) diagnosed with autism spectrum disorder (ASD).

Age of diagnosis (months)	36.76 ± 15.30
Gender	
Male	165 (81.7%)
Female	37 (18.3%)
Maternal age (years)	30.60 ± 5.84
Paternal age (years)	35.20 ± 6.43
Mother's education level	
Primary school and below	86 (42.6%)
Middle school	51 (25.2%)
High school	45 (22.3%)
University	20 (9.9%)
Father's education level	
Primary school and below	47 (23.3%)
Middle school	45 (22.3%)
High school	78 (38.6%)
University	32 (15.8%)
Parental status	
Married	189 (93.6%)
Separated/ Divorced	13 (6.4%)
Family structure	
Nuclear family	166 (82.2%)
Extended family	36 (17.8%)
Caregiver	
Mother	190 (94.0%)
Others	12 (6.0%)
Socioeconomic status	
Low	118 (58.4%)
Medium/ High	84 (41.6%)

Table II. Clinical and diagnostic features of the cases (n=202).

Who came to the diagnostic interviews?	
Mother	114 (56.4%)
Father	2 (1.0%)
Both	86 (42.6%)
Who referred family to the child and adolescent psychiatry clinic?	
Families' decision	118 (58.4%)
Pediatrician	33 (16.3%)
Family physician	28 (13.9%)
Other	23 (11.4%)
Who was the first person to suspect a developmental difference?	
Mother	126 (62.4%)
Father	18 (8.9%)
Other	58 (28.7%)
Has the patient been seen by a pediatrician in the last 6 months?	
Yes	165 (82.7%)
No	37 (17.3%)
Has a child psychiatry evaluation been recommended by the pediatrician?	
Yes	54 (32.7%)
No	111 (67.3%)
Has the patient been seen by the family physician in the last 6 months?	
Yes	151 (74.8%)
No	51 (25.2%)
Has a child psychiatry evaluation been recommended by the family physician?	
Yes	49 (32.5%)
No	102 (67.5%)
History of epilepsy	
Yes	11 (5.4%)
No	191 (94.6%)
History of premature birth	
Yes	23 (11.4%)
No	179 (88.6%)
History of regression	
Yes	47 (23.3%)
No	155 (76.7%)
Do parents (mother and/or father) have symptom denial?	
Yes	77 (38.1%)
No	125 (61.9%)

Table III. Complaints and its frequency in children diagnosed with ASD at time of application.

Speech delay	83.6%
Hyperactivity	43.0%
Inability to establish appropriate social relations with their peers	34.1%
Not responding when called by name	32.6%
Not taking instructions	24.7%
Being strictly attached to the certain routines	22.7%
Interest in rotating objects	20.8%
Turning around	19.3%
Swaying in place	17.3%
Preferring loneliness	15.3%
Inability to make eye contact	13.9%
Inability to play imaginary games	11.4%
Carefree going to strangers	8.9%
Toe walking	6.9%
Sensitivity to high sound	5.9%

In the correlation analysis, there was a positive and significant relationship between paternal age and age at diagnosis (ϱ = .235, p <.001). However, the relationship between maternal age and the age at diagnosis was not significant (ϱ = .079, p = .264) (Table V).

Discussion

In the present study, the mean age at diagnosis was 36.76 ± 15.30 months. A review of the literature, including 42 studies that were conducted between 1990 and 2012, reported that the mean age at diagnosis for ASD ranges from 38 to 120 months and that in recent years, ASD has indeed been diagnosed earlier.[13] A more recent meta-analysis, including studies between 2012 and 2019, also revealed a current mean age at diagnosis of 60.48 (30.90 - 234.57) months.[14] Studies investigating the age at diagnosis of ASD in Türkiye are scarce, although two studies reported a mean age at diagnosis of ASD of 40.70 (12 - 96) months and 32.39 (18 - 48) months, respectively.[15,16] Based on these findings, it can be said that the mean age at diagnosis has not decreased over time, as expected.

Table IV. Variables affecting age at diagnosis.

	<36 months (n)	>36 months (n)	
Gender			
• Female	22	15	
• Male	95	70	p=0.492
Socioeconomic status			
• Low	62	56	
• Medium/ High	55	29	p<0.05
Mother's education level			
• Middle school and below	77	60	
• High school and above	40	25	p=0.473
Father's education level			
• Middle school and below	47	45	
• High school and above	70	40	p<0.05
First to suspect ASD			
• Mother/ Father	97	47	
• Others	20	38	p<0.001
Parental refusal/denial			
• No	81	44	
• Yes	36	41	p<0.01
History of epilepsy			
• No	108	83	
• Yes	9	2	p=0.087
History of premature birth			
• No	105	74	
• Yes	12	11	p=0.553
History of regression			
• No	84	61	
• Yes	29	18	p=0.648
Has the patient been seen by a pediatrician in the last 6 months?			
• No	23	12	
• Yes	94	73	p=0.304
Has a child psychiatry evaluation been recommended by the pediatrician?			
• No	85	63	
• Yes	32	22	p=0.816
Has the patient been seen by the family physician in the last 6 months?			
• No	32	19	
• Yes	85	66	p=0.402
Has a child psychiatry evaluation been recommended by the family physician?			
• No	93	66	
• Yes	24	19	p=0.752

Chi square test

Table V. Relationship between age at diagnosis, maternal age and paternal age.

	1	2	3
1.Age of diagnosis (months)	-	-	-
2. Mother's age (years)	0.079	-	-
3. Father's age (years)	0.235*	0.541**	-

Spearman correlation analysis, *p<0.01, **p<0.001

Socio-familial factors

Socioeconomic status and parental education are known to be associated with age at diagnosis of ASD. Most of the families evaluated in the current study had a low socioeconomic status (SED). It was determined that the children of families with middle and higher SED were diagnosed significantly earlier. These results seem to be in line with some studies that found low household income to be a factor delaying the age at diagnosis[4,17], in contrast to studies that argue that there is no relationship between household income and age at

diagnosis.[18,19] It was thought that this result might be related to easier access to health services for families with medium-high SES. The relationship between the early diagnosis of ASD and the educational status of the parents was examined. It was determined that children of fathers who graduated from high school and university, were diagnosed significantly earlier than children of fathers with secondary school and lower education. This result is consistent with the literature.[20,21] Unlike fathers, there was no significant relationship between the education level of mothers and age at diagnosis. Different results have been reported in the literature regarding the effect of maternal education. In addition to studies that found higher maternal education to be associated with earlier diagnosis[22] and maternal education less than college to be associated with later diagnosis[23], there are also studies that did not find a relationship between maternal education and age at diagnosis.[24-26] The absence of a relationship between maternal education status and age at diagnosis in our study, suggests that mothers may be more sensitive to differences in the development of their children, and this sensitivity is independent of their education. On the other hand, sensitivity of fathers about the development of their children appears to be related to their education.

In our study, we investigated who initially noticed the signs of autism in their children. It was found that it was mothers in 62.4% of the cases, fathers in 8.9% and others (teachers, health care personnel) in 28.7% of cases. This finding was quite understandable considering that in the majority of the study group, the mother was the caregiver (94%). In a study in our country, it was found that those who first noticed ASD symptoms were mostly mothers (52.7%), and only 1.4% of fathers were the first to notice the symptoms.[16] The age at diagnosis was found to be significantly higher when the parents evaluated the symptoms as normal and the symptoms were noticed for the first time by individuals other than the parents. The failure of parents to notice the symptoms may be related to the fact that families do not have sufficient knowledge and experience about the development of their children. Another reason may be related to the severity of ASD. Symptoms of clinically milder cases may be normalized by families, or associated with non-autistic conditions.

In our study, the relationship between parental age and age at diagnosis was also examined. While a positive relationship was found between paternal age and age at diagnosis, no relationship was found between maternal age and age at diagnosis. In the literature, there is no definite data on the relationship between paternal age and age at diagnosis. In addition to studies claiming that older maternal age is associated with early diagnosis[27], there are also studies claiming that there is no relationship

between maternal age and diagnosis.[21,23] Further studies are needed to demonstrate the relationship between parental age and age at diagnosis.

Medical factors

In our study, 23.3% of the families stated that there was a regression accompanied by the loss of acquired skills in their children. These rates were similar to those found in the literature.[28,29] While the presence of regression was found to be associated with early diagnosis in the literature[22,23], there was no relationship between regression and age at diagnosis in our study. These results are provocative considering that the loss of acquired skills can be a serious concern for families and therefore pushes them to seek remedies more quickly. The most important reason for this situation may be that families perceive the regression period as temporary. Another reason may be related to the fact that families describing regression frequently apply to other departments, especially neurology, aside from the Department of Child Psychiatry, and the detailed neurometabolic examinations are time-consuming. Therefore, if deemed necessary it is suggested that conducting the Child Mental Health examinations, together with these examinations, without leaving them for after the neurometabolic examinations, would help prevent diagnostic delays.

Epilepsy was diagnosed in 5.4% of the cases. In other studies, it was reported that epileptic seizures accompany ASD at a rate of 5 to 30%, and this rate is approximately 20 times higher than the normal population.[15,30] The most important reason for the low rate of patients diagnosed with epilepsy in our study may be the exclusion of patients with overt neuromotor retardation and diagnosis of cerebral palsy. The age at diagnosis was similar between the group diagnosed with epilepsy and the group not diagnosed and this result is consistent with the literature.[27] A history of preterm birth was detected in 11.4% of the cases and these rates are also consistent with the literature, reporting that prematurity is one of the environmental factors that cause an increase in the incidence of ASD.[31] Follow-up studies on the relationship between autism and prematurity report that ASD is seen at a rate of 5 to 20% in prematurity, and these rates are 10 to 12 times higher than the prevalence of ASD in the general population.[32] In our study, no difference was found between the age at diagnosis between the ASD group with a history of prematurity and the group without. In the presence of a history of prematurity, it's common for families to experience more anxiety about the development of their children and to apply more readily to health centers. Therefore, the age at diagnosis of ASD may be expected to be earlier in this group. Despite this, the lack of difference may be related to the fact that these families' concerns are often about the physical development of their children and their search for remedies for the difference in their emotional development is more limited. The tendency of families to attribute the difference in the cognitive development of their children to prematurity can also be considered as another factor.

In our study, parents' approach to symptoms during the pre-diagnosis period was also questioned. It was found that in 38% of the patients, at least one of the parents denied the symptoms and insisted that their child's development was normal. The age at diagnosis was found to be significantly higher in this group. Similarly, in the literature, parents' concern about social skills development and worry about initial symptoms, are associated with early diagnosis.[25,33] The reason for this may be that parents cannot see the developmental differences in their children or that they reject the differences in their children, fearing that they will be accused of not raising "good" and "healthy" children. Therefore, persistent denial of the symptoms can be considered as a reflection of defence mechanisms on the part of the parents.

Factors associated with the health care system

Another finding of the study is regarding the examinations of the family physician and

pediatrician in the 6-month period prior to the diagnosis. According to the detailed history and electronic information systems analysis taken from the family, 165 of the children diagnosed with ASD were evaluated by a pediatrician and 151 by a family physician at least once. It was found that 32.7% of the cases evaluated by pediatricians and 32.5% of cases evaluated by family physicians, were referred to a child psychiatry examination. It was determined that some of the families applied for a child psychiatry examination without delay as a result of these referrals, while some of them did not comply with the recommendation. Although our results suggest that the recognition of ASD has increased compared to previous studies, it has been suggested that children with ASD are not yet sufficiently recognized by both physician groups that they frequently encounter. The most important reason for this situation may be that physicians are unable to allocate enough time to patients as a result of the workload they face, often focus on the complaint of the family and cannot find time to make a general evaluation. In fact, it is especially emphasized that the symptoms in children with ASD can only be noticed if sufficient examination times are allocated, otherwise they may be easily overlooked.[34,35] In addition, the lack of sufficient knowledge and experience on ASD was considered as another factor. In the studies conducted in our country, it was stated that the knowledge and skills of health professionals in recognizing autism spectrum disorder were insufficient.[36,37] It's reported that only 19% of children diagnosed with ASD had their autism symptoms noted by the pediatricians who examined them, and as a result, it was concluded that pediatricians displayed insufficient effectiveness in recognizing ASD symptoms and directing them.[16] In a study conducted with family physicians, deficiencies were reported in terms of recognizing and correctly guiding ASD.[38] As of December 2019, 98.8% of family physicians throughout the country were trained by a team of professionals, including child psychiatrists, within the scope of the nationwide program for the early recognition, orientation and follow-up of autism.[39] Considering that our study was conducted after this training program was completed, it can be said that there still remains a need for training activities for family physicians. In many studies, it has been stated that the concerns expressed by parents about the development of their children may lead to a delay in the diagnosis in cases, where the physicians did not adequately and carefully consider them.[36,40-42] When the family structures of children diagnosed with ASD were examined, it was found that 17.8% of them were extended families and 82.2% of them were nuclear families. In our study, it was determined that the family's structure didn't affect the age of diagnosis.

Complaints leading to diagnosis

The complaints expressed by the families of the cases diagnosed with ASD at the time of admission to the outpatient clinic were also compiled. It was hypothesized that knowing which symptoms are frequently expressed by families and which symptoms are less frequent, may be a warning for early diagnosis of ASD, especially for healthcare professionals. In our study, when the complaints of the family were evaluated in general, it was seen that they frequently expressed symptoms such as speech delay and hyperactivity, whereas they expressed autism-specific symptoms at a very low rate in most of the cases. Our most common complaint from the families was speech delay and, in a number of studies conducted with groups with ASD, it has been reported that the main reason for bringing the patient to the doctor is the delay in speaking.[6,26] Therefore, it's imperative to keep a diagnosis of ASD in mind in children with speech delay. The misconception that boys speak late compared to girls and that this is normal among family and some health professionals, causes delays in the diagnosis of ASD in our country. Another complaint frequently expressed by families diagnosed with ASD in our study was hyperactivity. In the

literature, excessive activity (hyperactivity) and its association with ASD have been reported as a very common condition.[43] This complaint, which is not specific for the diagnosis of ASD, is one of the most common reasons for presenting to Child Mental Health outpatient clinics. Easily recognizable symptoms such as not reacting to what is being said, not playing imaginary games, not making eye contact, turning around and rocking, were mentioned at a very low rate by our families. Often, these types of symptoms are interpreted by families incorrectly as "hyperactivity" and this may lead healthcare professionals away from the suspicion of ASD and cause delay in the diagnosis. This finding leads us to believe that the complaint of hyperactivity is perceived as an acceptable symptom for large social segments and is more easily expressed.

Our results reveal that the age at diagnosis of ASD is below the target level for early diagnosis, despite numerous studies shedding light on the topic. We have concluded that efforts should be focused on increasing the sensitivity of parents, especially fathers, on the subject in order to reduce the age at diagnosis in our country. There is a need to increase the awareness and experience of ASD-related healthcare professionals, who are likely to encounter children with ASD, and therefore have a critical role in early diagnosis.

Limitations and strengths

Not using a structured assessment tool for the diagnosis of autism, not evaluating the severity of autism symptoms, and collecting some data based on the family's statement are considered to be the most significant limitations.

The size of the sample, the diagnosis with at least two interviews, the participation of a second child psychiatry specialist in the diagnosis process, the use of screening scales during the diagnosis and the evaluation of all patients with a developmental test, are considered the strengths of the study.

Ethical approval

The study was approved by the Clinical Research and Ethics Committee of the Mersin City Training and Research Hospital, Mersin, Türkiye (Date of Approval: March 3, 2021; Reference number/Protocol No: 194). Written consents were obtained after detailed information was given about the subject and purpose of the study.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: KM, DP, AB, KMZ; data collection: KM, DP; analysis and interpretation of results: DP, AB, KMZ; draft manuscript preparation: KM, DP, AB, KMZ. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 789-800
https://doi.org/10.24953/turkjped.2022.1019

Original Article

Comparison of mothers of adolescents diagnosed with type 1 diabetes mellitus and mothers of healthy adolescents in terms of difficulty in emotion regulation, depression and anxiety levels and clinical variables

Büşra Olcay Öz[1], Dilşad Yıldız Miniksar[2], Şeyma Özge Kaban[1], Şengül Cangür[3], Fatma Yavuzyılmaz[4]

[1]Department of Child and Adolescent Psychiatry, Düzce University Faculty of Medicine, Düzce; [2]Child Psychiatry Clinic, Sağlık Bilimleri University, Dr. Sami Ulus Child Health and Diseases Training and Research Hospital, Ankara; [3]Department of Biostatistics and Medical Informatics, Düzce University Faculty of Medicine, Düzce; [4]Department of Pediatric Endocrinology, Düzce University Faculty of Medicine, Düzce, Türkiye.

ABSTRACT

Background. The aim of our study was to evaluate the difficulty in emotion regulation, depression and anxiety levels of mothers with a child diagnosed with type 1 diabetes mellitus (T1DM) compared to mothers of the non-T1DM control group.

Methods. Our study included 72 adolescents followed up with T1DM and 72 healthy adolescents and their mothers. Psychiatric evaluation of children was performed according to DSM-IV diagnostic criteria. All mothers were administered the "Difficulties in Emotion Regulation Scale-Brief Form (DERS-16)" and the "Hospital Anxiety-Depression Scale (HAD)".

Results. The most common psychiatric diagnoses in the T1DM group were attention deficit and hyperactivity disorder and anxiety disorders. The total and subscale scores of the DERS-16 and HAD scales of the mothers in the T1DM group were significantly higher than the control group. There was a statistically significant positive correlation between the DERS-16 total score and the HAD total and subscale scores of the mothers in the T1DM group. In the multivariate model found to be significant ($p<0.001$), only HbA1c levels an indicator of metabolic control, had significant and negative effects on emotion regulation, anxiety and depression ($p<0.05$), while sociodemographic characteristics did not have a significant effect ($p>0.05$)

Conclusions. Difficulty in emotion regulation and depression-anxiety levels were found to be higher in mothers of adolescents with T1DM compared to the control group. Difficulties in emotion regulation, depression and anxiety symptoms in the parent may reduce the treatment compliance of the adolescent with T1DM, which may result in worse metabolic control. Therefore, both adolescents and their parents should be evaluated in terms of psychiatric symptoms and necessary guidance should be given.

Key words: adolescents, depression, difficulty regulating emotion, mothers, type 1 diabetes mellitus.

Diabetes mellitus (DM) is a common metabolic disease in children. It is characterized by insulin deficiency or the effect of insulin causing hyperglycemia. Type 1 DM (T1DM) is insulin dependent and is usually first diagnosed in childhood or adolescence.[1] T1DM can be potentially life-threatening. As a result, emotional distress and psychiatric disorders may develop in both children and their parents. This is one reason why not only children but parents should also be treated, especially mothers.[2] The scientific literature shows that parents play an important role in developing

✉ Büşra Olcay Öz
drozbusra@gmail.com

Received 12th December 2022, revised 9th April 2023, 26th May 2023, 3rd July 2023, 25th July 2023, accepted 28th July 2023.

self-care and controlling blood sugar in children with diabetes from early diagnosis.[3] A child with T1DM needs care and support that requires constant effort and sacrifice from the family. Therefore, mothers play an important role in managing the health care and treatment regimen of children with T1DM.[4]

The standard parameter used to evaluate glycemic control in patients with DM is glycated hemoglobin (HbA1c). Good glycemic control in patients with type 1DM can prevent complications. In some studies, it has been shown that individuals with poor glycemic control have high levels of intrafamilial conflicts. Hospitalization, intensive medical treatments and the difficulty of treatment management negatively affect family members physically, emotionally and financially. Parents of children and adolescents with T1DM take responsibility for medical care (such as blood glucose monitoring, nutritional management, and insulin production).[5,6] Adolescence is the period when metabolic controls are at their worst in T1DM. In a study, it was shown that HbA1c levels were above the target (>7.5%) in 86% of adolescents with T1DM.[7] This situation develops as a result of many factors such as social influences, increased independence, irregular eating and exercise habits, decreased adherence to treatment, risky behaviors, weight gain and hormonal changes during adolescence. Adolescence-specific changes, as well as chronic disease diagnosis, treatment regimen, and challenging controls cause psychiatric symptoms in both adolescents and family members.[8] A recent meta-analysis showed a 30% prevalence for depression and 32% for anxiety among adolescents with T1DM, based on self-reported symptom severity.[9] Psychiatric problems are also common among the parents of adolescents with this disease.[10,11] Parents experience high levels of anxiety about their child's diagnosis of T1DM and the burden of daily treatment of T1DM. Anxiety is defined as a mental and emotional tension. Mothers of children with T1DM report more anxiety, lower satisfaction and self-confidence in caring for their children than fathers. Mothers feel more responsibilities than fathers in meeting the care and needs of children with chronic diseases.[12] In our study, this situation was effective in our purpose of evaluating mothers with such characteristics.

These psychiatric symptoms that develop in mothers are especially common due to inconsistent eating habits, frequent insulin administration, and the child's inability to recognize hypoglycemia attacks.[13] If adequate psychosocial support is provided to these children, treatment compliance, glycemic control, an increase of quality of life, and a decrease in disease-related complications can be observed.[14]

Difficulty in emotion regulation is defined by not accepting emotional reactions, lack of clarity, difficulty in behaving purposefully, inability to control impulses, lack of awareness, and difficulty in accessing emotion regulation strategies.[15] Difficulty in emotion regulation causes more anxiety and depression symptoms and an increase in internalization problems.[16] In the literature, it is seen that emotion regulation has important relations with anxiety and depression. Difficulty in emotion regulation causes negative emotional states to continue and depressive feelings to continue in individuals. The increase in negative mood and the inability of the person to control their emotions increase the negative affect from the features of major depressive disorder.[17] Living with T1DM can lead to difficulties in the management of emotions as well as psychiatric disorders in both the children who suffer from the disease and their parents. It is assumed that T1DM patients have difficulties in emotion regulation and their emotion regulation skills are weaker than healthy controls.[18] As far as we know, when we examined the literature, no study to date has investigated the difficulty levels of emotion regulation in parents of children with T1DM.

Anxiety and depression in parents of children and adolescents with T1DM often develop

because their child is diagnosed with T1DM. The depression, anxiety and stress experienced by the parents of children and adolescents with T1DM can negatively affect family dynamics and functions. As a result, problems may occur in children's compliance with T1DM treatment. There is no study in the literature investigating the relationship between difficulties in emotion regulation in mothers of adolescents with T1DM and HbA1c in adolescents. The main purpose of our work was therefore to compare the emotion regulation difficulty, depression and anxiety levels between the mothers of adolescents with T1DM and the mothers of healthy controls. Our secondary aim was to determine the relationship between T1DM disease duration and HbA1c levels of adolescents with T1DM and their mothers' emotional regulation difficulties, depression and anxiety symptoms.

Material and Methods

To obtain clinically and statistically significant difference with 5% significance level, 80% power and 0.48 effect size (medium), it was decided to randomly select a total of at least 140 individuals, with equal number of individuals in each group. Thus, our study included 72 adolescents aged 12-17 years who were followed up at the pediatric endocrinology outpatient clinic of Düzce University Hospital with the diagnosis of T1DM for at least one year, and their mothers. Adolescents with clinically normal intelligence and without chronic systemic disease other than T1DM were evaluated. 72 adolescents without T1DM diagnosis and their mothers who applied to the Pediatrics Department for routine check-up were determined as the control group. Siblings of adolescents with T1DM and control group included in the study did not have any chronic systemic disease or psychiatric disorder. All cases in the patient and control groups were evaluated by the child and adolescent psychiatrists who conducted the study. Psychiatric assessment of adolescents was performed using K-SADS-PL (Affective Disorders and Schizophrenia for School-Age Children, Current and Lifetime Version) and according to DSM-IV diagnostic criteria. The depression and anxiety levels of all mothers were evaluated with the "Hospital Anxiety-Depression Scale (HAD)" completed by the mothers, and the difficulty level in emotion regulation was evaluated with the "Difficulties in Emotion Regulation Scale-Brief Form (DERS-16)". T1DM-related variables (duration of disease, type of insulin delivery system [pump or injection]) and the last measured HbA1c level of adolescents with T1DM were recorded. According to the criteria set by the International Society for Pediatric and Adolescent Diabetes (ISPAD), the group with HbA1c values less than 7.5% was evaluated as having good glycemic control, and the group with more than 7.5% was evaluated as having moderate-poor glycemic control. BMI and BMI percentiles for weight and height of all adolescents were within the normal range according to World Health Organization parameters (https://www.who.int/growthref/who2007_bmi_for_age/en/, Accessed September 09, 2018). Adolescents and their mothers were verbally informed about the design of the study. Written informed consent was obtained from both adolescents and their mothers. Ethics committee approval was obtained for the study from the Non-Invasive Health Research Ethics Committee of Düzce University Faculty of Medicine (Decision No: 2022/05, Date: 17.01.2022).

Materials

Schedule for affective disorders and schizophrenia for school aged children, present and lifetime version (K-SADS-PL): K-SADS-PL is a semi-structured diagnostic interview developed to identify psychopathology in children and adolescents aged 6-18 years in accordance with DSM-IV.[19] As a result of the evaluation of the child's or adolescent's and parent's responses, the presence and severity of the symptoms are decided. Turkish validity and reliability were established by Gökler et al.[20]

Difficulties in Emotion Regulation Scale-Brief Form (DERS-16): The DERS-36 scale, developed

by Gratz and Roemer in 2004, consists of questions and 5 sub-dimensions (clarity, nonacceptance, impulse, strategies, and goals). A 16-item short form of the scale was created by Bjureberg et al. in 2016.[21] High scores on this scale indicate greater difficulty in emotion regulation. Turkish validity and reliability studies were performed by Yiğit et al.[22]

Hospital Anxiety Depression Scale (HAD): The scale, developed by Snaith and Zigmond in 1983, consists of 14 items. It was developed to evaluate the level and severity of depression and anxiety in patients.[23] Even number of items in the scale measure depression, odd items measure anxiety symptoms. The scale is a four-likert-type evaluation tool. Items are scored between 0-3. The patients can receive the lowest score of 0 and the highest score of 21 from the depression and anxiety subscales in the scale. The Turkish validity and reliability study of the scale was performed by Aydemir et al. in 1997.[24]

Statistical analysis

Appropriate descriptive statistics were calculated according to the type of variables examined in the study and the type of analysis applied. While quantitative variables were presented as median [Q1: 1st quartile, Q3: 3rd quartile], categorical variables were presented as numbers and percentages. Normality assumption control of quantitative variables was examined using the Shapiro Wilk test. Mann-Whitney U test was used for comparisons between groups. Spearman correlation coefficient was calculated to examine the relationships between the scales. Relationships between categorical variables were examined using the Pearson chi-square test. Although general regression assumptions such as independent observations and linearity of the relationship between dependent and independent variables were provided, the multivariate normality assumption which is one of the important assumptions of multivariate parametric regression analysis could not be provided. Hence, multivariate non-parametric regression (L1 spatial sign) analysis was applied to examine the factors affecting emotional regulation difficulties, depression and anxiety levels of mothers in T1DM group simultaneously. While Mardia's test and pairwise scatter plots were used to control the multivariate normality assumption, scatter plots were generated to check linearity assumption. SPSS 22 and R version 4.1.1 (MNM package) programs were used for statistical evaluations.[25] $p<0.05$ was considered statistically significant.

Results

50% of the 144 adolescents included in the study were T1DM and the other half were the control group. Of the adolescents with a median age of 14 [12-15], 61.1% (n=88) were female and 38.9% (n=56) were male. The groups were homogeneous in terms of gender and age ($p>0.05$). The characteristics of the adolescents according to the groups are given in Table I. In terms of height percentile and BMI percentile value, a significant difference was found between the groups ($p<0.001$ and $p=0.016$, respectively). While the BMI percentile value in the T1DM group was significantly higher than the control group, the opposite finding was obtained for the height percentile value ($p<0.05$ Table I).

54.2% of the adolescents in the T1DM group were treated with injection and 45.8% with a pump. It was determined that 30 (41.7%) of the adolescents with T1DM had a diagnosis of psychiatric disorder. The most common psychiatric disorders were anxiety disorder (15.3%), ADHD (15.3%), adjustment disorder (8.3%) and major depressive disorder (MDD, 5.6%), respectively. No psychiatric disorders were observed in children in the control group. The clinical characteristics of the participants are given in Table II in detail.

DERS-16 and HAD scale scores were obtained from the mothers of the adolescents in each group. In Table III, the descriptive statistics and

Table I. Sociodemographic characteristics of participants according to groups.

		Group									p-value
		T1DM (n=72)			Control (n=72)			Total (n=144)			
		Median	Q1	Q3	Median	Q1	Q3	Median	Q1	Q3	
Age (year)		13	12	15	15	12.5	15.5	14	12	15	0.051
Height persentil		40	15	60	65	50	80	55	30	75	<0.001
Weight persentil		55	30	80	60	35	80	55	32.5	80	0.725
BMI persentil		65	37.5	85	50	25	65	55	34	80	0.016
Gender*	Female	44 (61.1%)			44 (61.1%)			88 (61.1%)			0.999
	Male	28 (38.9%)			28 (38.9%)			56 (38.9%)			

Q1: 1st quartile, Q3: 3rd quartile, *: n (%). T1DM: Type 1 Diabetes Mellitus.

Table II. Distribution of adolescents in the T1DM group according to clinical characteristics.

	n	%
Injection	39	54.2
Insulin infusion pump	33	45.8
Psychiatric Disorders		
Absent	42	58.3
Present	30	41.7
Adjustment disorder	5	6.9
MDD	2	2.8
ADHD	5	6.9
ADHD+Adjustment disorder	1	1.4
ADHD+MDD	1	1.4
ADHD+Anxiety disorders	2	2.8
ADHD+Eating disorders	1	1.4
ADHD+Conduct disorder	1	1.4
Anxiety disorders	9	12.5
OCD	1	1.4
Encopresis	1	1.4
MDD+ Eating disorders	1	1.4
HbA1c level*	8.4 [7-9.4]	
DM Disease Duration (Year)*	3 [2-7]	

*Median [Q1: 1st quartile, Q3: 3rd quartile]. ADHD: attention deficit and hyperactivity disorder, MDD: major depressive disorder, OCD: obsessive compulsive disorder, T1DM: Type 1 Diabetes Mellitus.

comparison results of the total and subscale scores of the scales examined in the study according to the groups are given. There was a significant difference between the groups in terms of total and subscale scores of all the examined scales. Total and subscale scores of DERS-16 and HAD scales were significantly higher than those of the T1DM group compared to the control group ($p<0.05$, Table III).

In Table IV, the relationships between the scores of the mothers of the adolescents in the T1DM group are given from the scales. In the T1DM group, there was a significant positive correlation between the DERS-16 total scale score and HAD total (moderate), anxiety (moderate) and depression (poor) subscale scores. Similarly, a significant positive correlation was found between HAD total scale

Table III. Descriptive statistics and comparison results of the total and subscale scores of the scales according to groups.

	Group						p-value
	T1DM (n=72)			Control (n=72)			
	Median	Q1	Q3	Median	Q1	Q3	
DERS-16 Total	28	23	37	22	20	25	<0.001
Clarity	4	3	4	3	2	4	<0.001
Goals	6	5	8	5	4	6	0.005
Impulse	4.1	3	6	4	3	5	0.011
Strategies	8.5	6	10	6	5	8	<0.001
Nonacceptance	5	4	6	4	3	5	<0.001
HAD Total	14.5	10.5	18	6	3	8	<0.001
HAD Anxiety	8	6	10	4	2	5.5	<0.001
HAD Depression	6	4	9	2	1	4	<0.001

DERS: Difficulties in Emotion Regulation Scale-Brief Form, HAD: Hospital Anxiety Depression Scale, Q1: 1st quartile, Q3: 3rd quartile. T1DM: Type 1 Diabetes Mellitus.

Table IV. Relationships between the scales and subscales examined in the T1DM group.

		DERS-16 Total	HAD Anxiety	HAD Depressio n	HAD Total
HbA1c level	r	-0.19	-0.18	-0.04	-0.12
	p-value	0.117	0.124	0.730	0.330
DM Disease Duration	r	-0.07	-0.10	-0.24	-0.17
	p-value	0.558	0.429	0.046	0.158
HAD Total	r	0.53	0.88	0.79	1
	p-value	<0.001	<0.001	<0.001	-
DERS-16 Total	r	1	0.54	0.35	0.53
	p-value	-	<0.001	0.002	<0.001

DERS: Difficulties in Emotion Regulation Scale-Brief Form, DM: Diabetes Mellitus, HAD: Hospital Anxiety Depression Scale, r: Spearman correlation coefficient.

score and clarity (poor), goals (poor), impulse (poor), strategies (moderate), nonacceptance (moderate) subscale scores of DERS-16, anxiety (very strong), depression (strong). Moreover, there was a significant weak negative correlation between HbA1c level and impulse subscale scores while there was a significant weak negative correlation between DM disease duration and HAD depression scores ($p<0.05$ Table IV). There was no significant correlation between any of the other scales and subscales ($p>0.05$ Table IV).

Table V presents descriptive statistics and comparison results of the total and subscale scores of the scales according to treatment type. There was no significant difference between the treatment type in terms of total and subscale scores of all the examined scales ($p>0.05$ Table V).

The children in the T1DM group were divided into two groups according to their HbA1c levels ≤7.5 (n=23) and >7.5 (n=49). In Table VI, descriptive statistics and comparison results of the total and subscale scores of the scales according to HbA1c group are given. Except for the impulsive subscale score of DERS-16, there was no significant difference between the groups in terms of other scale scores ($p>0.05$). In the group with HbA1c level ≤7.5, the impulse subscale score was significantly higher than those with >7.5 ($p<0.05$ Table VI).

Table V. Descriptive statistics and comparison results of the total and subscale scores of the scales according to treatment type.

