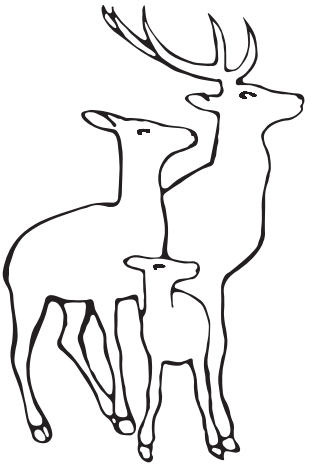
ISSN 0041-4301
Online ISSN 2791-6421
www.turkishjournalpediatrics.org

THE TURKISH JOURNAL OF PEDIATRICS

volume 66
issue 4
July-August 2024



THE TURKISH JOURNAL OF PEDIATRICS

www.turkishjournalpediatrics.org

Volume 66 ▪ Issue 4
July-August 2024

ISSN: 0041-4301
Online ISSN: 2791-6421

THE TURKISH JOURNAL OF PEDIATRICS

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

Cilt: 66 Sayı: 4, Temmuz-Ağustos 2024

KURUCU
İhsan DOĞRAMACI

EDİTÖR
Ali DÜZOVA

EDİTÖR YARDIMCILARI
Sinem AKGÜL
Gülen Eda UTİNE

İDARİ EDİTÖR
Yılmaz YILDIZ

YAYIN EDİTÖRLERİ
Özge BAŞARAN
Melis PEHLİVANTÜRK KIZILKAN
Leman AKCAN YILDIZ

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.2024

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: 66 Issue: 4, July-August 2024

FOUNDER
İhsan DOĞRAMACI

EDITOR-IN-CHIEF
Ali DÜZOVA

ASSOCIATE EDITORS
Sinem AKGÜL
Gülen Eda UTİNE

MANAGING EDITOR
Yılmaz YILDIZ

PUBLICATION EDITORS
Özge BAŞARAN
Melis PEHLİVANTÜRK KIZILKAN
Leman AKCAN YILDIZ

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.2024

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 published by Hacettepe University Institute of Child Health, in partnership with the Turkish National Pediatric Society 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. It publishes original articles, review articles, short communications, case reports, correspondence papers and letters to the editor. 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 Web of Science - Science Citation Index Expanded (SCIE), PubMed/MEDLINE, Scopus, Embase, CABI Abstracts, ProQuest, EBSCOhost, BIOSIS Previews, Türkiye Citation Index and TR-Index.

CONTENTS

VOLUME: 66 ISSUE: 4 JULY-AUGUST 2024

INVITED REVIEW

ORIGINAL ARTICLES

CONTENTS

VOLUME: 66 ISSUE: 4 JULY-AUGUST 2024

CASE REPORTS

The Turkish Journal of Pediatrics 2024; 66: 387-400
https://doi.org/10.24953/turkjpediatr.2024.4523

Invited Review

Child health in the first 100 years of Republic of Türkiye: a story of hope, labor and success

Canan Seren[1]

[1]*Department of Neonatology, Faculty of Medicine, Ondokuz Mayıs University, Samsun, Türkiye.*

ABSTRACT

The Republic of Türkiye commemorated its 100th year in 2023. Within one century, a battle weary, poor country has changed into a powerful, game changing leader in the world. This was accomplished by the motivation and overwork of the Turkish nation and a great leader, Mustafa Kemal Atatürk. The status of child health in 1923 can be summarized as high infant and under-five mortality rates, epidemic diseases and hardly any healthcare facilities and health-care professionals. Since a healthy, well educated workforce was one of the main requirements for the development of the young republic, child health was given a great emphasis. With the efforts of the whole nation, many children's hospitals were established, infant mortality decreased, and malaria, neonatal tetanus, polio and diphtheria were eradicated. In this article, the progression of child health in the first 100 years of the Republic of Türkiye will be reviewed.

Key words: Republic of Türkiye, child health, infant mortality rate, development, 100 years.

After the establishment of the Republic of Türkiye, the healthcare system mainly focused on child health and the eradication of infectious diseases like malaria, diphtheria, cholera and tetanus. Year by year, measures of health improved, infectious diseases were eradicated, and healthcare became widespread. Now, in the 100th Anniversary of the Republic, all Turkish children can access to free contemporary healthcare, equally. In this manuscript the breakthrough in child health in the first 100 years of the Republic of Türkiye will be reviewed in a chronological order.

Health in the beginning of the 20th century

The last years of the Ottoman Empire can be defined as a period of wars, epidemics and early deaths. During the First World War (WWI) 325,000 citizens had died and 400,000 were wounded.[1] In 1918, İstanbul had a population of 940,000 and nearly 10% of all deaths occurred due to tuberculosis. Cholera, typhus, diphtheria, influenza and rabies were also epidemic. Between 1870-1914, it was estimated that more than eight million children had died due to infectious diseases. These premature deaths due to infections touched the heart of the whole country as they were mentioned in Turkish literature: In the story named "Promise" (Ant) by Ömer Seyfettin, written in 1912, the death of a child due to rabies was described. Reşat Nuri Güntekin's well-known novel "The Wren" (Çalıkuşu) published in 1922 narrates the death of Munise due to diphteria when she was 14 years old. The malaria epidemic was jeopardizing the whole country. It was estimated that 70% of Antalya's population was infected with malaria.

There was no Ministry of Health (MoH) during the Ottoman Empire. The Quarantine Organisation (Karantina Örgütü) and "Meclis-i Tahaffuz" (Health Council, "Sıhhiye Meclisi") were established in 1840, and preventive health services were provided mainly in quarantine centers around the country.[2] In 1867, a non-military medical school "Mektep-i Tıbbiye-i Şahane" was established to train doctors. First,

✉ Canan Seren
drcananaygun@gmail.com

Received 31st Mar 2024, revised 8th May 2024, 12th Aug 2024, accepted 23th Aug 2024.

the education was both in French and Turkish. In 1870, medical education began to be taught completely in Turkish.

Dr. Marko Pasha, Dr. Abdullah Bey, Kırımlı Aziz Bey and Besim Ömer Pasha established "Mecrûhîn ve Mardâyı Askeriyeye İmdât ve Muavenet Cemiyeti" in 1868 and in 1877 this community was named as "Ottoman Hilâl-i Ahmer Association" (Kızılay, Red Crescent).[3] Dr. Giovanni Battista Violi was one of the famous pediatricians at that time who worked on smallpox and cholera vaccines. He established an international children's hospital in Şişli in 1905 (Hôspital et Clinique International pour les Maladies des Enfants a Chichli), serving children free of charge. This was the first hospital in which newborns were hospitalized. He also published the first pediatric journal in the Ottoman Empire in 1909: "La Pédiatrie en Turquie"/ "Türkiye'de Emrâz¬ı Etfâl". Being the fourth pediatric journal in Europe, it was pressed both in French and Turkish, whose publication ended with the explosion of WWI in 1914.[4]

Following the declaration of Constitutional Monarchy (Meşrutiyet, sharing of the governmental powers between sultan and people's council) "Müessesat-ı Hayriye-i Sıhhiye İdaresi" (Health Administration for Charitable Enterprises"), the head organization for hospitals was established in 1909. The "Şehremaneti İdare-i Sıhhiye" ("Municipal Health Administration") was also established the same year to organize public health, to take precautions for epidemics and to serve health care in İstanbul. The Health Directorate, led by the Ministry of Interior Affairs (MOIA) was responsible for health services. Laws regarding health were generally about emergency services during times of war and about the organization of health services like "Vilayati İdare-i Sıhhiye Nizamnamesi" in 1913. In 1916, a MoH (Sıhhıye Nezareti) was established that was working with the order of MOIA.[2] Health services were mainly located in the cities, and extension to rural areas was limited due to the paucity of doctors.[2]

In 1802 the first pediatric hospital of the Western World, "Hôpital des Enfants Malades" ("Hospital for Sick Children") was established in Paris, caring only for patients up to 15 years. This was followed by pediatric hospitals in Germany, the Russian Empire, Austria and England. The first children's hospital in the United States opened in Philadelphia in 1855. The first nursery was established in our lands in1892 by Besim Ömer Pasha in İstanbul, which served for 17 years.[5,6] The first pediatric hospital in our history is Hamidiye Etfal Hospital, that opened in June 1899 and was directed by İbrahim Pasha.[5,6] It was built by the order of Abdulhamid II, as a remembrance for his daughter Hatice Sultan, who had passed away after diphtheria when she was eight months old. An in-hospital laboratory prepared serums for scarlet fever, diphtheria and smallpox vaccines. The first sanatorium for children with 24 beds was also opened in this hospital in 1904. Treatment in this hospital was free of charge.[6]

In the 1900's Pediatrics was not a separate specialty and specific pediatrics departments did not exist. Pediatric healthcare was carried out under obstetrics and internal medicine departments. Newborns and infants less than three years were generally cared by obstetricians, while internists cared for children over three.[5] The first pediatrics clinic was opened as ten beds confined in a small part of İstanbul Kadırga Obstetrics Clinic (established in 1909).

In 1909, civil and military medical schools were united and started to serve as Faculty of Medicine ("Tıbbiye"). In this school, the first theoretical lectures on diseases of children ("Emrâz¬ı Etfâl") were started by Kadri Reşit Pasha (Anday) as one hour per week. He became the first professor of pediatrics of our country in 1917 and continued his service until 1933. Later in 1920's İhsan Hilmi Bey (Alantar) started lectures on Childcare ("*Puericulture*") in the medical school.[5,6]

According to the 1885 Ottoman Census, the total fertility rate (TFR) in İstanbul was 3.5 births per

woman and it was around 6 births per woman in 1920's in Anatolia.

Health During the War of Independence (1920-1923)

Only ten days following the foundation of the Grand National Assembly in Ankara, "Sıhhiye ve Muavenet-i İçtimaiye Vekâleti" (Ministry of Health and Social Service) was established on May 2nd 1920 (Law No: 3) and the first MoH was Dr. Adnan Adıvar.[2] The main responsibilities of the first MoH were to protect (Hıfzısıhha) and to rescue (treatment). Healthcare services were delivered at governmental and municipal facilities, quarantine offices and health posts, and the main goal was to heal the wounds of war. Malaria was epidemic, infecting nearly 40% of the soldiers in the army during the War of Independence, and three million people were infected with trachoma.

In 1921, İhsan Hilmi Bey (Alantar) who had practiced pediatrics in France and Germany established the first children's dispensary in Ankara and the second in Kayseri. He wrote a textbook named ''Child Care" ("Çocuk Bakımı") for students in teacher training schools.[5,6]

Many children were homeless and there were many orphans following the losses of Çanakkale, Balkan Wars and WWI, so "Himaye-i Etfal" Association ("Child Protection Association", "Çocuk Esirgeme Kurumu") was established in 1921. Great orphanages were opened in İstanbul, caring nearly for 2,500 children.

The education of midwives continued even during the War of Independence to reduce high maternal and neonatal mortality. In 1922, four-month courses on birth, puerperal and neonatal health were given to midwives in East Anatolia and Trabzon, led by Kazım Karabekir Pasha. Women's admission to medical faculties was also approved the same year.

Breastfeeding was encouraged to reduce infection-related deaths in infants and due to limited food supply. The breastfeeding rate was 95%, which had saved many lives.

Child Health After the Proclamation of the Republic of Türkiye (1923- 1935)

During this period, there were many reforms regarding education, industry, agriculture and social life, as well as health.[1] The healthcare workforce in 1923 included only 554 physicians (mainly from military service origin), 69 pharmacists, forty nurses, 560 health officers, 136 midwives and 86 hospitals with 6,437 beds. One doctor had to serve nearly 21,000 citizens.[2] Private practice was forbidden and doctors were obliged to work for the MoH. In the "Obligatory Service Law" of 1923, medical faculty students who admitted the obligatory service in rural parts were given encouraging advantages like free dormitories and their educational expenses were covered by the government.[2,7-9]

Statistics on the population, mortality and causes of death started after the establishment of the Statistics Institute in 1926.[10] In the 1930s, life expectancy was as low as 40 years, malaria being responsible for 2.3% of all deaths.[11] The first four leading causes of death for children were diarrhea, tuberculosis, pneumonia, congenital malformations and perinatal conditions.[12] Most of the population was living in rural areas and the authorities were not informed neither about the birth nor about the death of the children.[10,12] Extracting data about infant and child deaths from the registries was not possible and survey methods were used to understand the infant mortality rate (IMR).[10] The estimated IMR for 1923 was 250/1,000 and half of the births were lost in the first two years of life. Fuat Bey (Umay), the founder of "Himaye-i Etfal Association" visited four villages, recorded 402 births and 233 losses; with a death rate of 55%. Deaths were mainly due to neglect, hygiene issues, and infectious diseases.[10,12] In 1927, Prof. Camille Jacquart, chief of the Statistics Institute, reported the rate of birth to death as 48.6% in Kalecik by survey method. When we

consider these data, the IMR might be as high as 500/1,000 or higher.[12] For comparison, the IMR in 1923 in the United States was 78/1,000 and it was 73/1,000 in London. Child deaths were so important that Fuat Bey (Umay) in December 1926 gave a motion to the National Assembly to investigate their reasons. The same year, famous journalists Yunus Nadi and Falih Rıfkı wrote articles about child mortality to attract public attention.[12]

Child survival was synonym to the republic's survival, accordingly Himaye-i Etfal Association was always supported by Mustafa Kemal (the founder of Republic of Türkiye and the first president of the Republic) and the Turkish National Assembly. Türkiye was one of the first governments to sign the "Decleration of Children's Rights" in 1924.[5] In 1925, "Children's Day" started to be celebrated and in 1927 "Children's Day" was replaced with "Children's Week", to thoroughly discuss children's' problems.[5]

Due to high IMR, the government adopted pronatalist policies. Motherhood was advocated as a noble public service and the mothers were called "Mothers of the Republic". Girls were encouraged to marry at the appropriate age and raise many healthy children to serve the country. Medals and money were offered to women having more than six children and families having more than five children were freed from "road tax". Single men aged 25-65 were required to pay a tax, effective from January 1st 1927. Children were considered as the "Nations' Children" and future citizens, so child health was at the center of the health policy, together with the war against infectious diseases. Raising healthy children was advised and healthy children contests were established. A popular journal named "Gürbüz Çocuk" ("Robust Child") was published.

In 1926, the minimum age of marriage was lowered to 17 for women and 18 for men. The same year, the Turkish Civil Code classified abortion as a crime. So, TFR peaked at 7 in 1930 (for comparison, the TFR in 2023 was 1.51).

In 1927 an obstetrician, Dr. İsmail Derviş published a book named "Fenn-i Vilade" (Doğum) in which drawings of neonatal intubation by finger, incubator care and gavage feeding of newborns were included.[5]

There was a small children's service in Kadırga Obstetrics Hospital which moved to Haydarpaşa in 1923 and served nearly 40 children, consulted by Kadri Reşit Pasha. The number of beds increased to 54 in 1927.[5,6] The pediatric ward in Haseki Women's Hospital was augmented to 40 beds, consulted by Dr. Ali Şükrü Şavlı and a new division containing 100 beds was added subsequently.[5,13,14] Dr. Raif Yesari operated another pediatric clinic at Vakıf Gureba Hospital.[6]

In 1925 Zonguldak representative Tunalı Hilmi Bey stated in his parliamentary speech that the number of pediatricians should increase to decrease infant mortality and to educate the public about childcare and hygiene.[15]

In 1928, education on pediatrics started at İstanbul University Faculty of Medicine and after the university reform in 1933, the first chair of "Clinic for Child Care and Diseases" was established, and Dr. İhsan Hilmi Alantar was appointed as the clinic director.[16]

In those times, women were not included in the healthcare workforce, and most of the nurses were men. In 1924, Besim Ömer Pasha (whose nickname was the *midwife of the midwives*), Safiye Hüseyin Elbi, Akil Muhtar and Tevfik Sağlam established the first School of Nursery in İstanbul to start formal education for midwifery and nursery.

The Republic's first MoH, Dr. Refik Saydam (mission period: 1923- 1937) constructed and organized health services. The main targets of the health care system were to increase the number of doctors and midwives, establish preventive care, open new hospitals and dispensaries, make health organizations reach villages, and eradicate highly prevalent infectious diseases.[2,17] Institutions were created to combat common communicable diseases

like malaria, trachoma, leprosy and syphilis. Starting from 1924, maternity hospitals and child care institutions and country hospitals ("Numune Hospitals") were established first in Ankara, then in Erzurum, Diyarbakır, and Sivas.[2]

Breastfeeding was encouraged as a way of preventing infant deaths. In 1924, Dr. Ali Vahit (Yaşat) published a pamphlet named "How to Feed a Suckling Baby?" ("Memedeki Çocuk Nasıl Beslenir?") giving advice for common feeding problems in babies. He advised exclusive breastfeeding for eight months and pointed to gastrointestinal infections as the most common reason for child deaths. Growth charts were included in this pamphlet for growth monitoring and weighing of the babies monthly was suggested.[18]

In the first National Turkish Medical Congress (1925) malaria, tuberculosis and child deaths were identified as the most important health problems to battle. Tracking for diphtheria was also initiated in 1925.[19] In 1928, Hıfzıssıhha Institute and School was established and vaccine production for smallpox, tuberculosis and rabies started.[2] In 1930, the first immunization program started which was against smallpox. This meant that the young republic was one of the few countries that could produce its own vaccines. The cholera vaccine produced in Hıfzısıhha was even sent to China for an epidemic in 1938 and the typhus vaccine was widely used around the world in the Second World War (WWII).

Dispensaries against tuberculosis was established in İstanbul and Ankara.[2] Laws on "Practice of Medicine and Medical Sciences" (1928) and "Public Hygiene"" (1930) were passed. With the "Law of Public Hygiene," preventive medicine was established and the fight against infectious diseases was organized. In the first ten years of the Republic, 51 laws and 19 decrees with power of law ("Kanun Hükmünde Kararname") were enacted mainly to combat contagious diseases and to solve current health problems.[2]

Medical museums were established first in İstanbul and then in other big cities to give health education to citizens, especially primary school students.[2]

The "Turkish Pediatric Association" ("Türk Pediatri Kurumu") was established by doctors Kadri Raşit Anday, Mehmet Kamil Berk, Ali Şükrü Şavlı, İhsan Hilmi Alantar and Niyazi Ali Özsoy as "Council of Pediatricians" in May 1930 in İstanbul; just before the establishment of the American Academy of Pediatrics in June 1930. Dr. Sezai Bedrettin Tümay, who later established Cerrahpaşa Medical Faculty Pediatrics Clinic was also on the founding team.[5,6,14]

In 1933, the Department of Pediatric Surgery and Orthopedics was established at Hamidiye Etfal Hospital by Dr. Akif Şakar, within Istanbul University.[6] The same year, Moris Şinasi International Children's Hospital was established in Manisa. In 1930, the first Turkish medical journal on pediatrics was published: "Türk Pediatri Arşivi: İstanbul Çocuk Kliniği Dergisi" (still published as "Turkish Archives of Pediatrics").

In the first census in October 1927, the population was 13,648,270; with 77.7% of citizens living in rural areas.[2,10] In the second census in 1935, the population rose to 16,158,000; with a 2.1% increase in eight years. Low population was considered a health emergency, and specific emphasis was put on this topic.[10,11]

The number of doctors increased to 1,182 in 1930 and to 1625 in 1935. In the 1930's, there were only ten pediatricians in our country, which rose to 100 in 1943.[4] In 1935, the number of hospitals were 175 with 13,000 beds, there were 400 midwives and 202 nurses.

Child Health between 1936-1945

In those years, Türkiye was also affected by the world's economic crisis. The war against infectious diseases continued within the budgetary constraints. New hospitals were

established, and the number of patient beds increased. In 1940, there were 2,387 doctors (quadrupled in 17 years) and 405 nurses.

Since nutritional deficiency was an important reason for infant mortality, studies on nutrition were carried out. Preventive medicine, pre and post-birth hygiene and vaccination were given significance.[1,17] The economic constraints of WWII impacted the entire populace through poverty and food scarcity. Accordingly, the IMR which was 273/ 1,000 live births in 1935–1940 rose to 306/ 1,000 live births in 1940–1945.

Childcare was considered the whole nations' duty, but widespread traditional practices had to change to reduce infant mortality such as lying the babies on turd or soil ("höllük") which could lead to neonatal tetanus (NT). In 1938, the first Turkish Pediatrics Congress assembled during the 7th National Turkish Medical Congress in Ankara, 26 years after the first International Congress of Pediatrics (1912).[14] In this congress, Dr. İhsan Hilmi Alantar's lecture reflected the attitude toward children at that time: "Neither the mother nor the father alone can ensure that a child is raised properly. The government, cities, and charitable societies, the ones busy with health will do this".[16]

During that period, it is important to acknowledge the contributions of Dr. Albert Eckstein, a renowned German pediatrician who dedicated his services to the welfare of Turkish children. Dr Eckstein, being Jewish was exiled from Germany, accepted an invitation to establish the pediatrics clinic in Ankara Numune Hospital in 1935. During the two years following his arrival, his team (including Dr. İhsan Doğramacı) visited 188 villages in 25 provinces, interviewing almost 25,000 women in Anatolia. In his dairy, he wrote: "*Malaria holds the first place. We can also state that other diseases such as typhoid, typhus, whooping cough, measles, kala azar, leukemia and sarcoma also prepare the ground for noma by decreasing the organism's resistance. One-third of the babies born die before they reach one year of age. It is possible to decrease these deaths by some simple measures. However, we need educated people to spread these measures across Anatolia."*[20] He worked for 14 years in Ankara and established Ankara University Medical Faculty Pediatrics Clinic in 1945. After working here between 1945- 1949, he left the chair to Dr. Bahtiyar Demirağ, who led the clinic until 1981.[5,6,21]

In 1945, the "Extraordinary Law on Malaria Prevention" was passed and a comprehensive campaign was launched to combat malaria. Pamphlets, radio programs, and short movies were distributed, aiming to raise awareness about the transmission of malaria by mosquitos.[2] As a result of sustained efforts over several years, Türkiye has successfully eliminated local malaria cases since 2010.

Child Health during 1946-1960

In 1946, "First 10-Year National Health Plan", the first written health plan of the Republic was adopted in which maternal and child health were given priority. In 1950, the number of physicians rose to 3020, nurses to 721 and midwives to 1295. Since the average life-expectancy at birth was 43.6 years in 1950–1955, the governments continued pro-natalist policies. The biopolitics perspective in these years aimed at maintaining the TFR and ensuring child survival. Accordingly, Dr. İhsan Hilmi Alantar stated that every child was a social capital that belonged both to the parents and to the country.[16]

In 1947, Behçet Uz Children's Hospital with 150 beds was opened with the benefaction of Dr. Behçet Uz, who was the major of İzmir at the time. In 1941, Dr. Ziyaeddin Akbay had set up a children's unit in Zeynep Kamil Hospital. The mothers and babies were kept together in the nursery, with rooming-in philosophy, which was ahead of the time. In 1953, he established the first neonatal care unit at Zeynep Kamil Hospital, named as the "Preterm Child Care Center" and he also performed the first exchange transfusion for indirect hyperbilirubinemia in Türkiye. With the establishment of the "Preterm

Child Care Center", a reduction in the preterm death rate was observed.[22]

In 1952, the Maternal and Child Health Section was established at the MoH. In 1953, the "Maternal and Child Health Development Center" was inaugurated in Ankara with assistance from the United Nations International Children's Emergency Fund and the World Health Organization (WHO). Mother and child health centers were set up to provide prenatal and postnatal care. Ankara Children's Hospital (later named as Dr. Sami Ulus Children's Hospital) was established in 1956.[4]

The progress in child health in the 1950's is marked by the endeavors of Prof. Dr. İhsan Doğramacı.[23] While working at the university's Pediatrics Clinic, Prof. Doğramacı organized the constitution of the "Institute of Child Health" at Ankara University in 1955 and established Hacettepe University Children's Hospital with 150 beds in 1958. The same year he started the publication of "Journal of Child Health and Diseases" ("Çocuk Sağlığı ve Hastalıkları Dergisi") and "Turkish Journal of Pediatrics" (indexed in Science Citation Index Expanded), both of which are still published by Hacettepe University Institute of Child Health. The "Turkish National Pediatric Association" ("Milli Pediatri Derneği") was also established the same year by his initiatives. After the destruction of the first building of Hacettepe University Children's Hospital in 1961 by fire, a new hospital with 250 beds (both for children and adults) was built within six months. This hospital became the core of Hacettepe University Faculty of Medicine.[5,23]

In 1958 the first pediatric cardiac catheterization was performed by Prof. Dr. Ali Ertuğrul.

In 1959, the "School Nutrition Project" started, and milk powder and other foods were distributed to school children by the Ministry of National Education.

Universal BCG vaccination started in 1952. There was a big struggle against tuberculosis with mobile tuberculosis battle teams and dispanseries. The fight against smallpox was also successful, as the last case was diagnosed in 1957 and it was eradicated.[24]

Child Health during 1961-1980

This was the period of "Socialization of Health Services". The Nationalization of Health Care Delivery Law (Law No: 224) was passed in January 1961, and primary care based health services were given importance. Prof. Nusret Fişek was the mind behind this healthcare system philosophy. The aim was to extend health care, including preventive and environmental health services and health education to the whole country and to make it easily and equally accessible for every citizen. Health care services were organized to be delivered continuously and in accordance with the population's priorities, in a staged way. Twenty- six "Maternal and Child Health Centers" were opened. Health care for pregnant women and 0-6 age children were free of charge. The MoH worked together with Turkish Radio and Television for health education, especially by radio programs.

Türkiye's population was 27.8 million in 1960 and rose to 31.3 million in 1965, increasing 12.6% in five years. Accordingly, with the "Population Planning Law" enacted in 1965, the governmental policy shifted from pronatalist to antinatalist manner and family planning services were started. As a result, TFR, which was 5- 5.9 in 1960's, decreased to 4- 4.9 in 1970's.

Infant mortality rate decreased rather slowly. It was 200/1,000 in 1961 and 0- 4 year old children incurred 40% of all deaths. Contagious diseases like measles were still epidemic, prematurity and neonatal deaths were common. In 1960, a preterm and neonatal emergency care service was established at Zekai Tahir Burak Maternity Hospital, which was followed by the establishment of a "Preterm Ward" at Hacettepe University.[22]

In 1974 the first pediatric hemodialysis and in 1975 the first pediatric kidney transplant (by

Prof. Dr. Mehmet Haberal, Prof. Dr. Ümit Saatçi and their team) were performed at Hacettepe University Faculty of Medicine.[5]

Child Health during 1981-2000

During these years, Türkiye implemented many child survival activities to decrease IMR and to promote healthy growth of children, such as:

- Growth monitorization programs
- Expanded immunization programs: The elimination of polio and NT, reducing morbidity and mortality of measles and diphtheria
- Control of diarrheal diseases and oral rehydration therapy
- Prevention of deaths from pneumonia
- Safe motherhood projects: Family planning, nutrition and education of mothers
- Neonatal resuscitation program (NRP)
- The baby-friendly hospitals initiative and promotion of breast-feeding
- Salt iodization programs: In 1994, a strategy for developing a salt iodization program was initiated and iodization of table salts started in 1998.
- Elimination of vitamin A, D and iron deficiencies
- Neonatal screening programs: The first neonatal screening program was initiated by Prof. Dr. İmran Özalp for phenylketonuria in Hacettepe University (Table I).

With application of these strategies, the IMR which was 134/1,000 in 1978, declined to 67/1,000 between 1985- 1990, and further declined to 53/1,000 between 1988-1993, according to the State Planning Organization.[25] In 1993, the neonatal death rate was 29/1,000 live births (8/1,000 in developed, 36/1,000 in developing countries), post-neonatal death rate was 23/1,000, and under five-year mortality rate was 60.9/1,000.[26]

Table I. Neonatal screening programs in Türkiye

Start Year	Screening Program
1987	Phenylketonuria
2000: Regional 2004: National	Hearing screening
2006	Congenital hypothyroidism
2008	Biotidinase deficiency
2015	Cystic fibrosis
2017: Regional 2022: National	Congenital adrenal hyperplasia
2019	Ophtalmological screening • Neonatal: Red reflex, pupil reflex, asymmetry, cataracts • 36-48 months: LEA symbol test • 7 years: LEA symbol test
2022	Spinal muscular atrophy

Maternal-child health services were considered an integral part of primary health care by the governments and special effort was put into preventing and treating the common health problems for mothers and children. The ratio of lower respiratory tract infections as a reason for post neonatal deaths decreased from 35% in 1988 to 12.2% in 1991.[25] In 1996, the major causes of infant deaths were perinatal causes, meningococcal infections, and heart diseases.

The decline in IMR can be largely attributed to the implementation of NRP which started in 1991 with the collaboration of Hacettepe University, Ege University, İzmir Medical Chamber, the Turkish Neonatal Society and the MoH. First NRP courses were conducted in Ankara (Hacettepe University) and İzmir in 1991 and 1992.[22] Accordingly, perinatal mortality due to birth traumas and perinatal asphyxia decreased. Between 1990- 1995, the percentage of women receiving antenatal care increased from 43% to 63%.[22,26]

In 1989, the first pediatric continuous peritoneal hemodialysis was performed at Ankara University Faculty of Medicine. In 1994, Prof. Dr. Metin Karaböcüoğlu established the first pediatric intensive care unit (PICU), at İstanbul University Faculty of Medicine Emergency

Department with four beds. As of 2021, there are 878 PICU beds in Türkiye, 841 tertiary and 37 secondary level.[27]

In 1988 the first pediatric bone marrow transplantation (by Prof. Dr. Gündüz Gedikoğlu, Prof. Dr. Sema Anak and their team) and in 1990 the first pediatric living-related segmental liver transplantation (by Prof. Dr. Mehmet Haberal and his team, Ankara Başkent University) were performed.[6]

The Childhood National Immunization Program (NIP) by itself is a great endavour: In 1981, the Extended Immunization Program started to immunize population groups sensitive to vaccine-preventable diseases. The aim was to get a 90% vaccination rate for every infection, make 80% of 0-11-month-old children fully vaccinated and immunize all pregnant women against tetanus. In 1985, the Turkish National Immunization Campaign was launched and carried out in three rounds: September, October, and November-December. The aim was to immunize all 0- 60 month-old unvaccinated or under-vaccinated children against diphtheria, pertussis, tetanus, polio, and measles and to increase vaccination coverage. For poliomyelitis eradication, national immunization days were conducted in 1989 and 1995. In 1997, a polio mop-up program was implemented. The last case of poliomyelitis was recorded in 1998, and Türkiye was certified polio-free by WHO in 2002. A timeline of advances in pediatric healthcare is summarized in Table II.

Child Health in the 2000's and beyond

The population continued to increase and rose to 76,667,864 in 2013. Maternal and child health were given priority by the governments. Eventually, between 2003 and 2010, the ratio of pregnant women having four antenatal care visits increased from 54% to 82%.

From 2007 to 2012, Türkiye showed remarkable improvements in reducing infant and neonatal mortality rates (NMR): Infant and neonatal mortality rates, which were 16.4 and 12.2 in 2007 respectively, declined to 9.7 and 6.3 in 2012, with regional differences.[28] Prematurity, congenital abnormalities and congenital heart diseases were the three most common causes of infant deaths, which were in parallel with the developed countries. In 2009, 71% of infant deaths were of newborns. Dilli et al. reported that IMR and NMR significantly increased with the number of infants per pediatrician, doctor and midwife, while decreasing with the increased rate of hospital birth, antenatal care, infant follow-up, and staff with NRP certification.[28] The NRP certified health care professionals increased from 14.1/1,000 live births in 2007 to 24.0/1,000 live births in 2012. Neonatal intensive care unit (NICU) beds also doubled in that period: 2.2/1,000 in 2008 to 5.8/1,000 live births in 2012.[28]

The developments in neonatology also had a great effect on the reduction of IMR. The number of NICUs increased from 39 in 2002 to 116 in 2008, and the number of NICU beds increased from 665 to 4094 at the same period.[28]

In 2002, a special neonatal transport system was established in İzmir, which is still active.

In 2003, the MoH introduced the "Health Transformation Program" (HTP) in which good health and universal health coverage were considered a right and an integral part of citizenship. Preventive health care services were given weight through a family medicine system, in which every newborn child was assigned to a family medicine doctor (a general practitioner, GP) to perform well baby follow-up and childhood immunizations. Accordingly, pediatric health care changed from a mixed (both pediatrician and GP) care to a GP based system.[29]

In 2004, the "Iron Turkey Project" ("Demir gibi Türkiye Projesi") was launched to prevent iron deficiency anemia by iron supplementation for children and pregnant women.[30] In 2005, vitamin D supplementation for newborns started. Both iron and Vitamin D are given free of charge by the MoH.

Table II. Landmarks of childhealth in the first 100 years of Republic of Türkiye

Year	Landmarks
1920	Establishment of Ministry of Health and Social Service
1921	Establishment of "Himaye-i Etfal (Child Protection) Association"
1922	Women's admission to medical faculties
1923	Declaration of Republic
1924	Decleration of Children's Rights
1928	Establishment of Hıfzıssıhha Institute and School
1930	First national immunization programme (against smallpox)
	Establishment of Turkish Pediatric Association
	Publication of Türk Pediatri Arşivi (Turkish Archives of Pediatrics)
1933	First chair of Clinic for Child Care and Diseases, İstanbul University Faculty of Medicine
1938	Assembly of the first Turkish Pediatrics Congress
1945	Establishment of Ankara University Medical Faculty Pediatrics Clinic
1946	First 10-Year National Health Plan
1947	Establishment of Behçet Uz Children's Hospital, İzmir
1952	Establishment of Maternal and Child Health Section at Ministry of Health
	Universal BCG vaccination
1953	Establishment of Maternal and Child Health Development Center Preterm Child Care Center at Zeynep Kamil Hospital
	First exchange transfusion for hyperbilirubinemia
1955	Establishment of Hacettepe University Children's Hospital
	Publication of Journal of Child Health and Diseases (Çocuk Sağlığı ve Hastalıkları Dergisi) and Turkish Journal of Pediatrics
1957	Eradication of smallpox
1958	First pediatric cardiac catheterisation
1961	The Nationalization of Health Care Delivery Law
1974	First pediatric hemodialysis
1975	First pediatric kidney transplant
1981	Extended Immunization Program
1985	Turkish National Immunization Campaign
1988	First pediatric bone marrow transplantation
1990	First pediatric living-related segmental liver transplantation
1991	Neonatal Resuscitation Programme
1994	Establishment of the first pediatric intensive care unit
	Salt iodization program
2002	Polio-free certification by World Health Organization
2003	Health Transformation Programme
2004	Iron Turkey Project
2009	Elimination of neonatal tetanus
2011	Elimination of diphtheria

Due to successful immunization programs NT was eliminated in 2009 and diphtheria in 2011.[19] The measles elimination program started in 2002 and measles vaccination days were enacted at schools in 2003 and 2005. After this successful eradication program, there were nearly no

measles cases between 2007 and 2011. Following the 2011 Syrian war, an epidemic erupted in 2013 with 1,005 new cases. In 2023, there were nearly 5,000 cases of measles. Anyhow, Türkiye has been very successful regarding NIP, zero dose children decreasing from 3.1% in 1993 to 0.9 % in 2018, with regional disparities.[31] Türkiye's achievement in childhood immunization will be highlighted by stating that the percentage of zero doze children is 7.7% in 92 low and medium income nations.[32]

In 2015, there were 4025 pediatricians in Türkiye: 513 in university hospitals, 1694 in state hospitals, and 1818 in private practice. Approximately 2,000 residents were enrolled in pediatric residency programs. The ratio of pediatricians per child in Türkiye was 6.1/100,000, compared to 14.1/100,000 in European Union (EU) countries.[29]

In 2021, the first pediatric lung transplant was performed successfully in Ankara City Hospital, in a 15 year-old girl with cystic fibrosis.

Health Statistics of 2023 and Future of Turkish Children's Health

The Republic of Türkiye has experienced remarkable success in the past century in health services, but especially in children's health (Tables II and III). In 2023, 26% of the whole nation are between 0-17 years. The main reasons of death in 1-17 years group are external injuries and poisoning; not infectious diseases any more.[33] Although almost 509,6000 children younger than five years had died globally in 2021[34], IMR in 2022 had decreased to 9.2/1,000 in Türkiye and 99.7% of pregnant women received antenatal care.

BCG, hepatitis A and B, pentavalent vaccine (DTaP, inactive polio virus, Haemophilus influenzae type b), Streptococcus pneumoniae, oral polio, varicella and measles-mumps-rubella vaccines are included in the NIP, free of charge. Human papilloma virus vaccine is next in line to be included in the NIP.

As of 2022, 96,000 specialists, 109,000 GPs, 302,000 midwives/nurses are working for the health of the citizens all around Türkiye.[35] There are 6,835 pediatricians and 3,990 residents in pediatrics: 3,440 are working for the MoH, 445 in university hospitals and 2,500 in private practice. Obligatory service exits both after medical school graduation and after pediatric residency, which makes pediatric healthcare reach to the furthest parts of the country. A new and alarming problem for child health is that pediatrics was one of the least popular residencies for medical school graduates in 2022 and 2023 (second after emergency care). The problem also exists for subspecialties like neonatal and pediatric intensive care, pediatric nephrology and hematology/oncology who serve the most serious cases, have the highest malpractice risks and longest working hours and earn the least. If precautions are not taken immediately to encourage residency in pediatrics and labor intensive subspecialties, the workforce will shrink and children with the most serious illnesses will be at the greatest risk.

In 2023 life expectancy in Türkiye is close to 77,3 years, with an aging population. Deaths are mainly due to chronic diseases and cancer.[35] In 2022, TFR was 1.62, which was below the cut-off level of 2.10 to renew the population and decreased further to 1.51 in 2023.[36] For comparison, the average TFR in 27 EU countries in 2021 and 2022 were 1.53 and 1.46, respectively.[37] The population growth rate, which was 13.5/ 1,000 in 2019 decreased to 7.1/1,000 in 2022 and declined further to 1.1/1,000 in 2023. Besides, maternal age for the first birth is increasing (27.0 in 2023).[36] So, governmental policies should find a fine-tune between pro and antinatalist policies for optimum population growth.

One of the main problems of the near future is the high preterm birth rate. In 2022, the preterm birth rate was 12.9% and 129,557 preterm babies were born (preterm birth rates of 2022 in other parts of the world are: 10.4% in the USA, 7.6% in the United Kingdom, and

Table III. Child health statistics of Republic of Türkiye: 1923- 2023[10,33,40-42]

	Population	Total fertility rate (per woman)	Perinatal mortality rate (1/1,000)	Infant mortality rate (1/1,000)	Neonatal mortality rate (1/1,000)	Post Neonatal mortality rate (1/1,000)	Under five mortality rate (1/1,000)
1923	13,000,000			250			
1927	13,648,270	7					
1950	20,947,188			233			
1961	27,754,820	6.2		176			
1965	31,391,421			163			223
1978	41,953,105	4.3		134			
1988	52,125,597	3.4		77.7			
1990	56,473,035	3.08		60			80
1993	56,713,073	2.7	42	52.6	29.2	23.4	
1996	59,442,502			42.2			
1998	61,308,204	2.61		42.7	25.8	16.9	
2001	64,100,297	2.38			17		
2002	65,022,300			31.5			40
2003	65,938,265	2.2	24	29	17	12	37
2007	70,586,256	2.08	14.8	18.7	12.2	6.5	16.8
2008	71,517,100	2.2	14.1	14.8			14.1
2009	72,561,312		13.9	13.9	8.9		17.7
2010	73,722,988	2.08	12.0	10.1	6.6	3.5	18
2011	74,724,269	2.05	12.9	12	6.1	5.9	15
2012	75,627,384	2.11	12.6	9.7	6.3	3.4	11.0
2013	76,150,000	2.3	12.6	10.8	7	2.8	13.4
2015	78,741,053	2.16	11.5	10.2	7	3.2	12.4
2019	83,154,997	1.88	10.8	9.1	5.8	3.3	11.2
2020	83,380,000	1.77	10.6	8.6	5.5	3.1	10.6
2021	84,680,273	1.71	11.0	9.3	5.9	3.4	11.3
2022	85,279,553	1.62	10.5	9.2	5.7	3.5	11.2
2023	85,372,377	1.51		10.0			14.5

22% in India).[38,39] Preterm birth and associated lifelong consequences are major concerns for both the healthcare system and families, like cerebral palsy due to intracranial hemorrhage or blindness due to retinopathy of prematurity. Another concern is the survival of children with major congenital abnormalities and rare neurological/ metabolic disorders. To reduce these issues, we must educate families before conception about the risks of consanguineous marriages and conduct prenatal and neonatal screening similar to those for spinal muscular atrophy.

The development of telemedicine and similar technologies was one of the most exciting achievements of the new millennium; accelerating during the COVID-19 pandemic lockdowns. With widespread use of this new technology, every child might be able to reach a healthcare professional, regardless of where he lives, which might bring equality in healthcare.

Besides these achievements, we still have problems like child marriages, working children, refugee children, children affected by disasters, like many other parts of the world.

The trajectory pursued by the Republic of Türkiye in the realm of child health represents a remarkable tale of achievement. On behalf of the younger generation, I want to express my gratitude for the dedicated work of healthcare professionals over the past 100 years and the unwavering support received from all those involved in championing this cause. Our younger pediatricians are ready for healthier Turkish children and children all around the world.

Author contribution

The author confirms contribution to the paper as follows: Study conception and design: CS; data collection: CS; analysis and interpretation of results: CS; draft manuscript preparation: CS. The author reviewed the results and approved the final version of the manuscript.

Source of funding

The author declares the study received no funding.

Conflict of interest

The author declares that there is no conflict of interest.

REFERENCES

1. Özpekcan M. Türkiye Cumhuriyeti'nde sağlık politikası (1923-1933) [Yayımlanmamış Doktora Tezi]. İstanbul: İstanbul Üniversitesi, Atatürk İlkeleri ve İnkılâp Tarihi Enstitüsü; 1999.
2. Altıntaş A. Sıhhiye ve Muavenet-i İçtimaiye Vekâleti (Sağlık ve Sosyal Yardım Bakanlığı). Atatürk Ansiklopedisi. Available at: https://ataturkansiklopedisi.gov.tr/bilgi/sihhiye-ve-muavenet-i-ictimaiye-vekaleti-saglik-ve-sosyal-yardim-bakanligi
3. Kızılay Tarih. Tarihçe. Available at: http://kizilaytarih.org/tarihce.html
4. Dinç G, Etker Ş. Türkiye çocuk hekimliğinin ilk dergisi: La Pédiatrie En Turquie / Türkiye'de Emrâzı Etfâl. Osmanlı Bilimi Araştırmaları 2004; 2: 62-102.
5. Yurdakök M. Anadolu'da çocuk hekimliğinin tarihi. In: Yurdakök M, editor. Yurdakök Pediatri. Ankara: Güneş Tıp Kitapevi; 2017: 46-82.
6. Ünüvar E. Çocuk sağlığı ve hastalıkları tarihçesi. In: Yakıncı C, Tanrıverdi LH, Erkurt MA, Altuntaş F, Yiğenoğlu TN, editors. Cumhuriyetin 100. Yılında Tıp Dalları Tarihçesi. İstanbul: Nobel Tıp Kitabevi; 2023: 119-131.
7. Özpekcan M. Büyük Millet Meclisi tutanaklarına göre Türkiye Cumhuriyeti'nde sağlık politikası (1923-1933) (I. bölüm). Yeni Tıp Tarihi Araştırmaları 2001; 7: 105-60.
8. Terzioğlu A. Cumhuriyet dönemi Türk tıbbına ve tıp eğitimine kısa bir bakış. Yakın Dönem Türkiye Araştırmaları 2013; 2: 269- 307.
9. Republic of Türkiye Ministry of Health. History. Available at: https://www.saglik.gov.tr/EN-15600/history.html
10. Cumhuriyetin ilk sayımı. Şarman K, editor. In: Cumhuriyetin İlk Nüfus Sayımı. İstanbul: Türkiye İş Bankası Kültür Yayınları; 2023: 1-10.
11. Bakar C, Oymak S, Maral I. Turkey's epidemiological and demographic transitions: 1931-2013. Balkan Med J 2017; 34: 323-334. https://doi.org/10.4274/balkanmedj.2016.0960
12. Malkoç E. 1927 yılında hararetli bir tartışma: Türkiye'de çocuk ölümlerinin oranı. Toplumsal Tarih Dergisi 2018; 40-49.
13. Rasimoğlu CG. İki dünya savaşı arası dönemde Türkiye'de nüfus ve halk sağlığı tartışmalarının değerlendirilmesi. Mersin Üniversitesi Tıp Fakültesi Lokman Hekim Tıp Tarihi ve Folklorik Tıp Dergisi 2014; 4: 16-21.
14. Üstün Ç, Demirci Güngördü N, Özçiftçi S. Çocuk hekimliği tarihine ilişkin eski bir kitap incelemesi ve Dr. Sezai Bedrettin Tümay. Turk Pediatri Ars 2019; 54: 213-9. https://doi.org/10.14744/TurkPediatriArs.2019.91979
15. Meydan Acımış N, Kılıç BB. Cumhuriyet (1928-1973) döneminde sürdürülen sağlığın geliştirilmesi mücadelesinde halk sağlığı temaları. Mersin Üniversitesi Tıp Fakültesi Lokman Hekim Tıp Tarihi ve Folklorik Tıp Dergisi 2021; 11: 30-39. https://doi.org/10.31020/mutftd.784510
16. Kandal SC. Ihsan Hilmi Alantar and child welfare in early Republican Türkiye. Research on Education and Psychology (Rep) 2023; 7(Special Issue 2): 724-737. https://doi.org/10.54535/rep.1373079
17. Karabulut U. Cumhuriyet'in ilk yıllarında sağlık hizmetlerine toplu bir bakış, Dr. Refik Saydam'ın Sağlık Bakanlığı ve hizmetleri (1925-1937). Çağdaş Türkiye Tarihi Araştırmaları Dergisi 2007; 6: 151-160.

18. Özcan M, Demir Kürekçi H, Özer FE. Cumhuriyetin ilk yıllarında bebek ölümlerinin önlenmesine yönelik çalışmalara bir örnek: Memedeki çocuk nasıl beslenir? Akademik Bakış Dergisi 2016; 58: 642-655.

19. Özelçi P, Coşkun A, Kara A. Diphtheria epidemiology in Türkiye throughout history. J Pediatr Inf 2022; 16: e219-e235. https://doi.org/10.5578/ced.20229601

20. Akar N, Reisman A, Oral A. Albert Eckstein (1891-1950): Modernizer of Turkey's paediatrics in exile. J Med Biogr 2007; 15: 213-218. https://doi.org/10.1258/j.jmb.2007.06-54

21. Ankara University, Faculty of Medicine, Department of Child Health and Diseases. History. Available at: http://cocuksagligi.medicine.ankara.edu.tr/tarihce/

22. Kültürsay N, Satar M, Arsan S. Türkiye'de neonatolojinin gelişmesi. Available at: https://web.archive.org/web/20220706085259/http://www.neonatology.org.tr/wp-content/uploads/2016/12/tnd_tarihce-1.pdf

23. Yurdakök M. İhsan Doğramacı (1915-2010). Osmangazi Tıp Dergisi 2023; (Türk Tıbbına Hizmet Veren Bilim İnsanları Özel Sayısı): 76-83. https://doi.org/10.20515/otd.1400027

24. Kardaş, A. Cumhuriyet döneminde çiçek salgınları ve alınan önlemler. The Journal of Academic Social Science Studies 2020; 82: 309-321. https://doi.org/10.29228/JASSS.47189

25. Akın A, Köseli A. Improvements in mother-child health indicators in Turkey. Turk J Pediatr 1997; 39: 227-238.

26. Taşkın Ş, Atak N. Bebek ve çocuk ölümlerinin değerlendirilmesi: Türkiye nüfus ve sağlık araştırması, 1993 ve 1998. Ankara Üniversitesi Tıp Fakültesi Mecmuası 2004; 57: 1-12. https://doi.org/10.1501/Tipfak_0000000091

27. Yıldızdaş D, Aslan N. Current situation of pediatric intensive care specialty and pediatric intensive care units in Turkey: results of a national survey. Turk Arch Pediatr 2021; 56: 141-146. https://doi.org/10.14744/turkpediatriars.2020.26937

28. Dilli D, Köse MR, Gündüz RC, Özbaş S, Tezel B, Okumuş N. Recent declines in infant and neonatal mortality in Turkey from 2007 to 2012: impact of improvements in health policies. Cent Eur J Public Health 2016; 24: 52-57. https://doi.org/10.21101/cejph.a4097

29. Çullu F, Vural M. An overview on child health care in Turkey. J Pediatr 2016; 177(Suppl.): S213-S216. https://doi.org/10.1016/j.jpeds.2016.04.057

30. T.C. Sağlık Bakanlığı Ana Çocuk Sağlığı ve Aile Planlaması Genel Müdürlüğü. Demir Gibi Türkiye Projesi Genelgesi 2004 / 21. Available at: https://www.saglik.gov.tr/TR,11068/demir-gibi-turkiye-projesi--genelgesi-2004--21.html

31. Eryurt MA, Yalçin SS. Zero-dose children in Turkey: regional comparison of pooled data for the period 1990 to 2018. BMC Infect Dis 2022; 22: 421. https://doi.org/10.1186/s12879-022-07416-0

32. Cata-Preta BO, Santos TM, Mengistu T, Hogan DR, Barros AJD, Victora CG. Zero-dose children and the immunisation cascade: Understanding immunisation pathways in low and middle-income countries. Vaccine 2021; 39: 4564-4570. https://doi.org/10.1016/j.vaccine.2021.02.072

33. Tezel B, Aydın Ş. Sağlık Bakanlığının kuruluşunun 100. yılında Türkiye'de bebek ölümleri durum raporu. Available at: https://hsgm.saglik.gov.tr/depo/birimler/cocuk-ergen-sagligi-db/Dokumanlar/Kitaplar/Saglik_Bakanliginin_Kurulusunun_100._Yilinda_Turkiyede_Bebek_Olumleri_Durum_Raporu.pdf

34. Villavicencio F, Perin J, Eilerts-Spinelli H, et al. Global, regional, and national causes of death in children and adolescents younger than 20 years: an open data portal with estimates for 2000-21. Lancet Glob Health 2024; 12: e16-e17. https://doi.org/10.1016/s2214-109x(23)00496-5

35. T.C. Sağlık Bakanlığı 2023 Yılı Performans Programı. Available at: http://www.sp.gov.tr/upload/xSPRapor/files/I7Vfk+saglik_b_2023_pp.pdf

36. Türkiye İstatistik Kurumu. Doğum istatistikleri, 2023. Available at: https://data.tuik.gov.tr/Bulten/Index?p=Dogum-Istatistikleri-2023-53708

37. Eurostat. Fertility statistics. Available at: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Fertility_statistics

38. T.C. Sağlık Bakanlığı Halk Sağlığı Genel Müdürlüğü Çocuk ve Ergen Sağlığı Dairesi Başkanlığı. 17 Kasım Dünya Prematüre Günü. Available at: https://hsgm.saglik.gov.tr/tr/haberler-cocukergen/dunya-premature-gunu.html

39. CDC. Maternal Infant Health, Preterm Birth. Available at: https://www.cdc.gov/maternal-infant-health/preterm-birth/index.html

40. T.C. Sağlık Bakanlığı. İstatistikler, Bebek ve Çocuk Ölümleri. Available at: https://hsgm.saglik.gov.tr/depo/birimler/cocuk-ergen-sagligi-db/Dokumanlar/Istatistikler/Bebek_ve_Cocuk_Olumleri.pdf

41. Türkiye İstatistik Kurumu. Ölüm ve Ölüm Nedeni İstatistikleri, 2023. Available at: https://data.tuik.gov.tr/Bulten/Index?p=Olum-ve-Olum-Nedeni-Istatistikleri-2023-53709

42. Hamzaoğlu O, Özcan U. Türkiye Sağlık İstatistikleri 2006. Ankara: Türk Tabipleri Birliği Yayınları; 2005. Available at: https://www.ttb.org.tr/kutuphane/istatistik2006.pdf

The Turkish Journal of Pediatrics 2024; 66: 401-411
https://doi.org/10.24953/turkjpediatr.2024.4536

Original Article

Common viral respiratory infections in children with cancer during the COVID-19 pandemic: a multicenter study from Türkiye

Dilek Kaçar[ε1], Rejin Kebudi[ε2], Derya Özyörük[1], Deniz Tuğcu[3], Ayşenur Bahadır[4], Zeynep Canan Özdemir[5], Ali Aykan Özgüven[6], Mehmet Fatih Orhan[7], Ayşen Türedi Yıldırım[6], Canan Albayrak[8], İbrahim Kartal[8], Neriman Sarı[1], Hüseyin Tokgöz[9], Meryem Albayrak[10], Aylin Canbolat Ayhan[11], Nilgün Eroğlu[12], Sultan Aydın[13], Veysiye Hülya Üzel[14], Bülent Zülfikar[2], Ülkü Miray Yıldırım[2], Mustafa Büyükavcı[9], Hüseyin Gülen[6], Ersin Töret[5], Özcan Bör[5], Namık Yaşar Özbek[1], İnci Ergürhan İlhan[1], Neşe Yaralı[15]

[1]Department of Pediatric Hematology Oncology, University of Health Sciences Ankara Bilkent City Hospital, Ankara; [2]Department of Pediatric Hematology-Oncology, Oncology Institute, İstanbul University, İstanbul; [3]Department of Pediatric Hematology-Oncology, İstanbul University İstanbul Medical Faculty, İstanbul; [4]Department of Pediatric Hematology Oncology, Karadeniz Technical University Faculty of Medicine, Trabzon; [5]Department of Pediatric Hematology Oncology, Eskişehir Osmangazi University Faculty of Medicine, Eskişehir; [6]Department of Pediatric Hematology Oncology, Celal Bayar University Faculty of Medicine, Manisa; [7]Department of Pediatric Hematology Oncology, Sakarya University Faculty of Medicine, Sakarya; [8]Department of Pediatric Hematology Oncology, Ondokuz Mayıs University Faculty of Medicine, Samsun; [9]Department of Pediatric Hematology Oncology, Necmettin Erbakan University Faculty of Medicine, Konya; [10]Department of Pediatric Hematology Oncology, Kırıkkale University Faculty of Medicine, Kırıkkale; [11]Department of Pediatric Hematology Oncology, İstanbul Medeniyet University Faculty of Medicine, İstanbul; [12]Department of Pediatric Hematology Oncology, Ankara Etlik City Hospital, Ankara; [13]Department of Pediatric Hematology Oncology, Antalya Education and Research Hospital, Antalya; [14]Department of Pediatric Hematology Oncology, Dicle University Faculty of Medicine, Diyarbakır; [15]Department of Pediatric Hematology Oncology, Yıldırım Beyazıt University Faculty of Medicine, Ankara Bilkent City Hospital, Ankara, Türkiye.