	Treatment Type						p-value
	Injection (n=39)			Insulin infusion pump (n=33)			
	Median	Q1	Q3	Median	Q1	Q3	
DERS-16 Total	26	23	39	30	24	36	0.830
Clarity	4	3	4	4	3	4	0.360
Goals	6	4	9	6	5	8	0.681
Impulse	4	3	7	6	4	6	0.093
Strategies	8	6	10	9	6	10	0.860
Nonacceptance	5	4	6	5	4	6	0.679
HAD Total	13	10	20	15	11	17	0.865
HAD Anxiety	8	5	10	8	7	10	0.461
HAD Depression	7	4	10	6	5	8	0.654

Q1: 1st quartile, Q3: 3rd quartile, DERS: Difficulties in Emotion Regulation Scale-Brief Form, HAD: Hospital Anxiety Depression Scale.

Table VI. Descriptive statistics and comparison results of the total and subscale scores of the scales examined in the T1DM group according to HbA1c level.

	HbA1c						p-value
	≤7.5 (n=23)			>7.5 (n=49)			
	Median	Q1	Q3	Median	Q1	Q3	
DERS-16 Total	34	24	39	26	22	34	0.061
Clarity	4	4	5	4	3	4	0.432
Goals	7	6	9	6	5	8	0.145
Impulse	6	4	8	4	3	6	0.008
Strategies	9	6	11	8	6	10	0.414
Nonacceptance	5	4	7	4	3	6	0.053
HAD Total	16	12	20	13	10	17	0.187
HAD Anxiety	9	7	12	7	5	9	0.081
HAD Depression	7	5	9	6	4	8	0.372

Q1: 1st quartile, Q3: 3rd quartile DERS: Difficulties in Emotion Regulation Scale-Brief Form, HAD: Hospital Anxiety Depression Scale.

Table VII. Results of multivariate non-parametric regression analysis showing the effects of sociodemographic and clinical characteristic of children with T1DM on mothers' emotional regulation difficulties, depression and anxiety levels (n=72).

Parameter	DERS-16		*HAD Anxiety*		*HAD Depression*		Parameter
	B	SE	B	SE	B	SE	
(Intercept)	30.242	8.425	10.072	3.501	4.408	3.220	0.001
Age	0.759	0.493	0.135	0.205	0.009	0.188	0.422
HbA1c	-1.238	0.548	-0.476	0.228	-0.013	0.209	0.043
BMI	0.051	0.034	0.028	0.014	0.004	0.013	0.134
Gender*	-2.401	2.013	-1.061	0.836	1.039	0.769	0.112

The model is statistically significant with $\chi 2$= 74.001, df=15, p-value <0.001. *reference: female, B: regression coefficient, BMI: Body Mass Index, DERS: Difficulties in Emotion Regulation Scale-Brief Form, df: degree of freedom, HAD: Hospital Anxiety Depression Scale, SE: standard of error.

The results of the multivariate non-parametric regression model, in which the effects of some sociodemographic (age, gender, BMI) and clinical characteristic (HbA1c) of children with T1DM on the mothers' emotional regulation difficulties, depression and anxiety levels were examined simultaneously, are given in Table VII. In the multivariate model found to be significant ($p<0.001$), only HbA1c level had significant and negative effects on DERS-16, HAD Anxiety, HAD Depression ($p<0.05$), while sociodemographic characteristics did not have a significant effect. Moreover, HbA1c level had the greatest effect on DERS-16 ($p>0.05$ Table VII).

Discussion

In our study, difficulties in emotion regulation and depression-anxiety symptoms in mothers of adolescents with T1DM were evaluated. Secondly, we investigated the relationship between these parameters in children's mothers with HbA1c level, which affects metabolic control in T1DM, and T1DM disease duration. As a result of our study, the rate of psychiatric disorders was found to be high in adolescents with T1DM. These adolescents were found to be most frequently diagnosed with ADHD and anxiety disorders. It was determined that difficulties in emotion regulation, depression and anxiety levels were higher in mothers of adolescents with T1DM compared to the control group. There was a positive correlation between difficulty in emotion regulation and depression and anxiety levels in the T1DM group. There was no statistically significant relationship between the HbA1c level and DM disease duration of adolescents in the T1DM group and the difficulties in emotion regulation, depression-anxiety level of their mothers. No significant correlation was found between depression, anxiety and emotional dysregulation symptoms of mothers and good-moderate-poor glycemic control of T1DM children and adolescents. There was no significant relationship between the depression, anxiety and emotional dysregulation symptoms of mothers and the injection and pump methods used in the treatment of T1DM children and adolescents with T1DM. In the literature, there are studies evaluating depression and anxiety symptoms in mothers of children with T1DM, but no study evaluating difficulties in emotion regulation has been found. Our study evaluated the difficulties in emotion regulation, depression and anxiety symptoms in mothers of adolescents with T1DM. In the results of the multivariate non-parametric regression model, only HbA1c level was found to have significant negative effects on DERS-16, HAD Anxiety, HAD Depression, also HbA1c level had the greatest effect on DERS-16.

Compared to the general population, the incidence of psychiatric disorders is 2-3 times higher in children and adolescents with T1DM.[26] Fear, anxiety, anger, panic attacks and unhappiness are common in many adolescents after T1DM diagnosis.[27] Studies have reported that the most common psychiatric disorders in adolescents with DM are anxiety disorders, depression, ADHD, and eating disorders.[9,26,28] In a study, it was shown that the current and lifetime prevalence of psychiatric disorders in children with T1DM was 27% and 32%, respectively. In this study, it was shown that anxiety disorders, mood disorders and behavioral disorders are more common.[29] It has been reported that the most common psychiatric isorders in adolescent diabetics are anxiety, depression and eating disorders.[27] Similarly, in our study, the rate of psychiatric disorders in adolescents with T1DM was found to be higher than in the control group. Adolescents in the T1DM group were most frequently diagnosed with anxiety disorders, ADHD, adjustment disorder, depression and eating disorder. Additionally, it was determined that adjustment disorder and MDD, respectively were also prevalent.

Studies evaluating depression and anxiety symptoms in mothers of children with T1DM have reported different results. Many studies have shown that parents of children with T1DM

have higher depression and anxiety symptoms than parents of healthy children.[30-32] Another study investigating the anxiety levels of parents of children with T1DM showed that parents are vulnerable to high anxiety symptoms.[33] These studies included both parents of all participants. Another study reported that mothers of children with T1DM had high levels of anxiety symptoms.[34] In another study, no difference was found in terms of anxiety level in others of children with T1DM compared to the control group.[35] As a result of our study, we found that mothers of adolescents with T1DM had higher levels of depression and anxiety symptoms compared to the control group. We showed that there is a significant positive correlation between anxiety level and depression level. Our results support the literature in this respect. The presence of depression and anxiety symptoms in parents have a positive and significant effect on the depression and anxiety symptoms of their children with T1DM.

Parents of children with T1DM have many responsibilities, such as regular monitoring of blood sugar levels, administration of insulin therapy, physical activity, and appropriate diet regulation to prevent episodes of hypoglycemia and hyperglycemia. However, psychiatric problems that develop in parents can prevent parents from completing such responsibilities.[12] It has been shown that maternal anxiety and depression symptoms are associated with many negative outcomes in adolescents, including worsening glycemic control, lower quality of life, and depressive symptoms.[31,36] Therefore, mothers of adolescents with T1DM need coping strategies for diabetes-related depression and anxiety symptoms.

Difficulties in emotion regulation have been shown to be associated with psychiatric disorders. In particular, they increase the symptoms of depression and anxiety in individuals.[37,38] In our study, it was found that mothers in the T1DM group had more difficulties in emotion regulation. At the same time, it was determined that mothers who had difficulty in emotion regulation had higher depression and anxiety levels. Our results support the literature in this respect. However, no study has so far investigated the symptoms of difficulties in emotion regulation in parents of children with T1DM. Therefore, our study is valuable in terms of literature.

In a study, it was reported that mothers of children with T1DM had high levels of anxiety symptoms, but no relationship was found between the level of anxiety and the child's metabolic control.[34] In our study, no significant relationship was found between the HbA1c value, which is an indicator of metabolic control in adolescents with T1DM, and their mothers' depression-anxiety level and symptoms of difficulty in emotion regulation. There is a need for more comprehensive studies evaluating this situation.

The level of psychosocial support offered to parents of children and adolescents with T1DM is critical for their long-term coping skills. With adequate psychosocial support available, knowledge and trust can be built. This results in greater adherence to treatment, better glycemic control, better perception of overall quality of life, and reduced complications.

Evaluation of psychosocial support is important in the treatment of T1DM. Comprehensive treatment should include psychiatric problems in both the child and their parents to ensure well-being and prevent the development of complications. To create comprehensive programs that have an impact on individuals, the treatment team must include both child and adult mental health professionals. Today, diabetic patients lack supportive mental health services. Psychosocial support is necessary to create a normal life and a healthy environment at home, to have ideal physical development as well as emotional and cognitive maturity.

The limitations of our study are that the sample size is small, hyperglycemia, hypoglycemia and ketoacidosis attacks in adolescents with T1DM were not questioned, the sociodemographic

characteristics of the mothers were not evaluated, and the psychiatric histories of the mothers and adolescents before the diagnosis of T1DM were not known.

In conclusion, we showed that children and adolescents with T1DM have more psychiatric disorders than the healthy control group. In addition, we found that the mothers of children with T1DM had higher symptoms of depression, anxiety, and difficulty in emotion regulation. Depression and anxiety symptoms in the parent may decrease the treatment compliance of the adolescent with T1DM, which may result in worse metabolic control. Even if adolescents do not show signs of depression, parents should be evaluated for signs of depression and necessary guidance should be given. Large-scale longitudinal studies are needed to investigate difficulties in emotion regulation, depression and anxiety symptoms in parents of T1DM patients, and to clarify this causal relationship with the characteristics of the disease in their children. Considering the cross-sectional characteristics of our study, it is not possible to evaluate causality, so data analysis should be considered with care. Further large-scale studies are needed to establish causality.

Acknowledgements

We are grateful to all the children and their mothers who participated in the study.

Ethical approval

Ethics committee approval was obtained for the study from the Non-Invasive Health Research Ethics Committee of Düzce University Faculty of Medicine (Decision No: 2022/05, Date: 17.01.2022).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BÖ, DYM; data collection: ŞÖK, FY, BÖ, DYM; analysis and interpretation of results: ŞC, BÖ, ŞÖK; draft manuscript preparation: BÖ, ŞC. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 801-808
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Original Article

The etiologies and management of spinal cord compression in childhood cancers: Are we aware of the emergency of cord compression?

Arzu Okur, Özge Vural, Faruk Güçlü Pınarlı

Division of Pediatric Oncology, Department of Pediatrics, Gazi University School of Medicine, Ankara, Türkiye.

ABSTRACT

Background. The spinal cord compression causes irreversible long-term permanent neurological sequelae. This study aims to increase awareness of childhood cancers that cause cord compression by comparing histopathological diagnosis, treatments, and survival rates to the literature.

Methods. Seventy-three patients (38 male, 35 female) with spinal cord compression, among 1085 patients diagnosed with solid tumors at Gazi University Department of Pediatric Oncology between 1991 and 2021 were retrospectively evaluated.

Results. The mean time between the onset of complaints and diagnosis was 27.5± 24.9 (2-150) days. The first three most common tumors that caused cord compression; were central nervous system tumors in 22 (30%), neuroblastoma in 17 (23%), and malignant germ cell tumors in 8 (10%) cases. Of the patients, 46 (63%) had compression due to extradural masses, and 27 (37%) patients had an intradural compression. The most common symptoms were pain in 60 (82%), weakness in 57 (78%), and pins and needles in 28 (38%) patients, respectively. The clinical physical neurological examination findings were motor deficit in 62 (84%), and deep tendon reflex changes in 54 patients (73.9%). Compression findings were detected in 58 (79.5%) patients at diagnosis, and in 15 (20.5%) of them during follow-up. The most common level of compression was seen in the thoracolumbar region in 19 (26%) cases. In 65 (89%) patients with cord compression, corticosteroids were given as anti-edema treatment. Surgical excision was performed in 39 (53%) patients. Spinal radiotherapy was given to 35 patients (48%) with radiosensitive tumors. Chemotherapy protocols were started in 52 (71.2%) cases according to their diagnoses. Complete neurological recovery was achieved in 33 (45%) patients. The 5-year overall survival rates for solid tumors with extradural compression and intradural compression were 62% and 22%, respectively (p=0.002).

Conclusions. Neurological sequela-free recovery is possible with early diagnosis and urgent treatment. Spinal compression must be detected by detailed systemic and neurological examination and imaging methods. Patients should be rapidly transferred to pediatric oncology units after starting anti-edema treatment.

Key words: spinal cord compression, childhood cancers.

The spinal cord, conus medullaris or cauda equina compressions impair quality of life and cause irreversible, long-term, permanent neurological sequelae. The spinal cord may be compressed by a mass in the epidural space (extradural), or by metastatic spread to the cord parenchyma (intradural).[1,2] Although the incidence of acute cord compression may vary depending on the histopathology of the tumor, findings of cord compression occur in approximately 4-5% of childhood cancers at diagnosis.[1] While these rates in neuroblastoma, and in Hodgkin lymphoma were 9%, and 2% respectively, it was reported as 11-43% in medulloblastoma, at diagnosis.[1,3,4] In childhood

✉ Arzu Okur
arzu-okur@hotmail.com

Received 3rd April 2023, revised 7th July 2023, 27th August 2023, accepted 30th August 2023.

cancers, patients may only present with symptoms of cord compression.

Back pain is the most common presenting sign of spinal cord compression. Neurologic findings may vary depending on the spinal level of the lesion and the degree of compression. Weakness, ataxia, gait disturbance, and paraplegia can be observed. Sphincter dysfunction is seen most commonly as urinary retention or constipation. The localization of the level of epidural cord compression along the spine is determined by the specific effects on extremity strength, tendon reflexes, and sensory levels.[5-7] Treatment options for a patient with spinal cord compression include surgery (laminectomy or laminotomy), chemotherapy, anti-edema (steroid) therapy, and radiation therapy.[7]

The histopathology of the tumor is important in the choice of treatment for tumor-related cord compression. However, early and immediate interventions are essential, as is histopathology for complete recovery without neurological sequelae. Since most childhood malignant tumors are chemosensitive, complete neurological recovery can be achieved with rapid and appropriate treatment of the malignancy in pediatric cancer patients.[5,8]

This study aims to increase awareness of childhood cancers that cause cord compression by comparing histopathological diagnosis, treatment results, and survival rates to the literature.

Material and Methods

The clinical and demographic features, pathological characteristics, treatment modalities, survival rates, and functional neurological outcomes of pediatric patients who developed cord compression due to solid tumors at Gazi University, Department of Pediatric Oncology between 1991-2021 were evaluated retrospectively. This study was approved by the Ethics Committee of Gazi University (No. 2017120103-2).

Statistical analysis

Quantitative data were represented by mean ± standard deviation. Percentages described qualitative data and the comparison of these data was performed using the chi-square test. Patient and disease characteristics were compared between groups using Mann-Whitney U and chi-square/Fisher tests for numerical and categorical variables, respectively. Kaplan-Meier survival analysis was used to assess the median survival probability. Patient groups were compared in terms of survival duration using a log-rank test. All analyses were performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA), and $p<0.05$ was considered statistically significant.

Results

We retrospectively evaluated 73 patients (38 male, 35 female) with spinal cord compression out of 1085 patients diagnosed with solid tumors at Gazi University Department of Pediatric Oncology between 1991 and 2021. Cord compression was present in 6.7% of all childhood malignant tumors at our pediatric oncology unit. Patients with leukemia were not included in this study. The demographic findings of the patients including gender, age at diagnosis, duration of symptoms, and follow-up periods have been shown in Table I.

Table I. Distribution of patients by demographic findings.

		Patients (n:73)
Gender	Male	38 (52.1%)
	Female	35 (47.9%)
Age (years), mean ± SD (range)		4.92 ± 4.46 (0.31-16.27)
Symptom duration (days), mean ± SD (range)		27.5 ± 24.9 (2-150)
Follow-up period (years), mean ± SD (range)		3.26 ± 4.90 (0.1-22)

SD: standard deviation

In this study, 46 (63.1%) patients had extradural, and 27 (36.9%) patients had intradural cord compression. Spinal cord compression was observed in 58 (79.5%) patients at diagnosis and in 15 (20.5%) patients at relapse or progression. Twenty-two patients with intradural compression had central nervous system (CNS) tumors (medulloblastoma, astrocytoma, ependymoma), and 12 of them developed cord compression in progression or relapse. The histopathological diagnosis of patients and levels of cord compression are shown in Table II. The most common symptoms of the patients were pain in 60 (82%), weakness in 57 (78%), pins and needles in the extremities in 28 (38%), and urinary retention or constipation in 8 (10.9%) cases. The neurological examination findings were motor deficit (paralysis or plegia) in 62 (84%), deep tendon reflex changes or presence of pathological reflex in 54 (73.9%), sensory deficit in 13 (17%), sphincter dysfunction in 8 (10.9%), and tilt in 2 (2.7%) of the patients.

Compression findings of all patients were demonstrated by spinal magnetic resonance imaging (MRI) or vertebral computed tomography. Treatment modalities such as corticosteroids, chemotherapy, radiotherapy, and surgery were applied according to the diagnosis and clinical features of the patients. Approximately 90% of the patients received dexamethasone as an anti-edema treatment. Fifty-two patients (71%) promptly received chemotherapy protocols appropriate for their histopathological diagnosis. Radiotherapy was given to the spinal compression area of 35 patients who showed progression in neurological findings under chemotherapy. As a diagnostic surgical intervention, primary decompressive tumor excision and laminectomy were performed in 26 patients. The treatment modalities applied to the patients are listed in Table III.

When the neurological findings of 73 patients were evaluated after treatment, complete neurological remission was achieved in only 33 (45.2%) patients (Table IV).

Table II. Clinical features of malignant spinal cord compression.

Histopathological diagnosis	Number (%)
Central nervous system tumors	22 (30.1)
• Medulloblastoma	14 (19.1)
• Astrocytoma	6 (8.2)
• Ependymoma	2 (2.7)
Neuroblastoma	17 (23.2)
Malignant germ cell tumor	8 (10.9)
Ewing sarcoma/PNET	7 (9.5)
Langerhans cell histiocytosis	6 (8.2)
Rhabdomyosarcoma	6 (8.2)
Primary spinal cord tumors	5 (6.8)
Burkitt lymphoma	2 (2.7)
Levels of spinal cord compression	
• Cervical	9 (12.3)
• Cervicothoracic	12 (16.4)
• Thorax	18 (24.6)
• Thoracolumbar	19 (26.0)
• Lumbar-sacral	15 (20.5)

PNET: primitive neuroectodermal tumor

Table III. The treatment modalities applied to the patients.

Treatment	Number (%)
Corticosteroid	65 (89.0)
Chemotherapy	52 (71.2)
Surgery	39 (53.4)
• Primary decompressive tumor excision + laminectomy	26 (35.6)
- Complete	13
- Partial	9
- Biopsy	4
• Delayed surgery after chemotherapy	11
• Laminotomy	2
Radiotherapy	35 (47.9)

Table IV. Functional outcomes of 73 patients with malignant spinal cord compression.

Functional Outcomes	Number (%)
Neurological complete recovery	33
Motor deficit	
• Unable to walk	15
• Walking with support	15
Spinal deformity	6
Sphincter dysfunction	4

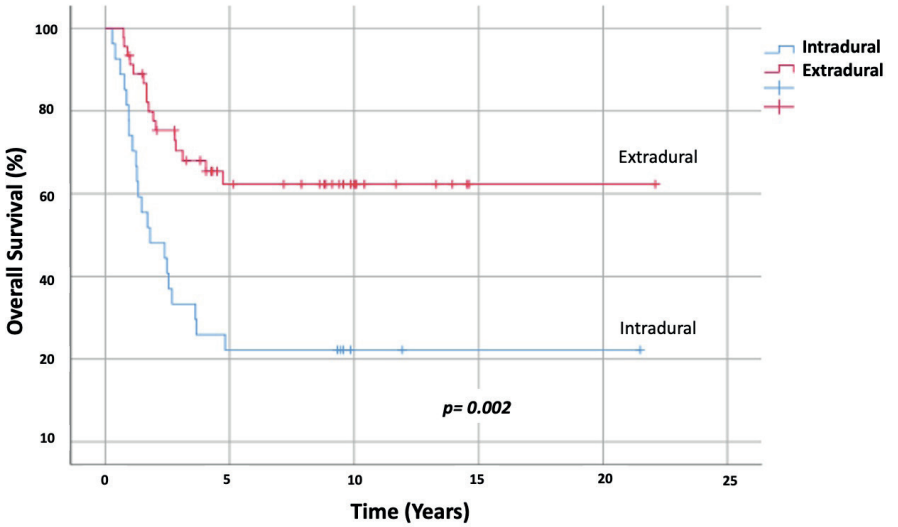

Fig. 1. The 5-year overall survival rates for patients with extradural compression and intradural compression.

While primary CNS tumors (22 patients) were the most common tumor with intradural cord compression, neuroblastoma (17 patients) was the most common solid tumor with extradural cord compression. Of the patients with neuroblastoma, 10 out of 17 were disease-free and in remission, and 7 out of the 10 patients had a complete neurological recovery. Disease-free remission and complete neurological recovery were achieved in 21 out of 46 patients with extradural cord compression. But most of the patients (18) with intradural compression died due to the rapid progression of the disease. When the survival analyses of all 73 patients in this study were evaluated, the 5-year overall survival rates for extradural compression and intradural compression were 62% and 22%, respectively (Fig. 1). Survival rates were significantly higher in patients with extradural tumors of spinal cord compression (p=0.002).

Discussion

The spinal cord, conus medullaris or cauda equina compressions impair quality of life and cause irreversible, long-term, permanent neurological sequelae. Tumor compression on the vertebral venous plexus causes vasogenic cord edema, venous hemorrhage, and ischemia, resulting in tissue damage and neurological deficit.[1,2] Cord compression is classified as extradural or intradural.[9,10] Extradural compression occurs due to the extension of paravertebral tumors through the intervertebral foramina. Extradural masses in children are usually caused by tumors such as neuroblastoma, Ewing sarcoma, rhabdomyosarcoma, lymphomas, germ cell tumors, and Langerhans cell histiocytosis.[11-13] Neuroblastoma is the most common extracranial solid tumor in childhood. It has been reported in various studies in the literature that approximately 10% of neuroblastoma cases have findings of compression at the time of diagnosis.[3,8,14] Seventeen (20%) out of 85 patients with neuroblastoma diagnosed in our center had neurological findings related to epidural compression. Neuroblastoma cases with compression findings were at a higher rate than in the literature in our group. We believe that it is important to raise the awareness of neurologic findings in childhood solid tumors in our country. De Martino et al.[2] reported that the leading cause of cord compression at the time of diagnosis was extradural tumors most commonly neuroblastoma (27.2%) followed by Ewing sarcomas (15.9%). As in the literature, the most common extradural compression tumors were neuroblastoma (23%), germ cell

tumor (11%), and Ewing sarcoma (10%) in the present series. Primary CNS tumors usually cause intradural compression through dropped metastases.[15-17] Primary CNS tumors were the most common malignancy in our series, probably due to being one of the referral centers for pediatric neurosurgery in the country. As primary high-grade CNS tumors with spinal metastases have a poor prognosis, 18 out of 22 patients with CNS tumors died due to the progression of the disease in this study.

Back pain or extremity pain due to tumor compression are usually the first and most prominent symptoms. Pain may be accompanied by motor and/or sensory deficits according to the compression area.[9,18] In the literature, Gunes et al.[11] reported that the most common symptom was pain (64%), and the most common finding on physical examination was motor deficit (53%) in 28 children with paravertebral malignant tumors. In our study, approximately 80% of patients had complaints of pain, motor deficits (paralysis or plegia), and deep tendon reflex changes at diagnosis. The high rate of neurological deficits was presumably due to delays in referral to pediatric oncology centers. Pediatric and/or non-pediatric physicians (orthopedics/neurosurgery/pediatric surgery) should be aware that all children experiencing back and/or extremity pain should have a thorough neurological and systemic assessment. Neurological findings of tilt symptoms, which were also present in two of our patients, in cervical spinal masses should be recognized by physicians. It is not easy for the family to notice the swelling in the sacrococcygeal region for germ cell tumors. We would also like to emphasize that it is important to remove the diapers of infants to perform a detailed examination. Since cord compression may be a finding in acute leukemia/lymphomas, the presence of hepatosplenomegaly, all regional lymph node examinations, and laboratory tests are important.[19,20] It must be known that a detailed evaluation is the cornerstone of the diagnosis.

Magnetic resonance imaging (MRI) is known as the most effective imaging method for the spinal cord and canal and also provides information about the origin of the tumor.[21,22] MRI was performed on almost all of our patients; however, computerized tomography was used when emergency MRI with anesthesia was not possible and /or before the 2000s in our center. We found the most common spinal cord compression in the thoracic region. It is important to note that an MRI should be done within 24-48 hours as irreversible permanent neurological sequelae may develop in children.

Treatment options for a patient presenting with spinal cord compression include anti-edema (steroid) therapy and tumor specific therapy like surgery, chemotherapy, and radiation therapy.[1,5,11] Although there is no clear information about the superiority of these treatment methods over each other, a multidisciplinary approach may be required depending on the type of tumor.[9,22,23] The histopathological diagnosis of the tumor is the determinant in the selection and success of the treatment.[14,24] High-dose dexamethasone reduces vasogenic cord edema and provides neurological improvement. In our series, approximately 90% of the patients were treated with steroids as anti-edema therapy. Since the patients had chemosensitive and high-grade tumors in this study, approximately 70% of the patients received chemotherapy according to the histopathological diagnosis and stage. Surgery may be required according to the histopathological diagnosis and/or progression of neurological findings despite chemotherapy and dexamethasone treatment for cord compression.[10,22,25] Surgery was performed in 39 patients in our study, and decompressive surgery and laminectomy (35%) were most commonly performed due to poor neurological clinical course. If the tumor is radioresponsive, radiation therapy is often the choice of treatment.[16,23] In the literature, 180-400cGy radiotherapy is recommended in radiosensitive tumors to provide spinal decompression.[9,16]

But we could not evaluate the dose or late sequels of radiotherapy given for compression due to the non-specificity of the study group. Because spinal radiotherapy was given to 35 (48%) patients according to whether the patients had previously received spinal radiotherapy, histopathology of the tumor, and progression of neurological findings in our series. Even though 22 patients with CNS tumors with spinal metastases received radiotherapy, 18 of them died due to the rapid progression of the disease. Spinal cord surgery and radiotherapy could cause serious growth problems, late side effects, and orthopedic spine defects in children, and 6 patients in our study had to use orthotic devices owing to spinal deformities. As our patients had wide heterogeneity in diagnosis, histologic classification, stage, treatment methods, and prognoses, we could not determine the best treatment. Moreover, spinal cord compression treatment requires a multidisciplinary approach in childhood cancers.

We found that the mean time to diagnosis was 27 days, but the duration of symptoms before diagnosis prolonged to 150 days in this study. Complete neurological recovery was achieved in 33 (45%) patients in the present series of patients. Tantawy et al.[25] found that neuroblastoma (29.2%) was the most common cause of malignant cord compression and reported that the time from symptom to diagnosis was 42 days and 75% of the patients had complete neurological improvements. They observed a high neurological recovery rate despite the delayed diagnosis due to their patients having chemosensitive tumors such as neuroblastoma and lymphoma. Furthermore, Kurucu et al.[12] reported that complete and partial recovery of neurologic deficits was achieved in 56% of patients with lymphomas in the literature. Unfortunately, we found that clinical neurologic sequelae were permanent in 55% of our patients. The high rate of permanent sequelae can be explained by the delay in diagnosis as well as the heterogeneity of histologic classification. One-third of the patients with high-grade CNS tumors were resistant to combined treatment, and also these patients had spinal metastases.

In our series, the mean follow-up period of the patients was 3.26±4.90 years (0.1-22 years). The 5-year overall survival rates for high-grade CNS tumors with intradural compression, and solid tumors with extradural compression were 22%, and 62%, respectively. Survival rates were similar to the literature in spinal cord compression due to extradural tumors.[18,25] But, the low survival rate of tumors with intradural compression might be explained by the high rate of metastatic CNS tumors in this series.

The spinal cord compression is an oncological emergency as irreversible permanent sequelae may develop. Physicians need to be made aware of symptoms owing to the important of early diagnosis and the effectiveness of urgent anti-edema treatment in cord compression.

The most important factors affecting the success of treatment for cord compression are early diagnosis and urgent treatment. It must be emphasized that back pain and extremity pain should be considered symptoms of cord compression. For this reason, every physician should perform a detailed systemic and neurological examination. In this study, we aimed to emphasize that delay in diagnosis or failure to manage or recognize symptoms may be important risk factors for permanent neurological sequelae. However, studies which are prospective, multicenter, and include patients with the same histological diagnosis are necessary to determine the most important prognostic factor and treatment.

Ethical approval

Gazi University Ethics Committee approved the study (report number: 2017120103-2).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AO, FGP; data collection: AO, ÖV; analysis and interpretation of results: AO, ÖV, FGP; draft manuscript preparation: ÖV. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 809-821
https://doi.org/10.24953/turkjped.2023.469

Original Article

The effect of granulocyte colony stimulating factor on genotoxicity in allogeneic peripheral blood stem cell transplantation donors: a prospective case-control study

Hasan Fatih Çakmaklı[1], Hafize Gökçe[1], Esma Söylemez[2], Pervin Topçuoğlu[3], Zeliha Kayaaltı[4], Dilber Talia İleri[1]

[1]Department of Pediatric Hematology, Ankara University Faculty of Medicine, Ankara; [2]Department of Pharmaceutical Toxicology, İstanbul Medipol University School of Pharmacy, İstanbul; [3]Department of Hematology, Ankara University Faculty of Medicine, Ankara; [4]Institute of Forensic Sciences, Ankara University, Ankara, Türkiye.

ABSTRACT

Background. Every year, thousands of donors are exposed to granulocyte-colony stimulating factor (G-CSF) for stem cell mobilization in hematopoietic stem cell transplantations (HSCT). Previous studies about the genotoxicity of G-CSF were inconclusive. In this study, the genotoxic effects of G-CSF in peripheral blood stem cell (PBSC) donors were evaluated prospectively by using three different validated and reliable methods for the first time in the literature to the best of our knowledge.

Methods. Donors of PBSC transplantation (n=36), who received G-CSF were evaluated for genotoxicity by micronucleus test (MNT), nuclear division index (NDI), and comet assay (CA). Genotoxic effects are expected to cause an increase in MNT and CA values and decrease in NDI. Blood samples were collected at three time-points (TP): before starting G-CSF (TP1), after G-CSF for five days (TP2), and one month after the last dose (TP3). Sixteen controls were included for baseline comparison of genotoxicity tests. CD34 cell counts and hemograms were also analyzed.

Results. MNT and CA parameters; comet and tail length, tail DNA%, and tail moment, showed no change in time whereas another CA parameter, Olive's tail moment (OTM) was increased significantly at TP3 compared to both baseline and TP2 ($p=0.002$ and $p=0.017$, respectively). Nuclear division index decreased significantly at TP2 ($p<0.001$), then increased above baseline at TP3 ($p=0.004$). Baseline comparison with controls showed higher MN frequency in donors without statistical significance ($p=0.059$). Whereas, CA results were significantly higher in controls. CD34 cell count showed moderate positive correlation with white blood cell count at TP2 (Pearson $R=0.495$, $p=0.004$).

Conclusions. Our results showed the genotoxic effect of G-CSF in healthy donors, in two of the three tests performed, short-term effect in NDI, and long-lasting effect in OTM. So, this study provides novel information for the debate about the genotoxicity of G-CSF and supports the need for further studies with a larger sample size and longer follow-up.

Key words: peripheral blood stem cell transplantation, granulocyte colony-stimulating factor, comet assay, micronucleus tests, genotoxicity tests, hematopoietic stem cell mobilization, hematopoietic stem cell donor, tissue donor.

✉ Hasan Fatih Çakmaklı
cakmaklihf@gmail.com

Received 25th June 2023, revised 8th July 2023, 26th July 2023, accepted 28th July 2023.

This study was presented as an oral presentation at the 46th Turkish National Hematology Congress (virtual) in 2020 and received an award in the field of Experimental Hematology and was published in abstract form in the proceedings of the congress.

Annually, > 90,000 hematopoietic stem cell transplantations (HSCT) are performed worldwide, including different indications for malignant and benign diseases.[1] Between two major methods of obtaining hematopoietic stem cells; peripheral blood stem cell (PBSC) collection by apheresis is the most common one (80% of all allogeneic HSCT) and generally preferred over bone marrow harvest, due to its advantages including faster engraftment, practicability for donor and medical staff, and lower risk of relapse for patients with high risk malignant disease.[2]

Granulocyte-colony stimulating factor (G-CSF) is the drug of choice for mobilizing stem cells. The short-term side effects of G-CSF are generally well-tolerated but the long-term effects remain unclear.[3] For many years, there have been some concerns that G-CSF may increase the risk of malignancy. Several preclinical and clinical studies performed to clarify this issue have been inconclusive regarding the increased genotoxicity associated with the use of G-CSF.[4] There are some anecdotal reports about acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) cases after PBSC donation.[5,6] But, in large series except one[7]; the incidence of cancer after PBSC and bone marrow transplantation was not different and did not detect an increase in the risk of MDS-AML.[8-10] The World Marrow Donor Association (WMDA) stated that there is not an increased risk of developing cancer after the use of G-CSF compared to donors not receiving G-CSF.[11] On the other hand, many centers continue to record the family history of leukemia and follow the donors for malignancies.[4]

Genotoxicity is defined as damage to genetic material by chemical, physical or biological agents. The relationship between genotoxicity and carcinogenicity has been clearly demonstrated. The detection of DNA damage in cells is fundamental for studying carcinogenesis. Therefore, genotoxicity studies are important in identifying carcinogens.[12] Today, different methods are used in genotoxicity studies. Micronucleus test (MNT), nuclear division index (NDI) and comet assay (CA) are the current reliable tests in 'International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use' classification but these methods have not been used in any previous study for evaluating the G-CSF genotoxicity.[13] An increase in MNT and CA values suggests there is a genotoxic effect, whereas NDI is expected to decrease with genotoxicity.[14,15]

In this prospective case-control study, our primary aim was to evaluate the possible genotoxic effects of G-CSF on healthy peripheral stem cell donors. For this purpose, donors were prospectively analyzed by MNT, NDI and CA at 3 time-points (TP), baseline (TP1), 5 days after G-CSF administration just before apheresis (TP2), and 1 month after stem cell collection (TP3). These tests were also performed on age- and sex-matched volunteers as a control group. Our secondary aim was to evaluate the effects of G-CSF on CD34 counts and hemograms.

Material and Methods

Participants and study design

Thirty-six HLA-matched related peripheral blood stem cell transplantation (PBSCT) donors and 16 age- and sex-matched healthy volunteers were included in the study. The data related to the donors and controls, including age, sex, body weight, smoking status and drugs being used, were recorded.

For mobilization of stem cells, the dose of G-CSF was 10 μg/kg/day subcutaneously for 5 days. Donors received two forms of G-CSF; filgrastim or lenograstim. On the 5th day, the G-CSF dose was applied at 6 a.m. and the apheresis was performed at 9 a.m.

Samples were collected for genotoxicity tests and hemograms at three time-points; TP1-3. Samples for genotoxicity tests were immediately transported to the laboratory and MNT and NDI were studied fresh and the samples for CA were stored frozen at -80°C.

The laboratory team were single-blinded during the genotoxicity studies. The CD34 counts after 5 days of G-CSF were measured as a routine procedure for transplantation at TP2. As a secondary outcome, for investigating the effect of G-CSF on hemogram and CD34 counts, results were recorded for the three TPs of the study.

To confirm the internal consistency and reliability, two samples one week apart were collected from 10/16 of controls.

The study, including sample collection and laboratory studies, were performed between May 2012- June 2013. The MNT and NDI were performed in the Pediatric Hematology Laboratory and the CA, in the Genetic Toxicology Laboratory of Forensic Sciences in Ankara University.

The study was approved by Ankara University Clinical Ethics Committee (Date: 28.05.2010, Number: 09-281-12). All samples were collected after written informed consent of donors and/or guardians before study entry.

Micronucleus test and nuclear division index

The micronucleus test was performed as defined in detail by Fenech et al.[14] In brief, for the preparation of cell cultures, fresh blood samples were added to the chromosome medium (Chromosome Medium B) and incubated at 37°C for 72 hours and cytochalazine-B was added to stop cytokinesis at 44 hours. At the end of previously defined procedures, the preparations were stained homogeneously in 5% Giemsa for the detection of micronucleus (MN) formations.

In these preparations, MN in 1000 binucleate cells were evaluated in total. During these examinations, 1000 binuclear cells were examined for each donor and those containing MN were noted. The following formula was used to calculate MN frequency and an increase in this value is interpreted as increased genotoxicity. "MN frequency=(1X 1MN)+(2X 2MN)+3X (3+4MN)/1000".[14]

Nuclear division index was calculated from the same preparations, after counting 500 cells and determining the number of cells with one, two, three and four nuclei and a decrease in NDI is interpreted as increased genotoxicity. The calculation was made according to the formula below. "NDI= [(1xN1)+(2xN2)+(3xN3)+(4xN4)] / n" (n: Total number of cells).[16]

Comet Assay

DNA damage was determined according to five CA parameters; comet length (CL), tail length (TL), tail DNA %, tail moment (TM), and Olive's tail moment (OTM). CA was conducted under alkaline Ph with some modifications from the original method as described earlier.[17-19] The levels of DNA damage were measured by the BAB Bs CA system and an increase in CA parameters is interpreted as increased genotoxicity. Approximately 15 regions of the preparation for each individual were scanned and 50 randomly selected lymphocytes were examined under an Olympus BX50 fluorescent microscope.

Pretest for genotoxicity tests: self-control

No significant difference was detected between two separate samples withdrawn at a one week interval from 10 volunteers in control group, confirming the internal consistency and reliability of genotoxicity tests ($p>0.05$).

Statistical analysis

All statistical analyses were performed using SPSS 21.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, USA). Since the test results obtained (MNT, NDI, CA) were repetitive measurements obtained from the same subjects, analysis of variance method was used for repeated measurements. Dependent groups T test was used to compare the results of the samples taken at different times from the control group. The results of the donors and controls were compared with the independent groups T-test. In the evaluation of the relationship between variables, correlation coefficients and

statistical significance were calculated with the Pearson test. The statistical significance limit was accepted as p<0.05 for all tests. The mean and standard deviation (SD) values were used as descriptives.

Results

Participants

The study group consisted of 36 donors (mean age±SD=32.3±14.5 years, min-max=8-66 years, M/F: 18/18) and 16 volunteers as the control group (mean age±SD=38±8.7 years, min-max=18-51 years, M/F: 8/8). Twenty-six of 36 donors (mean age±SD=32.7±14.6, min-max: 8-66 years, M/F: 13/13) completed all 3 sampling for genotoxicity tests, 10 donors who did not have three samples were excluded from the analysis of the genotoxicity tests. Eight of ten donors could not come for the third sampling due to geographic distance and the other two donors did not want to continue the study. Additionally, MNT could not be studied in two donors due to technical problems. As not all of the donors came for the originally planned first month control on time, the third samples were collected on average of the 42nd day (Median:36, IQR:10.8, min-max:30-148 days). The age and sex distributions of donors and controls were similar (p>0.05).

The drugs used for mobilization of stem cells were filgrastim (n=28) and lenograstim (n=8). CD34 cell count was available for 34 donors. Hemograms were evaluated in 26 donors who had three TP samples.

Micronucleus tests of donors

The MNT results were similar between the three TPs (TP1, TP2, TP3) (n= 24, p=0.819). (Fig. 1. A-C, Fig. 2, Table I).

Nuclear division index of donors

The nuclear division index of donors (n=24) was significantly decreased (p<0.001) at TP2 and significantly increased at TP3 compared to both TP1 and TP2 (p=0.004 and p<0.001, respectively) (Fig. 3 A-B, Fig. 4, Table I).

Comet assay of donors

Comet assay was studied with 5 parameters in 26 donors at three TPs. There was no significant difference between TPs for CL, TL, tail DNA%, TM. On the other hand, OTM at TP3 was

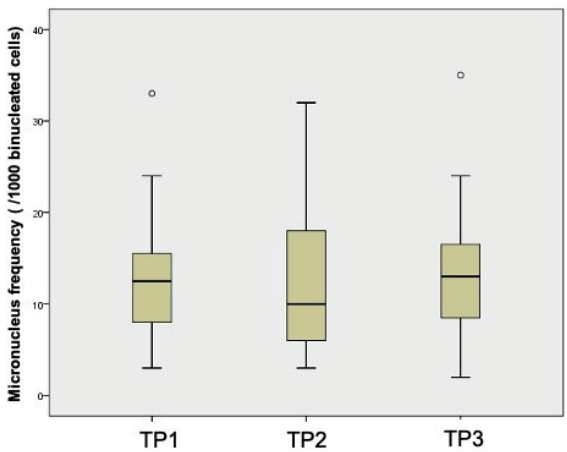

Fig. 2. Micronucleus frequency (/1000 binucleated cells) of donors at three time-points shown as a box-plot graph (TP1, TP2, TP3) (n= 24, p=0.819). TP: time-point.

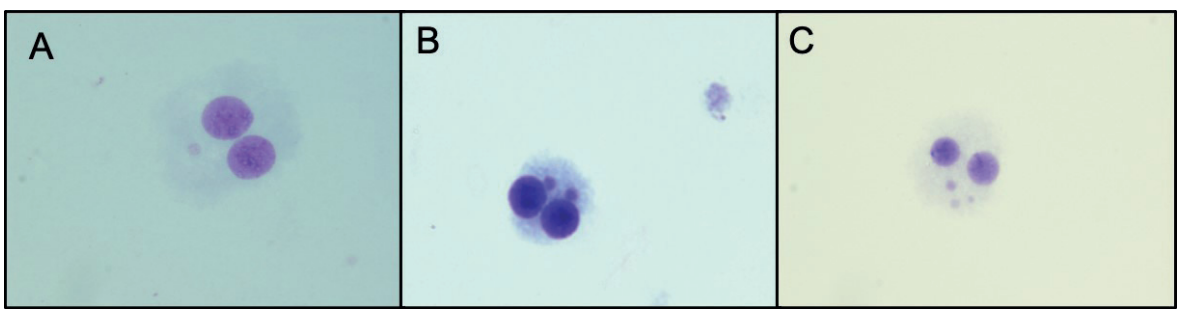

Fig. 1. Micronucleus tests (MNT). Micronuclei are seen as intracytoplasmic inclusions in two-nucleated cells, A. one micronucleus, B.two micronuclei, C. three micronuclei.

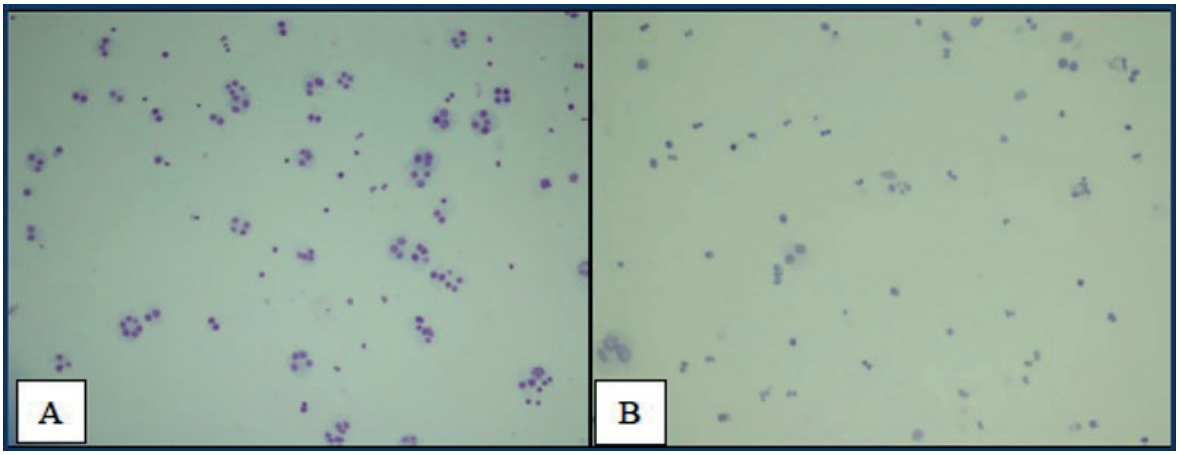

Fig. 3. Representative smear photographs used for calculating NDI of donors, A. before G-CSF, at TP1, normal induced dividing cells, B. after 5 days of G-CSF, at TP2, decreased nuclear division. G-CSF: granulocyte-colony stimulating factor, NDI: nuclear division index, TP: time-point.

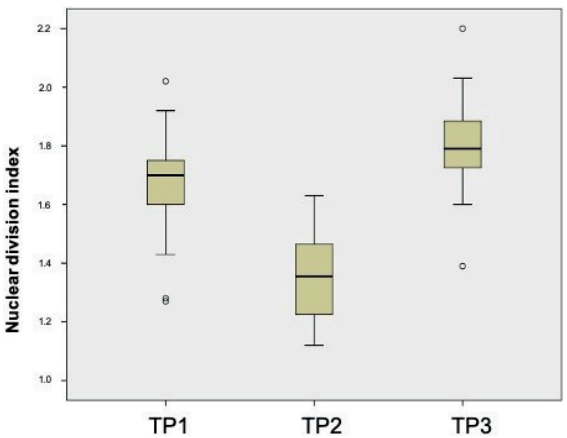

Fig. 4. Nuclear division index of donors at three time-points shown as a box-plot graph. (TP1 vs. TP2: p<0.001, TP2 vs. TP3: p<0.001, TP1 vs. TP3: p=0.004). TP: time-point.

significantly increased compared to both TP1 and TP2 (p=0.002 and p=0.017, respectively) (Fig. 5 A-E, Fig. 6, Table I).

Comparison of basal genotoxicity tests between donors and the control group

In terms of MNT, baseline results of donors (TP1) were higher compared to the control group (13.08±7.0 vs 9.19±4.82, respectively) without statistical significance (p=0.059) (Table II). For NDI, no statistically significant difference was found between groups (p=0.45) (Table II). In CA, results of controls, in terms of all parameters, were significantly higher than those of donors.

Factors affecting the CD34 count of donors

CD34 count of donors just before stem cell collection (TP2) was 86.6±46.0/µl (min-max: 16-246/µl). Age, body weight, gender and the form of G-CSF used (filgrastim or lenograstim) did not have a relationship with CD34 counts. Only the white blood cell (WBC) count at TP2 showed statistically significant and moderate correlation with CD34 count in the positive direction (Pearson R= 0.495, p=0.004) (Fig. 7, Table III).

Effects of G-CSF on hemogram parameters

Neutrophil, monocyte, basophil, eosinophil, and platelet counts showed significant increases at TP2 and decreased back to their baseline at TP3 (n=26). Lymphocyte counts, which had significantly increased at TP2, significantly decreased to even lower levels than the baseline count at TP3. There was no statistically significant change in hemoglobin levels (Table IV).

Table I. Genotoxicity test results of the control group at baseline and the donors at three TPs.

		TP	Donors*		Controls (n=16)	
			Mean±SD	Min-max	Mean±SD	Min-max
MNT (/1000 binucleated cells)		1	13.08±7.0	3.0-33.0	9.19±4.82	4.0-20.0
		2	12.31±6.94	3.0-32.0		
		3	13.3±7.75	2.0-35.0		
NDI		1	1.68±0.18	1.27-2.02	1.73±0.2	1.43-2.13
		2	1.35±0.16	1.12-1.70		
		3	1.81±0.16	1.39-2.20		
Comet	Comet length (μm)	1	26.43±4.37	20.19-30.39	31.82±3.27	28.4-39.56
Assay		2	26.60±4.51	19.30-35.85		
		3	27.63±5.96	11.09-36.40		
	Tail length (μm)	1	7.06±2.43	3.03-14.24	9.30±1.41	6.83-12.63
		2	6.73±1.60	3.86-10.20		
		3	7.51±2.22	2.65-12.56		
	Tail DNA%	1	72.20±10.9	48.1-88.82	78.65±4.98	67.82-84.72
		2	70.77±5.76	58.23-79.77		
		3	73.75±7.80	53.29-87.41		
	Tail moment	1	5.50±2.39	1.58-12.17	7.45±1.41	4.66-10.51
		2	5.03±1.42	2.47-8.08		
		3	5.90±2.13	1.91-11.16	24.95±1.07	19.85-32.91
	Olive's tail moment	1	19.99±3.80	12.47-26.33		
		2	20.39±5.33	10.18-30.88		
		3	23.21±4.12	13.73-35.85		

*: For MNT and NDI n=24, For Comet Assay n=26. MNT: micronucleus test, Min-max: minimum-maximum, NDI: nuclear division index, SD: standart deviation, TP: time-point

Table II. Comparison of the basal values of donors and the control group in terms of genotoxicity tests.

		Donors			Controls			p
		n	Mean±SD	Min-max	n	Mean±SD	Min-max	
MNT (/1000 binucleated cells)		24	13.08±7.0	3.0-33.0	16	9.19±4.82	4.0-20.0	0.059
NDI		24	1.68±0.18	1.27-2.02	16	1.73±0.2	1.43-2.13	0.45
Comet Assay	Comet length (μm)	26	26.43±4.37	20.19-30.39	16	31.82±3.27	28.4-39.56	<0.001
	Tail length (μm)	26	7.06±2.43	3.03-14.24	16	9.30±1.41	6.83-12.63	0.002
	Tail DNA%	26	72.20±10.9	48.1-88.82	16	78.65±4.98	67.82-84.72	0.013
	Tail moment	26	5.50±2.39	1.58-12.17	16	7.45±1.41	4.66-10.51	0.005
	Olive's tail moment	26	19.99±3.80	12.47-26.33	16	24.95±1.07	19.85-32.91	<0.001

MNT: micronucleus test, Min-max: minimum-maximum, NDI: nuclear division index, SD: standart deviation.

Table III. Comparison of CD34 counts by gender and G-CSF type.

	Sex			G-CSF		
	Female (n=17)	Male (n=17)	p	Filgrastim (n=27)	Lenograstim (n=7)	p
CD 34/ μl (mean±SD)	78.42±44.0	94.8±47.8	0.306	85.1±45.9	91.5±48.9	0.736

CD 34: CD 34 cell count, G-CSF: granulocyte-colony stimulating factor, SD: standart deviation.

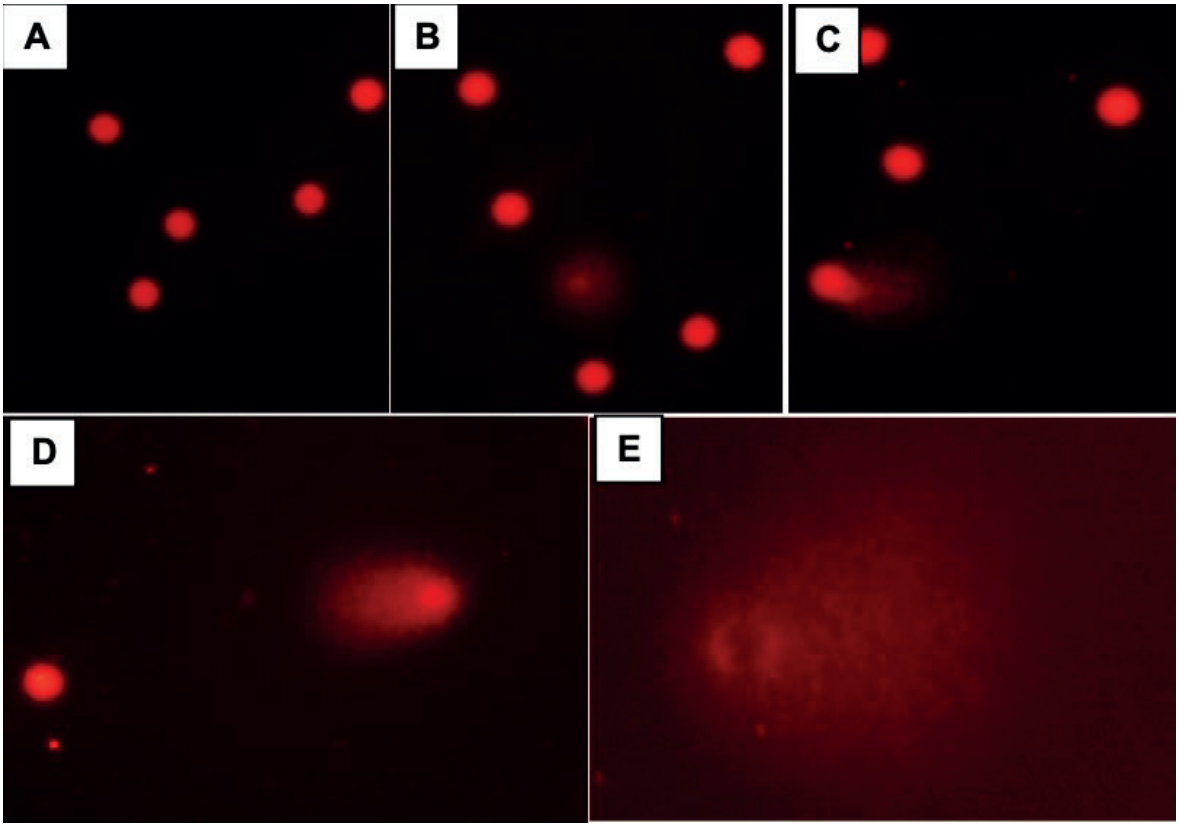

Fig. 5. Comet Assay, fluorescent microscobic images of comet assay showing increasing levels of DNA damage in lymphocytes in the order from A to E.

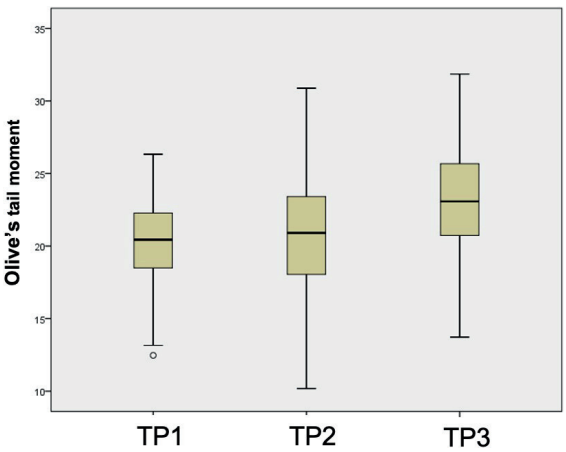

Fig. 6. Olive's tail moment of donors at three TPs shown as a box-plot graph. (TP1 vs. TP2: p=0.631, TP2 vs. TP3: p=0.017, TP1 vs. TP3: p=0.002). TP: time-point.

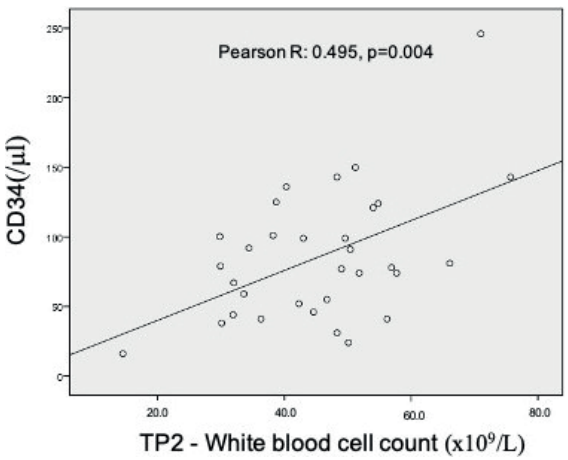

Fig. 7. Scattered dot graph showing the relationship between TP2 WBC count (x10^9/L) and CD34 count of donors (/µl). TP: time-point, WBC: white blood cell.

Table IV. Effect of Granulocyte-Colony Stimulating Factor on hemogram (n=26).

	TP1	TP2	TP3	P
WBC (x10^9/L)	5.340	46.807	45.80	*TP1 vs. TP2* <0.001
				TP1 vs. TP3 =0.043
				TP2 vs. TP3 <0.001
Neutrophil(x10^9/L)	4.396	33.024	4.013	*TP1 vs. TP2* <0.001
				TP1 vs. TP3 =0.380
				TP1 vs. TP3 <0.001
Lymphocyte (x10^9/L)	2.465	3.760	1.937	*TP1 vs. TP2* <0.001
				TP1 vs. TP3 =0.005
				TP2 vs. TP3 <0.001
Monocyte (x10^9/L)	0.440	9.197	0.451	*TP1 vs. TP2* =0.002
				TP1 vs. TP3 =0.781
				TP2 vs. TP3 =0.002
Eosinophil (x10^9/L)	0.155	0.464	0.126	*TP1 vs. TP2* <0.001
				TP1 vs. /TP3 =0.102
				TP2 vs. TP3 <0.001
Basophil (x10^9/L)	0.044	0.216	0.042	*TP1 vs. TP2* =0.047
				TP1/TP3 =0.908
				TP2/TP3 =0.056
Thrombocyte (x10^9/L)	251	221	249	*TP1/TP2* =0.007
				TP1/TP3 =0.746
				TP2/TP3 =0.008
Mean Platelet Volume (fL)	8.8	8.2	8.3	*TP1/TP2* =0.032
				TP1/TP3 =0.049
				TP2/TP3 =0.659
Hemoglobin (gr/dL)	14.1	13.9	13.8	0.414

TP: time-point, WBC: white blood cell

Discussion

In this study, the genotoxicity tests, namely MNT, NDI and CA, have been performed at 3 different TPs to explore whether G-CSF causes a genotoxic effect in healthy PBSCT donors for the first time in the literature. While NDI showed a short-term genotoxic effect at TP2 which normalized at TP3; OTM, one of the most sensitive components of CA for genotoxicity, revealed delayed genotoxic effect of G-CSF at TP3.[15,20,21] On the other hand, MNT did not detect any genotoxic effect.

The relationship between increased micronucleus formation due to known carcinogens, like gama radiation and ultraviolet light has been demonstrated.[22,23] Also, increased MN and elevated risk of cancer have been shown in clinical, prospective, and long-term follow-up studies.[24-26] Although MNT is a reliable method used in the detection of chromosomal damage, genome instability and cancer risk, it did not show a genotoxic effect in our study group.

Nuclear division index, which is a marker of cell proliferation, is expected to decrease with genotoxic effects. NDI was found to be lower in patients with lung cancer compared to healthy controls, and it was found to be lower in patients with colonic polyps or colon cancer compared to individuals with normal colonoscopy.[27,28] The NDI results of our donor group were

interpreted as G-CSF having an inhibitory effect on lymphocytes in the early period, but this effect was short-term. At TP3 this inhibitory effect disappeared and NDI increased above the baseline in a compensatory manner. Rutella et al.[29] showed that G-CSF inhibited the cell cycle progression in lymphocytes. Although our NDI results are in parallel with this finding, in our study this effect was reversible in about one month. The inhibiting effect on cell division may be an indicator of genotoxicity, but its disappearance at TP3 is in favor of reversibility.

The comet assay is a method that is frequently used in the evaluation of DNA damage and is used to investigate the possible genotoxic effects of newly defined drugs and chemicals.[15,30]As far as we know, this is the first study using CA to detect possible genotoxic effects of short-term G-CSF application in healthy individuals. The best parameters that predict genotoxicity in the CA are tail DNA% and Olive's tail moment.[15,20,21,31] In our study, CL, TL, tail DNA % and TM did not show a statistically significant change between the three TPs. On the other hand, OTM, which unites the TL and tail density as a single variable and therefore more sensitive in showing the genotoxic damage, was found to be increased at TP3 compared to TP1 and TP2. It is noteworthy that the genotoxic damage became obvious at TP3 in this assay, necessitating longer follow-up studies to determine the exact duration and the reversibility of this finding.

While no genotoxic effect was detected with MNT in our study, a statistically significant effect was demonstrated by OTM parameter of CA at TP3. On the contrary, a study comparing CA and MNT, found that the two methods had the same sensitivity in determining mutagenicity, while MNT was more powerful in determining low-level genotoxic damage potential, most likely because only the whole length parameters of CA was used in this study.[32] Our results showed that OTM, as one of the most sensitive parameter of CA, may be superior to MNT in detecting genotoxicity.

In the literature, laboratory studies investigating the possible genotoxic effect of G-CSF in healthy PBSCT donors with different methods provided various evidence which were inconclusive. Nagler et al.[33] detected asynchrony in the timing of allelic replication, changes in the capacity of DNA methylation, and aneuploidy that continued in the 6th month in lymphocytes. On the other hand, Schapira et al. found that DNA destabilization increased on the 5th day, returning to normal within 1-2 months.[34] According to Hirsch et al., G-CSF did not lead to any chromosomal instability and can be used safely.[35] More recent studies similarly found conflicting results such as; Baez et al.[36] showed that G-CSF treatment in healthy donors led to differential expression of a group of genes and microRNAs in CD34 cells, which was persistent after one year; whereas Leitner et al.[37] evaluated methylation in peripheral lymphocytes and did not find any significant change. As can be seen from these various studies, the methods of genotoxicity evaluation, target cells studied and time-points are not standard, long-term follow up data is lacking, leading to a confusion about the safety of G-CSF use in healthy PBSCT donors.

The comparison of basal genotoxicity tests between donors and controls showed that MN frequency was higher in donors at baseline. Although this was not statistically significant, it may support a possible increased genotoxicity in the donor group before G-CSF exposure. This may be due to the pre-procedural stress of the stem cell donation or cigarette smoking (there were no smokers in control group while 5 donors were smokers), which are possible genotoxic factors not present in control group.[38,39] On the other hand, unexpectedly, CA results of controls were significantly higher than the baseline values of donors. The samples of the donors and the controls arrived at the laboratory out of order and have been analyzed blindly by the investigator, excluding any possible technical problem responsible for these conflicting results. There may be other confounders such as air pollution, diet, alcohol

use, sedentary lifestyle and other genetic factors that are not easy to detect. In our study, as same individuals were evaluated longitudinally for possible genotoxic effects of G-CSF, we believe that the confounding effect of these factors has been minimized in the comparison of TPs in the donor group.

In our study, the the CD34 count showed a statistically significant, moderate and positive correlation with the WBC count at TP2 and there was no difference in CD34 mobilization effect of filgrastim and lenograstim consistent with the literature.[40,41] Additionally, the number of CD34 cells was not related to the donor's age, body weight or gender. However, there are controversial results in the literature.[42,43] It is thought that CD34 yield decreases, especially in females and with advanced age (>55 years).[44,45]

Following 5-day G-CSF, the WBC and lymphocyte counts increased significantly at TP2 compared to TP1, then returned to the normal range at TP3 but were significantly lower than baseline. Holig et al., in their study of 3928 unrelated donors, found that the WBC count was significantly lower than baseline in the 1st month after G-CSF administration, increased partially in the 4-year follow-up, but never reached baseline values. On the other hand it was found that the low lymphocyte count returned to normal one year later.[46] The reason for the prolonged slightly lower WBC counts relative to baseline is not clear but may be due to slow replacement of stem cells, "down-regulation" of G-CSF receptors, or other disturbances in cytokine linkages. In donors, leukocytosis secondary to possible pre-procedural stress may also be a reason for these values not returning to normal.[47] It was observed that the neutrophil, monocyte, basophil and eosinophil counts significantly increased at TP2 after G-CSF application, and decreased back to their previous values at TP3, consistent with the literature.[7,47]

In our study, there was a statistically significant decrease in platelet counts compared to baseline at TP2, coming back to normal at TP3. In large-scale studies, a decrease in platelet counts due to G-CSF use has been reported.[7] There may be two possible explanations; the partial suppression of platelet production secondary to the orientation of the stem cells to the myeloid series and hypersplenism secondary to the enlargement of the spleen due to G-CSF.[47]

There are some limitations to this study. First, fewer participants than planned at the beginning were recruited due to the difficulty of obtaining TP3 samples from individuals living in other cities. However, the sample size was found to be sufficient for this prospective study. Additionally, TP3 samples could not be collected homogenously on day 30 from all donors. The second limitation was not having a longer follow up of the donors, as there is no standard protocol for the follow-up of healthy donors in our country. Long-term follow-up of all donors and collection of follow-up data by the international study groups are recommended in order to understand the delayed effects of G-CSF. In France, these donors are covered by a 10-year health insurance and follow-up is planned for 10 years.[48] Last but not least, the conflicting results of CA in controls could not be clarified with additional experiments (/tests), as the sample collection part of the study was already completed.

In conclusion, in this study the genotoxic effects of G-CSF were detected in healthy PBSCT donors in two of the three tests perfomed (NDI and CA). Although NDI values decreased back to normal, OTM detected an actual persistent genotoxic effect at TP3. Future studies with larger donor groups with a longer follow-up are needed.

Acknowledgements

The donor group for the study was collected with the support of Ankara University Department of Pediatric Hematology and Oncology, Ankara University Department of Adult Hematology and Ankara University Department of Pediatric Immunology,

Hacettepe University Department of Pediatric Hematology and Oncology, Gülhane Military Academy Department of Pediatric Hematology. As the authors, we would like to express our gratitude to these centers. Special thanks to Assoc. Prof. Murat Akyıldız for his contribution in statistical analysis.

Ethical approval

Ankara University Clinical Ethics Committee (Date: 28.05.2010, Number: 09-281-12). All samples were collected after written informed consent of donors and/or guardians before study entry.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: HFÇ, Tİ; data collection: HFÇ, Tİ, HG, PT; analysis and interpretation of results: HFÇ, HG, ES, PT, ZK, Tİ, draft manuscript preparation: HFÇ, Tİ, ES, ZK. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

This study was supported by Ankara University Scientific Study Fund with project number 12B3330022.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 822-831
https://doi.org/10.24953/turkjped.2023.350

Original Article

Investigation of adolescents and their mothers in terms of nomophobia

Yusuf Selman Çelik, Burcu Ersöz Alan

Department of Child and Adolescent Psychiatry, Hacettepe University Faculty of Medicine, Ankara, Türkiye.

ABSTRACT

Background. Nomophobia (NoMP) is the fear of being unable to interact with others via mobile phones and is a current topic in adolescents' mental health. The purpose of this study was to investigate the association between NoMP in adolescents and their mothers' level of NoMP.

Methods. The levels of depression, anxiety, attention deficit/hyperactivity-impulsivity symptoms, NoMP, and perceived parental emotional availability were examined.

Results. One hundred fifty-five adolescents (60% girls) were included in the study with their mothers. Ninety-three (60%) adolescents (67.7% girls) and 64 (41.3%) mothers were classified as nomophobic. There was a positive correlation between the NoMP levels of adolescents and their mothers. Nomophobic girls perceived less paternal support. All psychopathologic symptoms were higher in the nomophobic mothers. There was no difference between nomophobic and non-nomophobic adolescents in terms of maternal psychopathologies.

Conclusions. We suggest that parental effects should be investigated during the assessment of NoMP, especially in adolescent girls. The phone usage habits of mothers and their relationship with their adolescent children were closely associated with adolescent NoMP.

Key words: nomophobia, adolescent, parent, emotional availability, psychopathology.

Nomophobia (No MobilePhone Phobia-NoMP) is a newly defined concept of the technological world where fear of being unable to be in contact with a mobile phone causes anxiety or provokes existing anxiety-related behaviors (e.g., compulsive checking of phones, social anxiety). The thought of being unable to access information and communicate when the mobile phone is unavailable causes discomfort, anxiety, and nervousness.[1] Nowadays, almost every mobile phone is 'smart', so the terms 'mobile phone' and 'smartphone' have been used interchangeably.

Smartphones provide many benefits in communication, information, education, entertainment, and business. On the other hand, becoming anxious when forgetting the mobile phone, when the battery of the phone is low, when the signal is lost, and/or carrying the mobile phone everywhere, checking it even if it is not ringing are the basic symptoms of NoMP.[2] These lead to physical problems (e.g., neckaches), traffic accidents, decreased sleep quality, and disturbances in social and psychological well-being.[3]

NoMP has not been considered a psychopathology. It demonstrates problematic behaviors and feelings due to the problematic use of a smartphone.[4] In the 20th century, mobile phones have become a part of daily life and NoMP is becoming more common among

✉ Burcu Ersöz Alan
burcuersoz02@gmail.com

Received 15th May 2023, revised 26th June 2023, 14th July 2023, 14th August 2023, accepted 14th August 2023.