ABSTRACT

Background. Microbiologic confirmation of respiratory tract infections gained importance during the coronavirus disease 2019 (COVID-19) pandemic. This study retrospectively evaluated seasonal distribution, clinical presentation, and complications of respiratory viral infections (RVIs) other than COVID-19 in children with cancer during and after the pandemic lockdown.

Methods. Two hundred and sixty-five inpatient and outpatient RVI episodes in 219 pediatric cancer patients confirmed by multiplex reverse transcriptase polymerase chain reaction (RT-PCR) panels from 13 centers were enrolled.

Results. Eighty-six (32.5%) of the total 265 episodes occurred in 16 months corresponding to the lockdowns in Türkiye, and the remaining 67.5% in 10 months thereafter. Human rhinovirus/enterovirus (hRE) (48.3%) was the most common agent detected during and after lockdown. Parainfluenza virus (PIV) (23.0%), influenza virus (9.8%), and respiratory syncytial virus (RSV) (9.1%) were the other common agents. The 28.7% of episodes were

✉ Rejin Kebudi
rejinkebudi@yahoo.com

ε The authors contributed equally to the research.

Received 30th Mar 2024, revised 27th Aug 2024, accepted 12th Sep 2024.

This study was presented as a poster at the 54th Congress of the International Society of Pediatric Oncology (SIOP) on September 28 - October 2022 in Barcelona, Spain.

lower respiratory tract infections (LRTIs), and complications and mortality were higher than upper respiratory tract infections (URTIs) (25.0% vs 5.3%). Bacteremia was identified in 11.5% of culture-drawn episodes. Treatment delay in one-third and death within four weeks after RVI in 4.9% of episodes were observed.

Conclusion. During the pandemic, fewer episodes of RVIs occurred during the lockdown period. Respiratory viruses may cause complications, delays in treatment, and even death in children with cancer. Therefore, increased awareness of RVIs and rapid detection of respiratory viruses will benefit the prevention and, in some cases, abrupt supportive and some antiviral treatment of RVI in children with cancer.

Key words: respiratory viral infections, children, cancer, COVID-19.

Respiratory viruses (RVs) are the most common infection agents in children and frequent causes of hospitalization in children with cancer.[1] After the first description of coronavirus disease 2019 (COVID-19) in December 2019, the pandemic mainly affected adults with immunosuppression and comorbidities. In March 2020, COVID-19 was first seen in our country, and the nationwide data of COVID-19 in children with cancer was reported.[2] Between January 2020 and May 2021, lockdowns and distance learning measures were taken, and the microbiologic confirmation of respiratory tract infections (RTIs) gained importance for extra precaution and isolation. Concurrently, our ability to ascertain RVs has improved with multiplex reverse transcriptase polymerase chain reaction (RT-PCR) platforms.

This study aimed to evaluate etiological agents, seasonal distribution, clinical pictures, complications, and mortality of non-COVID-19 respiratory viral infections (RVIs) during and after the pandemic lockdown in children with cancer in Türkiye.

Materials and Methods

In this retrospective multicenter study between January 1, 2020, and March 1, 2022, patients under 18 years of age with ongoing cancer chemotherapy or the ones who underwent hematopoietic stem cell transplantation (HSCT) with ongoing immunosuppressive therapy with viral upper respiratory tract infections (URTIs) or lower respiratory tract infections (LRTIs) were included. Episodes that occurred after four weeks from their last chemotherapy and following a year from HSCT without immunosuppression were excluded. Upper respiratory tract infections were defined as the presence of at least one of the following symptoms: fever, sore throat, rhinorrhea, nasal congestion, otitis media, and cough with normal chest examination findings. Lower respiratory tract infections were defined as the presence or absence of URTI symptoms accompanied by pathologic signs of auscultation or new pulmonary infiltrates observed on chest radiography or computed tomography (CT).

Viral respiratory infections were confirmed by multiplex RT-PCR panels from nasopharyngeal swabs. Assays could detect at least the following 19 viruses: influenza (A, H1N1, and B), human rhinovirus/enterovirus (hRE), coronavirus (229E, NL63, OC43, and HKU1), parainfluenza virus (PIV1, 2, 3, and 4), human metapneumovirus (A/B), human bocavirus (hBoV), respiratory syncytial virus (RSV A/B) and adenovirus. Some assays could also detect the following pathogens: *Mycoplasma pneumonia*, *Legionella pneumophila*, *Bordetella pertussis*, and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Viral types under a common taxonomy were classified together. For instance, PIV1, 2, 3, and 4 were collected under PIV. Detection of more than one agent by the same or concurrent PCR panel study was accepted as coinfection. Clinical episodes occurring in the same patient with the same virus after one month with complete clinical resolution were enrolled as separate episodes and classified as recurrent infections.

Demographic characteristics, malignancy types, presence of fever or respiratory symptoms, hospitalization status, the last chemotherapy date, steroid or rituximab usage, influenza vaccination, detected RVs and other agents in multiplex RT-PCR, blood cultures, antibiotic-antiviral usage, absolute neutrophil, lymphocyte, monocyte counts and C-reactive protein (CRP) levels on the previous or following two days of RVI diagnosis, chemotherapy delays, clinical progression, complications, and mortality within four weeks were assessed.

Febrile neutropenia (FN) was defined as an absolute neutrophile count of < 500 cells/mm^3 or < 1000 cells/mm^3 with an anticipated decline to < 500 cells/mm^3 in the following 48-hour period with a single oral temperature of > 38.3 °C or 38 °C sustained for > 1 hour.[3] The temperature measurement routes varied between centres such as axillary, tympanic and forehead scanner while every center adopted the measured temperature to the classical definition. In patients with FN, appropriate antibiotics were taken immediately after cultures were drawn. The ethics committee of Ankara Bilkent City Hospital approved the study (date: Jan. 4, 2023; number: E2-23-3106).

Statistical analysis

Mean, standard deviation, median, minimum-maximum values, frequency, and percentage were used for descriptive statistics. The distribution of variables was checked with the Kolmogorov-Simirnov test. Independent samples t-test and Mann-Whitney U test were used to compare quantitative data. The chi-square test was used for the comparison of the qualitative data. All tests adopted a value of $p \leq 0.05$ for statistical significance. Analyses were conducted using SPSS (version 20.0).

Results

Patient characteristics

A total of 265 RVI episodes of 219 children from 13 centers located in six geographical regions of Türkiye were included in the study. Forty (18.3%) patients had recurrent (2-4) episodes and the median age was 4.9 (0.4-17.8) years during the RVIs. Most of the episodes (55.0%) occurred in children with leukemia followed by solid tumors (27.9%), lymphoma (9.9%) and HSCT recipients (7.2%). All HSCT procedures were allogeneic, and all patients were still on immunosuppressive therapy, 10 for graft versus host disease (GVHD) prophylaxis and 9 for confirmed GVHD. One hundred and ninety-two patients (72.5%) were detected with RVs while they were followed up with an inpatient status. Seventy-three children were outpatients, and 57.5% of them were hospitalized during RVIs. The characteristics of episodes are shown in Table I.

Seasonal distribution of respiratory viruses

Eighty-six (32.5%) episodes occurred between January 2020 and May 2021, corresponding to Türkiye's lockdowns and distance learning period. The remaining 179 (67.5%) episodes occurred between 1st May 2021 (end of lockdowns) and 1st March 2022. Human rhinovirus/enterovirus was the most common virus observed yearlong in 48.3% (*n*=128). The other common virus was PIV, detected yearlong in 23.0% (*n*=61, PIV2: 1, PIV3: 51, PIV4: 9) of episodes, followed by influenza virus in 9.8% (*n*=26, influenza A: 15, influenza A H1N1: 2, influenza B: 9) and RSV in 9.1% (*n*=24) of episodes, which were mainly observed during winter months. Adenovirus, coronaviruses other than SARS-CoV-2, and hBoV were also seen yearlong with ratios 8.7% (*n*=23), 7.5% (*n*=20, coronavirus OC43: 12, coronavirus 229E: 6, coronavirus HKU: 1, coronavirus NL63: 1), and 4.9% (*n*=13), respectively. Human metapneumovirus was observed in 1.5% (*n*=4) during winter months. The monthly distribution of RVs is shown in Fig. 1. Coinfection occurred in 14% (*n*=37) of episodes (Fig. 2). In 57 of 85 (67.1%) recurrent RVIs, a diverse virus was detected, while in 13 (15.3%) episodes, the previous virus was detected with a new co-partner agent. A prior virus was detected

Table I. Demographic, clinical, and laboratory details of patients and RVI episodes.

	n	%	p-value
Median age (years)	4.9 (0.4-17.8)	56.6	
Male	150	43.4	
Female	115	72.5	
Inpatient	192	27.5	
Outpatient	73	57.5	
Hospitalization needs	42/73		
Diagnosis			
Leukemia[a]	146	55.0	
Solid tumors[b]	74	27.9	
Lymphoma[c]	26	9.9	
HSCT recipients[d]	19	7.2	
URTIs	189	71.3	
LRTIs	76	28.7	
Mean age (years)			
URTIs	6.5±4.5		0.319
LRTIs	5.9±4.4		
LRTIs at admission			
Leukemia	47	32.2	0.186
Solid tumors	17	23.0	
Lymphoma	5	19.2	
HSCT recipients	7	36.8	
Bacteremia			
URTIs	10/115	8.7	0.125
LRTIs	11/67	16.4	
Bacteremia			
FN	18/115	15.7	0.026
Non-FN	3/67	4.5	
Laboratory findings at diagnosis			
ANC (mm^3)			
URTIs	1200 (0-29860)		0.003
LRTIs	430 (0-33740)		
ALC (mm3)			
URTIs	680 (0-10760)		0.034
LRTIs	400 (0-11740)		
AMC (mm3)			
URTIs	225 (0-5400)		0.007
LRTIs	70 (0-21350)		
CRP (mg/L, N:0-5)			
URTIs	7 (0-300)		<0.0001
LRTIs	20 (0-208)		
Complications and mortality			
URTIs	10/189	5.3	<0.0001
LRTIs	19/76	25.0	
Complications and mortality			
Inpatients	23/192	12.0	0.381
Outpatients[e]	6/73	8.2	

Quantitatie data were presented as mean ± standard deviation, or median (min-max). ALC, absolute lymphocyte count; AMC, absolute monocyte count; ANC, absolute neutrophil count; CRP, C-reactive protein; FN, febrile neutropenia; HSCT, hematopoietic stem cell transplantation; LRTI, lower respiratory tract infection;
RVI, respiratory viral infection; URTI, upper respiratory tract infection.

[a] 130 acute lymphoblastic leukemia, 13 acute myeloid leukemia, 3 juvenile myelomonocytic leukemia

[b] 27 embryonal tumors, 19 central nervous system tumors, 18 bone and soft tissue tumors, 3 germ cell tumors, 7 others

[c] 22 non-Hodgkin lymphoma, 4 Hodgkin lymphoma

[d] 11 acute lymphoblastic leukemia, 4 juvenile myelomonocytic leukemia, 3 acute myeloid leukemia, 1 non-Hodgkin lymphoma

[e] at admission

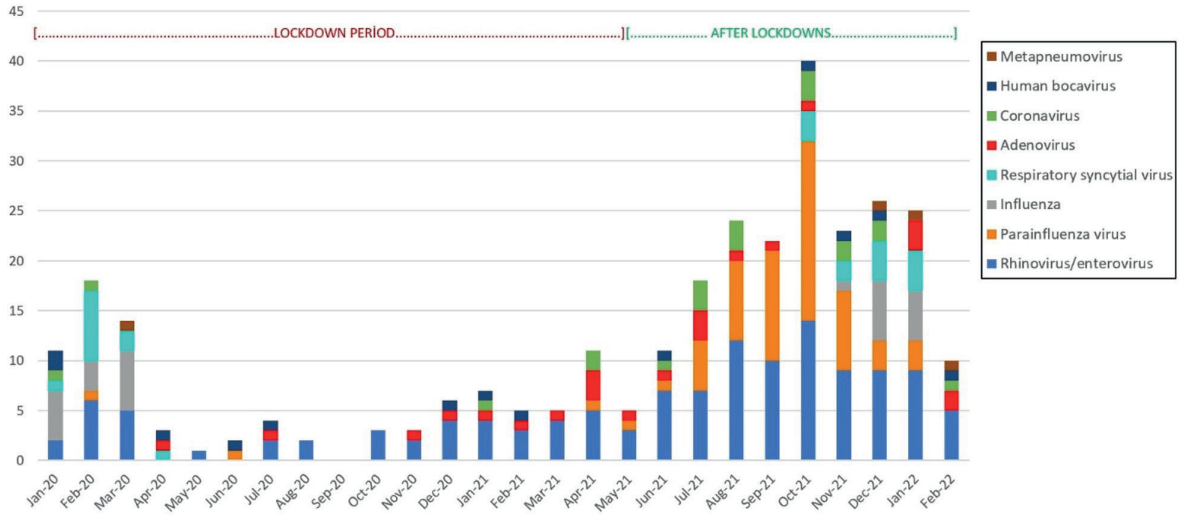


Fig. 1. Monthly distribution of detected viruses.

solely after complete recovery in 15 episodes (17.6%). SARS-CoV-2 PCR was examined in 220 episodes (83%) and detected in four episodes (1.8%) as a co-partner agent, which were all after the lockdown period. *Bordetella pertussis* in two episodes and *Legionella pneumophila* in one were also detected with concomitant hRE. Adenovirus was detected in 23 episodes, seven (30.4%) in HSCT recipients. It was the most common detected agent in HSCT recipients (36.8%).

Clinical presentations

Seventy-six episodes (28.7%) were diagnosed as LRTIs at first admission, and all virus types were related to LRTIs (Fig. 2). Lower respiratory tract infections were not associated with malignancy type, age, and coinfection (Table I). In 29.4% (*n*=78) of episodes, steroids had been used in the preceding two weeks, LRTI ratio was 32.1% in this group which was not higher than that of the steroid-free group (*p*=0.433). Rituximab was not used in any of the patients.

Fever accompanied 66% (*n*=175) of episodes, 43.4% (*n*=115) were classified as FN, and all those patients were treated in clinics. Antibiotics were used in 75.8% (*n*=201) via intravenous route in 182. Oseltamivir was used in all patients with influenza. Other antiviral agents were not used in any of the episodes. During the four weeks of follow-up, 94.7% (*n*=179) of URTIs and 75% (*n*=57) of LRTIs resulted in full recovery. The median chemotherapy delay was seven (2-80) days in 74 of 237 episodes (31.2%).

Laboratory and radiological results

At diagnosis of RVI, the median absolute neutrophil, lymphocyte, and monocyte counts were lower in LRTIs than in URTIs ($p<0.05$), whereas the median CRP level was significantly higher in LRTIs than in URTIs ($p<0.0001$) (Table I).

Blood cultures were obtained in 68.7% (*n*=182) of episodes, and bacteremia was identified in 11.5% (*n*=21), mostly in FN episodes. Five of the 21 bacteremia episodes were catheter-related bloodstream infections in FN episodes. There was no relation between bacteremia and the RVI site (Table I). The microbiological agents detected in blood cultures during episodes were as follows: *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Streptococcus pneumonia*, *Streptococcus oralis*, and *Candida albicans*.

Radiologic imaging, either posteroanterior chest radiography (*n*=113) or CT (*n*=43), was performed in 58.9% (*n*=156) at diagnosis. The

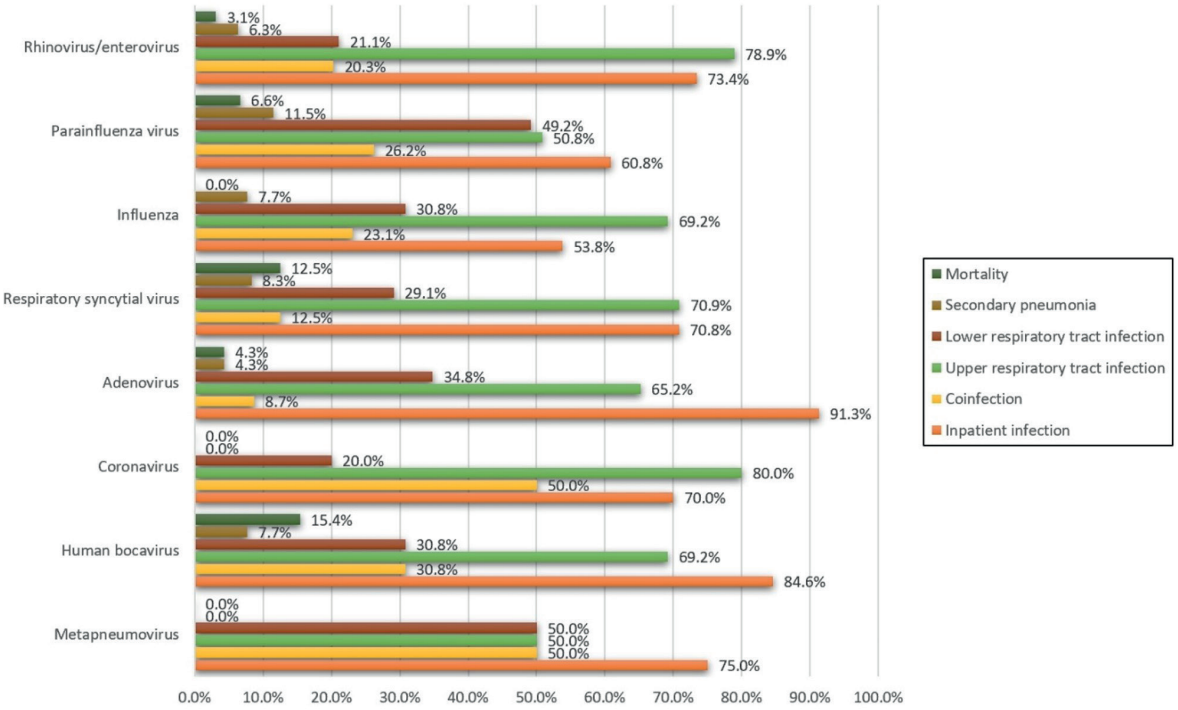


Fig. 2. Virus-specific clinical and prognostic characteristics.

most common findings were peribronchial thickening (35 episodes), ground-glass opacities (30 episodes), nodules (11 episodes), and consolidations (11 episodes).

Complications and mortality

During the follow-up, 5.3% of URTIs and 25.0% of LRTIs had complications or mortality (p<0.0001). The most common complication was secondary pneumonia, observed in 7.5% of the episodes (n=20). Secondary pneumonia was observed related with all the virus types except metapneumovirus and coronaviruses (Fig. 2). In these episodes, one hRE with *Legionella pneumophila* and one hRE with PIV were detected; the remaining 18 were single agents. These complicated 20 episodes were accompanied by bacteremia (n=4) (*S. epidermidis*, *E. faecium*, *S. lugdunensis*, *S. pneumonia)*, multiple organ failure (n=4), and myocarditis (n=2). Another critical complication, Guillian Barre Syndrome, was observed in two patients, in a relapsed acute lymphoblastic leukemia (ALL) patient after an adenovirus episode and in an ALL patient following an hRE episode complicated by *K. pneumonia* sepsis and myocarditis. Two URTIs were complicated by sinusitis (influenza virus, RSV), and one URTI by otitis media (hRE).

Overall, the mortality rate was 4.9% (n=13). The details of fatal episodes are shown in Table II. Most patients were either relapsed leukemia or at induction of leukemia with severe neutropenia and lymphopenia. The mortality rates of RVs are shown in Fig. 2.

Discussion

Viruses are important causes of RTIs in children with cancer and often cause similar clinical manifestations. Multiplex PCR is a sensitive method to detect viruses and fits with the "multi-etiological" nature of viral RTIs.[4] The COVID-19 pandemic has underscored the effects of RVIs, and the use of PCR for the diagnosis of RVIs has become more established. Besides, the diversity and prevalence of RVs changed because of the infection control measures taken during the pandemic.[5] Before the pandemic, the most

Table II. Clinical and laboratory details of mortal episodes.

viral agent/ agents	diagnosis	malignancy status/ post-HSCT duration	laboratory at the time of RVI				accompanying complications, comorbidities
			ANC/ mm^3	ALC/ mm^3	AMC/ mm^3	CRP mg/L (N:0-5)	
hRE	ALL	Remission	40	110	20	185	*Legionella pneumophila* coinfection
	ALL	Relapsed-refractory	20	220	0	183	Secondary pneumonia, invasive fungal infection
	HL	Relapsed	1100	10	10	204	Secondary pneumonia, multiple organ failure
RSV	ALL	Relapsed	20	70	0	125	Down S., secondary pneumonia, *S. epidermidis* bacteremia
	AML	Remission	210	20	0	4	
	ALL	Induction	100	290	0	3	
PIV3	ALL	Induction	760	380	150	0	Secondary pneumonia, myocarditis
	ALL	Relapsed-refractory	360	100	0	24	*K. pneumoniae* sepsis
	Osteosarcoma	Relapsed-refractory	8740	1260	490	208	Secondary pneumonia
hBoV	HSCT recipient (ALL)	Remission/ 122 days	80	430	20	40	*S. epidermidis* bacteremia, GI tract GVHD, adenoviremia
	HSCT recipient (JMML)	Remission/ 67 days	240	310	90	70	
Adenovirus	HSCT recipient (ALL)	Remission/ 50 days	120	30	20	204	Secondary pneumonia, multiple organ failure, *E. faecium* bacteremia, GI tract GVHD, adenoviremia
PIV3, hRE	HSCT recipient (ALL)	Remission/ 201 days	390	130	10	190	*S. pneumoniae* bacteremia, GI tract GVHD, adenoviremia

ALL, acute lymphoblastic leukemia; ALC, absolute lymphocyte count; AMC, absolute monocyte count; AML, acute myeloid leukemia; ANC, absolute neutrophil count; CRP, C-reactive protein; GI, gastrointestinal; GVHD, graft versus host disease; hBoV, human bocavirus; HL, Hodgkin lymphoma; hRE, human rhinovirus/enterovirus; HSCT, hematopoietic stem cell transplantation; JMML, juvenile myelomonocytic leukemia; PIV3, parainfluenza virus type 3; RSV, respiratory syncytial virus; RVI, respiratory viral infection.

common RVs were hRE, RSV, PIV, and influenza in pediatric cancer patients in our country.[6,7] A recent cohort showed rhinovirus was the most common RV agent in patients who were negative for SARS-CoV-2, and the frequency of influenza significantly decreased in the year 2020.[8] The significant findings observed in our study were that hRE viruses affect cancer patients yearlong despite lockdown and distance learning. SARS-CoV-2 had a low ratio of 1.9%, and influenza was not detected in any of the cases of children with cancer during the lockdown period. The lower rate of SARS-CoV-2 compared to other viruses can be attributed to frequent screening in patients, their caregivers, and health care providers, and those who test positive were immediately isolated. Srinivasan et al. detected rhinovirus in over 60% of pediatric cancer patients with URTI/LRTI, coinfection in 24% of patients, and coinfection did not increase the risk for LRTI.[9]

In our study, LRTIs were mostly related to hMPV, PIV, adenovirus, and RSV, which might be related to their high virulence. PIV was frequently associated with LRTIs and complicated with secondary pneumonia, in which mortality was higher. A study conducted with 74 pediatric cancer patients with PIV revealed the LRTI was present in 20.3% of these patients at the initial presentation, 20.3% of URTI progressed to LRTI, PIV-associated mortality was 18.5%, and 80% of infections were nosocomial.[10] PIV-3-related LRTIs in patients with hematologic malignancy were noted more frequently with higher viral loads than non-hematologic patients.[11]

RSV was reported as the third most common viral agent after hRE and PIV in children undergoing HSCT, with 10% mortality.[12] In adult HSCT recipients, ribavirin-based therapy was associated with decreased progression to LRTIs and improved mortality rates.[13] RSV was another frequent and mortal agent in our study; however, ribavirin was not used in any of the RSV episodes. Of interest, we didn't observe any RSV episodes in HSCT recipients. Adenovirus was the prominent agent with viremia in most HSCT patients. Following HSCT, activation of adenovirus and respiratory tract involvement may cause respiratory failure and death.[14]

An unexpected agent, hBoV, was detected in two mortal episodes in our HSCT recipients. A study from Türkiye revealed that one-third of pediatric patients with hBoV required intensive care due to severe acute LRTI.[15] However, in another study, hBoV was reported as an infrequent and non-serious respiratory pathogen in adult and pediatric HSCT recipients.[16]

According to the 2013 Infectious Diseases Society of America Guidelines, influenza vaccine is recommended for all immunocompromised patients aged six months or older[17] —none of the enrolled patients were known to be vaccinated against influenza. However, the vast usage of oseltamivir may have helped to no mortality from influenza.

Respiratory viruses are documented in 76.5% of FN attacks with respiratory symptoms and cause more extended hospitalization with antibiotic usage and higher mortality.[18] In the current study, almost half of the RVIs were seen during FN episodes, and the mortality was high in these patients. Koskenvuo et al. reported that half of the septic episodes in children with leukemia had an accompanying virus infection, mostly rhinovirus and RSV, and they had more severe clinical profiles.[19] However, Shinn et al. found that despite a high prevalence of RVIs in children with FN, the result of a respiratory multiplex PCR panel did not impact the length of hospital stay or bacteremia.[20] In our study, the bacteremia ratio was 15.7% in FN episodes, comparable with the reported ratios of 12.9-23.3% in children with FN.[21-23] The bacteremia ratio in non-FN episodes was 4.5%, higher than the reported ratios of 1.3-2.5% in community-acquired pneumonia but with similar agents like *S. pneumonia* and *S. aureus*.[24-26] Lymphopenia, corticosteroids, GVHD, and cytomegalovirus reactivation in HSCT recipients are also risk factors for viral LRTIs in hematological

patients.[27-30] In the present study, LRTIs were significantly associated with lower lymphocyte and monocyte counts but not higher in patients under steroids. The T cell-mediated immune response is fundamental in protection from viral infections. The patients notably lack T or B cell immunity due to immunosuppressive therapies and are susceptible to viral infections and related complications.[28]

Influenza, PIV, adenovirus, hMPV, measles, RSV, and coronavirus disrupt the mucosal barrier, impair ciliary and macrophage phagocytic functions, and have viral effects on the cytokine milieu commonly increasing the susceptibility of other viral, bacterial, and fungal infections and the development secondary pneumonia.[31] We observed secondary possible bacterial pneumonia as the most common complication. Indicating the bacterial infection predisposition of RVs, Peltola et al. found an association between rhinovirus circulation in the community and invasive pneumococcal disease in children younger than five years of age.[32]

In our study, 4.9% of episodes resulted in death. It is difficult to attribute these mortalities to viral infections because of other comorbidities. Furthermore, RVs can lead to delays in treatment. In one-third of episodes, approximately a week of planned cancer therapy delay was observed, similar to those reported in 22-33% and 6-9 days in other studies.[7,33]

Our study is a multicenter study, in which the changed frequency and seasonal distribution of RVIs with the pandemic were reported in a patient group with high morbidity and mortality. But it has some limitations. Firstly, it was retrospective, and it was challenging to detect patients' previous microbiological and clinical patterns, which might be related to morbidity and mortality. Also, the other risk factors and related clinical conditions leading to LRTI progression were unclear. The time between the onset of initial findings and admission may affect the need for hospitalization and the prognosis of outpatients. In addition, most RVs acquired through the community can also lead to nosocomial viral spread and outbreaks of centers. The epidemics of the centers were not investigated in our study.

Respiratory viruses may cause complications overlapping with other opportunistic infections and cause morbidity and mortality in children with cancer. Although the prevalence of RVs has changed since the COVID-19 pandemic, the distribution of RVs in pediatric cancer patients remain similar. Determining RVs may contribute to proper use of currently available antiviral drugs, isolation measures, reducing unnecessary antibiotics, and increasing appropriate therapy for complications. Specific recommendations for each virus will be possible by prospective studies revealing viral loads along with the development of vaccines and drugs.

Ethical approval

This study was approved by the Institutional Ethics Committee of Ankara Bilkent City Hospital (approval number: E2-23-3106). All procedures were under the Declaration of Helsinki and its later amendments or with comparable ethical standards. The local ethics committee did not require informed consent for this retrospective, non-invasive study. The local ethics committee permitted access to the raw data in the hospital database.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: DK, RK, DT, NE; data collection: DK, RK, DÖ, DT, AB, ZCÖ, AAÖ, MFO, ATY, CA, İK, NS, HT, MA, ACA, NE, SA, VHÜ, BZ, ÜMY, MB, HG, ET, ÖB, NYÖ, İEİ, NY; analysis and interpretation of results: DK, RK, DT, NE. NY; draft manuscript preparation: DK, RK, NY. 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.

REFERENCES

1. Hijano DR, Maron G, Hayden RT. Respiratory viral infections in patients with cancer or undergoing hematopoietic cell transplant. Front Microbiol 2018; 9: 3097. https://doi.org/10.3389/fmicb.2018.03097
2. Kebudi R, Kurucu N, Tuğcu D, et al. COVID-19 infection in children with cancer and stem cell transplant recipients in Turkey: a nationwide study. Pediatr Blood Cancer 2021; 68: e28915. https://doi.org/10.1002/pbc.28915
3. Freifeld AG, Bow EJ, Sepkowitz KA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the infectious diseases society of America. Clin Infect Dis 2011; 52: e56-e93. https://doi.org/10.1093/cid/cir073
4. Gabutti G, De Motoli F, Sandri F, Toffoletto MV, Stefanati A. Viral respiratory infections in hematological patients. Infect Dis Ther 2020; 9: 495-510. https://doi.org/10.1007/s40121-020-00313-6
5. Nagakubo Y, Hirotsu Y, Maejima M, et al. Non-pharmaceutical interventions during the COVID-19 epidemic changed detection rates of other circulating respiratory pathogens in Japan. PLoS One 2022; 17: e0262874. https://doi.org/10.1371/journal.pone.0262874
6. Büyükkapu-Bay S, Kebudi R, Görgün Ö, Meşe S, Zülfikar B, Badur S. Respiratory viral infection`s frequency and clinical outcome in symptomatic children with cancer: a single center experience from a middle-income country. Turk J Pediatr 2018; 60: 653-659. https://doi.org/10.24953/turkjped.2018.06.005
7. Aydin Köker S, Demirağ B, Tahta N, et al. A 3-year retrospective study of the epidemiology of acute respiratory viral infections in pediatric patients with cancer undergoing chemotherapy. J Pediatr Hematol Oncol 2019; 41: e242-e246. https://doi.org/10.1097/MPH.0000000000001418
8. Yiğit M, Özkaya Parlakay A. Decreased pediatric viral burden and increased rhinovirus infection during the COVID-19 pandemic. Turk J Pediatr Dis 2022; 16: 395-401. https://doi.org/10.12956/tchd.1026670
9. Srinivasan A, Gu Z, Smith T, et al. Prospective detection of respiratory pathogens in symptomatic children with cancer. Pediatr Infect Dis J 2013; 32: e99-e104. https://doi.org/10.1097/INF.0b013e31827bd619
10. Maeng SH, Yoo HS, Choi SH, et al. Impact of parainfluenza virus infection in pediatric cancer patients. Pediatr Blood Cancer 2012; 59: 708-710. https://doi.org/10.1002/pbc.23390
11. Lefeuvre C, Salmona M, Bondeelle L, et al. Frequent lower respiratory tract disease in hematological patients with parainfluenza virus type 3 infection. J Med Virol 2021; 93: 6371-6376. https://doi.org/10.1002/jmv.27243
12. Fisher BT, Danziger-Isakov L, Sweet LR, et al. A multicenter consortium to define the epidemiology and outcomes of inpatient respiratory viral infections in pediatric hematopoietic stem cell transplant recipients. J Pediatric Infect Dis Soc 2018; 7: 275-282. https://doi.org/10.1093/jpids/pix051
13. Shah JN, Chemaly RF. Management of RSV infections in adult recipients of hematopoietic stem cell transplantation. Blood 2011; 117: 2755-2763. https://doi.org/10.1182/blood-2010-08-263400
14. Ali S, Krueger J, Richardson SE, et al. The yield of monitoring adenovirus in pediatric hematopoietic stem cell transplant patients. Pediatr Hematol Oncol 2019; 36: 161-172. https://doi.org/10.1080/08880018.2019.1607961
15. Akturk H, Sık G, Salman N, et al. Atypical presentation of human bocavirus: severe respiratory tract infection complicated with encephalopathy. J Med Virol 2015; 87: 1831-1838. https://doi.org/10.1002/jmv.24263
16. Ogimi C, Martin ET, Xie H, et al. Role of human bocavirus respiratory tract infection in hematopoietic cell transplant recipients. Clin Infect Dis 2021; 73: e4392-e4399. https://doi.org/10.1093/cid/ciaa1149
17. Rubin LG, Levin MJ, Ljungman P, et al. 2013 IDSA clinical practice guideline for vaccination of the immunocompromised host. Clin Infect Dis 2014; 58: e44-100. https://doi.org/10.1093/cid/cit684
18. Meena JP, Brijwal M, Seth R, et al. Prevalence and clinical outcome of respiratory viral infections among children with cancer and febrile neutropenia. Pediatr Hematol Oncol 2019; 36: 330-343. https://doi.org/10.1080/08880018.2019.1631920

19. Koskenvuo M, Möttönen M, Rahiala J, et al. Mixed bacterial-viral infections in septic children with leukemia. Pediatr Infect Dis J 2007; 26: 1133-1136. https://doi.org/10.1097/INF.0b013e318146207c

20. Shinn K, Wetzel M, DeGroote NP, et al. Impact of respiratory viral panel testing on length of stay in pediatric cancer patients admitted with fever and neutropenia. Pediatr Blood Cancer 2020; 67: e28570. https://doi.org/10.1002/pbc.28570

21. Alali M, David MZ, Danziger-Isakov LA, Elmuti L, Bhagat PH, Bartlett AH. Pediatric febrile neutropenia: change in etiology of bacteremia, empiric choice of therapy and clinical outcomes. J Pediatr Hematol Oncol 2020; 42: e445-e451. https://doi.org/10.1097/MPH.0000000000001814

22. Kara SS, Tezer H, Polat M, et al. Risk factors for bacteremia in children with febrile neutropenia. Turk J Med Sci 2019; 49: 1198-1205. https://doi.org/10.3906/sag-1901-90

23. Haeusler GM, Thursky KA, Slavin MA, et al. Risk stratification in children with cancer and febrile neutropenia: a national, prospective, multicentre validation of nine clinical decision rules. EClinicalMedicine 2020; 18: 100220. https://doi.org/10.1016/j.eclinm.2019.11.013

24. Neuman MI, Hall M, Lipsett SC, et al. Utility of blood culture among children hospitalized with community-acquired pneumonia. Pediatrics 2017; 140: e20171013. https://doi.org/10.1542/peds.2017-1013

25. Youssef AS, Fanous M, Siddiqui FJ, et al. Value of blood cultures in the management of children hospitalized with community-acquired pneumonia. Cureus 2020; 12: e8222. https://doi.org/10.7759/cureus.8222

26. Fritz CQ, Edwards KM, Self WH, et al. Prevalence, risk factors, and outcomes of bacteremic pneumonia in children. Pediatrics 2019; 144: e20183090. https://doi.org/10.1542/peds.2018-3090

27. Hakim H, Dallas R, Zhou Y, et al. Acute respiratory infections in children and adolescents with acute lymphoblastic leukemia. Cancer 2016; 122: 798-805. https://doi.org/10.1002/cncr.29833

28. Marinelli T, Wee LYA, Rowe E, et al. Respiratory viruses cause late morbidity in recipients of hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2020; 26: 782-788. https://doi.org/10.1016/j.bbmt.2019.12.724

29. Damlaj M, Bartoo G, Cartin-Ceba R, et al. Corticosteroid use as adjunct therapy for respiratory syncytial virus infection in adult allogeneic stem cell transplant recipients. Transpl Infect Dis 2016; 18: 216-226. https://doi.org/10.1111/tid.12513

30. Pochon C, Voigt S. Respiratory virus infections in hematopoietic cell transplant recipients. Front Microbiol 2019; 9: 3294. https://doi.org/10.3389/fmicb.2018.03294

31. Prasso JE, Deng JC. Postviral complications: bacterial peumonia. Clin Chest Med 2017; 38: 127-138. https://doi.org/10.1016/j.ccm.2016.11.006

32. Peltola V, Heikkinen T, Ruuskanen O, et al. Temporal association between rhinovirus circulation in the community and invasive pneumococcal disease in children. Pediatr Infect Dis J 2011; 30: 456-461. https://doi.org/10.1097/INF.0b013e318208ee82

33. Dror T, Akerman M, Noor A, Weinblatt ME, Islam S, Glasser CL. Seasonal variation of respiratory viral infections: a comparative study between children with cancer undergoing chemotherapy and children without cancer. Pediatr Hematol Oncol 2021; 38: 444-455. https://doi.org/10.1080/08880018.2020.1871137

The Turkish Journal of Pediatrics 2024; 66: 412-420
https://doi.org/10.24953/turkjpediatr.2024.4512

Original Article

Safety and efficacy of COVID-19 vaccines in children and adolescents with cancer

Nilgün Kurucu[1], Tezer Kutluk[1], İbrahim Kartal[2], Şule Yeşil[3], Özge Vural[4], Oğuz S. Dinçer[2], Mehmet Ceyhan[5]

[1]Department of Pediatric Oncology, Faculty of Medicine and Institute of Cancer, Hacettepe University, Ankara; [2]Department of Pediatric Oncology, Faculty of Medicine, Ondokuz Mayıs University, Samsun; [3]Department of Pediatric Oncology, Ankara Dr. Sami Ulus Training and Research Hospital, Univeristy of Health Sciences, Ankara; [4]Department of Pediatric Oncology, Faculty of Medicine, Gazi University, Ankara; [5]Department of Pediatric Infectious Disease, Faculty of Medicine, Hacettepe University, Ankara, Türkiye.

ABSTRACT

Background. Children with cancer have a higher morbidity and mortality due to COVID-19. Vaccination of children with cancer is important. In this study, we aimed to investigate the effectiveness and side effects of the COVID-19 vaccines in children and adolescents with cancer.

Methods. Fifty-eight patients from four centers were included in the study. Antibodies to the SARS-CoV-2 spike protein levels were measured. Vaccine-related complaints were recorded.

Results. There were 33 male and 25 female patients. The mean age was 16.9±2.3 years. In 58.6% of cases, the diagnosis was hematological malignancies. Twenty patients were currently under treatment, while 38 had completed the treatment. Forty-eight patients received chemotherapy ± radiotherapy, 13 received immunotherapy, and 3 underwent stem cell transplantation. CoronoVac© and BNT162b2© vaccines were administered in 24% and 76%, respectively. The mean antibody level was lower in patients who received CoronaVac© than that of BNT162b2©, although the difference was not significant. The levels were within the protective limits in both groups. No significant difference was found in antibody levels according to diagnostic subgroups, treatment status, type of treatment, line of treatment, disease status and time between vaccines and measurement of antibody level. The most common side effects were pain at the injection site (37.9%) and malaise/weakness (17.2%), which were similar for both vaccines.

Conclusions. Our study showed that both mRNA and inactivated vaccines elicit an immune response in children with cancer. However, the seroconversion rate is significantly higher in mRNA vaccines. Side effects were similar to those seen in healthy children.

Key Words: cancer, COVID-19, vaccines, immunization.

As of the end of 2022, there are approximately a total of 650 million confirmed cases of coronavirus disease 2019 (COVID-19) and more than 6 million deaths reported by the World Health Organisation (WHO) globally.[1] In Türkiye, more than 17.000.000 cases of COVID-19 and 100.000 deaths have been reported.[2] At the beginning of the COVID-19 pandemic, the infection occurred infrequently in children, and most of them were asymptomatic or mildly symptomatic. However, new variants of the virus have resulted in significant changes in the clinical epidemiology of pediatric COVID-19. It was observed that the new variants affect a larger portion of the young population and that 18.8% of all COVID-19 infections were seen in children and adolescents.[3,4] Although

✉ Nilgün Kurucu
nlgnkrc@gmail.com

Received 8th Jan 2024, revised 3rd Mar 2023, 8th Aug 2024, accepted 8th Aug 2024.

This work was presented at the 55th Congress of the International Society of Paediatric Oncology (SIOP) Ottawa, Canada. October 11–14, 2023; Pediatr Blood Cancer 2023; 70 (S8): 416, EP544/#458\

the clinical signs in children became similar to those in adults, the frequency of serious and critical disease and the mortality rate due to COVID-19 remained low. Adults with cancer are reported to have a higher risk for COVID-19 and have more severe disease and higher mortality than the general population.[5] More than 25% of patients with cancer who catch the virus have died from the COVID-19 infection, while 0.9% of the normal population with COVID-19 have died.[1,5] At the end of 2022, there were 1814 children with cancer and laboratory confirmed COVID-19 infection reported from 51 countries.[6] Children with cancer are reported to have more severe and critical disease and a higher mortality rate (20% and 3.8%) due to COVID-19 than children without a cancer diagnosis (%12 and 1.9%).[4,6,7] Although several pharmacological agents are available, including antivirals (such as antiretrovirals, which are used for HIV infections, remdesivir, which was used in the Ebola epidemic), immunomodulatories, and monoclonal antibodies, a specific drug to cure COVID-19 has yet to be found.[3,8] It is now well established that vaccination is an optimal strategy to prevent infection or at least reduce its severity.[4]

Active immunization against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been shown to be highly effective at reducing the incidence, preventing severe illness and death from COVID-19. As of the end the 2022, approximately 50 vaccines were approved by at least one country, and at least one COVID-19 vaccine was approved in 201 countries.[9] On December 11, 2021, the U.S. Food and Drug Administration (FDA) approved an emergency use authorization for mRNA vaccine (PfizerBioNTech/Comirnaty) as a 2-dose series for the prevention of symptomatic COVID-19 in individuals aged ≥16 years.[9,10] Vaccination programs have been started by prioritizing high-risk groups in adults around the world[9] since December 2020 and in our country since January 2021[11]. In July 2021, the European Medicine Agency approved the use of the vaccine at the age of 12-17. Following this the age of immunization expanded to include age >5 years.[12] In the pediatric age, immunization is offered by a messenger RNA vaccine shot in the muscle of the upper arm. The schedule consists of a 2-dose primary series in children 5–11 years, with a booster dose in adolescents.[10,12-14] In our country, children over the age of 15 and between the ages of 12 and 15 with chronic diseases have been included in the vaccination program since 18 August 2021. Finally, as of September 6, the vaccination program has been expanded to include those over the age of 12 with the consent of the family. Three vaccines are in use in our country: mRNA BNT162b2© (Pfizer/BioNTech), inactivated CoronaVac© (Sinovac) and the inactivated Turkovac© (T.C. TUSEB).[11] After the experiences in adults, good efficacy and an acceptable security profile and effectiveness were demonstrated in children who received the COVID-19 vaccination.[13] Among children and adolescents, the safety of current COVID-19 vaccines is acceptable, and studies have suggested that mRNA vaccines can provide high protection against COVID-19 infection in pediatric age groups.[13,15]

Patients with cancer are at increased risk for greater morbidity and mortality due to COVID-19 infection.[5,6] Several studies have provided satisfactory evidence on the protective role of COVID-19 vaccines in patients with malignant disease.[16] In addition, COVID-19 vaccines are found to be safe and well tolerated in adults with cancer.[17] Based on this evidence, it was recommended that adults with cancer should receive the recommended dose and schedule of one of the COVID-19 vaccines. Among adults with cancer who were receiving active systemic chemotherapy, most exhibited an adequate antibody response to vaccines, although their antibody titers were lower than those of healthy controls, especially in cases with hematological malignancies.[16,17]

However, studies evaluating the immunogenicity and safety of COVID-19 vaccines in children with cancer are limited.[18,19] There are concerns regarding the protection of children with cancer through the standard

vaccination program and the safety of vaccines. In this regard, we performed this study to investigate the effectiveness and side effects of the COVID-19 vaccines.

Patients and Methods

The study included a total of 58 patients from four pediatric cancer centers. These patients had received at least two doses of the COVID-19 vaccine and had a minimum of 15 days since their previous immunization. The study protocol was approved by the Institutional Ethics Committee of Hacettepe University (2022/10-06). Written informed consent was obtained from the participants and/or their parents. Vaccine-related complaints observed by patients and parents were asked and recorded. A 5 ml venous blood sample was drawn from patients. The whole blood was allowed to clot by leaving it undisturbed at room temperature. This usually took 15–30 minutes. The clot was removed by centrifuging at 1,000– 2,000 g for 10 minutes. Following centrifugation, the serum was transferred into a clean polypropylene tube and stored at –20°C. Quantitative determination of antibodies to the SARS-CoV-2 spike protein levels was performed with Roche Elecsys© Anti-SARS-CoV-2S kit by electro chemiluminescence immunoassay method. The relationship between antibody response and vaccine type, diagnosis, disease status, treatment process, and treatment type were investigated.

Statistical analyzes were performed using IBM SPSS Statistics version 23.0. software. Categorical variables were recorded as numbers and percentages, and continuous variables were recorded as means ± standard deviations (SD) and median (interqunartile range) as appropriate. Compliance with normal distribution was examined with the Shapiro-Wilk test. The Mann-Whitney U test was used to compare the antibody levels between two vaccine groups. Antibody levels according to diagnostic groups (hematological malignancies and solid tumors), type of treatment (chemotherapy, chemoradiotherapy, immunotherapy ± chemotherapy), line of treatment (first line / second line or more), treatment status (completed / continued), disease status (active / remission), time between two vaccines (3-6 weeks / more than 6 weeks), and time between the last vaccine dose to the time point of measurement of antibody levels (less than 90 days/between 90 to180 days / between 180 to 270 days) were also compared. The comparison between two and three groups were performed with Mann-Whitney U and Kruskal Wallis tests, respectively. Frequencies of side effects in different groups were compared with Fisher's exact test. The *p*-value of less than 0.05 was considered significant.

Results

There were 33 male and 25 female patients with a mean age of 16.9±2.3 (12-21) years. The most common diagnoses were hematological malignancies (2 leukemia, 30 lymphomas and 2 Langerhans cell histiocytosis) in 34 patients (58.6%), followed by bone and soft tissue sarcomas in 13 (22.5%) (Table I). Twenty patients were currently on-treatment and they were vaccinated at the midpoint of the time between two courses (7-15 day before and after treatment). Patients taking oral agents did not interrupt their treatment. Treatment of 38 cases was completed (within the last 6 months in 13 patients, between 6 months and 1 year in 8 patients, and more than 1 year ago in 17 patients). Fifty-six patients recieved chemotherapy, 25 of those also received radiotherapy. In total, 12 patients received immunotherapy alone or in combination with chemotherapy. Seven patients received their vaccines with nivolumab (3), bevacizumab (1), entrectinib (1), sirolimus (1) and denosumab (1), and five patients received brentixumab (2), temsirolimus (1), nivolumab (1) and rituximab (1) before vaccination. Three cases underwent stem cell transplantation and have been followed in remission for 2-5 years before vaccination. Because of relapsed / refractory disease, twelve patients took second or more advanced line treatment. CoronoVac© and BNT162b2© COVID-19 vaccines were

administered in 24% and 76% of patients, respectively. At the time of the study, 86% of the patients were vaccinated with two doses, while only 13.8% received three doses. Antibody levels were lower than 300 U/ml in 35.7% and 6.8% of cases vaccinated with CoronoVac© and BNT162b2©, respectively (p: 0.015). The median time between the last dose of vaccine and measurements of antibody levels was 60 (15-270) days. The time between the last dose of vaccine and antibody measurement was less then 90 days in 35 (31.6%) cases, between 90 and 180 days in 17 (29.6%) cases, and between 180 and 270 days in 6 (17%) cases. Although levels in the third group (1204±1166.2 U/ml) was lower than that of second (1974.9±884.5 U/ml) and first group (2225.1±666.8 U/ml), the difference was not significant. When evaluated according to the vaccine type, the mean antibody level was found to be lower in those who received CoronaVac© compared to those who received BNT162b2© (1649.85±1143.04 vs 2172.22±682.10 U/ml). However, the difference was not statistically significant. It was determined that the mean antibody level during the treatment was within the protective limits for the infection (1890.93±972.47 U/ml), but it was lower than the antibody level in the patients whose treatment was discontinued (2128.21±756.57 U/ml) (p>0.05). No significant difference was found in antibody levels according to diagnostic subgroups, treatment status, type of treatment, line of treatment, disease status, and time between vaccines and measurement of antibody level.

The most common side effect was pain at the injection site (51.7%), followed by swelling/redness at the injection site (24.1%) and malaise/fatigue (18.9%). Although some side effects such as lymphadenopathy and headache, were seen only with BNT162b2© vaccine, there was no significant difference in the frequency of side effects in vaccine types (Table I). Side effects were not different between patients whose were on-treatment and off-treatment.

Discussion

Immunosuppressive diseases, cancer or treatment with anticancer drugs, and stem cell transplantation have suppressive effects on humoral, cell-mediated immunity and neutrophil function. It is known that children with cancer have an increased the risk of severe infections and complications caused by viral agents, such as adenovirus, respiratory syncytial virus, influenza and other agents.[20] Substantial evidence revealed that children with cancer have a higher risk for COVID-19 infection, and more severe disease and mortality. It was reported that almost 22% of the children with cancer had severe/critical disease and 18% necessitated intensive care.[5-7] Vaccination of patients with cancer became more important since they have higher morbidity and mortality due to the infection.[18,19] There have been numerous studies including meta-analyses in adults with a high number of patients. However, studies evaluating the immunogenicity and safety of vaccines in children with cancer are limited, although experts recommend them. The number of patients in those studies are small and the results are contradictory.[21-29]

The effectiveness of vaccines in children with cancer has been evaluated by measuring the T cell and/or most often B cell immune response, and/or by determining the rate of getting SARS-CoV-2 infection. We investigated immune response by measuring spike antibody levels and found that COVID-19 vaccines elicited an effective immune response. Miao et al.[21] compared the frequency of COVID-19 infection in vaccinated and unvaccinated children with cancer and showed that vaccination reduced the rate of infection and significantly improved clinical outcomes. In another study, none of the study subjects developed clinical disease at 12 weeks' follow-up after administration of the second dose of vaccine.[22] Revon-Riviere et al.[23] reported that 9 of 10 (90%) adolescent and young adult patients who were under cancer treatment had positive serology one month after the second vaccine injection; in addition, none

Table I. Characteristics of patients according to vaccine type.

Feature of Patients	Whole group	Type of Vaccine	
		m-RNA (BNT162b2©)	Inactivated (CoronaVac©)
Total number	58	44	14
Sex (M/F)	33/25	31/13	2/12
Age (median/range)	16.9±2.3 (12-21)	16.8±3.1 (12-21)	17.1±2.8 (12-21)
Diagnosis			
Hematological			
Leukemia	2 (3.4%)	-	2 (14.3%)
Hodgkin lymphoma	16 (24.1%)	10 (22.7%)	6 (42.8%)
Non-Hodgkin lymphoma	14 (31.8%)	12 (27.3%)	2 (14.3%)
Histiocytosis	2 (3.4%)	2 (4.5%)	-
Solid Tumors			
Central nervous system tumors	5 (8.6%)	4 (9.1%)	1 (7.1%)
Bone sarcomas	7 (20.5%)	6 (13.6%)	1 (7.1%)
Soft tissue sarcomas	6 (10.4%)	5 (11.3%)	1 (7.1%)
Germ cell tumors	3 (5.2%)	3 (6.8%)	-
Others	3 (5.2%)	2 (4.5%)	1 (7.1%)
Treatment Status			
On-treatment	20 (34.5%)	16 (36.4%)	4 (28.6%)
Off-treatment	38 (65.5%)	28 (63.3%)	10 (71.4%)
Type of treatment			
Chemotherapy	31 (53.4%)	23 (52.3%)	8 (57.1%)
Chemotherapy+radiotherapy	25 (43.1%)	20 (45.4%)	5 (35.7%)
Immunotherapy±chemotherapy	12 (20.6%)	8 (18.2%)	3 (21.4%)
Autologous haematopoietic stem cell transplantation	3 (51.7%)	3 (6.8%)	
Line of treatment			
First line	46 (79.3%)	35 (79.5%)	11 (78.6%)
Second line	8 (13.7%)	6 (13.6%)	2 (14.3%)
≥Third line	4 (6.9%)	3 (6.8%)	1 (7.1%)
Antibody level (U/ml)			
Mean±SD	2046.13±836.54	2172.22±682.10	1649.85±1143.04
Range	50-2500	136-2500	50-2340
Median (Interquartile range)	2500 (412.25)	2500 (2271.75)	2500 (252)
Side effects			
Arm pain	30 (51.7%)	22 (50%)	8 (57%)
Reaction in injection site	14 (24.1%)	9 (20.5)	5 (35.7%)
Malaise/fatigue	11 (18.9%)	9 (20.5)	2 (14.3%)
Myalgia	8 (13.7%)	7 (15.9%)	1 (7.1%)
Headache	5 (8.6%)	5 (11.4%)	-
Fever	6 (10.3%)	4 (9.0%)	1 (7.1%)
Lymphadenopathy	3 (5.1%)	3 (6.8%)	-
Bone and joint pain	2 (34.5%)	2 (4.5%)	1 (7.1%)

SD: standard deviation.

of the them developed COVID-19 infection. Lehrnbecher et al.[25] showed that seroconversion was achieved in 9 out of 11 (82%) adolescents with cancer after completion of two doses of vaccine and remained at effective levels for six months. Although we did not follow-up the antibody levels longitudinally, the median time between the last dose of the vaccine and measurements of antibody level was 60 days and reached to 270 days. Similar to most published reports, we evaluated the immunogenicity of the vaccine after two doses and found adequate antibody response. However, there are studies showing a significant increase in both the percentage of responsive patients and antibody titers after the 3rd dose.[25,26] It was found that detectable antibodies against SARS-CoV-2 were 76.2% (16/21) and 90% (18/20) after 2 and 3 doses, respectively.[25] Poparn et al.[22] compared the immune response to BNT162b2 mRNA vaccine in on-therapy and off-therapy pediatric cancer cases and found that the antibody levels were significantly low in the on-therapy group. Conversly, we did not find a significant difference between the antibody levels of patients receiving treatment and patients whose treatment was completed. Although the majority of the studies in the literature, including ours, showed COVID-19 vaccines provide adequate immune protection in children with cancer[21-26], the opposite results have rarely been reported. In a recent study, it was reported that only 36.4% of 11 cases showed adequate B-cell responses, while 77.8% showed adequate T-cell response.[27]

There are a some studies that include a healthy control group and children with cancer comparing the immune response to the COVID-19, but the results are contradictory.[27-29] Ma et al.[28] reported that 68.4% of children with cancer became seropositive after two doses of vaccine, but the seroconversion rate was significantly lower than that of healthy controls (86.5%). Additionally, antibody titers were found to be significantly lower in the patient group than in healthy children. In contrast, Martin et al.[29] reported that pediatric patients with cancer had similar immunoglobulin titers, antibody binding capacity, and effector function assay activity after vaccination against COVID-19 compared with healthy controls. Although we did not have a control group, we found that the effectiveness of the vaccines were similar to that reported in healthy children. In a meta-analysis of 88 articles on pediatric vaccination, the seroconversion rates after the second and third doses of the vaccines were found to be 96.5% and 99.8%, respectively.[15] In another systematic analysis it was reported that mRNA vaccines were found to be 91%–100% efficacious in preventing COVID-19 among children and adolescents.[13]

Vaccine studies in pediatric cancer patients in the literature have mostly been carried out with mRNA vaccines.[22-29] In a study conducted in China, it was shown that the inactivated vaccine was effective in children with hematology/oncology diseases.[21] We could not find any study comparing the different vaccine types in children with cancer. We compared an inactivated (CoronaVac) and a mRNA vaccine (BNT162b2) and showed that the mean antibody levels were within the protective limits in both groups.