Some of the data were presented as an oral presentation at the 13th International Congress on Psychopharmacology & International Symposium on Child and Adolescent Psychopharmacology, which took place in Antalya, Türkiye on November 9th-12th, 2022.

youth.[5] The factors related to NoMP are similar to those related to psychopathologies, especially anxiety disorders and addiction. Those factors related to psychopathologies could be divided into individual (young age, low self-esteem, having symptoms of depression/anxiety, etc.), familial (conflicts in the family, parental modeling, parenting style, etc.), and social (social pressure, social withdrawal, etc.) factors. Impulsivity[6], depression, stress, and anxiety[7] have been associated with NoMP in youth. Age is important, as NoMP is more common among people under the age of 20, like separation anxiety disorder, social anxiety disorder, and phobias.[2,8] According to the DSM-5 these disorders could be seen at any age, but with a lower rate in adults, and they aggregate within families. This could also be true for NoMP.

If smartphones are thought to be related to anxiety, familial factors should also be investigated. In this study, we aimed to investigate the levels of parental emotional availability, depression, anxiety, attention deficit/hyperactivity-impulsivity, and NoMP among mothers of adolescents. It was hypothesized that mothers of adolescents with NoMP were more depressed, had high levels of attention-deficit/hyperactivity-impulsivity and anxiety symptoms, and had lower emotional availability. Additionally, mothers with NoMP had high levels of psychopathological symptoms.

Material and Methods

This research has been approved by the Hacettepe University Non-Interventional Clinical Research Review Board (GO 22/205). All adolescents who applied to Hacettepe University Department of Child and Adolescent Psychiatry between December 2021 and March 2022 were invited. Adolescents and/or mothers who did not have a smartphone, could not complete the scales (due to physical problems, intellectual disability, or autism spectrum disorder) or were diagnosed with psychotic disorders were excluded. Also, adolescents whose mothers were deceased were not included. Among the 170 adolescents (aged 12-17 years) who met the inclusion criteria, five adolescents/mothers did not want to participate, and ten adolescents/mothers did not fill out the scales fully. Written consent was obtained from all adolescents and their mothers. The following scales were given.

Socio-demographic form: The questions pertained to socio-demographic characteristics and the daily time spent using a smartphone.

Nomophobia Questionnaire (NMP-Q): Yildirim & Correia developed and translated this scale into Turkish.[9] It is a 20-item questionnaire that evaluates nomophobia in four dimensions: Fear of not being able to communicate (fear of losing instant communication), fear of losing connectedness (thinking of being disconnected from friends and social media identity), fear of not being able to access information (feeling discomfort from not getting information via smartphones), and giving up convenience (feeling discomfort from any situation that distorts access to phones, like a low battery). It is a 7-point Likert-type scale with a cut-off score of 60. The Cronbach's alpha value of NMP-Q is 0.92 in its validity and reliability study, which was performed in a population with a mean age of 20.[9] It has been used in adolescents[10] and adults[11] with the same threshold values. Higher scores indicate a higher severity of nomophobia: 0-20: Absence of nomophobia, 21-59: Mild level, 60-100: Moderate level, and 101-140: Severe level. This study calculated the NMP-Q scores of adolescents (NMP-A) and mothers (NMP-M). The adolescents whose NMP-A<60 were referred to as absence/mild nomophobia (Without-NoMP), and those whose NMP-A≥60 were referred to as moderate/severe nomophobia (With-NoMP).

Lum Emotional Availability of Parents (LEAP): This scale rates the emotional availability of each parent. It has 15 items. Higher scores reflect higher levels of parental emotional availability. The highest possible score is 90.[12] Turkish validation of this scale was performed.[13] Adolescents filled out the scale. This study

calculated LEAP scores for mothers (LEAP-M) and fathers (LEAP-F).

Social Support Appraisal Scale for Children (SSAS-C): This scale evaluates the level of supportive behaviors from peers, family, and teachers. It has 41 items. Higher scores indicate a higher level of support. The highest possible score is 205.[14] The Turkish validation study of SSAS-C was performed.[15] Adolescents filled out the scale. Scores of family support were used in this study (SSAS-C-F).

Beck Depression Inventory (BDI): This inventory evaluates the severity of depressive symptoms with 21 items. The cut-off point is 17. The severity of depression is minimal (0-9), mild (10-16), moderate (17-28), and severe (29-63).[16] Its reliability and validity in Turkish were conducted.[17] The scale was given to mothers.

Beck Anxiety Inventory (BAI): This questionnaire evaluates the severity of anxiety symptoms. It has 21 items. The cut-off point is 16. The severity of anxiety is minimal (0-7), mild (8-15), moderate (16-25), and severe (26-63).[18] Its reliability and validity in Turkish were conducted.[19] The scale was given to mothers.

Adult Attention Deficit Hyperactivity Disorder Self-Report Scale (A-ADHD-SRS): The scale has been developed by the World Health Organization (WHO) for screening ADHD symptoms. It has 18 items: 9 inattention and 9 hyperactivity/impulsivity criteria.[20] Turkish reliability and validity of this scale were performed.[21] Mothers filled out the scale. Total score, scores of inattentiveness (A-ADHD-SRS-I) and hyperactivity/impulsivity (A-ADHD-SRS-HI) were calculated.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 26 was used for data analysis. The normality of the data was evaluated by Kolmogorov-Smirnov statistics. Descriptive analysis (mean, standard deviation [SD], percentage) was used. Student's t-test was performed to compare the two independent groups when parametric test assumptions were met. The Chi-square test was used to compare two categorical variables, and Fisher's exact test was performed when the sample size was small (i.e. psychiatric diagnosis, time spent on the phone, phone usage). The Pearson correlation coefficient was calculated to determine the relationship between two continuous variables. The Cronbach's alpha value for NMP-Q was calculated for internal consistancy. A p value of <0.05 was accepted as the level of statistical significance.

Results

A total of 155 adolescents (93 girls, 60%) and their mothers were evaluated. The mean age of adolescents was 14.87±1.47 years. The Cronbach's alpha value for NMP-A was 0.808 and indicated acceptable internal consistancy. Among the adolescents, 93 (60%) were in the With-NoMP group, while 62 (40%) were in the Without-NoMP group. There were significantly more girls in the With-NoMP group (n:63, 67.7% girls) ($p<0.05$). The mean NMP-A scores for girls and boys were 69.04±23.78 and 62.0±25.81, respectively ($p=0.083$). There were no statistically significant differences in sociodemographic variables (parents' age, occupational status, educational status, living place, number of siblings) between the two groups.

The mean age of mothers was 42.22±6.54 years (median:42.5, min:24, max:55). Sixty-four mothers (41.3%) were classified as nomophobic based on their NMP-M scores.

Results of scales

Nomophobic adolescents spent more time on smartphones than non-nomophobic adolescents. The level of nomophobia was higher in mothers of the With-NoMP group, and the emotional availability of fathers were lower in the With-NoMP group compared to the Without-NoMP group. (Table I) Pearson correlation analysis revealed a significant correlation between NMP-A and NMP-M (r:0.311, $p<0.001$). (Table II)

Table I. Variables among adolescents in terms of NoMP.

Variables	Without-NoMP (n:62)	With-NoMP (n:93)	p-values
Gender			
Girls	30 (48.4%)	63 (67.7%)	0.019*
Boys	32 (51.6%)	30 (32.3%)	
Psychiatric diagnosis			
Depressive disorders	16 (25.8%)	37 (41.1%)	0.06[a]
Specific phobia	6 (9.7%)	12 (13.3%)	0.613[a]
Social phobia	14 (22.6%)	23 (23.6%)	0.705[a]
Generalized anxiety disorder	13 (21%)	27 (30%)	0.262[a]
Obsessive compulsive disorder	5 (8.1%)	4 (4.4%)	0.487[a]
ADHD	21 (33.9%)	45 (50%)	0.07[a]
ODD	1 (1.6%)	8 (8.9%)	0.08[a]
Binge eating disorder	3 (4.8%)	11 (12.2%)	0.158[a]
Time spent on smartphone (hours/day)			
<1	7 (11.3%)	2 (2.2%)	0.032[a]*
1-2	19 (30.6%)	12 (13.3%)	0.013[a]*
2-4	17 (27.4%)	26 (28.9%)	1.000[a]
4-6	15 (24.2%)	25 (28.9%)	0.709[a]
>6	4 (6.5%)	25 (27.8%)	0.001[a]*
Phone usage main purposes			
Playing games	10 (16.1%)	23 (25.6%)	0.230[a]
Watching videos	25 (40.3%)	24 (26.7%)	0.08[a]
Social media	10 (16.1%)	24 (26.7%)	0.166[a]
Communication	11 (17.7%)	16 (17.8%)	1.000[a]
Education	6 (9.7%)	3 (3.3%)	0.160[a]
Age (year)	14.76±1.48	14.95±1.46	0.436[b]
NMP-A	41.87±11.03	82.46±16.75	<0.001[b]*
Not being able to communicate	9.0±3,42	15.63±5.53	<0.001[b]*
Losing connectedness	10.56±4.39	20.34±6.46	<0.001[b]*
Not being able to access information	15.16±6.28	31.14±7.27	<0.001[b]*
Giving up convenience	7.15±2.89	15.34±6.35	<0.001[b]*
NMP-M	45.08±16.13	55.30±23.56	0.003[b]*
BDI	12.55±8.34	13.19±7.81	0.628[b]
BAI	12.11±9.25	11.97±10.64	0.930[b]
A-ADHD-SRS			
Total	21.48±9.83	21.33±9.57	0.924[b]
Inattentiveness	10.13±5.62	10.86±5.45	0.420[b]
H/I	11.35±5.63	10.47±5.28	0.323[b]
SSAS-C-F	39.77±12.83	37.66±11.13	0.277[b]
LEAP-M	71.56±18.54	67.92±21.24	0.274[b]
LEAP-F	64.08±22.38	54.02±24.92	0.013[b]*

Data are presented as mean +/- SD or n (%), as appropriate; [a]Fisher's exact test, [b]Student's t-test, *Statistically significant, ADHD: Attention Deficit Hyperactivity Disorder, A-ADHD-SRS: Adult Attention Deficit Hyperactivity Disorder Self-report Scale, BAI: Beck Anxiety Inventory, BDI: Beck Depression Inventory, H/I: hyperactivity/impulsivity, LEAP-M: Lum Emotional Availability of Parents-Mothers, LEAP-F: Lum Emotional Availability of Parents-Fathers, NMP-A: Nomophobia Questionnaire-Adolescents, NMP-M: Nomophobia Questionnaire-Mothers, NoMP: nomophobia, ODD: Oppositional Defiant Disorder, SD: standard deviation, SSAS-C-F: Social Support Appraisal Scale for Children- Family.

Table II. Univariate analysis of the relationships between NMP-A and the other variables.

Variables	r[a]	p
NMP-M	0.308	*<0.001**
BDI	0.048	0.555
BAI	0.063	0.434
A-ADHD-SRS	0.043	0.595
SSAS-C-F	-0.144	0.074
LEAP-M	-0.050	0.540
LEAP-F	-0.157	0.055

[a]Pearson correlation analysis, *Statistically significant, A-ADHD-SRS: Adult Attention Deficit Hyperactivity Disorder Self-report Scale, BAI: Beck Anxiety Inventory, BDI: Beck Depression Inventory, LEAP-M: Lum Emotional Availability of Parents-Mothers, LEAP-F: Lum Emotional Availability of Parents-Fathers, NMP-A: Nomophobia Questionnaire-Adolescents, NMP-M: Nomophobia Questionnaire-Mothers, SSAS-C-F: Social Support Appraisal Scale for Children- Family

The only difference found when comparing nomophobic girls with nomophobic boys was in LEAP-F scores, which was lower in girls. (Table III) Nomophobic girls also had higher NMP-M scores (43.37±17.96 and 55.46±23.99, p:*0.016*) and lower LEAP-F scores compared to non-nomophobic girls (62.18±22.68 and 48.55±25.51, p:*0.018*).

Results of mothers

Mothers were considered nomophobic if their NMP-M score was ≥60. Table IV shows the differences between nomophobic and non-nomophobic mothers. The two groups differed in terms of time spent on smartphones, BDI, BAI, and A-ADHD-SRS.

Discussion

In this study, the prevalence of NoMP among adolescents was found to be 60%, which is consistent with the range reported in previous population-based studies in our country (40-80%).[10,22,23] The higher prevalence in this clinical sample may be due to the nature of the population studied.

In our study, the prevalence of NoMP among mothers was 41%, which was lower than the prevalence among adolescents. Studies of NoMP

Table III. Comparison of nomophobic girls with nomophobic boys.

Variables	Girls with-NoMP (n:63)	Boys with-NoMP (n:30)	p-values
NMP-A	81.94±16.45	83.57±17.60	0.663
Not being able to communicate	15.62±5.44	15.67±5.80	0.969
Losing connectedness	20.79±6.30	19.40±6.81	0.334
Not being able to access information	31.02±7.37	31.40±7.17	0.813
Giving up convenience	14.51±6.20	17.10±6.42	0.065
NMP-M	55.46±24.0	54.97±23.03	0.925
BDI	13.40±7.60	12.77±8.35	0.718
BAI	12.25±10.65	11.37±10.78	0.709
A-ADHD-SRS			
Total	21.73±10.23	20.50±8.09	0.565
Inattentiveness	11.06±5.97	10.43±4.22	0.605
H/I	10.67±5.53	10.07±4.77	0.611
SSAS-C-F	36.13±11.56	40.87±9.59	0.05
LEAP-M	65.89±21.62	72.20±20.11	0.182
LEAP-F	48.55±25.51	65.34±19.55	*0.002**

Data are presented as mean +/- SD or n (%), as appropriate; *Statistically significant, A-ADHD-SRS: Adult Attention Deficit Hyperactivity Disorder Self-report Scale, BDI: Beck Depression Inventory, BAI: Beck Anxiety Inventory, H/I: hyperactivity/impulsivity, LEAP-F: Lum Emotional Availability of Parents-Fathers, LEAP-M: Lum Emotional Availability of Parents-Mothers, NoMP: Nomophobia, NMP-A: Nomophobia Questionnaire-Adolescents, NMP-M: Nomophobia Questionnaire -Mothers, SD: Standard deviation, SSAS-C-F: Social Support Appraisal Scale for Children- Family

Table IV. Characteristics and levels of depression, anxiety, and attention/hyperactivity in mothers.

Variables	NMP-M<60 (n:91, 58.7%)	NMP-M≥60 (n:64, 41.3%)	p-value
Age (year)	42.40±6.33	42.98±5.35	0.613
Socioeconomic status (TL/month)			
Low (<5000)	26 (28.9%)	16 (25.0%)	
Moderate (5001-19999)	90 (68.9%)	45 (70.3%)	0.632
High (>20000)	2 (2.2%)	3 (4.7%)	
Living place			
Rural	3 (3.3%)	1 (1.6%)	0.643
Urban	88 (96.7%)	63 (98.4%)	
Educational status			
Primary school (4 years)	21 (23.3%)	7 (10.9%)	
Middle school (4 years)	5 (5.6%)	8 (12.5%)	0.125
High school (4 years)	41 (45.6%)	29 (45.3%)	
University	23 (25.6%)	20 (31.3%)	
Occupational status			
Not working	56 (61.5%)	36 (56.2%)	0.512
Working	35 (38.5%)	28 (43.8%)	
"Do you think you use the phone too much?"			
Yes	32 (35.2%)	35 (54.7%)	*0.021**
No	59 (64.8%)	29 (45.3%)	
Time spent on smartphone (hours/day)			
<1	36 (39.6%)	10 (15.6%)	
1-2	32 (35.2%)	25 (39.1%)	
2-4	18 (19.8%)	20 (31.3%)	*0.008**
4-6	4 (4.4%)	6 (9.4%)	
>6	1 (1.1%)	3 (4.7%)	
BDI	11.77±7.85	14.59±8.186	*0.03**
BAI	10.51±9.15	14.19±10.98	*0.02**
A-ADHD-SRS			
Total	19.35±9.37	24.30±9.33	*0.001**
Inattentiveness	9.38±5.37	12.25±5.30	*0.001**
Hyperactivity/Impulsivity	9.97±5.50	12.05±5.10	*0.018**
NMP-A	60.45±24.11	74.44±23.51	*<0.001**
Without-NoMP (n)	46 (50.6%)	16 (25%)	*0.001**
With-NoMP (n)	45 (49.4%)	48 (75%)	

Data are presented as mean +/- SD or n (%), as appropriate; *Statistically significant, A-ADHD-SRS: Adult Attention Deficit Hyperactivity Disorder Self-report Scale, BAI: Beck Anxiety Inventory, BDI: Beck Depression Inventory, NMP-A: Nomophobia Questionnaire-Adolescents, NMP-M: Nomophobia Questionnaire-Mothers, NoMP: Nomophobia, SD: Standard deviation, TL: Turkish lira.

among adults are scarce. In a population-based study among adults, moderate/severe NoMP was more common in younger participants, with a prevalence of 58%.[4] The prevalence of specific phobia and separation anxiety disorder in adults is 2.6-12.5% and 6.6%, respectively.[24,25] However, the prevalence of NoMP, even though it is considered a type of phobia or separation anxiety disorder, was much higher than both. In some studies, excessive smartphone usage due

to professional responsibilities was associated with NoMP among young adults[23], while in this study, we did not find any differences in sociodemographic variables.

Girls were found to be more nomophobic in our study, which is consistent with many previous studies.[9,10,22] It is possible that girls are more open to seeking psychiatric services, which could explain their higher representation in this clinical sample.[26] Moreover, the prevalence of anxiety disorders is generally higher in girls and the definition of NoMP is discussed based on anxiety.[1,27] Girls also tend to use smartphones more for communication purposes[28], and NoMP is rooted in the fear of being unable to interact with others. These factors may contribute to the gender differences observed. However, it should be noted that some studies have found males to be more nomophobic[29], and while others have found no significant gender differences.[30] These discrepancies may be attributed to the variations in sample ages or the countries where the studies were conducted.

Emotional availability of parents refers to the emotional elements of the child-parent relationship, including parental support, sensitivity, warmth, and closeness. LEAP measures adolescents' perception of maternal and paternal emotional accessibility in terms of these factors.[10] In general the fathers tend to score lower than mothers on the LEAP scale.[31] The effect of gender on paternal LEAP scores is still controversial, with some studies reporting lower scores in girls[31,32] and others finding no gender effect.[33] Maternal emotional availability is considered more significant to a child's functioning than paternal availability.[10] However, the perception of emotional availability from the opposite-gender parent is often more important than that from the same-gender parent, particularly in daughter-father interactions. Fathers play a crucial role in introducing the world to their children, and this role is believed to be more critical for daughters during adolescence as they strive to gain autonomy and independence. Daughters may experience a more restrictive attitude from their fathers during this separation-individuation process.[34] In this study, the emotional accessibility of fathers was found to be lower and significantly related to NoMP, particularly in girls. This finding suggests that emotional distance from the father may contribute to a disrupted separation-individuation process, leading to NoMP. Additionally, high levels of NoMP in mothers could potentially influence higher NoMP levels in adolescent girls through role modeling.

Perceived social support from family was similar between the two groups of adolescents in our study. A study conducted during the COVID-19 pandemic found that greater family support protected against NoMP.[35] Some studies have found no relationship between social support and NoMP[36], while others reported a negative relationship.[37] These varying results suggest that social support may have a moderated effect on NoMP rather than a direct one. In Turkish culture, which is generally collectivistic, family connections are highly valued.[34] Therefore, it is expected that perceived social support from the family would be higher overall in our country.

In this study, we found no differences in depression, anxiety, and ADHD levels between mothers of nomophobic and non-nomophobic adolescents. The lack of association between maternal psychopathology and adolescent NoMP is interesting and similar to the findings observed in children with specific phobias.[38] Further research is needed to explore the relationship between maternal psychopathology, maternal NoMP, and adolescent NoMP.

In our study, we found that nomophobic mothers had higher scores on depression, anxiety, and ADHD scales. The anxiety experienced by mothers with NoMP may be attributed to the difficulty of reaching family members during emergencies.[39] Mothers with higher anxiety levels tend to check and use their phones more frequently to connect with their children. NoMP is associated with loneliness among adolescents[40], which could

also be applicable to adults. Mothers with NoMP exhibited more psychopathologies, and it is possible that psychopathologies could lead to or result from loneliness. Additionally, fathers with lower emotional availability may contribute less to the family, leading mothers to feel more lonely and burdened. A study found a relationship between parental depression and internet addiction.[41] Dysfunctional family dynamics increase the risk of smartphone addiction among adolescents.[42] Furthermore, a positive parent-child relationship reduces the likelihood of technological dependence in adolescents.[43] The amount of time adolescents and parents spend on smartphones is also related.[44] A higher amount of time spent by mothers on smartphones may indicate dysfunctional parenting or serve as a model for the child.

This study has many limitations. Firstly, the cross-sectional design does not allow for casual relationships to be established. Secondly, the sample was collected from a single tertiary care center, which limits the generalizability of the results. Thirdly, the use of self-reported scales introduces the possibility of reporting bias. Fourth, although NMP-Q was used in studies with adolesents[10], and the Cronbach's alpha value for NMP-A showed acceptable internal consistency in this study, the grouping of adolescents would differ with different threshold values. This could increase the risk of type I errors. Finally, it would be important to investigate whether the mothers in the study had a psychiatric diagnosis, as the scales used do not provide a diagnostic assessment.

Future studies should investigate the effects of fathers, marital harmony, and parenting styles on NoMP. Considering the treatment of NoMP, it is recommended to assess the entire family, particularly in the case of girls. Additionally, studies on NoMP in adults would provide valuable information about the impact of NoMP on parenting.

Acknowledgements

The authors thank Prof. Devrim Akdemir for her valuable comments.

Ethical approval

This research has been approved by the Hacettepe University Non-Interventional Clinical Research Review Board (GO 22/205). Written consent was taken from all adolescents and their mothers.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: YSÇ, BEA; data collection: YSÇ; analysis and interpretation of results: YSÇ, BEA; draft manuscript preparation: YSÇ, BEA. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 832-844
https://doi.org/10.24953/turkjped.2022.573

Original Article

Possible effects of N-acetylcysteine in autism spectrum disorders: major clinical aspects, eating behaviors, and sleeping habits

Kevser Nalbant[1], Semih Erden[2]

[1]Department of Child and Adolescent Psychiatry, Hacettepe University Faculty of Medicine, Ankara; [2]Department of Child and Adolescent Psychiatry, Necmettin Erbakan University Meram Faculty of Medicine, Konya, Türkiye.

ABSTRACT

Background. N-acetylcysteine (NAC) is a promising agent for reducing irritability and hyperactivity and enhancing social responsiveness in children with autism spectrum disorders (ASD). This study aims to examine the effects of NAC on cardinal symptoms, eating, and sleeping habits in preschool children with autism.

Methods. The medical records of ASD patients were investigated retrospectively. 37 children with ASD who regularly received oral NAC in two divided doses per day (400-600 mg/day) for 8 weeks were included as the study group. The control group consisted of 21 children with ASD who were recommended NAC but never used it. The initial and second assessment scores after 8 weeks of regular use of the NAC group and control group on the Childhood Autism Rating Scale (CARS), Aberrant Behavior Checklist (ABC), Children Eating Behavior Questionnaire (CEBQ), and the Sleep Habits Questionnaire (CSHQ) were compared.

Results. Our findings suggested that oral NAC alleviated the intensity of cardinal autistic symptoms in areas of social withdrawal, interpersonal relationships, body use, listening response, and verbal communication. Corresponding problem behaviors such as irritability, stereotypic behavior, and hyperactivity were reduced. It was determined that there was no difference between the two groups in terms of eating behaviors and sleeping habits.

Conclusions. According to the results, NAC alleviated the severity of cardinal symptoms and reduced problem behaviors in autism. Additional trials with more systematic planning, controlling for confounding effects, and long-term follow-up should be provided in future studies.

Key words: autism spectrum disorder, child and adolescent psychiatry, N-acetylcysteine, eating behavior, sleep habits.

Autism spectrum disorder (ASD) is accompanied by adversities in social interaction and communication, limited interests, and stereotypical behaviors.[1] The prevalence of autism has increased in recent years and affects approximately 2% of children.[2] Although there is no definite explanation for the etiopathogenesis of autism, it is thought that the interaction of environmental and genetic variables play a role.[3,4] It is known that oxidative stress, which can increase in response to both genetic and environmental variables, triggers many different diseases[5,6] and is considered to have a prominent role in the etiopathogenesis of autism.[7-9] The deficiency of effective treatments and the widespread diagnosis of autism indicate that more studies are needed.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is a constitutional antioxidant that helps scavenge free radicals and buffer the reactive products of oxidative reactions.[10] It presents in two forms, the oxidized form (glutathione disulfide/GSSG) and

✉ Kevser Nalbant
drkevser@yahoo.com

Received 9th August 2023, revised 2nd September 2023, accepted 6th September 2023.

the reduced form (GSH). GSH gives its electrons to free radicals and prevents cell damage. It also plays a role in other critical functions such as protein and prostaglandin synthesis, transport of amino acids, and enzyme activation.[11,12] Furthermore, glutathione acts as a depot for neuronal glutamate.[13] The dysregulation of the glutamate-glutamine cycle between glial cells and glutaminergic neurons affects synaptic excitability.[13] In research exploring the role of glutathione in the etiopathogenesis of autism, it has been noted that people with autism tend to have low glutathione reserves and low plasma and cellular glutathione levels.[8] A study emphasized that the glutamate-glutamine ratio was elevated in the amygdala–hippocampus areas in individuals with autism[14], and it has been claimed that raised glutamate levels may result in neuronal excitotoxicity, which may lead to inadequate inhibition of the prefrontal cortex and hypersensitivity of the amygdala.[8,9] Additionally, it has been shown that oxidative stress in the brain may affect the pathogenesis of autism by decreasing glutathione levels in the temporal cortex and cerebellum.[15,16]

N-acetylcysteine (NAC) contains the amino acid cysteine, which is required in glutathione synthesis.[17,18] NAC is clinically used in the treatment of glutathione deficiency such as some genetic and metabolic disorders. It is further reported that NAC is useful as adjuvant therapy in different medical conditions such as chronic lung diseases, sleep apnea, parkinsonism, multiple sclerosis, acquired immune deficiency syndrome, schizophrenia, bipolar affective disorder, and obsessive-compulsive disorder.[19] NAC is thought to be a potential drug for alleviating autistic symptoms due to its functions overlapping with both glutaminergic and oxidative stress hypotheses, which are thought to be related to the pathophysiology of autism.[20] In an animal study investigating the effectiveness of an NAC valproate-induced model of autism, it was reported that the administration of NAC in male rats increased GSH levels and reduced repetitive and stereotypical behavior of the rats.[21] In another study, valproate-induced autism rat models were divided into two groups and NAC was given to one group and saline to the other for 10 days starting from day 21 postpartum. At the conclusion of the study, it was noted that the period and frequency of social interactions increased and anxiety-like attitudes decreased in rats given NAC.[22] When human studies researching the effect of oral NAC supplementation in autism were reviewed, it was seen that few studies were conducted in this field. In a meta-analysis of randomized placebo-controlled studies, it was found that NAC reduced hyperactivity and irritability and improved social responsiveness in children with ASD.[20] In addition, it was emphasized that NAC could be considered as an off-label drug because it was a well-tolerated and cheap drug with limited adverse effects.[20]

In this study, the possible effect of oral NAC on the eating behavior and sleeping habits of children with autism was also examined. When studies investigating the relationship between NAC and eating behaviors were reviewed, no study investigating this relationship was found in the literature. When studies exploring the connection between NAC and sleep habits were reviewed; a study reported that NAC had an effect on the central processes associated with obstructive sleep apnea and positively affected sleep[23], which led us to investigate the possible effect of NAC on the relationship between sleep and autism.

Although studies have reported the oxidative stress hypothesis and dysregulation in the glutaminergic system in the pathophysiology of autism, this study was planned because the effects of oral NAC on different clinical aspects in children with autism remain unclear and there are limited studies in this regard, the objective of this study was to assess the impact of oral NAC on cardinal autistic symptoms, and to investigate its possible effects on eating behaviors and sleeping habits.

Material and Methods

Study center, sample

The files of 318 children who presented to the outpatient clinic at the Necmettin Erbakan University Meram Faculty of Medicine Department of Child and Adolescent Psychiatry and were diagnosed as having ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM 5)[24] between February 2018 and February 2019 were included in the study.

The inclusion criteria for the NAC group and control group at initial assessment were being between the ages of 3 and 6 years and recommended NAC supplementation for one or more of the following symptoms: very short attention span, hyperactivity, irritability, restlessness, and sleep or eating problems. The inclusion criteria in the NAC group at the second assessment were to use oral NAC 400-600 mg/day regularly for eight weeks. The inclusion criteria in the control group at the second assessment were children who were recommended NAC but whose family did not use NAC for various reasons. The exclusion criteria were as follows: receiving any other treatment other than individualized special education, current or past use of any psychotropic medication, any individualized special education program change, routine rating scales not completed or missing at the initial assessment, and follow-up, the presence of any additional chronic disease (e.g., epilepsy, neurometabolic disorder), a genetic syndrome associated with autism (e.g., fragile X syndrome, tuberous sclerosis), and active infectious diseases or obesity.

It was determined that 53 of the children met the inclusion and exclusion criteria and used oral NAC 400-600 mg/day regularly. Of the 53 children with ASD who used NAC regularly, 16 more files were excluded from the study; eight files due to incomplete screening scales in the 8th-week evaluation, six files due to additional antipsychotic medication, and two files due to additional methylphenidate treatment. Finally, the files of 37 children with ASD who received 400-600 mg/day NAC for 8 weeks, who had no changes in individualized special education programs during the study period, and who did not use any additional drugs/agents were included in the study. It was determined that 25 of the children met the inclusion and exclusion criteria for control group. Of the 25 children with ASD who were recommended NAC but whose family did not use NAC, 4 more files were excluded from study; three files due to incomplete screening scales in the 8th-week evaluation and one file due to use of additional antipsychotic medication.

It was determined that the included children used effervescent tablets containing NAC in two divided doses per day, children under 15 kg used 2x200 mg/day, and children above 15 kg 2x300 mg/day of NAC. In the outpatient clinic of the researcher, clinical rating scales are routinely used for tracking each child with ASD in terms of cardinal symptoms, behavior, speech, eating habits, and sleep monitoring. Of these scales, the Turkish Version of the Childhood Autism Rating Scale (CARS) was applied by the researcher, and the Aberrant Behavior Checklist (ABC), the Children Eating Behavior Questionnaire (CEBQ), and the Sleep Habits Questionnaire (CSHQ) was completed by the parents. Approval for the study was acquired from the ethics committee by the Ethics Committee of the Necmettin Erbakan University Meram Faculty of Medicine Non-Pharmaceutical and Medical Device Research on April 16th, 2021 (Decision No: 2021/3202).

Instruments and measures

Demographic data and clinical history

All data were retrieved from Sociodemographic and Clinical Information files.

Childhood Autism Rating Scale (CARS)

The validation and reliability study of CARS was carried out by Schopler et al.[25] CARS

comprises 15 items, and each of these items contributes equally to the calculation of the total score. Each of the 15 items is rated using half-point increments, ranging from 1 to 4. CARS is typically assessed based on information gathered from both family interviews and direct observations of the child by physicians. The items assessed in the CARS are as follows:: 1. interpersonal relationships, 2. imitation, 3. emotional response, 4. body use, 5. object use, 6. adaptation to change, 7. visual response, 8. listening response, 9. taste and smell responses, 10. use of touch, 11. fear/nervousness, 12. verbal communication, 13. nonverbal communication, 14. activity level, 15. level of intellectual response, and 16. general impressions. A total score in the range of 15-29 typically suggests that the child does not have autism. A score falling between 30-36.5 indicates mild-to-moderate autism. A score ranging from 37-60 is indicative of severe autism. The adaptation of CARS to the Turkish language was first performed by Sucuoglu et al.[26], followed by Gassaloğlu et al. who extended the validity and reliability analysis.[27] The Cronbach's alpha coefficient for the total score of the scale was determined to be 0.95.[27]

Aberrant Behavior Checklist (ABC)

ABC is rated by the parent (or primary caretaker) and confirmed by a physician. The ABC tool is employed to characterize and quantify behavioral challenges commonly observed in children diagnosed with ASD. ABC has 58 items that range from 0 = no problem at all, to 3 = the problem is of a significant or intense magnitude.[28] ABC items are categorized and scored into five different subscales, which are as follows (1) Irritability, agitation, crying; (2) Lethargy, social withdrawal; (3) Stereotypical behavior; (4) Hyperactivity/incompatibility; (5) Inappropriate speech. The Turkish adaptation and validity and reliability study was performed by Karabekiroğlu and Aman.[29] The Turkish version of the ABC demonstrated satisfactory internal consistency. The Cronbach's alpha values were calculated as follows: Irritability, 0.94; Lethargy/Social Withdrawal, 0.92; Stereotypic Behavior, 0.87; Hyperactivity, 0.65; and Inappropriate Speech, 0.87.[29]

Children's Eating Behavior Questionnaire (CEBQ)

The CEBQ was designed to classify children's eating behaviors, with a particular focus on identifying early signs related to obesity and eating disorders.[30] The Turkish adaptation, as well as the assessment of validity and reliability, were carried out by Yılmaz et al.[31] Cronbach's alpha coefficients ranged from 0.61 to 0.84.[31] The CEBQ consists of 35 items. The CEBQ is formed by eight sub-scales. These subscales are responsiveness to food, emotional overeating, enjoyment of food, desire for drinks, satiety responsiveness, slowness in eating, emotional undereating, and food fussiness. These subscales are collected in two groups under the headings 'positive eating responsive' and 'negative eating responsive.' The positive eating-responsive subscales include food responsiveness, enjoyment of food, emotional overeating, and desire to drink, and the negative eating-responsive subscales include satiety responsiveness, slowness in eating, emotional undereating, and food fussiness.

Children's Sleep Habits Questionnaire (CSHQ)

The CSHQ was developed by Owens et al. with the aim of assessing the typical sleep patterns and sleep-related issues in children between the ages of 4 and 10 years.[32] The CSHQ comprised of a total 33 items. There are eight subscales that screen sleep disorders in children according to the international sleep classification. The subscales in the scale are listed as bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night wakings, parasomnias, sleep-disordered breathing, and daytime sleepiness. A cumulative score of 41 points is established as the threshold, with scores surpassing this value regarded as 'clinically significant'. The validity and reliability study was performed by Perdahlı Fiş et al.[33]

The Cronbach's alpha coefficient for the internal consistency of the scale was found to be 0.78.[33]

Data analyses

Statistical analysis of the data was conducted using the Statistical Package for the Social Sciences, SPSS 21.0 software. The normality of the data was assessed through the utilization of the Kolmogorov-Smirnov test and an examination of Skewness and Kurtosis statistics. The Mann-Whitney U test and Spearman correlation tests were used for non-parametric data. Before and after analyses of the scales were compared using the paired-samples T-test or Wilcoxon signed rank test according to whether parametric or non-parametric test assumptions were met, respectively. P-values less than 0.05 were accepted for statistical significance except for the CARS items. The CARS items Bonferroni corrected significance level P-value was 0.002.

Results

When the NAC group and the control group were compared in terms of sociodemographic data, no statistical difference was found between the two groups. Sociodemographic data and clinical features are given in Table I.

When the NAC group and control group were compared according to the scale scores in the initial assessment, it was seen that there was no statistically significant difference between each group (Table II).

In the NAC group, the scale scores in the initial assessment and second assessment were compared. A statistically significant decrease was found in the children's CARS total score ($p<0.001$) and C1 (interpersonal relationships) ($p<0.001$), C4 (body use) ($p<0.001$), C8 (listening response) ($p<0.001$), and C11 (verbal communication) ($p<0.001$) items score (Table III). A statistically significant decrease was found in the ABC subscale irritability ($p=0.001$), social withdrawal ($p<0.001$), stereotypic behavior ($p<0.001$), hyperactivity ($p<0.001$), inappropriate speech ($p=0.006$), and total scores ($p<0.001$) (Table IV). In the CEBQ, only a statistically significant increase was found in the emotional undereating subscale score ($p=0.003$) (Table IV). In the CSHQ, only a statistically significant increase was found in the 'sleep onset delay' subscale score ($p=0.012$) (Table IV).

When the scale scores were compared between the NAC group and control group, a statistically significant decrease was found in the children's CARS total score ($p<0.001$) (Table V). Also, a statistically significant decrease was found in the ABC subscale irritability ($p=0.001$), stereotypic behavior ($p<0.001$), and hyperactivity ($p<0.001$) and, total score ($p= 0.010$) (Table V).

In this study, when the initial assessments and second assessments of the CEBQ subscale scores were compared in the NAC group, a significant increase in the 'emotional undereating' subscale scores was found ($p=0.003$) (Table III). The relationship of this subscale with other scales and subscales scores was examined, no relationship was found. Also, no significant difference was found when the NAC group was compared with the control group (Table V).

Table I. Demographic and clinical characteristics of children with autism.

	NAC group (n=37)	Control group (n=21)	p
Age (month), mean (SD)	59.81 (23.26)	64.24 (21.88)	0.48
Gender, male/ female	30 / 7	18 / 3	
Age of diagnosis (month), mean (SD)	27.68 (7.43)	26.24 (4.21)	0.35
Beginning of individualized special education training time (month), median (min-max)	26 (2-93)	34 (3-70)	0.39
NAC usage dose (mg/day), median (min-max)	500 (400-600)	-	-

NAC: N-aceylcysteine, SD: standard deviation.

Table II. Aberrant Behavior Checklist, Children's Eating Behavior Questionnaire and Children's Sleep Habits Questionnaire scores at initial assessment in NAC and control groups.

	NAC group (n=37) Mean (SD), Median (min-max)	Control group (n=21) Mean (SD), Median (min-max)	t or z	p
CARS Total	38.33 (4.53)	37.73 (2.34)	-0.333	0.73
ABC				
Total score	63.40 (34.43)	55.71 (15.80)	8.701	0.25
Irritability	15.32 (8.94)	16.19 (5.29)	5.632	0.64
Social withdrawal	16.00 (0 - 43)	14.00 (2 - 23)	-1.648	0.10
Stereotypic behavior	10 (1-19)	7.00 (0 - 15)	-1.709	0.09
Hyperactivity	16 (1-38)	11 (3-35)	-1.269	0.21
Inappropriate Speech	4 (0-12)	5 (0-9)	-1.062	0.28
CEBQ				
Food responsiveness	8 (5-24)	6 (5-22)	-0.973	0.33
Emotional overeating	5 (4-20)	5 (1-9)	-0.797	0.42
Enjoyment of food	15(5-25)	15 (11-20)	-0.122	0.90
Desire to drink	8 (3-14)	7 (2-10)	-0.984	0.32
Satiety responsiveness	21 (10-32)	16 (10-28)	-1.581	0.11
Slowness in eating	9 (4-20)	7 (5-18)	-0.952	0.34
Emotional undereating	9 (4-18)	7 (2-15)	-1.352	0.17
Food fussiness	7 (3-14)	7 (3-14)	-0.824	0.41
CSHQ				
Bedtime resistance	13 (9-16)	11 (9-16)	-1.614	0.10
Sleep onset delay	3 (1-3)	2 (1-3)	-1.662	0.09
Sleep duration	7 (5-9)	6 (5-8)	-1.890	0.059
Sleep anxiety	8 (4-12)	7 (4-12)	-0.838	0.40
Night wakings	4 (3-8)	5 (3-7)	-0.083	0.93
Parasomnias	9 (7-20)	8 (2-12)	-1.744	0.08
Sleep disordered breathing	3 (3-8)	3 (0-6)	-0.325	0.74
Daytime sleepiness	12 (10-19)	12 (10-17)	-0.534	0.59

ABC: Aberrant Behavior Checklist, CEBQ: Children's Eating Behavior Questionnaire, CSHQ: Children's Sleep Habits Questionnaire, NAC: N-acetylcysteine, SD: standard deviation.

Table III. Childhood Autism Rating Scale scores at initial and second assessment in NAC group.

	Initial assessment (NAC group) Mean (SD)	Second assessment (NAC group) Mean (SD)	t	p
CARS Total	38.33 (4.53)	33.21 (4.61)	11.298	<0.001
C1 (interpersonal relationships)	3.01 (0.32)	2.24 (0.48)	-4.850	<0.001
C4 (body use)	2.82 (0.37)	2.33 (0.45)	-4.225	<0.001
C8 (listening response)	2.62 (0.47)	2.29 (0.47)	-3.592	<0.001
C11 (verbal communication)	2.74 (0.56)	2.28 (0.57)	-3.756	<0.001

CARS: Childhood Autism Rating Scale, NAC: N-acetylcysteine, SD: standard deviation.

Table IV. Aberrant Behavior Checklist, Children's Eating Behavior Questionnaire and Children's Sleep Habits Questionnaire scores at initial and second assessment in NAC group.

	Initial assessment (n=37) Mean (SD) Median (min-max)	Second assessment (n=37) Mean (SD) Median (min-max)	t or z	p
ABC				
Total score	63.40 (34.43)	44.32 (22.08)	4.382	<0.001
Irritability	15.32 (8.94)	11.13 (5.62)	3.641	0.001
Social withdrawal	16.00 (0 - 43)	10.97 (6.37)	4.471	<0.001
Stereotypic behavior	10 (1-19)	6 (0-17)	-3.672	<0.001
Hyperactivity	16 (1-38)	12 (1-22)	-3.551	<0.001
Inappropriate Speech	4 (0-12)	3 (0-9)	2.951	0.006
CEBQ				
Food responsiveness	8 (5-24)	9 (5-25)	-1.032	0.30
Emotional overeating	5 (4-20)	5 (4-20)	-0.284	0.77
Enjoyment of food	15(5-25)	16 (6-25)	-0.777	0.44
Desire to drink	8 (3-14)	7 (3-14)	-0.312	0.75
Satiety responsiveness	20.52 (6.99)	20.36 (6.02)	0.295	0.76
Slowness in eating	9 (4-20)	9.50 (4-20)	-0.169	0.86
Emotional undereating	9 (4-18)	9 (4-17)	-2.990	0.003
Food fussiness	7 (3-14)	6.50 (3-13)	-0.035	0.97
CSHQ				
Bedtime resistance	13 (9-16)	13 (10-15)	-0.264	0.79
Sleep onset delay	3 (1-3)	3 (1-3)	-2.500	0.012
Sleep duration	7 (5-9)	7 (5-8)	-0.546	0.58
Sleep anxiety	8 (4-12)	8 (4-12)	0.437	0.66
Night wakings	4 (3-8)	4.50 (3-8)	-0.484	0.62
Parasomnias	9 (7-20)	9 (7-15)	-0.733	0.46
Sleep disordered breathing	3 (3-8)	3 (3-9)	-0.000	1.00
Daytime sleepiness	12 (10-19)	12.5 (10-18)	-1.064	0.28

ABC: Aberrant Behavior Checklist, CEBQ: Children's Eating Behavior Questionnaire, CSHQ: Children's Sleep Habits Questionnaire, NAC: N-acetylcysteine, SD: standard deviation.

In the CSHQ, only a statistically significant increase was found in the 'sleep onset delay' subscale score (p=0.012) (Table III). When the relationship of this subscale with other scales and subscales scores was examined no relationship was found and, no significant difference was found when the NAC group was compared with the control group (Table V).

This study included only children whose special education conditions did not change during NAC use. However, we have seen that the total duration of special education hours that children with autism receive per month is not standard (duration of individualized special education training time, 26 hours/month (min-max 2-93). We analyzed whether there was any relationship between the total hours of special education the children received and our scale scores (CARS, ABC, CEBQ, and CSHQ) in the first and second evaluations. The study results indicated that there was no statistically significant correlation between the length of time a child received individualized special education and the outcomes assessed using our measurement scales.

Table V. Aberrant Behavior Checklist, Children's Eating Behavior Questionnaire, and Children's Sleep Habits Questionnaire scores at second assessment in NAC and Control groups.

	NAC group (n=37) Mean (SD) Median (min-max)	Control group NAC (n=21) Mean (SD) Median (min-max)	t or z	p
CARS Total	33.21 (4.61)	36.66 (2.22)	4.940	<0.001
ABC				
Total score	44.32 (22.08)	57.33 (14.86)	4.124	0.010
Irritability	11.13 (5.62)	15.09 (4.25)	1.754	0.007
Social withdrawal	10.97 (6.37)	14.00 (4.69)	1.900	0.063
Stereotypic behavior	6 (0-17)	8 (0-17)	-2.056	0.040
Hyperactivity	12.08 (6.93)	16.38 (7.55)	2.182	0.033
Inappropriate Speech	3.72 (2.11)	3.90 (2.49)	0.733	0.733
CEBQ				
Food responsiveness	9 (5-25)	10 (5-23)	-1.482	0.13
Emotional overeating	5 (4-20)	6 (1-17)	-0.431	0.66
Enjoyment of food	16 (6-25)	15 (12-20)	-0.233	0.81
Desire to drink	7 (3-14)	7 (2-12)	-0.548	0.58
Satiety responsiveness	20.50 (11-35)	20 (10-29))	-1.037	0.30
Slowness in eating	9.50 (4-20)	9.40 (5-18)	-1.097	0.27
Emotional undereating	9 (4-17)	8 (3-15)	-1.516	0.13
Food fussiness	6.50 (3-13)	6 (3-12)	-0.917	0.35
CSHQ				
Bedtime resistance	13 (10-15)	12 (9-14)	-1.428	0.15
Sleep onset delay	3 (1-3)	2 (1-3)	-0.893	0.37
Sleep duration	7 (5-8)	7 (5-8)	-1.021	0.30
Sleep anxiety	8 (4-12)	8 (4-12)	-0.336	0.73
Night wakings	4.50 (3-8)	4.70 (3-6)	-0.067	0.94
Parasomnias	9 (7-15)	7 (2-13)	-2.238	0.25
Sleep disordered breathing	3 (3-9)	3 (0-5)	-0.196	0.84

ABC: Aberrant Behavior Checklist, CEBQ: Children's Eating Behavior Questionnaire, CSHQ: Children's Sleep Habits Questionnaire, NAC: N-acetylcysteine, SD: standard deviation.

Discussion

This was a retrospective file review study researching the effects of oral NAC on autism symptoms, problem behaviors, eating behaviors, and sleep habits. The study compared children with autism aged 3-6 years whose parents reported that they used oral NAC for at least 8 weeks, and children with autism who were recommended oral NAC but did not use NAC for any reason.

When the results of our study were reviewed regarding the difference between the first and the second assessments in the NAC group, it was found that there was a statistically significant progression in interpersonal relationships, body use, listening response, and verbal communication scores and total score of CARS. Also, there was a statistically significant progression in all subscales scores and total scores of ABC. When the results about the difference between the NAC group and the control group, it was shown that there

was a progression in the total score of CARS and, there was a progression in the irritability, stereotypic behavior, and hyperactivity subscales scores and total score of ABC. In addition, it was determined that there was an increase in 'emotional undereating' in terms of eating behaviors, and a rise in the 'sleep onset delay' in terms of sleep habits. However, no significant difference was found when the NAC group was compared with the control group.

When the studies were reviewed in this area, in a randomized, placebo-controlled, double-blind study researching the effects of NAC in children with ASD, ABC, the Repetitive Behavior Scale-Revised, and the Social Responsiveness Scale were evaluated at baseline, 4th, 8th, and 12th weeks. It was reported that there were 14 children in the NAC group and 15 children in the placebo group aged 3-10 years in which oral NAC had few adverse effects and was well tolerated, and the ABC irritability subscale improved remarkably in the NAC group compared with placebo.[34] In our study, a substantial improvement was found in all of the ABC scale's total scores and subscale scores. In our study, the average age of the participants was 4.5 years, and a more homogeneous group was formed in our study in terms of the age range. However, investigator or parent bias could not be controlled due to the design of our study. Nevertheless, these results suggested that the effect of NAC use at younger ages on autism symptoms should be examined in more detail to benefit from NAC.

In another randomized, placebo-controlled double-blind research examining the augmentation of NAC in risperidone treatment, the effect of using 2x600 mg/day NAC + risperidone for 8 weeks was investigated using the ABC scale. Upon concluding the study, it was found that risperidone + NAC reduced irritability more than risperidone + placebo. It has been reported that the adverse effects of NAC are not common and are generally well tolerated, but do not alter the core symptoms of autism.[35] In another study by Nikoo et al., it was reported that there was a meaningful improvement in the irritability and hyperactivity/incompatibility subscale scores of the ABC scale and that NAC could be considered an adjuvant treatment in the treatment of autism.[36] In another randomized, placebo-controlled, double-blind, 12-week follow-up research involving 31 children (aged 4-12 years) with autism that evaluated the effectiveness, safety, and tolerability of NAC, the effectiveness of NAC was demonstrated using Clinical Global Impression (CGI) and venous blood samples were collected at baseline and at 12 weeks to explore the effect of NAC on markers of oxidative stress in blood. At the conclusion of the study, it was reported that NAC therapy was well tolerated and had the expected effect in increasing reduced form glutathione (GSH) output, but had no discernable effect on social difficulties in children with ASD.[37]

When all these studies were reviewed, in four studies, except for the study by Wink et al., it was observed that irritability scores on the ABC scale improved. In our research, it was found that there was a statistically significant advancement in interpersonal relationships, body use, listening response, and verbal communication item scores and total score of CARS, and there was a statistically significant progression in all subscale scores and total score of ABC. In our study, it was found that the irritability score, which was 14.15 (SD 9.15) at baseline, decreased to 10.23 (SD 6.21) in the 8th week. The CGI-I scale is a superficial assessment tool compared with CARS, where improvement is scored based on the physician's observation. In our study, autism symptoms were compared using CARS, enabling us to make a more detailed and autism-specific assessment in terms of comparison of change. However, the interpretation of the results should be made in the terms of the limitations of the study. The most obvious limitation of our study is the retrospective review of data and

the absence of a placebo-controlled comparison group. Although this situation makes it difficult to generalize the results of the study to all children with autism, it is thought that it will be more appropriate to use evaluation tools that screen symptoms in detail when investigating the effect of NAC on autism symptoms in future studies.

In our study, the possible effect of oral NAC on the eating behavior of children with autism was also examined. It has been reported that children with autism are extremely selective in their eating habits.[38-40] In a meta-analysis, it was reported that children with autism had a 5-times greater risk of nutritional problems compared with children without autism, and lack of nutritional diversity put individuals at risk for nutritional deficiencies.[40] Although food selectivity is considered to be associated with sensory hypersensitivity, the exact reason is unknown.[41] In a recent study investigating emotional eating behavior in autism, it was reported that children with autism were more prone to emotional overeating and emotional undereating behavior compared with their typically developing peers.[42] Therefore, in our study, it was planned to examine the possible effect of NAC on eating behaviors. In our research, a notable increase in scores was found in the emotional undereating subscale. However, no significant difference was found when the NAC group was compared with the control group. To the best of our knowledge, there has been no study that has specifically investigated the connection between NAC and eating behaviors. It is therefore important to investigate the relationship between NAC and emotional undereating in future studies to establish a cause-effect relationship.

In our study, we also explored the impact of oral NAC on the sleep patterns of children with autism. It is known that children with autism experience sleep disorders, especially insomnia, at much higher rates than the typical population, and the etiopathogenesis of this condition has not been fully elucidated.[43] A study reported that NAC had an effect on the central processes associated with obstructive sleep apnea and positively affects sleep[23], which led us to investigate the possible effect of NAC on the relationship between sleep and autism. When the literature was reviewed, no study was found that explored the relationship between NAC and sleep patterns in autism. In the present study, it was determined that children with autism in the NAC group fell asleep significantly later in their second evaluation compared with their first evaluation. Considering that there is no previous study in this area, it is thought that research with larger samples is needed to enlighten whether the prolongation of time to fall asleep was associated with the NAC group. However, no significant difference was found when the NAC group was compared with the control group.

The strongest aspect of our study is that the effects of oral NAC use on eating behaviors and sleeping habits in autism were also investigated. As far as we know, there is no study investigating the effects of NAC on the eating behaviors and sleep habits of children with autism. Another strength of our study is the indication of the severity of autism symptoms using CARS in the evaluations and the comparison between the control group. Previous studies were conducted in a wider age range in this area. In our study, a more homogeneous group was formed by including only children with autism in the 3-6 years age group because autism is an early-onset disorder, and oxidative stress, which can increase in response to both environmental and genetic factors, is considered to be the trigger of various diseases.[5,6] Compared with other animals, human neuronal development continues to progress rapidly after birth, increasing the impact of environmental factors on neuronal development.[44] Therefore, it is important to uncover the possible role of environmental agents in autism at younger ages because many environmental agents can be modified, regulated, and configured.

The most essential limitations of this study were having a retrospective chart review design, no placebo control group, and including only children who attended a tertiary psychiatric clinic. These limitations prevent generalizing the findings to all children with autism. Also, using self-report scales involve disadvantages such as presuming that parents understand the evaluation method and include bias in the responses. Follow-up studies on a larger sample will help enhance the levelevidence.

As a result, our findings suggested that oral NAC might reduce cardinal autistic symptoms and problematic behaviors. It is known that the emotional difficulties of children with autism are associated with behavioral problems and social difficulties, and also negatively affect vegetative symptoms such as sleep and eating patterns. Therefore, based on our findings, it was thought that the effect of NAC on the cardinal symptoms of autism should be examined in more detail in future studies. Aside from its limitations, this study offers important data for future studies in this field. NAC may be considered an adjuvant therapy with helpful therapeutic results for preschool children with autism. Larger samples, randomized controlled, and longer follow-up studies are needed to research the possible effects of NAC on autism.

Ethical approval

This research was approved by the Ethics Committee of the Necmettin Erbakan University Meram Faculty of Medicine Non-Pharmaceutical and Medical Device Research on April 16th, 2021 (Decision No: 2021/3202).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: KN, SE; data collection: SE; analysis and interpretation of results: KN, SE; draft manuscript preparation: KN, SE. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 845-852
https://doi.org/10.24953/turkjped.2022.344

Case Report

Compound heterozygous mutations in the helicase *RTEL1* causing Hoyeraal-Hreidarsson syndrome with Blake's pouch cyst: a case report

Min He, GuoLi Lian, HaiPeng Hu, Huan He, Miaomiao Wang

Department of Pediatric, the First Affiliated Hospital of Xi'an Jiaotong University, Xi 'an 710061, People's Republic of China.

ABSTRACT

Background. Telomeres inhibit DNA damage response at the ends of the chromosome to suppress cell cycle arrest as well as ensure genome stability. Dyskeratosis congenita (DC), a telomere-related disease, includes the classical triad involving oral leukoplakia, dysplastic nails, and lacy reticular pigment in the neck and/or upper chest. Hoyeraal–Hreidarrson syndrome (HHS), a severe manifestation of DC, frequently occurs during childhood, and patients with HHS often show short-term survival and thus do not exhibit all mucocutaneous manifestations or syndromic features.

Case. We report here a patient with HHS characterized by the proband's clinical attributes, such as growth delay, bone marrow failure, microcephaly, defects in body development, and the absence of cerebellar hypoplasia combined with Blake's pouch cyst. By using exome sequencing, novel compound heterozygous mutations (c.1451C>T and c.1266+3del78bp) were detected in the *RTEL1* (regulator of telomere elongation helicase 1) gene.

Conclusions. The DNA helicase RTEL1 plays a role in genome stability, DNA replication, telomere maintenance, and genome repair. Terminal restriction fragment length analysis revealed a significantly shorter telomere length of the proband. Our findings provided evidence that compound heterozygous *RTEL1* mutations cause HHS.

Key words: Blake's pouch cyst, dyskeratosis congenita, telomere length, Hoyeraal-Hreidarsson syndrome.

Hoyeraal-Hreidarsson syndrome (HHS) was first reported in 1995 in a child who presented with cerebellar hypoplasia, progressive pancytopenia, microcephaly, prenatal growth delay, and developmental delay.[1] HHS, which is a severe manifestation of dyskeratosis congenita (DC), also involves additional complications of intrauterine growth restriction (IUGR), cerebellar hypoplasia, microcephaly, immunodeficiency, as well as developmental delay. RTEL1 (regulator of telomere length 1), a critical DNA helicase, disassembles various secondary structures of DNA to enable 3R processes as well as ensure telomere integrity. Biallelic mutations in the gene *RTEL1*, either homozygous mutations or compound heterozygous mutations, induce extreme telomere deterioration, thereby resulting in a severe clinical phenotype that manifests as DC and HHS in early childhood. In this case report, we describe the clinical features and determine the relationship between these variants and their clinical phenotypes.

✉ Guoli Lian
lianguoli73@163.com

Received 26th April 2022, revised 11th December 2022, accepted 26th January 2023.

Case Report

A 4.5-year-old boy with aplastic anemia was admitted to our hospital. His birth history included microcephaly and IUGR. At 17 months of age, his walk was still unbalanced. A marginal state was indicated by the Gesell Developmental Scale, with a gross exercise scale of 59. His psychomotor development was significantly delayed. He also had mild anemia. Blood

and urine levels of organic acid metabolites were normal. Brain MRI revealed mild cerebellar hypoplasia. He therefore underwent language and motor training for 10 months. At 28 months of age, the patient developed thrombocytopenia and moderate anemia. Aplastic anemia, diagnosed by bone marrow aspirate and biopsy, showed severe reduction in the cellularity of hematopoietic tissues, and the adipose tissue did not display any apparent clonal or cytogenetic abnormalities. The patient's karyotype was 46, XY. A negative result in the chromosomal breakage study ruled out the presence of Fanconi anemia. Paroxysmal nocturnal hemoglobinuria (PNH) was excluded based on the findings of flow cytometry. The patient was therefore started on oxymetholone, cyclosporine, and Chinese herbal medicine for treating bone marrow failure (BMF). Although anemia and thrombocytopenia became gradually severe, the patient required intermittent blood transfusion after the age of three. Throughout the illness, he had apparent developmental delay and failure to thrive.

Physical examination

The height, weight, and head circumference percentiles of the patient were 3–15th percentiles, ≤15th percentile, and <3rd percentile, respectively. He had dysarthria, along with growth and developmental delays. Hemorrhagic spots and a few ecchymoses were distributed throughout the body, with the absence of skin pigmentation and admixed hypopigmented macules. His eyesight was normal, with no exudative retinopathy or conjunctivitis. The buccal mucosa was free of white plaques. The patient had normal fingernails and toenails at presentation. No signs of cerebellar ataxia or wide-based gait were noted.

Remarkable diagnostic findings

Abnormal laboratory values at the time of admission were as follows: hemoglobin 6.8 g/dL, absolute neutrophil count 0.93×10^9/L, and platelet count 1×10^9/L. The patient completed the Chinese Wechsler Intelligence Scale for Children. The IQ score was 52, indicating mild intellectual disability. Brain MRI revealed expansion of the fourth ventricle and cisterna magna. Neuroimaging specialists confirmed that the cerebellum had fused without hypoplasia, and a Blake's pouch cyst had developed (Fig. 1). Genetic testing demonstrated that the *RTEL1* gene had compound heterozygous mutations (Fig. 2). A missense variant, namely g.20: c.1451C>T (encoding p.Pro484Leu, NM_001283009) was detected at the site of exon 17, a variant of uncertain significance (PP1 + PM2_Supporting + PP4 + PP3).[5] Another mutation, i.e., g.20: c.1266+3del78bp (p.Ile398_Lys422del, NM_001283009), observed in the patient was a *de novo* mutation in the index

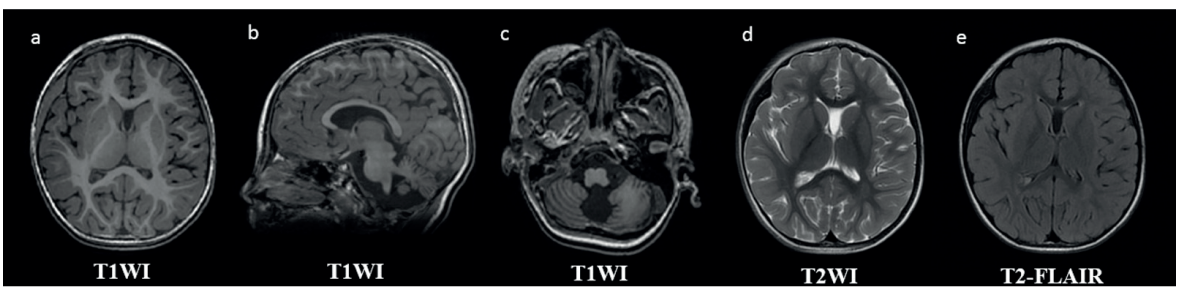

Fig. 1. Magnetic resonance imaging (MRI) of the brain: (a) Axial T1-weighted MRI of the brain shows normal findings; (b) Sagittal non-contrast T1-weighted MRI of the brain indicates the cerebellar volume; (c) Axial T1-weighted MRI demonstrates a relatively well-formed, nonrotated cerebellar vermis. The medial cerebellar hemisphere was symmetrically pressed, forming a typical keyhole-like fissure (Blake's pouch cyst); (d) Axial T2-weighted MRI of the brain demonstrates septum pellucidum; (e) Axial FLAIR images demonstrate normal findings.

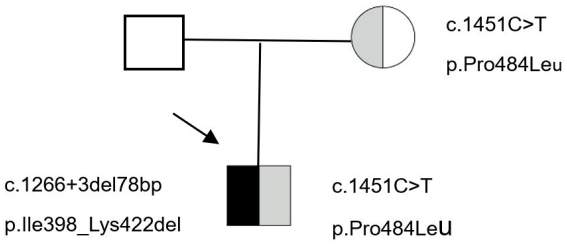

Fig. 2. Genealogical tree of the family. Arrow indicates the proband of the family. Open squares show noncarrier individuals. Half-filled circles represent individuals who are heterozygous for the *RTEL1* variant. Half-black and half-gray squares represent compound heterozygotes.

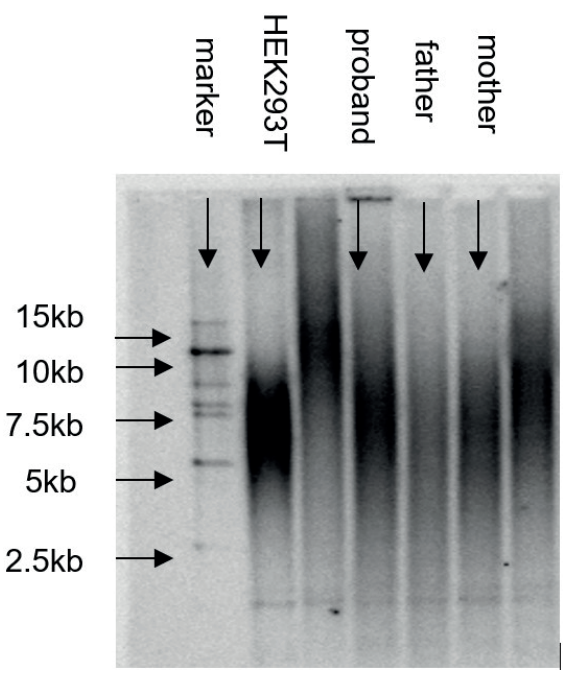

Fig. 3. Telomere lengths determined by terminal restriction fragment length analysis of HEK293T cell line and the proband and his father and mother.

case of this family. This mutation could not be identified even after bidirectional sequencing of both parents. Sanger sequencing and the work by Jullien et al.[3] demonstrated that c.1266+3del78bp results in the skipping of exon 15, thus causing an in-frame deletion of 25 amino acids (398-422), a pathogenic variant (PM3_Strong + PM4 + PS2_Moderate + PM2_Supporting + PP4).[3,4] Furthermore, to investigate the effects of *RTEL1* variants on telomere function, we determined the patient's overall TL, i.e., telomere length, in leukocytes by using terminal restriction fragment length analysis (Fig. 3). Visual inspection during electrophoresis is not easy to distinguish the difference in telomere length. The average telomere length of family members was shortened by database comparison, as determined by ImageQuant and ImagJ software calculations. According to the results, the proband had a telomere length distribution of 4.38-13.72 kb and an average telomere length of 6.88 kb. The laboratory database measured the length of telomeres in 3-year-old healthy children and found that the average length of 100 leukocytes was 7.32-7.62 kb (10th to 90th percentile). The proband's length of telomeres was below the 10th percentile. So we speculate that his telomeres are shortened. The distribution of the father's telomere length was 3.84-13.26 kb, with an average telomere length of 6.46 kb; the mother's distribution of telomere length was 4.10-13.54 kb, with an average telomere length of 6.63 kb. Their average telomere length was less than the laboratory database's average telomere length (6.75-7.05 kb), which did not match the biological age distribution (Figs. 4 and 5).

Oral oxymetholone and eltrombopag were administered. The platelet range was approximately 5–6×10^{10}/L. The patient did not undergo allogeneic BM transplantation until the 6-month follow-up.

Informed consent was received from the family for obtaining medical history and for publication of this case report.

Discussion

DC is a BMF-associated disorder and involves the triad of three classical manifestations of nail dystrophy, oral leukoplakia, and reticulate hyperpigmentation. Patients might not simultaneously exhibit these three

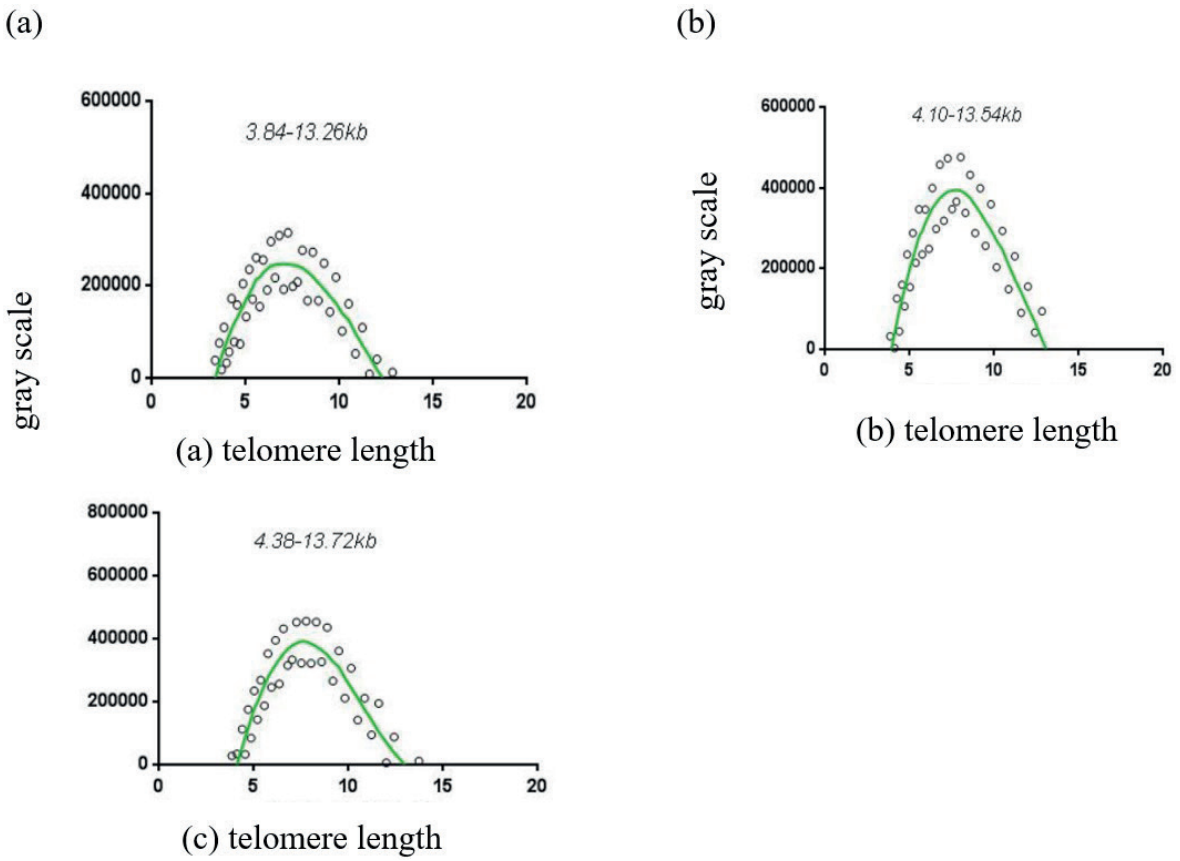

Fig. 4. Panels (a), (b), and (c) represent father, mother, and proband's telomere length, respectively.