Overall, COVID-19 vaccines were reported to be well tolerated. Approximately 50% of our patients had mild/moderate side effects. Pain and local reactions in the injection site were the most common complications reported in the literature similar to our study.[22,23,27] Although we did not have a healthy control group, we found that the frequency and distribution of side effects in our patients were not much different from those in studies conducted on healthy children. In a meta-analysis including 88 articles, the five adverse events with the highest incidence rates were tenderness (53%), injection site pain (51%), fatigue / asthenia / tiredness (24%), headache (20%), and myalgia / muscle pain (15%).[15] Ma et al.[28] showed that adverse events of the vaccines reported during follow-up were graded as I and II, and all adverse events were either self-limited or medically well-managed. The frequency and severity of side effects were

not found to be different between children with cancer and healthy children.

Our study has some limitations. Due to the lack of a control group, we did not have the opportunity to compare the results with healthy children. Since the vaccination process is not prospective, that is, the study was conducted on vaccinated patients, the number of doses, the time between doses, and the time of measurement of the antibody level were heterogeneous. In addition, we did not continue to monitor antibody levels, and hence we could not determine how long the immune protection provided by the vaccine lasted.

In conclusion, despite these limitations, our study provided some important implications. We showed that the seroprotective level of COVID-19 vaccines in children with cancer who receive anticancer treatment are effective but low compared with patients who completed the treatment. Both mRNA and inactivated vaccines produce immune responses, although antibody titers were lower with the inactivated vaccine than the mRNA vaccine.

Ethical approval

The study was conducted with the approval of the Ethics Committee of the Hacettepe University, (2022/10-06).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: NK,TK, MC; data collection: NK, İK, ŞY, ÖV, OSD; analysis and interpretation of results: NK, TK; draft manuscript preparation: NK, TK. All authors reviewed the results and approved the final version of the article.

Source of funding

The study was funded within the scope of project of "The UK Research and Innovation Global Challenges Research Fund (GCRF) Research for Health in Conflict in the Middle East and North Africa (R4HC-MENA) project; developing capability, partnerships and research in the Middle East and North Africa [ES/P010962/1]."

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. World Health Organization (WHO). Coronavirus disease (COVID-19) pandemic: WHO Coronavirus (COVID-19) Dashboard. Available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (Accessed on March 23, 2023).
2. Republic of Türkiye Ministry of Health. COVID-19 information platform. Available at: https://covid19.saglik.gov.tr/TR-66935/genel-koronavirus-tablosu.html (Accessed on March 23, 2023).
3. Habet V, Oliveira CR. Clinical epidemiology of pediatric Coronavirus disease 2019 and its postacute sequelae. Semin Respir Crit Care Med 2023; 44: 66-74. https://doi.org/10.1055/s-0042-1759566
4. Gupta SL, Tyagi R, Dhar A, Oswal N, Khandelwal A, Jaiswal RK. Children's SARS-CoV-2 infection and their vaccination. Vaccines (Basel) 2023; 11: 418. https://doi.org/10.3390/vaccines11020418
5. Saini KS, Tagliamento M, Lambertini M, et al. Mortality in patients with cancer and coronavirus disease 2019: a systematic review and pooled analysis of 52 studies. Eur J Cancer 2020; 139: 43-50. https://doi.org/10.1016/j.ejca.2020.08.011
6. Mukkada S, Bhakta N, Chantada GL, et al. Global characteristics and outcomes of SARS-CoV-2 infection in children and adolescents with cancer (GRCCC): a cohort study. Lancet Oncol 2021; 22: 1416-1426. https://doi.org/10.1016/S1470-2045(21)00454-X
7. Kebudi R, Kurucu N, Tuğcu D, et al. COVID-19 infection in children with cancer and stem cell transplant recipients in Turkey: a nationwide study. Pediatr Blood Cancer 2021; 68: e28915. https://doi.org/10.1002/pbc.28915
8. National Institutes of Health. COVID-19 treatment guidelines panel. Coronavirus disease 2019 (COVID-19) treatment guidelines. Available at: https://www.covid19treatmentguidelines.nih.gov (Accessed on March 23, 2023).

9. Basta NE, Moodie EMM on behalf of the VIPER (Vaccines, Infectious disease Prevention, and Epidemiology Research) Group COVID-19 Vaccine Development and Approvals Tracker Team. COVID-19 vaccine development and approvals tracker. 2020. Available at: https://covid19.trackvaccines.org

10. National Center for Immunization and Respiratory Diseases (NCIRD), Division of Viral Diseases. CDC COVID-19 Science Briefs. Atlanta (GA): Centers for Disease Control and Prevention (US); 2020-2021 Sep 15. Available at: https://www.ncbi.nlm.nih.gov/books/NBK570439/

11. Republic of Türkiye Ministry of Health. COVID-19 vaccines information platform. Available at: https://covid19asi.saglik.gov.tr/TR-77706/covid-19-asisi-ulusal-uygulama-stratejisi.html (Accessed on March 23, 2023).

12. Hause AM, Baggs J, Marquez P, et al. COVID-19 vaccine safety in children aged 5-11 Years - United States, November 3-December 19, 2021. MMWR Morb Mortal Wkly Rep 2021; 70: 1755-1760. https://doi.org/10.15585/mmwr.mm705152a1

13. Tian F, Yang R, Chen Z. Safety and efficacy of COVID-19 vaccines in children and adolescents: a systematic review of randomized controlled trials. J Med Virol 2022; 94: 4644-4653. https://doi.org/10.1002/jmv.27940

14. Bozzola E, Caffarelli C, Santamaria F, Corsello G. The year 2021 in COVID-19 pandemic in children. Ital J Pediatr 2022; 48: 161. https://doi.org/10.1186/s13052-022-01360-0

15. Gao P, Kang LY, Liu J, Liu M. Immunogenicity, effectiveness, and safety of COVID-19 vaccines among children and adolescents aged 2-18 years: an updated systematic review and meta-analysis. World J Pediatr 2023; 19: 1041-1054. https://doi.org/10.1007/s12519-022-00680-9

16. Kuderer NM, Hill JA, Carpenter PA, Lyman GH. Challenges and opportunities for COVID-19 vaccines in patients with cancer. Cancer Invest 2021; 39: 205-213. https://doi.org/10.1080/07357907.2021.1885596

17. Massarweh A, Eliakim-Raz N, Stemmer A, et al. Evaluation of seropositivity following BNT162b2 messenger RNA vaccination for SARS-CoV-2 in patients undergoing treatment for cancer. JAMA Oncol 2021; 7: 1133-1140. https://doi.org/10.1001/jamaoncol.2021.2155

18. Caniza MA, Homsi MR, Bate J, et al. Answers to common questions about COVID-19 vaccines in children with cancer. Pediatr Blood Cancer 2022; 69: e29985. https://doi.org/10.1002/pbc.29985

19. Furlong E, Kotecha RS, Conyers R, et al. COVID-19 vaccination in children and adolescents aged 5 years and older undergoing treatment for cancer and non-malignant haematological conditions: Australian and New Zealand Children's Haematology/Oncology Group consensus statement. Med J Aust 2022; 216: 312-319. https://doi.org/10.5694/mja2.51444

20. Kaltsas A, Sepkowitz K. Community acquired respiratory and gastrointestinal viral infections: challenges in the immunocompromised host. Curr Opin Infect Dis 2012; 25: 423-430. https://doi.org/10.1097/QCO.0b013e328355660b

21. Miao J, Zhang JY, Liang J, et al. Efficacy of inactivated SARS-CoV-2 vaccination in pediatric hematology/oncology patients: a real-world study. World J Pediatr 2023; 19: 1017-1021. https://doi.org/10.1007/s12519-023-00737-3

22. Poparn H, Srichumpuang C, Sosothikul D, et al. Immune response after 2 doses of BNT162b2 mRNA COVID-19 vaccinations in children and adolescents with cancer and hematologic diseases. Asian Pac J Cancer Prev 2022; 23: 2049-2055. https://doi.org/10.31557/APJCP.2022.23.6.2049

23. Revon-Riviere G, Ninove L, Min V, et al. The BNT162b2 mRNA COVID-19 vaccine in adolescents and young adults with cancer: a monocentric experience. Eur J Cancer 2021; 154: 30-34. https://doi.org/10.1016/j.ejca.2021.06.002

24. Donze C, Ninove L, de Lamballerie X, Saultier P, Andre N. Six-month BNT162b2 vaccine efficacy in adolescents and young adults with cancer. Pediatr Blood Cancer 2022; 69: e29547. https://doi.org/10.1002/pbc.29547

25. Lehrnbecher T, Sack U, Speckmann C, et al. Longitudinal immune response to 3 doses of messenger RNA vaccine against Coronavirus disease 2019 (COVID-19) in pediatric patients receiving chemotherapy for cancer. Clin Infect Dis 2023; 76: e510-e513. https://doi.org/10.1093/cid/ciac570

26. Schmidt KLJ, Dautzenberg NMM, Hoogerbrugge PM, et al. Immune response following BNT162b2 mRNA COVID-19 vaccination in pediatric cancer patients. Cancers (Basel) 2023; 15: 2562. https://doi.org/10.3390/cancers15092562

27. D'Souza AM, Thomas M, Gnanamony M, Nguyen TH, de Alarcon PA. SARS-CoV-2 vaccination response in pediatric oncology patients. Pediatr Blood Cancer 2024; 71: e30785. https://doi.org/10.1002/pbc.30785

28. Ma Y, Chen B, Wang Y, et al. Reducedhumoral response against variants of concern in childhood solid cancer patients compared to adult patients and healthy children after SARS-CoV-2 vaccination. Front Immunol 2023; 14: 1110755. https://doi.org/10.3389/fimmu.2023.1110755

29. Martin SD, Nziza N, Miozzo P, et al. Humoral profiling of pediatric patients with cancer reveals robust immunity following anti-SARS-CoV-2 vaccination superior to natural infection. Pediatr Blood Cancer 2023; 70: e30473. https://doi.org/10.1002/pbc.30473

The Turkish Journal of Pediatrics 2024; 66: 421-428
https://doi.org/10.24953/turkjpediatr.2024.4529

Original Article

Central line-associated bloodstream infection outbreak related to *Ralstonia pickettii*-contaminated saline in a pediatric hematopoietic stem cell transplant center

Safiye Suna Çelen[1], Suleimen Zhumatayev[1], Koray Yalçın[1], Manolya Kara[2], Murat Sütçü[3], Gülsün Karasu[1], M. Akif Yeşilipek[1]

[1]Department of Pediatric Hematology, School of Medicine, Göztepe Medical Park Training and Education Hospital, Bahçeşehir University, İstanbul; [2]Department of Pediatric Infectious Diseases, School of Medicine, Yeditepe University, İstanbul; [3]Department of Pediatric Infectious Diseases, School of Medicine, Bahcesehir Liv Training and Education Hospital, İstinye University, İstanbul, Türkiye.

ABSTRACT

Background. *Ralstonia pickettii* is an aerobic Gram-negative non-fermentative bacillus. It is an opportunistic pathogen that has recently prompted nosocomial outbreaks. Although it has low virulence, it can cause a wide range of invasive diseases in immunosuppressive patients. The characteristics of *R. pickettii*-related central line-associated bloodstream infection (CLABSI) outbreak in pediatric hematopoietic stem cell transplant (HSCT) recipients are presented in this study.

Materials and Methods. This was a single-center, retrospective analysis conducted at Bahcesehir University Goztepe Medicalpark Hospital . The clinical and laboratory characteristics of twelve children with Ralstonia-related CLABSIs were analyzed.

Results. Of the twelve patients with *R. pickettii* growth, seven were female. The median age was 12.1 (2-17) years. Autologous HSCT was performed in two of the patients and allogeneic HSCT was performed in ten patients for both malignant and non-malignant diseases. In the conditioning regimens, all patients were given myeloablative therapy. Clinical sepsis was the most common presentation. As a result of the investigations, *R. pickettii* growth was observed in saline solutions. All cases were successfully treated with the appropriate antibiotic regimen and the bacteria was not found in repeat cultures. Catheter removal was required in two patients. Mortality was not observed in any patient as the outcome of the infection episode.

Conclusion. The detection and control of the infectious source are critical in pediatric HSCT patients with severe immunosuppression, as medical equipment-related outbreaks can be life-threatening.

Key words: *Ralstonia pickettii,* stem cell transplantation, child, outbreak, infectious disease.

Ralstonia pickettii is a Gram-negative, aerobic, oxidase-positive, non-fermentative bacterium from the *Ralstonia* genus that has recently come to light due to its ability to cause nosocomial outbreaks.[1] It was previously described in the formation of biofilms in plastic industrial water pipes and has since been detected in a variety of water sources, including bottled water, standard purified water, laboratory-based high-purity water systems, and hospital water supplies.[2-5] It has the ability to reproduce in intravenous treatment solutions, posing a serious risk of bacteremia. *R. pickettii*-related central nervous infections and osteomyelitis have previously been reported.[6,7] Because of its low virulent nature, clinical infections almost exclusively occur in immunocompromised individuals, such as neonates, cancer patients, and patients in intensive care units.[1,8,9]

✉ Manolya Kara
manolya_kara@yahoo.com

Received 28th Aug 2023, revised 20th Apr 2024, 31st Jul 2024, accepted 15th Aug 2024.

Hematopoietic stem cell transplantation (HSCT) is a well-established treatment option for patients with a wide range of malignant and nonmalignant diseases. Patients undergoing HSCT require a central line (CL) for the administration of chemotherapy, blood products, and total parenteral nutrition during their long and tedious treatment.[10] Due to prolonged periods of immunosuppression, myeloablation, and indwelling catheter days, these patients are at a high risk of developing central line-associated bloodstream infections (CLABSIs).[11] Furthermore, since *R. pickettii* can form a biofilm layer, CLABSI is more likely to occur in the course of bacteremia. Although its mortality was found to be low due to its low virulence, the results of the outbreak can be disastrous if appropriate treatment is not promptly administered and precautions are not taken in HSCT patients who are severely immunosuppressed.[1,12] The purpose of this study is to present our experience with a *R. pickettii*-associated CLABSI outbreak in a pediatric HSCT unit.

Materials and methods

This was a retrospective analysis of an outbreak that occurred between May 2019 and August 2019 at the Pediatric HSCT Units of Bahcesehir University Goztepe Medicalpark Hospital.

Clinical setting & HSCT protocol

Our facility features a pediatric and hematology-oncology unit with 26 beds that serves as Türkiye's reference for auto and allo-HSCT. Every year, about 100 transplants are carried out. Each allogeneic HSCT recipient is housed in a single room equipped with high-efficiency (>99%) particulate air (HEPA) filters that can remove particles larger than 0.3 μm in diameter and more than 12 air exchanges per hour.

All patients have double-lumen tunneled central venous catheters (CVCs) named Broviac and Hickman catheters placed before HSCT. Needleless connectors and closed infusion systems are attached to the CVC. Central line care is provided by the nursing staff with hematology-oncology experience and trained in handling chemotherapy and infection control. The nurse-to-patient ratio is one for every three transplant beds. Standard practices for CVC care includes bathing every other day and cleaning the insertion site with chlorhexidine after every shower.

Closed infusion systems are changed every 72 hours. Access ports are scrubbed with chlorhexidine and accessed only with sterile devices. All lumens are covered with disinfecting hub caps.

Crystalloid fluids are continuously infused into central lines routinely. Central lines are flushed with saline solutions after every antibiotic infusion.

Systemic antimicrobials prophylaxis are not administered routinely before or after HSCT. Antifungal prophylaxis is routinely started on the date of CVC insertion and continued for 100 days after HSCT.

Surveillance data

Active surveillance of hospital-acquired infections is carried out by infection control nurses from the Hospital Infection Control Committee (HICC) in collaboration with infectious disease specialists in the HSCT unit of our hospital. The CDC and NHSN criteria are used to establish the diagnosis of CLABSI.[13]

As soon as an outbreak was suspected, HICC staff launched an investigation to determine the pathogen's source and transmission path. First, the newly purchased materials at the hospital were examined. Culture samples were taken from unsealed catheters, newly changed serum sale ampoules, distilled water, batticon, and any type of intravenous fluids and total parenteral nutrition (TPN) solutions. Samples were also taken from tap water and liquid soaps.

Identification of the bacteria

The culture samples were directly collected from the CVC and peripheral blood without saline flush. Determined samples were cultivated on blood agar, chocolate agar, and MacConkey agar by reduction method. If growth was observed after 24 hours of incubation in an oven at 35-37 °C for 24 hours, the identification phase was started (If no growth is observed, incubation is extended for up to 72 hours). Colony samples were taken and stained in a Gram staining device and the colonies were evaluated under the microscope, then suspended for delivery to the Vitek device where the McFarland ratio was adjusted. It was given to the device as the non-fermentative Gram negative group.

It was kept in incubation for 24 hours and turbidity was observed in liquid medium (If there was no change, it was extended for another 72 hours). As the image was blurred, the passage was performed on blood and MacConkey media. Following that, Gram-negative/positive separation on the medium was observed, and the colony was stained with Gram and examined under the microscope. After passage, the growths were placed in the device with the appropriate group to be identified based on colony morphology.

Statistical analysis

The statistical package for social science (SPSS) for Windows version 21.0 was used to analyze the data (SPSS 21.0, SPSS Inc. USA). Histogram graphics and Shapiro-Wilk tests were used to evaluate normality. The median, minimum, maximum, frequency, and percentage of the data are given. When the expected cell size was five, categorical variables between groups were compared using the two test or Fisher exact test. The student t-test was used to compare continuous variables that were normally distributed. Continuous variables that are not normally distributed were subjected to the Mann-Whitney U test. All p values are based on 2-tailed statistical analyses, and statistical significance was set at $p<0.05$. Univariate analysis and multivariate logistic regression analysis were performed to identify risk factors for mortality.

Ethical committee and informed consent

This study was performed with the permission of the Clinical Research Ethical Committee. Since it was a retrospective case-control study, no informed consent was taken.

Results

There were 12 patients diagnosed with *R. pickettii*-related CLABSI in this outbreak. Auto-HSCT was performed in two of the patients and allo-HSCT was performed in ten patients with both malignant and non-malignant disorders. In the conditioning regimen, all patients were given myeloablative therapy. The clinical and laboratory characteristics of the patients are detailed in Table I.

Antiviral (acyclovir 30 mg/kg/d IV or 100 mg/kg/d PO), antifungal (fluconazole 5 mg/kg/d PO or IV), and anti-*Pneumocystis jirovecii* pneumonia prophylaxis (trimethoprim - sulfamethoxazole 5 mg/kg/d PO 3 days/week) were given to all patients.

Of the 12 patients with *R. pickettii* growth, seven were female and five were male. The median age was 12.1 (2-17 years). All of the patients had a fever and a degree of clinical sepsis findings. Two patients required inotrope infusion for hypotension. The median absolute neutrophile count (ANC) was 22 (0-3690) / mm^3 and the median serum C-reactive protein (CRP) level was 93 (0.2-195) mg/L.

At the time of infectious episode, four patients had graft versus host disease (GvHD). All patients received meropenem, although some received combination therapy. The mean duration of treatment was fourteen days (7-30 days). The catheters of two patients were removed due to prolonged fever and hypotension. Mortality was not observed in any patient as an outcome of the infection episode.

Table I. The clinical and laboratory characteristics of the patients.

Patient number	Age, gender	Primary diagnosis	HSCT characteristics	GVHD prophylaxis	Time of growth, day	Clinical & laboratory characteristics	Presence of GVHD	Resistance	Treatment, duration	Infection Outcome
1	7 yrs Female	ALL	Allogenic 1st HSCT Source: BM Donor: MUD	MTX, tacrolimus	-77 d	Clinic sepsis ANC: 0 CRP: 177 mg/L	No	Colistin	Colisitn+ meropenem + G-CSF 10 d	Cured
2	16 yrs Male	ALL	Allogenic 1st HSCT Source: PBSC Donor: MUD	MMF MTX tacrolimus	4	Clinic sepsis, hypotension (inotrope support) ANC: 0 CRP: 91 mg/L	No	Gentamycin, Levofloxacin	Meropenem + amikacin 21 d	Cured
3	9 yrs Male	Fucosidosis	Allogenic 1st HSCT Source: BM Donor: MFD	MTX tacrolimus	-1	Fever+ tachycardia ANC: 3200/mm^3 CRP: 106 mg/L	No	None	Meropenem 20 d	
4	5 yrs Female	SCID	Allogenic 1st HSCT Source: BM Donor: MUD	MTX tacrolimus	19	Fever ANC: 230 /mm^3 CRP: 11 mg/L	No	Gentamycin, Amikacin	Meropenem 14 d	Cured
5	2 yrs Female	JMML	Allogenic 1st HSCT Source: BM Donor: MUD	MTX tacrolimus	46	Fever ANC: 1160 /mm^3 CRP: 0.2 mg/L	No	Gentamycin, Netilmicin	Meropenem 12 d	Cured (catheter is removed)
6	9 yrs Male	ALL	Allogenic 1st HSCT Source: PBSC Donor: MUD	MTX tacrolimus	4	Clinic sepsis ANC: 0 /mm^3 CRP: 188 mg/L	Grade 4 Gastrointestinal involvement	Gentamycin, Amikacin Netilmicin Ceftazidime	Meropenem 17 d	Cured

ALL; acute lymphoblastic leukemia AML, acute myeloid leukemia; ANC, absolute neutrophil count; BM, bone marrow; CRP, C-reactive protein; CSA, cyclosporine; SCID, Severe combined immune deficiency; JMML, juvenile myeloid monocytic leukemia; G-CSF, granulocyte colony stimulating factor; GVHD, graft versus host disease; HSCT, hematopoetic stem cell transplantation; MFD, Matched family donor; MMF, Mycophenolate mofetil; MSD, Matched sibling donor; MTX, Methotrexate; MUD, matched unrelated donor; PBSC, periphreal blood stem cell; PIP-TAZ, Piperacillin-tazobactam; Post-Cy, post transplant Cyclophosphamide.

Table I. Continued.

Patient number	Age, gender	Primary diagnosis	HSCT characteristics	GVHD prophylaxis	Time of growth, day	Clinical & laboratory characteristics	Presence of GVHD	Resistance	Treatment, duration	Infection Outcome
7	2 yrs Female	ALL	Allogenic 1st HSCT Source: BM Donor: MSD	MTX CSA	-28	Clinic sepsis, vomiting ANC: 0 /mm^3 CRP: 195 mg/L	No	Amikacin Gentamycin, PIP-TAZ	Colisitn+ meropenem + amikacin G-CSF 14 d	Cured
8	17 yrs Female	Ewing sarcoma	Autologous 1st HSCT	None	8	Fever ANC: 0 CRP: 172 mg/L	No	Netilmicin	Meropenem 9 d	Cured
9	7 yrs Female	ALL	Allogenic 1st HSCT Source: PBSC Donor: MUD	Post-CY tacrolimus Steroid	-86	Fever ANC: 420 /mm^3 CRP: 9 mg/L	Grade 2 Liver involvement	Gentamycin, Amikacin Netilmicin PIP-TAZ	Meropenem 17 d	Cured
10	10 yrs Male	AML	Allogenic 2nd HSCT Source: PBSC Donor: MUD	MTX CSA	-23	Fever, pneumonia ANC: 610 /mm^3 CRP: 95 mg/L	Grade 4 Gastrointestinal involvement	Gentamycin, PIP-TAZ	Colistin+ meropenem + amikacin 24 d	Cured (catheter is removed)
11	17 yrs Male	Hodgkin Lymphoma	Autologous 1st HSCT	None	2	Fever ANC: 44 /mm^3 CRP: 30 mg/L	No	None	Meropenem 7 d	Cured
12	17 yrs Female	Aplastic anemia	2nd HSCT Haplo Source: PBSC+BM Donor: Father	MMF Post-CY tacrolimus	2	Clinic sepsis, hypotension (inotrope support) ANC: 0 /mm^3 CRP: 55 mg/L	Grade 4 Liver involvement	None	Meropenem+amikacin 19 d	Cured

ALL; acute lymphoblastic leukemia AML, acute myeloid leukemia; ANC, absolute neutrophil count; BM, bone marrow; CRP, C-reactive protein; CSA, cyclosporine; SCID, Severe combined immune deficiency; JMML, juvenile myeloid monocytic leukemia; G-CSF, granulocyte colony stimulating factor; GVHD, graft versus host disease; HSCT, hematopoetic stem cell transplantation; MFD, Matched family donor; MMF, Mycophenolate mofetil; MSD, Matched sibling donor; MTX, Methotrexate; MUD, matched unrelated donor; PBSC, periphreal blood stem cell; PIP-TAZ, Piperacillin-tazobactam; Post-Cy, post transplant Cyclophosphamide.

As the result of the investigations, *R. pickettii* growth was observed in 50, 100, and 250 ml isotonic saline solutions. The use of the specific saline was immediately stopped by the Hospital Infection Control Committee.

Discussion

Ralstonia pickettii was discovered in 1973 and initially classified as *Pseudomonas* spp.[14] It was later reclassified into the genus Burkholderia, and the genus *Ralstonia* was named in 1995.[15] *R. pickettii* is the most common cause of invasive disease among *Ralstonia* species. The microorganism can survive in many water sources due to its low micronutrient requirement.[1] As it can contaminate medical treatment solutions, it causes hospital outbreaks.[1,4,9,12,16] Contamination occurs during the production line because bacteria can pass through the 0.2-micron filters used to sterilize pharmaceutical products.[17] As with saline, nosocomial infections have been reported due to magnesium vial, heparin flush, and intravitreal solutions.[18-20]

This was the first *R. pickettii* outbreak to impact our HSCT unit. When we encountered this unfamiliar microorganism, we immediately initiated surveillance screening. In accordance with the literature, we began by inspecting the newly purchased equipment, treatment solutions, and water supplies in the unit. As a result of our research, we discovered the growth of *R. pickettii* in isotonic solutions and instantly halted their use. Luckily we were able to control the outbreak without any loss. Recently, Bedir Demirdag et al.[12] from our country reported an outbreak of *R. pickettii* in pediatric oncology units. In that report, like ours, the source was determined to be the isotonic saline solution, and was taken under control without any mortality.

R. pickettii-associated pseudo-outbreaks have also been described in the literature due to the use of contaminated liquids in the laboratory process.[21] If a sample is collected by flushing with isotonic fluid during the culture collection process, it may result in false positivity. In our routine procedure, we collect culture samples directly from the CVC and peripheral blood without using a saline flush, excluding the possibility of contamination. Besides, all patients with *R. pickettii* growth exhibited fever and clinical signs of sepsis. Two patients required inotropes due to hypotension. Moreover, appropriate antibiotic treatment led to a rapid improvement in clinical findings.

The management of *R. pickettii* infections can be difficult due to the wide spectrum of antimicrobial susceptibility. Previous studies indicate a high level of β-lactam and aminoglycoside resistance in *Ralstonia* spp.[10,22] However, carbapenems and fluoroquinolones stand out as weapons in our arsenal.[23] In the present study, all of the isolates were susceptible to carbapenems. Levofloxacin resistance was detected in only one isolate. Most of the strains were resistant to aminoglycosides. During the outbreak, we used combination therapy for some patients until we obtained sensitivity results in culture antibiograms, at which point we used de-escalation. All patients responded well to the antibiotic regimen, and persistent bacteremia was not observed.

The decision to withdraw the catheter is critical in the treatment of CLABSI. Physicians hesitate to remove the catheter for a variety of reasons, particularly in pediatric patients due to the challenges of inserting a new catheter and the inadequacy and inconvenience of peripheral thin veins for continuation of treatment. However, rapid removal of the catheter is of vital importance in cases of clinical sepsis, where the appropriate antibiotic response cannot be obtained, and in the presence of microorganisms with high mortality that are difficult to eradicate.[24-26] During the infection episode, we had to remove the central catheter of two patients who had prolonged fever. No *R. pickettii* growth was observed in any cultures of removed catheters.

Although we cultivated *Ralstonia pickettii* in isotonic solutions as part of an epidemic control effort, we were unable to demonstrate the clonal relationship between the isolates from patients and the saline solution through pulse field electrophoresis. This was the limitation of this research.

In conclusion, it is crucial to identify and control the source of outbreaks, particularly in wards where critically ill patients like pediatric HSCT are admitted.

Ethical approval

This study was approved by the ethical committee of Bahcesehir University (2022-11/05).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SSC, SZ.; data collection: MS, KY; analysis and interpretation of results: MK, GK, MAY; draft manuscript preparation: MK. 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.

REFERENCES

1. Ryan MP, Adley CC. Ralstonia spp.: emerging global opportunistic pathogens. Eur J Clin Microbiol Infect Dis 2014; 33: 291-304. https://doi.org/10.1007/s10096-013-1975-9
2. Falcone-Dias MF, Vaz-Moreira I, Manaia CM. Bottled mineral water as a potential source of antibiotic resistant bacteria. Water Res 2012; 46: 3612-3622. https://doi.org/10.1016/j.watres.2012.04.007
3. Szymańska J. Bacterial decontamination of DUWL biofilm using Oxygenal 6. Ann Agric Environ Med 2006; 13: 163-167.
4. Kendirli T, Ciftçi E, Ince E, et al. Ralstonia pickettii outbreak associated with contaminated distilled water used for respiratory care in a paediatric intensive care unit. J Hosp Infect 2004; 56: 77-78. https://doi.org/10.1016/j.jhin.2003.09.011
5. Kulakov LA, McAlister MB, Ogden KL, Larkin MJ, O'Hanlon JF. Analysis of bacteria contaminating ultrapure water in industrial systems. Appl Environ Microbiol 2002; 68: 1548-1555. https://doi.org/10.1128/AEM.68.4.1548-1555.2002
6. Degeorges R, Teboul F, Belkheyar Z, Oberlin C. Ralstonia pickettii osteomyelitis of the trapezium. Chir Main 2005; 24: 174-176. https://doi.org/10.1016/j.main.2005.04.007
7. Bonatti H, Stelzmueller I, Laimer I, Obwegeser A. Ralstonia pickettii meningitis in a child with hydrocephalus. Eur J Pediatr Surg 2009; 19: 341-342. https://doi.org/10.1055/s-0029-1202252
8. Kismet E, Atay AA, Demirkaya E, et al. Two cases of Ralstonia pickettii bacteremias in a pediatric oncology unit requiring removal of the Port-A-Caths. J Pediatr Hematol Oncol 2005; 27: 37-38. https://doi.org/10.1097/01.mph.0000149960.89192.b0
9. Timm WD, Pfaff SJ, Land GL, et al. An outbreak of multi-drug resistant Pseudomonas pickettii pneumonia in a neonatal intensive care unit. In: Proceedings of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). 1995; 49.
10. Ryan MP, Adley CC. The antibiotic susceptibility of water-based bacteria Ralstonia pickettii and Ralstonia insidiosa. J Med Microbiol 2013; 62: 1025-1031. https://doi.org/10.1099/jmm.0.054759-0
11. McDonald MK, Culos KA, Gatwood KS, et al. Defining incidence and risk factors for catheter-associated bloodstream infections in an outpatient adult hematopoietic cell transplantation program. Biol Blood Marrow Transplant 2018; 24: 2081-2087. https://doi.org/10.1016/j.bbmt.2018.04.031
12. Bedir Demirdag T, Ozkaya-Parlakay A, Bayrakdar F, et al. An outbreak of Ralstonia pickettii bloodstream infection among pediatric leukemia patients. J Microbiol Immunol Infect 2022; 55: 80-85. https://doi.org/10.1016/j.jmii.2020.12.004
13. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control 2008; 36: 309-332. https://doi.org/10.1016/j.ajic.2008.03.002

14. Santos VM. To: Ralstonia pickettii bacteremia in hemodialysis patients: a report of two cases. Rev Bras Ter Intensiva 2016; 28: 488-489. https://doi.org/10.5935/0103-507X.20160082

15. Fluit AC, Bayjanov JR, Aguilar MD, et al. Characterization of clinical Ralstonia strains and their taxonomic position. Antonie Van Leeuwenhoek 2021; 114: 1721-1733. https://doi.org/10.1007/s10482-021-01637-0

16. Gardner S, Shulman ST. A nosocomial common source outbreak caused by Pseudomonas pickettii. Pediatr Infect Dis 1984; 3: 420-422. https://doi.org/10.1097/00006454-198409000-00006

17. Zhang L, Morrison M, Rickard CM. Draft genome sequence of Ralstonia pickettii AU12-08, isolated from an intravascular catheter in Australia. Genome Announc 2014; 2: e00027-14. https://doi.org/10.1128/genomeA.00027-14

18. Kimura AC, Calvet H, Higa JI, et al. Outbreak of Ralstonia pickettii bacteremia in a neonatal intensive care unit. Pediatr Infect Dis J 2005; 24: 1099-1103. https://doi.org/10.1097/01.inf.0000190059.54356.f3

19. Ross B, Steinmann J, Buer J, et al. Outbreak with Ralstonia pickettii caused by contaminated magnesium vials. Dtsch Med Wochenschr 2014; 139: 323-326. https://doi.org/10.1055/s-0033-1360059

20. Choudhury H, Jindal A, Pathengay A, Flynn HW. An outbreak of Ralstonia pickettii endophthalmitis following intravitreal methotrexate injection. Clin Ophthalmol 2015; 9: 1117-1120. https://doi.org/10.2147/OPTH.S81218

21. Costas M, Holmes B, Sloss LL, Heard S. Investigation of a pseudo-outbreak of 'Pseudomonas thomasii' in a special-care baby unit by numerical analysis of SDS-PAGE protein patterns. Epidemiol Infect 1990; 105: 127-137. https://doi.org/10.1017/s0950268800047725

22. Girlich D, Naas T, Nordmann P. OXA-60, a chromosomal, inducible, and imipenem-hydrolyzing class D beta-lactamase from Ralstonia pickettii. Antimicrob Agents Chemother 2004; 48: 4217-4225. https://doi.org/10.1128/AAC.48.11.4217-4225.2004

23. Celebi S, Sezgin ME, Cakır D, et al. Catheter-associated bloodstream infections in pediatric hematology-oncology patients. Pediatr Hematol Oncol 2013; 30: 187-194. https://doi.org/10.3109/08880018.2013.772683

24. Eggimann P, Sax H, Pittet D. Catheter-related infections. Microbes Infect 2004; 6: 1033-1042. https://doi.org/10.1016/j.micinf.2004.05.018

25. Gaynes R, Jacob JT. Intravascular catheter-related infection: epidemiology, pathogenesis, and microbiology. Available at: https://www.uptodate.com (Accessed on Aug 2022).

26. Aksoy BA, Kara M, Sütçü M, et al. Epidemiologic and microbiologic evaluation of catheter-line bloodstream infection in a pediatric hematopoietic stem cell transplant center. Am J Infect Control 2024; 52: 81-86. https://doi.org/10.1016/j.ajic.2023.08.010

The Turkish Journal of Pediatrics 2024; 66: 429-438
https://doi.org/10.24953/turkjpediatr.2024.4545

Original Article

Assessment of vitamin B12 and homocysteine levels in pregnant women admitted for delivery and cord blood samples of their newborn babies: a multicenter study

Zeynep Yıldız Yıldırmak[1], Dildar Bahar Genç[1], Alev Kural[2], Veli Mıhmanlı[3], Süleyman Salman[4], Keziban Doğan[5], Mehmet Ali Çiftçi[3], Nazlı Döktür Efeoğlu[4], Aliye Erdoğan[5], Neçirvan Çağdaş Çaltek[3], Emre Özgen[2], Ebru Kale[6]

[1]Department of Pediatric Hematology /Oncology, Sisli Hamidiye Etfal Training and Research Hospital, University of Health Sciences, İstanbul; [2]Department of Biochemistry, University of Health Sciences, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, İstanbul; [3]Department of Obstetrics and Gynecology, University of Health Sciences, Okmeydanı Training and Research Hospital, İstanbul; [4]Department of Obstetrics and Gynecology, University of Health Sciences, Gaziosmanpasa Training and Research Hospital, İstanbul; [5]Department of Obstetrics and Gynecology, University of Health Sciences, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, İstanbul; [6]Department of Biochemistry, University of Health Sciences, Dr. Lutfi Kirdar Kartal Training and Research Hospital, İstanbul, Türkiye.

ABSTRACT

Background. Vitamin B12, an indispensable micronutrient, is pivotal in numerous physiological processes, with particular significance during pregnancy and fetal development. The increasing adoption of vegetarian diets and the economic challenges associated with accessing animal-based food sources contribute to the prevalence of vitamin B12 deficiency. This study aims to examine the levels of vitamin B12 and homocysteine in pregnant women upon admission for delivery and to analyze corresponding cord blood samples from their newborn infants in a substantial sample within the Istanbul metropolitan area.

Materials and Methods. This cross-sectional multicenter study included women aged ≥16 years admitted for delivery and their newborns ≥34 weeks. The demographic data and the results of complete blood counts within the previous 24 hours before birth were recorded. Vitamin B12 and homocysteine levels were measured in maternal and cord blood samples. The study parameters were compared between the groups based on the mothers' and babies' homocysteine and vitamin B12 levels.

Results. The study included 832 pregnant women and 832 neonates. Anemia affected 36% of pregnant women, with a higher frequency in mothers with vitamin B12 deficiency. Seventy-eight mothers and 48.9% of neonates showed Vitamin B12 levels below 200 pg/mL, while elevated homocysteine levels were observed in 30% of mothers and 26% of neonates. Maternal vitamin B12 deficiency was significantly correlated with cord blood B12 deficiency and elevated homocysteine. The median cord blood vitamin B12 level was inversely correlated with the number of previous pregnancies.

Conclusion. Vitamin B12 deficiency is extremely common in pregnant women before delivery, significantly correlating to cord blood homocysteine and vitamin B12 levels. However, homocysteine alone is not a reliable marker for maternal vitamin B12 status. Implementing strategies to detect vitamin B12 deficiency and supplying

✉ Dildar Bahar Genç
baharbeker@yahoo.com

Received 30th Mar 2024, revised 5th Jun 2024, 20th Jun 2024, accepted 9th Aug 2024.

The preliminary results of this study were partially presented orally at the XIV. Eurasian Hematology-Oncology Congress held on October 11-14, 2023, and published as an abstract in Hematology, Transfusion and Cell Therapy 2023; 45(Suppl. 3): S23, https://doi.org/10.1016/j.htct.2023.09.038.

adequate vitamin B12 supplementation during pregnancy holds the potential to enhance maternal and neonatal health in Türkiye.

Key words: vitamin B12, homocysteine, pregnancy, cord blood.

Vitamin B12 (VB12) plays an essential role in various physiological processes, particularly during pregnancy and fetal development.[1] However, VB12 deficiency has become an important public health problem all over the world.[2] The limited consumption of animal foods owing to socioeconomic barriers, and ethical or health considerations contribute to the global increase in the prevalence of inadequate VB12 status.[3,4] During infancy, neonatal VB12 status reflects that of the mothers, in other words, babies born to mothers with VB12 deficiency are also born with insufficient VB12 stores[5], and clinical findings of VB12 deficiency, such as hypotonia, convulsions, growth retardation, anemia, and brain atrophy might become manifest during infancy due to insufficient levels of VB12 in breast milk.[6,7]

VB12 deficiency is generally defined as a VB12 level of less than 200 pg/mL, with threshold levels varying amongst laboratories and studies.[8] However, the signs and symptoms of early VB12 deficiency might be subtle, necessitating more sensitive diagnostic tests. Serum methylmalonic acid (MMA) and homocysteine are recognized as functional biomarkers for assessing VB12 deficiency[9], even in the absence of megaloblastic anemia.[8] A positive correlation between the elevated levels of homocysteine and the development of neurological dysfunction in infants due to VB12 deficiency has been reported as well.[6] However, the lack of agreement on cutoff values for each of the biomarkers continues to be a concern in diagnosing VB12 insufficiency.[10,11]

The objective of this study was to assess VB12 and homocysteine levels in a large group of pregnant women and to evaluate the impact of maternal VB12 status on the VB12 and homocysteine levels in newborns in Türkiye.

Materials and Methods

Healthy pregnant women aged ≥ 16 years admitted for delivery and their healthy babies ≥34 weeks were included in the study. Pregnant women under 16 years of age, babies requiring intensive care admission, babies born with anomalies, and twin pregnancies were not included.

This cross-sectional study was conducted in three tertiary centers in Istanbul. The demographic characteristics, namely, maternal age, mode of delivery, number of previous pregnancies, abortions and births, and drugs used during pregnancy, the height and weight of the neonates were recorded. The gestational week was determined by the last menstrual date or fetal ultrasonography. Whole blood count analysis was performed within 24 hours before delivery. Blood samples for VB12 and homocysteine from mothers (within one hour before delivery) and cord blood of babies were collected from September 2020 to September 2021. The samples were centrifuged, and the sera were stored at -20°C until the day of analysis.

VB12 analysis was performed by the "ECLIA" (electrochemiluminescence immunological test) method in COBAS-E immunological test analyzer. Homocysteine analysis was performed by the chemiluminescence method in Immulite 2000 device.

VB12 levels lower than 200 pg/mL were considered as a deficiency.[12] Homocysteine greater than 10 μmol/L was considered elevated and accepted as a reflection of VB12 functional status.[13,14] Study parameters were compared between groups dichotomized according to maternal VB12 (cut-off value of 200 pg/mL) levels.

Anemia is defined as a hemoglobin concentration of less than 110 g/L according to the World Health Organization Criteria for pregnant women.[15]

All study participants provided written informed consent. The research complied with all the relevant national regulations, and institutional policies, in accordance with the tenets of the Helsinki Declaration, and was approved by the author's institutional review board or equivalent committee (ethical approval number and date: 3132-2/2/2021).

Data were analyzed by SPSS statistical software v.22 program. Descriptive statistics were expressed as mean and standard deviation (SD), median and range for continuous variables, and number and percentage for categorical variables. The chi-square test was used to assess for comparisons of categorical variables between groups. One-way ANOVA and Mann-Whitney U tests were used for the comparison of groups with symmetrical and asymmetrical distribution, respectively. A p-value <0.05 was considered statistically significant.

Results

Two hundred fifty-eight patients were excluded from the study because they did not meet the research inclusion criteria, had insufficient information, or had hemolytic serum samples. Vitamin B12 and homocysteine levels in 832 pregnant women and cord blood samples that met the study criteria were assessed.

The study included 615 Turkish and 217 foreign pregnant women, mostly refugees from Syria. The median age of the study population was 28.1 (range 16.4-51.2) years. Eight percent of the pregnant women were under the age of 20, 25% were between the ages of 21 and 25, 29% were between the ages of 26 and 30, and 38% were between the ages of 31 and over. The average number of pregnancies per woman was three (1-9). 17, 53, 25, and five percent of women had first, 2-3, 4-5, and 6 or more pregnancies, respectively. The mean gestational age was 38.7±1.4 weeks. 46% of the pregnant women had vaginal delivery whereas 54% had Cesarean (C)-sections (Table I).

Sixty-seven percent of mothers received antenatal supplemental folic acid, while 75% received other supplements containing iron and/or multivitamins. 10.6% of pregnant women smoked during pregnancy. Thirty-six percent of the pregnant women were anemic, i.e., had hemoglobin less than 11 gr/dL before delivery. The frequency of anemia was higher in women with VB12 deficiency (p=0.00007). Twenty-one percent, 78%, and 1% of the women had mean corpuscular volume (MCV) values <80 fl, between 80 and 100 fl, and ≥100 fl, respectively.

The mean VB12 level of the pregnant women was found to be 157±75.3 pg/mL (range 27.3-882). Maternal VB12 levels revealed that 78.2% of pregnant women were VB12 deficient (<200 pg/mL), with 51.9% having VB12 levels less than 150 pg/mL. The mean homocysteine level of

Table I. Demographic characteristics of pregnant women.

Parameters	Results
Maternal age, yr, median (min-max)	28.1 (16.4-51.2)
Number of pregnancies, median (min-max)	3 (1-9)
Number of deliveries, median (min-max)	1 (0-8)
Time elapsed since the last birth, yr, mean±SD	3.69± 2.35
Duration since discontinuation of last breastfeeding episode, yr, mean±SD	2.69 ±2.32
Duration of gestation, days, mean±SD	270.81±11.32
Mode of delivery (Number of vaginal birth / Cesarean section)	388/444

SD: standard deviation.

the pregnant women was 8.56±5.51 (range 1.9-51.0) μmol/L while homocysteine levels were elevated in 26% of pregnant women, showing a functional deficiency (Table II).

According to the cut-off VB12 level of 200 pg/mL, maternal age, nationality, number of previous pregnancies and deliveries, time passed since the last birth, breastfeeding, gestational age, and history of smoking and receiving folate supplements during pregnancy were not statistically different between groups with and without VB12 deficiency (p>0.05). Vaginal deliveries and iron/multivitamin supplementation were associated with higher VB12 levels in the mothers (p=0.04 and p=0.01, respectively). Pregnant women who received antenatal iron/multivitamin supplementation had a mean VB12 level of 161.5 ± 75.3 pg/mL, compared to 145.1 ± 75.0 pg/mL in those who did not. Mothers with VB12 levels above 200 pg/mL had higher hemoglobin and hematocrit, higher MCV (within normal range) as well as lower homocysteine values, while their newborns had higher VB12 and lower homocysteine levels in their cord blood (p<0.05) (Table III).

The mean weight of the babies was 3271±435 g whereas the mean length was 49.5±2.2 cm. Mean cord blood VB12 and homocysteine levels were 234.7±13.2 pg/mL (range 30.6-971) and 9.13±5.75 (range 2-51) μmol/L, respectively. 48.9% of newborns were VB12-deficient (200 pg/mL), while homocysteine levels were elevated in 30%.

Vitamin B12 deficiency and elevated homocysteine levels were more frequent in the cord blood of babies born to mothers with VB12 deficiency (p=0.001, Table III). However, 44% of babies born to mothers with a VB12 level of <200 pg/mL had normal VB12 levels (Fig. 1). Cord VB12 levels negatively correlated with the increasing number of previous pregnancies (p=0.036) (Fig. 2). A significant relationship was found between both cord blood VB12 deficiency and maternal VB12 and homocysteine levels. The birth weight significantly correlated with cord blood VB12 levels, but the difference in mean birth weight between babies with and without VB12 deficiency was only 67 grams. The relationship between the gender of the baby and VB12 level was nonsignificant.

Discussion

Our study represents the most comprehensive evaluation of VB12 status in mothers and their newborn infants conducted in our country. The main outcome is that B12 deficiency is extremely common in pregnant women before delivery and that cord blood VB12 and homocysteine levels correlate strongly with maternal VB12 stores.

Table II. Vitamin B12 and homocysteine levels of babies and pregnant women.

Parameter	Maternal	Cord blood
Vitamin B12 , pg/mL (mean±SD)	157±75.3	234.7±13.2
Distribution of cases	78.2% deficient 21.8% sufficient	48.9% deficient 51.1% sufficient
<150	432 (51.9%)	230 (27.6%)
150-200	219 (26.3%)	177 (21.3%)
201-300	151 (18.1%)	259 (31.1%)
>301	30 (3.7%)	166 (20%)
Homocysteine , μmol/L (mean±SD)	8.56±5.51	9.13±5.75
≤10 (normal)	622 (74%)	589 (70%)
>10 (elevated)	210 (26%)	243 (30%)
VB12 <200 pg/mL and Homocysteine >10 μmol/L	179 (21%)	183 (21%)

SD: standard deviation.

Table III. Comparison of study parameters according to vitamin B12 status of mothers.

Study parameter	Maternal VB12 <200 pg/mL (n=652)	Maternal VB12 ≥200 pg/mL (n=180)	p-value
VB12 level, pg/mL, mean ± SD	129.1±40.4	261.6±81.2	0.000
Maternal age, yr, median (range)	27.8 (16.9-51.2)	29.25 (16.4-45.5)	0.06
Turkish / foreign nationality, n (%)	477 (73.1%) / 175 (26.9%)	138 (76.7%) / 42 (23.3%)	0.093
Number of pregnancies, median (range)	3 (1-9)	2 (1-9)	0.27
Number of births, median (range)	1 (0-8)	1 (0-8)	0.23
Number of abortus, median (range)	0 (0-5)	0 (0-5)	0.73
Time since last birth, yr, median (range)	3 (1-17)	3 (1-14)	0.73
Time since last breastfeeding, yr, median (range)	2 (0-15)	2 (1-12)	0.38
Folate usage (%)	66%	71%	0.28
Iron/other vitamin supplements (%)	73%	82%	0.01
Smoking (%)	10.1%	13%	0.17
Gestational age, wk, mean ± SD	38.7±1.3	38.6±1.4	0.33
Vaginal delivery / C/S, n (%)	292 (44.8%) / 360 (55.2%)	96 (53.3%) / 84 (46.7%)	0.04
Male / female infant, n (%)	350 (53.7%) / 302 (46.3%)	84 (46.7%) / 96 (53.3%)	0.10
Birth weight, g, median (range)	3282 (1735-4900)	3225 (2060-4445)	0.09
Birth height, cm, median (range)	50 (34-56)	50 (43-54)	0.24
Hemoglobin, g/dL, median (range)	11.3 (5.7-15,5)	11.8 (7.9-14.5)	0.00007
Hematocrit, %, median (range)	34 (21-46)	35 (25-43)	0.001
MCV, fL, median (range)	85 (59-106)	88 (62-106)	0.002
Leucocyte, /mm^3, median (range)	10600 (4430-31940)	10840 (5930-22080)	0.21
Neutrophil, /mm^3, median (range)	7890 (2000-29690)	7940 (3850-19560)	0.18
Lymphocyte, /mm^3, median (range)	1950 (210-8500)	1850 (760-4160)	0.13
Platelet, /mm^3, median (range)	222000 (45000-591000)	215000 (87000-547000)	0.20
Maternal homocysteine, µmol/L, median (range)	7.6 (1.9-51.0)	6.82 (2.0-39.3)	0.08
Cord VB12, pg/mL, median (range)	187 (40-971)	273 (30.6-906.7)	0.0001
Cord homocysteine, µmol/L, median (range)	8.02 (2.0-51.0)	6.77(2.0-47.9)	0.0001

C/S: Caesarean section, IQR: interquartile range, MCV: mean corpuscular volume, SD: standard deviation, VB12: vitamin B12

In our study, eight percent of pregnant women were under the age of 20, and half were second or third pregnancies. Our study's average age of pregnant women was comparable to other studies conducted in our country.[11,16,17] Although the age of pregnant women deficient in VB12 was younger than that of pregnant women with adequate levels, the difference was not statistically significant. Likewise, the number of pregnancies did not affect VB12 status. Beyoglu et al. related VB12 insufficiency to younger age at pregnancy, particularly between 18 and 24 years, and to gravida three or more.[18] In a study conducted in Türkiye's Southeastern Anatolia region, where the marital age is generally lower, 16% of pregnant women were under the age of 20, while the frequency of first pregnancies was higher than that of our study.[16] The age of the pregnant women and the number of pregnancies did not influence the VB12 status as well. The fact that Istanbul has the highest marital age in Türkiye and the southeastern region of Türkiye has the highest birth rate might elucidate the variations observed in our findings compared to other studies.[19]

Twenty-one percent of the pregnant women had microcytosis in their whole blood count. Microcytosis rates were similar in other studies conducted in our country.[20] Also, around two-thirds of pregnant women had anemia, with a

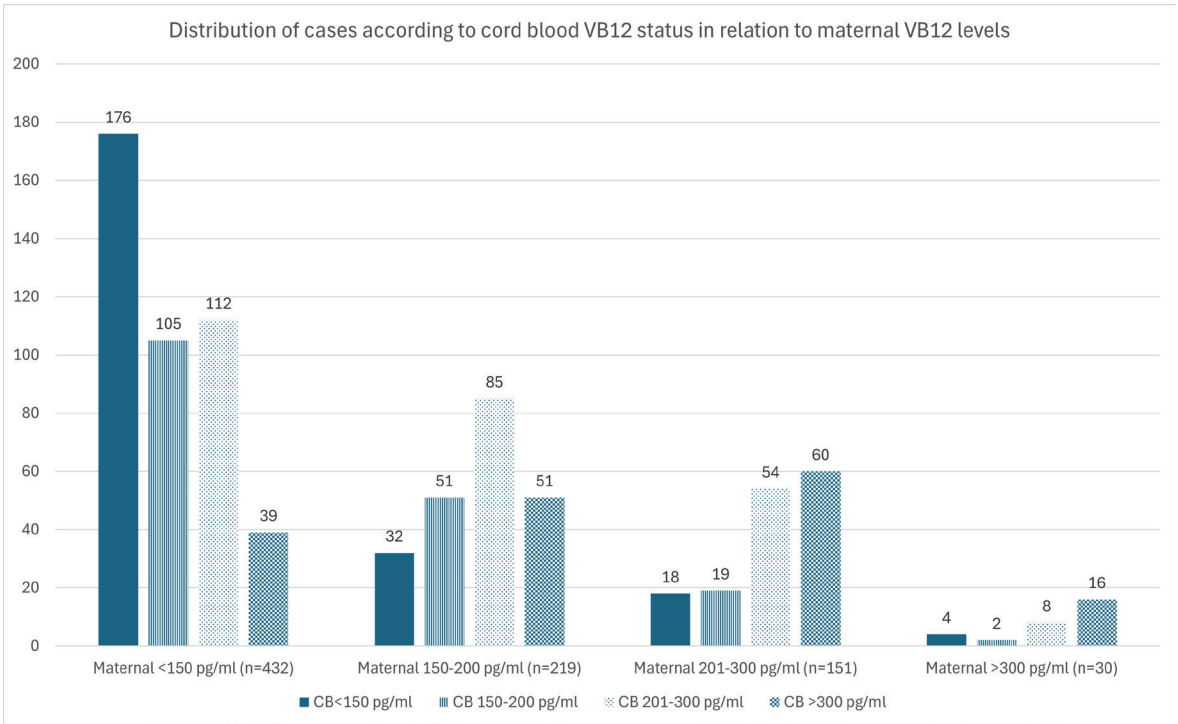


Fig. 1. Cord blood vitamin B12 and maternal vitamin B12 status relationship.