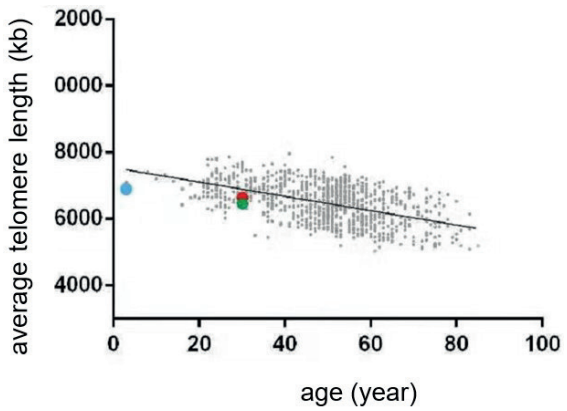

Fig. 5. Graph of mean telomere length and biological age. The proband's and parents' average TL were 6.88 kb, 6.46 kb, and 6.63 kb, respectively; all these values were shorter than the age-adjusted telomere lengths of unrelated, normal controls. The blue, green, and red circles show the proband, father, and mother, respectively.

features or may show other mucocutaneous findings, such as loss of dermatoglyphics, epiphora, palmoplantar hyperkeratosis, hyperhidrosis, and early graying and/or hair loss. HHS, which is a chronic variant of DC, involves the early onset of BMF, growth delay, immunodeficiency, developmental defects, microcephaly, and cerebellar hypoplasia. Our patient had some features of developmental delay, IUGR, cerebellar ataxia, microcephaly, and BMF; therefore, we suspected the diagnosis of inherited BMF.

By using exome sequencing, we identified compound heterozygous mutations were identified in the *RTEL1* gene. The *RTEL1* gene comprises 35 exons spanning approximately 40,000 bases of the genomic sequence on chromosome 20q13.33. Mutation in this gene can have a detrimental effect on DNA

repair and telomere integrity, resulting in the incorrect resolution of T-loops and thereby causing telomere shortening in humans. *RTEL1* is the first factor that was reported to be predominantly associated with HH rather than with DC.[5]

The missense mutation c.1451C>T (p.Pro484Leu) was one of the mutation sites. The proband's parents were asymptomatic, and his father was not a carrier. Sanger sequencing demonstrated that c.1451C>T was inherited from the mother. Stuart et al.[2] reported heterozygous *RTEL1* mutations (c.1451C>T, p.Pro484Leu) in patients with IPF (idiopathic pulmonary fibrosis). These results provided evidence that heterozygous *RTEL1* mutations cause IPF. Heterozygous *RTEL1* mutations are classified as an autosomal dominant trait in IPF and are thought to severely influence the stability and/or function of *RTEL1*.[6] Thus far, the autosomal dominant inheritance of *RTEL1* is reported only in IPF and is uncommon in hematological disorders.

Interestingly, c.1266+3del78bp, another mutation noted in this family, was a *de novo* mutation, which could not be identified by bidirectional sequencing of both parents. The same mutation site has been reported in a previous article, in which the compound heterozygous mutation of c.1266+3del78bp (p.Ile398_Lys422del) was detected in one patient, wherein the clinical features included microcephaly, global BMF, IUGR, cerebellar hypoplasia, growth delay, developmental delay, leukoplakia, and nail dystrophy.[4] By performing Sanger sequencing of exons 13–17, the authors confirmed that the mutation c.1266+3del78bp leads to exon 15 skipping and thereby results in an in-frame deletion of 25 amino acids (398-422).[7] The authors also identified 11 different *RTEL1* mutations in 10 patients from seven families, which was classified as an autosomal recessive trait. Biallelic mutations in *RTEL1*, either homozygous or compound heterozygous, induce extreme telomere deterioration and a severe clinical phenotype that manifests as HHS and DC in early childhood.[7-10]

Patients with HHS commonly show severely short telomeres in the blood. As the length of leukocyte telomeres typically shortens with age, children's telomeres are thought to be lengthier than those of their parents. In our case, the proband's TL in leukocytes was shorter than the age-adjusted TL. The TLs of his father and *RTEL1* heterozygous mother were also slightly shorter; however, his parents showed normal clinical phenotypes. Several observations manifest that their pathogenic effect in some of the carriers can be compensated by the functional wild-type allele *in vivo*. In addition, TL is also modified by other genetic variations and epigenetic modification, which might be involved in defects in telomere biology, telomere protection and/or replication. These identified telomere phenotypes and compound heterozygous mutations were consistent with the recessive compound heterozygous inheritance of HHS. The mutations of *RTEL1* are related to short leukocyte telomeres. TLs have a higher sensitivity and specificity for distinguishing patients with DC and HHS from those having other inherited BMF syndromes. Next, by using whole-exome sequencing, we investigated whether the parents carried mutations of other genes that can cause telomere shortening to determine the reason for the absence of clinical manifestation.

Thus far, 18 *RTEL1* mutations have been reported in 17 HHS patients from 14 families: 15 missense (M492I, E251K, A621T, E591D, L710R, I699M, V745M, G739V, R957W, K897Q, R974X, F964L, C1244R, R986X, and R1264H), 1 deletion (del398-422,422-446) and 2 intronic splicing mutations (IVS24+5G>A and c.102+2T>C).[4,7-9] Most *RTEL1* mutations detected were biallelic mutations, with either homozygous or (mostly) compound heterozygous autosomal recessive inheritance. The heterozygous *RTEL1* variants are associated with BMF. The mutations were located mostly in helicase, harmonin-like or C-terminal RING finger domains, which probably participates in ubiquitin transfer or protein–protein interactions. TL measurement alone might not be able to identify patients

carrying *RTEL1* variants and having telomere dysfunction.[12] An accurate genotype–phenotype study of patients carrying *RTEL1* mutations and a comprehensive functional and biochemical analysis of *RTEL1* mutant activities are needed to elucidate pathogenesis.

Blake's pouch cyst, a rare condition, belongs to the Dandy–Walker continuum. In 1900, J. Blake reported first reported that the tela choroidea of the fourth ventricle of a normal human embryo of 130 days old had a transient posterior evagination.[13] He recognized the foramen of Magendie that functions as an aperture and develops within a saccular expansion of the fourth ventricular cavity of the embryo.[13,14] Blake's pouch fails to regress following the nonperforation of the foramen of Magendie, with a subsequent expansion of the fourth ventricle and the supratentorial ventricular system.[15] As the larger foramen of Magendie does not exist, the ventricles remain enlarged with compression rather than underdevelopment of the cerebellar hemispheres and vermis. Because of growth factor or receptor expression, the choroid plexus fails to develop normally, along with the tela choroidea's fenestration delays.

In neuroimaging, Blake's pouch cyst can be easily misdiagnosed as cerebellar atrophy because of a lack of sufficient experience of the clinician. Typically, the radiological features of Blake's pouch cyst are as follows: (1) infra- or retrocerebellar localization of the cyst, (2) tetraventricular hydrocephalus, (3) a relatively well-formed, nonrotated cerebellar vermis, (4) cystic dilation of the fourth ventricle with no cisternal communication, and (5) compression on the medial cerebellar hemispheres to some extent.[15] Hence, the findings of the patient's brain MRI at 17 months were misdiagnosed as mild cerebellar hypoplasia, in which the cerebellar vermis was compressed. The differential diagnosis of Blake's pouch cyst includes all remaining posterior fossa cysts present in the Dandy–Walker complex, cyst-like malformations, and posterior fossa arachnoid cysts. The clinical presentation of Blake's pouch cyst varies, and it can be treated by shunting or an endoscopic third ventriculostomy.[16] Because our patient had no symptoms of intracranial hypertension and brain tissue compression, the neurosurgeon did not conduct any intervention. *ZIC2* mutations, haploinsufficiency of genes in the distal 13q region, and aneuploidy (47, XY, +18) have been reported to cause posterior fossa malformations. However, it remains unclear whether *RTEL1* mutations are associated with cysts.

Specific treatments must be tailored for each patient. Oral androgen agents such as oxymetholone have been successfully employed for more than 50 years for treating aplastic anemia, including BMF in Fanconi's anemia and DC; this treatment improves blood count, reduces transfusion dependence, as well as alleviates liver fibrosis and stabilizes lung function in patients with telomere biology disorders.[17-20]

Unlike acquired aplastic anemia, BMF in DC does not show any response to immunosuppressive therapy.[21] The thrombopoietin receptor (c-Mpl) agonist eltombopag is currently used as a frontline treatment in combination with immunosuppressive therapy for curing severe aplastic anemia. The systematic analysis of eltrombopag use in patients with telomere biology disorders remains to be conducted. Presently, there is a single report of two patients with DC who were treated with eltrombopag for BMF; however, both the patients did not show any therapeutic response.[22] BMF, the primary factor of mortality in DC patients, can be definitively treated with allogeneic HCT (hematopoietic cell transplantation). HCT is presently the sole curative approach for BMF; however, historically, it has dismal long-term efficacy.[23] Several other investigational therapies are useful for treating telomere biology disorders. In previous studies, PAPD5 inhibitors restored the activity of telomerase and TL in pluripotent stem cells induced by DC, which extended TL. TA-65, which is a small-molecule telomerase activator used in

telomerase gene therapy, can improve blood count as well as survival in mice with aplastic anemia.[24,25]

Acknowledgement

We are grateful to the patient's family for their support in sample collection and retrieval of information, which enabled to conduct this research. We thank the Kangso Medical for Genome sequence analysis for generating the data of exome sequences. We would like to thank TopEdit (www.topeditsci.com) for its linguistic assistance during the preparation of this manuscript.

Ethical approval

Informed consent was received from the family for obtaining medical history and for publication of this case report (No. XJTU1AF20221LSK-187).

Author contribution

Clinical data and preparation of the manuscript: Min He; laboratory analysis: Huan Huan; clinical data: HaiPeng Hu and MiaoMiao; statistical analysis: Min He; clinical advisor: Guoli. All authors reviewed the study results and have approved the final version of this manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 853-861
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Case Report

Cinacalcet therapy in a child with novel homozygous *CASR* p.Glu353Lys mutation causing familial hypocalciuric hypercalcemia type 1: case report and review of the literature

Serkan Bilge Koca

Division of Pediatric Endocrinology, Department of Pediatrics, University of Health Sciences, Kayseri City Hospital, Kayseri, Türkiye.

ABSTRACT

Background. Familial hypocalciuric hypercalcemia (FHH) is one of the conditions that should be considered in the differential diagnosis of hypercalcemia and normo-hypophosphatemia in childhood. Heterozygous Calcium-sensing receptor (*CASR*) gene mutations cause FHH, and homozygous *CASR* gene mutations cause neonatal severe primary hyperparathyroidism (NSHPT). Cinacalcet is an allosteric modulator of Calcium-sensing receptor (CaSR), and has been used in the treatment of these clinical entities in recent years.

Case. A 26-month-old boy was examined for a recurrent rash. During the evaluation, hypercalcemia (13.3 mg/dL), hypophosphatemia (2.3 mg/dL) and inappropriately normal PTH level (67 pg/mL) were observed. Neck and renal ultrasonography were normal. The parathyroid scintigraphy was unremarkable. The patient's family members were also evaluated, and hypocalciuria (fractional excretion of calcium were 0.01%, 0.04% on two separate tests) was detected concurrently with the patient's hypercalcemia. The mother's serum calcium was 10.2 mg/dL, the father's was 10.6 mg/dL, and the brother's was 12.8 mg/dL. *CASR* gene sequencing showed a novel homozygous mutation in exon 4 (c.1057G>A), which had generated a substitution of the amino acid glutamate to lysine at codon 353 (p.Glu353Lys). This mutation was homozygous in the children and heterozygous in the parents. Fluid hydration, furosemide, oral phosphorus, prednisolone, pamidronate and cinacalcet treatments were used in the management of hypercalcemia of the proband. A longer and more effective control was achieved with cinacalcet treatment.

Conclusions. FHH can be seen in heterozygous as well as homozygous *CASR* gene mutations. Different clinical findings may occur in different individuals from the same family. Cinacalcet therapy can be used successfully in the treatment of individuals with FHH.

Key words: calcium-sensing receptor (CaSR), cinacalcet, familial hypocalciuric hypercalcemia, hypercalcemia.

Calcium-sensing receptor (CaSR) is a receptor from the Class C family of the G protein coupled superfamily, encoded by exons 2-7 of the *CASR* gene located in the chromosome 3q13.3-21 region, consisting of 1078 amino acid residues and 3 structural domains.[1] CaSR and its signal transduction pathway detects changes in extracellular calcium levels, regulates parathormone (PTH) secretion, PTH gene expression, parathyroid cell proliferation, urinary calcium excretion, and controls calcium homeostasis.[2] *CASR* is mainly expressed in parathyroid chief cells, kidney tubule cells, thyroid C cells, bone, gastrointestinal tract, placenta, pancreas and brain cells.[3] The signal transduction pathway of CaSR also requires, the G-protein α-11 (Gα11) subunit encoded by the *GNA11* gene and the adapter-related protein complex 2s (AP2s) subunit encoded by the *AP2S1* gene, to be intact. Familial hypocalciuric hypercalcemia (FHH) is a group of metabolic diseases associated with CaSR

✉ Serkan Bilge Koca
kocaserkanbilge@yahoo.com.tr

Received 7th December 2022, revised 2nd May 2023, accepted 2nd July 2023.

and signaling pathways, characterized by persistent hypercalcemia, hypophosphatemia, hypermagnesemia, normal or mildly increased serum parathyroid hormone levels, and low urinary calcium excretion.[4] Neonatal severe hyperparathyroidism (NSHPT) is observed in those with homozygous loss of function mutations of the *CASR*, and FHH observed in individuals with heterozygous mutations.[5] Inactivating mutations of the *CASR* cause the calcium setting point to sense serum calcium at higher calcium levels, inappropriately increase PTH secretion, and increased renal tubular calcium reabsorption. FHH type 1 has a benign course and usually does not require medical treatment. In patients with hypercalcemia, it may not be possible to clinically distinguish NSHPT from FHH. Even in family members with the same mutation, clinical findings may be different.[6,7] Herein, we present siblings with moderate hypercalcemia who had a novel homozygous *CASR* mutation, and their parents with heterozygous mutations. In addition, we review the treatment management for hypercalcemia in these patients.

Case Report

Because standard care is applied, an ethics committee approval form is not required. This study was carried out in compliance with the terms of the Declaration of Helsinki. Informed consent form was obtained from the patient's family for the findings shared in this case presentation.

Case 1

A 26-month-old boy was admitted to the pediatric outpatient clinic due to recurrent widespread nonpruritic macular rashes on the body that had occurred in the last 2-3 days. He was referred to the endocrinology department for further evaluation and treatment with the diagnosis of hyperparathyroidism, since high serum calcium (13.3 mg/dL, reference range: 8.6-10.2 mg/dL) and low phosphorus levels (2.3 mg/dL, reference range: 3.8-6.5 mg/dL) were detected in the laboratory tests taken in his first evaluation, and the parathyroid hormone (PTH) level was inappropriately normal (PTH: 67 pg/mL, reference range: 15-65 pg/mL) while the serum calcium was high in the simultaneous repeated examinations. The patient was a Turkish male, born at 38 weeks of gestation after an uncomplicated cesarean section delivery. The patient's birth weight was 2800 g. The neurodevelopmental stages of the child and, eruption and replacement times of deciduous teeth were similar to those of his peers. There was no consanguinity between the mother and father. There was no family history of hypercalcemia, parathyroid or kidney disease, or urolithiasis. On physical examination at admission, his body weight was 13 kg (0.02 SDS), height was 82 cm (-1.73 SDS), body mass index was 19.3 kg/m^2 (1.76 SDS), and head circumference was 50 cm (0.77 SDS). Vital signs were as follows: pulse rate, 110 beats/min; blood pressure, 90/50 mmHg (63th and 82th percentile, respectively). His systemic examination was unremarkable except for rashes.

There were no findings suggestive of a bone lesion in the X-rays of the bilateral limbs, wrist and skull. Neck and kidney ultrasonographies were unremarkable. We prescribed fluid infusion, furosemide and oral phosphorus solution to treat his hypercalcemia and hypophosphatemia. However, repeated biochemical tests were consistent with hypercalcemia (13.5 mg/dL, reference range: 8.6-10.2 mg/dL), hypophosphatemia (2.3 mg/dL, reference range: 3.8-6.5 mg/dL), normomagnesemia (2.1 mg/dL, reference range: 1.6-2.6 mg/dL), inappropriately normal PTH level (64 pg/mL, reference range: 15-65 pg/mL), low TMP/GFR (1.65 mg/dL, reference range: 2.9-6.5), and low urinary calcium excretion (fractional excretion of calcium were 0.01%, 0.04% on two separate tests). Serum 25-OH vitamin D was 22 μg/L. Although the patient had biochemical blood tests compatible with

hyperparathyroidism and a low TmP/GFR ratio, he did not have hypercalciuria. Serum calcium level of the mother was 10.2 mg/dL and the father was 10.6 mg/dL, respectively. For this reason, we planned a genetic analysis for known genes for familial hypocalciuric hypercalcemia; *CASR*, G protein subunit alpha 11 (*GNA11*) gene, and adaptor related protein complex 2 subunit sigma 1 (*AP2S1*) gene. Predisolone was started because serum calcium was above 13 mg/dL in the follow-up. However, there was no decrease in serum calcium in the 1-week period. Therefore, a single dose of 1 mg/kg pamidronate was administered intravenously. Serum calcium was 10.7 mg/dL at the 48th hour after pamidronate treatment. However, in the first week control, serum calcium of 12.2 mg/dL, phosphorus of 1.3 mg/dL, magnesium of 2.5 mg/dL, alkaline phosphatase (ALP) of 119 U/L and PTH level of 467 pg/mL were detected. We also performed a parathyroid scintigraphy for parathyroid adenoma or hyperplasia, but the 99mTc-sesta MIBI tomoscintigraphy was negative. A diet program was set to provide the recommended daily amount of calcium for age.

CASR sequencing showed a novel homozygous mutation in exon 4 (c.1057G>A), which had generated a substitution of the amino acid glutamate to lysine at codon 353 (p.Glu353Lys). This single base exchange causes a missense mutation. The detected change has not been reported in another patient before in the literature (novel variant). The change detected according to the ACMG criteria was evaluated as a "variant of uncertain clinical significance" (VUS). We investigated mutant CaSR protein function prediction using the online protein prediction program Mutation T@sting (http://www.mutationtaster.org/) and Polyphen (http://genetics.bwh.harvard.edu/pph). Both of these predicted that the substitution of the amino acid glutamate at codon 353 for lysine would affect this protein's function and cause disease.

Due to the persistence of moderate hypercalcemia, off-label consent was obtained and cinacalcet treatment was initiated. Initially, cinacalcet therapy was administered as 15 mg per dose, in 2 doses per day. Since serum calcium was 12.9 mg/dL at the 1st month follow-up, his treatment was adjusted to 30 mg per dose, with a total daily dose of 60 mg. While his treatment continues in this way, serum calcium of 11.3, phosphorus of 3.5 mg/dL, ALP of 237 U/L, PTH of 72 pg/mL were detected in the 1st month of the treatment; and serum calcium of 10.6 mg/dL, phosphorus of 3.5 mg/dL, ALP of 561 U/L, and PTH of 48 pg/mL were detected in the 3rd month of the treatment. While the cinacalcet treatment was continuing, we noticed the elevation of ALP, therefore, vitamin D was included to the treatment at a maintenance dose (600 IU/day). When vitamin D was administered at a maintenance dose, ALP reached normal reference values. The patient has been successfully treated with cinacalcet for 12 months. We did not observe any adverse effects (nausea, vomiting, or hypocalcemia) while cinacalcet treatment was continued.

Case 2

The patient's 4 years 4 month old brother was also examined, it was observed that he also had hypercalcemia (12.8 mg/dL, reference range: 8.6-10.2 mg/dL), hypophosphatemia (3.6 mg/dL, reference range 3.7-5.6 mg/L), normomagnesemia (2.5 mg/dL, reference range: 1.6-2.6 mg/dL), inappropriately normal PTH level (37 pg/mL, reference range: 15-65 pg/mL), and hypocalciuria (fractional excretion of calcium was lower than 0.01%). 25-OH vitamin D was found to be 32 μg/L. The same gene variant was also detected in his sibling. We found that this gene variant was homozygous in both siblings, and heterozygous in both parents. Next generation sequencing results of the family members are shown in Fig. 1. Consequent Sanger sequencing results of the family members are shown in Fig. 2.

Fig. 1. Next generation sequencing results of the proband and family members for *CASR*.

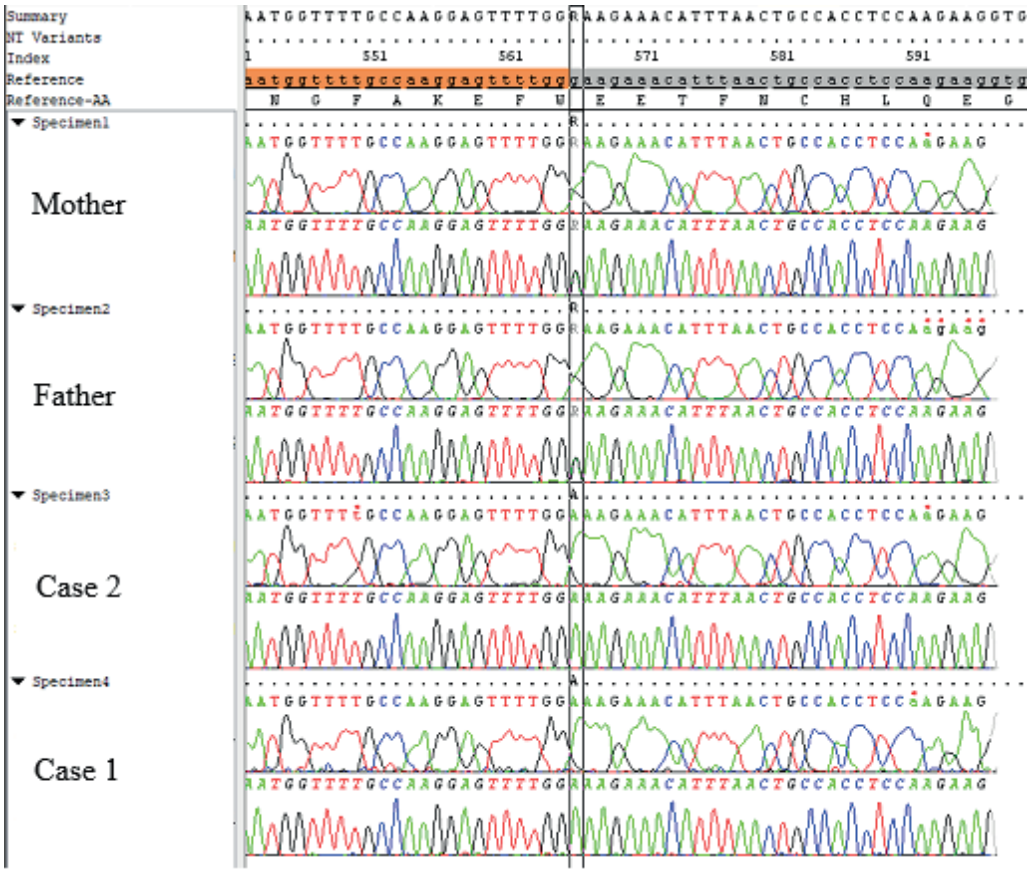

Fig. 2. Sanger sequencing results of the proband and family members for *CASR*.

Due to the persistence of moderate hypercalcemia (serum Ca: 12.7 mg/dL), off-label consent was obtained and cinacalcet treatment was initiated, with a procedure similar to that of his brother, with the consent of the family. Initially, cinacalcet therapy was administered as 45 mg per dose, in 2 doses per day, for a total daily dose of 90 mg. Serum calcium of 10.3 mg/dL, phosphorus of 5.1 mg/dL, ALP of 303 U/L, PTH of 36 pg/mL were detected in the 3rd month of the treatment.

The biochemical values of all family members at the time of first application are shown in Table I.

Table I. Biochemical values of family members at the time of application.

	CaSR p.Glu353Lys mutation	Ca (mg/dL)	P (mg/dL)	Mg (mg/dL)	ALP (U/L)	PTH (pg/mL)	Fractional excretion of calcium
Proband	Homozygous	13.5	2.3	2.1	196	64	0.01%
Sibling	Homozygous	12.8	3.6	2.5	285	37	<0.01%
Mother	Heterozygous	10.2	3.2	2.1	67	68	<0.01%
Father	Heterozygous	10.6	4	2.2	77	38	<0.01%

ALP: alkaline phosphatase, CaSR: calcium-sensing receptor, PTH: parathyroid hormone.

Discussion

In this case report, we present the differences in clinical and biochemical findings in family members with novel homozygous and heterozygous *CASR* mutations. This previously undescribed mutation was detected at codon 353 (p.Glu353Lys) in exon 4 of the *CASR*. We could not perform a functional study. However, unlike the parents, this mutation, which was found to be homozygous in both siblings, caused moderate hypercalcemia. As we found in our cases, missense mutations are the most common type of mutation.[8]

FHH is comprised of three genetically distinct conditions. *CASR*, *GNA11* and *AP2S1* gene mutations are responsible for FHH1, FHH2 and FHH3, respectively. The most common clinically observed type is *CASR* mutations.[9] Although the general opinion is that homozygous *CASR* mutations cause NSHPT and heterozygous mutations cause FHH type 1, it has been shown in the literature that some homozygous mutations cause FHH and some heterozygous mutations cause NSHPT.[5,10-13] Depending on how the mutation affects the function of the protein and which region it affects, the clinical findings and even the response to treatment may vary.[12,14]

Inactivating mutations in *CASR* lead to dysfunction through several different mechanisms. The expression of the receptor on the cell surface may be impaired, which may result from decreased biosynthesis and/or defects in its trafficking from the endoplasmic reticulum to the cell membrane. While the expression of the receptor on the cell surface is relatively normal, there may be a decrease in the ability of the receptor to bind to a ligand or to couple with G proteins. In addition, mutations can lead to increased degradation of the receptor, resulting in decreased expression of the mutant receptor on the cell surface.[1,15]

The majority of cases characterized by hypercalcemia are associated with hyperparathyroidism. It can be primary, secondary or tertiary. On the other hand, 95% of hyperparathyroidism is sporadic and 5% is associated with hereditary syndromes. These are NSHPT, isolated familial hyperparathyroidism, multiple endocrine neoplasia syndromes (MEN-1, MEN-2A, MEN 4), hyperparathyroidism-jaw tumor syndrome and FHH.[16] An increase in PTH levels can also be observed in vitamin D deficiency.

FHH is mostly asymptomatic. In some cases, nonspecific findings such as muscle weakness and fatigue, and in some cases more serious findings such as pancreatitis, chondrocalcinosis, and osteomalacia may be observed. Failure to thrive, hypotonia, respiratory distress, and life-threatening neonatal hypercalcemia may be observed in NSHPT cases. In addition, cases with hypercalciuria, renal calculi and parathyroid adenoma have also been reported.[17]

During the medical management of hypercalcemia, dietary calcium restriction, hydration, loop diuretics such as furosemide, oral phosphate, calcitonin, glucocorticoids, and bisphosphonates are used.[18] Calcitonin can reduce osteoclastic resorption in bones, but its effect is known to be transient. Bisphosphonates, which act by reducing bone resorption, are used in the treatment of hypercalcemia in children with FHH and NSHPT. Although short-term use of bisphosphonates is reasonable in this respect, our knowledge of their long-term adverse effects is limited. In addition, in some cases treated with bisphosphonates, a temporary decrease in calcium levels and then a re-increase has been reported.[19-21] A decrease in ionized calcium and an increase in PTH levels in the acute period after pamidronate treatment are expected findings in the clinical course and there are cases reported in the literature.[22]

Calcimimetic agents such as cinacalcet can alleviate hypercalcemia and reduce high PTH levels and have been used in the treatment of FHH and NSHPT in recent years. NSHPT cases with and without response to cinacalcet treatment have been reported in the literature.[23,24] It has also been suggested that if there is a mutation in

CASR that affects the Ca binding domain or the cinacalcet binding domain, cinacalcet treatment may not be effective regardless of whether the mutation is homozygous or heterozygous.[14]

In NSHPT, there are case reports where cinacalcet treatment has been used in the newborn or infant period in the dose range of 0.4 to 9.6 mg/kg/day or at doses of 20-202 mg/m2/day, and it has been observed to be successful.[12,14,19,24-26] A case with NSHPT was also reported regarding the safe use of cinacalcet monotherapy for 8 years up to parathyroidectomy.[17] A case of cinacalcet treatment given on the postnatal second day and treated successfully for 18 months was also reported from our country.[27]

Case series in which cinacalcet treatment was administered in relation to FHH in childhood were shared. Complaints such as constipation, enuresis, polyuria, nocturia, leg pain were reported in these cases.[28] Two 6- and 7-year-old boys, who were treated with cinacalcet for 4 years, and an 8-year-old girl who used it at a daily dose of 240 mg were reported.[28] However, although FHH is considered benign and asymptomatic, symptoms affecting daily activities can sometimes be overlooked.

In contrast to the homozygous *CASR* mutations presenting at an early age in the literature, clinically mild-moderate hypercalcemia was observed in our index case and his brother. In our proband, conditions such as having a higher calcium level than the older sibling and being symptomatic at an earlier age (recurrent rashes) were indications for using cinacalcet treatment. In addition, an elevation of more than 1 mg/dL above the upper limit of serum calcium in the index case and his sibling and its persistence also guided us in terms of giving calcimimetic treatment.[29]

In conclusion, familial hypocalciuric hypercalcemia is one of the conditions that should be kept in mind among the causes of hypercalcemia and hypophosphatemia, although it is rare in childhood. If the parathyroid gland scintigraphy is normal in clinically suggestive primary hyperparathyroidism, the *CASR* mutation should be the first genetic investigation step to be studied. FHH can be seen in heterozygous as well as homozygous *CASR* mutations. There may be different clinical findings and differences in the severity of disease among family members.[6] Although it differs according to the mutation type in cases with *CASR* mutation, cinacalcet treatment can provide a longer and more effective control than bisphosphonate treatment.

Ethical approval

Since this study is a case report, ethics committee approval is not required. Declaration of Helsinki criteria were taken into account. Written informed consent form was obtained from the family.

Author contribution

The author confirms contribution to the paper as follows: study conception and design: SBK; data collection: SBK; analysis and interpretation of results: SBK; draft manuscript preparation SBK.

Source of funding

The author declares the study received no funding.

Conflict of interest

The author declares that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 862-867
https://doi.org/10.24953/turkjped.2023.138

Case Report

Childhood borderline lepromatous leprosy: a case report

Grace Dumamawarni Hutahaean[1], Martin Susanto[1], Tiopan Napitupulu[1], Andre Marolop Pangihutan Siahaan[1], Beny Atmadja Wirjomartani[2]

[1]Faculty of Medicine, University of Sumatera Utara, Medan, Indonesia; [2]Faculty of Medicine, University of Padjadjaran, Bandung, Indonesia.

ABSTRACT

Background. Leprosy in children is a strong indicator of the recent failure of leprosy control and disease transmission programs. For twenty-two years, leprosy has been declared 'eliminated as a public health hazard,' yet new cases continue to emerge in endemic areas. The new case detection rate among the child population was recorded at 4.4 per million children. Because of their underdeveloped or neonatal immunity and exposure to intrafamilial contacts, children tend to be the most vulnerable population.

Case. We present a case of the borderline lepromatous type of leprosy in a 9-year-old Indonesian male patient with the chief complaint of three stiff fingers on his left hand that began four years ago and hypopigmented patches on the back and buttocks that began five years ago. In this case, there was a history of leprosy in his mother's sister, who had died. Leprosy in the patient was suspected of possibly being transmitted from his mother's sister who had intense contact with the patient. The results of bacteriological examination with Ziehl-Neelsen staining of tissue scrapings found acid-fast bacilli. He was treated with a multibacillary multidrug regimen for 12 months. Periodical observations after the patient received the treatment revealed no new spots on the patient's skin, some of the previous hypopigmented patches seemed to fade, especially those on the back.

Conclusions. In the absence of an effective vaccine, early diagnosis and treatment are critical in preventing disability and deformity and reducing the physical, psychosocial, and economic burden of the disease.

Key words: childhood, borderline lepromatous, Morbus Hansen, leprosy.

Leprosy (Morbus Hansen) is a chronic severe infectious disease caused by *Mycobacterium leprae*, that mostly affects the skin, mucosa, eyes, and nerves. Despite the availability of effective treatment, leprosy has become a major public health problem in many developing countries. For twenty-two years, leprosy has been declared 'eliminated as a public health hazard,' yet new cases continue to emerge in endemic areas. There were 127,558 new leprosy cases detected globally in 2020, according to official figures from 139 countries in the six World Health Organisation (WHO) regions. This includes 8,629 children under 15 years. The new case detection rate among the child population was recorded at 4.4 per million children. Among the new cases, 7,198 new cases were detected with grade 2 disabilities (G2D) and the new G2D rate was recorded at 0.9 per million population.[1] Because of their underdeveloped or neonatal immunity and exposure to intrafamilial contacts, children tend to be the most vulnerable population to *Mycobacterium leprae* infection. Leprosy is a master imitator, presenting as subtle hypopigmented patches on the face, arms, and cold parts of the skin before spreading extensively across the skin and causing neuromuscular symptoms such as sensory loss and muscle weakness. As a result, in locations where leprosy is still prevalent, it should be considered a differential diagnosis even in nonendemic areas not just by dermatologists, but also by doctors, neurologists, and pediatricians who care for children and adolescents.[2-4]

✉ Andre Marolop Pangihutan Siahaan
andremarolop@usu.ac.id

Received 1st March 2023, revised 30th May 2023, accepted 25th July 2023.

Case

We present a case of leprosy in a 9-year-old Indonesian male patient who came to the Haur Gading Health Center, South Kalimantan with the chief complaint of three stiff fingers on his left hand which had started four years ago. The patient's parents also complained of hypopigmented patches, which were neither itchy nor painful, on the patient's back and buttocks that began five years ago. The patient also complained of a bent finger that occurred three years ago. The patient lived with his parents and often met his mother's sister, who had passed away five years ago. His mother's sister was diagnosed with leprosy but had not taken medication regularly. Meanwhile, family members who live with the patient did not have any complaints. The patient had received complete basic immunizations including the Bacillus Calmette-Guerin (BCG) vaccine. A history of persistent coughing and other chronic diseases was denied by the patient's mother. The patient had not sought doctor's advice or received any medication before.

On general physical examination, no abnormalities were found. The patient was found to have compos mentis consciousness and a good general condition, weighting 17 kg. Eyebrows were normal and eyelids could open and close perfectly. The examination of the nose, ears, and throat found no abnormalities. There were no infiltrates in the right and left ear lobes. The extremities were warm, and there was no edema. Regional lymph node enlargement was not found. Dermatological status of vertebral location, right and left lumbar, and sacral area, showed hypopigmented plaque efflorescence, multiple, well-defined, geographic shape, and their sizes varied from 0.5 × 1 cm to 1 × 1.5 cm. Fig. 1 and Fig. 2 show the multiple hypopigmented patches on the left and right buttocks and back. A sensory examination of the leprosy lesions found a decrease in the sensations of pain, touch, and temperature on the lesions. On the tips of the fourth and fifth fingers of the left hand, there were reddish nodules and thickened nails (Fig. 3). A nerve examination revealed thickening and enlargement of the ulnar nerve and median nerve of the left hand. The third, fourth, and fifth fingers of the left hand look stiff and were stiff when moved. The voluntary muscle test (VMT) showed muscle weakness in the thenar and hypothenar muscles and numbness in the anterior fingers of the left hand (Table I). From the history and physical examination the patient was suspected of having borderline lepromatous (BL) leprosy. The patient was scheduled for a follow-up examination in the form of a slit-skin smear and laboratory examination. In the follow-up, the skin slit smear showed the presence of acid-

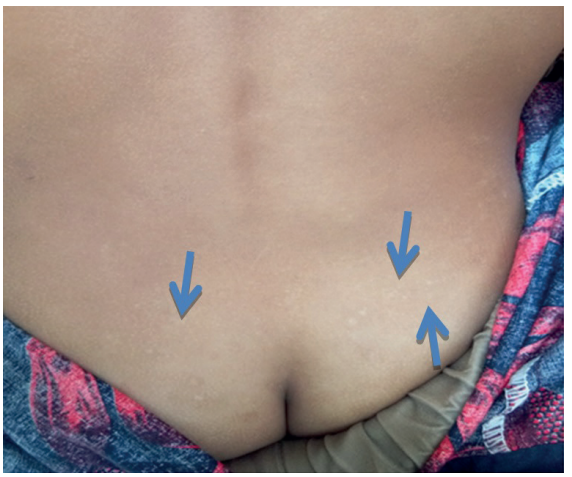

Fig. 1. Multiple hypopigmented patches on the left and right buttocks, which were not itchy and pain.

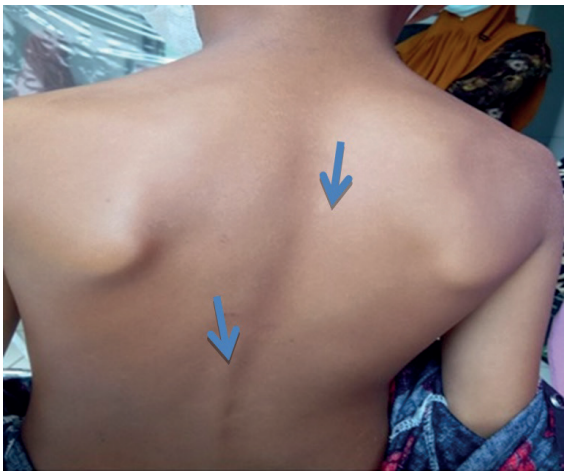

Fig. 2. Multiple hypopigmented patches on the back, which were not itchy and pain.

Fig. 3. Red nodule lesion on left hand before treatment.

fast bacilli (AFB) scrapings on the skin of the right and left ear lobes with a bacterial index of +3 (Fig. 4). The laboratory examination results revealed that the hemoglobin value decreased to 10.1 g/dl, while the white blood cell (WBC) and platelet counts were normal ($6.78\times10^3/mm^3$ and $325\times10^3/mm^3$). No abnormality was found in the blood glucose test (102 g/dl).

Based on the skin slit smear examination strengthened by the previous history and physical examination, the working diagnosis was confirmed as a borderline lepromatous type of leprosy. The prognosis for leprosy is good, as long as the patient has an early diagnosis and treatment. The management was given

Table I. Sensory and motor tests.

	Sensory Tests		Motor Tests	
	Right	Left	Right	Left
C5,6,7	Normal	Normal	Normal	Normal
C5,6	Normal	Normal	Normal	Normal
C7,8	Normal	Abnormal	Normal	Abnormal
C8, T1	Normal	Abnormal	Normal	Abnormal
T4	Normal	Normal	Normal	Normal
T10	Normal	Normal	Normal	Normal
T12	Abnormal	Abnormal	Normal	Normal
L1,2,3,4	Abnormal	Abnormal	Normal	Normal
L4,5	Abnormal	Abnormal	Normal	Normal
S1,2	Normal	Normal	Normal	Normal
S2,3,4	Normal	Normal	Normal	Normal

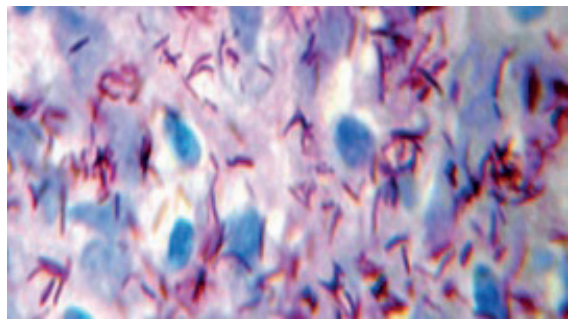

Fig. 4. Slit-skin smear result showing staining, slender bacilli.

as multidrug therapy (MDT) multibacillary (rifampicin 300 mg per month, dapsone 25 mg daily, and clofazimine 100 mg per month followed by 50 mg every 2 days) for 12 months. The patient's family was given education about the disease, the results of the examination, the treatment given, and a one-month regular control was advised. The patient was referred for regular physiotherapy. Periodical observations after the patient received treatment did not find new spots on the patient's skin, some of the previous hypopigmented patches seemed to fade, especially on the back. The fingers of the left hand were still stiff but the nodules had slightly faded (Fig. 5). The patient's general condition was better, and he acknowledged that all signs and symptoms had improved. The patient denied any history of fever or any adverse effects of the treatment. Regular physiotherapy for the patient is still ongoing.

Fig. 5. Fading nodular lesion on left hand after 1 month of treatment, though the fingers of the left hand are still stiff.

Discussion

Leprosy is a disease that can infect people of all ages. The prevalence of leprosy in children can serve as an indicator of the disease's prevalence in the general population, as well as a tool for determining how the disease is transmitted.[1,4,5] The incubation period for leprosy ranges from 2 to 4 years, although an incubation period of 3 months to 40 years has been reported.[2] Children are more susceptible to leprosy because their immune systems are not yet fully developed. The age of onset of this disease in children is between 5 and 14 years with the same prevalence in boys and girls.[4-7] Incidence in children under the age of one year has been documented by Brubaker, Meyers, and Bourland, who published two cases of a 6-month-old child with leprosy that were confirmed by histopathological examination.[8]

M. leprae bacteria are likely to enter the host through two routes: the skin and the upper respiratory tract.[9] Close contact with people with leprosy poses a significantly greater risk than those who do not live at home. The possibility of contracting the leprosy disease increases 4 times if there is contact with leprosy sufferers in the surrounding environment, the risk becomes 9 times greater in household contact and increases if the contact is a multibacillary type of leprosy patient.[10] In children, the source of leprosy infection is obtained from the sufferers with the untreated multibacillary type of leprosy in the family or community.[10,11] In a retrospective study conducted in India, more than one-third of leprosy cases in children (35%) had household contact with leprosy sufferers.[1,11]

The administration of the BCG vaccine as protection against *M. leprae* infection showed varying results, the effectiveness of the vaccine as protection against leprosy reached an average of 26%. A study in Brazil with a large sample size showed that the protective effect of this vaccine was 56% significant in the incidence of contact leprosy, with protection against multi-bacillary leprosy at 89% for children under 5 years, while the protective effect was not found in older children.[12] This indicates a protective effect of the BCG vaccine against the incidence of multibacillary type leprosy.[13] However, several factors also play a role in the incidence and type of leprosy. These factors include genetic, nutritional, and environmental factors (living in endemic areas).[14,15] In terms of environmental factors, according to Bakker et al's research in Flores, 4,774 people lived in the study region, of which 4,140 had leprosy, a figure that reached 87%. Where 39% were found to be multibacillary leprosy and 61% were single lesion paucibacillary or 2-5 lesions.[14] In this case, the patient was immunized with the BCG vaccine as an infant, and several factors contributed to the patient becoming infected with a multibacillary type of leprosy, including the patient living in a leprosy endemic area, genetic factors, and susceptibility to germs. The patient also made frequent contact with his mother's sister, who had multibacillary leprosy.

The diagnosis of leprosy was established based on the cardinal signs of leprosy through clinical examination, supported by AFB examination on a slit-skin smear.[4,8] Since 1996, WHO has recommended diagnosis of leprosy based on at least one of three cardinal signs: (i) hypopigmented skin patch with loss or reduced sensation; (ii) enlarged nerve; (iii) slit-skin smear-positive for leprosy bacilli. However, several studies on leprosy diagnostics, including on blood/serum samples have been carried out. Presently, confirmatory tests for leprosy (microscopy on slit-skin smears and biopsy) are usually carried out only in referral centers.[8] In this case, the patient had a hypopigmented skin patch with a loss of sensation, enlarged median nerve, and skin smear showed the presence of AFB. In 1962, Ridley and Jopling classified leprosy based on clinical features, which include typical tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), and lepromatous leprosy (LL).[7,13] Based on the patients' history, physical examination, and follow-up examination he was diagnosed with BL type leprosy.

Leprosy in children is usually in the form of hypoesthetic or asymptomatic lesions, while the patient rarely complains of neural manifestations. The appearance of leprosy in children is clinically different from that in adults. Lesions are usually fewer and less defined than those in adults and they predominate in exposed body areas.[1,16,17] Clinical aspects of leprosy type BL include the fact that the lesion typically begins with the macula. Small amounts at first, then rapidly spread throughout the body. The macula is more distinct and varies in shape. Although small, the papules and nodes are more defined, with the lesions distributed fairly symmetrically. Normal skin can be discovered between the lesions. Lesions differ in size and shape from one another. Infiltrates may show as plaques, particularly in the cheeks and ears. Nerve damage symptoms such as loss of feeling, hypopigmentation, decreased sweating, and hair loss emerge faster than in type LL. Nerve thickening may be palpated at the site of predilection. The AFB examination showed that many *M. leprae* bacteria were found on the BL spectrum.[8,13] Histopathological examination of the type of BL leprosy showed a collection of macrophage cells. These macrophages have a foamy cytoplasm as in the LL type. In addition, the presence of the grenz zone can also be seen and it is easy to find bacilli.[11,13,16]

WHO divides leprosy patients into 2 groups based on clinical criteria by using the number of skin lesions and nerves involved, as well as the examination of skin smears in determining the treatment of leprosy. This division includes paucibacillary type leprosy (1-5 skin lesions), and multibacillary type leprosy (more than 5 skin lesions). In addition, patients with smear-positive leprosy are also classified as a multibacillary type of leprosy, regardless of the clinical picture.[13] In this case, the patient had more than five skin lesions and the skin smear examination revealed a positive smear. Therefore, the patient was given multibacillary treatment. The diagnosis was confirmed by skin smears taken from the ear lobes which showed the presence of acid-fast bacilli. However, no biopsy was taken from the skin lesions. Hypopigmented macules and patches as seen in this boy were very nonspecific, especially in dark-skinned individuals. Many skin diseases can be listed in the differential diagnosis of such hypopigmented lesions in children, including postinflammatory hypopigmentation. Therefore, in the absence of histopathological confirmation, these lesions can not be defined as skin lesions of leprosy with certainty.

Treatment of leprosy based on WHO criteria which is called MDT, that consists of several antibiotics. Multibacillary leprosy is given a combination of rifampin, dapsone, and clofazimine.[7] In children aged 10-14 years, there is a special package treatment regimen that is distinguished from adults, with a duration of administration of 12 months. This regimen includes rifampin 450 mg monthly, dapsone 50 mg daily, and clofazimine 150 mg monthly followed by 50 mg every 2 days.[13,18] The incidence of disability in children is quite low compared to adults because the duration of the disease is shorter and the form of the disease is milder. However, the incidence of deformity increases with age and with long-standing disease.[15,18]

Deformity and disability result from the delay in diagnosis, having a substantial influence on the physical, emotional, and financial aspects of the child and his family. In this case, leprosy in the patient was suspected of being possibly transmitted from his mother's sister who had intense contact with the patient. The results of bacteriological examination with Ziehl-Neelsen staining of tissue scrapings found acid-fast bacilli. The treatment given was multibacillary multidrug therapy for children for 12 months. In the absence of an effective vaccine, early diagnosis and treatment are critical in preventing disability and deformity and reducing the physical, psychosocial, and economic burden of disease.

Ethical approval

The patient agreed and signed informed consent regarding publishing the case in an academic journal without exposing his identity.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: GDH, MS, RDTN, AMPS, BAW; data collection: GDH, MS, RDTN, AMPS, BAW; analysis and interpretation: GDH, MS; draft manuscript preparation: GDH, MS, RDTN, AMPS, BAW. All authors reviewed and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 868-873
https://doi.org/10.24953/turkjped.2022.935

Case Report

A rare complication of IgA vasculitis: renal and intestinal ischemia successfully treated with plasmapheresis

Şeyma Türkmen[1], Sevinç Taşar[2], Meryem Güzel[1], Hafize Emine Sönmez[3], Mustafa Çakan[1], Betül Sözeri[1]

[1]Department of Pediatric Rheumatology, University of Health Sciences, Ümraniye Training and Research Hospital, İstanbul; [2]Department of Pediatric Radiology, University of Health Sciences, Ümraniye Training and Research Hospital, İstanbul; [3]Department of Pediatric Rheumatology, Kocaeli University Faculty of Medicine, Kocaeli, Türkiye.

ABSTRACT

Background. IgA vasculitis (IgAV) is a multisystemic small vessel vasculitis and is the most common vasculitis in childhood. The characteristic findings of IgAV are palpable purpuric rash, abdominal pain, arthralgia or arthritis, and hematuria. Ischemic complications are very rare in IgAV. Thrombotic complications can be observed after a COVID-19 infection. Also in the presence of familial Mediterranean fever, IgAV may have an atypical or more severe course.

Case. We present a case of IgAV complicated with renal infarction and intestinal ischemia. There was no recent or distant history of COVID-19 in the patient or family members, but the patient's COVID-19 antibody was positive. In addition, *MEFV* gene analysis of the patient showed homozygous M694V mutation. The patient did not respond to enoxaparin, pulse methylprednisolone, intravenous immunoglobulin (IVIG), iloprost, and cyclophosphamide treatments. She was successfully treated with six sessions of plasmapheresis.

Conclusions. Plasmapheresis seems to be an effective treatment option in IgAV-related ischemic findings that do not respond to intensive immunosuppressive therapy.

Key words: IgA vasculitis, mesenteric ischemia, plasmapheresis, renal infarction, familial Mediterranean fever.

IgA vasculitis (IgAV), formerly called Henoch-Schönlein purpura or HSP, is a multisystemic small vessel vasculitis and it is the most common vasculitis in children.[1] It is considered an IgA-mediated autoimmune disease. The characteristic findings of IgAV, including palpable purpuric rash, abdominal pain, arthralgia or arthritis, and hematuria, are not always present at the same time.[2] Ischemic complications are very rare and renal infarction has been previously reported in only two pediatric cases.[1] Although mesenteric vasculitis is rare in IgAV patients, it is the most urgent complication of IgAV due to the risk of bowel necrosis and massive gastrointestinal hemorrhage.[2]

✉ Şeyma Türkmen
drpieni@hotmail.com

Received 2nd November 2022, revised 5th February 2023, 27th May 2023, accepted 14th July 2023.

SARS-CoV-2, causes a respiratory infection with symptoms ranging from a mild upper respiratory tract infection-like illness to severe pneumonia. It has also been reported to show extrapulmonary findings such as thrombotic, cardiac, and dermatological complications.[3]

Herein, we present a case diagnosed with IgAV complicated with renal infarction and intestinal ischemia. She did not have acute COVID-19 infection but was found to be positive for COVID-19 antibodies. Also, *MEFV* gene analysis showed homozygous M694V mutation.

Case Report

A 14-year-old female patient was referred to our center from an external hospital with complaints of purpuric rash on her lower extremities and abdominal pain for 3 days.

In the external center, severe abdominal pain had persisted despite steroid treatment (30 mg/day) and bilateral renal infarction was detected in abdominal computed tomography.

The patient's past medical history was unremarkable. In the family history, the father reported using colchicine because of familial Mediterranean fever (FMF) but the patient did not describe any previous history of recurrent abdominal pain or fever attacks. Physical examination revealed a rash typical of IgAV on her legs, severe abdominal pain and widespread tenderness in the abdomen. Other system examinations were normal with normal vital signs. The patient's pain visual analog scale (VAS) score was evaluated as 10 (Fig. 1). Complete blood count and biochemical tests were normal. Acute phase reactants were elevated (C-reactive protein 226 mg/L, erythrocyte sedimentation rate 83 mm/h). Proteinuria was detected with dipstick in urine analysis and the protein level in the 24-hour urine was 7.8 mg/m²/h, indicating mild proteinuria. Antinuclear antibody (ANA), anti-double stranded DNA (anti-dsDNA), anti-neutrophilic cytoplasmic antibody (ANCA) tests, and antiphospholipid antibody tests were all negative. While complement-3 (C3), and C4 levels were normal, von Willebrand factor (vWF) antigen was found to be high at 305% (normal value <100%). Adenosine deaminase 2 (ADA2) enzyme activity level was normal. Renal color Doppler ultrasonography and transthoracic echocardiography were normal. Abdominal computed tomography angiography and abdominal magnetic resonance angiography revealed infarcts in all parenchymal and corticomedullary areas of the kidneys, especially in the lower poles, and ischemia in the intestinal wall of the ileal segments and mesentery without any aneurysm or stenosis (Figs. 2 and 3). There was no recent or distant history of COVID-19 in the patient or family members, but the patient's COVID-19 antibody was positive (COVID-19 IgG 12.8 U/mL).

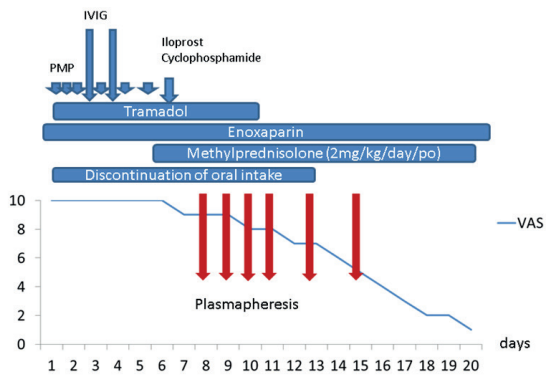

Fig. 1. Treatments used and pain visual analog scale (VAS) of the patient.

IVIG: intravenous immunoglobulin, PMP: intravenous pulse methylprednisolone

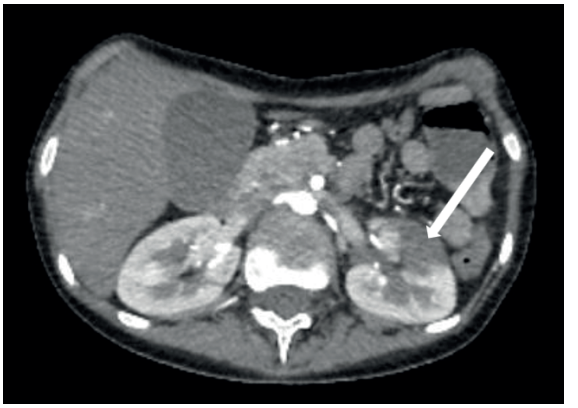

Fig. 2. Computed tomography angiography showing significant renal infarction in the upper pole of left kidney.

The patient was diagnosed as IgAV with renal and mesenteric vasculitis. Oral intake of the patient was discontinued. Intravenous hydration and antibiotics were started. Enoxaparin and pulse methylprednisolone treatment (30 mg/kg/day) was started. In the follow-up, six doses of pulse methylprednisolone (30 mg/kg/dose) were administered and steroid treatment was continued at a dose of 2 mg/kg/day. In the follow-up, abdominal pain did not improve. Total parenteral nutrition was started. Intravenous immunoglobulin (IVIG) (1 g/kg/day, 2 days) and iloprost treatments were given. Since abdominal pain continued

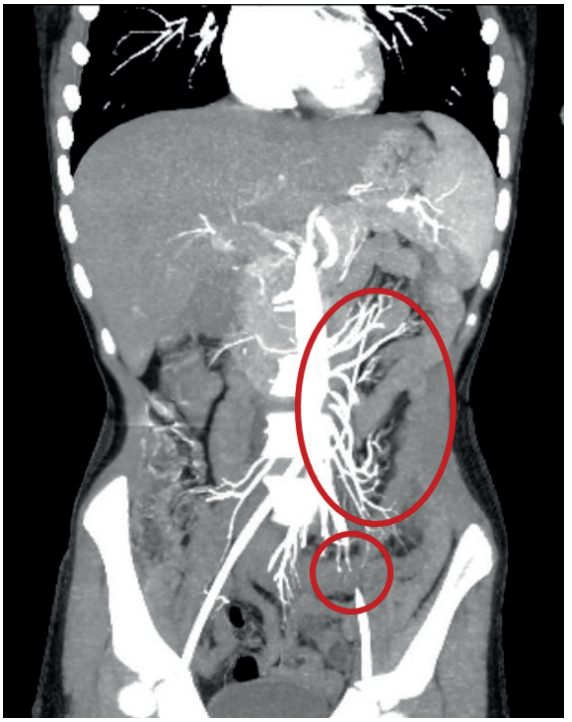

Fig. 3. Computed tomography angiography showing increased mesenteric vascularization at the level of the ileal loops in the left lower quadrant (upper ellipse) and contrast filling defect in the left iliac artery (lower circle).

a single dose of cyclophosphamide infusion was administered. Despite all these treatments, severe abdominal pain and need for narcotic analgesics continued. No significant regression was observed in VAS. Acute phase responses remained elevated and findings consistent with vascular involvement detected in magnetic resonance imaging continued in the control abdominal ultrasonography. For this reason plasmapheresis treatment (with fresh-frozen plasma replacement) was started and applied six times. On the 10th day of her hospitalization, after the 4th plasmapheresis, her abdominal pain started to regress and her pain VAS score gradually decreased. The patient tolerated oral feeding. Acute phase reactants became normal.

The patient was discharged with oral methylprednisolone (30 mg/day) and mycophenolate mofetil (500 mg/day). No mutation was detected in the *ADA2* gene, but *MEFV* gene analysis showed homozygous M694V mutation and colchicine treatment was started. Corticosteroids were discontinued on the 2nd month and she has been followed under remission with mycophenolate mofetil and colchicine treatments for five months.

Informed consent was received from the legal guardians of the child for publication.

Discussion

IgA vasculitis is the most common vasculitis in children. It is described by non-thrombocytopenic palpable purpura, arthritis or arthralgia, and gastrointestinal and renal findings. It is a systemic disease in which antigen-antibody (IgA) complexes enable the alternative complement pathway, resulting in inflammation and small vessel vasculitis.[4,5] Gastrointestinal involvement may be in the form of abdominal cramp-like pain, vomiting, hematemesis, hematochezia, melena, ischemic bowel, and rarely massive gastrointestinal bleeding and bowel obstruction.[2] Renal manifestations range from microscopic hematuria and mild proteinuria to nephrotic and nephritic syndrome and renal failure.[6,7]

Ischemic complications are very rare in IgAV. Talwalkar et al.[8] described renal infarction secondary to IgAV in a 12-year-old girl in 1984. Similarly, in 2014 Gracchi et al.[1] detected renal infarct in a 5-year-old male patient with IgAV. To the best of our knowledge, our case is the third case in the literature. Although mesenteric vasculitis, arterial and venous thrombosis due to IgAV are also very rare, they are associated with high mortality and are the most life-threatening complications.[2,9] In 2003, Wang et al.[2] described mesenteric vasculitis and intestinal ischemia in a 15-year-old male patient with IgAV. They reported that after pulse methylprednisolone treatment, the ischemic bowel symptoms and signs were improved without surgical operation. In 2020, Dhaliwal et al.[9] described a 15-year-old girl who presented with IgAV rash and developed diffuse

alveolar hemorrhage, intestinal ischemia, and venous thrombosis. The patient was treated successfully with pulse methylprednisolone, intravenous immunoglobulin, and intravenous cyclophosphamide.

The presence of increased factor VIII, homocysteine, lipoprotein A, vWF, and antiphospholipid antibodies are associated with prothrombotic events in IgAV. Together with the inflammatory event in IgAV, these factors increase the risk of thrombosis.[9] In 2011, Tayer-Shifman et al.[10] reported that systemic inflammation may increase procoagulant factors, decrease natural anticoagulants and fibrinolytic activity in untreated FMF patients, and therefore, more thrombotic events are expected in untreated FMF patients compared to healthy individuals, and colchicine may play a role in reducing inflammation and thus hypercoagulopathy. Our patient did not have any previous signs and symptoms compatible with FMF but *MEFV* gene analysis showed homozygous M694V mutation. We think that FMF may have contributed to the clinic presentation of our patient, because she had quite an atypical and severe disease course, complicated by thrombosis. It is also known that polyarteritis nodosa (PAN) is more commonly observed in children with FMF.[11] The absence of hypertension and aneurysm or stenosis in the angiography studies allowed us to exclude the diagnosis of PAN in our case. ADA2 deficiency is also a genetic disease characterized by thrombotic findings similar to PAN.[12] In this respect, enzyme and gene analysis were sent from the patient for differential diagnosis.

While COVID-19 is an important cause of hypercoagulopathy among adult patients, little information is available about thrombotic complications in children with COVID-19 to date.[13] In a cohort study published by Aguilera-Alonso et al.[13] in 2021, only 4 of 537 children diagnosed with COVID-19 developed thrombotic complications. Of these patients, 368 were hospitalized, 58 were followed up in the pediatric intensive care unit, and 47 cases were diagnosed with multisystemic inflammatory syndrome (MIS-C). Renal infarction in adults has been reported as a result of coagulopathy associated with COVID-19.[14,15] The youngest patient in the literature was a 37-year-old patient with no pre-existing comorbidities or risk factors who had bilateral renal infarction with COVID-19 pneumonia.[15] COVID-19 may cause intestinal ischemia via certain mechanisms. These are direct viral invasion of intestinal and vascular epithelium via angiotensin-converting enzyme 2 (ACE 2) receptors, systemic extend of pulmonary coagulopathy, complement-mediated vasculopathy, and platelet activation via spike protein binding to the ACE 2 receptor. However, the rarity of intestinal ischemia in the presence of COVID-19 limits our knowledge on this subject.[16] A case of COVID-19-associated neutrophilic arterial vasculitis has been reported in the literature, similar to thrombotic complications in PAN.[17] Thirteen cases of COVID-19-related acute mesenteric ischemia have been reported, including a 9-year-old girl.[18]

In 2019, Liu et al.[19] investigated the efficacy of a combination of methylprednisolone, cyclophosphamide, and plasmapheresis therapy versus pulse methylprednisolone and cyclophosphamide therapy in 60 children with IgAV nephritis. They reported that in the treatment of severe IgAV nephritis in children, plasmapheresis can further alleviate kidney damage, improve clinical outcome, and not increase the incidence of adverse reactions. In 2008, Acar et al.[20] treated a 13-year-old girl with severe gastrointestinal bleeding secondary to IgAV with plasmapheresis because she did not respond to pulse methylprednisolone and cyclophosphamide therapy. No gastrointestinal bleeding was observed after four sessions of plasmapheresis. They stated that plasmapheresis treatment can be an effective treatment in patients with IgAV who present with severe symptoms, including severe gastrointestinal symptoms. In 2006, Wortmann et al.[21] reported that they successfully treated a case with refractory intestinal vasculitis secondary to IgAV with plasmapheresis.

Our case with IgAV-related bilateral renal infarction and intestinal ischemia similarly did not respond to pulse methylprednisolone, IVIG and cyclophosphamide treatments, and was successfully treated with six doses of plasmapheresis.

Although very rare, the course of IgAV can be complicated by thrombotic and ischemic manifestations. Thrombotic complications can be observed after COVID-19 infection. In the presence of FMF, IgAV may be atypical and more severe, and thrombotic complications may occur. We think that the COVID-19 infection and FMF in our case triggered the common renal and mesenteric thrombosis associated with IgAV. Plasmapheresis seems to be an effective treatment option in IgAV-related ischemic findings that do not respond to intensive immunosuppressive therapy.

Ethical approval

Informed consent was received from the legal guardians of the child for the publication of the case report.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BS, ŞT; data collection: ŞT, MG; analysis and interpretation of results: BS, MÇ, SK, ŞT, HES; draft manuscript preparation: ŞT, BS. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 874-880
https://doi.org/10.24953/turkjped.2022.1067

Case Report

Hemoglobin cast nephropathy: a rare but serious complication of hemolysis in a pediatric patient

Demet Baltu[1], Nihan Avcu Oral[2], Selman Kesici[3], Rezan Topaloğlu[1], Osman İlhami Özcebe[4], Tekin Aksu[5], Diclehan Orhan[6], Fatih Özaltın[1]

[1]Division of Pediatric Nephrology, Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara; [2]Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara; [3]Division of Pediatric Intensive Care Unit, Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara; [4]Department of Internal Medicine, Hematology Unit, Hacettepe University Faculty of Medicine, Ankara; [5]Division of Pediatric Hematology, Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara; [6]Department of Pathology, Hacettepe University Faculty of Medicine, Ankara, Türkiye.

ABSTRACT

Background. Intravascular hemolysis is a serious and rare condition in children and causes the release of hemoglobin and heme into circulation, which have proinflammatory properties. These substances lead to inflammation, oxidative stress, apoptosis, and organelle dysfunction that lead to acute kidney injury (AKI). We report a pediatric case diagnosed with hemolysis-associated hemoglobin cast nephropathy due to autoimmune hemolytic anemia.

Case. A 4-year-old boy, who was admitted to another hospital with complaints of fever and dark urine for one day, developed anemia and kidney failure in the follow-up, was referred to our hospital. In physical examination, pallor and icterus on the sclera were noted. The patient had low hemoglobin and haptoglobin levels concomitant with high levels of serum lactate dehydrogenase, urea and creatinine. A peripheral blood smear showed marked spherocytes without schistocytes. A kidney biopsy was performed due to ongoing overt hemolysis and dialysis requirement, which showed findings consistent with hemoglobin cast nephropathy. Although the initial polyspecific direct antiglobulin test (DAT) was negative, due to persistent intravascular hemolysis DAT was studied monospecifically and showed IgM antibody positivity. Therefore, a diagnosis of autoimmune hemolytic anemia was made, and corticosteroid treatment was started. Hemolysis immediately ceased and the need for erythrocyte transfusion and dialysis disappeared.

Conclusions. Acute kidney injury associated with hemoglobin cast nephropathy is an extremely rare condition in childhood. Although the initial course is severe and potentially life-threatening, the prognosis is favorable with the treatment of the underlying cause and management of AKI. Therefore, pediatricians should be aware of this rare clinical entity during clinical practice.

Key words: acute kidney injury, autoimmune hemolytic anemia, intravascular hemolysis, hemoglobin cast nephropathy.

Intravascular hemolysis, which is characterized by red blood cell (RBC) lysis in circulation is an unusual but life-threatening condition and causes the release of proinflammatory hemoglobin and heme into circulation.[1] The underlying etiology is diverse, including autoimmune hemolytic anemia, infections, medications, toxins, transfusion of incompatible blood groups, paroxysmal nocturnal hemoglobinuria (PNH), mechanical, disseminated intravascular coagulation, thrombotic microangiopathy (TMA), and hemoglobinopathies.[2]

Hemolysis-associated acute kidney injury (AKI) occurs due to acute tubular injury as a result of inflammation, oxidative stress, apoptosis, and organelle dysfunction caused by molecules

✉ Demet Baltu
demetbaltu@gmail.com

Received 7th December 2022, revised 4th April 2023, accepted 1st May 2023.

such as hemoglobin, heme, and iron released from lysed RBCs.[3] Here, we report an extremely rare condition hemoglobin cast nephropathy, in a pediatric patient due to massive intravascular hemolysis that is associated with autoimmune hemolysis.