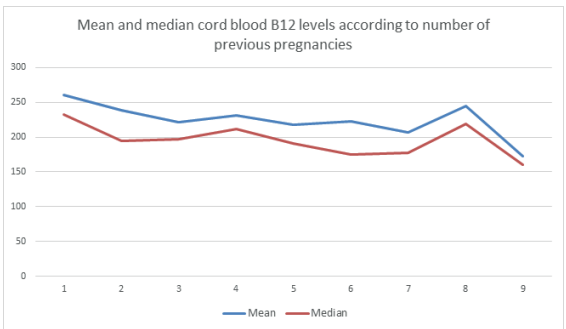


Fig. 2. Cord blood vitamin B12 levels according to number of previous pregnancies (pg/mL).

higher frequency in VB12-deficient mothers. Only 12 out of 832 patients had MCV ≥100 fl and four of 12 had anemia. In addition, MCV values were lower in women with VB12 deficiency, contrary to what would be expected, i.e., macrocytosis. These findings might be attributed to a possible concomitant iron deficiency in our study population. The ferritin levels were not measured in our study; therefore, an objective interpretation of this issue is impossible. These findings also highlight the fact that normal hemogram results in pregnant women were not a reliable indicator of VB12 insufficiency, as Roumeliotis et al. reported.[21]

The mean VB12 levels of the pregnant women in our study were quite low and VB12 deficiency afflicted approximately three-quarters of the pregnant population, with half suffering from levels below 150 pg/mL. Among the studies conducted on VB12 deficiency, there is no consensus on the threshold VB12 value that should be used to define the deficiency. Some studies consider VB12 levels of 200-300 pg/mL in asymptomatic individuals as a mild deficiency. Based on the threshold value of 300 pg/mL, it can be stated that 96.3% of the pregnant women (and 80% of the newborns) in our study had VB12 deficiency. Our findings were consistent with previous reports of VB12 insufficiency in Türkiye.[16,17]

Kalay et al. reported a negative correlation between neonatal VB12 levels and the number of births and birth weight.[17] We also observed a decrease in cord blood VB12 levels as the number of pregnancies increased. Similar to

our findings, a Norwegian study also noted a decline in neonatal VB12 levels with increased parity, in addition to a significant correlation between time since last birth and cord blood VB12 levels.[22] However, our study did not demonstrate an influence of period since last birth on cord blood VB12 levels.

The percentage of vaginal delivery was statistically higher in mothers with sufficient VB12 stores in our study. Zanardo et al. reported higher homocysteine concentrations in women undergoing elective C-sections under general anesthesia as well as in their babies.[23] Homocysteine elevation might result from maternal VB12 deficiency, but the study did not assess VB12 status, and the serum samples were collected after delivery. The authors assumed hyperhomocysteinemia to be caused by labor-induced hormonal changes and/or pharmacological interventions. The Turkish Ministry of Health strongly encourages vaginal birth and does not approve C-sections unless medically necessary. If C-sections were supposedly due to medical emergencies, and vaginal deliveries might have indicated a healthier pregnancy for both the mother and the fetus, one might assume that better prenatal care led to higher VB12 levels. In the current literature, maternal VB12 deficiency is linked to an increased risk of common pregnancy complications with adverse perinatal outcomes.[24] The reasons for C-sections were not investigated in our study. As a result, the reason for this correlation is unclear based on our current knowledge.

Mothers who received antenatal iron/multivitamin supplements had higher levels of VB12. However, irrespective of the supplementation, their mean VB12 levels remained below 200 pg/mL, and the difference was not reflected in maternal homocysteine, cord blood VB12, or homocysteine levels. Maternal VB12 levels directly correlate with neonatal VB12 stores, with an impact on neurological development. However, the effect of VB12 supplementation during pregnancy on offspring postpartum growth and neurodevelopment is still unknown. Srinivasan et al. showed that maternal VB12 supplementation during pregnancy had no effect on cognitive development in infants at 9 months of age, but higher maternal homocysteine levels were associated with poorer cognitive performance in some Bayley Scales of Infant Development-III subdomains.[25] Chandyo et al. demonstrated an improvement in maternal VB12 status but no improvement in infant growth and development in a double-blind randomized trial.[26]

Because of hemodilution and decreased haptocorrin production during pregnancy, serum VB12 concentrations fall, rendering this test inaccurate.[10] Hyperhomocysteinemia has been proposed as a measure of VB12 insufficiency in tissues, with controversial utility in diagnosing VB12 deficiency.[8] Despite the high prevalence of VB12 deficiency in our study, only about 26% of pregnant women exhibited a substantial increase in homocysteine levels, and none of them had evidence of VB12 deficiency-related signs or symptoms. This finding suggests that homocysteine alone is not always a reliable indicator of VB12 deficiency nor a suitable screening test for diagnosis, as Amarasinghe et al. previously reported.[27] Since serum VB12 assays estimate total VB12 rather than directly indicating metabolic utilization, it is challenging to definitively diagnose VB12 deficiency based on serum levels alone. There are no universally accepted cut-off values for holotranscobalamin, homocysteine, and methylmalonic acid as well.[28] Therefore, there is still a lack of consensus about the threshold value or the ideal combination of tests for diagnosing VB12 deficiency in pregnancy.

Despite the high prevalence of maternal VB12 deficiency, babies had a lower prevalence of VB12 deficiency. The same difference was observed in comparison to mean VB12 levels. In many studies from various countries, cord blood VB12 levels were found to be 27-100% higher than those of the mother.[1] In our study, this ratio was found to be 28%. This situation may be considered as a physiological response

for the baby's protection, similar to which has been reported for iron stores of babies in relation to maternal status.[29] Also, maternal VB12 measurement may not be a reliable determinant of VB12 deficiency, as circulating levels of holotrancobalamin (active VB12) might remain relatively stable during pregnancy.[9]

The relationship between VB12 status, birth weight, and gender remains controversial. Tanyildiz et al. reported lower birth weights in children with VB12 deficiency born to VB12 deficient mothers in comparison to those born to VB12 sufficient mothers. Conversely, a Norwegian study found lower cobalamin levels in heavier neonates and female neonates[22,30], our study identified a negative correlation between infants' birth weights and cord blood VB12 levels. However, it was not considered clinically important because the difference between the mean weights of newborns with and without VB12 deficiency was 67 g. There was no significant difference in the VB12 levels of male and female neonates' cord blood as well.

In our study, in babies of mothers with VB12 levels below 200 pg/mL, VB12 levels were significantly lower and homocysteine levels were significantly higher. These results are similar to findings reported from our country as well as developed countries[1,21,31] who recommend VB12 supplementation during pregnancy and lactation.[21]

The sample size and number of parameters evaluated were the study's strengths. The limitation of our study is the lack of assessment of nutrition and serum iron and folate status in mothers, as serum levels of homocysteine might also be influenced by folic acid, Vitamin B6, and betaine.

The different cut-off values and tests to diagnose VB12 deficiency, diverse maternal dietary habits, gastrointestinal risk factors, mode of birth, antenatal vitamin supplementation, and gestational age are the challenges to making a healthy comparison among studies on VB12 deficiency. As a result, each country may require a distinct approach to assessing VB12 status based on epidemiological studies and its specific socioeconomic and healthcare system. Our study revealed a significant VB12 deficiency in pregnant women and their babies, highlighting a need for a systematic exploration of health issues associated with insufficient VB12 in pregnant women and their babies, antenatal assessment of VB12 status, and VB12 supplementation if necessary.

Ethical approval

University of Health Sciences, Şişli Hamidiye Etfal Training and Research Hospital (approval number and date: 3132-2/2/2021).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: ZYY, DBG, AK, VM, SS, KD; data collection: ZYY, DBG, AK, VM, SS, KD, MAC, NDE, AE, NCC, EO, EK; analysis and interpretation of results: ZYY, DBG, AK; draft manuscript preparation: ZYY, DBG. All authors revised and approved the final version of the manuscript.

Source of funding

The Unit of Scientific Research Projects (USRP) of the University of Health Sciences (grant number 2020/106).

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Finkelstein JL, Kurpad AV, Thomas T, Srinivasan K, Duggan C. Vitamin B12 status in pregnant women and their infants in South India. Eur J Clin Nutr 2017; 71: 1046-1053. https://doi.org/10.1038/ejcn.2017.29

2. Shipton MJ, Thachil J. Vitamin B12 deficiency - A 21st century perspective. Clin Med (Lond) 2015; 15: 145-150. https://doi.org/10.7861/clinmedicine.15-2-145

3. Salehi G, Díaz E, Redondo R. Forty-five years of research on vegetarianism and veganism: a systematic and comprehensive literature review of quantitative studies. Heliyon 2023; 9: e16091. https://doi.org/10.1016/j.heliyon.2023.e16091

4. Pawlak R, Parrott SJ, Raj S, Cullum-Dugan D, Lucus D. How prevalent is vitamin B(12) deficiency among vegetarians? Nutr Rev 2013; 71: 110-117. https://doi.org/10.1111/nure.12001

5. Obeid R, Murphy M, Solé-Navais P, Yajnik C. Cobalamin status from pregnancy to early childhood: lessons from global experience. Adv Nutr 2017; 8: 971-979. https://doi.org/10.3945/an.117.015628

6. Ljungblad UW, Paulsen H, Mørkrid L, et al. The prevalence and clinical relevance of hyperhomocysteinemia suggesting vitamin B12 deficiency in presumed healthy infants. Eur J Paediatr Neurol 2021; 35: 137-146. https://doi.org/10.1016/j.ejpn.2021.10.008

7. Irevall T, Axelsson I, Naumburg E. B12 deficiency is common in infants and is accompanied by serious neurological symptoms. Acta Paediatr 2017; 106: 101-104. https://doi.org/10.1111/apa.13625

8. Hannibal L, Lysne V, Bjørke-Monsen AL, et al. Biomarkers and algorithms for the diagnosis of vitamin B12 deficiency. Front Mol Biosci 2016; 3: 27. https://doi.org/10.3389/fmolb.2016.00027

9. Green R, Allen LH, Bjørke-Monsen AL, et al. Vitamin B12 deficiency. Nat Rev Dis Primers 2017; 3: 17040. https://doi.org/10.1038/nrdp.2017.40

10. Sobczyńska-Malefora A, Delvin E, McCaddon A, Ahmadi KR, Harrington DJ. Vitamin B12 status in health and disease: a critical review. Diagnosis of deficiency and insufficiency - clinical and laboratory pitfalls. Crit Rev Clin Lab Sci 2021; 58: 399-429. https://doi.org/10.1080/10408363.2021.1885339

11. Yetim A, Aygün E, Yetim Ç, et al. Measurement of serum vitamin B12-related metabolites in newborns: implications for new cutoff values to detect B12 deficiency. J Matern Fetal Neonatal Med 2021; 34: 1260-1268. https://doi.org/10.1080/14767058.2019.1633301

12. Green R, Datta Mitra A. Megaloblastic anemias: nutritional and other causes. Med Clin North Am 2017; 101: 297-317. https://doi.org/10.1016/j.mcna.2016.09.013

13. Behere RV, Deshmukh AS, Otiv S, Gupte MD, Yajnik CS. Maternal vitamin B12 status during pregnancy and its association with outcomes of pregnancy and health of the offspring: a systematic review and implications for policy in India. Front Endocrinol (Lausanne) 2021; 12: 619176. https://doi.org/10.3389/fendo.2021.619176

14. Tangeraas T, Ljungblad UW, Lutvica E, et al. Vitamin B12 deficiency (un-)detected using newborn screening in Norway. Int J Neonatal Screen 2023; 9: 3. https://doi.org/10.3390/ijns9010003

15. World Health Organization (WHO). Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. WHO; 2023. Available at: https://www.who.int/publications/i/item/WHO-NMH-NHD-MNM-11.1

16. Koc A, Kocyigit A, Soran M, et al. High frequency of maternal vitamin B12 deficiency as an important cause of infantile vitamin B12 deficiency in Sanliurfa province of Turkey. Eur J Nutr 2006; 45: 291-297. https://doi.org/10.1007/s00394-006-0598-7

17. Kalay Z, Islek A, Parlak M, et al. Reliable and powerful laboratory markers of cobalamin deficiency in the newborn: plasma and urinary methylmalonic acid. J Matern Fetal Neonatal Med 2016; 29: 60-63. https://doi.org/10.3109/14767058.2014.986649

18. Beyoglu MM, Kostu B. Evaluation of vitamin B12 levels in pregnant population and relationship with nutritional deficiency. KSU Medical Journal 2022; 17: 77-82. https://doi.org/10.17517/ksutfd.971131

19. Turkish Statistical Institute. Birth Statistics 2022. Turkish Statistical Institute; 2023. Available at: https://data.tuik.gov.tr/Bulten/Index?p=Birth-Statistics-2022-49673 (Accessed on July 19, 2023).

20. Karabulut A, Güler ÖT, Karahan HT, Özkan S, Koyuncu H, Demirciler I. Premarital screening of 466 Mediterranean women for serum ferritin, vitamin B12, and folate concentrations. Turk J Med Sci 2015; 45: 358-363. https://doi.org/10.3906/sag-1401-25

21. Roumeliotis N, Dix D, Lipson A. Vitamin B12 deficiency in infants secondary to maternal causes. CMAJ 2012; 184: 1593-1598. https://doi.org/10.1503/cmaj.112170

22. Hay G, Clausen T, Whitelaw A, et al. Maternal folate and cobalamin status predicts vitamin status in newborns and 6-month-old infants. J Nutr 2010; 140: 557-564. https://doi.org/10.3945/jn.109.117424

23. Zanardo V, Caroni G, Burlina A. Higher homocysteine concentrations in women undergoing caesarean section under general anesthesia. Thromb Res 2003; 112: 33-36. https://doi.org/10.1016/j.thromres.2003.11.004

24. Finkelstein JL, Layden AJ, Stover PJ. Vitamin B-12 and perinatal health. Adv Nutr 2015; 6: 552-563. https://doi.org/10.3945/an.115.008201

25. Srinivasan K, Thomas T, Kapanee AR, et al. Effects of maternal vitamin B12 supplementation on early infant neurocognitive outcomes: a randomized controlled clinical trial. Matern Child Nutr 2017; 13: e12325. https://doi.org/10.1111/mcn.12325

26. Chandyo RK, Ulak M, Kvestad I, et al. The effects of vitamin B12 supplementation in pregnancy and postpartum on growth and neurodevelopment in early childhood: study protocol for a randomized placebo controlled trial. BMJ Open 2017; 7: e016434. https://doi.org/10.1136/bmjopen-2017-016434

27. Amarasinghe G, Jayasinghe I, Hettiarachchi A, et al. Can homocysteine be used to identify vitamin B12 or folate deficiencies during pregnancy in low resource settings? Curr Dev Nutr 2021; 5(Suppl 2): 707. https://doi.org/10.1093/cdn/nzab046_004

28. Stabler SP. Clinical practice. Vitamin B12 deficiency. N Engl J Med 2013; 368: 149-160. https://doi.org/10.1056/NEJMcp1113996

29. Kohli UA, Rajput M, Venkatesan S. Association of maternal hemoglobin and iron stores with neonatal hemoglobin and iron stores. Med J Armed Forces India 2021; 77: 158-164. https://doi.org/10.1016/j.mjafi.2019.11.002

30. Tanyildiz HG, Yesil S, Okur I, Yuksel D, Sahin G. How does B12 deficiency of mothers affect their infants? Iran J Pediatr 2017; 27: e12898. https://doi.org/10.5812/ijp.12898

31. Balcı YI, Ergin A, Karabulut A, Polat A, Doğan M, Küçüktaşcı K. Serum vitamin B12 and folate concentrations and the effect of the Mediterranean diet on vulnerable populations. Pediatr Hematol Oncol 2014; 31: 62-67. https://doi.org/10.3109/08880018.2013.829894

The Turkish Journal of Pediatrics 2024; 66: 439-447
https://doi.org/10.24953/turkjpediatr.2024.4579

Original Article

Diagnostic value and clinical significance of lncRNA *NEAT1* combined with miR-425-3p in children with viral myocarditis

Jielin Gao[1], Lili Qin[2], Qiaozhi Guo[2], Dongxia Zhao[3], Guomei Ma[3], Kuilong Zhou[4], Shuang Wang[5], Hengrui Hao[6]

[1]Department of Pediatrics, Xingtai People's Hospital, Xingtai; [2]Department of Nursing, Xingtai People's Hospital, Xingtai; [3]Department of Ultrasound, The First Affiliated Hospital of Xingtai Medical College, Xingtai; [4]Department of Traditional Chinese Medicine, Xingtai People's Hospital, Xingtai; [5]Department of Radiology, Xingtai People's Hospital, Xingtai; [6]Department of Pediatric Rehabilitation, Xingtai People's Hospital, Xingtai, China.

ABSTRACT

Background. Viral myocarditis (VMC) is common in children. Previous studies have reported the clinical value of nuclear paraspeckle assembly transcript 1 (*NEAT1*) and microRNA-425-3p (miR-425-3p) in certain diseases, but not in VMC. This article was designed to investigate the expression of long noncoding RNA (lncRNA) *NEAT1* and miR-425-3p in the serum of patients with VMC and their clinical significance.

Methods. We assessed VMC and healthy patients and analyzed differences in the expression levels of *NEAT1* and miR-425-3p. The correlation and targeting relationship between the two were reported by Spearman correlation analysis and luciferase reporter assay. ROC curves were plotted to reflect the diagnostic effect of both. In addition, according to the 12-month prognostic effect grouping, patients with VMC were separated into a group with good vs. poor prognosis, and the difference in the expression levels of *NEAT1* and miR-425-3p between the two groups were analyzed. The ability of the two markers in the prognosis of VMC was further analyzed by multiple logistic regression.

Results. *NEAT1* expression was up-regulated in VMC and miR-425-3p expression was down-regulated, and there was a negative correlation and targeting link between the two. The diagnostic efficacy of both *NEAT1* and miR-425-3p was higher than that of a single indicator. High expression of *NEAT1* and low expression of miR-425-3p were found in VMC patients with poor prognosis. Both were independent influencers of VMC prognosis.

Conclusion. *NEAT1* and miR-425-3p expressions were affected by VMC and had important clinical implications for VMC, indicating for the first time the clinical function of *NEAT1* and miR-425-3p in VMC.

Key words: NEAT1, miR-425-3p, viral myocarditis, diagnosis, prognosis.

Viral myocarditis (VMC), is a myocardial disease caused by viral invasion of cardiac tissue, resulting in myocardial cell degeneration, necrosis, and interstitial inflammatory changes.[1] The clinical manifestations of the disease are variable, and the preclinical symptoms are not obvious. Clinical studies have demonstrated that most VMC patients recover well after treatment, but continued progression can cause further damage to cardiomyocytes, leading to myocardial fibrosis, arrhythmias, heart failure, and even sudden death.[2-4] This type of VMC has a poor prognosis, with an annual survival rate of only 50% within 5 years.[5] Although the short-term mortality rate of fulminant myocarditis has been further reduced with the improvement of diagnostic and therapeutic modalities at present, there is a general lack of effective clinical

✉ Hengrui Hao
haohengruidr@163.com

Received 5th Dec 2023, revised 30th Nov 2023, 3rd Feb 2024, 28 Mar 2024, 1st Apr 2024, 23rd Jun 2024, 8th Jul 2024, accepted 8th Aug 2024.

treatments for VMC, and current treatments are mainly focused on controlling the complications caused by VMC.[6] Therefore, early diagnosis and prognostic monitoring are particularly important to reduce the mortality rate of VMC and improve the treatment outcome.

Long noncoding RNAs (lncRNAs) are long-chain RNAs that are not translated into proteins and have diverse biological activities.[7] Publications have reported that lncRNAs maternally expressed gene 3 (*MEG3*), regulator of reprogramming (*ROR*), and metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) can be involved in the development of myocarditis.[8-10] LncRNA nuclear paraspeckle assembly transcript 1 (*NEAT1*) is associated with the mechanism of action of lipopolysaccharide-induced myocardial injury.[11] *NEAT1* expression is upregulated in the peripheral blood of patients with myocardial infarction and has the potential to predict the development of myocardial infarction.[12] This disease is associated with myocardial damage closely related to VMC, but there is no relevant literature on the relationship between *NEAT1* and VMC. As competing endogenous RNAs (ceRNAs) of lncRNAs, microRNAs (miRNAs) likewise play important roles in VMC, for instance miR-21-5p and miR-1-3p.[13] miR-146b concentration is elevated in VMC patients and is associated with myocardial injury, independently predicting patient prognosis.[5] In myocardial injury tissues of the coxsackievirus type B3-induced VMC mouse model, reduced miR-425-3p expression levels suggest that miR-425-3p is associated with VMC.[14] The function of miR-425-3p on VMC was only researched in the mouse model with VMC.[14] However, its expression and clinical value in patients with VMC have not been previously reported. Therefore, this paper focuses on the clinical importance of *NEAT1* and miR-425-3p in VMC.

In this manuscript, we collected serum samples and clinical data from 108 patients with VMC and assessed the levels of *NEAT1* and miR-425-3p to explore the possibility as both diagnostic biomarkers as well as their prognostic value, and to provide references for early clinical diagnosis and therapeutic effects.

Materials and methods

Collection of research objects

Cases of children with VMC admitted to Xingtai People's Hospital from May 2019 to May 2022 were chosen for the study. All of them met the diagnostic criteria for VMC formulated by the Chinese Medical Association.[15] Specifically, the basis for the diagnosis of VMC involves clinical diagnostic findings and pathogen measurements. Clinical diagnostic bases include cardiac insufficiency, cardiogenic shock, or heart-brain syndrome; cardiac enlargement; electrocardiographic changes; and elevated creatine kinase-myocardial band (CK-MB) or positive cardiac troponin (cTnI or cTnT). Diagnosis of pathogens consists primarily of virus detection. Exclusion criteria included myocardial injury caused by congenital heart disease and rheumatic heart disease; metabolic diseases; and antiviral drugs and immunotherapy before admission. A further 102 cases of children who underwent health check-ups during the same time frame at our hospital were chosen for the purpose of constituting the control group. The clinical examination data of all subjects were collected, and all samples were collected and investigated with the informed consent of the guardian and signed for confirmation. The study was reviewed and approved by the ethics committee of Xingtai People's Hospital (Approval number: 2018-XPH-14).

Follow-up and prognosis outcome judgment

All patients received the routine treatment of VMC. Conventional treatment included antiviral therapy and intravenous gammaglobulin. Other measures included monitoring patients using electrocardiograms and oxygen therapy. All patients were followed up for 12 months and the prognosis within 12 months was summarized. Poor prognosis was defined as the

presence of recurrent myocarditis, readmission with arrhythmia, rehospitalization with heart failure, cardiac transplantation, and death. The remaining patients were included in the good prognosis group.

Measurement of serum NEAT1 and miR-425-3p level

Three ml of venous blood from VMC patients at admission was collected and centrifuged at 1800 r/min (centrifugal radius 15 cm) for 10 min to separate serum.

Extraction of total RNA from serum was used Trizol LS (ThermoFisher Scientific, California, America). The absorbance of the extracted solution was determined by an ultraviolet spectrophotometer, and the ratio of optical density (OD) 260/OD280 was calculated. RNA with a ratio between 1.8 and 2.0 was up to standard. Total RNA was reverse transcribed into complementary DNA (cDNA) using the first strand of cDNA synthesized premixed reagents (TIANGEN, Beijing, China) for *NEAT1* and miRNA First Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) for miR-425-3p. The expression of *NEAT1*, miR-425-3p, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and U6 was detected by a Talent fluorescence quantitative detection kit (TIANGEN, Beijing, China) on a BioRad fluorescence quantitative PCR instrument (BioRad, Hamburg, Germany). *GAPDH* and *U6* were detected as housekeeping genes. Cycle threshold (CT) values were obtained after the assay, and relative expression was obtained using $2^{-\text{deltadeltaCT}}$.

Levels of cTnI, CK-MB, and high-sensitivity C-reactive protein (hs-CRP)

The cTnI levels were detected by enzyme-linked immunosorbent assay (ELISA) using a Human cTnI ELISA Kit (Abcam, Cambridge, UK). CK-MB levels were detected using a DXC600 automatic biochemical analyzer. hs-CRP level was detected using a Human CRP ELISA Kit (Abcam, Cambridge, UK).

Detection of double luciferase reporter gene

NEAT1 wild-type vector (WT-*NEAT1*) and mutant vector (MUT-*NEAT1*) were constructed. miR-425-3p mimics, inhibitors, and negative controls were purchased from ThermoFisher Scientific (California, America). and WT-*NEAT1* and MUT-*NEAT1* were co-transfected with miR-425-3p mimic, inhibitors, and controls, respectively, into 293T cells. After 48 hours, luciferase activity was detected following the luciferase activity assay kit instructions (Biolab, Beijing, China).

Statistical analysis

GraphPad Prism (Version: 7.0) and IBM SPSS statistics (Version: 24) were used for the statistical analysis of data. Continuous variables were tested with the Kolmogorov-Smirnov inspector, and the data was confirmed to conform to normal distributions. Then the continuous variables were tested by t-test. The correlation between lncRNA and miRNA was identified by the Spearman method. The $\chi 2$ test was used for discrete data. Luciferase reported results were tested by two-way ANOVA followed by post hoc Bonferroni's test. The diagnostic value of *NEAT1*, miR-425-3p, and the combination of the two for VMC was determined using the subject's work characteristics (ROC) curve. Combined ROC curves were constructed using the predicted probability acquired through multivariate logistic regression of miR-425-3p and *NEAT1*. Multiple logistic analysis was used to analyze the influencing factors in the prognosis of VMC patients. The level of statistical significance was set at $p < 0.05$ in all statistical tests.

Results

Comparison of clinical symptoms between VMC and control groups

The clinical characteristics of all recruited patients and controls were gathered and estimated. There was no noteworthy

differentiation between the two groups of participants in relation to their age, gender, and BMI ($p > 0.05$, Table I). Patients in the VMC group possessed higher cTnl CK-MB, and hs-CRP levels ($p < 0.001$, Table I).

Serum levels of NEAT1 and miR-425-3p in VMC

The relative concentration of *NEAT1* was elevated in the VMC group, reflecting that *NEAT1* was involved in the development of VMC ($p < 0.001$, Fig. 1A). Diminished relative quantification of miR-425-3p was found in the VMC group ($p < 0.001$, Fig. 1B). A negative correlation between *NEAT1* and miR-425-3p was detected. As depicted in Fig. 1C-D, *NEAT1* and miR-425-3p were negatively correlated in both control group (R: -0.750, $p < 0.001$) and VMC patients (R: -0.777, $p < 0.001$), suggesting that there may be some correlation between the two.

Verification of the targeting relationship of miR-425-3p with NEAT1

The binding site of miR-425-3p to *NEAT1* was predicted using the RNAhybrid website (Fig. 2A). The luciferase intensity was significantly decreased in the miR-425-3p mimic and WT-*NEAT1* co-transfected group, and significantly increased in the miR-425-3p inhibitor and WT-*NEAT1* co-transfected group ($p < 0.001$, Fig. 2B). The variance in luciferase activity among groups co-transfected with MUT-*NEAT1* was not deemed statistically significant ($p > 0.05$, Fig. 2B). These results suggest that miR-425-3p might be a ceRNA of *NEAT1*.

Diagnostic significance of NEAT1 and miR-425-3p

The ROC curve showed that serum *NEAT1* predicted VMC patients with ROC of 0.875, sensitivity of 73.15%, and specificity of 87.25% (Fig. 3). miR-425-3p predicted patients with poor prognosis with a ROC of 0.832, sensitivity of 79.63%, and specificity of 77.45% (Fig. 3). The ROC, sensitivity, and specificity of the two combined were 0.901, 80.60%, and 87.30%, respectively (Fig. 3). The comparison of the area under the curve indicated that the combined diagnostic efficacy was better than the two single diagnostic indexes.

Comparison of basic data of VMC patients with different prognostic outcomes

There was no significance in the difference in age, BMI, and gender between the good and poor prognosis groups ($p > 0.05$, Table II). The cTnI, CK-MB, and hs-CRP levels of the poor prognosis group were all elevated ($p < 0.001$, Table II).

Serum levels of NEAT1 and miR-425-3p in VMC patients with different prognostic outcomes

The serum *NEAT1* levels in the good prognosis group were significantly lower than those in the poor prognosis group ($p < 0.001$, Fig. 4A). The

Table I. Demographics and baseline information of healthy and VMC groups.

Clinical features	VMC group (n = 108)	Control group (n = 102)	*p*-value
Age (year)	5.63 ± 2.15	6.22 ± 2.12	0.923
BMI (kg/m^2)	14.46 ± 1.98	14.33± 2.28	0.321
Duration (day)	4.86 ± 1.53	/	/
Gender (male/female)	50/58	52/50	0.497
cTnI (μg/L)	0.49 ± 0.11	0.06 ± 0.01	< 0.001
CK-MB (U/L)	33.48 ± 5.81	8.91 ± 2.18	< 0.001
hs-CRP (mg/L)	4.32 ± 0.88	1.17 ± 0.19	< 0.001
NEAT1	1.00 ± 0.29	1.47 ± 0.30	< 0.001
miR-425-3p	1.00 ± 0.31	0.59 ± 0.26	< 0.001

Note: BMI: Body-mass index; CK-MB: Creatine kinase myocardial band; cTnI: Cardiac troponin I; hs-CRP: Hypersensitive C-reactive protein; VMC: Viral myocarditis.

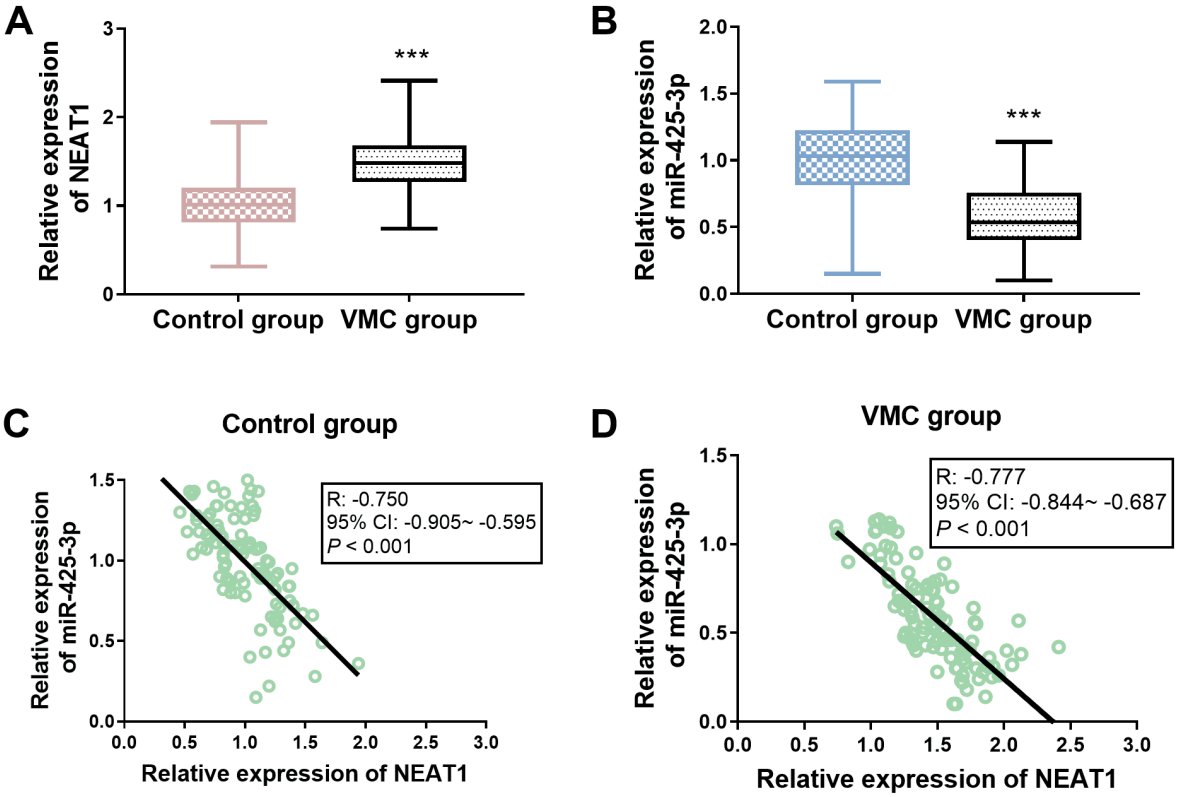


Fig. 1. (A) The concentration of NEAT1. **(B)** Reduced miR-425-3p in VMC patients. **(C)** Negative relationship between NEAT1 and miR-425-3p in control group. **(D)** Negative relationship between NEAT1 and miR-425-3p in VMC group. ***p < 0.001, compared to control group. VMC: viral myocarditis.

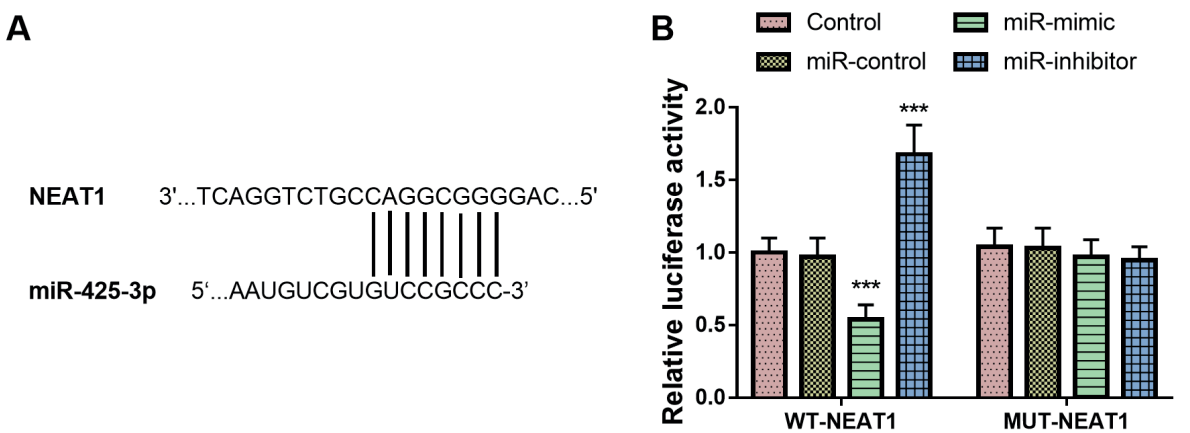


Fig. 2. miR-425-3p targeted NEAT1. **(A)** Targeted bases between *NEAT1* and miR-425-3p. **(B)** Luciferase reporter assay certified targeted interconnection between *NEAT1* and miR-425-3p. ***P < 0.001, compared to control group.

declined levels of miR-425-3p were observed in the poor prognosis group ($p < 0.001$, Fig. 4B). These results displayed that the concentration of *NEAT1* and miR-425-3p was influenced by the prognosis of VMC.

Possibility of NEAT1 and miR-425-3p as independent predictors of VMC

A multiple logistic regression model was developed to analyze the independent influencing factors. Baseline characteristics

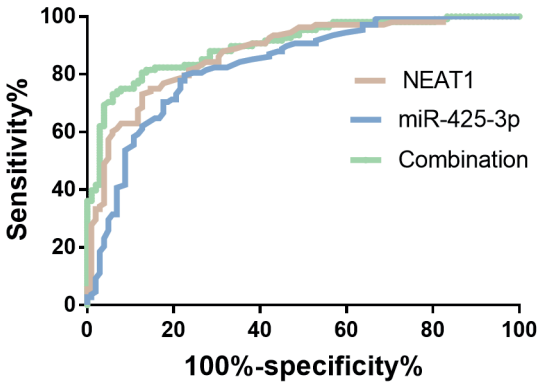


Fig. 3. Diagnostic significance of NEAT1, miR-425-3p, and their combination.

were categorized into 9 variables that were all entered into logistic regression models. The model's goodness of fit was evaluated with the Hosmer-Lemeshow test (p = 0.228). The results showed that elevated cTnI (odd ratios: 3.883, 95% confidence interval: 1.275-11.825), elevated CK-MB (odd ratios: 3.671, 95% confidence interval: 1.194-11.285), elevated *NEAT1* (odd ratios: 9.714, 95% confidence interval: 3.204-29.453) and reduced miR-425-3p (odd ratios: 0.159, 95% confidence interval: 0.049-0.512) were independent risk factors for poor prognosis in VMC patients (all $p < 0.05$, Table III).

Table II. Demographics and baseline information of patients with VMC.

Clinical features	Good prognosis group (n = 62)	Poor diagnosis group (n = 46)	p-value
Age (year)	6.13 ± 2.29	6.35 ± 1.90	0.599
BMI (kg/m^2)	14.68 ± 2.09	14.18 ± 1.79	0.201
Duration (day)	4.88 ± 1.44	4.84 ± 1.67	0.191
Gender (male/female)	31/31	19/27	0.437
cTnI (μg/L)	0.43 ± 0.09	0.57 ± 0.09	< 0.001
CK-MB (U/L)	30.87 ± 5.12	36.99 ± 4.79	< 0.001
hs-CRP (mg/L)	4.04 ± 0.73	4.69 ± 0.94	< 0.001
NEAT1	1.37 ± 0.31	1.61 ± 0.23	< 0.001
miR-425-3p	0.73 ± 0.23	0.40 ± 0.17	< 0.001

Note: BMI: Body mass index; CK-MB: Creatine kinase myocardial band; cTnI: Cardiac troponin I; hs-CRP: Hypersensitive C-reactive protein; VMC: Viral myocarditis.

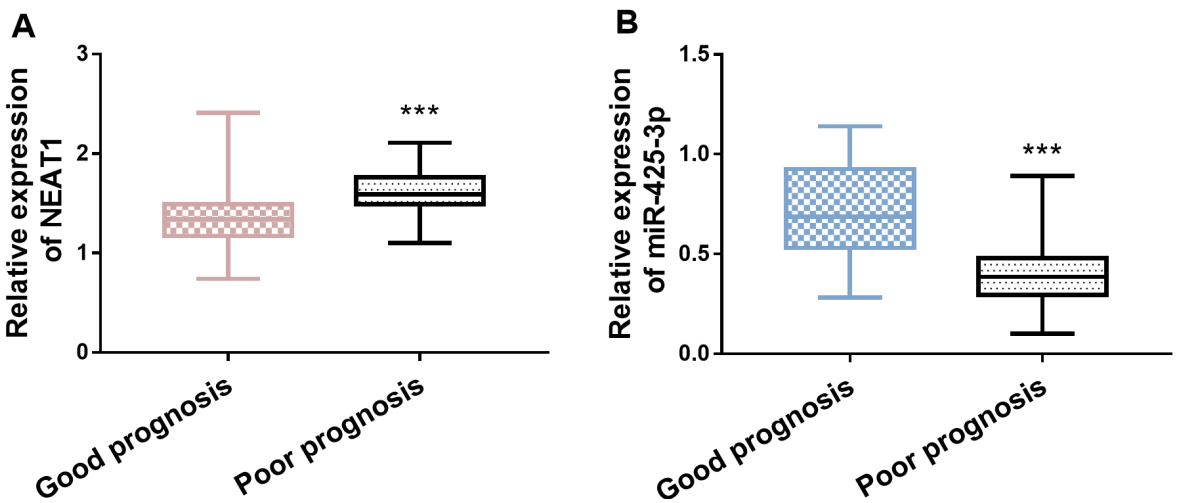


Fig. 4. (A) Elevated NEAT1 levels in poor prognosis group **(B)** Declined miR-425-3p levels in poor prognosis group ***p < 0.001, compared to good diagnosis group.

miR-425-3p: microRNA-425-3p; NEAT1: nuclear paraspeckle assembly transcript 1.

Discussion

VMC has high morbidity and mortality.[16] At present, the diagnosis of VMC is mainly based on the history of viral infection, clinical features, and serum myocardial enzyme levels, but the sensitivity of these is low.[17] The gold standard for clinical diagnosis of VMC is endomyocardial biopsy, but it is difficult to obtain an accurate diagnosis in the early stage of the disease.[5,18] Therefore, screening for appropriate markers is necessary for the clinical management of VMC.

LncRNAs are closely related to heart diseases.[19] LncRNA *HIF1A* antisense RNA 1 (*HIF1A*-AS1), *AK085865*, and *GBP9* are both influenced by the VMC and participate in the development of VMC, indicating the potential roles of lncRNAs in VMC.[20-22] *NEAT1* is involved in a variety of illnesses, including heart failure and myocardial infarction. Wang et al.[23] show that the expression level of *NEAT1* is significantly increased in the mice of myocardial infarction after coronary heart disease, and the high expression of *NEAT1* together with the diminished miR-22-3p may become targets for myocardial infarction. Ge et al.[24] report that NEAT1 is improved in patients with heart failure and can facilitate the progression of fibrosis in vitro models, indicating that NEAT1 is associated with the pathological process of heart dysfunction. All these articles suggest that NEAT1 might be involved in heart disorders. The present study determined that the relative expression level of serum NEAT1 was improved in the VMC group and further elevated in VMC patients with poor prognosis, suggesting that NEAT1 might be involved in the development of VMC. Elevated serum expression levels of *NEAT1* are valuable for the diagnosis of VMC. In addition, *NEAT1* can be used as an independent biomarker to detect the disease progression of VMC. Taken together, these findings pinpointed that the expression of *NEAT1* was affected by the development of VMC and could predict the diagnosis and prognosis of VMC.

miRNAs have been proven to regulate the expression process of host and virus genes, which plays a certain role in many diseases.[25] In children with VMC, the concentration of miR-155 and miR-381 is regulated by VMC, indicating these miRNAs are closely linked to VMC.[26,27] The results of this study verified that the content of miR-425-3p in the VMC group was decreased and its expression level further declined in patients with a poor prognosis. The ROC curve showed that miR-425-3p was a prognostic marker. The multiple logistic regression analysis model showed that the decrease of miR-425-3p was an independent indicator for adverse prognosis in patients with VMC. Our findings further certified the diagnostic and prognostic significance of miR-425-3p for patients with VMC. MiR-425-3p is a member of the miRNA gene cluster which has been studied a lot. In myocardial tissues of mice with VMC, the concentration of miR-425-3p is down-regulated, reflecting that miR-425-3p might be a mediator in VMC.[14] The reduced miR-425-3p expression is observed in patients with heart failure and it can serve as a biomarker, lending evidence that miR-425-3p is associated with the occurrence of heart diseases.[28] In addition, the targeting relationship between *NEAT1* and miR-425-3p was further confirmed. miR-425-3p concentration changes altered the luciferase intensity of *NEAT1*-WT, suggesting that, miR-425-3p is a downstream regulator of *NEAT1*. The value of the combined diagnosis of NEAT1 and miR-425-3p was also evaluated by the ROC, which showed that the combined diagnosis significantly improved the predictive efficacy. However, there are some limitations in this study, such as a lack of in-depth mechanism research, a relatively small sample size, and a single research center.

To sum up, the concentration of NEAT1 in the serum of patients with VMC was up-regulated, while the expression of miR-425-3p was down-regulated. There was a negative relationship between NEAT1 and miR-425-3p and miR-425-3p was possibly a target of NEAT1. The combination of NEAT1 and miR-425-3p had a good diagnostic value for VMC. Both NEAT1 and miR-425-3p were independent prognostic biomarkers.

Ethical approval

The study protocol was approved by The Ethics Committee of Xingtai People's Hospital (Ethical approval number: 2018-XPH-14, 02 April 2018) and followed the principles outlined in the Declaration of Helsinki. In addition, informed consent has been obtained from the participants involved.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: JG, HH; data collection: LQ, QG, DZ; analysis and interpretation of results: GM, KZ, SW; draft manuscript preparation: JG, HH. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

This study was funded by Scientific Research Plan of Hebei Administration of Traditional Chinese Medicine(2021453).

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Zhou J, Xu J, Li P, et al. Necroptosis and viral myocarditis: tumor necrosis factor α as a novel biomarker for the diagnosis of viral myocarditis. Front Cell Dev Biol 2022; 10: 826904. https://doi.org/10.3389/fcell.2022.826904
2. Deckx S, Johnson DM, Rienks M, et al. Extracellular SPARC increases cardiomyocyte contraction during health and disease. PLoS One 2019; 14: e0209534. https://doi.org/10.1371/journal.pone.0209534
3. Yue-Chun L, Gu XH, Li-Sha G, et al. Vagus nerve plays a pivotal role in CD4+ T cell differentiation during CVB3-induced murine acute myocarditis. Virulence 2021; 12: 360-376. https://doi.org/10.1080/21505594.2020.1869384
4. Narovlyanskaya O, Winokur EJ. Viral myocarditis. Dimens Crit Care Nurs 2020; 39: 75-80. https://doi.org/10.1097/DCC.0000000000000402
5. Yan M, Wang J, Wang S, Zhang Y, Liu L, Zhao H. Expression levels of microRNA-146b and anti-cardiac troponin I in serum of children with viral myocarditis and their clinical significance. Iran J Public Health 2021; 50: 510-519. https://doi.org/10.18502/ijph.v50i3.5592
6. Liu H, Li M, Song Y, Xu W. TRIM21 restricts coxsackievirus B3 replication, cardiac and pancreatic injury via interacting with MAVS and positively regulating IRF3-mediated type-I interferon production. Front Immunol 2018; 9: 2479. https://doi.org/10.3389/fimmu.2018.02479
7. Zhang C, Xiong Y, Zeng L, et al. The role of non-coding RNAs in viral myocarditis. Front Cell Infect Microbiol 2020; 10: 312. https://doi.org/10.3389/fcimb.2020.00312
8. He F, Liu Z, Feng M, et al. The lncRNA MEG3/miRNA-21/P38MAPK axis inhibits coxsackievirus 3 replication in acute viral myocarditis. Virus Res 2024; 339: 199250. https://doi.org/10.1016/j.virusres.2023.199250
9. Xue Y, Ke J, Zhou X, et al. Knockdown of LncRNA MALAT1 alleviates coxsackievirus B3-induced acute viral myocarditis in mice via inhibiting Th17 cells differentiation. Inflammation 2022; 45: 1186-1198. https://doi.org/10.1007/s10753-021-01612-x
10. Zhang N, Sun Y. LncRNA ROR facilitates myocardial fibrosis in rats with viral myocarditis through regulating C-Myc expression. Eur Rev Med Pharmacol Sci 2019; 23: 10982-10988. https://doi.org/10.26355/eurrev_201912_19803
11. Wang SM, Liu GQ, Xian HB, Si JL, Qi SX, Yu YP. LncRNA NEAT1 alleviates sepsis-induced myocardial injury by regulating the TLR2/NF-κB signaling pathway. Eur Rev Med Pharmacol Sci 2019; 23: 4898-4907. https://doi.org/10.26355/eurrev_201906_18078
12. Zhao J, Chen F, Ma W, Zhang P. Suppression of long noncoding RNA NEAT1 attenuates hypoxia-induced cardiomyocytes injury by targeting miR-378a-3p. Gene 2020; 731: 144324. https://doi.org/10.1016/j.gene.2019.144324
13. Marketou M, Kontaraki J, Patrianakos A, et al. Peripheral blood microRNAs as potential biomarkers of myocardial damage in acute viral myocarditis. Genes (Basel) 2021; 12: 420. https://doi.org/10.3390/genes12030420

14. Li J, Tu J, Gao H, Tang L. MicroRNA-425-3p inhibits myocardial inflammation and cardiomyocyte apoptosis in mice with viral myocarditis through targeting TGF-β1. Immun Inflamm Dis 2021; 9: 288-298. https://doi.org/10.1002/iid3.392

15. Wu T. The diagnostic standard of viral myocarditis. Chinese Journal of Practical Pediatrics 2000; 5: 315.

16. Olejniczak M, Schwartz M, Webber E, Shaffer A, Perry TE. Viral myocarditis-incidence, diagnosis and management. J Cardiothorac Vasc Anesth 2020; 34: 1591-1601. https://doi.org/10.1053/j.jvca.2019.12.052

17. Putschoegl A, Auerbach S. Diagnosis, evaluation, and treatment of myocarditis in children. Pediatr Clin North Am 2020; 67: 855-874. https://doi.org/10.1016/j.pcl.2020.06.013

18. Adeboye A, Alkhatib D, Butt A, Yedlapati N, Garg N. A review of the role of imaging modalities in the evaluation of viral myocarditis with a special focus on COVID-19-related myocarditis. Diagnostics (Basel) 2022; 12: 549. https://doi.org/10.3390/diagnostics12020549

19. Xue Y, Ke J, Zhang J, et al. Analysis of long noncoding RNAs and messenger RNAs expression profiles in the hearts of mice with acute viral myocarditis. J Med Virol 2023; 95: e28473. https://doi.org/10.1002/jmv.28473

20. Cao H, Yang B, Zhao Y, Deng X, Shen X. The pro-apoptosis and pro-inflammation role of LncRNA HIF1A-AS1 in Coxsackievirus B3-induced myocarditis via targeting miR-138. Cardiovasc Diagn Ther 2020; 10: 1245-1255. https://doi.org/10.21037/cdt-20-545

21. Xue Y, Zhang J, Ke J, et al. LncGBP9 knockdown alleviates myocardial inflammation and apoptosis in mice with acute viral myocarditis via suppressing NF-κB signaling pathway. Inflamm Res 2022; 71: 1559-1576. https://doi.org/10.1007/s00011-022-01644-5

22. Zhang Y, Li X, Kong X, et al. Long non-coding RNA AK085865 ablation confers susceptibility to viral myocarditis by regulating macrophage polarization. J Cell Mol Med 2020; 24: 5542-5554. https://doi.org/10.1111/jcmm.15210

23. Wang L, Wang L, Wang Q. Constitutive activation of the NEAT1/miR-22-3p/Ltb4r1 signaling pathway in mice with myocardial injury following acute myocardial infarction. Aging (Albany NY) 2021; 13: 15307-15319. https://doi.org/10.18632/aging.203089

24. Ge Z, Yin C, Li Y, et al. Long noncoding RNA NEAT1 promotes cardiac fibrosis in heart failure through increased recruitment of EZH2 to the Smad7 promoter region. J Transl Med 2022; 20: 7. https://doi.org/10.1186/s12967-021-03211-8

25. Ferragut Cardoso AP, Banerjee M, Nail AN, Lykoudi A, States JC. miRNA dysregulation is an emerging modulator of genomic instability. Semin Cancer Biol 2021; 76: 120-131. https://doi.org/10.1016/j.semcancer.2021.05.004

26. Zhang Y, Sun L, Sun H, et al. MicroRNA-381 protects myocardial cell function in children and mice with viral myocarditis via targeting cyclooxygenase-2 expression. Exp Ther Med 2018; 15: 5510-5516. https://doi.org/10.3892/etm.2018.6082

27. Zhang Z, Dai X, Qi J, Ao Y, Yang C, Li Y. Astragalus mongholicus (Fisch.) Bge improves peripheral treg cell immunity imbalance in the children with viral myocarditis by reducing the levels of miR-146b and miR-155. Front Pediatr 2018; 6: 139. https://doi.org/10.3389/fped.2018.00139

28. Wang L, Liu J, Xu B, Liu YL, Liu Z. Reduced exosome miR-425 and miR-744 in the plasma represents the progression of fibrosis and heart failure. Kaohsiung J Med Sci 2018; 34: 626-633. https://doi.org/10.1016/j.kjms.2018.05.008

The Turkish Journal of Pediatrics 2024; 66: 448-456
https://doi.org/10.24953/turkjpediatr.2024.4638

Original Article

Characterization of lingual microbiota in pediatric geographic tongue

Yi You[1], Yuan He[2], Peicheng Huang[1]

[1]Department of Stomatology, Hunan Children's Hospital, Hunan; [2]Hunan Key Laboratory of Oral Health Research & Hunan 3D Printing Engineering Research Center of Oral Care & Hunan Clinical Research Center of Oral Major Diseases and Oral Health & Academician Workstation for Oral-maxilofacial and Regenerative Medicine & Xiangya Stomatological Hospital & Xiangya School of Stomatology, Central South University, China.

ABSTRACT

Background. Geographic tongue is an oral mucosal lesion affecting the tongue. The association between geographic tongue and the mucosal microbiota in children remains unclear.

Method. To characterize the feature of lingual microbiota in pediatric geographic tongue, lingual swabs were collected from lesion sites and healthy sites of 25 patients with geographic tongue (14 males and 11 females; age 5.21 ±2.94 years) and 19 controls (10 males and 9 females; age 5.31±2.82 years). DNA was extracted and the 16S rRNA was amplificated, sequenced and analyzed.

Results. The lingual microbiota composition was significantly different between children with geographic tongue and the healthy cohort; *Streptobacillus* was reduced in geographic tongue, while *Catonella, Bacillus* and *Oribacterium* were overrepresented. When the lesions and the normal mucosa were compared, an increased abundance of *Prevotella oris* was observed.

Conclusion. Our results provided new insight into the association between oral microbiota and pediatric geographic tongue.

Key words: geographic tongue, children, microbiota, 16S rRNA.

Geographic tongue (GT), also known as benign migratory glossitis, is a common inflammatory condition that affects the mucous membranes of the tongue.[1] It manifests as irregular, map-like patches on the tongue's surface with papillary atrophy, surrounded by a whitish peripheral zone. These patches may change in shape, size, and location over time. Generally, GT is asymptomatic, while in some cases, symptoms such as soreness, sensitivity, and burning sensations, are triggered by acidic drinks and spicy foods.[2] Epidemiological studies have reported varying prevalence rates, ranging from 1% to 3% in the general population[3], with a slightly higher incidence in women compared to men[4], while in the pediatric population it ranges from 0.37% to 14.3%.[1]

The pathogenesis of geographic tongue involves complex interactions between genetic and environmental factors.[5] Genetic predisposition plays a significant role, as the condition often demonstrates a familial tendency.[6] Mutations in genes involved in immune regulation and epithelial cell function, such as *IL36RN*[7] and human leukocyte antigen (HLA) genes[8], have been implicated in GT development. Association with other conditions like psoriasis[6] and juvenile diabetes[9] has also been observed.

Environmental factors, including stress, hormonal changes, allergic reactions, certain dietary factors, and oral microbiota, may contribute to GT development. Papillary atrophy, a structural change in the tongue associated with GT, may alter the bacterial

✉ Yi You
yuyudany@sina.com

Received 15th Apr 2024, revised 30th Jul 2024, 8th Aug 2024, accepted 9th Aug 2024.

community, leading to inflammation. Elevated levels of inflammatory mediators like interleukin (IL)-8 and calprotectin in GT suggest a reciprocal relationship between microbiota dysbiosis and inflammation.[10,11]

The presence of fungi had been reported to be associated with GT.[12,13] The development of Next-generation sequencing (NGS) allowed the study of microbial communities.[14] Recent research has highlighted the role of oral microbiota in oral mucosal lesions like oral cancer[15], oral submucous fibrosis[16], oral leukoplakia[17] and oral lichen planus.[18] Studies on the lingual microbiota profiles of adults with GT identified *Microbacterium*, *Leptospira*, *Methylotenera*, and *Lactococcus* as bacteria associated with GT lesion sites.[19]

The oral microbiome undergoes changes with age, and microbial profiles in children differ from those of other age groups.[20] Bacterial diversity and richness increase during early childhood.[21] These findings suggest that the oral microbiota in children has distinct characteristics. However, the association between oral microbiota and GT in children remains unclear.