Case Report

A previously healthy 4-year-old boy was admitted to another hospital with complaints of fever and dark urine for one day. Two weeks before the presentation, he had a fever, cough, runny nose, and watery diarrhea, and all these symptoms resolved within 3-4 days. No medication was taken. Within 24-hour of admission, he had become pale and anuric, and a rapid decrease in the hemoglobin level and acute increase in serum urea and creatinine levels (from 0.72 mg/dl to 1.29 mg/dl within 9 hours) were observed. The patient was referred to our hospital for further investigation and management with a preliminary diagnosis of hemolytic uremic syndrome (HUS).

At admission, the patient was afebrile, his blood pressure was 120/60 mmHg (95th percentile for age, gender and height 113/70 mmHg), heart rate 107/min, respiratory rate 24/min, and oxygen saturation was 95%. The physical examination was unremarkable except for pallor and icterus that was visible on the sclera. Laboratory tests were as follows: hemoglobin 7.6 g/dl, mean corpuscular volume (MCV) 78.9 fl, reticulocyte count 3.04%, white blood cell count 16,500/mm^3, platelet count 359,000/mm^3, total bilirubin 1.9 mg/dl (normal: 0.3-1.2), direct bilirubin 0.3 mg/dl (normal: 0-0.2), lactate dehydrogenase (LDH) 2,843 U/L (normal: 110-295) and creatinine kinase 355 U/L (normal: <171). Acute phase reactants were high namely C-reactive protein 14.2 mg/dl (normal: 0-0.8) and procalcitonin 161 ng/ml (normal: 0-0.1). AKI was diagnosed with high blood urea nitrogen (49.6 mg/dl, normal: 5-18) and serum creatine levels (1.9 mg/dl, normal: 0.26-0.50). Estimated glomerular filtration rate was calculated as 24.3 ml/min/1.73m^2 by the modified Schwartz formula.[4] Microscopic examination of the peripheral blood smear revealed marked spherocytes without schistocytes. Serum haptoglobin level was <5.83 mg/dl (normal: 36-196), and direct antiglobulin test (DAT) and indirect antiglobulin tests studied via the polyspecific antiglobulin test were negative. Urine analysis demonstrated 1+ protein and trace blood with a specific gravity of 1013. In the microscopic evaluation, five red blood cells per high power field were seen. No pathological findings were found in the kidney ultrasound. On the day of admission, hemodialysis was performed due to anuria and progressive deterioration of kidney functions. The patient needed intermittent hemodialysis for two weeks and received seven RBC transfusions due to ongoing hemolysis. A comprehensive study was performed to detect the etiology of hemolysis including ADAMTS13 activity, coagulation studies, direct and indirect agglutination, hemoglobin electrophoresis, and enzyme assays for pyruvate kinase deficiency and glucose-6-phosphate dehydrogenase deficiency, osmotic fragility testing, and flow cytometry for PNH. All of these tests were found to be within normal limits.

Respiratory pathogens panel for bacteria and viruses as well as SARS-CoV-2 PCR were negative. Serologic tests for cytomegalovirus, Epstein-Barr virus, hepatitis B and C viruses, human immunodeficiency virus (HIV), anti-nuclear antibody, and anti-double-stranded DNA were negative. However decreased levels of complement 3 (59.2 mg/dl, normal: 79-152) and complement 4 (8.50 mg/dl, normal: 16-38) were detected. Due to concern about a possible malignancy, a bone marrow aspiration was performed, and any hematological malignancy was excluded.

HUS was considered in the differential diagnosis because of the presence of anemia, elevation of LDH, and AKI in the initial evaluation of the patient, however absence of both thrombocytopenia and peripheral smear findings of TMA (i.e. schistocytes, fragmented erythrocytes) ruled out HUS. A kidney biopsy

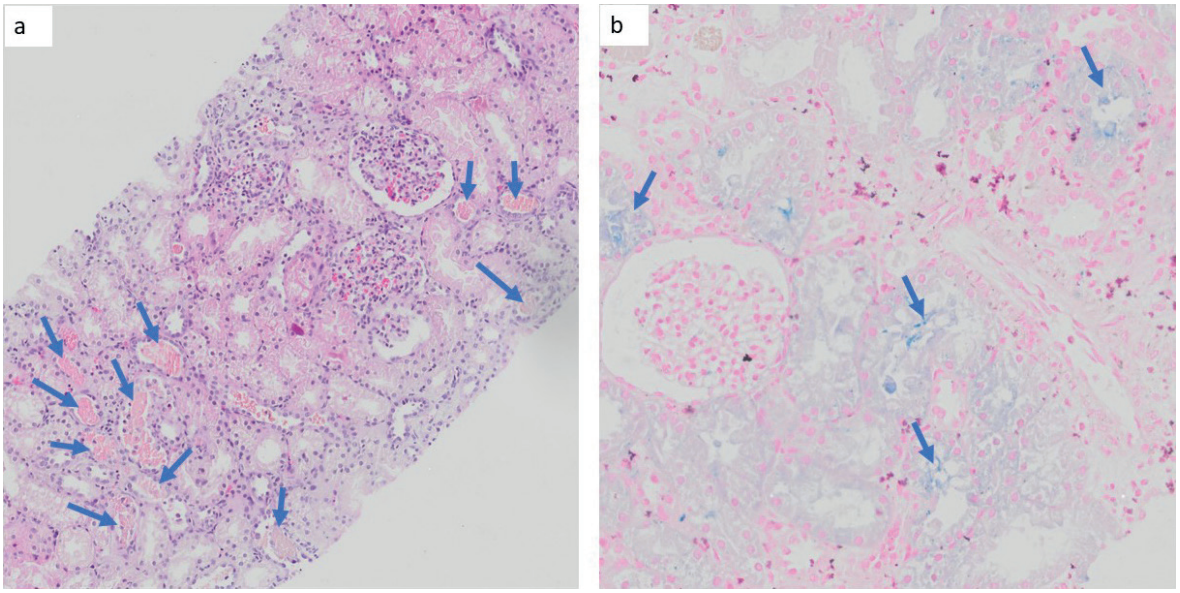

Fig. 1. Pathological findings from kidney biopsy. (a) Histopathologic examination of the renal biopsy revealed abnormal casts (arrows) in the tubules of the kidney. The casts appeared granular, eosinophilic with hematoxylin and eosin (H&E) stain, pale on periodic acid-Schiff (PAS) stain, there were some fragmented cell nuclei in the casts. There was no giant cells or inflammatory cells (H&E stain). (b) Presence of iron in the casts (arrows), consistent with hemolysis in the etiology (Prussian blue stain).

was performed on the 7th day of admission due to lack of improvement in kidney functions and ongoing dialysis requirements. Forty glomeruli were sampled. Glomeruli were otherwise normal except for segmental mesangial cellular proliferation alone. Despite extensive clinical overlap with HUS, there was no histological evidence of acute vascular endothelial damage or TMA. However, tubular findings were most striking including vacuolar degeneration, loss of brush borders, and granular, sometimes globoid-shaped pigmented casts that were predominantly observed in the proximal tubules (Fig. 1a). Interstitial fibrosis and tubular atrophy were absent. Prussian blue staining clearly demonstrated hemosiderin accumulation in the casts (Fig. 1b). Immunohistochemical staining for hemoglobin could not be performed due to unavailability of the dye in Türkiye. Immunofluorescence examination, using IgA, IgG, IgM, C1q, C3, C4, kappa, albumin, and fibrinogen was negative. Taken together, the biopsy was reported as a hemoglobin cast nephropathy with presence of intratubular hemoglobin casts that led to acute tubular necrosis.

Because of rapid declining of hemoglobin levels and of laboratory findings suggesting intravascular hemolysis, DAT was performed manually and mono specifically. Although repeated polyspecific antiglobulin tests were negative, the monospecific antiglobulin test showed IgM antibody positivity. Therefore, autoimmune hemolytic anemia was considered, and corticosteroid (prednisolone 2 mg/kg/day) treatment was started on the 15th day of admission. Hemoglobin level immediately stabilized within 1 day after initiation of corticosteroid concomitant with a rapid decline in blood LDH levels and rapid improvement in kidney function. As such, requirement of both erythrocyte transfusion and dialysis ended. Serum creatinine level decreased to 0.4 mg/dl within 2 weeks (Fig. 2).

Written informed consent was received from the parents of the patient for publication.

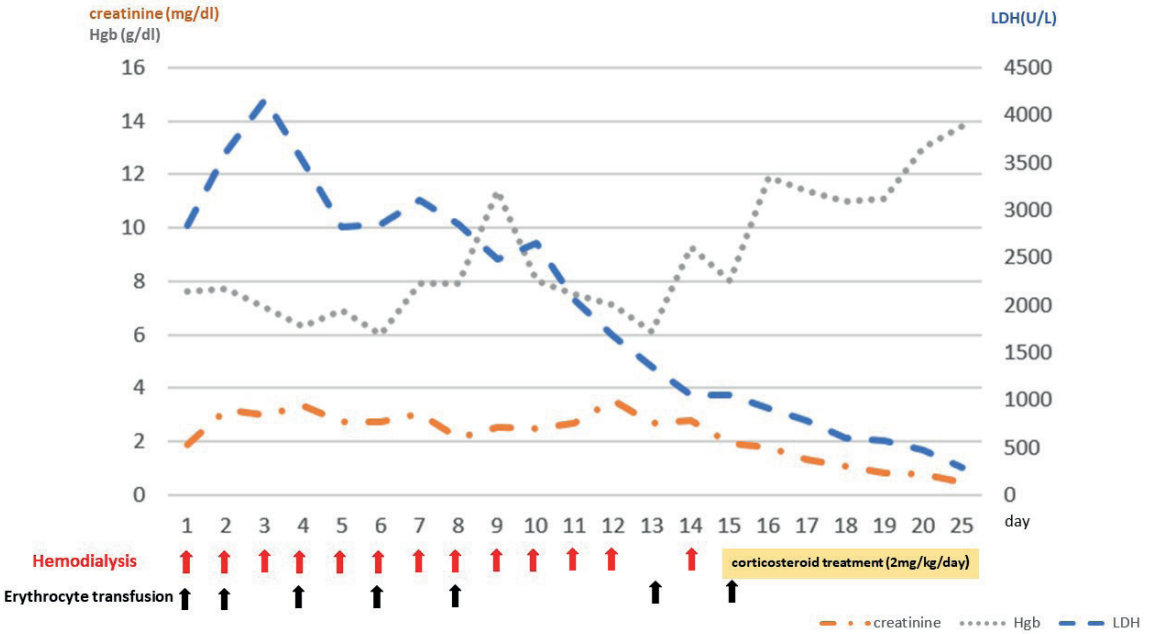

Fig. 2. Levels of hemoglobin (Hgb), lactate dehydrogenase (LDH), and serum creatinine from admission to diagnosis and treatment. Red arrows show hemodialysis sessions and black arrows show erythrocyte transfusions.

Discussion

Here we present a pediatric patient with a hemoglobin cast nephropathy, which is an extremely rare condition that requires special attention in pediatrics practice.

Acute hemolytic anemia is usually a rapidly progressing, severe, and life-threatening condition. The first step is to determine whether the disease is immune-mediated or not. Autoimmune hemolytic anemia is an acquired form of hemolytic anemia, which is caused by the host's immune system acting against its RBC antigens and is mostly defined as hemolytic anemia with a positive DAT. Most commercial DATs are routinely performed by a polyspecific method, which is able to detect IgG and complement (C3d), but not IgA or IgM antibodies. Therefore, it may cause false negative results as was the case in our patient. Despite the obvious clinical and laboratory findings of severe intravascular hemolysis, DAT was persistently negative in our patient. Therefore, monospecific DAT was studied manually and was found to be positive, which was the rationale for starting corticosteroid therapy that resulted in rapid improvement. Our observation emphasizes the importance of monospecific DAT in the presence of unexplained intravascular hemolysis.

Although acute and chronic nephrotoxicity of hemoglobin and heme have been defined, the exact mechanism of nephrotoxicity has not been fully elucidated, but it is most likely to be multifactorial.[3] Cell-free hemoglobin, heme, and iron, which are released from lysed RBCs have pro-inflammatory properties. Normally hemoglobin binds to haptoglobin in circulation. In overt hemolysis, large amounts of hemoproteins exceed the capacity of the endogenous scavengers; these free hemoproteins in plasma are filtrated by the kidney. Free hemoglobin, heme, and iron cause oxidative stress, nitric oxide (NO) depletion, inflammation, and eventually cell death in kidney tissue. Experimental studies have shown that mitochondria are particularly vulnerable to heme-mediated damage and

heme accumulation within mitochondria results in organelle dysfunction and concomitant kidney injury. In addition, hemoproteins cause endothelial activation and vascular injury. Ultimately, tubular necrosis, the formation of intratubular casts due to the interaction of hemoglobin with Tamm-Horsfall protein, and, decreased kidney perfusion due to lack of depletion have been suggested to be responsible for hemoglobin cast nephropathy.[5-7]

Clinically hemolysis-associated nephropathy can resemble other etiologies of AKI, such as acute tubular necrosis, acute interstitial nephritis, and TMA. The main cause of TMA in children is HUS. HUS is the most important differential diagnosis due to its similar clinical presentation and laboratory findings such as anemia, elevation of LDH, and a decrease in haptoglobin level. While the presence of schistocytes suggests the diagnosis of HUS, spherocytosis is present in autoimmune hemolytic anemia in the peripheral smear. The antiglobulin test is therefore critical in determining whether hemolytic anemia is immune-mediated or not. Direct antiglobulin test is negative in HUS except for *Streptococcus pneumoniae*-associated HUS. Kidney biopsy is the gold standard for diagnosis and is necessary to rule out TMA, as there are significant differences in the management of these two distinct entities. Histopathologically, TMA is characterized by microvascular damage in the form of endothelial swelling, fibrinoid necrosis and/or fibrin thrombus in capillaries and other small-sized blood vessels, whereas hemoglobin cast nephropathy is characterized by the presence of prominent intratubular casts, especially in the proximal tubules, and signs of acute tubular injury as in our case.

Several histopathologic entities can mimic hemoglobin cast nephropathy. Histological differential diagnosis includes myoglobin casts, degenerating RBC casts, bile casts, light chain type casts, and acute tubular necrosis.[8] Myoglobin and hemoglobin are structurally similar molecules, therefore cast formation of these molecules is indistinguishable by light microscopy; specific staining with immunohistochemistry (IHC) is the definite way to differentiate them. Clinical data can be a guide if hemoglobin IHC stain is unavailable, as was the case in our patient. We thought that the casts observed in our patient were most likely hemoglobin casts as there was strong evidence of intravascular hemolysis. In contrast, myoglobin casts are typically seen in the setting of rhabdomyolysis and those patients frequently have elevated creatinine kinase levels. RBC casts can also be seen in the presence of glomerular hematuria and are often accompanied by glomerulonephritis. Both RBC casts and hemoglobin casts stain positive for hemoglobin by IHC. The most important feature that distinguishes RBC casts from hemoglobin casts is the presence of residual fragmented RBCs, so-called RBC 'ghosts'. We excluded this possibility with kidney biopsy findings. Other reasons of cast nephropathy are intratubular bile casts that are also associated with liver failure and in this case, total bilirubin is often >20 mg/dl. Light chain cast nephropathy accompanies plasma cell dyscrasia, therefore, is often observed in adulthood.[9]

Hemolysis-associated hemoglobin cast nephropathy is a rare condition and has been reported mostly in adults. Dvanajscak et al.[10] reported the largest case series including 27 adult patients. The remaining data are in the form of case reports in the literature. To the best of our knowledge, among these reported cases in the literature, only three biopsy-proven pediatric patients are present. The First patient was a 2-year-old girl with PNH and AKI who required hemodialysis[11], the other was a 17-year-old boy who presented with AKI and acute hemolysis after exercise[12], and the last patient was a 7-year-old girl with Evans syndrome who developed AKI in the setting of intravascular hemolysis.[13] In the first and second patients, renal functions returned to normal within 2 weeks and 1 month, respectively after controlling of hemolysis. Unfortunately, the last patient died in the acute disease period.

The primary treatment of hemoglobin cast nephropathy is to eliminate the triggering factor of intravascular hemolysis and to prevent ongoing hemolysis. Conditions such as autoimmune hemolytic anemia, drugs, toxins, PNH, and disseminated intravascular coagulation (DIC) that may cause intravascular hemolysis should be quickly reviewed and the underlying cause should be treated. This is critical for the recovery of kidney functions. In addition, supportive treatments such as blood transfusion and management of AKI are essential steps in the treatment. Although most patients need kidney replacement therapy, the prognosis is often favorable and complete resolution is usually expected. All 27 patients in the report of Dvanajscak et al.[10] presented with AKI and 57% of them required temporary hemodialysis. Kidney functions returned to normal at 78% during a mean follow-up period of 9 months. Likewise, although our patient initially presented with severe AKI requiring hemodialysis, kidney functions returned to normal within two weeks following cessation of hemolysis after starting specific treatment for autoimmune hemolytic anemia.

In conclusion, acute intravascular hemolysis is a severe and life-threatening condition. Concurrent acute kidney injury is also an extreme condition in the pediatric age. Despite severe initial clinical course and mostly requirement of dialysis, the prognosis is favorable with the treatment of the underlying condition causing hemolytic episodes and with appropriate AKI management. Therefore, in the case of AKI accompanied by severe hemolysis, hemoglobin cast nephropathy should be considered in the differential diagnosis of HUS if there are no specific TMA findings such as schistocyte, fragmented erythrocyte in the peripheral smear and thrombocytopenia is not accompanied. In this case, the absence of acute vascular endothelial damage or TMA findings in the kidney biopsy and the presence of acute tubular damage findings accompanied by intratubular casts stained with hemoglobin immunohistochemically were diagnostic. Although the inability to perform hemoglobin staining is a limitation, our case is a good example of this extremely rare condition with its clinical, laboratory, and histopathological findings. Moreover, this case report also highlights that monospecific antiglobulin tests should also be run when antiglobulin tests give negative results in cases whereby there is presence of clinical and laboratory evidence of persistent hemolysis.

Ethical approval

Informed consent was obtained from the family for the publication of the case report.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: DB, FÖ; data collection: DB, NAO, SK, RT, OİÖ, TA, DO, FÖ; analysis and interpretation of results: DB, FÖ; draft manuscript preparation: DB, FÖ. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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The Turkish Journal of Pediatrics 2023; 65: 881-885
https://doi.org/10.24953/turkjped.2022.512

Case Report

Late-presenting congenital diaphragmatic hernia in a child with gastric perforation and acute pancreatitis

Qing Li, Caixia Liu

Department of Cardiothoracic Surgery, Children's Hospital of Shanxi Province, Taiyuan, Shanxi, China.

ABSTRACT

Background. Late-presenting congenital diaphragmatic hernia occurs beyond the neonatal period, and is relatively rare, presenting with nonspecific respiratory and gastrointestinal symptoms.

Case. We report a rare case of late-presenting congenital diaphragmatic hernia in a 7-year-old girl, who presented with abdominal pain, shortness of breath and fever on admission. Work-up revealed intrathoracic gastric perforation, acute pancreatitis and septic shock with a diaphragmatic defect. Due to the high content of amylase in pleural effusion, we suspected the presence of a pancreaticopleural fistula, and we were also puzzled whether the gastric perforation was caused by a pleural indwelling catheterization, but this was ruled out. We about performed a laparotomy to reposition the herniated organs, repair the hernia and the gastric perforation, and undergo the gastrostomy. The girl had an uneventful post-operative recovery.

Conclusions. Late-presenting congenital diaphragmatic hernias are often misdiagnosed. Clinicians should combine multiple imaging modalities to make a definite diagnosis and perform surgery as soon as possible to avoid severe complications.

Key words: late-presenting congenital diaphragmatic hernia, hydropneumothorax, gastric perforation, acute pancreatitis, children.

Congenital diaphragmatic hernia (CDH) is characterized by an embryonic defect of the diaphragm, resulting in the abdominal organs herniating into the thoracic cavity, with a reported incidence of 1 in 2500-5000 live births.[1,2] The majority of CDHs suffer from respiratory distress in the neonatal period due to pulmonary dysplasia and pulmonary hypertension. About 5-20% of the cases occur beyond the neonatal period, which is called late-presenting CDH. They present with nonspecific respiratory and gastrointestinal symptoms and are always misdiagnosed, sometimes even fatal.[3] Here we report a pediatric case of late-presenting CDH with gastric perforation, acute pancreatitis and septic shock.

✉ Caixia Liu
lcxzr0351@163.com

Received 31st May 2022, revised 22nd October 2022, 7th May 2023, accepted 20th August 2023.

Case Report

A 7-year-old girl presented to our pediatric emergency department with progressive abdominal pain accompanied by shortness of breath for 1 day and fever for 10 hours. There was no history of trauma or surgery. Her vital signs showed tachycardia (196 beats/minute) with a fever of 39.5°C, hypotension (56/32 mmHg), and tachypnea (60 breaths/minute) with a transcutaneous oxygen saturation of 83%. On physical examination, she was lethargic, cyanotic and her capillary refill time was delayed. She had slight epigastric tenderness, muffled cardiac sounds and her breath sounds on the left hemithorax were diminished on auscultation. Blood gas analysis showed a PaO_2/FiO_2 ratio less than 300 mmHg and metabolic acidosis with lactate of 7.21 mmol/L. Emergency fluid resuscitation and endotracheal intubation were performed, and she was admitted to the pediatric intensive care unit.

Initial chest X-ray in supine position revealed the left lung field hyperlucent with the mediastinum shifting to right, which suggested pneumothorax (Fig. 1). Instant thoracentesis released a large amount of gas and 400 ml of brown turbid pleural effusion, then a thoracic drainage tube was placed. The girl's vital signs improved apparently. The laboratory test showed the leukocyte count was within the normal limit, but c-reactive protein (130.73 mg/L) and procalcitonin (41.22 ng/ml) were significantly elevated. In addition, serum amylase was 498 U/L, serum lipase was 600 U/L, and amylase in pleural effusion was 28108.1 U/L. They were all above normal. We suspected the presence of a pancreaticopleural fistula, but the abdomen ultrasound was consistent with acute pancreatitis and didn't detect a fistula. We gave her conservative treatment, including antibiotics, and somatostatin analogues. One day later, the level of serum amylase (382 U/L) and serum lipase (69 U/L) decreased, but the patient's condition did not improve significantly. The subsequent X-ray showed an air-filled oval-shaped outline of the left lower hemithorax in continuity with the abdomen, and the rightward mediastinal shift remained. The bedside thorax ultrasound detected some solid tissue echo in the left thoracic cavity, but the sonographer on duty didn't identify it due to a lack of experience. So we performed an upper gastrointestinal series at bedside with iodic contrast medium injected through a nasogastric (NG) tube, demonstrating the intrathoracic stomach filled with contrast, and contrast leaking into the thoracic cavity (Fig. 2). At that time, the diagnosis of a left late-presenting CDH with gastric perforation, acute pancreatitis and septic shock was basically clear.

An exploratory laparotomy was performed urgently, and a defect of about 5cm×3cm in the left posterolateral diaphragm (Bochdalek hernia) was found. Surgeons enlarged the tight hernia ring and repositioned the herniated abdominal organs, including the stomach, spleen, a small portion of the omentum, transverse colon and proximal small intestine. Exploring the left thoracic cavity revealed mild pulmonary dysplasia, then they closed the

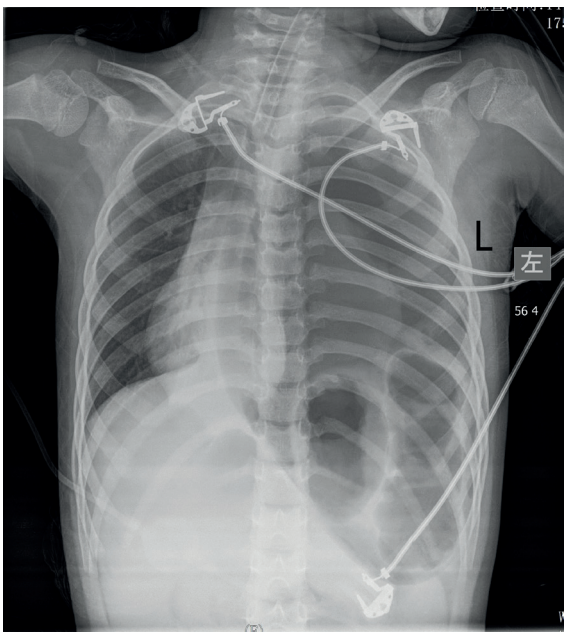

Fig. 1. Initial chest X-ray in supine position showing the left lung field is hyperlucent with a rightward mediastinal shift.

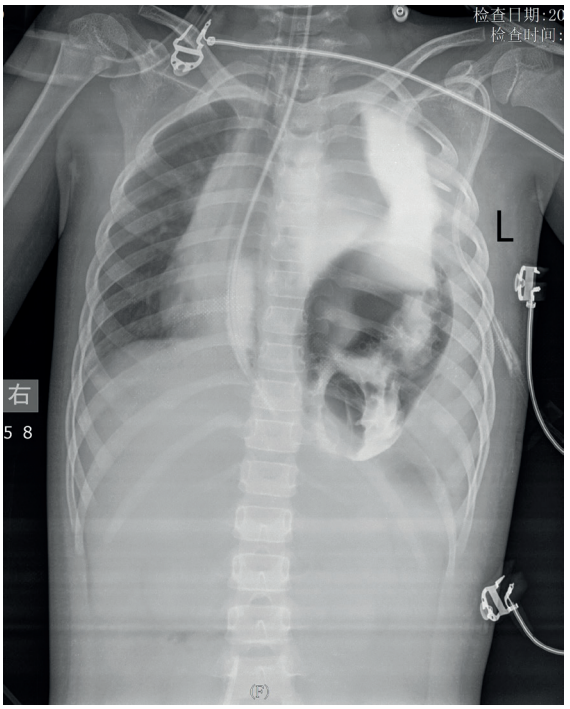

Fig. 2. The upper gastrointestinal series demonstrating the intrathoracic stomach filled with contrast, and contrast leaking into the thoracic cavity.

diaphragmatic defect with 2-0 non-absorbable interrupted sutures and found high tension at the suture site, so they reinforced it with a bovine pericardial patch. The herniated organs appeared congested, edematous, and closely adhered to each other. They released the adhesions and detected two gastric perforations with surrounding necrosis on the lesser curvature (3cm×2cm) and fundus (4cm×3cm) respectively, and multiple focal ischemia could be seen on the gastric wall (Fig. 3 and Fig. 4). There was no evidence that the thoracic drainage tube had damaged the stomach, surgeons repaired the gastric perforation and performed a gastrostomy and implanting a mushroom head tube into the stomach through the skin, and found no evidence of a pancreaticopleural fistula intraoperatively. The girl had an uneventful post-operative recovery, she was discharged on the 10th post-operative day and followed up for 6 months asymptomatically. Informed consent was obtained from the girl's family for this case report.

Discussion

Late-presenting CDH is less common than neonatal CDH despite the fact that the diaphragmatic defects are both congenital in anatomy, but the clinical characteristics are quite different from each other.[3] Late-presenting CDH has no obvious symptoms in the early stage that may be attributed to the small amount of herniated organs in the thoracic cavity or without any herniation.[4] Intrathoracic pressure increases while the abdominal organs suddenly herniate into the chest cavity, accompanied by pulmonary compression, contralateral mediastinal displacement and incarcerated intestinal obstruction, resulting in diverse clinical manifestations.[3]

According to the radiological findings on admission, pneumothorax was the most important emergency diagnosis for this patient. Considering that trauma can also lead to pneumothorax, we repeatedly confirmed the trauma history of this case and ruled it out. We also suspected the presence of a pancreaticopleural fistula due to the high amylase content in the pleural effusion. It has been documented that a pleural effusion amylase level higher than 50,000 IU/L could be directly diagnosed as a pancreaticopleural fistula.[5] But our case had only an intermediately increased amylase level of 28108.1U/L in the pleural effusion, and we eventually disproved that suspicion because there was no further indication of the fistula. It is also inappropriate to analyze the pneumothorax and pleural effusion separately in our case. High levels of amylase in pleural effusion can be found in other pathologies, including acute pancreatitis.[5]

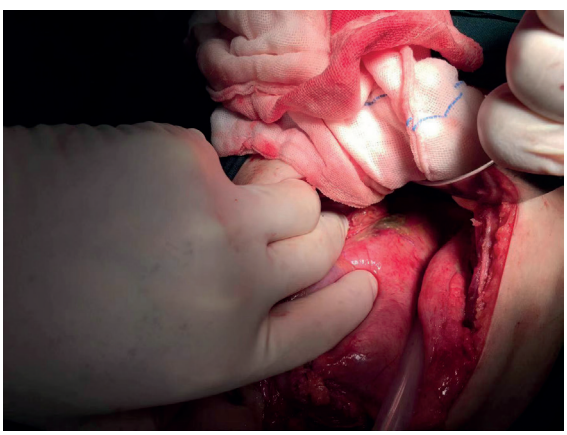

Fig. 3. Intraoperative photograph showing a gastric perforation of about 3cm×2cm on the lesser curvature of the stomach with surrounding necrosis.

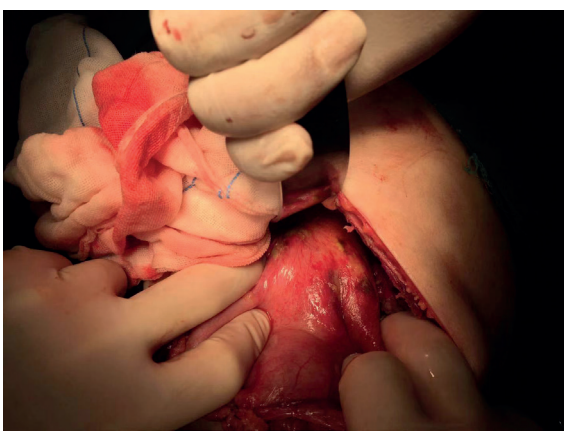

Fig. 4. Intraoperative photograph showing multiple focal ischemia on the gastric wall.

The leakage of salivary amylase into the thorax was probably due to the gastric perforation. Acute pancreatitis secondary to diaphragmatic hernia is relatively rare. Harrington et al.[6] reported an adult Bochdalek hernia case with acute pancreatitis, but the pancreas was not the hernia content, and the acute pancreatitis was considered to be due to traction. Our case was similar in that pancreatitis was triggered by mechanical traction of other hernia content.

Chest and abdomen X-rays are usually the initial imaging studies performed in late-presenting CDH, but they are not enough to make a definitive diagnosis. CDH may even be misdiagnosed as another disease due to the resemblance of radiological features like pneumothorax, pneumonia, pleural effusion, or congenital pulmonary airway malformation.[3] As a gradually popularized diagnostic method, the point-of-care ultrasound can be used independently for the diagnosis of pneumothorax or diaphragmatic hernia, which provides real-time dynamic images and is easily repeatable, but requires appropriate training and quality assurance.[7,8] Although, in this case, bedside ultrasound did not provide valid diagnostic information due to the inexperience of the young sonographer at the time, more attention should be paid to the role of ultrasound. The upper gastrointestinal series is also a helpful diagnostic modality, which played a crucial role in our case. The computed tomography (CT) can clearly detect the diaphragmatic defect and intrathoracic viscera, which is the more important examination.[3] This case was not transferred for CT because of her critical condition. So, apart from perfect medical history collection, appropriate imaging examination is the key to a correct diagnosis.

However, the misdiagnosis rate for late-presenting CDH is as high as 38.2%.[3] Interventions following misdiagnosis may lead to iatrogenic complications, such as gastric perforation resulting from thoracentesis and pleural indwelling catheterization.[9,10] Clinicians should be more cautious about these interventions while they seem reasonable. Fortunately, in our case, the surgical exploration verified that the two large gastric perforations had no relation to the thoracic drainage tube. We speculate that the gastric perforations may have existed before admission and resulted from the high tension of the intrathoracic stomach, which was the severest complication in our case.

Once the late-presenting CDH is clearly diagnosed, surgical treatment should be performed promptly, laparotomy is superior to dealing with cases that have visceral complications.[11] In this case, we performed a gastrostomy in addition to the repair to provide effective decompression and adequate drainage of the dilated stomach in order to prevent secondary perforation. In addition, considering there was a risk of recurrence of a diaphragmatic hernia, we used a bovine pericardial patch to reinforce the primary repair because of the high tension at the suture site. It has been documented that a biologic mesh has a better application prospect in terms of low reherniation, low calcification and infection rate.[12]

In conclusion, late-presenting CDH in children presenting as gastric perforation, acute pancreatitis and septic shock is rare. Clinical and imaging misdiagnosis is much more common. Clinicians should combine multiple imaging modalities to make a definite diagnosis and perform surgery as soon as possible to avoid severe complications.

Acknowledgements

We would like to extend our sincere thanks to our colleague, Ning Zhang, who helped us collect the radiographic images.

Ethical approval

Informed consent to publish the case report has been obtained. And this report does not contain any personal information that could lead to the identification of the patient.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: QL, CL; data collection: QL; analysis and interpretation of results: QL, CL ; draft manuscript preparation: QL. Both authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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Corrigendum to "Successful treatment of post-pericardiotomy syndrome via C1 inhibitor replacement therapy in a hereditary angioedema patient with Marfan syndrome" [Turk J Pediatr 2023; 65: 338-343]

Ezgi Topyıldız[1], Handan Duman Şenol[1], Figen Gülen[1], Esen Demir[1], Nihal Mete Gökmen[2]

[1]Division of Pediatric Allergy and Clinical Immunology, Department of Pediatrics, Ege University Faculty of Medicine, İzmir; [2]Division of Allergy and Clinical Immunology, Department of Internal Medicine Allergy, Ege University Faculty of Medicine, İzmir, Türkiye.

In the article "Topyıldız et al., Successful treatment of post-pericardiotomy syndrome via C1 inhibitor replacement therapy in a hereditary angioedema patient with Marfan syndrome. Turk J Pediatr 2023; 65: 338-343" the authors would like to correct the dose of colchicine from 1 mg/kg/day to 1 mg/day. The authors would like to apologise for any inconvenience this may have caused.

DOI of original article: https://doi.org/10.24953/turkjped.2022.637

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CORRIGENDUM

Printed by METEKSAN in Ankara, Turkey