Our study characterizes the microbiota profiles of children with GT at the lesion and surrounding healthy sites. By comparing these profiles to the bacterial community of healthy control children, we aim to shed light on the role of microbiota in pediatric GT.

Materials and Methods

Ethics approval and consent to participate

The study was examined and given approval by the Ethics Committee of Hunan Children's Hospital. All the methods were carried out in line with relevant guidelines and regulations. The participants provided informed consent prior to taking part in the study.

Patients and sample collection

Children (n=25) with only primary untreated GT between 2 and 10 years of age fulfilling diagnostic criteria were recruited at the Hunan Children's Hospital. Healthy controls (n=19) were also recruited from Hunan Children's Hospital. All individuals were free of antibiotic therapy for a month prior to the study. Specimens of GT lesion and normal mucosa on the opposite side from the GT participant were collected unstimulated by swab in accordance with the Manual of Procedures for Human Microbiome Project. This was done at least 1 hour after eating or drinking. If the patient had eaten within one hour, mouth rinsing was done and then a wait of 10 minutes was observed before sample collection. Swabs were collected in 1.5 mL tubes and stored at −80 °C before use.

DNA extraction

Microbial DNA was extracted from each sample using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The DNA concentrations were measured by using the Qubit quantification system (Thermo Scientific, Wilmington, DE, US). The extracted DNA was stored at -20 °C until use.

16S gene amplicon sequencing

The 16S ribosomal RNA (rRNA) gene amplification procedure included two polymerase chain reaction (PCR) steps. Briefly, For the first PCR reaction, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC).

DNA was amplified in 96-well plates with a reaction mixture of 1X KAPA HiFi Hot start Ready Mix, 0.1μM primer 341 F, 0.1 μM primer 805 R, and 12.5 ng template DNA, totaling a total volume of 50 μL per sample. Reactions were conducted in a T100 PCR thermocycle (BIO-RAD) according to the following cycling program: 3 minutes of denaturation at 94 °C, followed by 18 cycles of 30 seconds at 94 °C (denaturing), 30 seconds at 55 °C (annealing), and 30 seconds at 72 °C (elongation), with a final

extension at 72 °C for 5 minutes. Subsequently, the amplified products were examined by 2% agarose gel electrophoresis and quantified by the Qubit quantification system (Thermo Scientific, Wilmington, DE, US). In the second PCR step, sequencing primers and adaptors were added to the amplicon products. Specifically, 2 μL of the diluted amplicons were mixed with a reaction solution consisting of 1×KAPA HiFi Hotstart ReadyMix, 0.5μM fusion forward and 0.5μM fusion reverse primer, and 30 ng Meta-gDNA (total volume 50 μL). The PCR was run following the previous cycling program except with a cycling number of 12. The amplification products were purified with AMPure XP Beads (Beckman Coulter Genomics, MA, USA) according to the manufacturer's instructions and quantified as described earlier. Equimolar amounts of the amplification products were pooled into a single tube, and the concentration of the pooled libraries was measured by the Qubit quantification system. 2 × 250 bp paired-end sequencing with dual-index reads were carried out at MiSeq Reagent Kits v2 (Illumina Inc.) on the Illuimina MiSeq System (Illumina Inc., CA, USA).

Data processing

Fastq-files were demultiplexed by the MiSeq Controller Software (Illumina Inc.). Then the amplification primers, diversity spacers, and sequencing adapters, merge-paired were trimmed and the low quality reads were filtered by USEARCH. Operational taxonomic unit (OTU)s equaling or above 97% were clustered by UPARSE. The RDP classifier were used for assignment of taxonomy of the OTUs and alignment for the sequences\. The OTUs were analyzed by phylogenetic and OTU methods in the Quantitative Insights into Microbial Ecology (QIIME) software version 2. α-diversity (Observed OTU number, Shannon index, Simpson index, Chao1 index, observed species, ACE index) and β-diversity (Unweight UniFrac distances and Weight UniFrac distances) were calculated based on the rarefied OTU counts. Function prediction was performed by PICRUSt2, and differentially abundant function predictions were identified by STAMP.

Statistics and data analysis

Differential α-diversity analysis was performed utilizing the Wilcoxon rank-sum or Kruskal-Wallis test. The software linear discriminant analysis (LDA) Effect Size (LEfSe) was used to identify taxa characterizing the differences between conditions.

Results

Characteristics of the bacterial communities

To characterize the oral microbiome community in GT, oral swapping specimens were collected from GT children (n= 25) and healthy controls (n= 19) (Table I). Samples were sequenced on an Illumina Miseq system, a total of 5.98×10^6 raw sequences were obtained. Samples with a low number of combined paired reads (<10000) were excluded in the following analysis. According to the rarefaction data (Fig. S1), subsets of 10000 reads (this quantity was sufficient to identify

Table I. Baseline characteristics of the cohort.

	Heathy control	Geographic tongue	P value
Age	5.31±2.82	5.21 ±2.94	0.9
Female / Male	9 / 10	11 / 14	1
Raw reads	91403.21±3364.51	GT-H: 83715.2±11027.8	
		GT-L: 86048.88±8133.74	<0.01
Filtered reads	75787.32±3511.34	GT-H: 70408.4±9561.95	
		GT-L: 71325.76±6551.8	<0.01

GT-H, Healthy tongue area of geographic tongue patients; GT-L, Lesions of geographic tongue patients.

most of the bacterial community members as the rarefaction curve of the observed OTUs reached a plateau at this point) were picked randomly to normalize sequencing depth for subsequent community composition analysis. The clustering of the picked reads with a 97% sequence identity led to 2985 unique OTUs in the datasets, which were classified into 17 distinct bacterial phyla, 69 distinct bacterial families, and 123 distinct bacterial genera (Table S1). Nearly half of the OTUs were shared by the groups (Fig. 1A), and the counts of OTUs mapped to different levels of taxon were comparable (Fig. 1B). At the phylum, bacteria from *Firmicutes* (44.7%), followed by Proteobacteria (26.4%), Bacteroidetes (14.9%), Actinobacteria (8.3%) and *Fusobacteria* (5.3%) dominated (Fig. S2A). For genera, *Streptococcus* (23.6%), *Neisseria* (12.9%), *Haemophilus* (11.5%), *Veillonella* (9.9%), *Rothia* (5.7%) and *Granulicatella* (4.2%) compromised the most predominant genus, and these predominant taxa show no difference between groups. (Fig. S2B).

Richness and diversity of the microbiota comparison by groups

Alpha diversity indices did not differ by gender or age. No significant difference was observed between healthy controls with normal lingual mucosa in GT patients or between normal mucosa with GT lesions (Fig. S3).

The samples in GT groups seemed to cluster together based on OTU abundance (Fig. S4). And a scatter plot based on principal coordinate analysis (PCoA) or non-metric multidimensional scaling (NMDS) used weighted or unweighted Unifrac distance was utilized to reveal bacterial community composition. Cluster of samples of different conditions was observed, especially at weighted Unifrac distance (Fig. S5B, D). A minor shift of bacterial community was found from normal mucosa or lesions in GT patients to healthy controls (Fig. S5). According to multi-response permutation procedures (MRPPs, Adonis, Anosim), the differences between groups was statistically significant, even though they seem to be small (Table S2). Partial Least Squares Discriminant Analysis (PLS-DA) could separate those samples of different conditions (Fig. S6).

Differences in the compositions of the microbiota in GT patients and healthy controls

Then the bacterial communities were analyzed at different taxonomic levels by LEfSe based on taxonomy data. Compared to healthy controls, *Streptobacillus* was depleted in the normal lingual mucosa in GT patients, while *Catonella, Oribacterium, Bacillus, Prevotella scopos* and *Prevotella nanceiensis* were overrepresented (Fig. 2A, 2B, Table S3), *Prevotella oulorum* was underrepresented, while the abundance of *Bacillus* was increased in GT lesion compared to

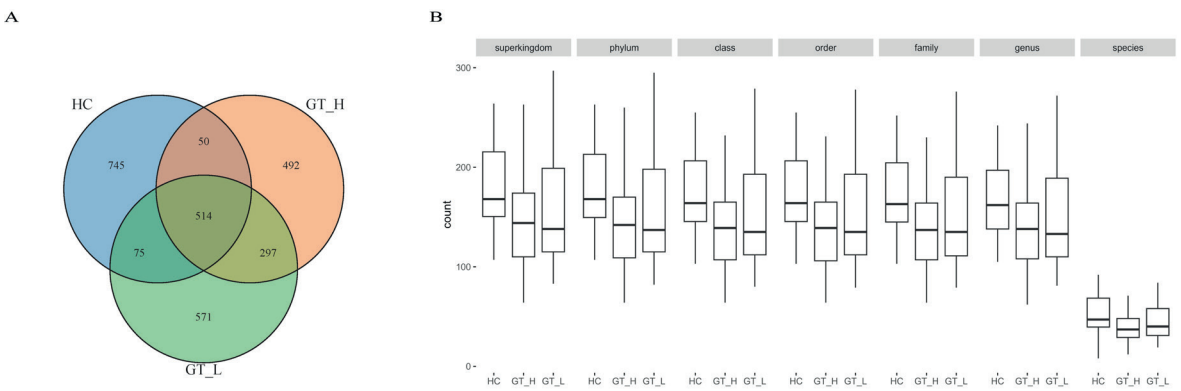


Fig. 1. The distribution of OTUs detected. A: Venn diagram showed the overlap of OTUs in different groups. B: Number of taxa at different levels assigned by OTUs. HC indicated healthy control, GT-H indicated the normal lingual area in geographic tongue patients, GT-L indicated the lesion in geographic tongue patients. OTU: operational taxonomic unit.

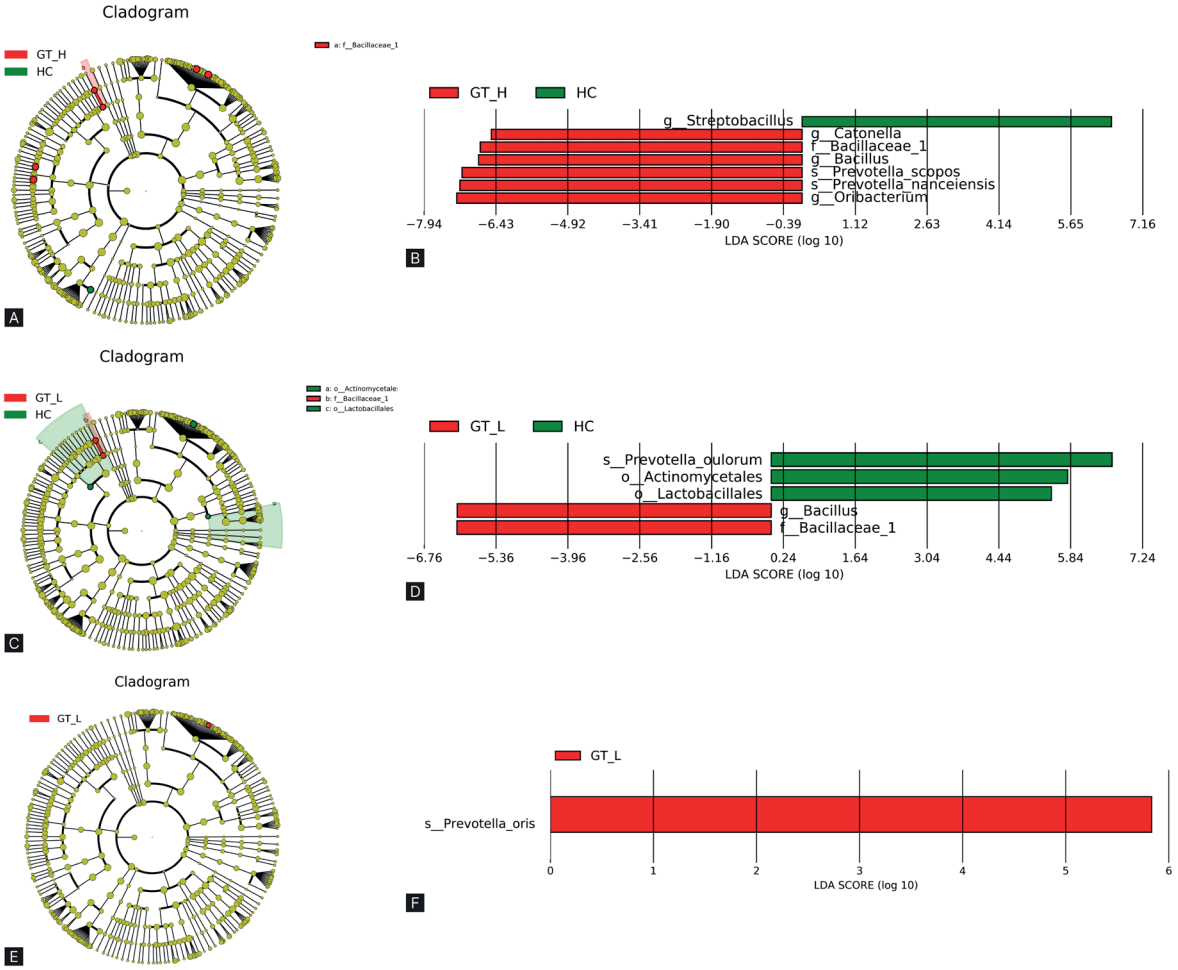


Fig. 2. The differentially enriched bacteria in different groups were determined by linear discriminant analysis (LDA) effect size (LEfSe) analysis. A: Cladogram using the LEfSe method indicating the phylogenetic distribution of microbiota exhibited different abundance in the tongues of heathy controls (HC) and geographic tongue patients (GT-H). B: Histogram of the LDA scores was calculated for the selected taxa which showed the significant bacterial difference in heathy controls and geographic tongue patients. C: Cladogram showed the phylogenetic distribution of microbiota exhibited different abundance at normal lingual site (GT-H) or lesion (GT-L) in geographic tongue patients. D: Histogram of the LDA scores for the selected taxa which showed the significant bacterial difference at healthy lingual or lesion in geographic tongue patients.

healthy controls (Fig. 2C, 2D, Table S3). When the normal mucosa was compared to lesions, increased abundance of *Prevotella oris* was observed (Fig. 2E, 2F, Table S3).

PICRUSt2 was utilized for function prediction, and differentially abundant KEGG pathways were observed, microbiota at normal mucosa in GT had increased function of phosphonate and phosphonate metabolism, apoptosis, glycosaminoglycan degradation, and Vibrio cholerae infection, while decreasing function of synthesis and degradation of ketone bodies (Fig. 3A). Compared to normal mucosa, the bacteria at the lesion had decreased linoleic acid metabolism (Fig. 3B), and the bacteria communities at the lesion had increased function of *Vibrio cholerae* infection, endocytosis, while reduced valine, leucine and isoleucine degradation and synthesis and degradation of ketone bodies was revealed (Fig. 3C). Distinct function profiles of COG (Fig. S7), KO (Fig. S8), and METACYC (Fig. S9) were also observed.

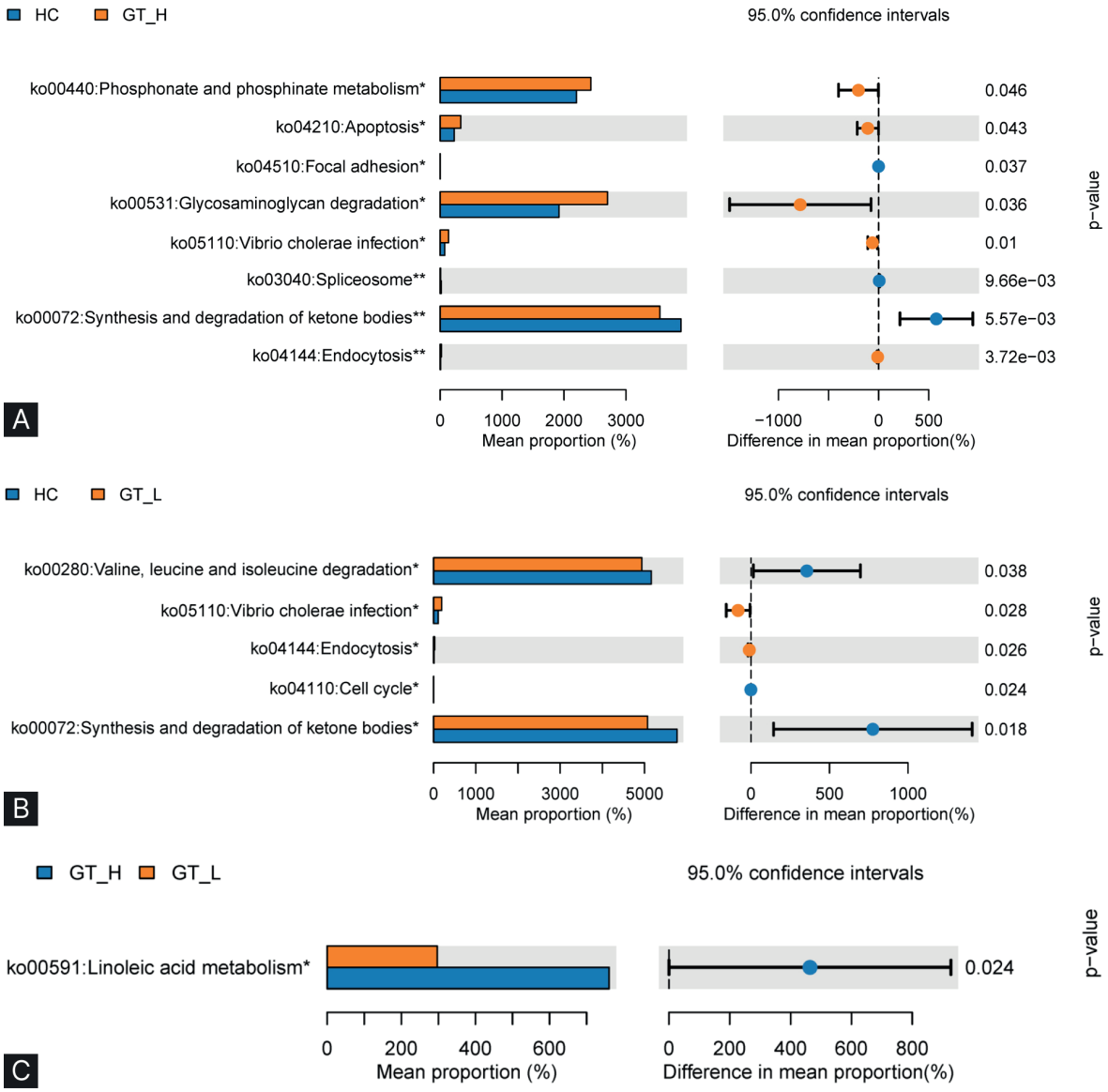


Fig. 3. The differentially enriched KEGG pathway predicted by PICRUSt2 was determined by STAMP. A: Differentially enriched KEGG pathway at lingual in heathy controls (HC) and geographic tongue patients (GT-H). B: Differentially enriched KEGG pathway at normal lingual (GT-H) or lesion (GT-L) sites in geographic tongue patients.

Discussion

We characterized the bacterial community of the GT lesion and normal mucosa in children, revealing an association between GT and dysbiosis of the lingual microbiota. These findings suggest that the oral microbiome may play a role in GT pathogenesis.

The normal tongue structure, characterized by papillae, provides a niche for the colonization of oral microbiota. One of the most striking features of GT is the loss of filiform papillae[22], potentially leading to the loss of bacterial niches and resulting in microbiota dysbiosis. A reciprocal cause-and-effect relationship between dysbiosis and inflammation is

possible[23], with inflammation fueling the selective growth of dysbiotic communities and dysbiosis exacerbating inflammation.[24-26]

The oral microbiome develops during early childhood and undergoes dynamic changes over time. Species richness increases, and the relative abundance of dominant bacteria changes with age[21], resulting in distinct microbiota patterns between adults and children.[27] Therefore, the microbiota of children may exhibit unique features under pathological conditions compared to adults.

In previous studies of adult GT patients, Shannon and Simpson diversity indices were found to be increased in the healthy mucosa of GT patients compared to GT lesions or healthy controls.[19] However, in our cohort, no significant difference in alpha diversity was observed, although the average values of observed species, Chao1, and ACE indices were decreased in the GT lesion and nearby healthy mucosa, while the Shannon and Simpson indices showed no such trend. This discrepancy may be attributed to the distinct characteristics of the children's microbiota. Based on PCoA or NMDS of beta diversity, a shift between healthy controls, healthy mucosa, and GT lesions was observed, indicating alterations in bacterial communities. Nevertheless, the high variability of tongue microbiota resulted in scattered subject distribution.

The abundance of *Catonella*, *Oribacterium*, *Bacillus*, *Prevotella scopos* and *Prevotella nanceiensis* was elevated in the normal mucosa of GT patients compared to healthy controls. Higher relative abundance of *Catonella* in subgingival dental plaque has been associated with periodontitis[28,29], and Catonella species have been implicated in endodontic infections and oral cancer.[30] *Oribacterium* has been found to be enriched in proliferative verrucous leukoplakia (PVL) patients and may serve as a biomarker for PVL.[31] Significantly higher abundances of *Oribacterium* were observed in oral rinse samples of oral cavity cancer (OCC) and oropharyngeal cancers.[32] *Bacillus* was strongly associated with chronic erosive gastritis (CEG) patients with typical yellow tongue coating (YTC).[33] Children of parents with periodontitis had over 6- and 4-fold increases in *Prevotella scopos*.[34] *Prevotella nanceiensis* was more abundant in children with Henoch-Schönlein purpura and positively correlated with the amount of IgA.[35] Thus, increased abundance of these taxa may be associated with inflammatory responses.

Additionally, we found *Prevotella oris* to be overrepresented in GT lesions. *Prevotella oris* has been detected at high abundance in bronchoalveolar lavage fluid samples from some cystic fibrosis individuals[36] and is considered a likely pathogen capable of causing lung infections.[37] However, its association with pathological status remains unclear.

It is noteworthy that the differentially abundant taxa differ from those reported in previous studies of adult GT patients, as the oral bacterial profiles in children are under development and influenced by diet and living habits. Upon reviewing the dietary habits of the study group, it was observed that a subset of children (3 out of 25 in the patient group and 2 out of 19 in the control group) had a history of consuming chili peppers. However, the specific quantities consumed were not documented. It was reported that chili peppers can induce changes in the gut microbiota[38], yet our understanding of their impact on oral microbiota remains limited. Unfortunately, the precise meal plans for the cohort were not accessible. Nonetheless, it was noted that rice and rice noodles were the primary dietary staples, accompanied by common dishes such as eggs, fried potatoes, tofu, dried bean curd, boiled fish, Kung Pao chicken, stir-fried lettuce stem slices, and Chinese sponge gourd. Further research is warranted to elucidate the intricate relationship between diet and oral microbiota. Functional prediction using PICRUSt2 indicated alterations in the overall metabolism of microbiota in GT patients and GT lesions, which could act as a mediator between the bacteria and inflammation.

Our study demonstrates an association between microbiota imbalance and GT in children. Several taxa showed differential abundance between normal mucosa in GT patients and healthy controls, as well as between GT lesions and normal mucosa in GT patients. Establishing the reciprocal relationship between microbiota dysbiosis and inflammation requires further investigation.

Supplementary materials

Supplementary materials for this article are available online at https://doi.org/10.24953/turkjpediatr.2024.4638

Ethical approval

This study was approved by the Medical Ethics Committee of Hunan Children's Hospital (No. KY-2021-57).

Author contribution

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

Source of funding

The project was supported by the Natural Science Foundation of Hunan Province China (Grant No. S2021JJKWLH0132).

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Pinto A, Haberland CM, Baker S. Pediatric soft tissue oral lesions. Dent Clin North Am 2014; 58: 437-453. https://doi.org/10.1016/j.cden.2013.12.003
2. Ugar-Cankal D, Denizci S, Hocaoglu T. Prevalence of tongue lesions among Turkish schoolchildren. Saudi Med J 2005; 26: 1962-1967.
3. Shulman JD. Prevalence of oral mucosal lesions in children and youths in the USA. Int J Paediatr Dent 2005; 15: 89-97. https://doi.org/10.1111/j.1365-263X.2005.00632.x
4. Jainkittivong A, Langlais RP. Geographic tongue: clinical characteristics of 188 cases. J Contemp Dent Pract 2005; 6: 123-135.
5. Dafar A, Çevik-Aras H, Robledo-Sierra J, Mattsson U, Jontell M. Factors associated with geographic tongue and fissured tongue. Acta Odontol Scand 2016; 74: 210-216. https://doi.org/10.3109/00016357.2015.1087046
6. Picciani B, Santos VC, Teixeira-Souza T, et al. Investigation of the clinical features of geographic tongue: unveiling its relationship with oral psoriasis. Int J Dermatol 2017; 56: 421-427. https://doi.org/10.1111/ijd.13460
7. Liang J, Huang P, Li H, et al. Mutations in IL36RN are associated with geographic tongue. Hum Genet 2017; 136: 241-252. https://doi.org/10.1007/s00439-016-1750-y
8. Darling MR, Su N, Masen S, et al. Geographic tongue: assessment of peripheral nerve status, Langerhans cell, and HLA-DR expression. Oral Surg Oral Med Oral Pathol Oral Radiol 2017; 124: 371-377.e1. https://doi.org/10.1016/j.oooo.2017.05.516
9. Wysocki GP, Daley TD. Benign migratory glossitis in patients with juvenile diabetes. Oral Surg Oral Med Oral Pathol 1987; 63: 68-70. https://doi.org/10.1016/0030-4220(87)90342-2
10. Garsjö V, Dafar A, Jontell M, Çevik-Aras H, Bratel J. Increased levels of calprotectin in the saliva of patients with geographic tongue. Oral Dis 2020; 26: 558-565. https://doi.org/10.1111/odi.13258
11. Dafar A, Bankvall M, Garsjö V, Jontell M, Çevik-Aras H. Salivary levels of interleukin-8 and growth factors are modulated in patients with geographic tongue. Oral Dis 2017; 23: 757-762. https://doi.org/10.1111/odi.12654
12. Kullaa-Mikkonen A, Kotilainen R. The prevalence of oral carriers of Candida in patients with tongue abnormalities. J Dent 1983; 11: 313-317. https://doi.org/10.1016/0300-5712(83)90115-x
13. Dudko A, Kurnatowska AJ, Kurnatowski P. Prevalence of fungi in cases of geographical and fissured tongue. Ann Parasitol 2013; 59: 113-117.
14. Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. Oral Dis 2017; 23: 276-286. https://doi.org/10.1111/odi.12509

15. Su SC, Chang LC, Huang HD, et al. Oral microbial dysbiosis and its performance in predicting oral cancer. Carcinogenesis 2021; 42: 127-135. https://doi.org/10.1093/carcin/bgaa062

16. Zhong X, Lu Q, Zhang Q, He Y, Wei W, Wang Y. Oral microbiota alteration associated with oral cancer and areca chewing. Oral Dis 2021; 27: 226-239. https://doi.org/10.1111/odi.13545

17. Hernandez BY, Zhu X, Goodman MT, et al. Betel nut chewing, oral premalignant lesions, and the oral microbiome. PLoS One 2017; 12: e0172196. https://doi.org/10.1371/journal.pone.0172196

18. Chen J, Liu K, Sun X, Shi X, Zhao G, Yang Z. Microbiome landscape of lesions and adjacent normal mucosal areas in oral lichen planus patient. Front Microbiol 2022; 13: 992065. https://doi.org/10.3389/fmicb.2022.992065

19. Dafar A, Bankvall M, Çevik-Aras H, Jontell M, Sjöberg F. Lingual microbiota profiles of patients with geographic tongue. J Oral Microbiol 2017; 9: 1355206. https://doi.org/10.1080/20002297.2017.1355206

20. Willis JR, Saus E, Iraola-Guzmán S, et al. Citizen-science reveals changes in the oral microbiome in Spain through age and lifestyle factors. NPJ Biofilms Microbiomes 2022; 8: 38. https://doi.org/10.1038/s41522-022-00279-y

21. Dzidic M, Collado MC, Abrahamsson T, et al. Oral microbiome development during childhood: an ecological succession influenced by postnatal factors and associated with tooth decay. ISME J 2018; 12: 2292-2306. https://doi.org/10.1038/s41396-018-0204-z

22. Assimakopoulos D, Patrikakos G, Fotika C, Elisaf M. Benign migratory glossitis or geographic tongue: an enigmatic oral lesion. Am J Med 2002; 113: 751-755. https://doi.org/10.1016/s0002-9343(02)01379-7

23. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. Nat Rev Microbiol 2018; 16: 745-759. https://doi.org/10.1038/s41579-018-0089-x

24. Diaz PI, Hoare A, Hong BY. Subgingival microbiome shifts and community dynamics in periodontal diseases. J Calif Dent Assoc 2016; 44: 421-435.

25. Herrero ER, Fernandes S, Verspecht T, et al. Dysbiotic biofilms deregulate the periodontal inflammatory response. J Dent Res 2018; 97: 547-555. https://doi.org/10.1177/0022034517752675

26. Nassar M, Tabib Y, Capucha T, et al. GAS6 is a key homeostatic immunological regulator of host-commensal interactions in the oral mucosa. Proc Natl Acad Sci U S A 2017; 114: E337-E346. https://doi.org/10.1073/pnas.1614926114

27. Burcham ZM, Garneau NL, Comstock SS, et al. Patterns of oral microbiota diversity in adults and children: a crowdsourced population study. Sci Rep 2020; 10: 2133. https://doi.org/10.1038/s41598-020-59016-0

28. Li Y, He J, He Z, et al. Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients. ISME J 2014; 8: 1879-1891. https://doi.org/10.1038/ismej.2014.28

29. Siqueira JF, Rôças IN. Catonella morbi and Granulicatella adiacens: new species in endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 102: 259-264. https://doi.org/10.1016/j.tripleo.2005.09.021

30. Yang J, He P, Zhou M, et al. Variations in oral microbiome and its predictive functions between tumorous and healthy individuals. J Med Microbiol 2022; 71: 10.1099/jmm.0.001568. https://doi.org/10.1099/jmm.0.001568

31. Herreros-Pomares A, Llorens C, Soriano B, et al. Oral microbiome in Proliferative Verrucous Leukoplakia exhibits loss of diversity and enrichment of pathogens. Oral Oncol 2021; 120: 105404. https://doi.org/10.1016/j.oraloncology.2021.105404

32. Lim Y, Fukuma N, Totsika M, Kenny L, Morrison M, Punyadeera C. The performance of an oral microbiome biomarker panel in predicting oral cavity and oropharyngeal cancers. Front Cell Infect Microbiol 2018; 8: 267. https://doi.org/10.3389/fcimb.2018.00267

33. Ye J, Cai X, Yang J, et al. Bacillus as a potential diagnostic marker for yellow tongue coating. Sci Rep 2016; 6: 32496. https://doi.org/10.1038/srep32496

34. Monteiro MF, Altabtbaei K, Kumar PS, et al. Parents with periodontitis impact the subgingival colonization of their offspring. Sci Rep 2021; 11: 1357. https://doi.org/10.1038/s41598-020-80372-4

35. Chen B, Wang J, Wang Y, et al. Oral microbiota dysbiosis and its association with Henoch-Schönlein Purpura in children. Int Immunopharmacol 2018; 65: 295-302. https://doi.org/10.1016/j.intimp.2018.10.017

36. O'Connor JB, Mottlowitz MM, Wagner BD, et al. Divergence of bacterial communities in the lower airways of CF patients in early childhood. PLoS One 2021; 16: e0257838. https://doi.org/10.1371/journal.pone.0257838

37. Fernández Vecilla D, Roche Matheus MP, Iglesias Hidalgo G, Ugalde Zárraga E, Unzaga Barañano MJ, Díaz de Tuesta Del Arco JL. Two cases of Prevotella oris causing serious pleuropulmonary infections. Rev Esp Quimioter 2023; 36: 439-441. https://doi.org/10.37201/req/001.2023

38. Xiang Q, Tang X, Cui S, et al. Capsaicin, the spicy ingredient of chili peppers: effects on gastrointestinal tract and composition of gut microbiota at various dosages. Foods 2022; 11: 686. https://doi.org/10.3390/foods11050686

The Turkish Journal of Pediatrics 2024; 66: 457-464
https://doi.org/10.24953/turkjpediatr.2024.5096

Original Article

Determination of thiol-disulphide homeostasis in premenstrual syndrome during adolescence

Laden Jaferi[1], Melis Pehlivantürk Kızılkan[2], Demet Aygün Arı[3], Özcan Erel[4], Sinem Akgül[2]

[1]Department of Pediatrics, Bayındır Hospital, Ankara; [2]Division of Adolescent Medicine, Department of Pediatrics, Hacettepe University, Ankara; [3]Department of Pediatrics, University of Health Sciences, Ankara Dr. Sami Ulus Children's Health and Diseases Training and Research, Hospital, Ankara; [4]Department of Biochemistry, Yıldırım Beyazıt University, Ankara City Hospital, Ankara, Türkiye.

ABSTRACT

Background. Premenstrual syndrome (PMS) characterized by cyclic symptoms during the luteal phase of the menstrual cycle, presents an uncertain etiology in adolescents involving hormonal fluctuations and serotonin-related neurotransmitters with a limited existing literature on the impact of oxidative stress. This study aimed to explore the potential association between PMS and oxidative stress in adolescents.

Methods. In a cross-sectional study conducted at a university hospital, involving 45 adolescent girls aged 12 to 18, participants were categorized based on the presence or absence of PMS using the cut-off point of 110 on the PMS Scale developed by Gençdoğan. Oxidative stress was assessed through dynamic thiol-disulfide homeostasis. The shift from the balance towards disulfide form is associated with oxidative stress, whereas towards thiol it shows a greater antioxidant capacity.

Results. Thirty out of the forty-five participants were found to have PMS with a mean age of 15.5 years. The PMS group demonstrated a significant increase in antioxidant markers, specifically elevated native (631.6±57.55 vs 598.2±41.08, p=0.048) and total thiol levels (675.15±3.4 vs 639.3±44.9, p=0.031). Despite a significant increase in thiol, thiol to disulfide ratio was not found to be significant (p=0.849).

Conclusion. Contradictory to other studies in adults, we have demonstrated an increase in the antioxidant markers in adolescents with PMS. Elevated antioxidant status in adolescents with PMS may be an adaptive response to acute cyclic inflammation in the adolescent period, which might decrease with the progression of age. Further research is needed to investigate the complex interaction between oxidative stress and PMS across different age groups.

Key words: premenstrual syndrome, thiol-disulphide homeostasis, adolescence, oxidative stress.

Premenstrual syndrome (PMS) is a complex and prevalent condition characterized by cyclic physical, behavioral, and psychological symptoms during the luteal phase of the menstrual cycle, typically resolving with menstruation onset. PMS induces various discomforts in the days preceding menstruation, including breast tenderness, headaches, joint pain, mood changes (irritability or mood swings), and behavioral alterations in sleep patterns or appetite.[1]

PMS etiology remains unknown; however, some studies suggest that the hormonal fluctuations or sensitivity to these changes during the menstrual cycle play a significant role in the manifestation of PMS symptoms. Symptoms have also been shown to be associated with serotonin and other related neurotransmitters. Studies have also demonstrated associations between PMS and lifestyle factors, including eating and drinking habits.[2] Additionally, there

✉ Laden Jaferi
drladenjafari@gmail.com

Received 16th Jul 2024, revised 22nd Aug 2024, accepted 24th Aug 2024

is growing evidence indicating the involvement of inflammatory processes in PMS.[3]

Inflammation is a vital immune response, but when it becomes dysregulated, it can contribute to the development and aggravation of various health issues. It is suggested that progesterone and estrogens exhibit anti-inflammatory effects, however, excessive doses may have a pro-oxidative impact and lead to oxidative stress. Although individuals with PMS have hormone levels similar to those of healthy women, they may be more sensitive to hormonal changes during the luteal phase. Additionally, estrogen conversion products, such as catechol estrogens, can generate oxygen radicals. Inflammatory molecules in PMS may trigger the release of pain-associated prostaglandins and influence neurotransmitters, potentially worsening premenstrual symptoms and contributing to mood disturbances.[4]

Thiols are organic compounds containing a sulfhydryl group (-SH) that can be oxidized to form disulfides. A disulfide is a chemical bond formed between two sulfur atoms of the sulfhydryl groups from two thiol molecules. This thiol-disulfide exchange is crucial in protein structure and function, acting as a switch to regulate protein activity and maintaining cellular redox homeostasis. The comparative analysis of thiol and disulfide levels is a biochemical indicator that can be utilized to assess the cellular oxidative stress status and overall antioxidant defense mechanisms. The abundance of thiols in cells may indicate the active nature of these defense mechanisms and their ability to provide protection against potential oxidative damage. If thiol levels are higher than disulfide levels, it generally indicates that cells are in a reduced state rather than experiencing oxidative stress. In other words, the redox balance in cells leans towards a more reduced (reductive) state, suggesting a high antioxidant capacity and a lower risk of oxidative damage. On the contrary, if disulfide levels are higher than thiols, this situation indicates that cells are exposed to oxidative stress and are in a more oxidized state. Oxidative stress can lead to damage in cells and tissues and may play a role in the development of chronic diseases such as PMS.[4,5]

The association between PMS and different parameters related to inflammation, oxidative stress, and antioxidative stress has previously been studied. Limited evidence indicates that women experiencing PMS may exhibit higher levels of inflammatory parameters, lower antioxidant status, and higher oxidation levels compared to those without PMS although results are inconsistent. These findings may suggest a potential link between oxidative stress, inflammation, and the development of PMS.[3,6] However, the majority of these studies have predominantly focused on adult women, there was high heterogeneity in the study designs, and the diagnosis of PMS was not always made with a validated method.[3]

Focusing on the adolescent age group can provide a unique framework for investigating the relationship between PMS and oxidation in this period of life.[7,8] Therefore, this study aimed to explore the potential association between PMS and oxidative stress in adolescent girls.

Materials and Methods

Study participants

The participants included in this cross-sectional study were healthy females with regular menstrual cycles, aged between 12-18 years, who presented to the adolescent medicine clinic at Hacettepe University, İhsan Doğramacı Children's Hospital between January 2022 and October 2022 for a well-child visit. Regular menstrual cycles were defined as lasting between 21 and 45 days, a range chosen based on established criteria in the literature for defining menstrual regularity among adolescent females.[9] As PMS is triggered by ovulation, we only included participants with regular menstrual cycles to ensure that they were ovulating. Adolescents with a history of physical or mental illnesses and/or using any medication were excluded from the study.

Written informed consent was acquired from the parents and written assent was obtained from all adolescents. Study approval (GO 21/67) was obtained from the Hacettepe University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee.

Heights of the adolescents were measured by a Harpenden stadiometer, and body weights were measured by a digital scale. Body mass index (BMI, kg/m^2) was then calculated by dividing the body weight (kg) with the square of the height (m). Age of menarche was noted.

Evaluation of PMS

The diagnosis of PMS was determined using the Premenstrual Syndrome Scale developed by Gençdoğan in 2006.[10] It is a 44-item, five-point (none, very little, sometimes, often, always) Likert-type questionnaire with nine subgroups. A score of "one point" was assigned to the "none" option, and a score of "five points" was assigned to the "always" option. The minimum and maximum total scores that could be obtained from each subscale were as follows: 7-35 for depressive mood, 7-35 for anxiety, 6-30 for fatigue, 5-25 for irritability, 7-35 for depressive thoughts, 3-15 for pain, 3-15 for appetite changes, 3-15 for sleep changes, and 3-15 for bloating. The lowest possible score that could be obtained from the entire scale is 44, and the highest score is 220. An increase in scores indicates the intensity of PMS. The presence of PMS is assessed based on whether the total and subscale scores exceed 50% of the maximum possible score. The reliability of the scale, assessed by Cronbach's Alpha, is 0.75. Participants who scored 110 or higher on the scale were assigned to the PMS group, while those scoring lower than 110 were assigned to the control group.

Assessment of oxidative stress parameters

In this study, blood samples were collected for the determination of thiol and disulfide values and prepared for laboratory analyses. We utilized thiol and disulfide levels as biomarkers to estimate oxidative stress in this study. To assess thiol-disulfide hemostasis parameters, a blood sample was collected from subjects into plain tubes following an eight-hour fasting period. Following rapid centrifugation of blood samples at a speed of 1500 rpm for a duration of 10 minutes, the plasma and serum components were separated. The serum samples were subsequently stored at a temperature of -80 °C. Blood samples were collectively sent to the Medical Biochemistry Laboratory of Ankara Bilkent City Hospital's for the analysis of oxidative stress parameters. The whole research cohort was examined collectively during a single session. The study investigated the balance of serum thiol-disulfide levels using a completely automated analytical technique established by Erel and Neselioglu.[11] The dynamic disulfide level was calculated by dividing the difference between total thiol and native thiol into two. Following the process of dynamic disulfide, we measured the levels of native and total thiol. We then computed the ratios of disulfide to native thiol, disulfide to total thiol, and native thiol to total thiol. Measurements were analyzed using the spectrophotometric method developed by Erel. According to the Erel method[11], the levels of plasma disulfide were measured to be 17.29±5.32 μmol/L, native thiol levels were 397±62 μmol/L, and the ratio of disulfide to native thiol percentage was 4.32±1.49 in healthy subjects. Blood was not drawn at a particular phase of the menstrual cycle.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 21. The distribution of data was assessed using the Kolmogorov-Smirnov test. For the comparison of independent variables showing a normal distribution, independent t- test was used, while the Mann-Whitney U test was employed for data that did not exhibit a normal distribution. Qualitative data were evaluated using the chi-square test and Fisher's exact test. Pearson or Spearman correlation tests were conducted as applicable. A p value <0.05 was accepted as statistically significant.

Results

A total of 53 patients were considered eligible for this study. However, three patients were excluded after being diagnosed with other illnesses during the study, three others declined to provide blood samples, and two patients were excluded due to incomplete PMS scale responses. Our research examined a cohort of 45 adolescent girls with a mean age of 15.4±1.4 years. While 30 adolescents (66.6%) reported a PMS score over 110 with a mean score of 150.6±27.5, 15 (33.4%) reported a PMS score lower than 110 with a mean score of 87.2±21.8 (P<0.001). The clinical parameters of the PMS (+) and PMS (-) groups are presented in Table I, and a comparative analysis of the anthropometric and clinical data between the two groups did not yield any statistically significant differences.

The comparison of native thiol, total thiol and disulfide values, along with the comparison of these values are presented in Table II. The results indicated a significant elevation in native (p=0.048) and total thiol (p=0.031) levels in the PMS group. We did not find a significant difference in disulfide levels between the two groups.

The Pearson correlation analysis was utilized because the variables demonstrated a normal distribution. The analysis revealed no strong linear correlations, whether positive or negative, between PMS scores and thiol, disulfide, or thiol-disulfide ratios. The associations observed between PMS scores and these biochemical parameters were generally weak. Although low positive correlations were identified between PMS scores and native thiol, total thiol, and various disulfide ratios, none of these reached statistical significance (p > 0.05). Notably, the correlation between disulfide and PMS score had a relatively higher coefficient (r = 0.262) compared to other variables, but this too did not achieve statistical significance (p = 0.093). Overall, the findings do not indicate a strong relationship between PMS scores and the biochemical measurements evaluated. The correlation analysis results are presented in Table III.

Table I. The clinical parameters of the adolescents with and without PMS.

Clinical variables	PMS(+) (n=30)	PMS(-) (n=15)	P value
Age, mean±SD (years)	15.5±1.3	15.3±1.5	0.544
Body weight, mean±SD (kg)	61.7±11.3	67.7±20.7.5	0.232
Height, mean±SD (cm)	160.2±4.6	162.6±6.3	0.168
Body mass index, mean±SD (kg/m^2)	24.1±4.6	25.3±6.5	0.476
Menarchial age, mean±SD (years)	11.8±1.3	11.5±1.2	0.594
PMS score, mean±SD	150.6±27.5	87.2±21.8	<0.001

PMS: premenstrual syndrome

Table II. Comparison of oxidative stress in adolescents with and without PMS.

Laboratory variables	PMS (+) (n=30)	PMS (-) (n=15)	P value
Native thiol (μmol/L)	631.6±57.55	598.2±41.08	**0.048**
Total thiol (μmol/L)	675.1±53.4	639.3±44.9	**0.031**
Disulfide (μmol/L)	21.7±5.5	20.5±5.7	0.503
Disulfide / native thiol	3.5±1.0	3.4±0.9	0.839
Disulfide / total thiol	3.3±0.9	3.2±0.8	0.847
Native thiol / total thiol	93.5±1.7	93.6±1.6	0.849

PMS: premenstrual syndrome

Table III. Thiol-disulfide levels and PMS correlation.

Variables	Correlation Coefficient (r)	P value
PMS score vs. native thiol	0.155	0.326
PMS score vs. total thiol	0.213	0.176
PMS score vs. disulfide	0.262	0.093
PMS score vs. (disulfide / native thiol) * 100	0.199	0.207
PMS score vs. (disulfide / total thiol) * 100	0.197	0.210
PMS score vs. (native thiol / total thiol) * 100	-0.197	0.210

PMS: premenstrual syndrome. Pearson correlation analysis was used to evaluate the relationships between PMS scores and biochemical parameters.

Discussion

In our study, we demonstrated that the PMS group displayed a statistically significant increase in both native and total thiol levels. The difference was not statistically significant for disulfide levels. These results suggest that adolescents with PMS have higher antioxidant status compared to the control group.

Oxidation and inflammation are closely interrelated biological processes. Oxidative stress is a condition where there is an imbalance between oxidants such as free radicals and reactive oxygen species and the body's antioxidant defense system. These reactive species can cause oxidation of lipids, proteins, and DNA, leading to cellular damage and inflammation. Numerous studies have been conducted regarding the relationship between various gynecological issues and oxidative stress.[12] Studies focusing on the relationship between PMS and oxidative stress, utilized a broad range of different biomarkers directly indicating inflammation or oxidative stress.[3]

Among inflammatory markers, interleukins, tumor necrosis factor α, interferon γ, high-sensitivity C-reactive protein, granulocyte-macrophage colony-stimulating factor, and anti-heat shock protein 27 have been evaluated. While some studies have reported statistically significant results regarding increased inflamation, there are also other studies that have not found a significant association.[13,14] Oxidative stress markers, including total oxidative stress indicators, lipid peroxidation levels, protein oxidation products, and other related parameters, have been studied in relation to PMS. In the research conducted by Incebiyik et al., total oxidative stress was assessed using total oxidant status and the oxidative stress index. However, no statistically significant differences were observed between the PMS group and the control group.[15] Non-enzymatic antioxidant parameters and total antioxidant capacity (TAC) have been investigated between PMS and control groups. In one study, a statistically significantly lower TAC was found in women with PMS compared to the control group, but there are also studies that did not find such a difference.[16,17] These findings are inconsistent and do not directly indicate a higher inflammatory and oxidant status in PMS cases, suggesting that the relationship between PMS and oxidative stress related inflammation is not firmly established.[3]

Thiols and disulfides are used as antioxidant markers in many various studies, and to the best of our knowledge, there is just one study that evaluated these markers in PMS, and it included adult women.[16] In this study, oxidant status was evaluated using lipid hydroperoxide (LHP), malondialdehyde (MDA), and protein carbonyl (PC), while antioxidant status was evaluated using total thiol and TAC. Biomarkers were examined on the follicular (3rd day) and luteal (21st day) phases of the menstrual cycle, and no statistically significant differences were found between the study and control groups during the follicular phase. During the luteal phase, although no significant differences were observed between the groups in terms of MDA, PC, and total thiol levels, it was noted that LHP

levels increased in the study group compared to the control group, and TAC levels decreased.[16] In contrast to this study, we have demonstrated a significant elevation of antioxidant levels in the PMS group.

Our findings lack alignment with the limited information in the literature showing either an increase or no change in oxidant levels and a decrease or no change in antioxidant status, which can be explained in several ways. Population differences should be considered in the obtained results. Adolescence, characterized by distinct and unique features from adulthood, accompanies significant biological, psychological, and social changes, indicating that the etiological causes of PMS might differ during the adolescent period.[18-20] While prolonged and severe stress conditions often lead to a decrease in thiol levels, in certain cases, cells may elevate thiol levels to cope with this stress, possibly by activating defense mechanisms or regulating signals.[3,18] During adolescence, the rise in antioxidant levels might suggest the presence of acute onset inflammation during the premenstrual phase, resulting in a reactive and activated response of the antioxidant system rather than chronic, prolonged stress observed in adults. Similarly, the oxidation and antioxidation processes change with aging. As individuals age, the antioxidant defense mechanisms in the body generally weaken, and coping with damage caused by free radicals may become less effective. Additionally, as metabolism slows down with age, the repair processes of cells can change, making the body more vulnerable to oxidative damage. Enzymes functioning as antioxidants may decrease in production or become less effective.[19,21] All these factors can explain the age-related oxidative stress parameter differences between adolescents and adults with PMS.

Our study has several limitations. The small sample size and blood samples obtained irrespective of the menstrual cycle phase are the major limitations. Considering the complex hormonal fluctuations in the menstrual cycle, it can be assumed that oxidation levels may vary during different menstrual phases, particularly the premenstrual period. Another limitation is the lack of oxidant status measurements. Future studies examining both oxidant and antioxidant biomarkers at follicular and luteal phases of the cycle might provide additional data. Another limitation is the lack of nutritional habits, exercise levels, and healthy lifestyle preferences of adolescents, which may have both affected the PMS symptomatology and oxidative stress status.[22,23] Lifestyle factors such as a balanced and healthy diet, regular exercise, limiting tobacco and alcohol consumption, and stress management can support the body's capacity to cope with oxidative stress and help reduce oxidative damage.[24] Factors such as family support, education, and cultural influences may also play important roles in shaping adolescents' experiences with PMS.

In conclusion, there is uncertainty regarding the role of increased oxidative stress in PMS, and data specific to the adolescent period is very limited. Our study demonstrating elevated antioxidant status in adolescents with PMS might occur in response to acute cyclic inflammation during the premenstrual phase and act as an adaptive mechanism in reaction to increased oxidative stress. As age progresses the antioxidant capacity might decrease resulting in some studies demonstrating heightened oxidative stress and decreased antioxidant status in adults with PMS. Future research with more robust methodologies, including longitudinal studies with larger and more diverse age populations, is needed to better understand the complex interaction between oxidative stress and PMS.

Ethical approval

The study was approved by Hacettepe University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee (date: 19.01.2021, number: 2021/02-47).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SA, DA; data collection: DA, LJ, ÖE; analysis and interpretation of results: MPK, LJ, ÖE; draft manuscript preparation: LJ. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Takeda T. Premenstrual disorders: Premenstrual syndrome and premenstrual dysphoric disorder. J Obstet Gynaecol Res 2023; 49: 510-518. https://doi.org/10.1111/jog.15484
2. Siminiuc R, Ţurcanu D. Impact of nutritional diet therapy on premenstrual syndrome. Front Nutr 2023; 10: 1079417. https://doi.org/10.3389/fnut.2023.1079417
3. Granda D, Szmidt MK, Kaluza J. Is Premenstrual syndrome associated with inflammation, oxidative stress and antioxidant status? A systematic review of case-control and cross-sectional studies. Antioxidants (Basel) 2021; 10: 604. https://doi.org/10.3390/antiox10040604
4. Dandekar A, Mendez R, Zhang K. Cross talk between ER stress, oxidative stress, and inflammation in health and disease. Methods Mol Biol 2015; 1292: 205-214. https://doi.org/10.1007/978-1-4939-2522-3_15
5. Erel Ö, Erdoğan S. Thiol-disulfide homeostasis: an integrated approach with biochemical and clinical aspects. Turk J Med Sci 2020; 50: 1728-1738. https://doi.org/10.3906/sag-2003-64
6. Sultana A, Rahman K, Heyat MBB, Sumbul, Akhtar F, Muaad AY. Role of inflammation, oxidative stress, and mitochondrial changes in premenstrual psychosomatic behavioral symptoms with anti-inflammatory, antioxidant herbs, and nutritional supplements. Oxid Med Cell Longev 2022; 2022: 3599246. https://doi.org/10.1155/2022/3599246
7. Bahrami A, Bahrami-Taghanaki H, Khorasanchi Z, et al. Menstrual problems in adolescence: relationship to serum vitamins A and E, and systemic inflammation. Arch Gynecol Obstet 2020; 301: 189-197. https://doi.org/10.1007/s00404-019-05343-1
8. Bahrami A, Gonoodi K, Khayyatzadeh SS, et al. The association of trace elements with premenstrual syndrome, dysmenorrhea and irritable bowel syndrome in adolescents. Eur J Obstet Gynecol Reprod Biol 2019; 233: 114-119. https://doi.org/10.1016/j.ejogrb.2018.12.017
9. Itriyeva K. The normal menstrual cycle. Curr Probl Pediatr Adolesc Health Care 2022; 52: 101183. https://doi.org/10.1016/j.cppeds.2022.101183
10. Gençdoğan B. Premenstruel sendrom için yeni bir ölçek. Türkiye'de Psikiyatri Derg 2006; 8: 81-87.
11. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin Biochem 2014; 47: 326-332. https://doi.org/10.1016/j.clinbiochem.2014.09.026
12. Drejza MA, Rylewicz K, Majcherek E, et al. Markers of oxidative stress in obstetrics and gynaecology-a systematic literature review. Antioxidants (Basel) 2022; 11: 1477. https://doi.org/10.3390/antiox11081477
13. Foster R, Vaisberg M, Bachi ALL, et al. Premenstrual syndrome, inflammatory status, and mood states in soccer players. Neuroimmunomodulation 2019; 26: 1-6. https://doi.org/10.1159/000494559
14. Bertone-Johnson ER, Ronnenberg AG, Houghton SC, et al. Association of inflammation markers with menstrual symptom severity and premenstrual syndrome in young women. Hum Reprod 2014; 29: 1987-1994. https://doi.org/10.1093/humrep/deu170
15. Incebiyik A, Camuzcuoglu A, Hilali NG, et al. Serum oxidative stress, visfatin and apelin in healthy women and those with premenstrual syndrome. J Obstet Gynaecol 2015; 35: 188-192. https://doi.org/10.3109/01443615.2014.948399
16. Duvan CI, Cumaoglu A, Turhan NO, Karasu C, Kafali H. Oxidant/antioxidant status in premenstrual syndrome. Arch Gynecol Obstet 2011; 283: 299-304. https://doi.org/10.1007/s00404-009-1347-y
17. Fathizadeh S, Amani R, Haghighizadeh MH, Hormozi R. Comparison of serum zinc concentrations and body antioxidant status between young women with premenstrual syndrome and normal controls: a case-control study. Int J Reprod Biomed 2016; 14: 699-704.
18. Ertürk C, Altay MA, Büyükdoğan H, Çalışkan G, Erel Ö. Thiol/disulfide homeostasis as a novel indicator of oxidative stress during the treatment process of patients with septic arthritis. Jt Dis Relat Surg 2020; 31: 502-508. https://doi.org/10.5606/ehc.2020.71982

19. Boss GR, Seegmiller JE. Age-related physiological changes and their clinical significance. West J Med 1981; 135: 434-440.

20. Michaud PA, Fombonne E. Common mental health problems. BMJ 2005; 330: 835-838. https://doi.org/10.1136/bmj.330.7495.835

21. Eckert MA, Harris KC, Lang H, et al. Translational and interdisciplinary insights into presbyacusis: a multidimensional disease. Hear Res 2021; 402: 108109. https://doi.org/10.1016/j.heares.2020.108109

22. Aleksandrova K, Koelman L, Rodrigues CE. Dietary patterns and biomarkers of oxidative stress and inflammation: a systematic review of observational and intervention studies. Redox Biol 2021; 42: 101869. https://doi.org/10.1016/j.redox.2021.101869

23. Powers SK, Deminice R, Ozdemir M, Yoshihara T, Bomkamp MP, Hyatt H. Exercise-induced oxidative stress: Friend or foe? J Sport Health Sci 2020; 9: 415-425. https://doi.org/10.1016/j.jshs.2020.04.001

24. Sharma V, Mehdi MM. Oxidative stress, inflammation and hormesis: the role of dietary and lifestyle modifications on aging. Neurochem Int 2023; 164: 105490. https://doi.org/10.1016/j.neuint.2023.105490

The Turkish Journal of Pediatrics 2024; 66: 465-472
https://doi.org/10.24953/turkjpediatr.2024.4580

Original Article

Cost-saving approach with screening of selected variants in genetic diagnosis in Turkish pediatric familial Mediterranean fever patients: a single center longitudinal study

Betül Sözeri[1], Şengül Çağlayan[1], Taner Coşkuner[1], Yasemin Kendir-Demirkol[2]

[1]*Department of Pediatric Rheumatology, University of Health Sciences, Umraniye Training and Research Hospital, İstanbul;*
[2]*Department of Pediatric Genetics, University of Health Sciences, Umraniye Training and Research Hospital, İstanbul, Türkiye.*

ABSTRACT

Background. The aim of this study was to investigate whether a short exon screening consisting of selected variants could confirm the diagnosis in patients with a preliminary diagnosis of familial Mediterranean fever (FMF), thus providing a cost-saving alternative to a comprehensive MEditerranean FeVer (*MEFV*) gene sequence analysis test.

Methods. This observational study on pediatric patients focused on clinically suspected FMF cases without prior genetic analysis. Participants met the Turkish pediatric FMF criteria. They underwent short exon screening for M694V, M680I, V726A, and E148Q variants. Those who were heterozygous or negative on short exon screening received further *MEFV* gene sequence analysis.

Results. The study involved 1557 patients. Pathogenic variants in both alleles of the *MEFV* gene were found in 611 patients (39.2%), and a high-penetrance variant in heterozygosity or an E148Q variant on the other allele was found in 643 patients (41.3%). A further 189 patients (12.1%) had one or two E148Q variants. Short-exon screening was negative in 114 patients (7.6%). Of the 876 patients who underwent *MEFV* gene sequence analysis, additional variants were found in 72 of the 762 initially heterozygous patients. Of the 114 initially negative patients, 34 had homozygous or compound heterozygous variants, and 74 had heterozygous variants. Ultimately, only 6 patients yielded negative results in the *MEFV* gene sequence analysis.

Conclusion. The short exon screening for common *MEFV* mutations offers a practical and cost-saving alternative to comprehensive *MEFV* gene sequence analysis in populations with a high prevalence of FMF.

Key words: Familial Mediterranean fever, genetics, diagnosis, cost-saving.

The most common autoinflammatory disease in the world is familial Mediterranean fever (FMF), which is particularly prevalent in populations originating from the Eastern Mediterranean region.[1] The prevalence of FMF has been reported to be 1:1000 worldwide, displaying significant regional variations, and Türkiye is most likely the nation with the highest prevalence.[2,3]

✉ Betül Sözeri
drbetulsozeri@gmail.com

Received 31st Mar 2024, revised 25th Jun 2024, 20th Aug 2024, accepted 26th Aug 2024.

Recurrent episodes of fever, sterile peritonitis, arthritis, pleuritis, and erysipelas-like erythema (ELE) are its defining features. The disease can present with many different clinical phenotypes and the diagnosis is primarily based on clinical symptoms.[4] The Tel Hashomer criteria was developed as the first diagnostic criteria for the adult population.[5] Later, Livneh et al.[6] developed a criteria set, and this criterion was found to have low specificity for pediatric cases. For this reason, new FMF criteria were formulated for the pediatric group in 2009, known as the Turkish FMF Pediatric criteria.[4] Although the validity of these criteria remains

limited for other ethnic groups, they are highly relevant among the Turkish population, which is considered an endemic community. In Turkish children, these criteria have demonstrated a sensitivity and specificity of 93.4% and 84.1%, respectively.[7]

FMF is known to be inherited in an autosomal recessive manner, but a substantial number of heterozygous individuals who express phenotypic characteristics are present.[8] It results from gain-of-function mutations located on the MEditerranean FeVer (*MEFV*) gene. The *MEFV* gene, comprising 10 exons, is located on chromosome 16 (16p13.3) and encodes a 781 amino acid protein called pyrin.[9,10] Multiple sequence variants in the *MEFV* gene have been associated with FMF, with 397 sequence variants identified in the *MEFV* gene according to the INFEVERS database (http://fmf.igh.cnrs.fr/infevers/) since its initial definition in 1997.[9] These variants based on current evidence are categorized according to their potential association with the disease phenotype as benign, likely benign, likely pathogenic, or pathogenic.[11] Additionally, there exist a considerable number of variants whose clinical associations remain unclear, and these are termed variants of uncertain significance (VUS).[11,12]

Identifying the correct patient solely based on diagnostic criteria may not always be possible. A study conducted by Ben-Chetrit E. and colleagues[13], which involved the analysis of 446 patients for *MEFV* mutations, found that only 43% of patients referred by a general practitioner were genetically confirmed, while 76.4% of patients referred by specialists received genetic confirmation. However, even in countries with a high prevalence of FMF, the diagnosis can still be missed, delayed, or misdiagnosed. In addition, some patients may present with atypical symptoms. In these groups of patients or in those who may carry pathogenic or sequence variants but remain asymptomatic, genetic testing is crucial for accurate guidance on prophylaxis and treatment.

Predominantly, mutations in exon 10 of the *MEFV* gene, including M694V, M680I, V726A, and M694I, have the highest allele frequencies among different ethnic groups and are considered pathogenic variants.[14] However, the classification of the E148Q variant, which is frequently observed in certain populations, as a disease-causing mutation remains controversial.[15]

The aim of this study was to investigate whether a short exon screening consisting of selected variants could confirm the diagnosis in patients with a preliminary diagnosis of FMF based on clinical findings, thus providing a cost-saving alternative to a comprehensive *MEFV* gene sequence analysis test.

Patients and methods

This is a longitudinal observational study conducted on pediatric patients with clinically suspected FMF at the University of Health Sciences, Umraniye Training and Research Hospital, Department of Pediatric Rheumatology, Türkiye, from June 2019 to October 2023. A total of 1557 patients with clinically suspected FMF who had not yet undergone genetic analysis were included. All participants who were referred to our tertiary care pediatric rheumatology center were of Turkish descent and met the criteria outlined in the Turkish pediatric FMF criteria.[4] We excluded patients with any other associated autoinflammatory diseases and rheumatic disease. A comprehensive approach was taken, including detailed medical histories, physical examinations, and analysis of laboratory tests performed during both symptomatic and asymptomatic periods. Our team then implemented a short exon screening protocol created by us, targeting the M694V, M680I, V726A, and E148Q variants. All patients included in the study underwent initial screening with the short exon test. Patients identified as heterozygous or negative based on the screening were subjected to a *MEFV* gene sequence analysis.

During the course of the study, it was determined that the *MEFV* gene sequence analysis cost was 4.2 times higher than that of the short exon screening kit.

The study protocol was reviewed and approved by the Ethics Committee of University of Health Sciences, Umraniye Training and Research Hospital (Approval No:B.10.1.TKH.4.34.H.GP.0.01/46, Approval Date: 20/03/2019) in line with the ethical principles stated in the Declaration of Helsinki. Written informed consent was obtained from legal guardians.

Statistical analysis

The statistical analyses were conducted using SPSS version 25.0. The variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov - Smirnov test) to determine whether or not they were normally distributed. In the descriptive analysis, normally distributed variables were presented as mean, +/- standard deviation (SD), and non-normally distributed variables were presented as median and interquartile range (Q1-Q3). Categorical variables were compared with the chi square test. The Mann–Whitney U test was used to compare the non-normally distributed variables between two independent groups. One-way ANOVA was used to compare the parameters between the groups. An overall p-value of less than 0.05 was considered to show a statistically significant difference.

Results

In this study, a total of 1557 patients were involved. Among these patients, 793 (50.9%) were female, and 764 (49.1%) were male. The median age of the patients at diagnosis was 6 years (interquartile range, IQR: 4-9). The median age at onset of symptoms was 4 years (IQR: 2-7.5). The median number of annual attacks experienced by the patients was 12 (IQR: 6–24), and the mean duration of the episode was 3 ± 1.7 days. There was consanguinity in 331 patients (21.2%).

The most common symptom observed in the study was abdominal pain, in 1400 patients (89.9%). After abdominal pain, the most common symptoms in the study were fever (88%), and musculoskeletal involvement, in the form of arthralgia (59%), myalgia (52.7%), and arthritis (23%). Additionally, amyloidosis was reported in three patients. The demographic characteristics of the patients, clinical manifestation and characteristics of attack episodes are presented in Table I.

In the short exon screening analysis, we identified two pathogenic variants in both alleles of the *MEFV* gene in 611 patients (39.2%). Meanwhile, 643 patients (41.3%)

Table I. Demographic characteristics, clinical manifestations, and characteristics of attack episodes in patients with familial Mediterranean fever (N=1557).

Gender (F / M)	793 / 764
Age at diagnosis (years), median (IQR)	6 (4-9)
Age at onset of symptoms (years), median (IQR)	4 (2-7.5)
Number of annual attacks, median (IQR)	12 (6-24)
Duration of the episode (days), mean ± SD	3 ± 1.7
Clinical characteristics	
Abdominal pain, n (%)	1400 (89.9%)
Fever, n (%)	1370 (88%)
Arthralgia, n (%)	932 (59.9%)
Myalgia, n (%)	821(52.7%)
Arthritis, n (%)	358 (23%)
Exertional leg pain, n (%)	402 (25.8%)
Chest pain, n (%)	285 (18.3%)
Diarrhea, n (%)	268 (17.2%)
Vomiting, n (%)	226 (14.5%)
Headache, n (%)	197 (12.7%)
Erysipelas-like erythema, n (%)	175 (11.2%)
Constipation, n (%)	94 (6%)
Protracted febrile myalgia	15 (1%)
Orchitis, n (%)	7 (0.4%)
Amyloidosis, n (%)	3 (0.2%)

IQR, interquartile range; SD, standard deviation.

possessed either one high-penetrance variant in heterozygosity or E148Q variant in the other allele. Furthermore, 189 patients (12.1%) had either one or two E148Q variants, as detailed in Table II. A total of 114 patients (7.6%) yielded negative results in the genetic tests.

MEFV gene sequence analysis was conducted in a total of 876 patients (56.2%) who either showed no mutation or had heterozygous variants as identified in the short-exon screening. Out of a total of 762 patients who initially presented with heterozygous variants, 72 were found to have additional variants in the other allele. In the remaining 690 patients, no mutations were observed in the second allele. The most frequently detected variants in the second allele were R761H and P369S variants. Detailed results of the variant analysis for these patients are presented in Table III. In the group of 114 patients where no mutation was initially detected in the short-exon screening, 34 patients were subsequently found to have either homozygous or compound heterozygous variants. Furthermore, heterozygous variants were detected in 74 patients. As a result of this analysis, the *MEFV* gene sequence analysis yielded a negative result in the case of 6 patients. The distribution of patients according to short exon screening and *MEFV* gene sequence analysis results is shown in Fig. 1. The genotype results of patients whom no mutation was detected in the short-exon screening are reported in Table IV.

In terms of allele frequencies, M694V was the most common variant (42.8%), followed by M680I (9.1%), E148Q (8.3%), V726A (7.9%), R761H (2.4%), and P369S (1.4%).

Table II. Most Common mutations identified with short exon screening analysis.

Mutations	n (%)
M694V / M694V	340 (21.8)
M680I / M680I	29 (1.9)
V726A / V726A	13 (0.8)
E148Q / E148Q	13 (0.8)
M694V / M680I	111 (7.1)
M694V / V726A	90 (5.8)
M680I / V726A	28 (1.8)
M694V / E148Q	44 (2.8)
M680I / E148Q	7 (0.4)
V726A / E148Q	6 (0.4)
M694V / -	409 (26.3)
M680I / -	80 (5.1)
V726A / -	97 (6.2)
E148Q / -	176 (11.2)
No mutation	114 (7.4)

Table III. Variants detected in second allele from MEFV gene analysis in patients with heterozygous variants identified by short-exon screening.

Mutations	n (%)
R761H	32 (4.1)
P369S	14 (1.8)
H478Y	1 (0.1)
A744S	3 (0.4)
M694I	6 (0.8)
I591T	5 (0.6)
F479L	1 (0.1)
R653H	1 (0.1)
K695R	2 (0.2)
L110P	5 (0.6)
T267I	1 (0.1)
R408Q	1 (0.1)

Discussion

This study makes a significant contribution to the field of diagnosis, especially in a population like Türkiye, where FMF is common. We aimed to provide an innovative approach that reduces cost without disrupting diagnosis, by implementing a short exon screening protocol focusing on common *MEFV* gene mutations. Our findings provide favorable insight into the practicality and effectiveness of this short exon screening method in the real world. This longitudinal study was designed to be limited to a single center, primarily due to the challenges of conducting affordable genetic evaluation in populations where FMF is commonly suspected.

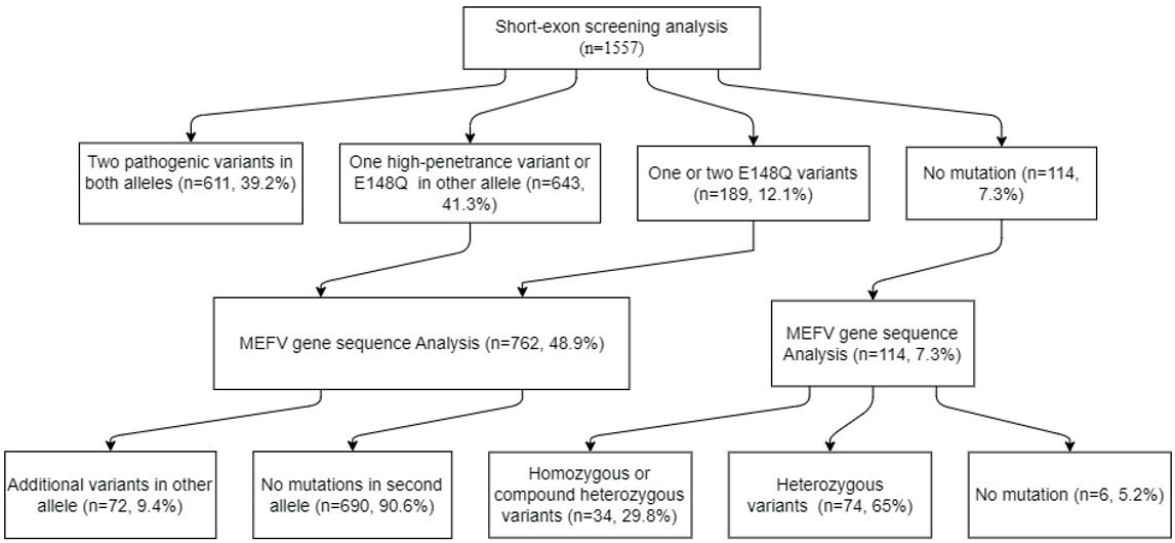


Fig. 1. Flowchart of *MEFV* gene analysis: Short exon screening and sequence analysis results.
MEFV, MEditerranean FeVer

In Türkiye, the diagnosis of FMF is often considered in pediatric patients with recurrent fever, leading to an increase in the frequency of genetic testing. However, the high costs associated with genetic testing place a significant economic burden on the country. Since FMF is an autosomal recessive disease, its definitive genetic diagnosis is made by identifying two pathogenic variants in the *MEFV* gene. However, the presence of at least one pathogenic variant is regarded as corroborative for accurately classifying FMF patients exhibiting a consistent phenotypic presentation.[16,17]

Table IV. The genotype results of patients with no mutation detected in short exon screening.

Mutations	n (%)
R761H / -	27 (23.7)
P369S / R408Q	18 (15.8)
A744S / -	15 (13.1)
P369S / -	8 (7)
K695R / -	7 (6.1)
R761H / R761H	7 (6.1)
I591T / -	5 (4.4)
F469L / -	3 (2.6)
T267I / -	3 (2.6)
E230K / -	3 (2.6)
P369S / P369S	2 (1.7)
K695R / R761H	2 (1.7)
R653H / A289E	1 (0.8)
Y471X / -	1 (0.8)
R354W / -	1 (0.8)
F479L / F479L	1 (0.8)
I641F / I641F	1 (0.8)
R761H / M694I	1 (0.8)
F479L / M694I	1 (0.8)
V487M / -	1 (0.8)
No mutation	6 (5.2)

Most pathogenic or likely pathogenic variants in FMF are located on exon 10. In regions where FMF is endemic, the M694V variant is the most common. Other common exon 10 variants, such as M694I, V726A and M680I, account for approximately 75% of all FMF cases.[18,19] In the present study, the frequency of these variants was found to be 80%, consistent with the literature.

Despite the classification of the E148Q variant as non-pathogenic, it has been included in the short exon screening kit. This decision was based on its notable prevalence in the Turkish population and in some other populations, as well as its documented association with clinical findings in several studies.[15,20,21]

Familial Mediterranean fever is typically inherited in an autosomal recessive pattern,

yet approximately 30% of patients present with a monoallelic disease harboring a single pathogenic variant.[17] In our cohort, a single variant was identified in 762 patients (48.9%). Upon conducting a comprehensive gene analysis in these patients, the frequency of identifying an additional variant was found to be 9.4%. The low incidence of detecting a second variant indicates that, in endemic populations, short-exon screening may yield sufficiently accurate results.

In the study by Kilim et al.[22], a short screening kit targeting five main mutations (E148Q, M680I, M694I, M694V and V726A) was used in a cohort of 1637 patients suspected of having FMF. The results showed that 812 patients (49.6%) had no detectable mutations, 581 patients (35.5%) had one mutation, 241 patients (14.7%) had two mutations (including 122 homozygous and 119 compound heterozygous), and 3 patients (0.2%) had three mutations. Subsequently, genetic testing was conducted on the patients, including R761H, A744S, K695R, M680I(c/t), and P369S, and additional mutations were identified in 68 patients (%4.3).

Moradian et al.[23] performed a study on 1299 FMF patients, employing a short screening kit for 12 *MEFV* mutations (E148Q, P369S, F479L, M680I (G/C), M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S, and R761H). A subset comprising 23 heterozygous, 20 homozygous, and 20 asymptomatic individuals (63 in total) underwent *MEFV* gene sequence analysis. The analysis revealed that 4.3% of the heterozygous patients exhibited additional mutations undetectable by the initial sequencing.

Mattit et al.[24] included a total of 83 patients diagnosed with FMF and 242 healthy Syrian controls in their study. All participants were screened for the five most common *MEFV* mutations (M694V, M694I, M680I, V726A, and E148Q). In 25 patients (30%) who exhibited only one or no mutations, sequencing of exon 10 was performed, and in three patients additional mutations were identified (12%).

In the aforementioned studies, the frequency of additional mutations ranged from 4.3% to 12%, which is consistent with our findings. Generally, studies have shown that short exon screening kits offer high accuracy rates in diagnosing FMF. This is particularly beneficial in regions such as Türkiye, where the economic burden is significant.

In countries with a high prevalence of FMF and a significant rate of consanguineous marriages, the accumulation of affected individuals across consecutive generations may give the impression of dominant inheritance. However, this phenomenon is, in reality, a form of pseudo-dominant transmission. The frequency of consanguineous marriages within our country is 24%, which was comparable with our study.[25] This may explain the clinical manifestation observed in 48.9 % of our patients who possess a heterozygous mutation. Nevertheless, several *MEFV* variants exhibiting true dominant inheritance patterns have also been identified.[8,26]

Although this study makes significant contributions, it has limitations, primarily due to its focus on a specific ethnic group. This may limit the generalizability of the findings to other populations. Future research could aim to validate these findings in diverse ethnic backgrounds. As our study focused on common *MEFV* gene mutations, it may limit the comprehensiveness of our diagnostic approach, especially in cases with atypical genetic profiles. Furthermore, our reliance on Turkish pediatric FMF criteria may not cover all phenotypic variations of the disease.

In conclusion, the short exon screening for common *MEFV* mutations offers a practical and 4-fold lower cost alternative to comprehensive genetic testing in populations with a high prevalence of FMF. This approach can facilitate early diagnosis and the timely initiation of treatment, improving patient outcomes. As FMF is a complex and variable condition, an integrated approach that combines clinical assessment with targeted genetic testing is essential for optimal patient management.

Ethical approval

The study protocol was reviewed and approved by the Ethics Committee of the University of Health Sciences, Umraniye Training and Research Hospital (Approval No:B.10.1.TKH.4.34.H.GP.0.01/46).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BS, ŞÇ, and TC; data collection: ŞÇ, and TC; analysis and interpretation of results: BS, YKD, and ŞÇ; draft manuscript preparation: BS and ŞÇ. 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.

REFERENCES

1. Ben-Chetrit E, Touitou I. Familial Mediterranean fever in the world. Arthritis Rheum 2009; 61: 1447-1453. https://doi.org/10.1002/art.24458
2. Ozalkaya E, Mir S, Sozeri B, Berdeli A, Mutlubas F, Cura A. Familial Mediterranean fever gene mutation frequencies and genotype-phenotype correlations in the Aegean region of Turkey. Rheumatol Int 2011; 31: 779-784. https://doi.org/10.1007/s00296-010-1383-8
3. Ozen S, Karaaslan Y, Ozdemir O, et al. Prevalence of juvenile chronic arthritis and familial Mediterranean fever in Turkey: a field study. J Rheumatol 1998; 25: 2445-2449.
4. Yalçinkaya F, Ozen S, Ozçakar ZB, et al. A new set of criteria for the diagnosis of familial Mediterranean fever in childhood. Rheumatology (Oxford) 2009; 48: 395-398. https://doi.org/10.1093/rheumatology/ken509
5. Sohar E, Gafni G, Pras M. Tel Hashomer criteria for the diagnosis of FMF. In: Sohar E, Gafni G, Pras M, editors. Proceedings of the First International Conference on FMF. London and Tel Aviv: Freund Publishing House; 1997: 207.
6. Livneh A, Langevitz P, Zemer D, et al. Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum 1997; 40: 1879-1885. https://doi.org/10.1002/art.1780401023
7. Sag E, Demirel D, Demir S, et al. Performance of the new 'Eurofever/PRINTO classification criteria' in FMF patients. Semin Arthritis Rheum 2020; 50: 172-175. https://doi.org/10.1016/j.semarthrit.2019.08.004
8. Stoffels M, Szperl A, Simon A, et al. MEFV mutations affecting pyrin amino acid 577 cause autosomal dominant autoinflammatory disease. Ann Rheum Dis 2014; 73: 455-461. https://doi.org/10.1136/annrheumdis-2012-202580
9. The International FMF Consortium. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. Cell 1997; 90: 797–807. https://doi.org/10.1016/S0092-8674(00)80539-5.
10. French FMF Consortium. A candidate gene for familial Mediterranean fever. Nat Genet 1997; 17: 25-31. https://doi.org/10.1038/ng0997-25
11. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015; 17: 405-424. https://doi.org/10.1038/gim.2015.30
12. Van Gijn ME, Ceccherini I, Shinar Y, et al. New workflow for classification of genetic variants' pathogenicity applied to hereditary recurrent fevers by the International Study Group for Systemic Autoinflammatory Diseases (INSAID). J Med Genet 2018; 55: 530-537. https://doi.org/10.1136/jmedgenet-2017-105216
13. Ben-Chetrit E, Urieli-Shoval S, Calko S, Abeliovich D, Matzner Y. Molecular diagnosis of FMF: lessons from a study of 446 unrelated individuals. Clin Exp Rheumatol 2002; 20: S25-S29.
14. Tufan A, Lachmann HJ. Familial Mediterranean fever, from pathogenesis to treatment: a contemporary review. Turk J Med Sci 2020; 50: 1591-1610. https://doi.org/10.3906/sag-2008-11
15. Öztürk K, Coşkuner T, Baglan E, et al. Real-life data from the largest pediatric familial Mediterranean fever cohort. Front Pediatr 2022; 9: 805919. https://doi.org/10.3389/fped.2021.805919

16. Booth DR, Gillmore JD, Lachmann HJ, et al. The genetic basis of autosomal dominant familial Mediterranean fever. QJM 2000; 93: 217-221. https://doi.org/10.1093/qjmed/93.4.217

17. Booty MG, Chae JJ, Masters SL, et al. Familial Mediterranean fever with a single MEFV mutation: where is the second hit? Arthritis Rheum 2009; 60: 1851-1861. https://doi.org/10.1002/art.24569

18. Touitou I. The spectrum of familial Mediterranean fever (FMF) mutations. Eur J Hum Genet 2001; 9: 473-483. https://doi.org/10.1038/sj.ejhg.5200658

19. Yaşar Bilge Ş, Sarı İ, Solmaz D, et al. The distribution of MEFV mutations in Turkish FMF patients: multicenter study representing results of Anatolia. Turk J Med Sci 2019; 49: 472-477. https://doi.org/10.3906/sag-1809-100

20. Dundar M, Kiraz A, Emirogullari EF, et al. A molecular analysis of familial Mediterranean fever disease in a cohort of Turkish patients. Ann Saudi Med 2012; 32: 343-348. https://doi.org/10.5144/0256-4947.2012.343

21. Kırnaz B, Gezgin Y, Berdeli A. MEFV gene allele frequency and genotype distribution in 3230 patients' analyses by next generation sequencing methods. Gene 2022; 827: 146447. https://doi.org/10.1016/j.gene.2022.146447

22. Kilim Y, Magal N, Shohat M. Expanding the panel of MEFV mutations for routine testing of patients with a clinical diagnosis of familial Mediterranean fever. Isr Med Assoc J 2011; 13: 206-208.

23. Moradian MM, Sarkisian T, Ajrapetyan H, Avanesian N. Genotype-phenotype studies in a large cohort of Armenian patients with familial Mediterranean fever suggest clinical disease with heterozygous MEFV mutations. J Hum Genet 2010; 55: 389-393. https://doi.org/10.1038/jhg.2010.52

24. Mattit H, Joma M, Al-Cheikh S, et al. Familial Mediterranean fever in the Syrian population: gene mutation frequencies, carrier rates and phenotype-genotype correlation. Eur J Med Genet 2006; 49: 481-486. https://doi.org/10.1016/j.ejmg.2006.03.002

25. Koç İ. Türkiye'de akraba evliliklerinin yaygınlığının değişimi ve dirençli grupların belirlenmesi: 2018 Türkiye Nüfus ve Sağlık Araştırması'ndan evlilik kuşaklarına göre analizler. Turkish Journal of Public Health 2022; 20: 423-438. https://doi.org/10.20518/tjph.1114922

26. Rowczenio DM, Youngstein T, Trojer H, et al. British kindred with dominant FMF associated with high incidence of AA amyloidosis caused by novel MEFV variant, and a review of the literature. Rheumatology (Oxford) 2020; 59: 554-558. https://doi.org/10.1093/rheumatology/kez334

The Turkish Journal of Pediatrics 2024; 66: 473-480
https://doi.org/10.24953/turkjpediatr.2024.4921

Original Article

Cholecystectomy in children: indications, clinical, laboratory and histopathological findings and cost analysis

Aysel Ünlüsoy Aksu[1], Nebiyye Genel[2], Gülseren Şahin[1], Ferda Özbay Hoşnut[1], Ayşegül Tok[1], Ayşe Karaman[3]

[1]Department of Pediatrics, Pediatric Gastroenterology, Hepatology and Nutrition, Dr. Sami Ulus Maternity and Child Health and Diseases Training and Research Hospital, Ankara; [2]Department of Pathology, Dr. Sami Ulus Maternity and Child Health and Diseases Training and Research Hospital, Ankara; [3]Department of Pediatric Surgery, Dr. Sami Ulus Maternity and Child Health and Diseases Training and Research Hospital, Ankara, Türkiye.

ABSTRACT

Background. The most common indication for cholecystectomy in children is cholelithiasis, and routine histopathological examination is performed on all gallbladder specimens. Currently, selective histopathological examination is suggested instead of routine examination due to the low frequency of gallbladder cancer in adults. The purpose of this study was to evaluate the indications, clinical, laboratory and histopathological findings of the cholecystectomy in children. We also questioned the contribution and cost-effectiveness of routine histopathological evaluation in diagnosis and treatment.

Methods. A total of 114 children underwent cholecystectomy between the years 2008 and 2022. The clinical findings, laboratory, and imaging results of the patients and histopathological findings of the gallbladder specimens were evaluated retrospectively.

Results. Cholelithiasis were diagnosed in 71%, choledochal malformation in 15.8%, hydrops of gallbladder and/or biliary sludge in 12.3%, and hypoplasia of gallbladder in 0.9% of the patients. Histopathologically significant findings were observed in only 3 patients (2.6%); adenomyomatosis in 2 and angiodysplasia and pyloric metaplasia in 1. While the cost of a cholecystectomy and histopathologic examination combined amounted to 27.77% of the minimum wage in Türkiye in 2024, the histopathologic examination alone constitutes just 0.67% of the minimum wage and 2.4% of the operation fee.

Conclusion. In children undergoing cholecystectomy, histopathological examination does not provide any significant contribution to the patient's diagnosis and follow-up management. In children, selective gallbladder histopathological examination might reduce health costs and save time for pathologists.

Key words: cholecystectomy, histopathology, cholelithiasis, adenomyomatosis, children.

With the improved availability of ultrasonography, the prevalence of cholelithiasis in children has increased from 1.9% to 4% between the years 1959 and 2011.[1] At the same time, the number of cholecystectomy procedures performed has increased by 213% over a 9 year period ending in 2012.[2] There is a notable increase in the number of cholecystectomies performed on children, mostly due to the rising incidence of obesity.[3] The symptoms of cholelithiasis are not specific, and examination findings may be subtle in children.[4] In addition, ultrasound findings may show differences between intra- and inter-observers.[4]

Following cholecystectomy, all specimens removed during the operation are routinely sent for histopathological examination in adults, as there is a potential risk of carcinoma. Currently, selective histopathological examination is

✉ Aysel Ünlüsoy Aksu
ayselun@gmail.com

Received 3rd Jul 2024, revised 9th Aug 2024, accepted 12th Aug 2024.

The work was presented as an abstract at the 15th National Pediatric Gastroenterology, Hepatology and Nutrition Congress in Trabzon, Türkiye, 30 May-2 June 2024.

suggested instead of routine examination due to the low frequency of gallbladder cancer in adults.[5] Incidental carcinoma is found in 0.5-1.1% of cholecystectomies performed for cholelithiasis.[5] Individuals with a gallbladder wall thickness of >3 mm and enhanced vascularity have a higher cancer risk.[6] Preoperative abdominal ultrasound and computed tomography studies of gallbladder wall thickness estimation are found to be linearly correlated with histopathology, especially for severe wall thickening (>7 mm).[7]

The occurrence of complications due to underdiagnosis and incorrect treatment of cholelithiasis has an annual risk of 1-2%.[8] Cholelithiasis/cholecystitis is the second most costly disease per hospitalization in pediatric patients after congenital malformation. In the United States, the median total charge per hospitalization for cholelithiasis/cholecystitis is 37,607 US dollars. Healthcare expenditures for biliary tract disease is 16.9 billion US dollars annually, including both adults and children.[9]

The purpose of this study was to evaluate the indications, clinical, laboratory and histopathological findings of cholecystectomy in children. We also questioned the contribution and cost-effectiveness of routine histopathological evaluation to diagnosis and treatment.

Methods

This study included pediatric patients under the age of 18 years who underwent cholecystectomy at Dr. Sami Ulus Maternity and Child Health and Diseases Training and Research Hospital between January 2008 and December 2022. Data were collected retrospectively, including patients' age at the time of cholecystectomy, sex, body mass index (BMI) Z-score, indications for cholecystectomy, presence of single or multiple stones, underlying hematological diseases, accompanying symptoms, complete blood count (CBC) and biochemical parameters. There were no patients who underwent cholecystectomy for acute cholecystitis.

Additionally, macroscopic features of the gallbladder, such as cystic lesions, fibrotic, fragmented, irregular and diffuse wall thicknesses, were noted from pathology reports. Hydrops of the gallbladder refers to an enlarged gallbladder due to obstruction of the cystic duct, typically caused by an impacted stone or stricture. Moreover, microscopic features such as inflammation (granulomatous, xanthogranulomatous, follicular), epithelial atrophy or hyperplasia, cholesterolosis, dysplasia, metaplasia (pyloric, intestinal), eosinophilic or lymphoeosinophilic infiltration, stones, ulceration, erosion in the gallbladder, presence of denuded epithelium, serositis, adenoma (tubular), adenocarcinoma, adenomyomatosis, gangrene, ischemia, cholesterol polyp, hyperplastic polyp, mucocele, pancreatic heterotopia, hyperplastic Luschka ducts, and porcelain calcification were evaluated by hematoxylin and eosin staining. The gallstones, composed mainly of cholesterol, appears yellow and the dark brown and black stones contain mostly bilirubin. So, the colors of the gallstones were also noted.

Statistical analysis

The descriptive statistics for the data included the median, 25th to 75th interquartile range for gallbladder macroscopic features including longest gallbladder dimension and gallbladder wall thickness, CBC and biochemical parameters; mean and standard error values were reported for BMI Z-score. The parametric assumptions were tested using the Shapiro-Wilk Test and the Levene Test. The Mann-Whitney U test was performed for variables where the parametric assumptions were not met, chi-square test was used for quantitative data, and the Student t test was performed for variables for which the parametric assumptions were met at the 0.05 level of significance. The program IBM SPSS Statistics for Windows, version 22.0. Armonk, NY: IBM Corp, 2013 was used for statistical analyses.

This study was approved by Ankara Bilkent City Hospital Ethics Committee (Date: 01.03.2023, Number: E2-23-3472).

Results

A total of 114 children underwent cholecystectomy, of whom 100 (88%) were female and 14 (12%) male. Fourteen of these patients underwent cholecystectomy in the first 5 years (January 2008-December 2012), and 100 patients underwent cholecystectomy in the last 10 years (January 2013-December 2022). The most common indication for cholecystectomy was cholelithiasis (Table I). The median age and BMI Z-score of patients were higher in those with cholelithiasis, and cholelithiasis was more frequently observed in girls (p<0.05) (Table II and Table III). Hemolytic anemia was present in 12 (10.5%) patients. Among these cases, 8 patients had hereditary spherocytosis, one had hereditary elliptocytosis, one had sickle cell anemia, one had transfusion dependent beta-thalassemia and one had congenital dyserythropoietic anemia. Out of 83 children with cholelithiasis, the color of the stones was noted in 62 patients. Among these, 23 had green stones, 20 had yellow stones, and 19 had black stones. Among the patients with hemolytic disease, green and/or green-black stones were observed in 7 patients, black stones in 3 patients and yellow stones in one patient, one patient however was missing color information. Green gallstones were observed in 2 patients with choledochal malformations type 1 or type 2.

There was no statistically significant difference in the presence of hemolytic anemia, and symptoms of nausea and vomiting, between patients with or without cholelithiasis (p> 0.05). However, the rate of abdominal pain was higher in those with cholelithiasis (p< 0.05) (Table II). The laboratory results indicate that individuals with cholelithiasis had reduced levels of platelet count, alkaline phosphatase and aspartate aminotransferase (p< 0.05) (Table III).

Histopathological examination revealed chronic cholecystitis in all but 7 patients. Of these 7 patients without chronic cholecystitis, 2 had multiple gallstones, 1 had a hypoplastic gallbladder, and 3 had a type 1 choledochal malformation. One patient with overt clinical symptoms of cholecystitis underwent cholecystectomy, and the histopathology of the gallbladder was found to be normal. The macroscopic pathological examination indicated the presence of hydrops in 3 cases and the histopathological examination indicated cholesterolosis in 12 patients with cholelithiasis. Two patients without cholelithiasis exhibited hydrops, and two others showed

Table I. Indications for cholecystectomy (N=114).

Preoperative Diagnosis	Number (%)
Multiple gallstones	60 (52.6%)
Single gallstone	21 (18.4%)
Choledochal cyst type 1	16 (14.0%)
Hydropic gallbladder and/or biliary sludge	14 (12.3%)
Choledochal cyst type 2	2 (1.8%)
Hypoplastic gallbladder	1 (0.9%)

Table II. Comparison of sex, diagnosis of hemolytic anemia, and symptoms in patients with and without cholelithiasis.

		Gallstone		Total	P
		Absent n=31 (27.2%)	Present n=83 (72.8%)		
Sex (%)	Female	24 (24%)	76 (76%)	100	0.041
	Male	7 (50%)	7 (50%)	14	
Hemolytic anemia (%)	Negative	30 (29.4%)	72 (70.6%)	102	0.121
	Positive	1 (8.3%)	11 (91.7%)	12	
Abdominal pain (%)	Negative	19 (46.3%)	22 (53.7%)	41	0.001
	Positive	12 (16.4%)	61 (83.6%)	73	
Nausea and/or vomiting (%)	Negative	21 (29.2%)	51 (70.8%)	72	0.535
	Positive	10 (23.8%)	32 (76.2%)	42	

Table III. Comparison of age, BMI Z-scores, complete blood count, biochemical parameters, and macroscopic pathological examination of the gallbladder in patients with and without cholelithiasis.

	Gallstone						P
	Absent			Present			
	Q1	Median	Q3	Q1	Median	Q3	
Age, years	3	7	13	11	15	17	**< 0.001**
BMI Z score (mean±SD)		-0.4±0.2			0.7±0.2		**< 0.001**
Hemoglobin g/dl	11.7	12.5	13.7	12.4	13.2	14	0.093
White blood cell, $x10^6$/L	6700	7450	8700	5780	7160	8760	0.226
Platelets, $x10^9$/L	260	320	405	236	289	334	0.020
Eosinophil count, $x10^6$/L	100	180	220	90	140	220	0.259
Eosinophil, %	1.7	2.1	3.2	1.2	2.0	2.6	0.333
ALT, U/L (Normal range 0-32)	13	19	36	11	15	21	0.055
AST, U/L (Normal range 0-36)	24	31	42	17	22	25	**< 0.001**
Alkaline Phosphatase, U/L (Normal range 128-420)	156	228	275	86	144	185	**< 0.001**
GGT, U/L (Normal range <73)	12	14	142	11	14	21	0.147
Total bilirubin, mg/dL (Normal range 0.3-1.2)	0.3	0.6	1.1	0.5	0.6	1	0.388
Direct bilirubin, mg/dL (Normal range 0-0.2)	0.1	0.2	0.3	0.1	0.1	0.3	0.567
Gallbladder longest dimension, cm	5	6	8	5.5	6.5	8	0.122
Gallbladder wall thickness, cm	0.2	0.3	0.4	0.2	0.2	0.3	0.116

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyl transferase, Q1: 25th quartile, Q3: 75th quartile; SD, standard deviation.

cholesterolosis, both of whom had a choledochal malformation. In a patient with a choledochal malformation with pancreaticobiliary junction Todani 1A, Komi 3 anomaly; mononuclear inflammatory cell infiltration and Rokitansky-Aschoff sinus, and pyloric metaplasia were observed in the mucosa. In the mucosa and the submucosa, diffuse increased vascularization, dilatation of venous vessels and thick-walled arterial structures were observed, indicating angiodysplasia. The surface epithelium of hypoplastic gallbladder was not visible in most areas and the epithelium was flattened partially in a patient. Fibrosis was observed in the subepithelial area and Rokitansky-Aschoff sinus as well as inflammatory cells in the fibrous tissue. Another patient with a choledochal malformation, showed an enlarged and cystic appearance of the Rokitansky-Aschoff sinus. Adenomyomatous hyperplasia was noted in 2 patients: one presented with a polyp in the gallbladder and the other presented with multiple stones. Both patients had symptoms of abdominal pain, and the length of the removed gallbladder was 5 cm in both. The wall thickness of the gallbladder of the patient who underwent surgery for the polyp was 0.3 cm and the wall thickness of the gallbladder of the other patient was 0.5 cm. In a patient with a choledochal malformation, areas of hemorrhage and ischemia were observed in the serosa. A gallbladder perforation was observed in a patient diagnosed with calculous cholecystitis. Elevated levels of amylase and lipase were seen in 5 patients, while 4 patients were diagnosed with type 1 choledochal malformation and one patient had multiple gallbladder stones.

There was no significant difference in gallbladder wall thickness and length between patients with and without cholelithiasis (p> 0.05) (Table III). In 9 patients, the gallbladder wall was thick (>0.3 cm) on ultrasound, and on macroscopic examination the thickness of the gallbladder wall was 0.3-0.4 cm in 8 patients and 0.7 cm in one. Hydrops of the gallbladder were observed in 7 patients on ultrasound and macroscopically the size of

the gallbladder was 5-7.5 cm in 5 patients and 9.5-10 cm in 2. Our patients did not exhibit any clinical manifestations such as xanthogranulomatous inflammation, dysplasia, eosinophilic or lymphoeosinophilic infiltration, ulceration in the gallbladder, tubular adenoma, adenocarcinoma or pancreatic heterotopia.

In Türkiye, the cost of a histopathologic study of the gallbladder alone amounts to 114 Turkish Liras (TL) (0.67% of minimal wage), equivalent to around 3.5 US dollars, reimbursed by the Turkish Social Security Institution. However, when considering the combined expenses of cholecystectomy and histopathologic investigation, the entire cost reaches 4722 TL, equivalent to approximately 147.5 US dollars and 27.77% of the minimum wage in Türkiye (17002 TL, approximately 528 US dollars). Hence, histopathologic examination constitutes 2.4% of the total expenditure.

Discussion

Cholecystectomy is indicated in children for symptomatic cholelithiasis, asymptomatic cholelithiasis with hemolytic diseases, acute cholecystitis that does not respond to medical management, chronic cholecystitis causing recurrent symptoms, gallbladder polyps larger than 1 cm or symptomatic polyps, gallbladder anomalies, and cholecystitis with pancreatitis.[10] Prolonged symptom duration, signs of systemic inflammation, history of lithotherapy, and gallbladder wall thickening of 3 mm or more have been identified as indications for immediate surgery.[10] A recent systematic review and meta- analysis showed that, the most common indications for laparoscopic cholecystectomy in children are cholelithiasis, cholecystitis, and biliary dyskinesia.[11] In the present study the most common indication for cholecystectomy was cholelithiasis followed by choledochal cyst and hydropic gallbladder and/or biliary sludge.

The current study revealed that individuals with cholelithiasis had a higher median age, more frequent abdominal pain, and a higher BMI Z-score (p<0.001). Also, cholelithiasis was more frequently observed in girls (p<0.05), in line with other pediatric studies.[2] Black pigment gallstones, also known as calcium bilirubinate stones, are typically associated with hemolytic disease.[12] However, in our cohort, green and green-black pigment stones were more common among patients with hemolytic disease. Two patients with choledochal malformation had green gallstones, despite the usual description of brown gallstones in such patients.[12]

The length and wall thickness of the gallbladder did not differ significantly (p>0.05) between those with and without cholelithiasis. The gallbladder wall is defined as thick and suspicious for cancer if it measures more than 0.3 cm on ultrasound or macroscopic examination.[13] The median gallbladder wall thickness was 0.3 cm or less in patients with and without cholelithiasis in this study.

The study revealed that Rokitansky-Aschoff sinus was present in three children (2.6%) and adenomyomatous hyperplasia was present in two children (0.17%). These rates are much lower compared to adults, where Rokitansky-Aschoff sinus has been reported in 65% and adenomyomatous hyperplasia in 5% of cases.[14,15] There was no muscular layer hypertrophy and epithelial proliferation in the patients with Rokitansky-Aschoff sinus. Adenomyomatosis is rare in children, and progression to carcinoma has not been reported.[16] Carcinoma has been observed in 0.06% of dysplasia-preceding pyloric metaplasia cases and in 0.04% of intestinal metaplasia cases in cholecystectomy specimens from adolescents and young adult patients aged 11-20 years.[17] In this study, one patient (0.87%) aged 3.5 years with an anomaly of the pancreaticobiliary junction anomaly (Todani 1A, Komi 3), had pyloric metaplasia, increased vascularization in the mucosa and submucosa and angiodysplasia of the gallbladder. As documented in prior studies, 3 patients were found to have angiodysplasia in the gallbladder.[18] One of these patients was a 78-year-old male in whom multiple

angiodysplasias were also observed in the gastrointestinal tract. The other two female patients were 29 and 36 years old and one of them had an association with cholelithiasis.[18] Apart from these cases, angiodysplasia of the gallbladder has not been reported in the literature. According to our findings, 2 patients with adenomyomatous hyperplasia and one patient with angiodysplasia and pyloric metaplasia did not develop any further clinical manifestations subsequent to cholecystectomy.

The criteria for diagnosing gallbladder hydrops typically include gallbladder distention with an anterior-posterior diameter >5 cm and with clear fluid content, and irrespective of acute inflammation and gallbladder wall thickness.[12] In this study, 5 patients had gallbladder hydrops upon macroscopic inspection. Among them, 2 presented with a solitary gallstone, 2 presented with several stones, and one presented with a choledochal malformation. Since the size of the gallbladder in children varies according to age, height, weight and body surface area, it is not appropriate to use parameters such as a diameter >4.5-5 cm to diagnose a hydropic gallbladder.[4] In our research, we assessed the length of the gallbladder and observed no difference between the patients with and without cholelithiasis. A review of 134 cholecystectomy specimens identified 8 cases (6%) of eosinophilic cholecystitis and 3 cases (2.2%) of lymphoeosinophilic cholecystitis in children.[19] In our study, we did not encounter any patients exhibiting eosinophilic or lymphoeosinophilic infiltration.

For a comprehensive histopathological evaluation, specimens from the fundus, corpus, and parts of the cystic duct-neck should be examined. One study found that taking samples from all three parts with longitudinal sectioning led to a higher number of mucosal lesions and preinvasive lesions.[20] In our study, classical sampling was performed with samples from three parts placed in one cassette, which may have resulted in the failure to detect some mucosal lesions. In fact, in both types of sampling, samples are taken from three parts of the gallbladder, and if dysplasia is detected in these samples, examination of the entire gallbladder is recommended.[21]

Even in Asian countries, where incidental gallbladder carcinomas are common, histopathological examination is recommended in selected cases.[22] Routine histopathological examination is also not recommended by the Dutch national guidelines devised in 2014.[23] However, in 2018 a study conducted in the Netherlands found that the rates of histopathological examination of cholecystectomy specimens did not decrease between 1990 and 2015.[23] The histopathology rate could be reduced by 90% by sending macroscopically suspicious specimens for further evaluation.[24]

The study's limitations stem from its reliance on retrospective histopathology reports, where each gallbladder sample was evaluated by more than one pathologist. The reporting pathologists may have considered some histopathological abnormalities as unimportant and not worth reporting. Despite conducting the study in a single center, the population encompassed patients from various regions of Türkiye, thereby reflecting the country's demographic structure.

Implementing histological investigations selectively, based on clinical and macroscopic observations before and during surgical procedures, can potentially reduce healthcare costs. In children undergoing cholecystectomy, histopathological examination does not provide any significant contribution to the patient's diagnosis and follow-up management. Despite this, pathology departments routinely receive samples removed during surgery and are exposed to excessive workload. Although the financial burden of histopathological examination is negligible in Türkiye, it may be high in other countries. In low-risk areas for gallbladder cancer; more than half of the studies implemented a more selective policy;

while studies in high-risk areas emphasize that all gallbladder specimens should be evaluated by a pathologist.[24] Even in high-risk areas, the incidence of truly incidental gallbladder cancer is 0.44% and if surgeons examine gallbladders systematically, it drops to 0.08%.[24] Surgeons might miss incidental cancer, and they might fear medicolegal consequences. The FANCY study showed that 0.22% of 10041 gallbladder specimens had cancer. Surgeons could have held back histopathologic examination for 78.1% of the specimens, saving €70.35 per person by using selective instead of routine examination.[25] Studies are needed to pinpoint suspicious macroscopic features in gallbladder specimens from children and to limit histopathological examination to these specific cases. In children, selective gallbladder histopathological examination might reduce health costs and save time for pathologists.

Ethical approval

The study was approved by Ankara Bilkent City Hospital Ethics Committee (date: 01.03.2023, number: E2-23-3472).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AÜA, NG, AK, data collection: AÜA, NG, GŞ,; analysis and interpretation of results: FÖH,AT, draft manuscript preparation: AÜA, NG, AK. All authors reviewed the results and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Mehta S, Lopez ME, Chumpitazi BP, Mazziotti MV, Brandt ML, Fishman DS. Clinical characteristics and risk factors for symptomatic pediatric gallbladder disease. Pediatrics 2012; 129: e82-e88. https://doi.org/10.1542/peds.2011-0579
2. Zdanowicz K, Daniluk J, Lebensztejn DM, Daniluk U. The etiology of cholelithiasis in children and adolescents-a literature review. Int J Mol Sci 2022; 23: 13376. https://doi.org/10.3390/ijms232113376
3. Walker SK, Maki AC, Cannon RM, et al. Etiology and incidence of pediatric gallbladder disease. Surgery 2013; 154: 927-933. https://doi.org/10.1016/j.surg.2013.04.040
4. Pelizzo G, Bussani R, De Silvestri A, et al. Laparoscopic cholecystectomy for symptomatic cholecystic disease in children: defining surgical timing. Front Pediatr 2020; 8: 203. https://doi.org/10.3389/fped.2020.00203
5. Limaiem F, Sassi A, Talbi G, Bouraoui S, Mzabi S. Routine histopathological study of cholecystectomy specimens. Useful? A retrospective study of 1960 cases. Acta Gastroenterol Belg 2017; 80: 365-370.
6. Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome. Clin Epidemiol 2014; 6: 99-109. https://doi.org/10.2147/CLEP.S37357
7. Kokoroskos N, Peponis T, Lee JM, et al. Gallbladder wall thickness as a predictor of intraoperative events during laparoscopic cholecystectomy: a prospective study of 1089 patients. Am J Surg 2020; 220: 1031-1037. https://doi.org/10.1016/j.amjsurg.2020.03.007
8. Li ZZ, Guan LJ, Ouyang R, Chen ZX, Ouyang GQ, Jiang HX. Global, regional, and national burden of gallbladder and biliary diseases from 1990 to 2019. World J Gastrointest Surg 2023; 15: 2564-2578. https://doi.org/10.4240/wjgs.v15.i11.2564
9. Peery AF, Crockett SD, Murphy CC, et al. Burden and cost of gastrointestinal, liver, and pancreatic diseases in the United States: update 2021. Gastroenterology 2022; 162: 621-644. https://doi.org/10.1053/j.gastro.2021.10.017
10. Destro F, Pierucci UM, Durante E, et al. Laparoscopic cholecystectomy in children: the experience of two centers focusing on indications and timing in the era of "New Technologies". Children (Basel) 2023; 10: 1771. https://doi.org/10.3390/children10111771
11. Mattson A, Sinha A, Njere I, Borkar N, Sinha CK. Laparoscopic cholecystectomy in children: a systematic review and meta-analysis. Surgeon 2023; 21: e133-e141. https://doi.org/10.1016/j.surge.2022.09.003

12. Lam R, Zakko A, Petrov JC, Kumar P, Duffy AJ, Muniraj T. Gallbladder disorders: a comprehensive review. Dis Mon 2021; 67: 101130. https://doi.org/10.1016/j.disamonth.2021.101130

13. Bazoua G, Hamza N, Lazim T. Do we need histology for a normal-looking gallbladder? J Hepatobiliary Pancreat Surg 2007; 14: 564-568. https://doi.org/10.1007/s00534-007-1225-6

14. Parolini F, Indolfi G, Magne MG, et al. Adenomyomatosis of the gallbladder in childhood: a systematic review of the literature and an additional case report. World J Clin Pediatr 2016; 5: 223-227. https://doi.org/10.5409/wjcp.v5.i2.223

15. Terada T. Histopathologic features and frequency of gall bladder lesions in consecutive 540 cholecystectomies. Int J Clin Exp Pathol 2013; 6: 91-96.

16. Pasierbek M, Korlacki W, Grabowski A. Adenomyomatosis of the gallbladder in an adolescent, a very rare condition. Arch Argent Pediatr 2020; 118: e43-e47. https://doi.org/10.5546/aap.2020.eng.e43

17. Ghartimagar D, Shrestha MK, Jhunjhunwala A, et al. Non-neoplastic lesions of gallbladder among cholecystectomy specimens of a tertiary care center: a descriptive cross-sectional study. JNMA J Nepal Med Assoc 2021; 59: 970-974. https://doi.org/10.31729/jnma.5861

18. Švagelj I, Vučko M, Hrskanović M, Švagelj D. Angiodysplasia of the gallbladder: an unknown risk factor for cholecystolithiasis. Case Rep Pathol 2020; 2020: 7192634. https://doi.org/10.1155/2020/7192634

19. Garzón G LN, Jaramillo B LE, Valero H JJ, Quintero C EM. Eosinophilic cholecystitis in children: case series. J Pediatr Surg 2021; 56: 550-552. https://doi.org/10.1016/j.jpedsurg.2020.05.039

20. Argon A, Yağcı A, Taşlı F, et al. A different perspective on macroscopic sampling of cholecystectomy specimens. Korean J Pathol 2013; 47: 519-525. https://doi.org/10.4132/KoreanJPathol.2013.47.6.519

21. Abdullazade S, Akarca FG, Esendagli G, et al. The contribution of additional sampling in cholecystectomy materials: a multicenter prospective study. Turk Patoloji Derg 2020; 36: 188-194. https://doi.org/10.5146/tjpath.2020.01483

22. Deng YL, Xiong XZ, Zhou Y, Shrestha A, Li FY, Cheng NS. Selective histology of cholecystectomy specimens-is it justified? J Surg Res 2015; 193: 196-201. https://doi.org/10.1016/j.jss.2014.07.039

23. Olthof PB, Metman MJH, de Krijger RR, Scheepers JJ, Roos D, Dekker JWT. Routine pathology and postoperative follow-up are not cost-effective in cholecystectomy for benign gallbladder disease. World J Surg 2018; 42: 3165-3170. https://doi.org/10.1007/s00268-018-4619-5

24. Bastiaenen VP, Tuijp JE, van Dieren S, et al. Safe, selective histopathological examination of gallbladder specimens: a systematic review. Br J Surg 2020; 107: 1414-1428. https://doi.org/10.1002/bjs.11759

25. Bastiaenen VP, van Vliet JLP, de Savornin Lohman EAJ, et al. Safety and economic analysis of selective histopathology following cholecystectomy: multicentre, prospective, cross-sectional FANCY study. Br J Surg 2022; 109: 355-362. https://doi.org/10.1093/bjs/znab469

The Turkish Journal of Pediatrics 2024; 66: 481-489
https://doi.org/10.24953/turkjpediatr.2024.5072

Case Report

Clinicopathological features and treatment of aggressive natural killer cell leukemia: case series and literature review

Yongan Ni[1], Lei Li[2], Yuping Wang[3], Lirong Sun[1]

[1]Department of Pediatric Hematology and Oncology, the Affiliated Hospital of Qingdao University, Qingdao, Shandong; [2]Department of Respiratory & Cardiovascular Pediatrics, the Affiliated Hospital of Qingdao University, Qingdao, Shandong; [3]Department of Pediatrics, Qingdao Women and Children's Hospital, Qingdao, Shandong, China.

ABSTRACT

Background. Aggressive natural killer cell leukemia (ANKL) is rare and difficult to diagnose in early stages, with no standard treatment and a poor prognosis.

Case presentation. Two adolescents with ANKL presented with hemophagocytic lymphohistiocytosis (HLH), with Case-1 presenting as refractory HLH and Case-2 with lung involvement. The morphology of bone marrow showed an increase in unidentified cells, which mainly expressed CD56. Cytogenetic analysis showed complex karyotypes. Both patients received intensive combined chemotherapy based on pegaspargase and anthracyclines. Case-1 died of tumor lysis syndrome. Case-2 underwent hematopoietic stem cell transplantation and is currently alive and disease-free.

Conclusions. HLH can serve as the initial manifestation of ANKL. Leukemia cells of ANKL have significant variations in the morphology and mainly express CD56. Intensive combination chemotherapy based on pegaspargase and anthracyclines may be considered for ANKL.

Key words: aggressive NK cell leukemia, asparaginase, anthracyclines, hemophagocytic lymphohistiocytosis.

Aggressive natural killer cell leukemia (ANKL) is a rare systemic mature natural killer (NK) cell proliferating tumor with an aggressive and fulminant clinical course.[1,2] It usually presents with fever, systemic symptoms, leukemic hemogram, and elevated serum lactate dehydrogenase levels and may be accompanied by hemophagocytic lymphohistiocytosis (HLH), disseminated intravascular coagulation (DIC), or multiple organ failure.[1,2] The commonly involved sites include bone marrow, peripheral blood, lymph nodes, liver, and the spleen, while the involvement of skin, soft tissue, lung, and omentum is rare.[2] ANKL occurs mainly in adults between 30 and 50 years of age, and there are only occasional reports of cases in children.[1,2]

✉ Lirong Sun
sunlr@qdu.edu.cn

Received 11th Jul 2023, revised 6th Nov 2023, 19th Apr 2024, accepted 21st Apr 2024.

The early diagnosis of ANKL is difficult mainly due to the complexity of clinicopathological manifestations (mimicking many syndromes or diseases) and the lack of specific immunophenotype and molecular biological characteristics.[3,4] At present, the conventional treatment is chemotherapy based on L-asparaginase (L-ASP) and allogeneic hematopoietic stem cell transplantation (allo-HSCT).[1,2] However, the complete remission (CR) rate of chemotherapy is below 36%.[5-7] The 1-year cumulative incidences of relapse or progression has been found to be 55.5% after allo-HSCT.[7] The prognosis is poor, and the median survival time is less than 2 months.[1,2,6,8]

Herein, we report the diagnosis and treatment of two cases of ANKL manifested as HLH in children, of which one case with lung involvement has survived for 19 months after allo-HSCT.

Case Presentations

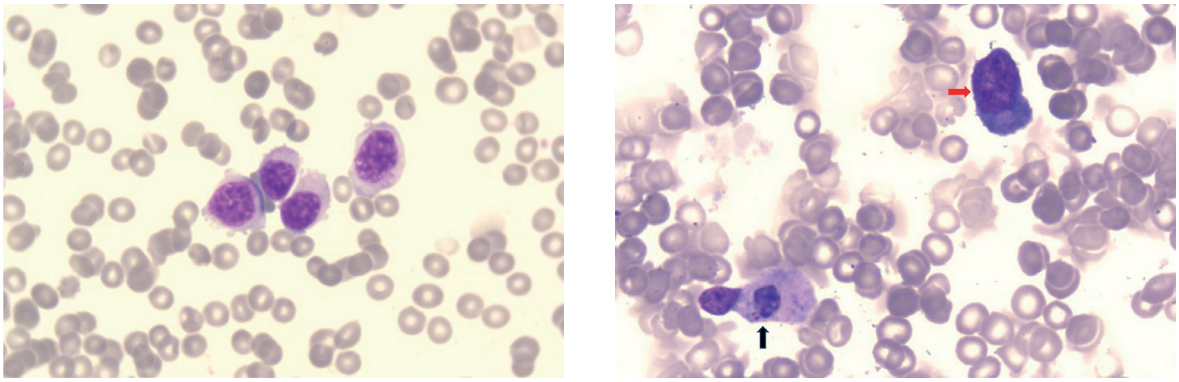
Case-1

A 15-year-old female patient was admitted to our hospital for recurrent fever of three months. Before admission, Epstein-Barr virus (EBV) infection-associated HLH was diagnosed due to recurrent fever, splenomegaly, pancytopenia, elevated triglycerides (4.69 mmol/L, range: 0-1.7 mmol/L), elevated ferritin (7,876 ng/mL, range: 30-400 ng/mL), hemophagocytosis in the bone marrow, elevated soluble CD25 (20,881.06 pg/mL, range: 410-2,623 pg/mL), and EBV-DNA 3.17×10^4 copies/mL (range: ≤ 5.0×10^3 copies/mL). The tests of NK cell activity and cerebrospinal fluid were normal. The detection of mutations in primary HLH-related genes was negative. 18F-fluorodeoxyglucose positron emission tomography/computed tomography (18F-FDG-PET/CT) showed splenomegaly with diffuse metabolic elevation (maximum standardized uptake value [SUVmax]: 3.5). The treatment response was partial remission after five weeks of chemotherapy of the HLH-2004 protocol (dexamethasone, etoposide, and cyclosporine). Subsequently, she received two cycles of the COP regimen (cyclophosphamide, vindesine, and prednisone) and two cycles of the L-DEP regimen (pegaspargase, adriamycin, etoposide, and methylprednisolone). However, she still had recurrent fever, splenomegaly, and ferritin levels exceeding 3500 ng/mL during chemotherapy. Empirical anti-infection treatments including meropenem, linezolid, and voriconazole did not improve the recurrent fever.

After admission, physical examination revealed anemia, skin ecchymosis, and splenomegaly. Peripheral blood cell counts showed leucocyte count 1.52 × 10^9/L, neutrophil count 1.41 × 10^9/L, hemoglobin concentration 62 g/L, and platelet count 51 × 10^9/L. Laboratory tests showed ferritin 58,250 ng/mL, triglycerides 4.4 mmol/L, decreased NK cell activity (0.57%, range: ≥ 15.11%), soluble CD25 4,846.49 pg/mL, hemophagocytosis in the bone marrow, and EBV-DNA 5.74×10^3 copies/mL. C-reactive protein, procalcitonin, and erythrocyte sedimentation rates were 75.54 mg/L (range: <5 mg/L), 0.23 ng/mL (range: <0.05 ng/mL), and 103.0 mm/h (range: ≤15 mm/h), respectively. The tests of tubercle bacillus, 1,3-β-D-glucan, and galactomannan were negative. No evidence of rheumatic immune diseases and malignancies was found. Re-induction therapy with HLH-2004 protocol was given. Meanwhile, empirical anti-infection treatments including meropenem, linezolid, and voriconazole were given. However, the patient still experienced recurrent fever, pancytopenia, splenomegaly, ferritin levels > 45,000 ng/mL, and triglyceride levels > 5.5 mmol/L during the re-induction treatment. Bone marrow aspiration was performed again after four weeks of re-induction therapy, and bone marrow smear showed 80% of unidentified cells (Fig. 1A). Immunophenotype of abnormal cells by flow cytometry was CD56+, CD2+, CD94+, CD30+, CD38+, CD3-, CD4-, CD8-, CD5-, CD7-, $TCR_{\alpha\beta}$-, $TCR_{\gamma\delta}$-, CD16-, CD10-, CD25-, CD57-, and CD161-. The diagnosis of ANKL was given. VDLP (vincristine, idarubicin, pegaspargase, and prednisone) regimen was adopted for induction chemotherapy. During the induction chemotherapy, she experienced tumor lysis syndrome and finally died of multiple organ failure.

Case-2

A 14-year-old male patient was admitted to our hospital due to fever with a dry cough for two weeks and chest pain for three days. Physical examination revealed anemia, hepatomegaly, and splenomegaly. Peripheral blood cell counts showed leucocyte count 0.83 × 10^9/L, neutrophil count 0.38 × 10^9/L, hemoglobin concentration 93 g/L, and platelet count 43 × 10^9/L. Laboratory tests showed fibrinogen 1.35 g/L (range: 2-4 g/L), triglycerides 4.12 mmol/L (range: 0.3-1.92), ferritin 25,297 ng/mL, NK cell activity 15.68% (range: ≥ 15.11%), soluble CD25 34,957 pg/mL, and EBV-DNA 2.2×10^5 copies/mL. C-reactive protein, procalcitonin, and erythrocyte sedimentation rates were 32.04 mg/L, 0.45 ng/mL, and 8 mm/h, respectively. The tests



A. B.

Fig. 1. The morphology of bone marrow. **A.** Abnormal cells in the bone marrow smear of Case-1 (Wright Giemsa stain, ×1,000). The abnormal cells were irregular in shape and large in size, with granules in the cytoplasm, some of which had pseudopodia. **B.** Abnormal cells and hemophagocytosis (the black arrow) in the bone marrow smear of Case-2 (Wright Giemsa stain, ×1,000). The abnormal cells (the red arrow) had a large size, abundant basophilic cytoplasm, and fine nuclear chromatin.

of 1,3-β-D-glucan, galactomannan, tubercle bacillus, and bacterial culture (blood) were negative. The bone marrow smear showed 22.5% of unidentified cells and hemophagocytosis (Fig. 1B). Immunophenotype of abnormal cells by flow cytometry was CD45++, CD56++, CD2+, CD94+, CD33+, HLA-DR+, CD81+, CD16-, CD117-, CD34-, CD13-, CD10-, CD19-, CD11b-, CD7-, CD5-, CD36-, CD64-, CD4-, CD8-, sCD3-, cyMPO-, cyCD79a-, cyCD3-, and CD123-. High resolution computed tomography of the chest showed multiple space-occupying lesions in both lungs. The manifestations of 18F-FDG-PET/CT images (Fig. 2) were as follows: (1) There were multiple soft tissue density masses and nodules in both lungs, with the largest located in the anterior segment of the superior lobe of the right lung (the maximum cross-section of approximately 44 × 50mm), lobulation at the edge, low-density necrosis at some centers, and increased radioactive uptake (SUVmax: 11.8). (2) Bones of the whole body showed uneven increased radioactive uptake (SUVmax: 11.3), but computed tomography showed no bone destruction. Serum tumor markers such as carcinoembryonic antigen, carbohydrate antigen 72-4, neuron specific enolase, squamous cell carcinoma antigen, and pro-gastrin-releasing peptide were all negative. The pathology of the lung mass puncture biopsy showed a large area of coagulative necrosis, the peripheral residual small focal fibrous tissue presented as chronic inflammation with collagenization and fibrous exudation, and the severe tissue necrosis. The diagnosis of HLH and ANKL was given. The detection of mutations in primary HLH-related genes was negative. The treatment response was partial remission after three weeks of chemotherapy of HLH-2004 protocol (dexamethasone, etoposide). However, there was no improvement in pulmonary space-occupying lesions. Subsequently, for ANKL, the patient underwent allo-HSCT, after chemotherapy with EDCH+P regimen (vincristine, cyclophosphamide, dexamethasone, liposome doxorubicin, etoposide, and pegaspargase; two cycles), DDGP regimen (cisplatin, dexamethasone, gemcitabine, and pegaspargase), and chidamide combined with etoposide and dexamethasone regimen. Currently, the patient is alive and disease-free.

Discussion

The diagnosis of ANKL in children meets numerous challenges. First, the clinical manifestations are diverse and mimic a variety of syndromes and diseases, making early diagnosis difficult.[3,4] Second, the morphology

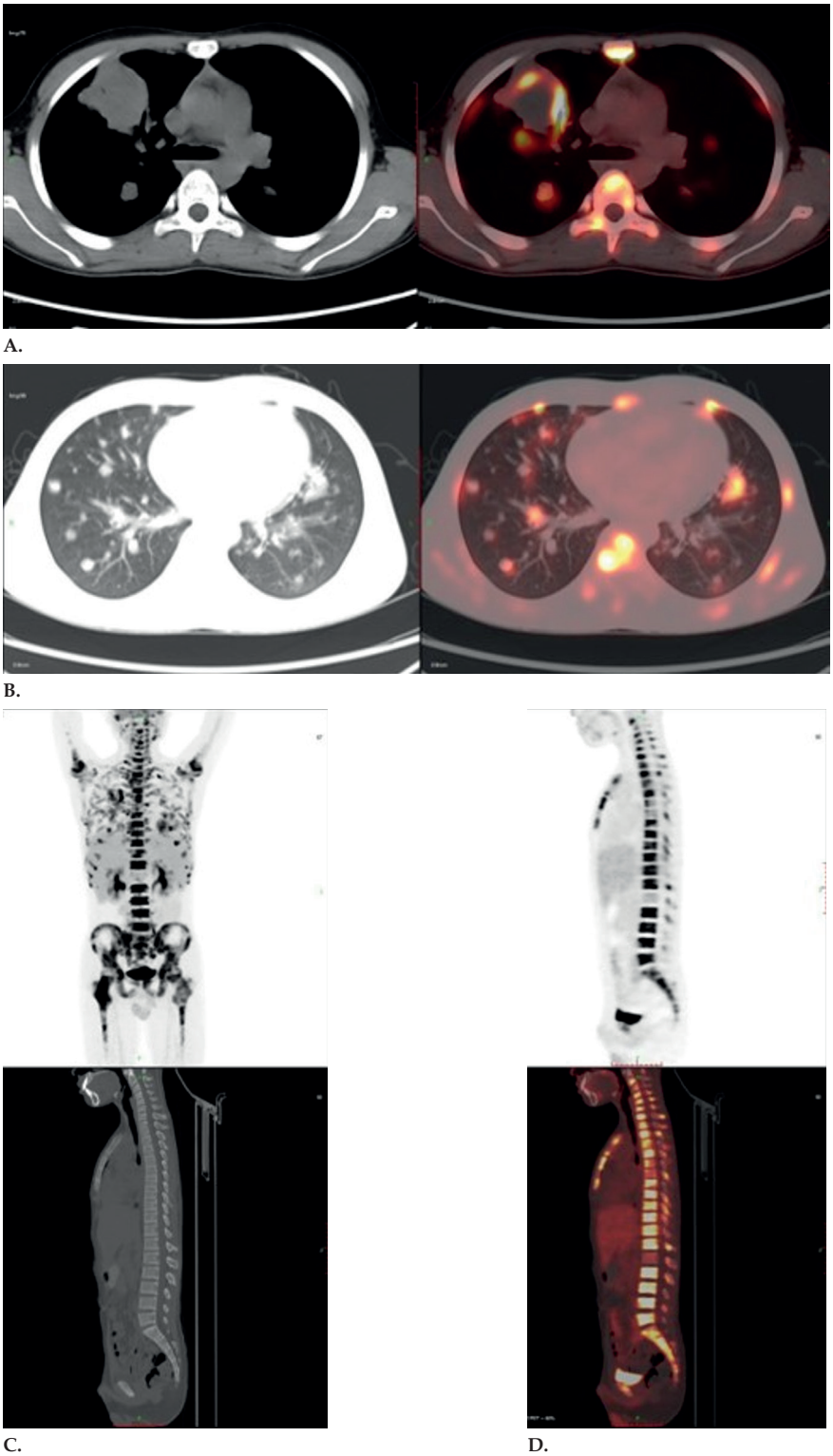


Fig. 2. The manifestations of 18F-FDG-PET/CT of Case-2. **A.** and **B.** Masses and nodules in lungs, with increased radioactive uptake (SUVmax: 11.8). **C.** and **D.** Bones of the whole body, with uneven increased radioactive uptake (SUVmax: 11.3), but no bone destruction in the computed tomography.

of leukemic cells of ANKL are significantly different.[9,10] Third, abnormal NK cells in ANKL mainly expressed CD56, but with no specific immunophenotype, and CD56-negative cases accounted for 16.7%.[11,12] Fourth, cytogenetics show complex karyotypes without any specificity.[13,14] Finally, no specific fusion gene or mutant gene may be found.

Therefore, we systematically analyzed the clinical and pathological manifestations, bone marrow morphology, immunophenotyping, cytogenetic and molecular biology characteristics, and treatment of 19 children with ANKL (including our two cases and 17 cases[3,8,15-29] from China Biology Medicine disc and Pubmed) in this study. General clinical characteristics of these 19 cases are summarized in Table I.

ANKL had a variety of clinical manifestations. The median age was 14 years (range: 0.75–18 years), and adolescent patients accounted for 63.2%. Most patients presented with fever (89.5%), splenomegaly (89.5%), hepatomegaly (57.9%), and anemia (68.4%). Leukopenia, thrombocytopenia, and elevated serum lactate dehydrogenase level were identified in 61.1% (11/18), 68.4% (13/19), and 100% (12/12) of the patients, respectively. Nine patients (56.3%) were positive for EBV. Six patients (31.6%) developed HLH, of which two patients initially presented with relapsed/refractory HLH. DIC occurred in five patients (26.3%), three of which had experienced it before chemotherapy. Three patients had medullary masses. Detailed data can be found in Supplementary Table I.

The morphological, immunophenotypic, cytogenetic, and molecular biological characteristics of bone marrow in 19 children with ANKL are summarized as follows. First, the morphology of bone marrow was mainly characterized by the increase in the number of unidentified cells or large granular lymphocytes. The abnormal cells were irregular in shape, medium or large in size, and medium to rich in basophilic cytoplasm containing azurophil granules. The cytoplasm of some cells had vacuolization, pseudopodium-like apophyses, or tails. The shape of the nucleus was irregular, the karyoplasmic ratio was large, the chromatin was diffuse, and the nucleolus was visible. Second, abnormal cells mainly expressed CD56 (100%) and CD2 (76.9%), partially expressed CD7 (45.5%) and CD16 (40.0%). No abnormal cell population was detected by flow cytometry in two patients (10.5%), but the immunohistochemistry of solid tissues (testes or bone marrow) showed that CD56 was positive. Third, the cytogenetic analysis showed that ANKL mainly manifested complex karyotype (63.6%), but specific chromosomal abnormalities were lacking. Finally, no specific fusion genes or mutant genes were found. Detailed data can be found in Supplementary Tables II and III.

Ten out of the 19 children with ANKL underwent intensive combined chemotherapy based on L-ASP and/or anthracyclines. CR occurred in four patients (40%) and partial remission (PR) in two patients (20%). Two patients received allo-HSCT and achieved disease-free survival. Tumor lysis syndrome occurred in 10.5% of patients during the course of treatment. Four patients (21.1%) were lost at follow-up, and nine patients (60%) died. Detailed data can be found in Supplementary Table IV.

Thus, combining the condition and diagnostic process of our two patients, the diagnosis of ANKL mainly relies on the morphology and immunophenotype of bone marrow. When the diagnosis of ANKL is difficult, the following measures help confirm the diagnosis: (1) The pathology and immunohistochemistry of bone marrow or extramedullary mass biopsy[30], because the immunophenotype of abnormal NK cells in different sample types (bone marrow, peripheral blood, and lymph nodes) is consistent.[14] (2) For CD56-negative patients, CD94 and CD335 help in identifying NK cell tumors because both of them are highly expressed in NK cell tumors.[31,32]

Table I. General clinical characteristics of 19 enrolled ANKL patients.

Characteristics	No. of patients available	No. of abnormal patients (%)
Age, years		
Median (range): 14 (0.75-18)	19	
≥13		12 (63.2)[3,15,16,20-22,24,25,27,28]
<13		7 (36.8)[8,17-19,23,26,29]
Sex	19	
Male		14 (73.7)[3,8,16,18-21,23-26,28,29]
Female		5 (26.3)[15,17,22,27]
Country	19	
Asia		15 (78.9)[15-21,24-29]
Non-Asia		4 (21.1)[3,8,22,23]
Clinical manifestation		
Fever	19	17 (89.5)[3,8,16-24,26-29]
Anemia	19	13 (68.4)[8,16-20,22,25-27,29]
Hemorrhage	19	5 (26.3)[17,18,21,29]
Hepatomegaly	19	11 (57.9)[3,8,17,19,20,23,24,26-28]
Splenomegaly	19	17 (89.5)[3,8,16-19,21-29]
Lymphadenopathy	19	5 (26.3)[15,19,20,24,27]
Extramedullary mass	19	3 (15.8)[15,26]
Skin lesion	19	1 (5.3)[19]
Hyperleukocytosis (leukocyte count > 100×10^9/L)	19	1 (5.3)[8]
Leukopenia (leukocyte count < 4×10^9/L)	18	11 (61.1)[15-17,22,23,25,27-29]
Thrombocytopenia	19	13 (68.4)[3,16-18,22-25,27-29]
Elevated serum lactate dehydrogenase level	12	12 (100)[3,15-20,22,27,29]
Epstein-Barr virus positive	16	9 (56.3)[8,15,17,20,22,23,28]
Disseminated intravascular coagulation	19	5 (26.3)[3,17,21,24]
Hemophagocytic lymphohistiocytosis	19	6 (31.6)[3,17,23,28]
Central nervous system leukemia (newly diagnosed patients)	19	0 (0)
Bone marrow examination		
Morphology	19	17 (89.5)
Unidentified cells	17	11 (64.7)[8,15,16,19,20,23,26,27,29]
Large granular lymphocytes	17	4 (23.5)[17,18,22,28]
Immunophenotype	19	18 (94.7)
CD56	18	18 (100)[3,8,15-20,22-29]
CD2	13	10 (76.9)[3,17-20,22-24]
CD7	11	5 (45.5)[3,16,17,20,24]
CD16	10	4 (40)[3,20,22,24]
cCD3	11	3 (27.3)[23-25]
CD8	10	2 (20)[23,28]

ANKL, aggressive natural killer cell leukemia.

Table I. Continued.

Characteristics	No. of patients available	No. of abnormal patients (%)
Cytogenetics	19	11 (57.9)
Complex karyotype	11	7 (63.6)[17,22-24,27]
Common abnormal chromosomes		
Chromosome 8	11	4 (36.4)[17,22,26]
Chromosome 21	11	4 (36.4)[17,27]
Chromosome 7	11	3 (27.3)[17,23]
Molecular biology	19	5 (26.3)
Specific fusion gene	5	0 (0)
Specific mutant gene	5	0 (0)
Treatment	19	18 (94.7)
Chemotherapy based on L-asparaginase and/or anthracyclines	18	10 (55.6)[8,15,16,24-28]
Complete remission	10	4 (40)[24,25,27,28]
Partial remission	10	2 (20)[8]
Relapse	10	1 (10)[26]
Hematopoietic stem cell transplantation	18	2 (11.1)[28]
Tumor lysis syndrome	18	2 (11.1)[8]
Prognosis	19	15 (78.9)
Loss at follow-up	19	4 (21.1)[15,20,21,26]
Deaths	15	9 (60)[3,16-19,22,23,29]

ANKL, aggressive natural killer cell leukemia.

Combining the treatment of our two patients and the literature review mentioned above, the intensive combination chemotherapy based on L-ASP and anthracyclines may be considered for the initial treatment of ANKL to achieve a better response. A multicenter retrospective study in adults showed that 3 of 13 patients receiving chemotherapy based on anthracycline/anthraquinone achieved CR, compared with none of the 8 patients who received chemotherapy without anthracycline.[33] Meanwhile, the overall survival (OS) rate of patients receiving L-ASP-based combined chemotherapy significantly improved[6] because L-ASP was not affected by P-glycoprotein (encoded by *MDR1*, a multidrug resistance gene) highly expressed by NK cell tumor cells. In addition, the AIEOP-95 high-risk acute lymphoblastic leukemia (ALL) regimen[25] and ALL-BFM95 regimen[27], both based on L-ASP and anthracyclines, resulted in long-term disease-free survival.

In conclusion, HLH can serve as the initial manifestation of ANKL. The diagnosis of ANKL is challenging and depends mainly on the morphology and immunophenotype of bone marrow. Intensive combination chemotherapy based on asparaginase and anthracycline may be considered for ANKL.

Supplementary materials

Supplementary materials for this article are available online at https://doi.org/10.24953/turkjpediatr.2024.5072

Ethical approval

The study was approved by Medical Ethics Committee of Affiliated Hospital of Qingdao University (date: 28.07.2021, number: QYFY WZLL 26613 and date: 11.07.2023, number: QYFY WZLL 27941). Informed consent of our two cases was obtained from their parents.

Author contribution

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

Source of funding

The authors declares the study received no funding.

Conflict of interest

The authors declares that there is no conflict of interest.

REFERENCES

1. Chan JKC, Jaffe ES, Ko YH. Aggressive NK-cell leukaemia. In: Swerdlow SH, Campo E, Harris NL, et al, editors. WHO Classifification of Tumours of Haematopoietic and Lymphoid Tissues. Revised 4th ed. IARC: Lyon; 2017: 353-354.
2. Hue SS, Oon ML, Wang S, Tan SY, Ng SB. Epstein-Barr virus-associated T- and NK-cell lymphoproliferative diseases: an update and diagnostic approach. Pathology 2020; 52: 111-127. https://doi.org/10.1016/j.pathol.2019.09.011
3. Lepe-Zuniga JL, Jeronimo-Lopez FJ, Hernandez-Orantes JG, Mendez-Cigarroa AO. Non-EBV-related aggressive NK-Cell leukemia: an oncohematological great imitator. J Hematol 2018; 7: 163-166. https://doi.org/10.14740/jh462
4. Jin C, Bandovic J, Brody J, Hsu P, Sheikh-Fayyaz S, Zhang X. Aggressive natural killer cell leukemia mimicking interstitial lung diseases with the activation of the mitogen-activated protein kinase pathway. Int J Clin Exp Pathol 2018; 11: 5502-5508.
5. Qian WS, Shen L, Wulipan F, et al. Clinical characteristics and prognosis of patients with aggressive NK cell leukemia. Zhonghua Xue Ye Xue Za Zhi 2022; 43: 424-426. https://doi.org/10.3760/cma.j.issn.0253-2727.2022.05.013
6. Tang YT, Wang D, Luo H, et al. Aggressive NK-cell leukemia: clinical subtypes, molecular features, and treatment outcomes. Blood Cancer J 2017; 7: 660. https://doi.org/10.1038/s41408-017-0021-z
7. Fujimoto A, Ishida F, Izutsu K, et al. Allogeneic stem cell transplantation for patients with aggressive NK-cell leukemia. Bone Marrow Transplant 2021; 56: 347-356. https://doi.org/10.1038/s41409-020-01009-8
8. Rey-Helo E, Pérez-Velásquez RD, Cortés-Alva D. Agressive NK-cell leukemia in a pediatric patient. Bol Med Hosp Infant Mex 2019; 76: 188-192. https://doi.org/10.24875/BMHIM.19000089
9. Syrykh C, Péricart S, Lamaison C, Escudié F, Brousset P, Laurent C. Epstein-Barr virus-associated T- and NK-Cell lymphoproliferative diseases: a review of clinical and pathological features. Cancers (Basel) 2021; 13: 3315. https://doi.org/10.3390/cancers13133315
10. Ishida F. Aggressive NK-Cell leukemia. Front Pediatr 2018; 6: 292. https://doi.org/10.3389/fped.2018.00292
11. Guerreiro M, Príncipe F, Teles MJ, et al. CD56-negative aggressive NK cell leukemia relapsing as multiple cranial nerve palsies: case report and literature review. Case Rep Hematol 2017; 2017: 3724017. https://doi.org/10.1155/2017/3724017
12. Wang LP, Yu YP, Mei JG, Song P, An ZM, Zhou XG. Clinical and pathological characteristics of aggressive natural killer cell leukemia patients. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2021; 29: 1093-1100. https://doi.org/10.19746/j.cnki.issn.1009-2137.2021.04.012
13. Yang CF, Hsu CY, Ho DM. Aggressive natural killer (NK)-cell leukaemia and extranodal NK/T-cell lymphoma are two distinct diseases that differ in their clinical presentation and cytogenetic findings. Histopathology 2018; 72: 955-964. https://doi.org/10.1111/his.13463
14. Li Y, Wei J, Mao X, et al. Flow cytometric immunophenotyping is sensitive for the early diagnosis of de novo aggressive natural killer cell leukemia (ANKL): a multicenter retrospective analysis. PLoS One 2016; 11: e0158827. https://doi.org/10.1371/journal.pone.0158827
15. Gu Y, Guo FL, Yang L, Ma L. A case of natural killer cell leukemia with perineal mass as the main manifestation. J Leuk Lymph 2008; 17: 378-379. https://doi.org/10.3760/cma.j.issn.1009-9921.2008.05.023
16. Chen DD, Yu ZH, Guo Z, Chen HR, He XP. Aggressive NK cell leukemia: a case report and literature review. Chin J Clinicians (Electronic Edition) 2010; 4: 222-225. https://doi.org/10.3969/j.issn.1674-0785.2010.02.025
17. Liu ZQ, Liu R, Shi XD. Aggressive NK cell leukemia: a case report. Chin J Hematol 2010; 31: 359. https://doi.org/10.3760/cma.j.issn.0253-2727.2010.05.022
18. Zhu RM, Yang J. A case of childhood acute aggressive NK cell leukemia. Shenyang Budui Yiyao 2011; 24: 83.

19. Ma SX, Zhao K, Zhang Y. A case of childhood aggressive NK cell leukemia with rash as the main manifestation. Shandong Medical Journal 2012; 52: 102.

20. Wang XC, Ding KY, Wang ZH, Wang J, Wang XQ, Li Q. Clinical characteristics of five cases of aggressive NK cell leukemia. Shandong Medical Journal 2017; 57: 98-101. https://doi.org/10.3969/j.issn.1002-266X.2017.30.032

21. Wei XY, Huang KK, Huang HY, Cui LY, Zhang MJ. Two cases of aggressive NK cell leukemia complicated with multiple organ dysfunction syndrome. Guangdong Medical Journal 2017; 38: 1949. https://doi.org/10.3969/j.issn.1001-9448.2017.12.046

22. Skorupa A, Chaudhary UB, Lazarchick J. Cold agglutinin induced autoimmune hemolytic anemia and NK-cell leukemia: a new association. Am J Hematol 2007; 82: 668-671. https://doi.org/10.1002/ajh.20876

23. Petterson TE, Bosco AA, Cohn RJ. Aggressive natural killer cell leukemia presenting with hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2008; 50: 654-657. https://doi.org/10.1002/pbc.21358

24. Huang J, Ni W, Wang L, Zhou W, Jin J. A rare case of co-existent aggressive natural killer cell and acute monocytic leukemia with cytomegalovirus infection. Int J Hematol 2008; 87: 553-556. https://doi.org/10.1007/s12185-008-0078-3

25. Patel AP, Ghatak SB, Patel JA. Long term survival in aggressive NK cell leukemia. Indian Pediatr 2010; 47: 807-808. https://doi.org/10.1007/s13312-010-0108-0

26. Yadav SP, Kharya G. Aggressive natural cell leukemia in an infant with bilateral testicular mass. Indian Pediatr 2011; 48: 79-80.

27. Park JA, Jun KR, Nam SH, Ghim TT. Favorable outcome in a child with EBV-negative aggressive NK cell leukemia. Int J Hematol 2013; 97: 673-676. https://doi.org/10.1007/s12185-013-1319-7

28. Kim BK, Hong KT, Kang HJ, et al. Successful treatment of pediatric Epstein-Barr virus-positive aggressive natural killer-cell leukemia. J Pediatr Hematol Oncol 2019; 41: e336-e337. https://doi.org/10.1097/MPH.0000000000001237

29. Pan M, Huang JX, Wu Q, et al. Misdiagnosis of invasive NK cell leukemia in children: a case report. J China Pediatr Blood Cancer 2020; 25: 236-238. https://doi.org/10.3969/j.issn.1673-5323.2020.04.013

30. Gao LM, Zhao S, Liu WP, et al. Clinicopathologic characterization of aggressive natural killer cell leukemia involving different tissue sites. Am J Surg Pathol 2016; 40: 836-846. https://doi.org/10.1097/PAS.0000000000000634

31. Fang H, Wang W, Kadia TM, El Hussein S, Wang SA, Khoury JD. CD94 expression patterns in reactive and neoplastic T-cell and NK-cell proliferations. Leuk Res 2021; 108: 106614. https://doi.org/10.1016/j.leukres.2021.106614

32. Freud AG, Zhao S, Wei S, et al. Expression of the activating receptor, NKp46 (CD335), in human natural killer and T-cell neoplasia. Am J Clin Pathol 2013; 140: 853-866. https://doi.org/10.1309/AJCPWGG69MCZOWMM

33. Suzuki R, Suzumiya J, Nakamura S, et al. Aggressive natural killer-cell leukemia revisited: large granular lymphocyte leukemia of cytotoxic NK cells. Leukemia 2004; 18: 763-770. https://doi.org/10.1038/sj.leu.2403262

The Turkish Journal of Pediatrics 2024; 66: 490-498
https://doi.org/10.24953/turkjpediatr.2024.4702

Case Report

A case with short stature and proteinuria: atypical presentation of a family with m.3243A>G mutation

Gönül Büyükyılmaz[1], Mihriban İnözü[2], Büşranur Çavdarlı[3]

[1]Department of Pediatric Endocrinology, Ankara Bilkent City Hospital, Ankara; [2]Department of Pediatric Nephrology, Ankara Bilkent City Hospital, Ankara; [3]Department of Medical Genetics, Ankara Bilkent City Hospital, Ankara, Türkiye.

ABSTRACT

Background. The mitochondrial DNA (mtDNA) m.3243A>G mutation is one of the most common pathogenic mtDNA variants. The phenotypes associated with this mutation range from asymptomatic induviduals to well-defined clinical syndromes, or non-syndromic mitochondrial disorders. Variable clinical features in pediatric cases may cause difficulty in diagnosis. Kidney involvement in this mutation is uncommon and reported on a case-by-case basis. Here, we report on a patient with m.3243A>G mutation, who presented with short stature and proteinuria, and his family, who share the same genotype but exhibit different heteroplasmy levels in different tissues and variable phenotypes.

Case presentation. A 15-year-old male patient was admitted to the pediatric endocrinology department with short stature. His examinations revealed nephrotic range proteinuria, hearing loss, impaired glucose tolerance, and Wolf-Parkinson-White syndrome. From family history, it was learned that diabetes mellitus (DM) and progressive sensorineural hearing loss were common in this family. The patient's mother, who had chronic kidney disease, DM, and hearing loss, had died suddenly for an unknown reason. Considering the family history, a genetic analysis was performed for mitochondrial disease. Mitochondrial DNA analysis revealed a m.3243A>G mutation with 47% heteroplasmy in blood, 62% heteroplasmy in buccal cells, and 96% heteroplasmy in urothelial cells in our patient.

Conclusions. Short stature without any other complaint and renal involvement are rare findings in m.3243A>G mutation. In patients presenting with proteinuria, in the presence of conditions affecting many systems such as endocrine system pathologies, hearing loss, and cardiac pathologies, and in the presence of individuals with a similar family history of multiple organ involvement, mitochondrial diseases should be considered, and examined from this perspective. Our case illustrates the value of a detailed medical and family history.

Key words: maternally inherited diabetes and deafness, mitochondrial disease, m.3243A>G, proteinuria, short stature.

Short stature, one of the most common reasons for referral to pediatric clinics, resulting from reduced growth plate chondrogenesis may be due to growth plate-specific factors or factors elsewhere in the body that may affect the growth plate.[1] Therefore, patients with short stature should be carefully evaluated. Urinalysis is also recommended for evaluation.[2] Proteinuria is a common finding in primary care practice. Abnormal findings resolve in repeated tests in most children with proteinuria, but if proteinuria persists, further investigation is required. One of the important and rare causes of proteinuria is mitochondrial disease.[3]

Patients with mitochondrial diseases may present with a broad clinical spectrum. However, in mitochondrial diseases, some organs with a high energy demand, such as the nervous system, and skeletal and cardiac muscles are expected to be more affected as a result of insufficient ATP production to meet energy demands.[4] Mutations in mitochondrial DNA (mtDNA) or mitochondria-associated nuclear DNA (nDNA) genes can cause mitochondrial diseases. Thus, mitochondrial

✉ Gönül Büyükyılmaz
gonulgulal@hotmail.com

Received 4th May 2024, revised 30th Jul 2024, 13th Aug 2024, accepted 27th Aug 2024.

diseases can exhibit maternal inheritance attributable to mtDNA mutations; autosomal or X-linked inheritance associated with nDNA mutations, and a pattern of inheritance associated with de novo mutations that can occur in both genomes.[5] Pathogenic variants of mtDNA usually only affect a proportion of mtDNA molecules (heteroplasmy).[6] This means the presence of a mixture of mutated and wild-type mitochondrial genomes within individual cells. Since the range of heteroplasmy can vary from cell to cell, organ to organ, and individual to individual, clinical variability can be observed even with the same mtDNA mutation.[7] Although the mutant load at birth may be similar in different tissues, this changes with age progresses. Postmitotic cells, such as skeletal muscle or urinary epithelial cells, and mitotic cells, such as hair follicles and buccal mucosa, tend to have higher and more stable levels of heteroplasmy with age. Blood cells generally have the lowest levels of heteroplasmy.[8,9]

The mtDNA *MT-TL1* m.3243A>G mutation, one of the most common pathogenic mtDNA variants, presents with complex genetic, pathogenic molecular mechanisms and phenotypes.[9] The first identified and best described m.3243A>G-associated phenotype is mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). In 1990, the m.3243A>G mutation was found in the *MT-TL1* gene that encodes mitochondrial leucine transfer RNA 1.[10] More than 16 in 100,000 individuals carry the m.3243A>G mutation. Carriers of the m.3243A>G mutation are clinically heterogeneous. Its phenotypes range from asymptomatic to well-defined clinical syndromes, or non-syndromic-mitochondrial disorders. Well-defined syndromes are MELAS, myoclonic epilepsy and ragged-red fibers (MERRF), maternally inherited diabetes and deafness (MIDD), and chronic progressive external ophthalmoplegia. Hearing loss, short stature, nephropathy, underweight, enteromyopathy, hypertrophic cardiomyopathy, and cluster headaches are non-syndromic-mitochondrial disorders.[9,11-15]

Sometimes, due to atypical presentation, diagnosis and classification may be difficult in mitochondrial diseases. Here, a patient with a *MT-TL1* m.3243A>G mutation, who presented with short stature and proteinuria, and his family, who have the same genotype with different heteroplasmy levels in different tissues and variable phenotypes, are reported.

Case Presentation

A 15-year-old male patient (III-2 in Fig. 1) was referred to our endocrine department with a complaint of short stature. The patient was born at term, with a birth weight of 3,000 g, from a nonconsanguineous marriage. His neuromotor development was consistent with his peers. His family history included individuals with diabetes mellitus (DM), sensorineural hearing loss, chronic kidney disease, stroke, psychosis, and sudden death from an unknown cause (Table I) (Fig. 1). When the patient applied to the endocrinology department, his body weight was measured as 38.4 kg (–2.94 standard deviation score [SDS]), his height was 154 cm (–2.3 SDS), and body mass index (BMI) was 16.1 kg/m² (–2.2 SDS). Bone age was 15 years. Blood pressure was 110/65 mmHg. The patient was Tanner stage V and other systemic examinations including the neurological system were normal. No edema was detected. The laboratory test results were compatible with impaired glucose tolerance, abnormal urinalysis with proteinuria, normal renal, and liver functional tests, and electrolytes (Table II). Thyroid function tests, insulin-like growth factor-1, and insulin-like growth factor binding protein-3 levels checked for short stature were normal. There was no acidosis or alkalosis. No pathology was detected in the examinations performed for short stature. No further examination was performed because the epiphyses were closed. When questioned about polyuria, polydipsia, and weight loss, it was learned that he had no additional complaints. The patient was negative for type-1 DM autoantibodies. Kidney ultrasonography and

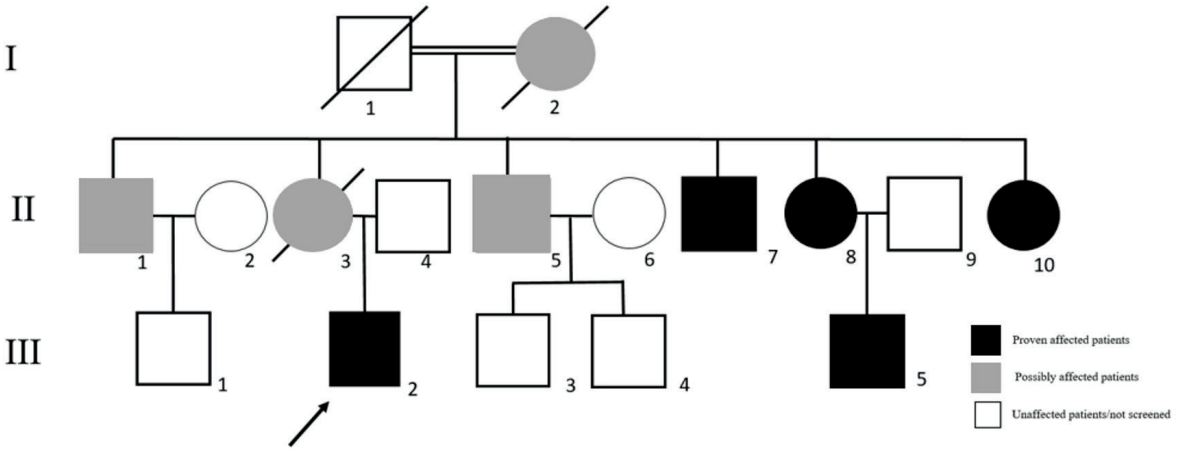


Fig. 1. Pedigree of this family with *MT-TL1* (NC_012920.1:3230_3304): m.3243A>G mutation. Circles, female patients; squares, male patients. Solid symbols represent individuals harboring the m.3243A>G mutation; shaded symbols represent individuals with a clinically suggestive history but no genetic confirmation; open symbols represent unaffected individuals. Roman numerals indicate the generation and the Arabic numerals indicate the individual. A line through the symbols indicates that the subject is deceased. Since the family history of the upper generations in the pedigree is not informative for the disease, it was not drawn. Maternal grandparents of the index case (individuals I-1 and I-2) were first-cousins.

Table I. General characteristics of the family members.

Patient no (see Fig. 1)	Sex	Age at diagnosis (years)	Short stature	Malnutrition	Proteinuria	Chronic kidney disease	Diabetes mellitus	Wolf Parkinson White syndrome	Muscle cramps	Stroke and seizures	Progressive sensorineural hearing loss	Psychosis	Other features
I-2	F	Death at 66				+	+				+		
II-1	M	48					+				+		
II-3	F	Death at 46				+	+				+		
II-5	M	45					+						
II-7	M	42						+					
II-8	F	41									+	+	
II-10	F	40	+	+			+		+	+	+		
III-2	M	15	+	+	+			+			+		IGT
III-5	M	17	+	+								+	

IGT: impaired glucose tolerance

Doppler ultrasonography were normal. Hearing test revealed mild bilateral sensorineural hearing loss. The ophthalmic examination was normal. It was learned that the genetic analysis ordered with the preliminary diagnosis of Alport due to hearing loss and proteinuria was normal.

Considering the family history, the mitochondrial whole genome was sequenced via next generation sequencing system (Myseq, Illumina®) with the DNA extracted from peripheral lymphocytes. A missense known variant on *MT-TL1* gene, m.3243A>G was found with a 47% heteroplasmy level in blood.

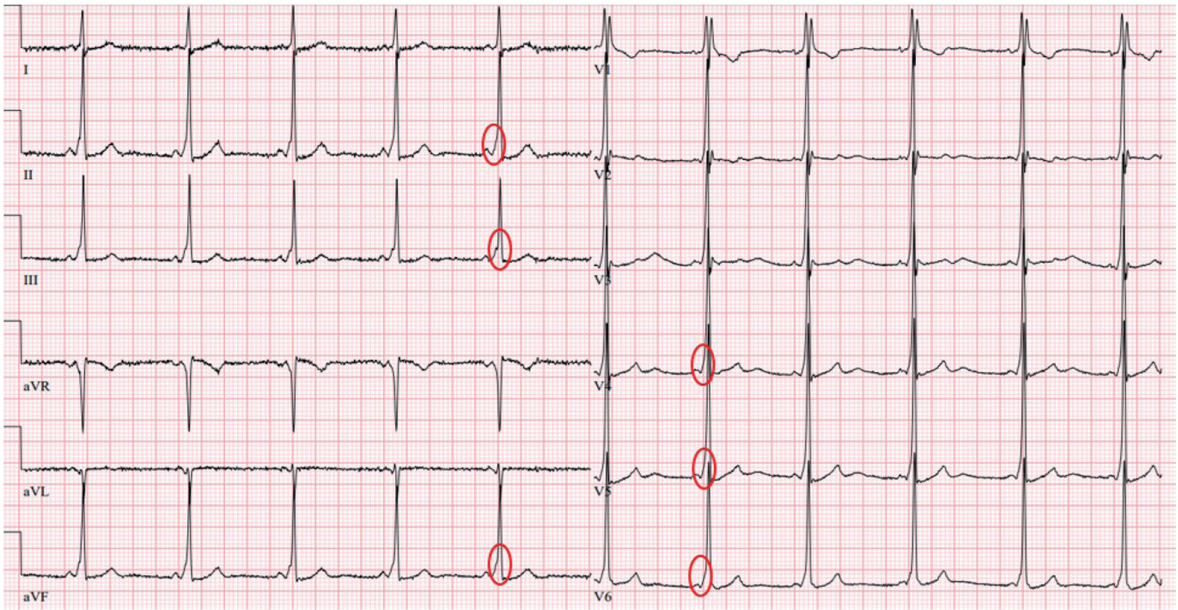


Fig. 2. Electrocardiogram of index case (III-2) with Wolff-Parkinson-White pattern. Elliptical red markings highlight the delta waves.

On cardiac examination, the patient had normal echocardiographic findings but was found to have Wolff-Parkinson-White syndrome (Fig. 2). He underwent radiofrequency ablation (RFA) for Wolff-Parkinson-White syndrome. ECG findings resolved after RFA and no any residual ECG abnormalities were detected again during the follow-up.

The patient was also evaluated by the Divisions of Pediatric Neurology and Metabolism. Metabolic investigations involving very long-chain fatty acids, free carnitine, urinary organic acids, urinary and plasma amino acids, pyruvic acid, and ammonia levels were normal. The laboratory analyses revealed intermittently mildly elevated lactate (27.9-28.9 mg/dL, controls < 19.8), and the creatine kinase (CK) level was found to be high once, but the repeat tests were found to be within the normal range (Table II). Brain magnetic resonance imaging (MRI) showed that the 4th ventricle was larger than normal, cerebellar folia were enlarged incompatible with the patient's age, and perimedullary-pericerebellar extra-axial cerebrospinal fluid spaces were enlarged. Magnetic resonance spectroscopy was found normal. As a result of all these evaluations, a treatment consisting of enalapril and coenzyme Q_{10} as well as thiamine, riboflavin, and carnitine was initiated. The patient, who has been followed for 2 years, has had no additional complaints, and the latest laboratory values are given in Table II. During follow-up, nephrotic-range proteinuria was detected in the patient.

Other family members were referred to relevant departments for clinical and genetic assessment. The same m.3243A>G heteroplasmic pathogenic variant in the *MT-TL1* gene was detected with the mitochondrial genome sequencing from peripheral lymphocytes in some family members whereas some did not have it (Table III). Some family members could not be evaluated because they were either deceased or unwilling to participate in the study. It was learned that the aunt's (II-10) hearing loss became apparent at the age of 18 and a cochlear implant was placed at the age of 25. She was diagnosed with DM at the age of 18 and ordered for genetic analysis for maturity-onset diabetes of the young (*HNF1A, HNF4A, HNF1B* and *GCK* gene), which was found to be normal. The aunt (II-10) had a stroke and seizure at the

Table II. The laboratory findings of the case

Parameters	On admission	Second visit	Latest visit	Reference ranges
Fasting plasma glucose (mg/dL)	89	75	72	<100
2. hour glucose during an OGTT (mg/dL)	151	NA	NA	<140
HbA1c (%)	5.6	5.1	5.2	<5.7
Total cholesterol (mg/dL)	NA	159	NA	107-202
HDL (mg/dL)		50		>40
LDL (mg/dL)		89		<100
TG (mg/dL)		102		47-210
CK (U/L)	553	123	108	32-294
Na/K/Cl (mEq/L)	137/4.7/100	138/4/99	138/4.1/101	132–146/3.5–5.5/98–109
Urea (mg/dL)	39	34	39	17-43
Creatinine (mg/dL)	0.66	0.67	0.63	0.67-1.25
eGFR (ml/min/1.73 m²)	97	95	102	>90
Uric acid (mg/dL)	5.2	5.2	4.7	3.7-9.2
Albumin (g/L)	48	45	46	32-48
Blood pH	7.35	7.35	7.38	7.35-7.45
Bicarbonate (mmol/L)	26.3	24.3	23.8	21-26
Lactate (mg/dL)	27.9	23.3	28.9	4.5-19.8
Urine pH	6	5.5	5.5	4.5-8
Urine protein	3+	3+	3+	Negative
Spot urine protein/creatinine (mg/mg)	1.7	2.3	3.5	<0.2
24-hour urinary protein (mg/m²/hour)	21	42	44.1	<4
24-hour urine microalbumin (mg/day)	422	810	882	<30
Spot urine beta-2 microglobulin (mg/L)	NA	0.19	0.16	0-0.2
24-hour urinary beta-2 microglobulin (mg/day)	0.36	NA	NA	0.03-0.37

CK, creatine kinase; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OGTT, oral glucose tolerance test; TG, tryglyseride. e-GFR was calculated using the modified Schwartz formula.

age of 39. In laboratory results, renal functional tests, electrolytes, urinalysis, lactate, and CK were found to be normal. HbA1c was 6.7% (normal: <5.7%). The cardiological evaluation was normal. Our patient's cousin (III-5) was 17 years old and was evaluated in our clinic. His height was 159 cm (–2.4 SDS), and his BMI was 17.4 kg/m² (–2.3 SDS). Systemic examinations including the neurological system were normal. In laboratory results, renal functional tests, electrolytes, urinalysis, lactate, CK, and cardiological evaluation were normal.

Finally, considering the heteroplasmic distribution of the mitochondrial variants among different tissues, mitochondrial genome analyses for the known pathogenic variant were repeated using the DNA extracted from buccal swabs and urine in the patients who accepted to give samples. The variant was detected with a 72% heteroplasmy level from the urine sample of the 42-year-old uncle (patient II-7) with a history of Wolff-Parkinson-White syndrome, whose blood and buccal samples were normal (Table III).

Table III. Heteroplasmy levels at different tissues of the *MT-TL1* gene m.3243A>G variant in family members

Patient number	Blood (%) / Reading depth	Buccal cells (%) / Reading depth	Urothelial cells (%) / Reading depth
II-7	Normal	Normal	71 / 452
II-8	30 / 2736	NA	NA
II-10	36 / 1756	35 / 341	55 / 162
III-2 (index)	47 / 955	62 / 596	96 / 999
III-5	52 / 1273	NA	NA

NA, not available.

Discussion

The mutation m.3243A>G in the mitochondrial *MT-TL1* gene results in a heterogeneous disorder due to its significant overlap and diverse spectrum of clinical presentations. We describe the phenotypic features and molecular diagnosis of a family with *MT-TL1* m.3243A>G mutation. The presenting symptoms of the proband in the family were short stature and proteinuria, which were reported less frequently. In contrast, the symptoms were distributed in a wide range of DM, chronic kidney disease, Wolff-Parkinson-White syndrome, sensorineural hearing loss, psychosis, stroke to sudden death history in the current family. It was learned that although there were multiple affected individuals in the family and they were followed up in different departments, and they were never examined for mitochondrial disease. This case highlights the atypical presentation of *MT-TL1* gene m.3243A>G mutation in children, the importance of detailed family history, and the possibility of metabolic disease in cases of unexplained multisystem involvement.

The nervous system is the most affected system in patients with m.3243A>G, and the other affected tissues or organs are skeletal muscles, heart muscles, ears, eyes, kidneys, liver, and the endocrine system.[16] The clinical features of m.3243A>G mutation have been described in several reports. Xia et al.[17] evaluated one hundred pediatric patients with symptomatic mitochondrial disease harboring the m.3243A>G mutation. They found that seizure (76%) and short stature (73%) were the most common symptoms. They also showed that elevated plasma lactate (70%), weight loss (69%), abnormal MRI changes (68%), vomiting (55%), decreased vision (52%), headache (50%), and muscle weakness (48%) were other symptoms. In their study, they reported that while most of the patients were multi-symptomatic, only two patients had one symptom, and five patients manifested two symptoms.[17] In another study evaluating 35 patients with the m.3243A>G mutation, short stature was detected in 22.9% of the cases.[18] Short stature is frequently observed in individuals who carry the m.3243A>G mutation. Nevertheless, our case, who presented no symptoms and was identified only based on short stature during the investigation, has not been documented in the literature. Abnormally low body weight and stunting have also been observed in individuals with mitochondrial diseases.[19] Although the etiology of growth retardation is not fully known, it is thought that it may be related to many medical comorbidities such as central nervous system, cardiac, gastrointestinal and renal diseases beyond endocrinopathies. It should also be noted that it may be due to growth hormone deficiency.[18]

The other endocrine system involvements in the m.3243A>G mutation include the pancreas, thyroid, parathyroid, pituitary, and gonads.[20] MIDD is the most common phenotype involving the endocrine system.[21] Clinical characteristics related to MIDD include early onset DM with short stature, a normal or low BMI, and bilateral hearing impairment that occurs at about the same time as the onset of DM.[22] Although patients may present differently, insulin-dependent DM and sensorineural

hearing loss usually occur by the age of 30-40.[23] Cardiomyopathy, neuropsychiatric disorders, myopathy, and renal dysfunction are other clinical comorbidities associated with this disease.[23] Another study showed that the m.3243A>G mutation may be a contributing genetic factor in the development of end-stage kidney disease in patients with diabetes.[24] DM, kidney disease, and hearing loss were common in the family who had never been evaluated for mitochondrial disease. Our case illustrates the value of a detailed medical and family history as it can help in the diagnosis of mitochondrial diseases on clinical suspicion alone.

Since mitochondria are also abundant in the kidneys, mitochondrial dysfunction can also cause renal damage. Kidney involvement should not be overlooked as it has a potential impact on morbidity and mortality.[25] Patients with MELAS have been reported to have worsening renal function and progression into end-stage renal disease.[26] Yang et al. found the prevalence of nephropathy to be 13% in their study.[14] Previous reports have shown that kidney involvement in carriers of the m.3243A>G mutation is usually in the form of Fanconi syndrome or focal segmental glomerulosclerosis (FSGS).[26,27] These patients usually present with signs of proximal tubular dysfunction and nephrotic-range proteinuria. Since tubular function has a high energy demand, tubular dysfunction is the most common renal symptom in mitochondrial diseases.[28] Fanconi syndrome is believed to be caused by impaired activity of ATP-dependent sodium-potassium pumps, which are essential in the tubular reabsorption process.[25] Previous studies have found abnormal mitochondrial build-up in podocytes in the kidneys of patients with FSGS-associated mitochondrial disease. These findings have been attributed to a compensatory increase in mitochondria due to mitochondrial dysfunction. Mitochondrial dysfunction in podocytes leads to damage in glomerular epithelial cells, resulting in proteinuria and ultimately glomerulosclerosis.[28] Accurate identification of the mitochondrial origin of renal disease in these patients is essential for the selection of adequate treatment and appropriate supportive care. Although genetic analysis could not be performed because our patient's mother and grandmother died, it is thought that their chronic kidney disease may have been due to the m.3243A>G mutation. Kidney function tests and blood pressure monitoring of this family should be performed regularly.

Our patient had bilateral mild sensorineural hearing loss in addition to proteinuria. Sensorineural hearing loss that accompanies renal disease initially suggests Alport syndrome. But haematuria is a key feature of Alport syndrome in contrast to m.3243A>G associated nephropathy. Sensorineural hearing loss in patients with m.3243A>G mutation often begins gradually, occurs bilaterally, and may become severe over time and men are affected more than women.[29] In a study 238 cases of m.3243A>G were evaluated, and hearing impairment was detected in 81% of the cases.[30]

Another system affected in our patient was the cardiovascular system. Cardiac manifestations in m.3243A>G mutation patients are common and serious. Although hypertrophic cardiomyopathy is the most commonly reported cardiovascular disease in patients with m.3243A>G mutation, cardiac conduction disorders such as Wolff-Parkinson-White syndrome have also been reported.[31] Sudden adult death syndrome is a frequent occurrence in patients with m.3243A>G[32] so it is important to conduct regular cardiac arrhythmia surveillance and cardiac echocardiography in m.3243A>G mutation carriers.

In our case report, mutation load was also investigated in different tissues in some family members. We found that the proportion of mutant genomes was markedly higher in the DNA from urothelial cells than in the DNA from blood and buccal cells. While the uncle (patient II-7) had no detectable mutant genomes in blood and buccal cells, these were present in urothelial cells. This is related to the fact that, as we have emphasized before, urine

epithelial cells tend to have higher and more stable heteroplasmy levels than blood cells with increasing age.[8] This finding suggests that when the index of suspicion for a mitochondrial DNA mutation is high, DNA from multiple tissues should be screened.

In conclusion, the current family showed intra-familial clinical diversity. Maternal family history of chronic kidney disease, DM, stroke, psychosis, and hearing loss in some of the family members led to a suspicion of mitochondrial disease. While patients without typical findings are generally diagnosed with mitochondrial diseases based on screening results due to their relatives, in our family, the entire family was diagnosed after the patient who presented with atypical findings was diagnosed. It should be remembered that only short stature may be the first clinical symptom at presentation in these patients. Our case illustrates the value of a detailed medical and family history.

Ethical approval

We have obtained informed consent from the patient's parent or guardian and family members to publish this case report as it includes a detailed family history.

Author contribution

Study conception and design: GB, Mİ, BÇ; data collection: GB, Mİ, BÇ; analysis and interpretation of results: GB, Mİ, BÇ; draft manuscript preparation: GB, Mİ, BÇ. 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.

REFERENCES

1. Baron J, Sävendahl L, De Luca F, et al. Short and tall stature: a new paradigm emerges. Nat Rev Endocrinol 2015; 11: 735-746. https://doi.org/10.1038/nrendo.2015.165
2. Braun LR, Marino R. Disorders of growth and stature. Pediatr Rev 2017; 38: 293-304. https://doi.org/10.1542/pir.2016-0178
3. Viteri B, Reid-Adam J. Hematuria and proteinuria in children. Pediatr Rev 2018; 39: 573-587. https://doi.org/10.1542/pir.2017-0300
4. El-Hattab AW, Adesina AM, Jones J, Scaglia F. MELAS syndrome: clinical manifestations, pathogenesis, and treatment options. Mol Genet Metab 2015; 116: 4-12. https://doi.org/10.1016/j.ymgme.2015.06.004
5. Gorman GS, Chinnery PF, DiMauro S, et al. Mitochondrial diseases. Nat Rev Dis Primers 2016; 2: 16080. https://doi.org/10.1038/nrdp.2016.80
6. Schon KR, Ratnaike T, van den Ameele J, Horvath R, Chinnery PF. Mitochondrial diseases: a diagnostic revolution. Trends Genet 2020; 36: 702-717. https://doi.org/10.1016/j.tig.2020.06.009
7. Grady JP, Pickett SJ, Ng YS, et al. mtDNA heteroplasmy level and copy number indicate disease burden in m.3243A>G mitochondrial disease. EMBO Mol Med 2018; 10: e8262. https://doi.org/10.15252/emmm.201708262
8. Shanske S, Pancrudo J, Kaufmann P, et al. Varying loads of the mitochondrial DNA A3243G mutation in different tissues: implications for diagnosis. Am J Med Genet A 2004; 130A: 134-137. https://doi.org/10.1002/ajmg.a.30220
9. Li D, Liang C, Zhang T, et al. Pathogenic mitochondrial DNA 3243A>G mutation: from genetics to phenotype. Front Genet 2022; 13: 951185. https://doi.org/10.3389/fgene.2022.951185
10. Goto Y, Nonaka I, Horai S. A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 1990; 348: 651-653. https://doi.org/10.1038/348651a0
11. van den Ouweland JM, Lemkes HH, Ruitenbeek W, et al. Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. Nat Genet 1992; 1: 368-371. https://doi.org/10.1038/ng0892-368
12. Sotiriou E, Coku J, Tanji K, Huang HB, Hirano M, DiMauro S. The m.3244G>A mutation in mtDNA is another cause of progressive external ophthalmoplegia. Neuromuscul Disord 2009; 19: 297-299. https://doi.org/10.1016/j.nmd.2009.01.014

13. Shen X, Du A. The non-syndromic clinical spectrums of mtDNA 3243A>G mutation. Neurosciences (Riyadh) 2021; 26: 128-133. https://doi.org/10.17712/nsj.2021.2.20200145

14. Yang M, Xu L, Xu C, et al. The mutations and clinical variability in maternally inherited diabetes and deafness: an analysis of 161 patients. Front Endocrinol (Lausanne) 2021; 12: 728043. https://doi.org/10.3389/fendo.2021.728043

15. Fabrizi GM, Cardaioli E, Grieco GS, et al. The A to G transition at nt 3243 of the mitochondrial tRNALeu(UUR) may cause an MERRF syndrome. J Neurol Neurosurg Psychiatry 1996; 61: 47-51. https://doi.org/10.1136/jnnp.61.1.47

16. El-Hattab AW, Almannai M, Scaglia F. MELAS. In: Adam MP, Feldman J, Mirzaa GM, et al, editors. GeneReviews(®). Seattle, WA: University of Washington, Seattle; 1993.

17. Xia CY, Liu Y, Liu H, Zhang YC, Ma YN, Qi Y. Clinical and molecular characteristics in 100 chinese pediatric patients with m.3243A>G mutation in mitochondrial DNA. Chin Med J (Engl) 2016; 129: 1945-1949. https://doi.org/10.4103/0366-6999.187845

18. Chin J, Marotta R, Chiotis M, Allan EH, Collins SJ. Detection rates and phenotypic spectrum of m.3243A>G in the MT-TL1 gene: a molecular diagnostic laboratory perspective. Mitochondrion 2014; 17: 34-41. https://doi.org/10.1016/j.mito.2014.05.005

19. Wolny S, McFarland R, Chinnery P, Cheetham T. Abnormal growth in mitochondrial disease. Acta Paediatr 2009; 98: 553-554. https://doi.org/10.1111/j.1651-2227.2008.01148.x

20. Al-Gadi IS, Haas RH, Falk MJ, Goldstein A, McCormack SE. Endocrine disorders in primary mitochondrial disease. J Endocr Soc 2018; 2: 361-373. https://doi.org/10.1210/js.2017-00434

21. Robinson KN, Terrazas S, Giordano-Mooga S, Xavier NA. The role of heteroplasmy in the diagnosis and management of maternally inherited diabetes and deafness. Endocr Pract 2020; 26: 241-246. https://doi.org/10.4158/EP-2019-0270

22. Suzuki S, Oka Y, Kadowaki T, et al. Clinical features of diabetes mellitus with the mitochondrial DNA 3243 (A-G) mutation in Japanese: maternal inheritance and mitochondria-related complications. Diabetes Res Clin Pract 2003; 59: 207-217. https://doi.org/10.1016/s0168-8227(02)00246-2

23. Guillausseau PJ, Massin P, Dubois-LaForgue D, et al. Maternally inherited diabetes and deafness: a multicenter study. Ann Intern Med 2001; 134: 721-728. https://doi.org/10.7326/0003-4819-134-9_part_1-200105010-00008

24. Iwasaki N, Babazono T, Tsuchiya K, et al. Prevalence of A-to-G mutation at nucleotide 3243 of the mitochondrial tRNA(Leu(UUR)) gene in Japanese patients with diabetes mellitus and end stage renal disease. J Hum Genet 2001; 46: 330-334. https://doi.org/10.1007/s100380170068

25. Sproule DM, Kaufmann P. Mitochondrial encephalopathy, lactic acidosis, and strokelike episodes: basic concepts, clinical phenotype, and therapeutic management of MELAS syndrome. Ann N Y Acad Sci 2008; 1142: 133-158. https://doi.org/10.1196/annals.1444.011

26. Seidowsky A, Hoffmann M, Glowacki F, et al. Renal involvement in MELAS syndrome - a series of 5 cases and review of the literature. Clin Nephrol 2013; 80: 456-463. https://doi.org/10.5414/CN107063

27. Alcubilla-Prats P, Solé M, Botey A, Grau JM, Garrabou G, Poch E. Kidney involvement in MELAS syndrome: description of 2 cases. Med Clin (Barc) 2017; 148: 357-361. https://doi.org/10.1016/j.medcli.2017.01.029

28. Güçer S, Talim B, Aşan E, et al. Focal segmental glomerulosclerosis associated with mitochondrial cytopathy: report of two cases with special emphasis on podocytes. Pediatr Dev Pathol 2005; 8: 710-717. https://doi.org/10.1007/s10024-005-0058-z

29. Manwaring N, Jones MM, Wang JJ, et al. Population prevalence of the MELAS A3243G mutation. Mitochondrion 2007; 7: 230-233. https://doi.org/10.1016/j.mito.2006.12.004

30. Pickett SJ, Grady JP, Ng YS, et al. Phenotypic heterogeneity in m.3243A>G mitochondrial disease: the role of nuclear factors. Ann Clin Transl Neurol 2018; 5: 333-345. https://doi.org/10.1002/acn3.532

31. Niedermayr K, Pölzl G, Scholl-Bürgi S, et al. Mitochondrial DNA mutation "m.3243A>G"-Heterogeneous clinical picture for cardiologists ("m.3243A>G": A phenotypic chameleon). Congenit Heart Dis 2018; 13: 671-677. https://doi.org/10.1111/chd.12634

32. Ng YS, Grady JP, Lax NZ, et al. Sudden adult death syndrome in m.3243A>G-related mitochondrial disease: an unrecognized clinical entity in young, asymptomatic adults. Eur Heart J 2016; 37: 2552-2559. https://doi.org/10.1093/eurheartj/ehv306

The Turkish Journal of Pediatrics 2024; 66: 499-504
https://doi.org/10.24953/turkjpediatr.2024.4817

Case Report

Disseminated cryptococcosis in a child with liver transplantation: a case report

Doğan Barut[1], Bora Kunay[1], Sema Yıldırım Arslan[2], Ayça Aydın Uysal[3], Zümrüt Şahbudak Bal[2], Pınar Yazıcı[4], Miray Karakoyun[1], Sema Aydoğdu[1]

[1]Division of Gastroenterology, Hepatology and Nutrition Disease, Department of Pediatrics, Medical School of Ege University, İzmir; [2]Division of Infectious Disease, Department of Pediatrics, Medical School of Ege University, İzmir; [3]Department of Microbiology, Medical School of Ege University, İzmir; [4]Division of Pediatric Intensive Care, Department of Pediatrics, Medical School of Ege University, İzmir, Türkiye.

ABSTRACT

Background. *Cryptococcus neoformans* causes cryptococcosis, primarily affecting immunocompromised individuals, including solid-organ transplant recipients, and, less frequently, immunocompetent people.

Case. A 15-year-old male with congenital hepatic fibrosis, portal hypertension, and cirrhosis underwent orthotopic liver transplantation. He received perioperative antimicrobial and antifungal prophylaxis and continued immunosuppressive treatment. Thirty months post-transplant, he presented with fever, hypertension, and sacroiliac joint pain. Peripheral blood cultures showed *C. neoformans*, confirmed by pan-fungal polymerase chain reaction assay and latex agglutination tests. Despite initial treatment with intravenous (IV) fluconazole, his condition worsened, necessitating intubation for acute hypoxic respiratory failure. Magnetic resonance imaging and computed tomography scans indicated disseminated cryptococcosis with lymphadenitis, possible meningitis, and pneumonia. Treatment was escalated to IV liposomal amphotericin B and 5-flucytosine, while reducing immunosuppressive treatment. Despite negative fungal cultures on the tenth day, the patient deteriorated, developing pancreatitis, pneumonia, and massive gastrointestinal bleeding, leading to death on the 35th day of hospitalization.

Conclusion. This case shows the severity and complexity of managing disseminated cryptococcosis in pediatric liver transplant recipients. Aggressive therapy and early identification are essential for improving outcomes in these high-risk patients.

Key words: cryptococcosis, liver transplantation, immunocompromised patients.

Opportunistic fungal infections, such as cryptococcosis, primarily impact immunocompromised and rarely immunocompetent patients. *Cryptococcus neoformans* is the most common species, and inhalation is the primary route of infection.[1] The infection is typically related to immunosuppression in patients who have had solid organ transplants (SOT) and hematopoietic progenitor transplants.[2]

✉ Miray Karakoyun
miraykarakoyun@hotmail.com

Received 6th Jun 2024, revised 4th Jul 2024, 19th Jul 2024, accepted 22nd Jul 2024.

Patients who are SOT recipients are at risk of cryptococcosis, with an incidence that reaches 5.3%.[3] It is usually a late-onset infection (after the first year posttransplant).[4] However, it may begin earlier, particularly in liver and lung transplant patients compared to kidney transplant patients. This may be attributed to the intensity of the immunosuppressive treatment. The lung and central nervous system (CNS) are the most commonly affected systems, but up to 61% of liver transplant patients have disseminated disease, with extrapulmonary involvement in 75% of cases. Other less frequently affected sites include the skin (nodules, papules, ulcers), soft tissue, and the osteoarticular system in 6% to 12% of patients. The liver, kidneys, and

prostate are less frequently involved. In SOT, the mortality of cryptococcosis can reach 50% with CNS involvement. Early diagnosis, timely and adequate antifungal treatment, reduction of immunosuppression, and increased intracranial pressure management are essential to improve post-transplant survival.[6]

We report a case of disseminated cryptococcosis presenting in a patient 30 months after orthotopic liver transplantation.

Family consent and permission were obtained for the case presentation.

Case presentation

A 15-year-old male patient who had been diagnosed with congenital hepatic fibrosis and developed complications of portal hypertension and cirrhosis underwent orthotopic liver transplantation 30 months ago. According to institutional protocol, he received antimicrobial and antifungal prophylaxis in the perioperative period with piperacillin-tazobactam for 48 hours, trimethoprim-sulfamethoxazole and fluconazole for 90 days, and immunosuppressive treatment (prednisolone at 20 mg/day, conventional tacrolimus at 5 mg every 12 hours). He was discharged from the hospital 18 days after his transplant. The Patient followed up regularly at Ege University Pediatric Gastroenterology, Hepatology and Nutrition Clinic, and Kent Hospital Transplant Clinic.

On admission (30 months after liver transplantation), the patient was febrile (39°C), awake, and hypertensive (145/95 mmHg). Physical examination revealed bilateral sacroiliac joint pain and motion range limitation without meningeal signs.

The hemogram disclosed a hemoglobin level of 12.8 g/dL and a leukocyte count of 17.99x10^3 µl with 82.8% segments. Laboratory data is shown in Table I. Serology for herpes simplex virus, human herpesvirus 6, Epstein-Barr virus, cytomegalovirus, and hepatotropic viruses (hepatitis A, B, C virus) were negative. In the microbiology laboratory, yeast cells more significant than normal were observed in wet preparation of the blood culture plate. Intravenous (IV) fluconazole therapy was therefore initiated empirically. The capsule was imaged using Indian ink. Color change was detected on Christensen's urea agar at the 5th hour. The brown colonies were determined

Table I. Laboratory data of the patient.

Day of hospitalization	CRP (mg/L)	AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	WBC (10^3/µl)	Platelet count (10^3/µL)	Cryptococcus antigen	Treatment	Tac concentration (µg/L)	Culture
At admission	95	56	106	52	1.54	17.99	456			5.32	
Day 1	91	48	86	51	1.64	16.85	378			3.32	
Day 2	127	42	90	41	1.47		424			1.23	
Day 3	80	27	61	28	1.53	15.27	443			0	
Day 7	66	44	56	43	1.79	13.64	383	Positive	Fluconazole		positive
Day 14	28	24	37	21	1.71	14.5	329	Positive	L-AmB		positive
Day 21	424	15	13	29	1.49	22.1	311	Positive	L-AmB		
Day 28	124	113	35	57	1.05	33.1	98	Positive	L-AmB		
Day 35	240	1859	222	129	2	44.1	19	Positive	L-AmB		

ALT: alanine transaminase, AST: aspartate transaminase, CRP: C-reactive protein, L-AmB: liposomal amphotericin B, Tac: tacrolimus, WBC: white blood count.

on the 4th day on Staib agar. Identification of *Cryptococcus neoformans* was done by using the VITEK®MS (bioMérieux, Marcy I'Etoile, French) technique. Peripheral blood culture showed *C. neoformans* growth with susceptibility to fluconazole (minimum inhibitory concentration < 2 μg/mL) and amphotericin B (AmB) (minimum inhibitory concentration < 2 μg/mL). A pan-fungal polymerase chain reaction assay was positive for *C. neoformans.* The latex agglutination test for cryptococcal polysaccharide antigen was positive in serum. Diagnostic testing was negative for mycobacteria, and blood cultures for aerobes were also negative. A lumbar puncture could not be performed because the parents did not provide consent. The family later confirmed that they owned chickens, and his uncle had a parrot. However, the death of all the chickens ten days ago prevented the collection of their excrement. Because they thought the parrot was in excellent health, his parents did not give samples of the parrot to the laboratory. Abdominal magnetic resonance imaging (MRI) showed multiple intraperitoneal and retroperitoneal enlarged lymph nodes. Neck tomography showed multiple supraclavicular and infraclavicular lymphadenopathies. Brain MRI detected a low signal in T2 FLAIR hyperintense foci, which are evaluated primarily in favor of chronic ischemia, which was observed in the right frontal lobe deep white matter adjacent to the posterior horn of both lateral ventricles and the periventricular white matter (Fig. 1). In contrast, acute ischemia signs were not detected. Disseminated cryptococcosis with cryptococcemia was confirmed, although we could not confirm meningeal involvement. The absence of cerebrospinal fluid (CSF) findings precluded the confirmation of possible meningitis due to the headache and hypertension. Antifungal induction therapy was switched to IV liposomal AmB (5 mg/kg/day) plus 5-flucytosine (5-FC) (100 mg/kg/day) immediately. In addition, the dose of immunosuppressant therapy was reduced to 2.5 mg tacrolimus every 12 hours, 2.5 mg/day prednisolone, and 500 mg mycophenolate mofetil every 12 hours.

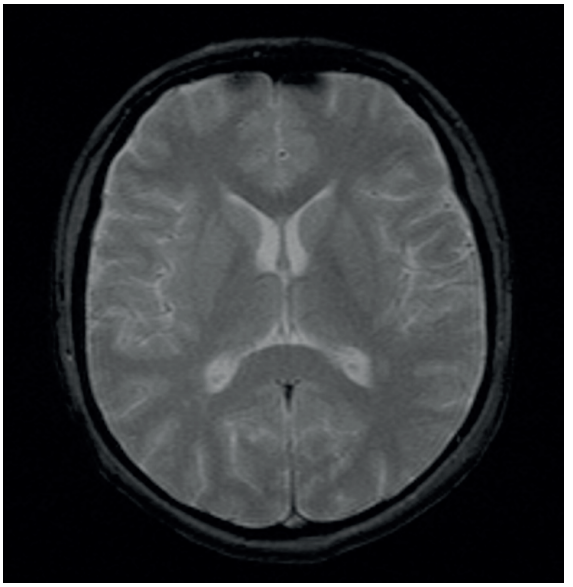


Fig. 1. Cranial MRI Spect (Day 3). Brain MRI detects the low signal in T2 FLAIR hyperintense foci in the right frontal lobe deep white matter adjacent to the posterior horn of both lateral ventricles and the periventricular white matter.

On the tenth day of treatment, fungal cultures were negative. However, the patient's general condition did not improve on the 20th day. The patient was agitated, diaphoretic, and in respiratory distress. He was intubated for acute hypoxic respiratory failure. Laboratory tests were significant for C-reactive protein 225 mg/L, procalcitonin 2.59 μg/L, urea 28 mg/dL, creatinine 1.75 mg/dL, AST 18 U/L, ALT 18 U/L, lipase 1300 U/L, amylase 890 U/L, leukocyte count 26.11x10^{3}/μl, hemoglobin 9.2 g/dL, thrombocyte 436x10^{3}/μl. Echocardiography showed no abnormality. The chest X-ray revealed multiple lung lesions, prompting the start of broad-spectrum antimicrobials and the continuation of antifungal therapy. Computed chest tomography revealed pleural effusion (3 cm), atelectasis, pneumonia with a right lobe cavitary nodule, and no mediastinal lymphadenopathy and heterogeneity of the pancreas (Fig. 2). A bronchoscopy was not performed because the parents did not provide consent. In the intensive care follow-up, respiratory distress worsened rapidly. There was no growth of other bacterial or fungal

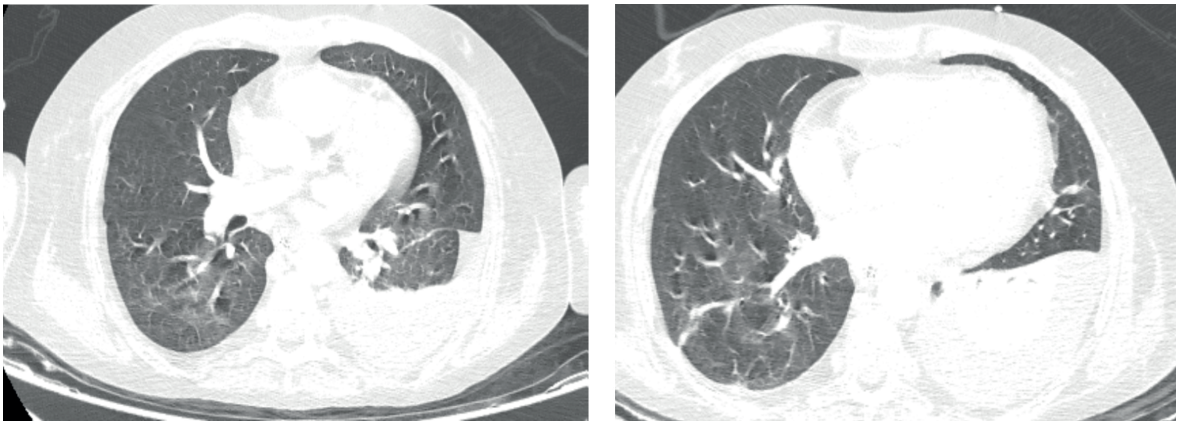


Fig. 2. Computed tomography chest. Computed tomography of the chest demonstrates a cavitary nodule, pleural effusion, and atelectasis.

microorganisms in the patient's blood culture, urine culture, or catheter culture. The patient was primarily diagnosed with pneumonia and pancreatitis, followed by massive gastrointestinal bleeding and shock, and died on the 35th day of hospitalization.

Discussion

Cryptococcosis is a widespread invasive mycosis that causes severe morbidity and mortality. *Cryptococcus neoformans* is a common environmental and human pathogen capable of causing significant illness.[7] This yeast-like encapsulated basidiomycetous fungus has been detected in various environmental sources, including bird droppings, vegetable matter, wood, soil, and dairy products.[8] The fungus typically enters the body through the respiratory system via inhalation of basidiospores or yeast cells, which can be sequestered in sanctuary locations, such as alveolar macrophages. Most immunocompetent people can independently clear the organism, but a small percentage of individuals enter a state of latent infection following inhalation that can then progress to pulmonary infection, hematogenous spread, and extrapulmonary illness.[9,10] There have been few reports of infection acquired by SOT.[11] Studies have shown that cryptococcosis in SOT recipients typically manifests as symptomatic disease and may progress much more quickly than previously.[12,13]

Cryptococcus spp. cause up to 8% of fungal illnesses after SOT and are the third most common cause of fungal infection after *Candida* and *Aspergillus spp.*[14] *Cryptococcus spp.* are observed in up to 5.3% of liver transplants and more commonly appear in kidney transplant recipients than in other patients.[3,5]

Most cases of cryptococcal infection in SOT recipients occur after the first year following transplantation.[4] In a large surveillance study, the median time to onset cryptococcosis following transplantation was 575 days.[14] The possibility of an unexplained pretransplant infection and graft transmission should be explored in early-onset infection, especially if it develops within the first 30 days following transplantation.[15,16] In our patient, the disease was late-onset, occurring after 30 months. Late-onset infections most likely result from primary infection rather than from reactivation.[13] In our case, the point source was possibly a chicken or parrot. However, no samples were taken from the chickens or parrot; thus, we cannot confirm transmission from bird to human. However, the findings strongly support zoonotic transmission.

The CNS is the most common site of symptomatic cryptococcosis, affecting approximately 80% of patients with the disease and typically presenting as subacute or chronic

meningitis.[15,16] Headache, fever, and confusion are the most common symptoms, and ataxia, amaurosis, and cranial nerve palsies can also occur.[11] The second most common manifestation of cryptococcosis is respiratory disease, which can appear in the form of respiratory consolidations, nodular or cavitary infiltrates, miliary patterns, or pleural effusion. In the case presented, disseminated involvement (meningeal, pulmonary, and lymphatic) by *Cryptococcus neoformans* was documented.

Direct microscopy of sputum, bronchoalveolar lavage, CSF, blood, urine, peritoneal lavage, or organ biopsy can aid in cryptococcosis diagnosis. Fungal cultures can be collected from these types of samples. *Cryptococcus* antigens in serum and CSF can be detected using a latex agglutination test. In patients with cryptococcosis affecting the CNS, cranial imaging may reveal leptomeningeal enhancement, encephalomalacia, infarcts, cerebellitis, hydrocephalus, and transverse myelitis.[7,17,18] In our case, the CSF sample could not be obtained because a lumbar puncture and bronchoalveolar lavage could not be performed; however, *Cryptococcus neoformans* was detected in the peripheral blood culture, and the antigen test was positive. There was no growth of other bacterial or fungal microorganisms in the patient's blood, urine, or catheter cultures, so an additional nosocomial infection was not considered. Therefore, the patient's death was thought to be most likely due to cryptococcal infection.

Treatment recommendations for cryptococcosis vary depending on the site of infection and the host's immunological status. According to the Infectious Diseases Society of America (IDSA), the recommended induction and consolidation therapy for CNS and disseminated disease is AmB deoxycholate (1 mg/kg per day IV) plus flucytosine (100 mg/kg per day orally in 4 divided doses) for two weeks (for the non–human immunodeficiency virus-infected, non-transplant population, follow the treatment length schedule for adults), followed by fluconazole (10–12 mg/kg per day orally) for eight weeks; for AmB-intolerant patients, either liposomal AmB (5 mg/kg per day) or AmB lipid complex (5 mg/kg per day).[15] For solid transplant recipients with cryptococcosis, reducing the immunosuppressive drug regimen to the lowest possible level is an essential step in antifungal therapy. Following the detection of cryptococcus in our patient, we arranged his treatment according to the literature and reduced immunosuppressive agents.

The mortality rate in disseminated cryptococcosis is around 17% and increases to almost 50% when the CNS is involved, particularly in the presence of factors predictive of 90-day mortality, such as cryptococcosis, compromise in the state of consciousness, and intracranial hypertension.[1,19] Our patient's response to treatment was unfavorable and he experienced complications, leading to death.

Cryptococcosis is a rare infection among SOT children. We recommend that immunocompromised patients avoid owning birds and regions contaminated with bird droppings due to the risk of cryptococcosis transmission from pets to humans.

Ethical approval

Family consent and permission were obtained for the case presentation.

Author contribution

Study conception and design: DB, PY, MK; data collection: BK, SYA, SA; analysis and interpretation of results: GKA,PY, ZŞB; draft manuscript preparation: PY, DB, SA. All authors revised the manuscript and approved the final version of the article.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Pappas PG. Cryptococcal infections in non-HIV-infected patients. Trans Am Clin Climatol Assoc 2013; 124: 61-79.
2. Williamson PR, Jarvis JN, Panackal AA, et al. Cryptococcal meningitis: epidemiology, immunology, diagnosis and therapy. Nat Rev Neurol 2017; 13: 13-24. https://doi.org/10.1038/nrneurol.2016.167
3. Sun HY, Wagener MM, Singh N. Cryptococcosis in solid-organ, hematopoietic stem cell, and tissue transplant recipients: evidence-based evolving trends. Clin Infect Dis 2009; 48: 1566-1576. https://doi.org/10.1086/598936
4. Husain S, Wagener MM, Singh N. Cryptococcus neoformans infection in organ transplant recipients: variables influencing clinical characteristics and outcome. Emerg Infect Dis 2001; 7: 375-381. https://doi.org/10.3201/eid0703.010302
5. Singh N, Alexander BD, Lortholary O, et al. Cryptococcus neoformans in organ transplant recipients: impact of calcineurin-inhibitor agents on mortality. J Infect Dis 2007; 195: 756-764. https://doi.org/10.1086/511438
6. Baddley JW, Forrest GN; AST Infectious Diseases Community of Practice. Cryptococcosis in solid organ transplantation. Am J Transplant 2013; 13(Suppl 4): 242-249. https://doi.org/10.1111/ajt.12116
7. Curi ALL, Lazera M, Vasconcelos-Santos DV. Cryptococcosis. In: Zierhut M, Pavesio C, Ohno S, Orefice F, Rao N, editors. Intraocular Inflammation. Berlin, Heidelberg: Springer; 2016: 1277-1283. https://doi.org/10.1007/978-3-540-75387-2_123
8. Ellis DE, Zaoutis T, Thibault DP, Crispo JAG, Abraham DS, Willis AW. Readmissions after hospital care for meningitis in the United States. Am J Infect Control 2020; 48: 798-804. https://doi.org/10.1016/j.ajic.2019.10.025
9. Ferreira GSA, Watanabe ALC, Trevizoli NC, et al. Transmission of cryptococcosis by liver transplantation: a case report and review of literature. World J Hepatol 2020; 12: 253-261. https://doi.org/10.4254/wjh.v12.i5.253
10. Wu G, Vilchez RA, Eidelman B, Fung J, Kormos R, Kusne S. Cryptococcal meningitis in solid organ transplant recipients in a tertiary medical center. Neurology 2000; 54: A286-A287.
11. Vilchez RA, Fung J, Kusne S. Cryptococcosis in organ transplant recipients: an overview. Am J Transplant 2002; 2: 575-580. https://doi.org/10.1034/j.1600-6143.2002.20701.x
12. Pappas PG, Alexander BD, Andes DR, et al. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 2010; 50: 1101-1111. https://doi.org/10.1086/651262
13. Baddley JW, Schain DC, Gupte AA, et al. Transmission of cryptococcus neoformans by organ transplantation. Clin Infect Dis 2011; 52: e94-e98. https://doi.org/10.1093/cid/ciq216
14. Sun HY, Alexander BD, Lortholary O, et al. Unrecognized pretransplant and donor-derived cryptococcal disease in organ transplant recipients. Clin Infect Dis 2010; 51: 1062-1069. https://doi.org/10.1086/656584
15. McHale TC, Boulware DR, Kasibante J, Ssebambulidde K, Skipper CP, Abassi M. Diagnosis and management of cryptococcal meningitis in HIV-infected adults. Clin Microbiol Rev 2023; 36: e0015622. https://doi.org/10.1128/cmr.00156-22
16. Tugume L, Ssebambulidde K, Kasibante J, et al. Cryptococcal meningitis. Nat Rev Dis Primers 2023; 9: 62. https://doi.org/10.1038/s41572-023-00472-z
17. Perfect JR, Dismukes WE, Dromer F, et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis 2010; 50: 291-322. https://doi.org/10.1086/649858
18. Kwizera R, Omali D, Tadeo K, et al. Evaluation of the dynamiker cryptococcal antigen lateral flow assay for the diagnosis of HIV-associated cryptococcosis. J Clin Microbiol 2021; 59: e02421-e02420. https://doi.org/10.1128/JCM.02421-20
19. Miwa T, Okamoto K, Ikeuchi K, et al. The role of frequent screening or diagnostic testing of serum cryptococcal antigen in liver transplant recipients: a descriptive epidemiology. Open Forum Infect Dis 2024; 11: ofae255. https://doi.org/10.1093/ofid/ofae255

The Turkish Journal of Pediatrics 2024; 66: 505-510
https://doi.org/10.24953/turkjpediatr.2024.4655

Case Report

Non-syndromic perspective on a unique progressive familial intrahepatic cholestasis variant: *ZFYVE19* mutation

Coşkun Fırat Özkeçeci[1], Melike Arslan[1], Edibe Gözde Başaran[1], Yasin Maruf Ergen[1], Önder Bozdoğan[2], Necati Balamtekin[1]

[1]Division of Pediatric Gastroenterology, Department of Pediatrics, Gülhane Training and Research Hospital; [2]Department of Pathology, Gülhane Training and Research Hospital, Ankara, Türkiye.

ABSTRACT

Background. *ZFYVE19* mutation has been recently identified as one of the non-syndromic causes of cholestasis. It is associated with elevated gamma-glutamyl transferase levels and is likely a cause of neonatal-onset and intrahepatic cholestasis.

Case. Here, we report a rare case of *ZFYVE19* defect, confirmed by whole exome sequencing (WES). Our patient, who is currently 4 years old, presented to us at the age of 2 years with elevated levels of serum transaminases and bilirubin. WES revealed a homozygous *ZFYVE19* mutation despite preserved synthetic liver function. This gene has recently been identified in the literature as a cause of non-classical progressive familial intrahepatic cholestasis (OMIM # 619849). Treatment with an appropriate dose of ursodeoxycholic acid resulted in the regression of elevated liver enzymes and itching. The patient's body mass index progressively increased throughout the treatment period. No medication side effects were observed at any point. Currently, the patient remains asymptomatic during follow-up.

Conclusion. We have identified the *ZFYVE19* mutation as a variant that is not accompanied by any other symptoms. However, we have limited knowledge about the progression of the disease and are closely monitoring the patient for potential liver-related issues. Using WES in cases of undiagnosed liver enzyme elevations or cholestasis can help identify new genes and improve our understanding of the underlying pathophysiology.

Key words: cholestasis, progressive familial intrahepatic cholestasis, ursodeoxycholic acid, whole exome sequencing.

Cholestasis is defined as the accumulation of bile products in the liver due to a decrease in the formation of bile in hepatocytes or a decrease in the excretion of bile products into the bile ducts/intestinal lumen. It can be acute, chronic, or recurrent, and is seen in approximately 1 in 2500 live births.[1] It can occur in all age groups, including the neonatal period, and can be caused by either intrahepatic or extrahepatic factors. Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of diseases that originate in the hepatocytes and have autosomal recessive inheritance. It is considered one of the causes of intrahepatic cholestasis.[2] The incidence of this group of diseases is estimated to be 1 in 50,000 to 1 in 100,000 live births, and both sexes are affected equally. It is known that the three classical types of PFIC are caused by mutations in hepatocyte transport genes involved in bile acid formation.[3]

In this case report, we present a 4-year-old male patient with a rare genetic form of cholestasis not caused by the classical PFIC genes.

Case description

A male patient presented to our clinic at the age of 2 years with complaints of jaundice and itching. In the patient's medical history,

✉ Coşkun Fırat Özkeçeci
coskunfirat3@hotmail.com

Received 19th Apr 2024, revised 30th Jun 2024, accepted 5th Jul 2024.

it was noted that he was monitored due to prolonged jaundice during the neonatal period. At three months of age, he was diagnosed with cholestatic liver disease based on hepatosplenomegaly, elevated transaminases, and jaundice. The patient's parents had a history of first-degree consanguinity. The patient had been using ursodeoxycholic acid (UDCA) for approximately 2 years, and the family discontinued UDCA usage two months prior to the current visit due to a lack of improvement in symptoms. At the age of two years, the patient's anthropometric measurements were as follows: weight -1.4 SDS, and body mass index -1.3 SDS. On physical examination, the spleen was palpable approximately 2 cm below the costal margin. Other system examinations were unremarkable. The family conducted the Caregiver Impression of Severity (CaGIS) assessment to evaluate the patient's itching.[4] The assessment ranged from 1 to 5, with 1 indicating no itching and 5 indicating very severe itching. The CaGIS score was evaluated as 4. Following the patient's presentation to our clinic, a comprehensive etiological screening for cholestasis was initiated through primary and secondary investigations.[5] At the time of admission, Alanine aminotransferase (ALT) was 258 U/L, Aspartate aminotransferase was 279 U/L, Gamma-glutamyl transferase (GGT) was 254 U/L, total bilirubin was 3.2 mg/dL, direct bilirubin was 1.9 mg/dL (Fig. 1a, 1b), fasting blood sugar was 82 mg/dL, albumin was 4.4 g/dL, international normalization ratio (INR) was 1.1, hemoglobin (HGB) was 12.9 g/dL, platelet count (PLT) was 89,000 cells/μL, and white blood cell count (WBC) was 5,200 cells/μL. Detailed metabolic, endocrinological and infectious tests were negative. Celiac disease antibodies yielded negative results. Serum bile acids could not be assessed due to the patient's two-year history of UDCA usage. Concurrently, 20 mg/kg/day of ursodeoxycholic acid was initiated. Abdominal ultrasound revealed coarse, granular liver parenchyme with irregular contours, and an enlarged spleen measuring 142 mm in vertical length. The parenchymal echo was heterogeneous, and increased echogenicity was noted at the portal hilum. Portal venous doppler ultrasound was normal. Upper gastrointestinal endoscopy was performed to investigate possible complications of liver cirrhosis. Grade III esophageal varices and fundic varices were observed so the endoscopic band ligation was applied for the esophageal ones and Propranolol was initiated at a dose of 1 mg/kg/day. Magnetic resonance cholangiopancreatography was normal. The common bile duct also appeared normal. Next generation sequencing (NGS) evaluation was conducted for the patient with no identified etiology, excluding classical PFIC subtypes, potential congenital metabolic disorders, and structural cholestatic diseases. A liver biopsy revealed moderate inflammation, interpreted as cirrhosis with significant fibrosis (fibrosis stage 6/6). Marked ductal proliferation was also observed at the portoparenchymal border. However, a definitive etiological interpretation could not be made (Fig. 2).

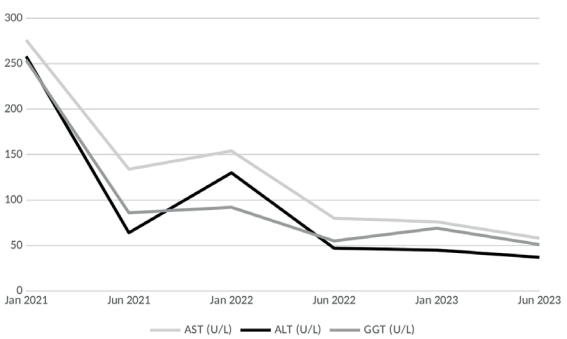


Fig. 1. a. Baseline and follow up values in relation to therapy (ALT: alanine transaminase, AST: aspartate transaminase, GGT: gamma-glutamyl transferase).

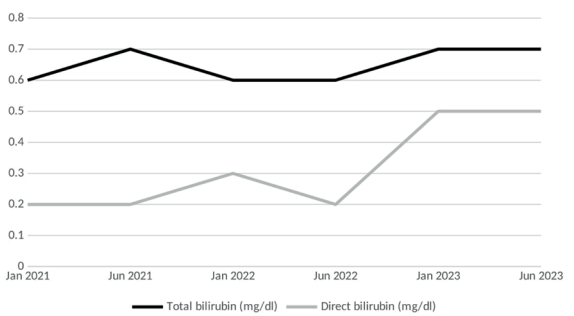


Fig. 1. b. Baseline and follow up values in relation to therapy (Total bilirubin, direct bilirubin).

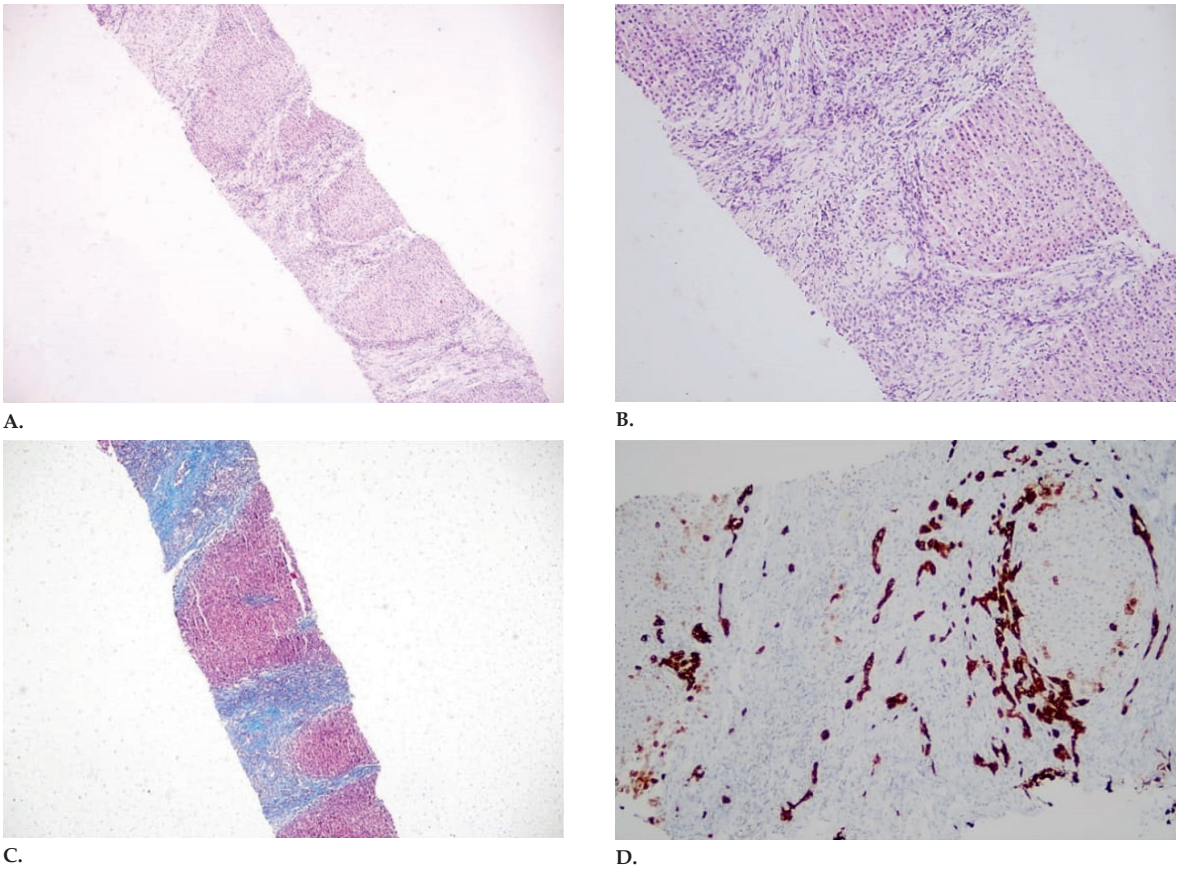


Fig. 2. Liver biopsy pathology sections of the patient. In hematoxylin and eosin sections of the liver biopsy sample, **(A, B)** fibrosis was observed, accompanied by moderate inflammation and proliferation of bile ducts. It is noteworthy that fibrosis is more descriptive in the trichrome stain **(C)**, and it can be interpreted as cirrhosis. **(D)** The immunohistochemical study of CK7 indicates significant proliferation of bile ducts. A: Original microscopic level, B: X100, C: X40, D: X200.

The patient followed up for cryptogenic cirrhosis underwent whole exome sequencing (WES) analysis. A homozygous mutation of c.547C>T (p.Arg183Ter) was identified in the *ZFYVE19* (zinc finger FYVE-type containing 19) gene. This homozygous mutation in the gene led to the diagnosis of a rarely defined genetic cholestasis type (non-syndromic phenotype) known as PFIC9 (OMIM # 619849). The same mutation was detected in both parents as heterozygous. For treatment, the patient was administered ursodeoxycholic acid (20 mg/kg/day), vitamin K (10 mg/week), propranolol (1 mg/kg/day), and fat-soluble vitamin supplementation. Throughout the follow-up period, the patient's clinical and laboratory findings did not deteriorate.

At present, the patient is 4 years old, with normal transaminase levels, serum albumin, and INR. Serum conjugated bilirubin levels were normal. An upper gastrointestinal endoscopy performed for control purposes revealed grade I esophageal varices, with no fundic varices observed. The patient did not experience any gastrointestinal bleeding, and the itching complaint was resolved with current treatments. The family conducted the CaGIS assessment to evaluate the patient's itching for the second time; the CaGIS score was 1. No side effects were observed in the patient while receiving all these treatments. The body mass index increased from -1.3 SDS to -0.49 SDS during the follow-up period. After starting UDCA treatment at an appropriate

dose, liver transaminases and bilirubin values gradually decreased, and the itching complaint completely regressed. Our follow-ups, ongoing for two years, continue without any complaints from the patient. However, hepatosplenomegaly persists. During follow-ups, renal and neurological functions, as well as psychomotor development, were evaluated by specialists and found to be within normal limits. Written informed consent was obtained from the patient's parents for the publication of this case report.

Discussion

PFIC is a genetic disease that can progress to liver cirrhosis due to defective transport of the bile ducts. It can manifest either asymptomatically, such as incidentally detected hypertransaminasemia, or as an end-stage liver disease.[6] In symptomatic cases, treatment options include a few effective drugs, biliary diversion procedures to ensure bile flow or liver transplantation. Medications used for symptom treatment include rifampicin (5 mg/kg/day), UDCA (20-30 mg/kg/day), and odevixibat (40 μg/kg/day).[7] Diagnosis is established through clinical, biochemical, radiological, and histopathological findings, supported by genetic studies. Subtypes are traditionally categorized into PFIC1, PFIC2, PFIC3, PFIC4, and PFIC5, with corresponding genes identified as *ATP8B1, ABCB11, ABCB4, TJP2*, and *NR1H4*, respectively.[6,8] With the increasing focus on genetic studies, new and rare subtypes of PFIC have been identified. These include PFIC6, PFIC7, PFIC8, PFIC10, PFIC11, and PFIC12, with mutations attributed to *SLC51A, USP53, KIF12, MYO5B, SEMA7A*, and *VPS33B*, respectively.

PFIC9 is an autosomal recessive genetic disorder that typically begins in infancy or childhood and is characterized by an increase in serum gamma-glutamyl transferase. Mutations in the *ZFYVE19* gene have been identified as a key regulator of cytokinetic abscission. This gene plays a crucial role in disrupting the bridge between post-mitotic sister cells, leading to the development of the condition.[9] Jaundice, hepatosplenomegaly, portal hypertension, or upper gastrointestinal bleeding may manifest clinically. Patients often benefit from UDCA therapy, with liver enzymes occasionally remaining within the normal range. However, some patients may still require liver transplantation despite treatment. Liver biopsies may reveal micronodular cirrhosis, portal dilation accompanied by fibrosis, bile duct proliferation, and ductal plate malformation.[10] Significant bile duct proliferation was observed in our case, and there was a noticeable moderate lymphocyte-rich inflammation in the portal areas. Liver histopathology revealed a cirrhotic process with marked fibrosis.

Pepe et al.[11] described the case of a 6-year old female patient with a homozygous *ZFYVE19* mutation, previously treated with rifampicin and UDCA but still experiencing resistant itching. They successfully tried odevixibat as an alternative. In our case, we started our treatment with UDCA as medical treatment and we are since continuing.

The choice of medical treatment should be evaluated based on the patient's clinical presentation. However, how long patients can continue using their liver with the selected medical treatment, or if it is possible to preserve the patient's liver, is a question that remains unanswered.

In this diagnosis, we anticipate that the frequency of the "idiopathic neonatal hepatitis" diagnosis will decrease with the assistance of emerging next-generation techniques.[12] PFIC9, recently identified as a new cause of hypertransaminasemia, was diagnosed in our patient through WES analysis. Following cytokinetic abscission, midbodies move toward the cell surface and participate in ciliogenesis. Therefore, any intervention in ZYFVE19 expression can lead to abnormal chromosome segregation and DNA damage.[9] It has been reported that individuals with *ZFYVE19* mutations may also experience cilia dysfunction. Molecular results even indicate that cilia dysfunction is observed in

unaffected cells in individuals with *ZFYVE19* mutations, suggesting a broader impact beyond cholestasis.[13] Unfortunately, due to the unsuitability of laboratory conditions, we were unable to assess investigations into cilia dysfunction in our patient. As new-generation techniques continue to aid in the understanding of genetic disorders, we believe that the identification of specific genetic causes, such as PFIC9, will contribute to more accurate and targeted diagnoses, reducing the prevalence of previously labeled idiopathic conditions like idiopathic neonatal hepatitis.

The important point to consider here is that the *ZFYVE19* mutation is the cause of a newly defined disease called PFIC9, which is also one of the causes of idiopathic neonatal hepatitis to date. It is important to continue to monitor these patients because we do not yet know how much and how it will affect the liver, how and when it will affect other organs that are not phenotypically affected, or whether it will involve other systems. In addition, In addition, WES analysis increases the diagnosis rate in cases of cholestasis with unknown cause, it should be used more widely. Even if it is not applied routinely, it will increase current knowledge and, as a result, studies for the treatment of the newly defined genes.

Acknowledgments

We thank Dr. Serdar Ceylaner for his review of our genetic result.

Ethical approval

Written informed consent was obtained from the patient's parents.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: CFO, NB; data collection: CFO, EGB, MA, YME; analysis and interpretation of results: CFO, OB, MA, NB; draft manuscript preparation: CFO, MA, EGB, MA, YME. 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.

REFERENCES

1. Feldman AG, Sokol RJ. Neonatal cholestasis: updates on diagnostics, therapeutics, and prevention. Neoreviews 2021; 22: e819-e836. https://doi.org/10.1542/neo.22-12-e819
2. Jacquemin E. Progressive familial intrahepatic cholestasis. Clin Res Hepatol Gastroenterol 2012; 36(Suppl 1): S26-S35. https://doi.org/10.1016/S2210-7401(12)70018-9
3. Davit-Spraul A, Gonzales E, Baussan C, Jacquemin E. Progressive familial intrahepatic cholestasis. Orphanet J Rare Dis 2009; 4: 1. https://doi.org/10.1186/1750-1172-4-1
4. Gwaltney C, Ivanescu C, Karlsson L, Warholic N, Kjems L, Horn P. Validation of the PRUCISION instruments in pediatric patients with progressive familial intrahepatic cholestasis. Adv Ther 2022; 39: 5105-5125. https://doi.org/10.1007/s12325-022-02262-7
5. Fawaz R, Baumann U, Ekong U, et al. Guideline for the evaluation of cholestatic jaundice in infants: joint recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr 2017; 64: 154-168. https://doi.org/10.1097/MPG.0000000000001334
6. Vinayagamoorthy V, Srivastava A, Sarma MS. Newer variants of progressive familial intrahepatic cholestasis. World J Hepatol 2021; 13: 2024-2038. https://doi.org/10.4254/wjh.v13.i12.2024
7. Baker A, Kerkar N, Todorova L, Kamath BM, Houwen RHJ. Systematic review of progressive familial intrahepatic cholestasis. Clin Res Hepatol Gastroenterol 2019; 43: 20-36. https://doi.org/10.1016/j.clinre.2018.07.010
8. Amirneni S, Haep N, Gad MA, Soto-Gutierrez A,

Squires JE, Florentino RM. Molecular overview of progressive familial intrahepatic cholestasis. World J Gastroenterol 2020; 26: 7470-7484. https://doi.org/10.3748/wjg.v26.i47.7470

9. Nigg EA, Holland AJ. Once and only once: mechanisms of centriole duplication and their deregulation in disease. Nat Rev Mol Cell Biol 2018; 19: 297-312. https://doi.org/10.1038/nrm.2017.127
10. Luan W, Hao CZ, Li JQ, et al. Biallelic loss-of-function ZFYVE19 mutations are associated with congenital hepatic fibrosis, sclerosing cholangiopathy and high-GGT cholestasis. J Med Genet 2021; 58: 514-525. https://doi.org/10.1136/jmedgenet-2019-106706
11. Pepe A, Colucci A, Carucci M, et al. Case report: add-on treatment with odevixibat in a new subtype of progressive familial intrahepatic cholestasis broadens the therapeutic horizon of genetic cholestasis. Front Pediatr 2023; 11: 1061535. https://doi.org/10.3389/fped.2023.1061535
12. Ananth R. Neonatal cholestasis: a primer of selected etiologies. Pediatr Ann 2018; 47: e433-e439. https://doi.org/10.3928/19382359-20181018-01
13. Mandato C, Siano MA, Nazzaro L, et al. A ZFYVE19 gene mutation associated with neonatal cholestasis and cilia dysfunction: case report with a novel pathogenic variant. Orphanet J Rare Dis 2021; 16: 179. https://doi.org/10.1186/s13023-021-01775-8

CASE REPORTS

Printed by METEKSAN in Ankara, Turkey